DEFECTIVE SPERM FUNCTION IN HUMAN MALE INFERTILITY

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DECLARATION

I declare that this thesis has been composed by myself.

The work described in this thesis was undertaken by me whilst a member of a research group. All of the work described herein was undertaken by me, with the exception of the zona-free hamster oöcyte penetration tests described in Chapters 3 and 4, most of which were performed by Dr R J Aitken.

Much of the material contained in Chapters 3, 4 and 5 of this thesis has been published as follows :

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Irvine D S, Aitken R J, Measurement of intracellular calcium in human spermatozoa., Gamete Res, 1986,15, 57-71.

Irvine D S, Aitken R J, Predictive value of in-vitro sperm function tests in the context of an AID service., Human Reprod, 1986, 1: 539-545.

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ABSTRACT

Infertility, the failure of a couple to conceive after 12 months of unprotected intercourse, is a common clinical problem, with available estimates suggesting that as many as one couple in six may be affected. The aetiology of this problem is poorly understood, however it would appear that an abnormality is detectable in the male partner in some 43% of infertile couples, and thet defective sperm function is the commonest single abnormality detected during the investigation of infertility.

The conventional criteria of semen quality are purely descriptive, providing information on the volume of semen , its physical characteristics, and on the concentration, motility and morphology of the spermatozoa present. A survey of semen quality amongst men of recently proven fertility has suggested that values in excess of 20 x 10^6/ml for sperm concentration, and 40% for motility represent a good definition of normality (being around the 5th centile), but that these criteria are of very little clinical value in terms of the diagnosis of male fertility.

The accurate diagnosis of male infertility was found to be facilitated by the use of *in-vitro* tests of human sperm function, including the zona-free hamster oöcyte penetration test, and the photomicrographic study of sperm movement characteristics. When combined with the conventional criteria of semen quality in a retrospective study of semen used in therapeutic donor insemination, such tests were found to distinguish with clinically useful accuracy between ejaculates which were and those which were not able to initiate pregnancy, providing a predictive accuaracy of around 80%. In contrast, the measurement of semen adenosine triphosphate levels was not found to be of value. The design of the zona-free hamster oöcyte penetration test employed in this study involved the use of the divalent cation ionophore A23187, to activate the cells by means of a calcium influx.

Studies using $^{45}Ca^{2+}$ demonstrated that the plasma membrane of the human spermatozoon is permeable to calcium, and that a significant proportion of calcium entering the cell is sequestered in the mitochondrion. Studies using quin-2 provided the first measurements of free intracellular calcium in human spermatoazoa, with resting levels of 1-200nM being obtained. Evidence was obtained to suggest that extracellular calcium enters the cell passively, rather than through voltage sensitive calcium channels, and that the sperm actively extrudes this calcium. The level of free calcium was found to be affected by the level of extracellular calcium and by temperature. A23187 was shown to cause a significant increase in intracellular calcium. In addition measurements of intracellular pH were obtained with the indicator BCECF, with resting values being around 7.02 .

CHAPTER ONE

INTRODUCTION

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"Be fruitful and multiply and replenish the earth "

Genesis, 1, v.28.

1.1 Objectives

Infertility, the failure of a couple to conceive and give birth, is a common and distressing human problem. Available estimates suggest that about 15% of couples of reproductive age, perhaps as many as 50 - 80 million couples world-wide, may suffer from infertility. Almost half of this infertility is thought to be due in whole or in part to male reproductive dysfunction. Despite the extent of the problem, our ability to accurately diagnose the existence of male infertility, our understanding of the aetiology of male infertility, and our ability to provide proven effective treatment remains extremely poor. Only once reliable diagnosis is possible, and only when we are able to understand the causes of male infertility, can we hope to begin to develop and evaluate rational therapeutic strategies. The principal objectives of this thesis, therefore, are the evaluation of new approaches to the diagnosis of male infertility and the understanding of the causes of male infertility by examining human spermatozoal function at the level of the cell biology of the spermatozoon.

1.2 Infertility

The motivations for parenthood are a complex and poorly understood combination of instinct and social conditioning. Whilst it is likely that social conditioning is a major factor in a couple's decision to attempt to have a family, in that children learn that childbearing is expected of them, other competing influences may include the need of the individual to prove sexual

potency and virility, the desire of the couple to compete on an equal footing with other couples, their wish to play the adult mother-father roles, and their need to have a child to love and to provide companionship (Kerr and Parboosingh, 1974). From a more practical point of view, in many communities children are required to help with the work of the family, and to provide a source of security in old age, whilst at an emotional level, many women clearly desire the emotional experiences of pregnancy, childbirth and childrearing per se. For women and for men, children represent an achievement, both personally and as an expression of their sexuality (Pfeffer and Woollett, 1983)

In addition to these internal pressures, there are many powerful external pressures for childbearing, including the horror of childlessness and the importance of childbearing expressed in the Judaeo-Christian religious tradition :

> "Rachel envied her sister Leah and cried out to her husband, Jacob, 'give me children or I shall die'".
>
> Holy Bible
> Genesis, 30, 14.

> "As arrows are in the hand of a mighty man, so are children of the youth; happy is the man that hath his quiver full of them."
>
> Psalms 127, v 3-5.
> The Holy Anglican Bible

> "First, it was ordained for the procreation of children."
>
> The Book of Common Prayer
> Solemnisation of Matrimony

"Adam was not deceived, but the woman was deceived and became a transgressor. Yet woman will be saved through bearing children, if she continues in faith and love and holiness, with modesty."

Holy Bible
1 Timothy, 2, 14-18.

Other religions take an equally strong stance, and in many Moslem countries childlessness, in addition to being seen as a tragedy, is a valid basis for divorce and for taking a second wife. In addition to the religious pressures felt by couples, there are the secular pressures of would-be grandparents and of the reproductive norms presented by the media. These forces are felt strongly by couples who have no children, and those who are intentionally childless may be categorized as selfish and emotionally lacking, whilst those whose childlessness is is unwanted may, in addition, be treated with a condescending pity and be driven to feelings of sexual inadequacy, immaturity and incompetence. In any scientific discussion of infertility, it is all too easy to become distanced from the individual tragedy of childlessness.

1.3 Epidemiological Definitions

In the dictionary, fertile (from the latin fertilis or the old french fertil) is defined as "capable of conceiving and giving birth", infertile is defined simply as "not fertile", while, from a clinical point of view, fertility has an additional precise demographic definition, namely the number of live births per 1000 women aged 15-44 years (Last, 1970). It is rather more difficult to provide a clinical definition of infertility, and one might begin by looking at patterns of natural fertility.

Various estimates of natural fertility are available. Studies of couples not using contraception suggest that approximately 25% will conceive within 1 month, 60% within 6 months, 75% within 9 months and 90% within 18 months (Peel and Wood, 1972; Kerr and Parboosingh, 1974) (Figure 1.1, p 49). Southam (1960) estimated that after 6 months of trying to conceive, 41% of women will remain non-pregnant, that after 12 months this figure falls to 27%, finally falling to 18% remaining non-pregnant after 24 months of trying to conceive. Tietze (1956; 1968), studying women attempting to conceive for the first time without ever having used contraception, and women attempting to conceive following the discontinuation of contraception, found that approximately 90% had conceived within 12 months. The results of these four studies are summarised graphically in Figure 1.1 (p 49). For practical clinical purposes therefore, it is generally considered that if a couple fail to conceive after 12 months of normal unprotected sexual intercourse, then a potential problem exists which merits investigation and treatment (Hudson et al, 1980a; Cates et al, 1985; Hull et al, 1985). Other authorities, including the World Health Organization Scientific Group studying the epidemiology of infertility (World Health Organization, 1975) have suggested that a period of 2 years of exposure to pregnancy should elapse before a couple are considered to be suffering from infertility.

Failure of conception after a period of unprotected sexual intercourse, must be clearly distinguished from the failure of a woman to carry a pregnancy to term, having successfully conceived, although the consequences of this for the couple are very similar. Likewise, in cultures with a strong preference for male offspring, the failure of a woman to produce sons rather than daughters is seen as a special form of infertility, and therefore in some

African countries, the term primary infertility is applied when a woman has no live children, secondary infertility when she has no live male children (Pawson, 1986).

The epidemiological terminology surrounding infertility was set out by a recent World Health Organization Scientific Group as follows (World Health Organization, 1975) :

1. Primary Infertility : Failure to conceive despite cohabitation and exposure to pregnancy for a period of two years.

2. Secondary Infertility : The woman has previously conceived, but is subsequently unable to do so despite cohabitation and exposure to pregnancy for a period of two years. If the woman has breast fed an infant, the period of exposure to pregnancy should be calculated from the end of the period of lactational amenorrhoea.

3. Pregnancy Wastage : The woman is able to conceive, but unable to produce a live birth. Loss of pregnancy during the first 28 weeks of gestation is referred to as early and intermediate fetal loss, or abortion, and may be spontaneous or induced. Beyond 28 weeks of gestation and up to term, such losses are referred to as late fetal deaths or stillbirths.

4. Child Mortality : All deaths of children born alive, up to their fifth birthday. Perinatal mortality (stillbirths plus all deaths of offspring during the first week after birth) may be recorded separately.

5. Unproven Fertility : This refers to problems sometimes perceived by individuals or couples as infertility, or included as infertility in demographic surveys, whereas in fact the woman is virtually not at risk of conception.

For present purposes however, infertility will be defined as the failure of a couple to conceive despite twelve months of normal unprotected sexual intercourse.

1.4 Prevalence of Infertility

> "Infertility is a major medical and social problem of global dimensions. Epidemiological studies carried out by the Program indicate that up to 15% of couples of reproductive age, perhaps as many as 50-80 million couples, may have infertility problems."
>
> (Diczfaluzy, 1986)

Estimates of the magnitude of the problem of infertility will vary depending upon the database used for their compilation, either demographic data, or health services based data commonly being used. In either case, the conclusions reached concerning the size of the problem will be innaccurate (Belsey and Ware, 1986) and this is illustrated in Figure 1.2 (p 50). Demographically based surveys estimate only primary infertility, and rarely distinguish between involuntary infertility, voluntary infertility, and unknown fertility. Health services based data on the other hand provides a poor assessment of the scale of the problem unless account is taken of every affected couple attending services providing any form of infertility care, including the primary care sector. In addition, health services based data assumes that the majority of couples suffering from involuntary infertility

seek medical attention (Hull et al, 1985) and it is difficult to know the extent to which this is the case.

Recent surveys by the World Health Organization (Diczfaluzy, 1986) into the prevalence of primary and secondary infertility, pregnancy wastage and infant and child mortality, have found considerable regional variation. In a survey of the Southern Sudan, primary infertility was found amongst 24% of women aged 20-29 and amongst 40% of those aged 30-44. Surveys in other countries found much lower rates of primary and secondary infertility, ranging between 2 and 13% (Diczfaluzy, 1986). The most recent data concerning the prevalence of infertility in the UK is perhaps that published by Hull and colleagues (Hull et al, 1985; 1986). This group studied 708 couples presenting to specialist infertility services from within a population of residents of a single health district, and excluded referrals from outwith this defined area. From a population of 400,000 residents, referrals were received at a rate of 472 new couples per year, representing an annual incidence of 1.2 couples per year per 1000 population. These workers estimated that 1 couple in 6 (17%) would request help with infertility problems at some time in their lives, and that for primary infertility this figure was 1 couple in 8 (13%) (Figure 1.3, p 51), with seventy-one percent of all couples presenting suffering from primary infertility. Further studies will be required to confirm these findings. The average duration of infertility at presentation was 29 months (range 1-13 years), while the average age of the women was 28 years (18-46) and that of the men 31 years (18-64) (Hull et al, 1985). It is interesting to compare this distribution of age, type and duration of infertility with that of a recent World Health Organization survey (Cates et al, 1985) (Table 1.1, p 39). This latter group of workers studied 7596 couples presenting with infertility, 3902 from within the developed

countries and 3694 from the developing countries. Within the developed countries they observed that the vast majority of couples were aged between 25 and 34 at presentation and that 71% were complaining of primary infertility. In 83% of couples, the period of infertility at presentation was in excess of 1.5 years, and in 54% was greater than 2.5 years.

It is clear therefore, that infertility is a very common problem amongst young couples, and that the majority of couples affected experience problems in starting their first pregnancy. We therefore need to try to understand the causes of infertility if we are to be able to help those affected, and while our understanding of the aetiology of female infertility is comparatively good, our understanding of the aetiology of male infertility remains very poor, largely as a result of our relative inability to accurately assess whether or not a given individual is subfertile.

1.5 Aetiology of Infertility

The literature is replete with studies on the causation of infertility in men and women. Unfortunately, the available literature is very difficult to interpret, reflecting as it often does the specialist interests, prejudices, referral bases, available facilities and diagnostic protocols employed by the authors concerned. Thus data collected on the aetiology of infertility varies greatly in its reliability and uniformity. The situation was well summarised in a recent World Health Organization Report (World Health Organization, 1984) :

> "Often the information collected is excessive and irrelevant for the diagnosis and appropriate therapy and in other instances insufficient, and furthermore may differ from patient to patient even within the same centre. The lack of uniformity, the extent of the investigation of the

couple, the selection of the infertile patient for treatment, the choice of therapy, the treatment scheme, the monitoring and the follow-up seldom allows a meaningful comparison of data reported from different centres."

In an attempt to circumvent some of these problems, the World Health Organization promulgated a scheme for the investigation of the infertile couple (World Health Organization, 1984) which, although able to be criticised in many respects, has resulted in the generation of interesting data concerning the present state of knowledge, or more often lack of knowledge, concerning the aetiology of infertility (Cates et al, 1985; Diczfaluzy, 1986). This survey involved data collection from 33 centres in 25 countries over the period 1979 -1984, during which time over 8500 couples were enrolled, of whom over 5600 (69%) completed investigation to the point of a diagnosis being made in both partners. Couples were admitted to the study if they had been infertile for at least one year, and were subjected to a diagnostic protocol involving a detailed history and clinical examination of both partners, together with a number of laboratory tests, including at least two semen analyses, and in most cases requiring diagnostic laparoscopy and endocrine investigations.

The diagnoses arrived at in the female partners of the couples studied are listed in Table 1.2 (p 40), the commonest diagnosis being that of tubal disease, occurring in 40.6% of cases, followed by endocrine disorders (including failure of ovulation), which were found in 37.6% of cases. All of the other defined causes (including endometriosis, ovarian, uterine and cervical abnormalities, sexual dysfunction and congenital, systemic and iatrogenic causes of infertility) had very low incidences (less than 6.0% each), and in 35.1% of cases there was no demonstrable cause for infertility

in the female partner within the limits of the investigational protocol. The superiority of diagnostic laparoscopy over hysterosalpingography in the diagnosis of female infertility was clearly shown in this study (Table 1.3, p 41) in so far as hysterosalpingography detected tubal pathology in 26.5% of cases compared to 65.1% in the case of diagnostic laparoscopy and this finding has been supported by a more recently published study (World Health Organization,1986). The diagnoses arrived at in the assessment of the male partners in the same study are listed in Table 1.4 (p 42). The commonest "diagnostic" category was that of "no demonstrable cause", which was reached in 42.8% of cases. By implication therefore, a diagnosis was reached in 57.2% of cases, however, unlike their female counterparts, there were no single large diagnostic categories amongst the male partners, but rather a large number of diagnostic categories with relatively few cases in each. Varicocele was the largest single group, comprising 12.6% of cases, followed by primary idiopathic testicular failure (11.2%) and accessory gland infection (6.9%), all other causes (including other abnormalities of semen analysis, immunological problems, congenital abnormalities, sexual dysfunction, systemic, iatrogenic and endocrine causes) each accounting for less than 6.0% of cases. Multiple pathology was found in only 4.3% of male cases, in contrast to 29.2% of cases in the female group.

Cates and colleagues (Cates et al, 1985), working from a portion of the same database subdivided the couples studied by region, which revealed interesting differences in the patterns of causation of infertility between the developed countries and the various developing regions studied. This study then allows us to consider the aetiology of infertility in the developed countries in isolation, in contrast to that cited above, and, as can be seen

from Table 1.5 (p 43), within this region a male cause alone was found in 22% of couples and a female cause alone in 31% of couples. Causes were found in both partners in 21% of couples and no cause was found in either partner of 14% of couples. From this data it follows that a male problem was identified in at least 43% and a female problem in 52% of couples in the developed countries. As before, the commonest female diagnoses were tubal disease (36%) and endocrine problems (33%), however, in this survey, there was no demonstrable cause for infertility in 40% of women studied in the developed countries. Amongst the male group, again, no demonstrable cause was identified in 49% of cases, with varicocele accounting for 11%, primary idiopathic testicular failure for 10% and accessory gland infection for only 7% (Table 1.6, p 44). As a result of this experience, the World Health Organization has recently modified its recommended protocol for the investigation of infertility (World Health Organization, 1986) and has concomitantly reorganized the possible diagnoses which can be reached under this scheme (Tables 1.7 and 1.8, pp 45-46)(Comhaire et al, 1987). It can be seen that many of the male diagnoses do not have a proven causative relationship to infertility, in contrast to the majority of female diagnoses (Table 1.9, p 47).

Within the UK, there have been a number of surveys of the aetiology of infertility (Templeton and Peney, 1982; West et al, 1982), the most recent being that by Hull and colleagues (Hull et al, 1985; 1986), although of course this study did not employ exactly the same investigational protocol as those discussed above, and is therefore not directly comparable. This group identified ovulatory failure in 21% of couples and tubal damage in 14%. Sperm defects or dysfunction (defined by these workers as an abnormal semen analysis or failure of sperm to penetrate normal cervical

mucus) were identified in 24% of couples, while 28% of couples were found to have no demonstrable abnormalities and were therefore described as suffering from unexplained infertility (Table 1.10, p 48). Thus this group found that sperm dysfunction was the single commonest defined cause of infertility in the U K.

Three main points emerge from these studies of the aetiology of infertility. Firstly, a significant proportion of all infertility is due, either in whole or in part, to abnormalities within the male partner. The proportion of infertility apparently due to male reproductive dysfunction appears to lie between 26 and 53% within the developed countries, with 43% probably being the best available estimate (Cates et al, 1985). Secondly, the diagnoses commonly arrived at in the male partners of infertile couples are generally descriptive only and do not imply any understanding of the pathophysiology of the condition or of its actual causal relationship to infertility. Indeed in the case of many diagnoses, evidence supporting a causal link with infertility is notably lacking, perhaps the best example being that of varicocele, the commonest male diagnostic category in the studies cited above (Cates et al, 1985; Diczfaluzy, 1986), and in the case of which there is much debate concerning its relationship with infertility , its effects on sperm function, and the putative benefits of treatment (Comhaire, 1983; Cockett et al, 1984; Vermeulen and Vanderweghe, 1984; Comhaire and Kunnen, 1985). Many of the less common diagnostic categories are of a purely descriptive nature, although in some cases there does exist evidence linking the descriptive diagnosis concerned with infertility; (for example isolated teratozoospermia, which was found in 8.0% of cases in one study (Cates et al, 1985), good evidence of impaired sperm fertilizing ability has been provided (Shalgi et al, 1985)). Thirdly, a significant proportion of infertility, between 12 and 28%,

is commonly designated as "unexplained" when conventional diagnostic methods are used (Cates et al, 1985; Hull et al, 1985).

This difficulty in the accurate and meaningful diagnosis of male infertility is central to the dilemmas which confront clinical andrologists. It has been said that the three principles of treatment are firstly diagnosis, secondly diagnosis and thirdly diagnosis. In the field of male infertility, not only do we often fail to establish **what** is wrong with the patient, we often fail to establish **whether** or not there is anything wrong with the patient. It will be helpful at this point to consider briefly the conventional approach to the diagnosis of infertility, and to male infertility in particular, prior to considering in depth the problems encountered in attempting to diagnose male infertility and the possible solutions to these problems.

1.6 Investigation of Infertility

From ancient times, mankind has been concerned with the investigation of infertility (Aiman, 1984). The great civilizations, nurtured in the fertile valleys of the rivers Nile in Egypt, Tigris and Euphrates in Messopotamia and Indus in Northern India were all concerned with the evaluation of fertility :

> "To distinguish her who shall conceive from her who shall not conceive, pour thou fresh oil.....examine her."
>
> Kahun Papyrus
> Circa 2000 B.C.

> "To know a woman who will bear from a woman who will not bear : Watermelon pounded and bottled with the milk of a woman who has borne a male child; make it into a dose. To be swallowed by the

woman. If she vomits, she will bear. If she belches, she will never bear.

Babylon
Circa 1200 B.C.

"If a woman does not conceive, and wished to ascertain whether she can conceive, having wrapped her in blankets, fumigate below with oil of roses, and if it appear in the nostrils and mouth, know that of herself she is not unfruitful."

Hippocrates
460-377 B.C.

The realistic evaluation of the infertile couple did not begin to become a practical possibility until the explosion of knowledge in reproductive biology which took place in the mid-seventeenth century, with the publication of William Harvey's **"De Generatione Animalium"** in 1651, Regner de Graaf's description of the ovarian follicle in 1672, and Anthony van Leeuwenhoek's description of spermatozoa in 1677 in his **"de Natis e semini genitali Animalculis"** (Van Leeuwenhoek, 1678). From these beginnings evolved the contemporary approach to the evaluation of the infertile couple and the outline of this which follows is based upon the schemes promulgated by the World Health Organization (World Health Organization, 1984,1986).

The Evaluation of the Female Partner

The assessment of each partner is normally considered in terms of the history, physical examination and investigations performed.

History : Within the history, it is usual to note both the duration of marriage or cohabitation as well as the duration of infertility complained of, and

whether this is primary or secondary. The previous reproductive history of the patient is particularly important, including full details of previous pregnancies and their outcome (whether live-births, spontaneous or induced abortions, stillbirths, molar or ectopic pregnancies), and whether these were to the current or to a previous sexual partner. Any post-partum or post abortion complications are of relevance, and the patient's contraceptive history is of interest, particularly in respect of previous use of the intrauterine contraceptive device or of hormonal contraception. Amongst aspects of the patient's medical history which should be noted should be included a history of systemic disease, including tuberculosis, diabetes or thyroid disease, any history of pelvic inflammatory disease together with its outcome, and any past history of sexually transmitted disease. A history of medical treatment, of excessive consumption of alcohol, tobacco or drugs, or of exposure to occupational or environmental toxins should be noted. Any history of previous abdominal surgery is of interest, including appendicectomy or other pelvic surgery, and particularly any history of tubal, ovarian or uterine surgery. The menstrual history is of course of prime importance, including details of the age at menarche, whether primary or secondary amenorrhoea exists, and if not then the frequency and regularity of menses at present should be clearly established. The presence of dysmenorrhoea, intermenstrual bleeding or post-coital bleeding should be assessed and any abnormal vaginal discharge noted, as should abnormal discharge from the nipple. The patient's sexual history should be taken, covering the frequency and timing of intercourse, the use of lubricants, the degree of penetration and the presence of dyspareunia.

Examination : The examination of the female partner should include assessment of the height, weight and blood pressure and the general

physical examination should cover the thyroid gland, and the major systems. Hair distribution should be assessed, and if thought to be abnormal should be objectively quantified using the scoring system devised by Ferriman and Gallwey (Ferriman and Gallwey, 1961). The development of the breasts should be examined and the presence of galactorrhoea sought. The reproductive system should of course be examined in detail. The vulva should be inspected, the size of the clitoris noted and the state of the hymen, vagina and cervix noted, with particular reference to evidence of the presence of infection. The cervix, uterus and adnexae should be evaluated by bimanual palpation.

Investigations : Investigations performed upon the female partner should include haemoglobin estimation and urinalysis. Circulating levels of prolactin and possibly of follicle stimulating hormone (FSH) , luteinising hormone (LH) , oestradiol and thyroxine should be measured, as should progesterone in the mid-luteal phase of at least two menstrual cycles, as an index of the patient's ovulatory status. In certain cases, karyotyping, radiology of the pituitary fossa and perimetry of the visual fields may be indicated. The state of the pelvis should be assessed by diagnostic laparoscopy (Templeton and Kerr, 1977), which will allow the evaluation of tubal disease and patency, the presence of adhesions or of endometriosis. The configuration of the uterine cavity should be assessed by hysterosalpingography, and a biopsy of endometrium taken for histological examination and for culture for evidence of infection including tuberculosis.

The above constitutes the basic investigations of the female partner and will allow the establishment of a range of common diagnoses (Table 1.9, p 47), most of which have a well documented pathophysiology and relationship

with infertility, such as tubal damage and occlusion, endometriosis and failure of ovulation. This is in striking contrast to the situation in the male, although the investigation of the male partner follows the same lines of history, examination and investigations.

The Evaluation of the Male Partner

History : As with the female partner, the duration of the present union and the duration of infertility complained of should be established at the outset, and again, a family history of endocrine disease and of tuberculosis (Wang and Jones, 1983) should be sought. In the patient's past medical history, areas which should receive special attention are a history of mumps virus infection, the age at which this occurred and whether or not there was an associated orchitis (Werner, 1950a; b). A history of respiratory disease should be carefully sought, including recurrent respiratory tract infections, sinusitis, bronchiectasis or cystic fibrosis, as these conditions can be associated with ciliary dysfunction, and therefore with impaired sperm motility, as in Kartagener's syndrome (Kartagener, 1933; Afzelius et al, 1975; Eliasson et al, 1977), or with obstructive azoospermia in Young's syndrome. A history of diabetes, uraemia or of other systemic illness should be sought (Green and Kelalis, 1968; Chopp and Mendez, 1978; Wang and Jones, 1983), as should any history of recent pyrexial illness as this may compromise spermatogenesis for many weeks (Heller et al, 1964). Parasitic diseases, such as schistosomiasis and filiariasis are rare but must be borne in mind as potential causes of excurrent duct obstruction and prostatovesiculitis (Plorde, 1977).

Any symptomatology related to the urinary tract, such as dysuria, urethral discharge, frequency or haematuria is of self-evident importance (Mobley,

1975; Fowler and Kessler, 1983; Lunenfeld, 1986). Likewise, aspects of the specific reproductive history are important, including any history of testicular maldescent (Lipshultz, 1976; Lipshultz et al, 1976), injury (Frey and Rajfer, 1984), torsion (Bartsch et al, 1980; Ransler and Allen, 1982) or epididymo-orchitis (Werner, 1950a; b; Morley et al, 1977), and any history of surgery which may have compromised the genital tract, such as herniorrhaphy, orchidopexy, drainage of hydrocele, ligation of a varicocele or bladder neck surgery (Smith, 1969; Lunenfeld, 1986). Other specific conditions may impair reproductive performance, and a group of patients now requiring infertility investigations are those who have survived treatment for testicular or lymphatic malignancy, and who may suffer the consequences of chemotherapy, radiotherapy or retroperitoneal lymph node dissection (MacLeod et al, 1964; Orecklin et al, 1973). A history of drug ingestion, including sulphasalazine (Levi et al, 1979; Gwercman and Skakkebæk, 1986), cimetidine (Van Thiel et al, 1979) or nitrofurantoin (Nelson and Steinberger, 1952), or of exposure to other toxins, including alcohol and tobacco, known to impair spermatogenesis (Van Thiel et al, 1975; Stillman, 1986) is important, as is the occupational history in terms of exposure to toxic chemicals and hyperthermia (Lipshultz et al, 1980). The sexual history should endeavour to cover the adequacy of erectile function and if there is doubt, the presence of early morning and masturbatory erections should be enquired into in order to differentiate organic from psychogenic impotence (Bancroft and Wu, 1985). The occurrence of intravaginal ejaculation should be established (Green and Kelalis, 1968), and again if there is doubt, the occurrence of nocturnal or masturbatory ejaculation sought, in addition to which the characteristics of ejaculation such as associated pain, prematurity or delay should be established. A substantial number of couples attending infertility clinics will be infertile as a consequence of sexual dysfunction

(Dubin and Amelar, 1972), and a number of couples with sexual dysfunction will present to infertility clinics seeking primary help. Of course, any history of sexually transmitted disease and its outcome is of note (Lipshultz et al, 1983), as is a history of drug abuse or of other factors exposing the patient to high risk of infection with human immunodefficiency virus (HIV), due to the problems which this poses for the couple in terms of the risks of transmission and pregnancy, and in terms of the problems presented to laboratory staff handling blood and semen samples (Stewart et al, 1985).

Examination : The examination of the male partner should include a general medical examination covering height and weight, blood pressure and all of the major systems. The secondary sexual development of the patient must be assessed, and signs of hypogonadism sought (Klinefelter et al, 1942; Paulsen et al, 1968), including examination of the visual fields, to assess pituitary enlargement, and examination of the sense of smell to exclude Kallmann's syndrome (Kallman, 1944). Gynaecomastia should be specifically examined for. Turning to the urogenital examination, the penis is examined for evidence of phimosis, hypo- or epi-spadias or the characteristic plaques of Peyronie's disease (Smith, 1966). The scrotum should be examined and the site of the testes determined, following which their volume in milliliters should be determined with the aid of a Prader orchidometer, and their consistency evaluated (Lipshultz and Howards, 1983; Lunenfeld, 1986). Any tenderness of the gonads should be noted and the epididymides carefully palpated from caput to cauda, to exclude thickening, tenderness or cystic lesions, or the atrophy or absence of the epididymides (Kaplan et al, 1969; Comhaire et al, 1980). The vasa deferentia should next be palpated to establish their presence, and any thickening or induration noted. Scrotal swellings, such as hydrocele or

hernia should be noted and the presence and grade of varicocele established by asking the patient to perform Valsalva's manoeuvre (Comhaire, 1983). The inguinal regions should be inspected for hernia, scarring or the presence of lymphadenopathy. A rectal examination should be performed to assess the state of the prostate and seminal vesicles (Mobley, 1975; Comhaire et al, 1980; Fowler and Kessler, 1983; Suomonen et al, 1983).

Investigation : The laboratory investigation of the male partner should include measurement of haemoglobin and white cell counts, together with routine urinalysis. The circulating levels of LH, FSH, testosterone and prolactin may be measured (Segal et al, 1976; Lunenfeld, 1986). The single most important investigation is the semen analysis, a minimum of two being undertaken, to take account of the considerable inter-ejaculate variability which is known to occur (World Health Organization, 1980; 1987). In its most elementary form, semen analysis should include evaluation of the volume, appearance and viscosity of the sample, followed by an assessment of the sperm concentration and of the proportions of motile and morphologically normal spermatozoa. The presence of agglutinated spermatozoa or of immature germinal cells or leucocytes is of note. Other investigations which may be required of the male partner under particular circumstances include radiological examination of the pituitary fossa, examination of the visual fields and pituitary function studies in patients suspected of having pituitary disease. Examination of the karyotype, of a testicular biopsy, or of the presence of fructose, citrate or acid phosphatsase in the seminal fluid may be required in cases of azoospermia. Bacteriological or virological examination of seminal fluid, prostatic fluid or

urethral swabs may be indicated (Mobley, 1975) as may screening of serum or seminal plasma for the presence of antisperm antibodies.

The above constitutes an outline of the basic clinical and laboratory examination of the potentially infertile male. It will allow the detection of a number of relatively uncommon disorders known to cause infertility in the male and of a much larger number of disorders whose relationship to infertility is speculative and whose pathophysiology remains obscure. A list of the diagnostic classifications which can be arrived at under the original version of the Word Health Organization protocol (and upon which the studies of Cates et al, (1985) and Diczfaluzy (1986) were based) is given in Table 1.7 (p 45), from which it can be seen that many of the possible diagnoses, although rare, are undoubtedly causally related to infertility, such as the immotile cilia syndrome, sexual dysfunction, retrograde ejaculation, gonadotrophin deficiency and ductal obstruction. However, many possible diagnoses, including varicocele, immunologic infertility or male accessory gland infection, while being associated with infertility are not necessarily causally related in any given case. The diagnosis of "primary idiopathic testicular failure", a major category in numeric terms (Cates et al, 1985; Diczfaluzy, 1986), is no more than a descriptive term for patients with abnormalities detected in semen analysis for which no cause or putative cause can be found. The diagnostic categories possible under the terms of the WHO protocol have recently been revised to take account of such criticisms (World Health Organization, 1986; Comhaire et al, 1987) and a list of these is given in Table 1.8 (p 46). A number of categories whose relationship to infertility is uncertain remain, but the relationship between an abnormal semen analysis and infertility is no longer taken for granted.

1.7 Conclusions

It can be seen therefore, that infertility is a common problem, one which has concerned mankind for centuries, and one which may now affect one couple in six in this country, and as many as 50 - 80 million couples world-wide. It is a problem which causes great distress to those affected, that is those couples who fail to conceive despite 12 months of unprotected sexual intercourse. Our knowledge of the aetiology of infertility is imperfect, but it is clear that substantial reproductive dysfunction can occur in both men and women. Whilst our understanding of female reproductive function and dysfunction, and our ability to evaluate and to subsequently intervene and influence female reproductive dysfunction, is comparatively advanced, our understanding of male reproductive function and dysfunction is very poor, and therefore our ability to evaluate disorders of male reproductive function and to subsequently intervene with benefit is very limited.

Unlike the situation in the female, the male role in reproduction is largely limited to the production and delivery of gametes, and the success of the male's contribution to the initiation of a pregnancy depends upon the quality of the gametes which he produces. These gametes are, by their nature, directly available for assessment, and for this reason, the analysis of semen has assumed great importance in the evaluation of the infertile couple. The subject of semen analysis will be discussed at length later in this thesis. It is clear that the conventional history and examination of the infertile male identifies only a small number of comparatively rare causes of infertility and it will become clear that the conventional semen analysis is greatly flawed as a tool for evaluating the ability of an individual to achieve fertilization *in-vivo*. There is therefore a great need to develop appropriate investigations

which will permit the accurate evaluation of the *in-vivo* fertilizing ability of an individual, that is, investigations which will permit the evaluation of the functional competence of the gametes produced by an individual. Given that it becomes possible to accurately identify spermatozoa whose functional competence is impaired, it is then necessary to understand why such spermatozoa are defective, in so far as this understanding will be the logical prelude to therapeutic strategies aimed at correcting spermatozoal dysfunction.

The studies outlined in this thesis will aim therefore to describe and evaluate techniques for the accurate evaluation of spermatozoal function, and to investigate the mechanisms regulating spermatozoal function at the level of the cell biology of the spermatozoon. The conventional criteria of semen quality will first be examined in detail, following which newer tests sperm function, including semen ATP concentration, zona-free hamster oöcyte penetration testing and sperm movement characteristics will be discussed and their clinical usefulness evaluated. Lastly, the effects of reagents used to optimise these diagnostic assays upon cellular physiology, in particular upon intracellular calcium and pH will be examined.

Table 1.1

Patterns of Infertility : Ages of Couples, Type and Duration of Infertility

(Percentages of Couples) (Data From : Cates et al, 1985)

Characteristic	Developed	Developing			
		Africa	Asia	Latin America	Eastern Mediterranean
(n =	3902	842	1192	1228	432)
Age of Woman (yrs)					
<24	25	42	22	24	24
25-34	68	52	70	65	69
>35	7	6	8	11	7
Age of Man (yrs)					
<24	10	5	4	11	2
25-34	72	60	70	62	53
>35	18	35	26	27	45
Type of Infertility					
Primary	71	48	77	60	84
Secondary	29	52	23	40	16
Duration of Infertility					
<1.5	17	7	11	11	9
1.5-2.5	29	23	23	24	15
2.5-4.5	29	33	31	29	22
4.5-8.0	18	21	22	24	26
>8.0	7	16	13	12	28

Table 1.2

Aetiology of Female Infertility : WHO Study

Diagnoses reached in 5857 women of 8546 couples studied.

(Data From : Diczfaluzy, 1986)

Diagnosis	n	Percentage	
		Diagnoses	Cases
Tubal Abnormalities	2380	31.4	40.6
Endocrine Causes	2205	29.1	37.6
No Demonstrable Cause*	2058	27.2	35.1
Endometriosis	343	4.5	5.9
Acquired Ovarian, Uterine or Cervical Abnormalities	285	3.8	4.9
Sexual Dysfunction	103	1.4	1.8
Congenital Abnormalities	85	1.1	1.5
Systemic Causes	50	0.7	0.9
Iatrogenic Causes	34	0.4	0.6
Endometrial Tuberculosis	27	0.4	0.5
	7570	100.0	129.2

* Includes women who did not undergo laparoscopy

Table 1.3

Aetiology of Female Infertility : WHO Study

Diagnoses reached with hysterosalpingography and diagnostic laparoscopy in 5636 women.

(Data From : Diczfaluzy, 1986)

Diagnosis	Hysterosalpingography		Laparoscopy	
	n	%	n	%
No demonstrable cause	1457	43.6	589	25.6
Tubal abnormalities	884	26.5	1495	65.1
Endometriosis	2	0.1	341	14.8
Acquired uterine, ovarian or cervical abnormalities	134	4.0	141	6.1

Table 1.4

Aetiology of Male Infertility : WHO Study

Diagnoses reached in 6407 men of 8546 couples studied.

(Data From : Diczfaluzy, 1986)

Diagnosis	n	Percentage	
		Diagnoses	Cases
No demonstrable cause	3127	46.8	42.8
Varicocele	806	12.1	12.6
Primary idiopathic testicular failure	717	10.7	11.2
Accessory gland infection	441	6.6	6.9
Abnormal sperm morphology	376	5.6	5.9
Other semen abnormality	273	4.1	4.3
Low sperm motility	252	3.8	3.9
Suspected immunological factors	193	2.9	3.0
Congenital abnormalities	138	2.1	2.2
Sexual dysfunction	127	1.9	2.0
Systemic or iatrogenic causes	127	1.6	2.0
Obstructive azoospermia	64	1.0	1.0
Endocrine causes	41	0.6	0.6
	6682	100.0	104.3

Table 1.5

Aetiology of Infertility by Region: General Diagnostic Categories (Percentages of Couples) (Data From : Cates et al, 1985)

Category	Developed	Developing			
		Africa	Asia	Latin America	Eastern Mediterranean
Became pregnant	12	15	16	13	15
No cause found in either	14	5	13	10	3
Female cause only	31	37	34	25	25
Male cause only	22	8	13	22	19
Causes found in both	21	35	24	30	38

Table 1.6

Aetiology of Infertility by Region: Specific Diagnostic Categories
(Percentages of Couples) (Data From : Cates et al, 1985)

Diagnosis*	Developed	Developing			
		Africa	Asia	Latin America	Eastern Mediterranean
Female Diagnosis					
No demonstrable cause	40	16	31	35	26
Bilateral tubal occlusion	11	49	14	15	20
Pelvic adhesions	13	24	13	17	13
Acquired tubal abnormality	12	12	12	12	9
Anovular regular cycles	10	14	9	9	15
Anovular oligomenorrhoea	9	3	7	9	11
Ovular oligomenorrhoea	7	4	11	5	8
Hyperprolactinaemia	7	1	10	3	1
Endometriosis	6	1	10	3	1
Male Diagnosis					
No demonstrable cause	49	46	58	41	28
Varicocele	11	20	10	19	12
Primary idiopathic testicular failure	10	7	1	13	25
Accessory gland infection	9	11	3	12	3
Abnormal morphology	8	5	3	4	3
Low sperm motility	3	1	5	8	5

* Not all categories listed

* Some patients had more than one diagnosis made

Table 1.7

Evaluation of the male partner of an infertile couple.

Diagnoses achievable under the original WHO investigational protocol.

Normal
Sexual dysfunction
Iatrogenic causes
Systemic causes
Congenital causes
Varicocele
Ejaculatory disturbance
Retrograde ejaculation
Abnormal morphology
Immotile cilia syndrome
Immunological infertility
Male accessory gland infection
Pituitary lesion
Gonadotrophin deficiency
Primary idiopathic testicular failure
Kleinefelter's syndrome
Partial ductal obstruction
Ductal obstruction

Table 1.8

Evaluation of the male partner of an infertile couple.

Diagnoses achievable under the modified WHO investigational protocol.

Sexual and / or ejaculatory dysfunction
No demonstrable cause
Isolated seminal fluid abnormalities
Iatrogenic causes
Systemic causes
Congenital abnormalities
Acquired testicular damage
Varicocele
Male accessory gland infection
Immunological causes
Endocrine causes
Idiopathic infertility with oligozoospermia
Idiopathic infertility with asthenozoospermia
Idiopathic infertility with teratozoospermia
Obstructive azoospermia
Idiopathic infertility with azoospermia

Table 1.9

Evaluation of the female partner of an infertile couple.

Diagnoses achievable under the modified WHO investigational protocol.

Sexual dysfunction
Hyperprolactinaemia
Organic lesions of the hypothalamo-pituitary region
Amenorhoea with elevated FSH
Amenorrhoea with adequate endogenous oestrogen
Amenorrhoea with low endogenous oestrogen
Oligomenorrhoea
Irregular menses or ovulation
Anovulation with regular cycles
Congenital abnormalities
Bilateral tubal occlusion
Pelvic adhesions
Endometriosis
Acquired uterine or cervical lesions
Acquired tubal lesions
Acquired ovarian lesions
Endometrial tuberculosis
Diagnosis unknown (no laparoscopy)
Iatrogenic causes
Systemic causes
Abnormal post-coital test
No demonstrable cause

Table 1.10

Annual causes of infertility as initially classified by presenting features in new couples.
(Data From Hull et al, 1985)

Cause of Infertility	% of Couples (n=472)
Ovulatory failure	21
Tubal damage	14
Endometriosis	6
Mucus defect / dysfunction	3
Sperm defect / dysfunction *	24
Other male infertility	2
Coital failure / suspected coital failure	6
Unexplained infertility	28
Others	11
All male diagnoses	**26**

13% of couples have two or more causes of infertility

* Oligospermia treated as normal if mucus penetration normal

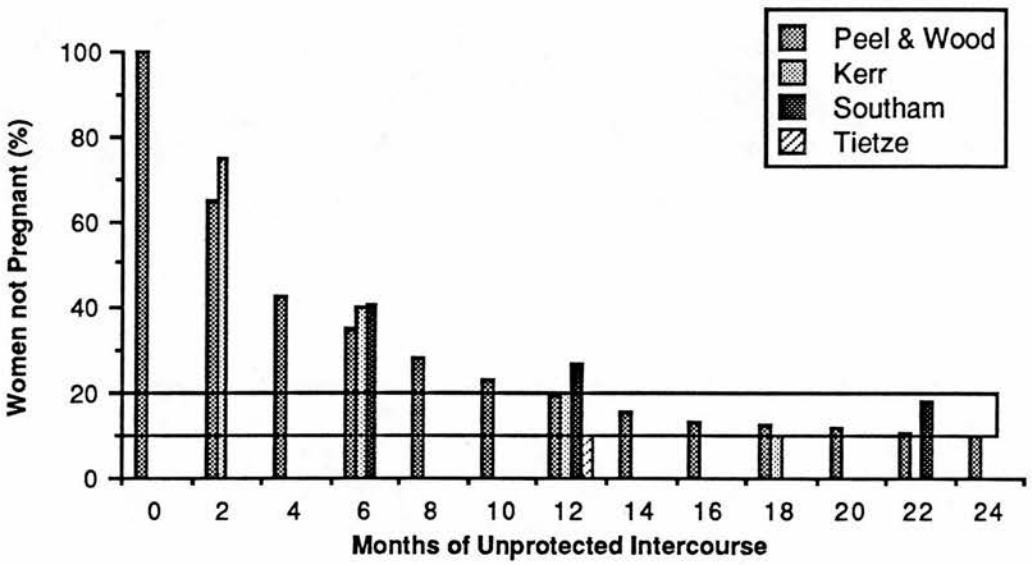


Figure 1.1
Natural Fertility

Women remaining non pregnant after varying periods of marriage. (Data from : Peel and Wood, 1972; Kerr and Parboosingh, 1974; Southam, 1960; Tietze, 1968)

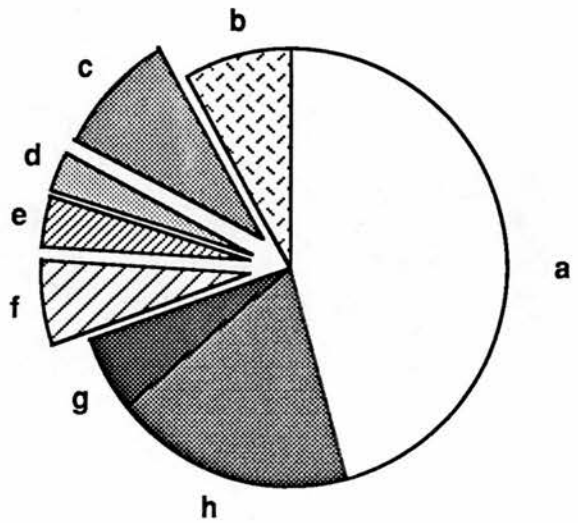


Figure 1.2

The population of couples of reproductive age.

a = Fertile couples
b = Fertile, self perceived as infertile
c = Primary Infertility, Requests care
d = Secondary Infertility, Requests care
e = Primary Infertility, No health services contact
f = Secondary Infertility, No health services contact
g = Voluntary Infertility
h = Unknown Fertility

Demographically based studies of the prevalence of infertility will include only groups C, E, G, and H. Health Service based data will include only groups C and D.
(Data from : Belsey and Ware, 1986)

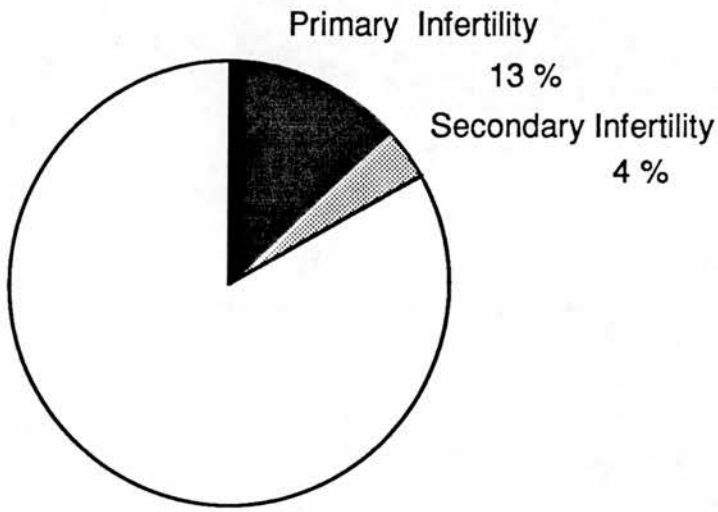


Figure 1.3
Prevalence of Infertility

Prevalence of primary and secondary infertility in the UK.
(Data from : Hull et al, 1985)

CHAPTER TWO

SEMEN QUALITY IN NORMAL MEN

"The enumeration of spermatozoids has seldom been practiced. How useful it may prove either as an index of sexual or general health I do not know."

Benedict, 1910

2.1 History of Semen Analysis

The story of semen analysis begins in the town of Delft in Holland in 1632. This was the birthplace of Anthoni van Leeuwenhoek, probably the first person to observe spermatozoa in the human ejaculate with the aid of his rudimentary microscope. Leeuwenhoek reported his first observations of motile spermatozoa in his dramatic letter to The Royal Society of November 1677, **"de Natis e semini genitali Animalculis"** (Van Leeuwenhoek, 1678), and, in a further letter dated 1685 he went on to speculate that the existence of spermatozoa, or 'animalcules' as he called them, in semen was associated with its fertilizing ability, and moreover that the absence of fertilizing ability could be due either to the absence of spermatozoa, or to their having reduced function :

"Now when a man is unable to beget children by his wife, although his virility is unimpaired, he is said in common parlance to have a cold nature.To my mind, however, it would be more apt to say that no living animalcules will be found in the seed of such a man, or that, should any living animalcules be found in it, they are too weakly to survive long enough in the womb."

(Van Leeuwenhoek, 1685)

As will become apparent, contemporary andrology has been slow to realise the implications of this observation for the diagnosis of male infertility.

Charles Bonnet, writing almost one hundred years later was less certain as to the role of spermatozoa in semen :

> "They are, of all animalculi of liquids, those which have most excited my curiosity : the element in which they live, the place of their abode, their figure, motion, their secret properties; all, in a word, should interest us in so singular a kind of minute animate beings.
> Why do they appear only at the age of puberty; where did they exist before this period ? Do they serve no purpose but to people the fluid where they are so largely scattered ? How far are we from being able to answer any of these questions ? And how probable it is that future age will be as ignorant of the whole as our own !"
>
> (Bonnet, 1771)

The first quantitative studies on seminal fluid were probably performed by Lazzaro Spallanzani in 1780 (Zorgniotti, 1975), who found that the seminal fluid of amphibia fertilized ova even in extreme dilutions, and the later work of Prévost, Dumas and Hertwig began to elucidate the mechanism of fertilization, establishing that one sperm was necessary to fertilize each egg (Zorgniotti, 1975). Little further progress was made in the quantitative study of seminal fluid until the mid-nineteenth century when clinical attention began to turn to the assessment of vaginal fluids. Around 1844, the French microscopist Donné, whilst studying the mucosa of the vagina and uterus, found spermatozoa in the vaginal mucus of a patient, at the Hôpital de Lourcine, who had been admitted the previous day (Donné, 1844). The postcoital evaluation of vaginal and cervical fluids in a clinical context was first undertaken by a gynaecologist, J Marion Sims, who was interested in the simple presence of motile spermatozoa rather than their number (Sims, 1866), although the technique was further developed by Max Hühner, an

American urologist, who began to evaluate the number as well as the motility of spermatozoa within the vagina (Hühner, 1913).

The direct quantitative study of the human ejaculate did not begin until the early part of the present century. Benedict ,working in New York, published a brief report on the counting of spermatozoa in semen using a blood counting chamber as early as 1902 (Benedict, 1910), with the following comment :

> "The enumeration of spermatozoids has seldom been practiced. How useful it may prove either as an index of sexual or general health I do not know."

Only in 1929 was the quantitation of spermatozoa in human semen placed on a scientific footing with the publication of "The Spermatozoa Count." by Macomber and Sanders (1929) in which they described the (now established) technique of diluting semen with a solution of bicarbonate and formalin prior to counting the spermatozoa present using a 'blood counting chamber'. They began to evaluate the range of sperm concentrations in semen associated with fertility, and concluded that, while pregnancy could occur with sperm concentrations below 60 x 10^6/ml of ejaculate, conception was more likely with sperm concentrations higher than this. Their conclusions held sway until the 1950's when John MacLeod, an Edinburgh graduate, began his extensive studies of semen quality in a wide variety of groups of fertile and potentially infertile men (Macleod and Gold, 1951; 1956) from which he concluded that sperm concentrations in excess of 20 x 10^6/ml were associated with fertility, which figure holds to the present day. Since this time a vast literature has accumulated on the subject of semen analysis, the techniques involved in its undertaking (World Health

Organization, 1980, 1987; Lipshultz and Howards, 1983; Sokol and Swerdloff, 1984; Glezerman and Bartoov, 1986), the values observed in normal populations (MacLeod and Gold, 1951; 1956; Ulstein 1973; Schwartz et al, 1979; Hudson et al 1980b; Åbyholm, 1981; Jouannet et al, 1981; Leto and Frensilli, 1981; Aitken et al, 1982a; Thorneycroft et al, 1984; Wang et al, 1985; Poland et al, 1986), and in various groups of potentially infertile men (Van Zyl et al, 1975; Smith et al, 1977; Cockett et al, 1979; Glass and Ericsson, 1979; David et al, 1980; Bostofte et al, 1981; Fariss et al, 1981; Aitken et al, 1982b; c)

2.2 Methodology of Semen Analysis

Much has recently been written on the subject of the methodology of semen analysis (World Health Organization, 1980; 1987; Lipshultz and Howards, 1983; Sokol and Swerdloff, 1984; Glezerman and Bartoov, 1986) which methodology has, until recently, varied greatly from one institution to another. The methodology promulgated by the World Health Organization represents the most recent attempt to standardize the laboratory procedures involved in the performance of semen analysis (World Health Organization, 1987) and this will now be outlined in detail, since it is of some importance to the arguments which follow. According to this methodology, the parameters which should be measured are :

Appearance
Volume
Consistency
pH
Motility
Sperm concentration

Cellular elements other than spermatozoa
Agglutination
Viability
Normal morphology

The most important parameters measured are those of sperm concentration, motility and morphology. In addition, semen culture and biochemical analysis of seminal plasma and spermatozoa may be performed.

Sample Collection and Delivery :

The sample should be collected after a minimum of 48 hours and not longer than 7 days sexual abstinence. Two semen samples should be collected for initial evaluation, not less than 7 days or more than 3 months apart. This is in order to take account of the effects of inter-ejaculate variability and length of abstinence that are known to occur (Schwartz et al, 1979; Poland et al, 1986). The sample should be collected by masturbation into a clean wide mouthed glass or plastic container, and delivered to the laboratory within one hour of collection, if it is not possible for the patient to produce the sample at the laboratory.

Initial Evaluation :

Appearance : The sample should be evaluated by inspection and should normally be grey-opalescent, homogenous and should liquefy within 60 minutes. Abnormalities of appearance or failure of liquefaction should be noted.

Volume : This should be measured by aspirating the sample into a graduated syringe or pipette, or by using an appropriate measuring cylinder.

Consistency : The "viscosity" of the liquefied sample should be evaluated by expelling the semen gently through a 21G needle, or by introducing and

withdrawing a glass rod. A normal sample should fall as discrete drops, not as threads of greater than 2cm length.

pH : This is measured using pH paper, and should be in the range 7.2-7.8.

Microscopic Evaluation :

Motility : A fixed volume of 10-15µl of semen is placed on a clean glass slide and covered with a cover slip of 20 x 20 to 24 x 24mm square. This is then examined at x400 - x600, preferably using phase contrast optics. One hundred spermatozoa are examined and their motility assessed, either as (a) motile and (b) immotile, or as (1) rapid and linearly progressive motility, (2) slower and sluggish linear or non-linear movement, (3) non-progressive motility , and (4) immotile. In either case, the percentage of spermatozoa in each category is scored.

Estimation of Sperm Concentration : This can be done approximately at this stage by examining several fields under the x40 objective, the number of spermatozoa per field being roughly equal to their concentration in millions per milliliter (10^6/ml).

Cellular Elements other than Spermatozoa : Epithelial cells, spermatogenic cells and white blood cells may be seen, their concentration in a normal sample being less than 1 x 10^6/ml. If more are present, a specific stain should be performed for peroxidase positive white blood cells.

Agglutination : The adherence of spermatozoa to each other, head-head, head-tail, tail-tail or in mixed fashion should be noted.

Sperm Viability : If the percentage of immotile spermatozoa exceeds 60%, the proportion of live spermatozoa may be determined by using supra-vital staining techniques, whereby dead cells with damaged plasma membranes take up stains such as eosin.

Sperm Concentration : This should be accurately determined by diluting a portion of the semen sample 1:20 with a diluent composed of $NaHCO_3$ and formalin to immobilize the spermatozoa, and then loading this diluted preparation into a standard haemocytometer (eg the Improved Neubauer), covering with a coverslip, and allowing to settle for 1-5 minutes. The number of spermatozoa is counted by standard techniques, and the result expressed as the concentration of spermatozoa (x 10^6/ml).

Morphology of Spermatozoa : A smear slide of semen is prepared using 10-15µl of semen, which is then dried and stained by the Giemsa, modified Papanicolau or Bryan-Leishman methods (for details see World Health Organization, 1987), and examined by light microscopy for the various morphological abnormalities which are described. The result is expressed as the percentage of spermatozoa which are morphologically normal.

Testing for Antibody Coating of Spermatozoa : This may be undertaken by the MAR-test or Immunobead method (Bronson et al, 1984).

In addition to the above, a number of additional procedures may be undertaken in the evaluation of human semen, including semen culture (Mobley, 1975), and biochemical evaluation for quantitation of acid phosphatases, citric acid and zinc (as markers of prostatic function) (Suominen et al, 1983), fructose and prostaglandins (as markers of seminal vesicular function) (Comhaire et al, 1980) and free l-carnitine (as a marker of epididymal function).

The World Health Organization has issued a statement of the commonly accepted normal values for the parameters discussed above (Table 2.1, p

75), although making the point that it is preferable for each laboratory to establish its own normal ranges for each variable, by evaluating semen from individuals of proven fertility.

2.3 Application of Semen Analysis to Fertile and Infertile Men

Having considered in some detail the methodology of the conventional semen analysis, one can consider the results obtained by its application to various defined groups of normal individuals and potentially infertile patients.

A number of andrological studies of men of proven fertility have been undertaken within this decade. Åbyholm (1981) studied 51 Norwegian men whose wives were pregnant, and who had conceived during the first 6 months of attempting to do so. This group had a mean age of 29.5 years (range 21-43 years), and submitted semen samples for analysis between 2 and 9 months (mean 5.1 months) after the first day of the wife's last menstrual period, and after 1 - 14 days (mean 4.5 days) of sexual abstinence. It was observed that the mean semen volume was 3.90ml (range 0.5-12.5, SD = 2.06), the mean sperm concentration was 89 x 10^6/ml (range 2-363, SD = 68), the mean percentage of motile spermatozoa was 53% (range 10-80, SD = 9), and the mean percentage of morphologically normal cells was 40% (range 3-84, SD = 18). The frequency distributions of sperm concentration and motility observed in this study are shown in Figure 2.1 (p 79), from which it can be seen that these are markedly skewed, and that a significant proportion of recently fertile men fall below the standards of "normality" set by WHO (World Health Organization, 1987) (Table 2.1, p 75).

A similar study was performed by Jouannet and co-workers in France at the same time as that cited above (Jouannet et al, 1981). This group studied 484 men of proven fertility, regardless of the interval that had elapsed since their previous conception, and produced data on the various parameters of semen analysis in 324 ejaculates produced after a period of abstinence of 5 days or less. They observed that the mean sperm concentration was 95.4 x 10^6/ml, (5th, 10th, 90th and 95th percentiles being 14, 25, 180, and 217 x 10^6/ml respectively), the mean percentage of motile cells was 71.8% (5th, 10th, 90th and 95th percentiles being 55, 59, 80, and 83% respectively), and the mean percentage of morphologically normal cells was 61.3% (5th, 10th, 90th and 95th percentiles being 44, 48, 75, and 76% respectively) (Jouannet et al, 1981). Again, these workers observed non-normal distributions, but with rather smaller numbers of men falling outwith the WHO criteria for normality.

Both of the studies of men of alleged proven fertility cited above were based upon European populations. Wang and colleagues have recently reported on a survey of semen parameters in 1239 normal Chinese men (Wang et al, 1985), of whom 1157 were of unknown fertility, and 82 were of proven fertility. They found that semen volume, sperm concentration, sperm motility and morphology were similar to those described for Caucasian subjects, with the mean semen volume for the entire group being 2.86ml (SD = 1.45, range = 0.5 - 9.0ml), the mean sperm concentration 83.0 x 10^6/ml (SD = 64, range = 1 - 250 x 10^6/ml), the mean sperm motility at 2 hours 56.5% (SD = 15, range = 2 - 87%), and the mean percentage of morphologically normal spermatozoa being 66.0% (SD = 10.2, range = 30 - 90%). For their subgroup of proven fertility, these mean figures were (±SD) semen volume,

2.71 (±1.68) ml, sperm concentration 71.0 (± 58) x 10^6/ml, sperm motility 56.8 (± 17.0)%, and normal morphology 62.6 (± 10.7)%.

Several other studies have recently been published concerning the semen quality of men of alleged proven fertility (Nelson and Bunge, 1974; Rehan et al, 1975; Smith and Steinberger, 1977; David et al, 1980; Fariss et al, 1981; Aitken et al, 1982a), and their principal findings are summarised in Table 2.2 (p 76). The average semen volume in these 10 studies ranged from 2.5-3.6ml, the average sperm concentration from 48-140 x 10^6/ml, the average percentage motility from 45.8-78.5%, and the average percentage normal morphology from 40.0-74.0%. From these various studies it can be seen that there is considerable variation between the several laboratories involved, emphasizing the importance of a given laboratory establishing its own range of normal values, by applying standard techniques in its own hands to the relevant local population. A number of the studies cited above have provided reasonably detailed data on the frequency distributions of sperm concentrations in the populations under study, and Figure 2.2 (p 80) represents data collated from 4 such studies (MacLeod and Gold, 1951; Nelson and Bunge, 1974; Rehan et al, 1975; Smith and Steinberger, 1977). From this figure, as from figure 2.1 (p 79), it can be seen that significant numbers of men of proven fertility have sperm concentrations below 20 x 10^6/ml, casting doubt on the value of a conventional semen analysis in assessing the fertility of an individual.

A number of studies are also available concerning the semen quality of groups of infertile men. MacLeod and Gold (1951) surveyed 1000 men of proven fertility, and compared the distribution of their sperm concentrations

with those of 1000 husbands of infertile marriages. Their results are summarised in Figure 2.3 (p 81) which shows that only 5% of their group of fertile men had sperm concentrations below 20 x 10^6/ml, in contrast to 16% of infertile husbands. Nevertheless, this means that 84% of husbands of infertile marriages in this study had normal concentrations of spermatozoa. A different approach was taken by Smith and colleagues (Smith et al, 1977), who looked at sperm concentrations in 189 couples presenting to an infertility clinic. Of their group of 189 couples, 140 had some form of treatment, and of these, 81 achieved pregnancies, and from the data given it is possible to look at the distribution of overall pregnancy rates with respect to sperm concentration (Figure 2.4, p 82). Although pregnancy rates were higher when sperm concentrations rose above 60 x 10^6/ml, couples with sperm concentrations below 20 x 10^6/ml achieved pregnancy rates of between 20 and 38%. A similar study was undertaken by van Zyl and co-workers (Van Zyl et al, 1975), who looked at 157 male partners of apparently normal females over a seven year period and found a pregnancy rate of 51.9% amongst men with sperm counts below 10 x 10^6/ml and 73.7% amongst men with sperm counts between 10 and 20 x 10^6/ml Whilst these pregnancy rates may seem unusually high for such a group of patients, the data nevertheless serves to emphasize the point that the semen analysis *per se* is no more capable of identifying infertility with accuracy than it is of identifying fertility with accuracy.

2.4 Semen Quality amongst Normal Men

As was stated above, it is important for each laboratory to establish its own standards of normality of semen quality, by the application of standard

techniques to its local population. In order to provide current data for the purposes of this thesis, a study was undertaken of semen quality amongst a group of semen donors in Edinburgh.

2.5 Materials and Methods

A group of 202 semen donors were recruited, principally from amongst male university students and from amongst the male partners of couples attending antenatal parentcraft classes in a large local maternity hospital, and whose female partners were currently pregnant. Of these 202 individuals, 55 were of recently proven fertility, and the remainder were of unknown or of unproven fertility. Over the three year period from January 1984 to January 1987, 1362 semen samples were collected and studied, of which 512 were from individuals of proven fertility.

In addition, 107 of these donors, including all 55 individuals of proven fertility, underwent a detailed clinical assessment, including a reproductive history and examination, and during which testicular volumes were examined and the presence of clinically apparent varicocele was sought for by examining the subject standing whilst he performed Valsalva's manoeuvre. Varicoceles were graded into Grade 0 = no clinically detectable varicocele; Grade 1 = dilation of the pampiniform plexus during valsalva's manoeuvre; Grade 2 = palpable dilation of the pampiniform plexus at rest; Grade 3 = visible and palpable dilation of the pampiniform plexus (Comhaire, 1983).

Semen analyses were performed according to the general techniques described by the World Health Organization (World Health Organization, 1980; 1987) and described above. Semen samples were produced by masturbation following two or more days of sexual abstinence, and were collected in clean wide-mouthed plastic containers (Sterilin Limited, Feltham, England), which had previously been tested and shown to be non-toxic to spermatozoa. Samples were analysed within 2 hours of production and were kept in an incubator at 37°C during analysis, as was all equipment used in their analysis. Completeness of liquefaction and viscosity were first assessed by simple inspection and the volume of the sample was next determined by aspirating the sample into sterile graduated 10ml pipette (Falcon 7530F, Becton Dickinson Labware, Oxnard, USA). Motility was determined by placing a 10µl drop of the well mixed semen sample onto a glass microscope slide which was prewarmed to 37°C, and gently lowering a cover slip onto this, before examining the sample at x 400 magnification using a phase contrast microscope (Leitz Instruments Limited, Luton, England). The percentage of motile cells was scored by counting 100 cells, and classifying each as motile or immotile without regard to the quality of the motility, the result being expressed simply as percentage motility. The concentration of spermatozoa in the sample was determined by mixing the sample thoroughly and then adding 10µl of semen to 190µl of sperm diluting fluid (50g $NaHCO_3$, 10ml 35% formalin/litre). This was mixed by vortexing and 10µl drops loaded into each side of an Improved Neubauer haemocytometer chamber (Weber Scientific Instruments, Sussex, England), allowed to settle for a few minutes before the spermatozoa present were counted routinely under x100 magnification, the result being expressed as 10^6 spermatozoa/ml of semen. The percentage of morphologically normal

spermatozoa was not routinely determined.

Statistical analysis was performed according to standard techniques (Armitage, 1971) using the procedures described in the Statistical Package for the Social Sciences (Nie et al, 1975)

2.6 Results

Clinical Evaluation

Considering firstly the ages of the semen donors, the ages of the 107 donors who underwent clinical assessment ranged from 18 - 46, with the mean age being 27.1 years (SD 7.1, SEM 0.68, median 26.9, mode 19). The frequency distribution of the ages of these donors is shown in Figure 2.5 (p 83).

Left testicular volumes ranged from 10 - 30ml, with a mean of 21.2ml (SD 4.5, SEM 0.44), while for right testicular volumes the mean was 21.4ml (SD 4.4, SEM 0.42). For the sub-population of donors of proven fertility, the mean left testicular volume was 22.2ml (SD 4.7, SEM 0.62), and the mean right testicular volume was 22.3ml (SD 4.8, SEM 0.63), with only 5.3% of fertile men having left testicular volumes of 12ml or below, and 12.3% having left testicular volumes in excess of 25ml. The frequency distributions of left and right testicular volumes for the groups discussed above are shown in Figure 2.6 (p 84).

A clinically apparent varicocele (Grade 1 - 3) was found on the left in 30.5% of the whole group and in 31.6% of the proven fertile sub-population.

Comparable figures for the presence of a right sided varicocele were 14.3% of the whole group and 14.0% of the fertile sub-population. These clinically apparent varicoceles are shown by grade in Figure 2.7 (p 85), from which it can be seen that the majority were grade 1, with 16.2% of all donors having a grade 1 left sided varicocele, compared to 7.6% having grade 2 left varicoceles, and only 6.7% having grade 3 varicoceles. Amongst fertile donors, 19.3% were found to have a grade 1 left sided varicocele, 7.0% were grade 2 and 5.3% were grade 3. Right sided varicoceles were much less common, with only 12.4% and 1.9% of all donors having grade 1 and 2 right sided varicoceles, and amongst fertile donors, these figures were 10.5% and 3.5% respectively. No grade 3 right sided varicoceles were detected.

Semen Analyses

A total of 1362 semen samples from 202 donors were analysed in terms of volume, concentration of spermatozoa and percentage of motile spermatozoa. Of these, 512 were from 55 donors of proven fertility.

Considering firstly abnormalities of liquefaction or viscosity, it was noted that these parameters were abnormal in 23.1% of all samples, and in 18.8% of samples from fertile donors. When semen volume was measured, it was noted that this was not normally distributed, having a coefficient of skewness of 1.144 for the whole group and 1.173 for the fertile subpopulation (Figure 2.8, p 86). The mean semen volume was 2.9ml amongst all samples, with the 5th centile being 0.9ml and the 95th centile being 6.0ml. Amongst semen samples from fertile donors, the mean volume was 3.0ml, with the 5th centile being 0.9ml, and the 95th centile being 6.1ml (Table 2.3, p 77).

When the concentration of spermatozoa, or sperm density as it is commonly referred to, was examined, it was again noted that the distribution was non-normal, with a coefficient of skewness of 2.535 amongst all samples and 1.625 amongst the fertile subpopulation. The frequency distribution of sperm concentrations for the whole population and for the fertile sub-population is shown in Figure 2.9 (p 87), emphasizing this point. The mean sperm concentration was 103.8 x 10^6 spermatozoa/ml for the whole group, with 5th and 95th centiles of 16 x 10^6/ml and 260 x 10^6/ml respectively. For the fertile sub-population, the mean sperm concentration was 114.6 x 10^6/ml, with 5th and 95th centiles of 23 x 10^6/ml and 260 x 10^6/ml respectively (Table 2.3, p 77).

Finally, the percentage of motile spermatozoa in each sample was examined, and in this case the distribution of motilities was closer to normal, with coefficients of skewness of -0.709 overall and -0.488 for the fertile subpopulation (Figure 2.10, p 88). The mean percentage motility for the whole group was 55.0% (SEM 0.34) with 5th and 95th centiles of 34% and 73% respectively. For the fertile subpopulation, the mean value for motility was 54.6% (SEM 0.50) with 5th and 95th centiles of 36% and 71% respectively (Table 2.3, p 77).

It is also of interest to examine the phenomenon of inter-ejaculate variability (Poland et al, 1986) in our own population. Figures 2.11 and 2.12 (pp 89-90) show the values of sperm concentration, total sperm concentration, semen volume and percentage motility obtained in a series of 34 ejaculates from one semen donor over a period of approximately 80 weeks. It is seen that the volume of this individual's semen varies from 0.4 to 4.7ml and his sperm

concentration from 46 to 198 x 10^6 spermatozoa/ml. In contrast, the motility of his spermatozoa varies much less, ranging from 44 to 90%, and it is seen therefore that in terms of sperm concentration and motility, his semen quality, although showing considerable variation from time to time is within normal limits at all times. This does not hold true for all individuals, and Figure 2.13 (p 91) shows the values of sperm concentration and percentage motility of a series of seven semen samples provided by one semen donor over a ten week period. It is seen from these graphs that his sperm concentration is below the threshold level of 20 x 10^6/ml on two occasions, and his percentage motility is below 40% on two occasions, however, three semen samples are completely within normal limits. This emphasizes the importance of examining at least two and possibly several semen samples from any one patient before deciding whether his semen quality is within "normal" limits or not.

2.7 Discussion

The common nature of the problem of infertility, and the importance of male reproductive dysfunction as a contributory cause of this problem has been stressed in Chapter 1, together with the central role of the semen analysis in the routine assessment of male reproductive dysfunction. In the present chapter, the methodology of the semen analysis has been described in detail, and this methodology has been applied to an Edinburgh population, to provide a background of normal values against which to work. If one employs the 5th and 95th centiles of the various parameters as the limits of normality, then the normal limits for sperm concentration are 23 - 260 x 10^6/ml and for percentage of motile spermatozoa, 36 - 71%. For

convenience, it is appropriate to think in terms of lower acceptable limits, and to round these to the easily remembered values of **20 x 10^6 spermatozoa /ml** for density and **40% for motility** (Table 2.4, p 78). Whilst these figures are in agreement with the WHO standard for sperm concentration (also 20 x 10^6/ml) they fall quite short of this standard for motility, WHO requiring 50% or more spermatozoa with forwardly progressive motility before a semen sample is accepted as within normal limits (World Health Organization, 1987).

To define standards of normality of semen within which 90% of fertile men will fall is of only very limited use. The pioneering work of Macleod and Gold (1951, 1956) has demonstrated that between 78 and 84% of the husbands of infertile marriages will have sperm concentrations in excess of 20 x 10^6/ml, and several groups of workers (Van Zyl et al, 1975; Smith et al, 1977; Bostofte et al, 1981) have demonstrated that significant pregnancy rates are observed amongst infertile men with semen quality below the limits of normality described above. Similarly, prospective studies of infertile couples carried out by Aitken and colleagues (Aitken et al, 1984a; Aitken, 1985) demonstrated a 37% pregnancy rate after 2 years of follow up of a group of couples with unexplained infertility, in whom semen quality was conventionally normal, and a 25.9% pregnancy rate after 4 years of follow up of a group of infertile couples with oligozoospermia (sperm concentrations below 20 x 10^6/ml).

It is clear, therefore, that the conventional criteria of semen quality provide only a very poor guide to the fertility of an individual. The clinician, faced in the infertility clinic with the male partner of an infertile couple, requires an

answer to the question "Are the gametes produced by my patient capable of establishing a pregnancy *in-vivo* ?". What is required therefore is a test, or combination of tests, that evaluates the integrity of sperm function, in contrast to the purely descriptive approach of the conventional semen analysis. It will be helpful, therefore, to consider the functional attributes which a spermatozoon must possess in order to be capable of establishing a pregnancy *in-vivo* .

2.8 Normal Sperm Function

At the moment of ejaculation, an average of 3 milliliters of semen, containing approximately 350 million spermatozoa are deposited in the region of the external cervical os and in the posterior fornix of the vagina. Most of these spermatozoa, only about 50% of which will be motile, are contained within the first portion of the ejaculate, are mixed mainly with secretions from the prostate gland (Mortimer, 1983), and are thus protected to some extent from the deleterious effects of the secretions of the seminal vesicles and the acidic pH of the vaginal secretions around mid-cycle (Moghissi et al, 1972). The buffering capacity of seminal plasma is such that the pH of the vaginal environment is elevated to around pH 7 very rapidly (Fox et al, 1973), and within 15-20 minutes most of the spermatozoa which will do so have penetrated the cervical mucus (Tredway et al, 1975; 1978), the remainder being rapidly inactivated and rendered incapable of penetrating cervical mucus (Perloff and Steinberger, 1963; Wallace-Haagens et al, 1975). The exact role of the coagulation and liquefaction of semen in this process is not clear, since following coagulation, the liquefaction of semen takes between 5 and 20 minutes (Sobrero and MacLeod, 1962; Tauber et al, 1980),

however spermatozoa are found within cervical mucus within 1.5 minutes of ejaculation (Sobrero and MacLeod, 1962). This ability of spermatozoa to escape from seminal plasma and to penetrate cervical mucus represents a major aspect of sperm function, and it appears that this ability is dependant upon the motility patterns of the spermatozoa (Aitken et al, 1986; Mortimer et al, 1986). The motility of spermatozoa has been shown to be regulated at least in part by cyclic adenosine 3',5' monophosphate (cyclic AMP) and calcium (Tash and Means 1982; 1983) and the availability of adenosine triphosphate is therefore also of importance (Hicks et al, 1972).

Having penetrated the mucus within the cervical canal, spermatozoa must next traverse this to enter the cavity of the uterus and thereby gain access to the fallopian tube, the site of fertilization. There is much debate concerning the events during this phase of sperm transport (Mortimer, 1983). At some stage during their sojourn through cervical mucus and through the lumen of the female genital tract, spermatozoa undergo the process commonly referred to as capacitation, whereby they acquire the ability to fertilize oöcytes, an ability which they lacked at the time of ejaculation (Bedford, 1970; 1983; Fraser 1984; Langlais and Roberts, 1985). The few hundred spermatozoa which reach the site of fertilization must make contact with the oöcyte, and penetrate the investments of the oöcyte, namely the cumulus oöphorus and the zona pellucida, and during this process, they must undergo the acrosome reaction and thereby generate a fusogenic equatorial segment to permit fusion with the oölemma (Decker et al, 1976; Fleming and Yanagimachi, 1981; Bedford, 1983; Fraser, 1984; Langlais and Roberts, 1985; Murphy et al, 1986). This acquisition of the ability to fuse with the oölemma, following as it does the successful completion of the

processes of capacitation and the acrosome reaction, is central to sperm function, for unless this is successfully achieved, fertilization is impossible. The central importance of calcium and pH in the sequence of events comprising capacitation and the acrosome reaction has been described by a number of workers (Yanagimachi and Usui, 1974; Singh et al, 1978; Triana et al, 1980; Christen et al, 1982; Fraser, 1982; Yanagimachi, 1982; Murphy and Yanagimachi, 1984; Nagae and Srivastava, 1986; Roldan et al, 1986).

2.9 Conclusions

The shortcomings of the conventional semen analysis have been outlined, demonstrating that it is a very blunt instrument for the prediction of the likely fertilizing ability of an individual. The clinical need is for tests of sperm function which will accurately predict *in-vivo* fertilizing ability, and such tests must clearly be based upon our existing knowledge of normal sperm function. A number of key areas of sperm function have been identified above, such as the requirement for spermatozoa to penetrate cervical mucus, to penetrate the investments of the oöcyte, and to fuse with the oölemma, and the importance of adenosine triphosphate, calcium and pH in this process has been suggested. These key areas of sperm function can be used as the focal point for the development of *in-vitro* tests of sperm function, and subsequent chapters in this thesis will discuss such tests in detail, with a view to examining critically their likely clinical usefulness. From these tests of sperm function follows an ability to examine the sub-cellular control of sperm function in detail, and thus to begin to understand the defects which might be present in individuals with impaired sperm function,

such understanding being the necessary prelude to the development of therapeutic strategies for their correction.

Table 2.1

Semen Analysis : Commonly Used Criteria for Normality
(From : World Health Organization, 1987)

Parameter	Normal Value
Volume	2.0ml or more
pH	7.2 - 7.8
Sperm Concentration	20 x 10^6 spermatozoa/ml or more
Total Sperm Count	40 x 10^6 spermatozoa or more
Motility	50% or more with forward progression; or
25%	or more with rapid linear progression
within 60	minutes after collection
Morphology	50% or more with normal morphology
Viability	50% or more live, i.e. excluding dye
White blood cells	less than 1 x 10^6/ml
Zinc (total)	2.4 micromoles or more per ejaculate
Citric acid (total)	10 mg or more per ejaculate
Fructose (total)	13 micromoles or more per ejaculate
MAR-test	less than 10% spermatozoa with adherent particles
Immunobead test	less than 10% spermatozoa with adherent beads

Table 2.2

Summary of published information on conventional semen parameters in men of proven fertility.

Study	n	Volume (ml)	Density (x 10^6/ml)	Motility (%)	Morphol (% normal)
Åbyholm, 1981*	51	3.9 ± 2.06	89.0 ± 68	53.0 ± 9.0	40.0 ± 18.0
Aitken et al, 1982a**	35	2.8 ± 0.2	129.3 ± 13.8	62.0 ± 2.0	54.6 ± 1.2
David et al, 1980*	167	3.6 ± 1.3	140.0 ± 75	78.5 ± 8.6	---
Jouannet et al, 1981*	324	3.6	95.4	71.8	61.3
Fariss et al, 1981**	25	---	95.4 ± 13.4	45.8 ± 3.2	50.3 ± 2.7
Macleod & Gold 1951*	1000	3.4 ± 1.9	107.0 ± 74	---	---
Nelson & Bunge, 1974*	386	2.8 ± 1.6	48.0 ± 40.1	---	74.0 ± 12.0
Rehan et al, 1975*	1300	3.2 ± 1.4	79.0 ± 57	65.0 ± 22	---
Smith & Steinberger, 1977*	2000	2.5 ± 1.5	70.0 ± 65	---	---
Wang et al, 1985*	82	2.7 ± 1.68	71.0 ± 58	56.8 ± 17	62.6 ± 10.7

* (Means ± standard deviations)

** (Means ± standard error of the mean)

Table 2.3

Study of Semen Quality amongst a group of 202 normal semen donors, including 55 donors of proven fertility, Edinburgh, 1984 - 1987.

Descriptive statistics.

Parameter	Volume		Density		Motility	
Group	All	Fertile	All	Fertile	All	Fertile
No Samples	1361	512	1362	512	1362	512
No Donors	202	55	202	55	202	55
Mean	2.9	3.0	103.8	114.6	55.0	54.6
SEM	0.04	0.07	2.32	3.41	0.34	0.50
SD	1.60	1.56	85.62	77.10	12.66	11.42
Skewness	1.144	1.173	2.535	1.625	-0.709	-0.488
Median	2.6	2.7	81.2	97.5	56.0	54.8
Mode	2.1	2.1	46.0	92.0	59.0	54.0
5th Centile	0.9	0.9	16.0	23.0	34.0	36.0
10th Centile	1.1	1.3	28.0	37.0	40.0	41.0
90th Centile	5.0	5.1	201.0	209.0	69.0	67.0
95th Centile	6.0	6.1	260.0	260.0	73.0	71.0

Table 2.4

Semen Analysis : Criteria for normality based upon normal fertile donors in the Edinburgh Area, 1984-1987.

Parameter	Units	Normal Value
Volume	ml	1.0 - 6.0
Sperm Concentration	10^6/ml	> 20.0
Motility	% motile	> 40.0
Morphology	% normal	> 40.0

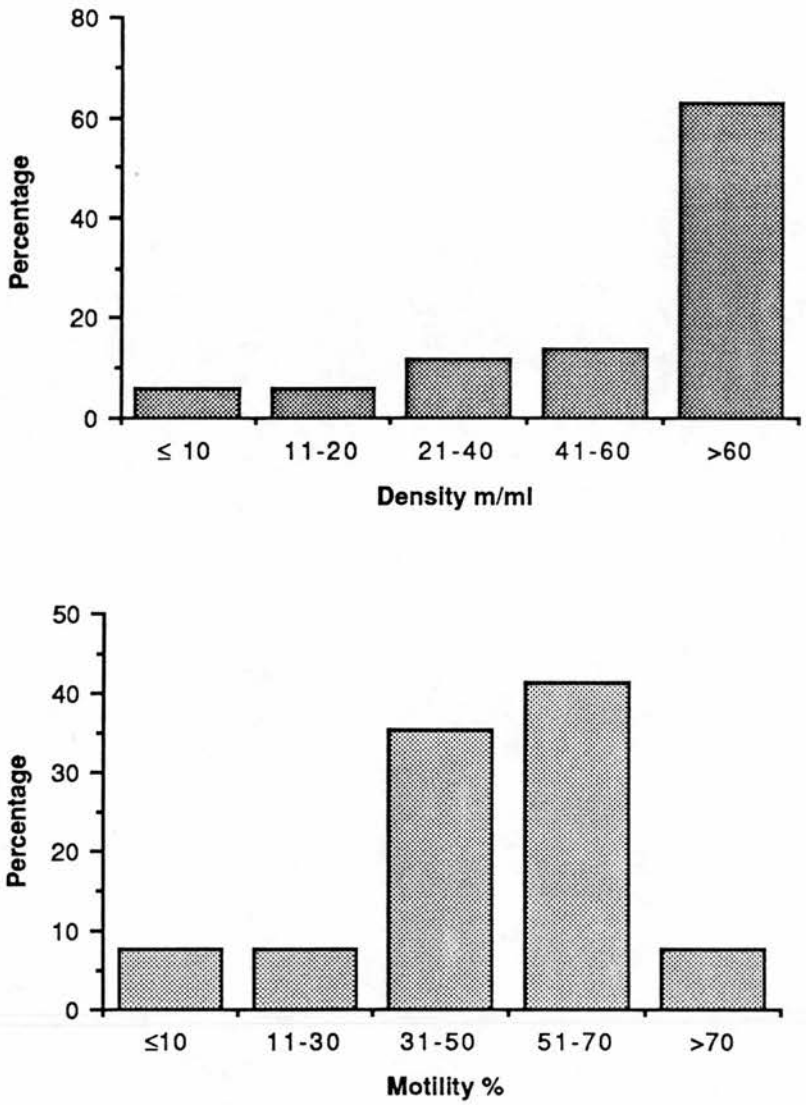


Figure 2.1

Semen Analysis in Normal Fertile Men

This figure shows the frequency distribution of sperm concentration (10^6 spermatozoa/ml) and sperm motility (% motile) in a group of 51 Norwegian men of recently proven fertility. The frequency distributions are markedly skewed, and a significant proportion of individuals fall below the standards of "normality" set by WHO.

(Data From : Åbyholm, 1981)

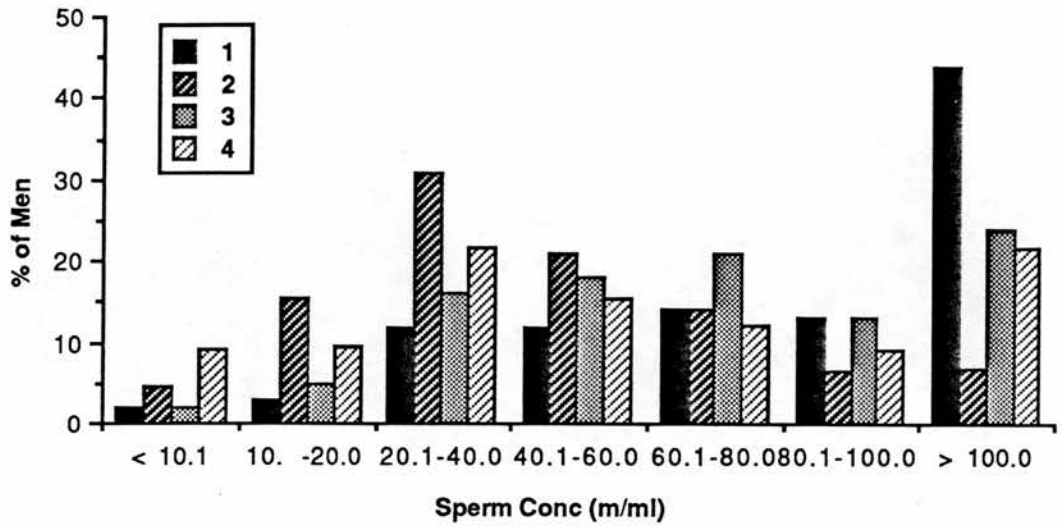


Figure 2.2

Semen Analysis in Normal Fertile Men

This figure shows the frequency distribution of sperm concentration (x 10^6 spermatozoa/ml) in four studies of normal fertile men.

Study 1 : Macleod and Gold, 1951

Study 2 : Nelson and Bunge, 1974

Study 3 : Rehan et al, 1975

Study 4 : Smith and Steinberger, 1977

Again it can be seen that significant numbers of fertile men have sperm concentrations of below 20 x 10^6/ml.

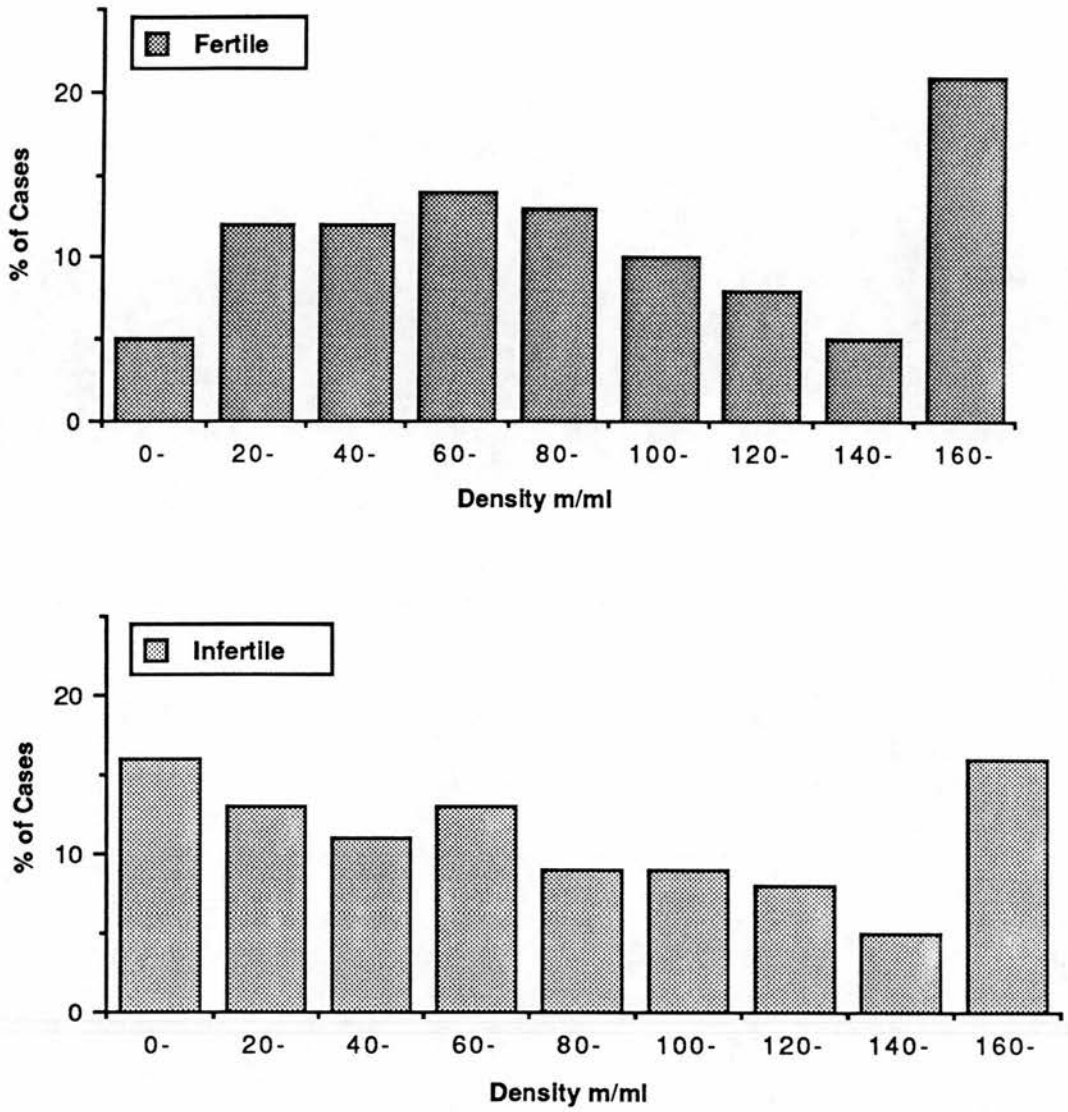


Figure 2.3

Semen Analyses in Fertile and Infertile Men

This figure shows the frequency distribution of sperm concentration in 1000 men of proven fertility and 1000 male partners of infertile marriages.
(Data from : Macleod and Gold, 1951)

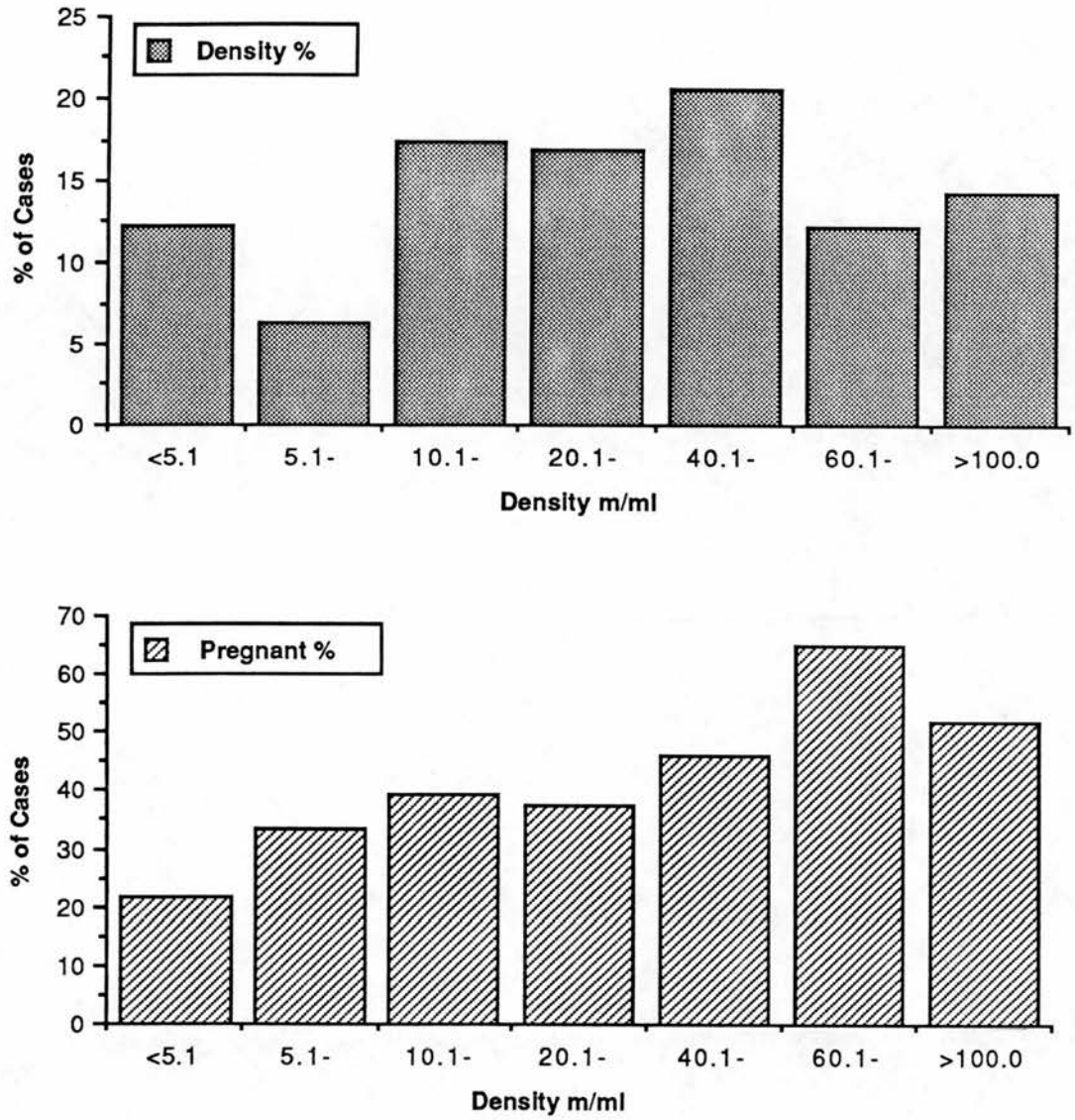


Figure 2.4

Semen Analysis and Pregnancy Rates In Infertile Men

This figure shows the frequency distribution of sperm concentrations (10^6 spermatozoa/ml) in 189 couples presenting to an infertility clinic. Of these 49 couples were unsuitable for treatment, and 140 were treated. Of these 81 achieved pregnancies, and the overall pregnancy rate in relation to sperm concentration is shown.
(Data from : Smith et al, 1977)

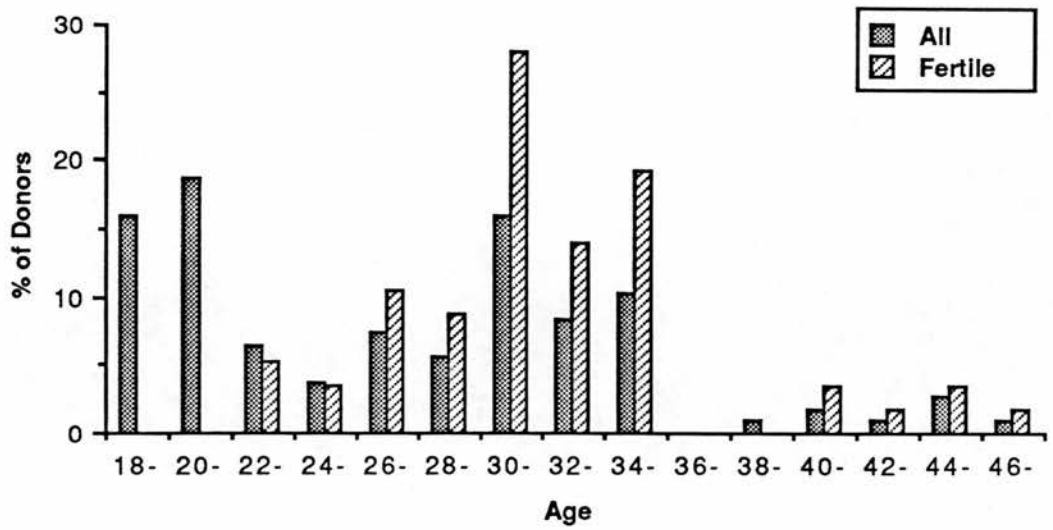


Figure 2.5

Semen Donor Survey : Ages of Donors

Frequency distribution of ages of donors studied, for whole group (=**All**, n=107, and for sub-population of proven fertility (=**Fertile**, n=55).

Whole Group	Mean age = 27.1 years
	Range = 18 - 46 years
Fertile Group	Mean age = 31.7 years
	Range = 22-46

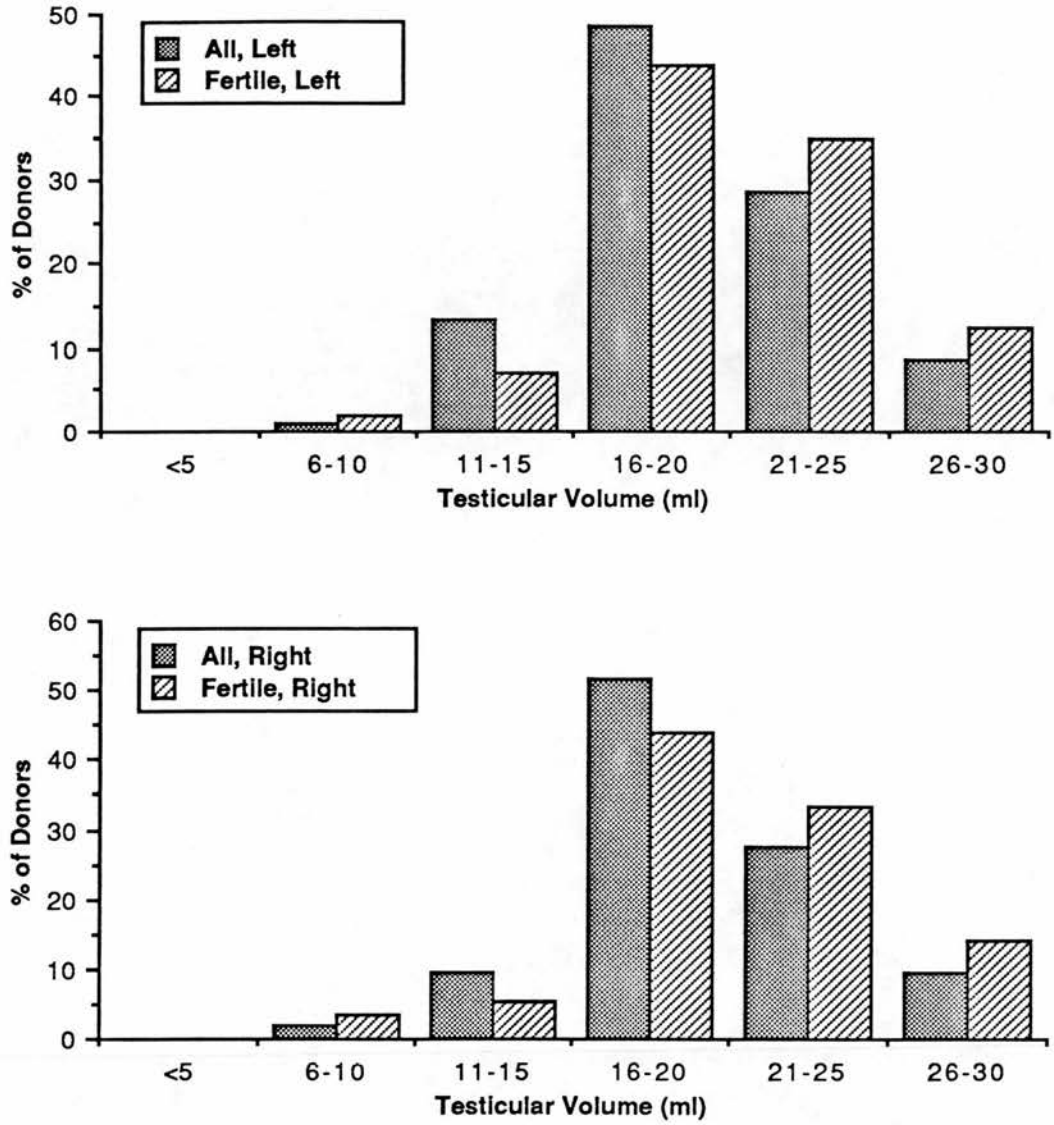


Figure 2.6

Semen Donor Survey : Testicular Volumes

Frequency distributions of Left (upper graph) and Right (lower graph) testicular volumes.

Whole group	Mean Left testicular volume = 21.2ml
	Mean Right testicular volume = 21.5ml
Fertile group	Mean Left testicular volume = 22.2ml
	Mean Right testicular volume = 22.3ml

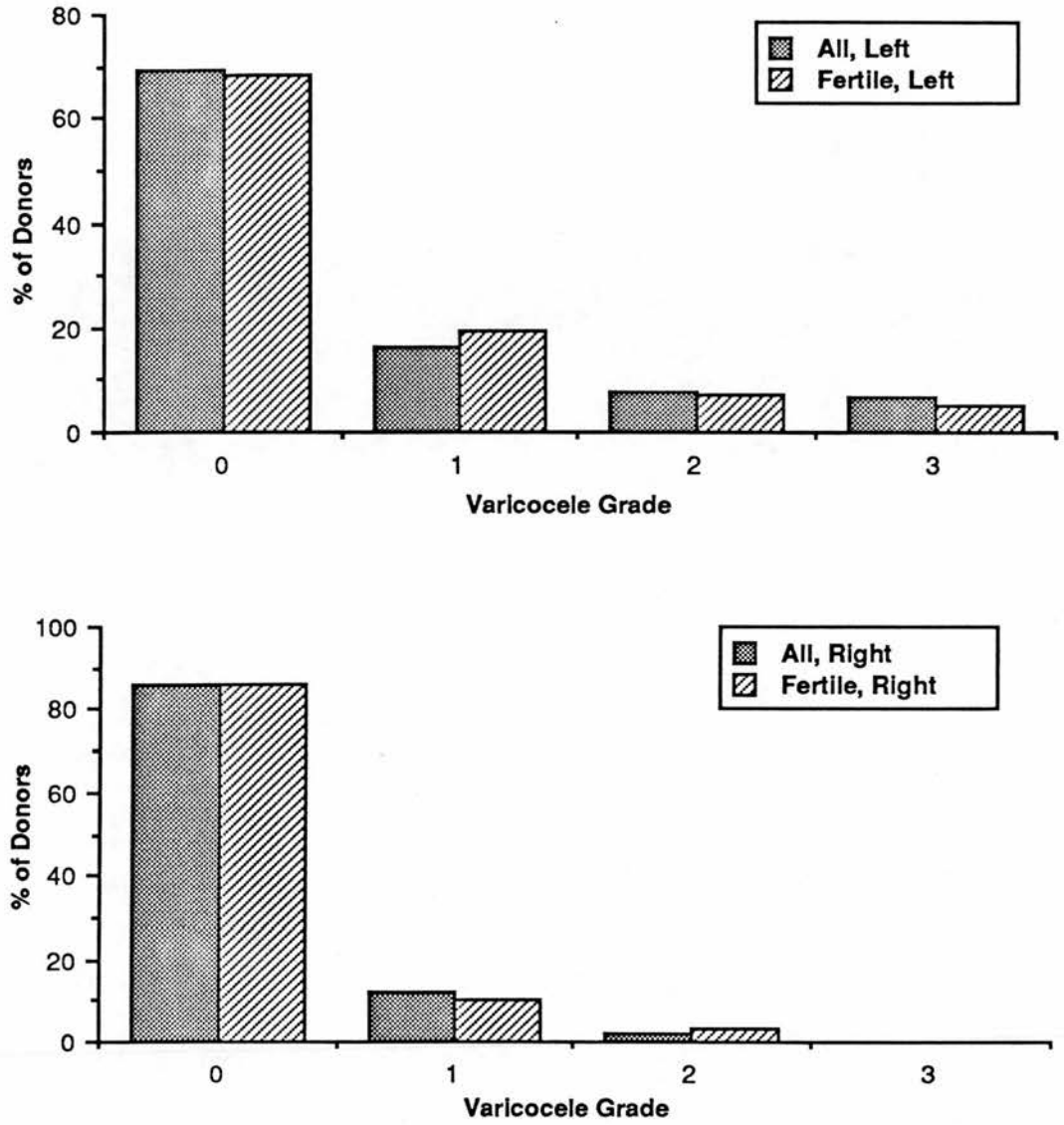


Figure 2.7

Semen Donor Survey : Incidence of Clinical Varicocele

Frequency of clinical varicocele amongst semen donors. Upper graph = left sided varicoceles, Lower graph = right sided varicoceles. Overall, on the left side, 16.2% of donors had grade 1 varicocele, 7.6% grade 2 and 6.7% grade 3. On the right these figures were 12.1%, 1.9%, 0.0%. Amongst fertile donors, on the left side, 19.3% had grade 1, 7.0% grade 2, and 5.3% grade 3. On the right, these figures were 10.5%, 3.5%, and 0.0%.

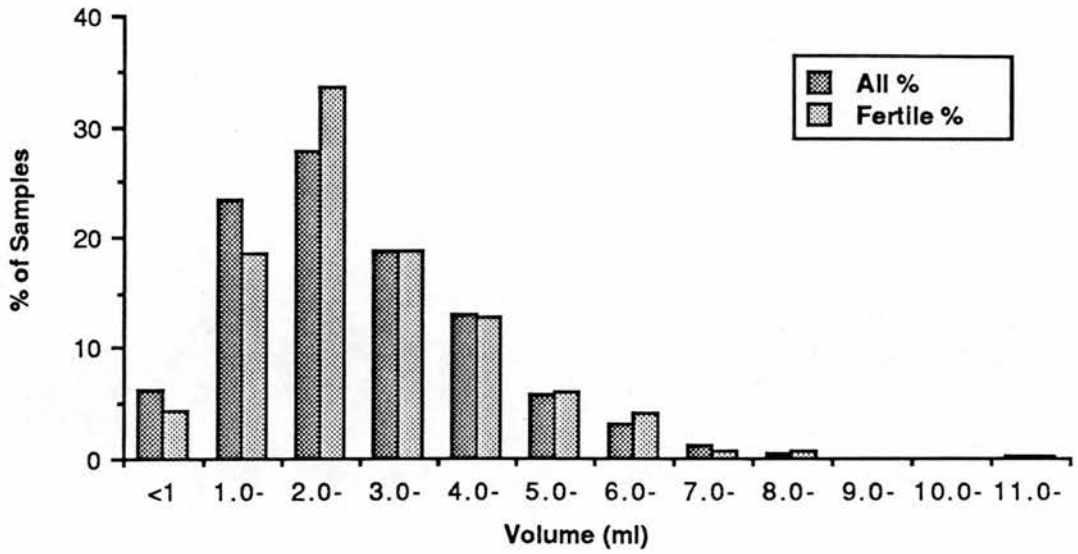


Figure 2.8

Semen Donor Survey : Semen Volume

The graph shows the frequency distribution of semen volume amongst 1362 semen samples from 202 normal donors, including 512 semen samples from 55 donors of proven fertility, also shown separately.

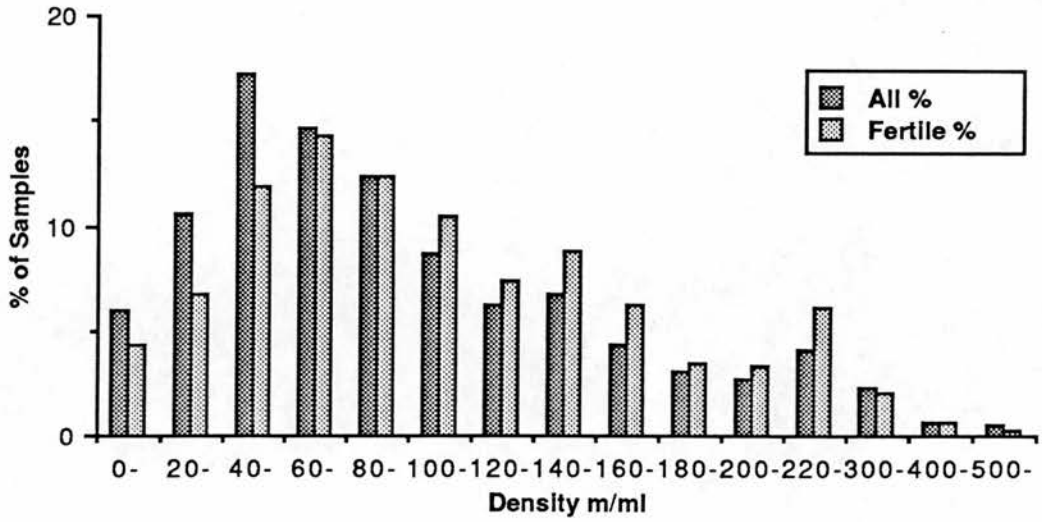


Figure 2.9

Semen Donor Survey : Concentration of Spermatozoa

The graph shows the frequency distribution of sperm concentration amongst 1362 semen samples from 202 normal donors, including 512 semen samples from 55 donors of proven fertility, also shown separately.

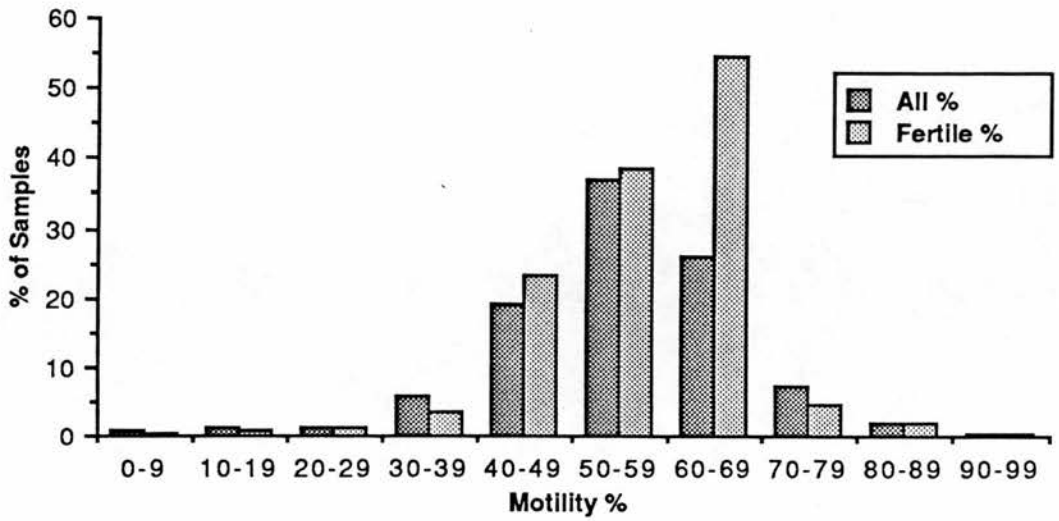
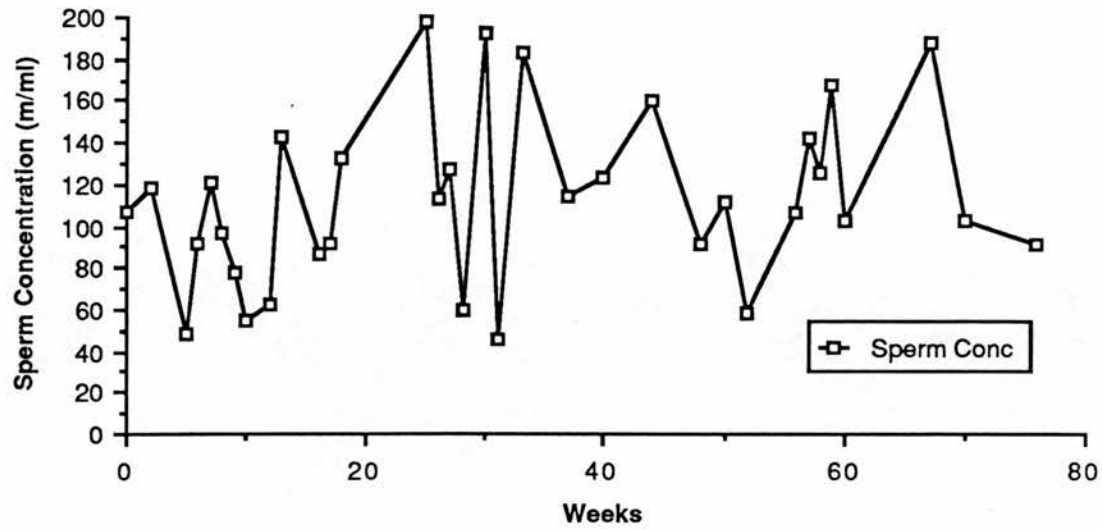


Figure 2.10

Semen Donor Survey : Percentage of Motile Spermatozoa

The graph shows the frequency distribution of percentage motility amongst 1362 semen samples from 202 normal donors, including 512 semen samples from 55 donors of proven fertility, also shown separately.



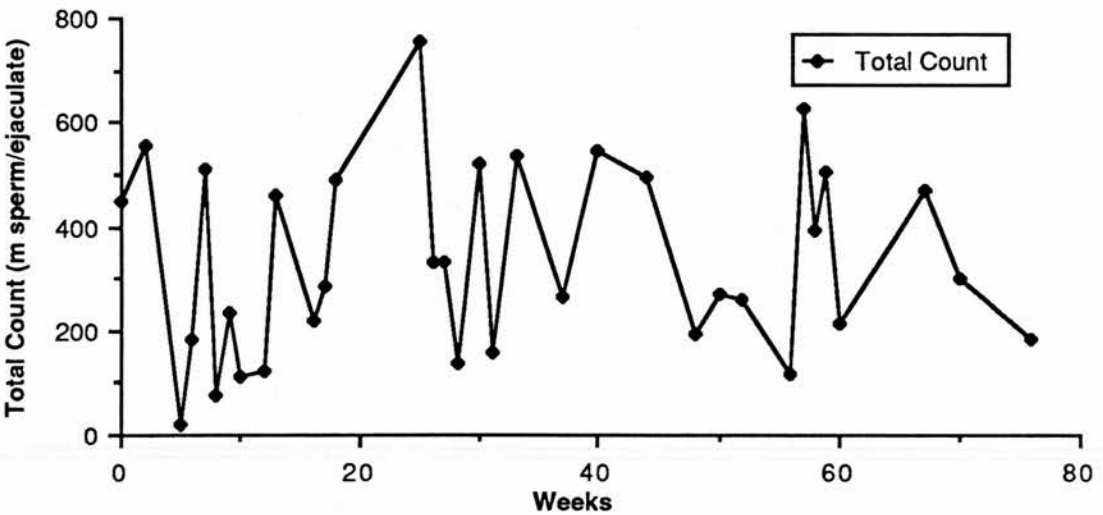


Figure 2.11

Semen Donor Survey : Inter-Ejaculate Variability

The graphs above show the values obtained for sperm concentration (10^6 spermatozoa/ml of ejaculate) and for total sperm count (10^6 spermatozoa/ejaculate) from a single semen donor who provided 34 ejaculates at intervals over a period of approximately 80 weeks.

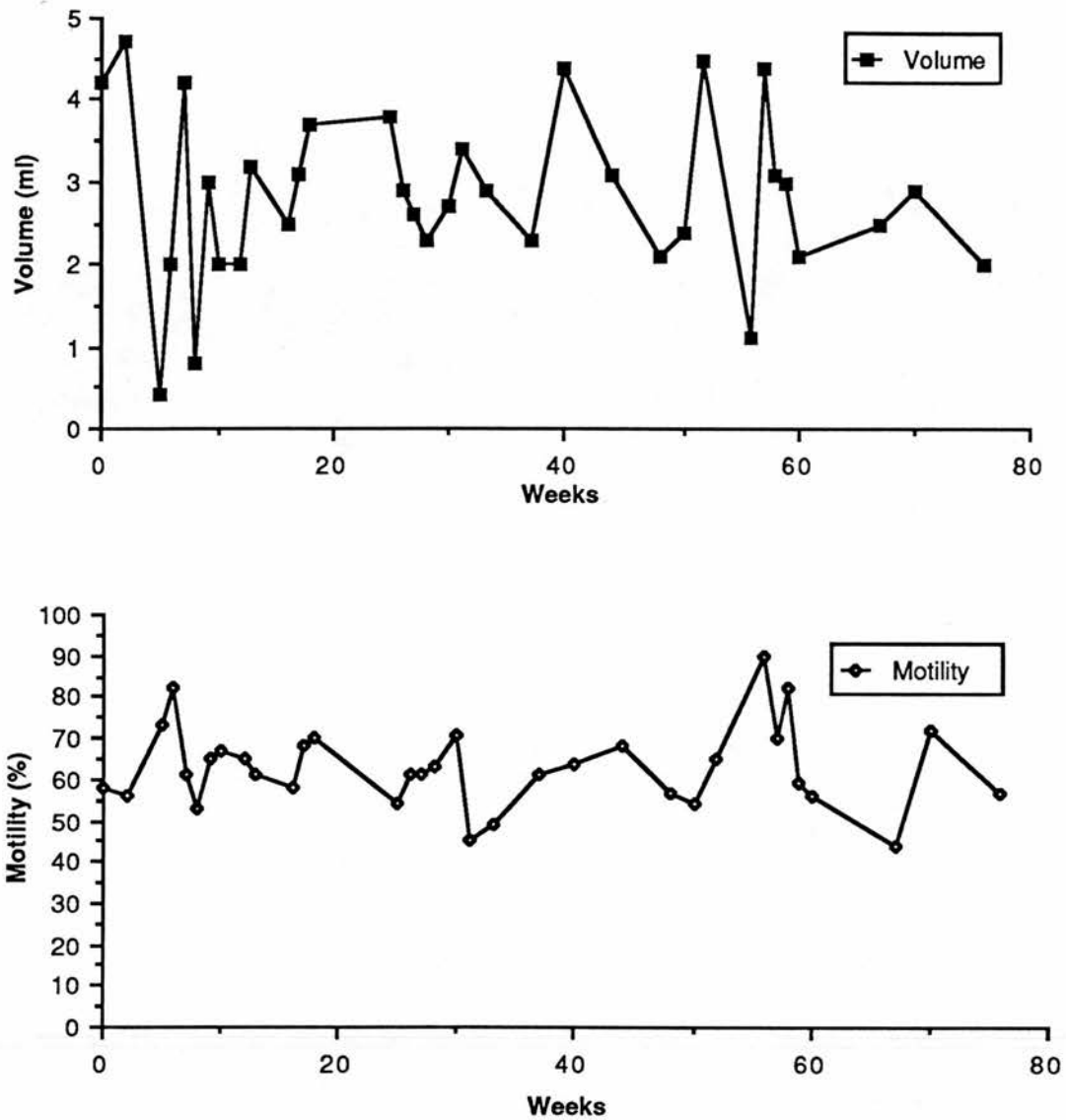


Figure 2.12

Semen Donor Survey : Inter-Ejaculate Variability

The graphs above show the values obtained for semen volume (ml) and percentage of motile spermatozoa from a single semen donor who provided 34 ejaculates at intervals over a period of approximately 80 weeks.

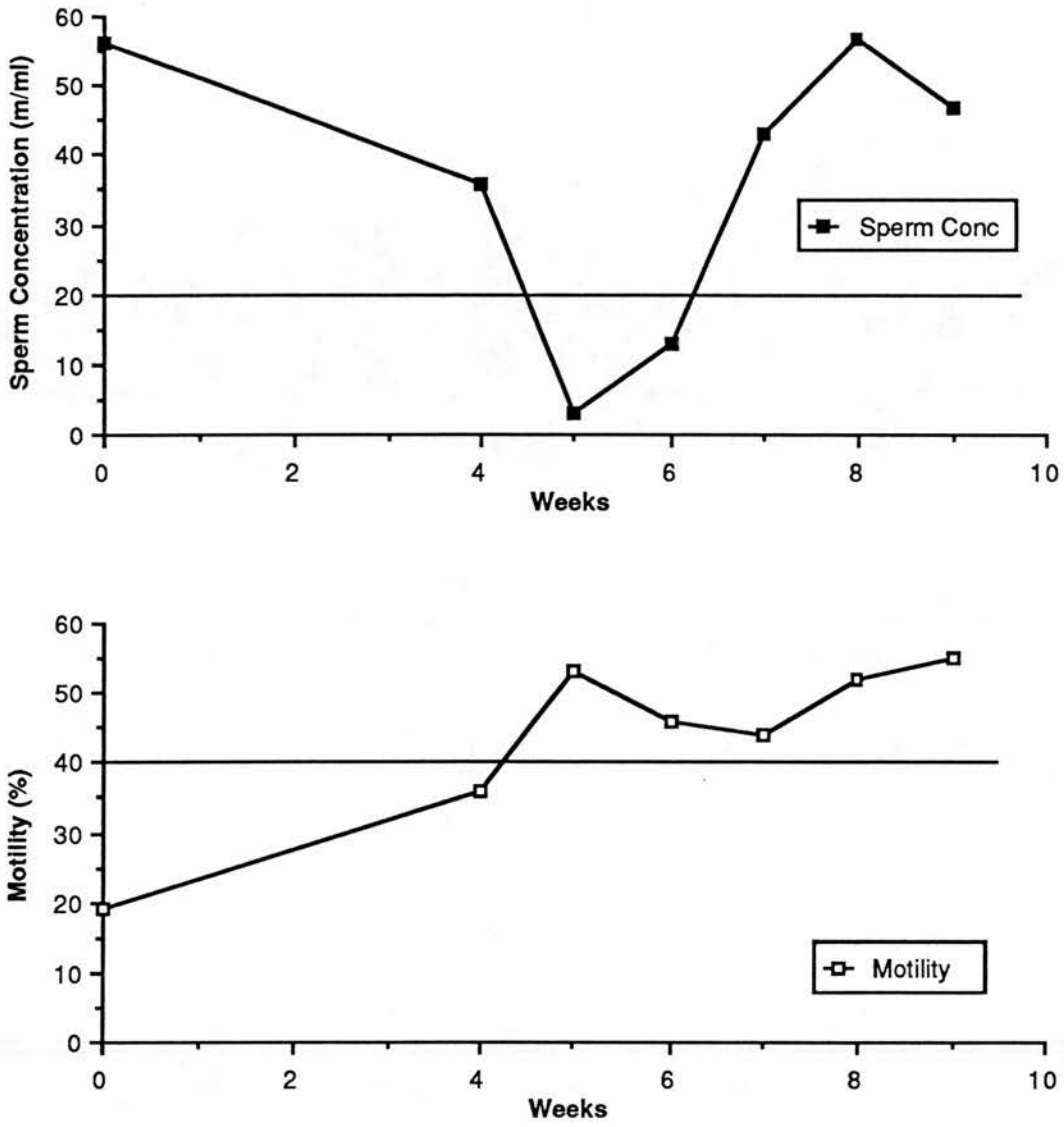


Figure 2.13

Semen Donor Survey : Inter-Ejaculate Variability

The graphs above show the values obtained for sperm concentration and percentage of motile spermatozoa from a single semen donor who provided 7 ejaculates at intervals over a period of approximately 10 weeks. It can be seen that his semen quality is occasionally below the standards of normality discussed in the text. Three of the seven samples are normal.

CHAPTER THREE

THE VALUE OF SEMEN ADENOSINE TRIPHOSPHATE (ATP) MEASUREMENTS IN ASSESSING THE FERTILIZING ABILITY OF HUMAN SPERMATOZOA

3.1 Introduction

As was indicated in Chapter Two, the information provided by the conventional criteria of semen quality, sperm number, motility and morphology, is of only limited value in assessing the fertilizing potential of an ejaculate, and this diagnostic problem is central to the dilemmas which confront clinical andrologists. In response to this problem, a number of *in-vitro* tests of sperm function have been devised, which aim to provide clinically useful data on the likely *in-vivo* fertilizing potential of a given ejaculate, and which are based upon our existing understanding of what constitutes normal sperm function. Perhaps foremost amongst these tests is the zona-free hamster oöcyte penetration test which was originally described by Yanagimachi and colleagues in 1976 (Yanagimachi et al, 1976), and depends upon the fact that the eggs of the golden hamster, *Mesocricetus auratus* , can be deprived of their ability to preserve species specificity at the time of sperm-egg fusion by the removal of the zona pellucida. This renders them capable of being penetrated by the spermatozoa of a wide variety of species, and this fact has been used to enable the development of a bioassay of human sperm fertilizing ability which has been widely applied in a clinical context (Barros et al, 1979; Rogers et al, 1979; Binor et al, 1980; Matthews et al, 1980; Overstreet et al, 1980; Hall, 1981; Karp et al, 1981; Tyler et al, 1981; Aitken et al, 1982a; b; c; 1983a; b; c; 1984a; b; Martin et al, 1982; Stenchever et al, 1982; Templeton et al, 1982; Rogers, 1983; 1985; Wickings et al, 1983; Aitken and Elton, 1984; 1986; Blasco, 1984; Yanagimachi, 1984; Aitken , 1985; Shalgi et al, 1985). Prospective clinical studies of the ability of the zona-free hamster oöcyte penetration test to predict the probability of a given couple achieving a pregnancy have shown it to be significantly superior to the conventional

criteria of semen quality (Aitken et al, 1984a; Aitken, 1985), although the current methodology for the performance of this test is imperfect (Aitken et al 1984b). Another major area of sperm function which has been used as the basis for the development of an *in-vitro* test has been the penetration by sperm of ovulatory cervical mucus. This can be quantitatively examined (Katz et al, 1980 ;Schats et al, 1984) and studies on the movement characteristics of human spermatozoa (Overstreet et al, 1979; David et al 1981; Katz and Overstreet, 1981; Katz et al, 1981; Serres et al, 1984) have suggested that variations in these characteristics may account for variation in the ability of sperm to penetrate the barrier of cervical mucus and the investments of the oöcyte (Hull et al, 1984; Aitken et al, 1985; 1986; Feneux et al, 1985; Mortimer et al, 1986).

The clinical value of the zona-free hamster oöcyte penetration test and of sperm movement characteristics in predicting the *in-vivo* fertilizing ability of a given ejaculate will be assessed in detail in the following chapter. These bioassays suffer from the major drawback of being complicated, expensive and time-consuming, and it is unlikely that the widespread evaluation of potentially infertile men by such techniques will ever become feasible. Therefore, there is a great need for the development of a method for the assessment of the functional competence of human spermatozoa that is reliable, simple, reproducible and meaningful. In this regard, attention has recently been focussed on the potential use of adenosine triphosphate (ATP) measurements as a possible marker of human sperm fertilizing potential (Comhaire et al, 1983)

Human spermatozoa generate ATP principally by glycolysis (Peterson and Freund, 1970; 1974; Hammerstedt and Lardy, 1983) and to a much lesser

extent by oxidative phosphorylation (Ford and Harrison, 1981). This ATP is utilised for a variety of cellular functions, including the provision of energy for motility (Young and Nelson,1969; Summers, 1974; McGrady, 1979; Suter et al, 1979), the maintenance of ionic gradients (Durr et al, 1972; Borle, 1981) and as a source of cyclic AMP (Menon and Gunaga, 1974).

The presence of ATP in semen was originally described by Mann in 1945 (Mann, 1945), since when a number of workers have measured the ATP present in mammalian (Brooks, 1970) and human (Fiorelli et al, 1982) semen and have published data on the concentrations of ATP observed in fertile men (Calamera et al, 1979; Vilar et al, 1980; Caldini et al, 1982). It has been claimed that a relationship exists between the content of ATP and the motility of a population of spermatozoa (Calamera et al, 1982; Orlando et al, 1982), although not all workers have been able to establish this relationship (Levin et al, 1981) and in addition, claims have been made for the existence of a relationship between ATP concentration and the morphological normality of a population of spermatozoa (Schirren et al, 1979).

In the light of these observations concerning the importance of ATP to human sperm function, and concerning the relationship between ATP measurements and other aspects of semen quality, it has been suggested (Comhaire et al, 1983) that the measurement of ATP in semen may be a useful predictor of sperm fertilizing ability. Given the pressing need for a simple test of sperm fertilizing ability, a study was undertaken to evaluate the usefulness of ATP measurements in assessing the fertilizing capacity of human spermatozoa *in-vivo* and *in-vitro* .

3.2 Materials and Methods

Patients

Semen samples were obtained by masturbation either from a panel of semen donors who were involved in a program for the treatment of infertile couples by therapeutic donor insemination (AID), or from the male partners of infertile couples currently undergoing investigations within the Infertility Clinic, Royal Infirmary, Edinburgh. In the case of the latter, their female partners had been demonstrated to be normal within limits previously defined (Templeton et al, 1982; Aitken et al, 1984a), and thus had normal findings on routine history and examination (see Chapter 1), regular menstrual cycles within the limits 28 ± 4 days, ovulatory levels of progesterone in the luteal phase of the cycle (documented in at least two cycles) and normal pelvic anatomy with bilateral tubal patency demonstrated at diagnostic laparoscopy and dye hydrotubation (Templeton and Kerr, 1977). Screening for antisperm antibodies was not undertaken.

Conventional Semen Analysis

Semen samples were delivered to the laboratory within 60 minutes of production and were allowed to liquefy for at least 30 minutes prior to analysis. Assessment was made of volume, viscosity, completeness of liquefaction, sperm concentration (10^6/ml) and motility (%) by the use of standard laboratory techniques (World Health Organization, 1980; 1987) as described in detail in Chapter 2. Chemical reagents used throughout were supplied by the Sigma Chemical Co, St Louis, MO, or BDH Chemicals unless stated otherwise.

Adenosine Triphosphate Measurements

ATP was measured with the aid of a simple bioluminescent assay. (Lundin and Thore, 1975; Comhaire et al, 1983) In outline, 25µl of the semen sample or washed sperm preparation under analysis was mixed with an equal volume of a 10% solution of trichloracetic acid (TCA) and to this was added 850µl of a Tris(hydroxymethyl)aminomethane-ethylenediamine-tetracetic acid (Tris-EDTA) buffer (0.1 M Tris, 2mM EDTA, pH 7.5). Bioluminescence was measured in a Berthold luminometer (Laboratory Impex Ltd, Twickenham, England) after the addition of 100µl of luciferin-luciferase reagent (LKB-Wallac, Turku, Finland). The assay was calibrated with the use of a standard solution of ATP (LKB) and light scattering due to turbidity was corrected by the addition of an internal standard solution where appropriate. This was generally performed in the case of measurements made on whole semen samples, and not in the case of measurements made on washed suspensions of spermatozoa.

Zona-Free Hamster Oöcyte Penetration Testing

This was performed as originally described by Yanagimachi and colleagues (1976) and according to the detailed methodology of Aitken and colleagues (Aitken et al, 1983a; 1984b). The semen sample was first transferred to a sterile centrifuge tube and diluted with modified Biggers, Whitten and Whittingham (BWW) medium (Biggers et al, 1971) (Appendix 1), containing 20mM Hepes buffer and 3 mg/ml human serum albumin. The sample was centrifuged at 500 x g for 5 minutes following which the supernatant was discarded and the sample washed a further two times in 5ml volumes of fresh medium BWW. The washed sample was finally resuspended at a concentration of 10 x 10^6/ml in four separate aliquots that contained, respectively, either BWW medium, BWW medium made hyperosmotic (410

mOsm/kg) by the addition of NaCl (Aitken et al, 1983a), or BWW medium containing either a 50μM or 100μM aqueous suspension of the Ca^{++}, Mg^{++} salt of the divalent cation ionophore A23187 (Calbiochem, Bishops Stortford, England) (Aitken et al, 1984b). The samples were then maintained in an incubator at 37°C in an atmosphere of 5% CO_2 in air for a period of three hours, after which the spermatozoa were centrifuged at 500 x g for 5 minutes and resuspended in fresh medium BWW at a density of 10 x 10^6 spermatozoa/ml before the introduction of zona-free hamster oöcytes.

Adult female Golden hamsters (*Mesocricetus auratus*) were induced to superovulate by an intraperitoneal injection of 40 IU pregnant mare serum (PMS) (Folligon, Intervet Laboratories Ltd., Cambridge, England) on day 1 of the estrus cycle, followed by an equivalent dose of human chorionic gonadotrophin (hCG) (Pregnyl, Organon Laboratories Ltd., Morden, England) on day 3. Autopsies were performed on the animals 17 to 18 hours after hCG administration, and the cumulus masses were released from the tubal ampullae into medium BWW at 37°C. The cumulus cells were subsequently dispersed by 0.1% hyaluronidase in BWW, and the ova washed two times in fresh medium before being transferred to 0.1% trypsin to remove the zona pellucidae. Once the zonae had dissolved, the oöcytes were carefully washed two times in BWW before being transferred to 50μl droplets of sperm suspension under paraffin oil. The sperm and zona-free hamster oöcytes were co-incubated for 3 hours at 37°C in 5% CO_2 in air, at the end of which time the ova were washed to remove any loosely attached spermatozoa, transferred to a clean glass microscope slide, and compressed to a depth of about 30μm under a 22 x 22 mm coverslip on 4 paraffin wax supports. The oöcytes were examined under phase contrast microscopy for the presence of decondensing sperm heads with an

attached or closely associated tail within the vitellus. Between 20 and 40 ova were scored per sample and the percentage of ova containing such swollen sperm heads was recorded as the penetration rate.

Semen Cryopreservation and Therapeutic Donor Insemination

The semen samples used for therapeutic donor insemination were frozen and stored according to the techniques described by Jackson and Richardson (1977). The cryoprotective medium was composed of glycerol (16% vol/vol), egg yolk (28% vol/vol), fructose (1.24% wt/vol), and 56% (vol/vol) of a 3.2% (wt/vol) stock solution of sodium citrate in distiled water. This was gradually added to the semen at room temperature in 0.5ml aliquots until a 1:1 dilution was obtained, and the samples were then frozen in straws containing approximately 0.5ml at an average rate of 7.8°C/minute in the vapour phase of a flask containing liquid nitrogen until a temperature of at least -120°C was attained, at which point they were immersed in the liquid nitrogen. Insemination was performed intracervically with this buffered sperm preparation, after allowing it to warm to 37°C. Insemination was performed only one time in each cycle and was timed to coincide with ovulation, which was determined with the use of daily plasma luteinising hormone radioimmunoassays (Djahanbakhch et al, 1981). An ejaculate was regarded as successful if a pregnancy was achieved and unsuccessful if the ejaculate failed to produce a pregnancy after four or more inseminations into different recipients. For analysis, two straws (0.8ml) of each cryostored ejaculate were thawed, mixed with 1.6ml of BWW medium, and layered over four 1.5ml columns of BWW containing 7.5% human serum albumin which were then incubated at 37°C in 5% CO_2 in air. After 90 minutes incubation, the lower 1.0ml of each of these columns was recovered and pooled before being centrifuged at 500 x g for 5 minutes and

the pelleted spermatozoa resuspended in BWW medium. This procedure is modified from that described by Ericsson and colleagues (Ericsson et al, 1973), and resulted in a preparation of motile spermatozoa, free from the cryopreservative buffer, and without the need to resort to the amount of centrifugation required to prepare spermatozoa from semen, centrifugation being poorly tolerated by cryopreserved spermatozoa. Sperm concentration (10^6/ml), motility (%) and ATP were measured on both the frozen/thawed semen sample and on the column separated sperm preparations, to allow for any confounding effect of the presence of the cryopreservative medium.

Statistical Analysis

Comparisons between groups were performed with the use of the *t*-test, once the normal distribution of the data had been checked and the equality of variances assessed by the variance ratio; only differences with $P < 0.05$ were considered significant. Where unequal variances were found, the comparison was made with the use of the t* statistic, which employs separate rather than pooled variance estimates. Relationships between variables were examined with Pearson's correlation coefficient (*r*) and by multiple regression analysis. Discrimination between groups employed a multivariate discriminant analysis following Rao's method, with the aid of the Statistical Package for the Social Sciences (SPSS) (Nie et al, 1975)

3.3 Results

This analysis of the role of ATP measurements in the diagnosis of human sperm function is set out in two parts; firstly in the context of the laboratory evaluation of the male partner of a couple in which the female partner is normal within the limits of conventional investigation, and secondly, in the

context of a donor insemination programme, where relationships with *in-vivo* fertility can be critically examined.

Adenosine triphosphate in the laboratory evaluation of male infertility

Thirty-nine ejaculates from men with normal partners were analysed. Of these 39 semen samples, 6 were oligozoospermic (that is, had sperm concentrations < 20 x 10^6/ml); seven were from patients with clinical varicoceles (Comhaire, 1983), and 26 samples were from couples with 'unexplained' infertility (thus, in addition to the normality of the female partner, the male partner had semen with in the limits of : sperm concentration ≥ 20 x 10^6/ml, motility ≥ 40%, normal morphology ≥ 40%). Semen samples were assessed for sperm concentration (10^6/ml), motility (% motile spermatozoa), ATP (μM, uncorrected and corrected for turbidity), and zona-free hamster oöcyte penetration (% of eggs penetrated) under the four conditions described above, namely, normosmotic BWW, hyperosmotic BWW, and two concentrations of Ca^{++}, Mg^{++} A23187. Each washed sperm preparation was again assessed for percentage motility and ATP (μM) immediately before the addition of hamster oöcytes.

The effects of the four different incubation conditions employed (normosmotic, hyperosmotic, 50μM and 100μM A23187) are illustrated in Figures 3.1 and 3.2 (pp 122-123), from which the capacity of A23187 to maximise the level of sperm-oöcyte fusion whilst causing only minimal alterations to sperm motility is clearly seen. Motility in the normosmotic control incubations was 46.5 ± 2.65% (mean ± SEM), whilst in the presence of 50μM A23187 motility was 44.0 ± 2.71%, falling to 33.1 ± 5.34% in the presence of 100 μM A23187. The percentage of zona-free hamster oöcytes

penetrated in the control incubation was only 4.65 ± 1.77% (mean ± SEM), rising to 9.52 ± 2.85% under hyper osmotic conditions and to 38.56 ± 5.67% and 31.80 ± 5.34% in the presence of 50 and 100μM A23187, respectively. These latter two differences were found to be significant ($P < 0.001$) on paired *t* - testing. In addition, the levels of ATP measured on these washed sperm suspensions following 3 hours of incubation in the conditions described were found to be significantly altered, from 2.94 ± 0.45 μM (mean ± SEM) in the normosmotic control to 2.39 ± 0.40, 2.41 ± 0.49 and 2.40 ± 0.45 μM in the hyperosmotic, and calcium ionophore stimulated incubations, respectively (all $P < 0.02$ on paired *t* -testing with the control).

The concentration of ATP in semen was strongly correlated with the concentration of spermatozoa ($r = 0.697$, $P < 0.001$, $n = 38$) and with the concentration of motile spermatozoa ($r = 0.745$, $P < 0.001$, $n = 38$) (Figure 3.3, p 124), and this relationship remained when correction was made for semen turbidity ($r = 0.59$, $r = 0.623$, respectively, $P < 0.001$, $n = 36$) (Figure 3.4, p 125). In contrast, there was no relationship between motility and either uncorrected ATP ($r = 0.282$, P = not significant, $n = 38$) or ATP corrected for turbidity ($r = 0.359$, P = not significant, $n = 35$) (Figure 3.5, p 126).

Relationships with the outcome of zona-free hamster oöcyte penetration testing are shown in Table 3.1 (p 113), and in Figures 3.6 and 3.7 (pp 127-128). For each correlation, the maximum number of data pairs available were used. From the data shown in Table 3.1 (p 113), it can be seen that none of the parameters measured in semen, namely, sperm concentration, motility or ATP concentration, was related to the outcome of the egg penetration test (expressed as percentage of oöcytes penetrated) when the

latter was performed under normosmotic or hyperosmotic conditions. In contrast, there were significant positive correlations between each of the variables listed and the outcome of the egg penetration test performed in the presence of the calcium ionophore A23187. The strongest relationship seen was between ATP (uncorrected for turbidity) and the percentage of zona-free hamster eggs penetrated in the presence of 100μM A23187 (r = 0.674, P < 0.001, n =36). In addition, there were significant positive correlations between the percentage of zona-free oöcytes penetrated in the presence of 100μM A23187 and the sperm concentration and motility observed in the original semen sample (r = 0.534, P < 0.01, n =35, and r = 0.403, P < 0.05, n =36, respectively). These three relationships, between the percentage of zona-free oöcytes penetrated and the sperm concentration, sperm motility and ATP concentration, were also found in the case of the 50μM A23187 stimulated test (all p < 0.001). Motility and ATP measured on the washed spermatozoa at the time of egg penetration testing were also unrelated to the outcome of the egg penetration test except in the cases of the 50μM and 100μM ionophore tests, in which motility at the time of testing was correlated with the percentage of zona-free oöcytes penetrated (r = 0.583, P < 0.001, n =31, and r = 0.403, P < 0.02, n =36, respectively).

Three main points emerge from this analysis. Firstly, the concentration of ATP in semen is positively correlated with the number of spermatozoa present. Secondly, the concentration of ATP in semen is positively correlated with the percentage of zona-free oöcytes penetrated in the 50- and 100 μM A23187 stimulated incubations. Thirdly, both the concentration of spermatozoa in semen and sperm motility are positively correlated with the percentage of zona-free oöcytes penetrated in the 50- and 100 μM

A23187 stimulated incubations. This raises the possibility that the positive correlation observed between ATP in semen and sperm function as measured by these ionophore stimulated incubations is simply due to the significance of the concentration of motile cells in the outcome of these assessments.

This possibility was investigated further by performing multiple regression analyses with the use of the percentage of oöcytes penetrated as the dependent variable. In the case of the 100μM A23187 penetration test, sperm concentration and motility together explained 35.37% of the variance in the percentage of oöcytes penetrated (R = 0.595), and the inclusion of the ATP concentrations in this relationship explained an additional 13.81% of this variance. Thus all three variables together were able to account for 49.81% of the variance in the outcome of oöcyte penetration testing (R = 0.701). In the case of the 50μM A23187 penetration test, sperm concentration and motility together explained 64.89% of the variance in the percentage penetration (R = 0.806), and the inclusion of the ATP concentration only improved this by 3.81%, making the percentage of variance explained by all three variables together 68.70% (R = 0.829). These results suggest that the majority of the the information contributed by ATP in its relationship with the outcome of the oöcyte penetration test is simply information on density and motility.

It was thought that the relationships found might have been artefactual, arising from the fact that the patient group studied was heterogeneous and included a small number of oligospermic patients who would tend to have low ATP levels, because of their reduced sperm numbers, and who would have intrinsically poor sperm function (Aitken et al, 1982c; Aitken, 1985).

However, when those patients with sperm concentrations below 20 x 10^6/ml (n = 6), were eliminated from the analysis, the relationship between ATP in semen and the percentage of zona-free oöcytes penetrated in the 100μM A23187 stimulated incubation persisted (r = 0.708, P < 0.001, n = 30).

The importance of sperm number in explaining the above correlations is again emphasized if we consider the relationship between ATP in semen, uncorrected for light scattering, and the outcome of the egg penetration test in the presence of 100μM ionophore, for which r = 0.674 (P <0.001, n = 36). When the ATP measurement was corrected for light scattering (which will be affected by density and motility), the correlation was reduced to r = 0.551 (P <0.001, n = 36). However, when the ATP measurement was corrected for density and turbidity, and the result expressed as picomoles ATP/10^6 cells, the relationship became insignificant (r = 0.196, P = not significant) (Figure 3.8, p 129). This would tend to support the view that the amount of information contributed by ATP, per se, to the apparent relationship with the oöcyte penetration test is small.

Adenosine triphosphate in the assessment of the fertility of cryostored donor semen

One hundred and two cryostored ejaculates were analysed. Measurements were made of sperm concentration, motility, and ATP concentration on the thawed samples, and after the isolation of the spermatozoa on albumin columns, these measurements were repeated. From the group of 102 ejaculates, 27 had succeeded in producing pregnancies, and were therefore classified as 'successful', whereas 21 ejaculates had been unsuccessful in producing pregnancies, despite four or more cycles of insemination into different recipients, and were therefore classified as

'unsuccessful'. It is of course recognised that this distinction is not absolute and that, in any case, fertility is a relative, rather than an absolute, concept. Nevertheless, it was felt that this classification would provide a useful basis from which to approach the statistical analysis of the data. The remaining 54 ejaculates had failed to achieve pregnancies, but at the time of analysis, had been inseminated for three cycles or fewer, and were for this reason excluded from the analysis of individual ejaculates.

Comparisons were made between the successful and unsuccessful groups of ejaculates with regard to the various criteria of semen quality presented in this study. This analysis indicated that no significant differences existed between the fertile and unsuccessful groups with respect to density, motility, ATP or ATP corrected for light scattering, density or motility before or after isolation of the spermatozoa on the albumin columns (Tables 3.2 and 3.3, pp 114-115). However, a significant difference was observed between the groups in respect to the density of motile cells in the cryostored preparation ($t = 2.20$, $P < 0.05$) (Table 3.2, p 114).

In a further attempt to distinguish between the successful and unsuccessful donors to the AID programme on the basis of ATP measurements, a multivariate discriminant analysis was employed. The same group of 48 ejaculates was analysed, comprising 27 successful and 21 unsuccessful ejaculates; the variables available for the analysis were as above, namely, conventional criteria of semen quality and ATP (uncorrected and corrected for light scattering, density and motility), both before and after the separation of spermatozoa on albumin columns (Table 3.4, p 116). With the use of these variables, and with a stepwise selection method with maximization of Rao's V, it was not possible \to achieve significant discrimination between

the successful and unsuccessful groups. The overall percentage of grouped cases correctly classified was 66.6% (P = 0.0813).

Because significant differences in semen quality exist between ejaculates from the same individual (Aitken and Elton, 1984; Poland et al, 1986), the practice of averaging the results obtained for several different ejaculates from one donor is not statistically valid. For this reason, each ejaculate was treated as an individual unit in the above analysis. However, because a positive correlation has been reported (Comhaire et al, 1983) between the mean ATP concentrations recorded for multiple ejaculates from individual semen donors and their relative fertility, this form of analysis was repeated on the present data set.

The 102 ejaculates analysed had been produced by a panel of 25 donors, 13 of whom had achieved pregnancies by AID and 12 of whom had failed to do so, all donors having been used for a minimum of 3 cycles of insemination. A mean value for each criteria of semen quality was calculated for each donor, as described by Comhaire and colleagues (Comhaire et al, 1983), and these mean values were then used for comparison of those donors who had achieved a pregnancy with those who had not achieved a pregnancy. Again, simple t -testing failed to reveal any meaningful differences between the successful and unsuccessful donors (Tables 3.5 and 3.6, pp 117-118), and a multivariate discriminant analysis was equally unable to discriminate between them. Relative fertility was then calculated from the number of pregnancies produced by a given donor and the number of cycles of insemination over which his ejaculates had been used, and this was expressed as a percentage (pregnancies/cycle). With the use of this index of relative fertility as the dependent field, linear

regression analysis was performed for each of the measured parameters, but again, no significant relationships were found (Table 3.7, p 119).

If those donors who succeeded in achieving pregnancies were considered separately, as in the report of Comhaire et al (1983), then a significant positive correlation was found between fertility and the concentration of ATP measured after separation of the spermatozoa on the albumin columns (r = 0.65, P <0.02, n = 13) (Table 3.8, p 120). This relationship was examined further in a multiple regression analysis (Table 3.9, p 121). From these results, it can be seen that the motility and concentration of spermatozoa in the original samples explained only 10.0% of the variance in fertility and that the ATP concentrations improved this figure by only 0.3%. However, the concentration and motility of cells recovered from the albumin columns explained a further 26.3% of this variance, and the ATP measured on these samples improved this figure by a further 28.8%. Finally, when all six variables were employed in a multiple regression equation, 65.5% of the variation in relative fertility of a group of successful donors could be explained (R = 0.809) (Table 3.9, p 121).

3.4 Discussion

The subcellular biochemical economy of the sperm cell is complex, and although ATP is undoubtedly a major component of this economy, there are many others; it would be surprising if all defective sperm function could be reflected in variations in the level of a single biochemical parameter. As has been mentioned above, human sperm generate ATP principally by glycolysis (Peterson and Freund, 1970), to a much lesser extent by oxidative phosphorylation (Ford and Harrison, 1981), and they utilize this

ATP as a source of energy for motility, for the maintenance of ionic gradients and as a source of cyclic AMP (Durr et al, 1972; Menon and Gunaga, 1974; Suter et al, 1979). Although its has been shown that the ATP concentration of an ejaculate is positively correlated with the concentration of spermatozoa and their motility (Calamera et al, 1982), given that there is no fixed relationship between the concentration of motile spermatozoa in an ejaculate and fertility (Smith et al, 1977; Glass and Ericsson, 1979; Aitken et al, 1984a), then there is no deductive reason to expect a relationship between ATP and fertility.

The present study has confirmed the established finding of a strong positive correlation between ATP concentration in semen and both sperm concentration and motile sperm concentration. In addition, Comhaire et al (1883) have reported the existence of a strong positive correlation between the concentration of ATP per milliliter of fresh ejaculate and the percentage of zona-free hamster oöcytes penetrated by the spermatozoa. They found that this relationship was true for both successful donors and the male partners of normal wives. The present study of a group of ejaculates from the male partners of normal wives has also revealed the existence of a similar correlation between ATP concentration and hamster oöcyte penetration. Furthermore, this relationship was shown to be independent of the inclusion of oligozoospermic patients within the study group. Despite the ability of the present study to confirm the existence of a positive correlation between ATP levels and the outcome of the hamster oöcyte penetration test, the multiple regression analyses presented above indicate that the majority of the relationship between ATP and the outcome of the egg penetration test is dependent on the strong positive relationships that exist between ATP and sperm number. The inclusion of ATP, per se, could only improve our

capacity to explain the outcome of the egg penetration test by between 3.8% and 13.8%. This is in agreement with the fact that the relationship between ATP and the outcome of the egg penetration test becomes non-significant when the ATP measurement is corrected for both turbidity and sperm concentration. Thus it is apparent that although the measurement of ATP does contain some useful information on likely sperm function, its usefulness in this regard is limited. Nevertheless, it is noteworthy that 68.7% of the variation in percentage egg penetration can be explained, in the case of the 50μM A23187 test, by the combination of sperm density, motility and ATP level, and this fact may be of value in situations in which zona-free hamster oöcyte penetration testing is not available.

This raises a point of interest with regard to the methods used to perform the zona-free hamster oöcyte penetration test. All of the relationships which were found (sperm concentration, motility and ATP) with the outcome of this test existed only for those tests performed in the presence of the ionophore A23187, and no relationships were observed in the absence of A23187, regardless of whether the incubations were performed in normosmotic or hyperosmotic media. These results would tend to support the view that such a methodology for the performance of the egg penetration test maximises the ability of the test to examine the full functional competence of the male gamete (Aitken et al, 1984b). Comhaire et al (1983) have reported the existence of significant differences between successful donors and the husbands of normal wives attending an infertility clinic in respect of their mean semen ATP levels. The present comparison of successful and unsuccessful ejaculates used in an AID program was unable to confirm this finding, and, moreover, a multivariate discriminant analysis employing conventional criteria of semen quality and ATP measurements was unable

to distinguish significantly between these two groups. Likewise, it has been reported (Comhaire et al, 1983) that a positive correlation exists between the relative fertility *in-vivo* (expressed as percentage pregnancies per cycle) of a semen donor and the mean ATP concentration of a group of his ejaculates. Again, the present study of successful and unsuccessful donors was unable to confirm this finding, and it was noted that neither comparative statistics nor multivariate discriminant analysis revealed any significant differences between the groups. Nevertheless, when successful donors alone are considered, a combination of data based on conventional criteria of semen quality and ATP levels before and after isolation of the spermatozoa on albumin columns can yield a significant relationship with relative fertility (R = 0.809). Although interesting, this information is of little practical value, because in a clinical setting one is more interested in distinguishing between those ejaculates that can and those that cannot achieve a pregnancy than in obtaining a quantitative assessment of the relative fertility of a successful ejaculate.

In conclusion, the present study has confirmed several aspects of the study by Comhaire et al (1983) in revealing positive relationships between ATP levels and the performance of spermatozoa in both the hamster oöcyte penetration test and an AID programme. The clinical value of these relationships is, however, questionable. In the case of the egg penetration test, the significance of the ATP determinations appeared to be largely due to the correlations that exist between this factor and sperm number. If the results were expressed in terms of sperm concentration (ATP/10^6 spermatozoa), then the correlations with hamster egg penetration disappeared. Although a correlation was observed with the outcome of use of semen in AID, it applied only to the relative fertility of successful donors.

ATP measurements were not capable of distinguishing successful from unsuccessful donors and for this reason may be of limited practical value in routine andrologic assessments.

Table 3.1

Relationship between various parameters measured in human semen and the outcome of zona-free hamster oöcyte penetration testing. Correlation coefficients (*r*) obtained by simple linear regression.

Parameter	Correlation Coefficients *r* (*n*)			
	Normos-motic	Hyperos-motic	50μM A23187	100μM A23187
Sperm Concentration (10^6/ml)	0.266 (32)	0.022 (34)	0.562 (30)[a]	0.534 (35)[b]
Motility (%)	0.306 (32)	0.115 (35)	0.583 (31)[a]	0.403 (36)[c]
ATP (μM)	0.191 (32)	0.187 (35)	0.566 (31)[a]	0.674 (36)[a]
ATP corrected for turbidity (μM)	0.007 (30)	0.172 (33)	0.435 (29)[d]	0.551 (34)[b]

[a] $P < 0.001$
[b] $P < 0.01$
[c] $P < 0.05$
[d] $P < 0.02$

Table 3.2

Comparisons between a group of successful (*n* = 27) and unsuccessful (*n* = 21) ejaculates used in an AID programme in terms of the parameters measured on the frozen-thawed samples. (*t* - test or *t - statistic)[a].**

Parameter	Successful Ejaculates (Mean ± SEM)	Unsuccessful Ejaculates (Mean ± SEM)	Comparison *t*	*P*
Sperm concentration (10^6/ml)	50.22 ± 5.03	44.67 ± 8.21	0.60	NS
Motility (%)	32.22 ± 2.29	27.62 ± 2.38	1.38	NS
Concentration of motile cells (10^6/ml)	15.24 ± 1.49	10.52 ± 1.49	2.20	<0.05
Uncorrected ATP (μM)	3.41 ± 0.40	2.69 ± 0.42	1.21	NS
Corrected ATP (μM)	5.02 ± 0.75	5.64 ± 1.32	-0.41	NS
ATP/10^6 cells (pM)	111.66 ± 17.13	146.63 ± 29.30	-1.08	NS
ATP/10^6 motile cells (pM)	378.91 ± 59.13	602.99 ± 141.34	-1.46	NS

[a]SEM = standard error of the mean; NS = not significant

Table 3.3

Comparisons between a group of successful (*n* = 27) and unsuccessful (*n* = 21) ejaculates used in an AID programme in terms of the parameters measured after albumin column separation. (*t* - test or t^* - statistic)[a].

Parameter	Successful Ejaculates (Mean ± SEM)	Unsuccessful Ejaculates (Mean ± SEM)	Comparison *t*	*P*
Sperm concentration (10^6/ml)	20.18 ± 2.30	17.62 ± 4.34	0.55	NS
Motility (%)	57.26 ± 2.29	55.28 ± 3.16	0.48	NS
Concentration of motile cells (10^6/ml)	11.72 ± 1.29	8.38 ± 1.74	1.44	NS
ATP concentration (nM)	364.4 ± 47.00	383.1 ± 70.00	0.99	NS
ATP/10^6 cells (pM)	20.60 ± 2.83	21.95 ± 4.19	-0.28	NS
ATP/10^6 motile cells (pM)	37.48 ± 5.40	41.46 ± 8.61	-0.41	NS

[a]SEM = standard error of the mean; NS = not significant

Table 3.4

Multivariate discriminant analysis of ejaculates used in an AID programme : Variables available to the analysis

Frozen / thawed ejaculate :		
	Concentration of spermatozoa	10^6/ml
	Motility	%
	Concentration of motile spermatozoa	10^6/ml
	Uncorrected ATP	µM
	ATP corrected for turbidity	µM
	ATP /10^6 spermatozoa	nM
	ATP /10^6 motile spermatozoa	pM
Following Column Separation :		
	Concentration of spermatozoa	10^6/ml
	Motility	%
	Concentration of motile spermatozoa	10^6/ml
	Uncorrected ATP	µM
	ATP /10^6 spermatozoa	pM
	ATP /10^6 motile spermatozoa	pM

Table 3.5

Comparisons between the mean values for several ejaculates from a group of successful (*n* = 13) and unsuccessful (*n* = 12) donors contributing to an AID programme in terms of the parameters measured on the frozen-thawed samples. (*t* - test or *t - statistic)[a].**

Parameter	Successful Donors (Mean ± SEM)	Unsuccessful Donors (Mean ± SEM)	Comparison *t*	*P*
Sperm concentration (10^6/ml)	53.56 ± 6.08	49.38 ±11.69	0.32	NS
Motility (%)	28.53 ± 1.83	31.27 ± 3.69	-0.67	NS
Concentration of motile cells (10^6/ml)	14.23 ± 1.33	15.20 ± 4.16	-0.22	NS
Uncorrected ATP (μM)	3.50 ± 0.40	2.67 ± 0.52	0.81	NS
Corrected ATP (μM)	5.50 ± 0.75	5.14 ± 1.02	0.29	NS
ATP/10^6 cells (pM)	114.75 ±13.42	132.85 ±24.56	-0.66	NS
ATP/10^6 motile cells (pM)	462.61 ±50.20	481.70 ±91.414	-0.19	NS

[a]SEM = standard error of the mean; NS = not significant

Table 3.6

Comparisons between the mean values for several ejaculates from a group of successful (*n* = 13) and unsuccessful (*n* = 12) donors contributing to an AID programme in terms of the parameters measured after albumin column separation. (*t* - test or t^* - statistic)[a].

Parameter	Successful Donors (Mean ± SEM)	Unsuccessful Donors (Mean ± SEM)	Comparison *t*	*P*
Sperm concentration (10^6/ml)	28.16 ± 4.84	22.34 ± 5.32	0.81	NS
Motility (%)	54.70 ± 2.04	56.85 ± 3.26	-0.57	NS
Concentration of motile cells (10^6/ml)	14.37 ± 2.19	11.38 ± 2.30	0.94	NS
ATP concentration (nM)	366.3 ±44.0	391.8 ± 79.0	-0.29	NS
ATP/10^6 cells (pM)	18.59 ± 2.94	22.57 ± 3.61	-0.86	NS
ATP/10^6 motile cells (pM)	34.82 ± 5.52	41.11 ± 5.92	-0.78	NS

[a]SEM = standard error of the mean; NS = not significant

Table 3.7

Linear regression analysis (Pearson correlation coefficients) of various measurements on cryostored semen samples; Mean values per donor related to pregnancies per cycle percent for all donors (*n* =25)

Parameter	*r*	*P*
Frozen/thawed samples :		
Sperm concentration (10^6/ml)	-0.002	0.496
Motility (%)	-0.026	0.451
Concentration of motile cells (10^6/ml)	-0.022	0.457
Uncorrected ATP (μM)	0.140	0.252
Corrected ATP (μM)	0.020	0.463
ATP/10^6 cells (pM)	-0.072	0.365
ATP/10^6 motile cells (pM)	-0.076	0.359
Following albumin column separation :		
Sperm concentration (10^6/ml)	0.143	0.247
Motility (%)	0.033	0.439
Concentration of motile cells (10^6/ml)	0.236	0.128
ATP concentration (nM)	0.158	0.225
ATP/10^6 cells (pM)	-0.045	0.416
ATP/10^6 motile cells (pM)	-0.054	0.399

Table 3.8

Linear regression analysis (Pearson correlation coefficients) of various measurements on cryostored semen samples; Mean values per donor related to pregnancies per cycle percent for donors achieving pregnancies only (*n* =13)

Parameter	*r*	*P*
Frozen/thawed samples :		
Sperm concentration (10^6/ml)	-0.185	0.272
Motility (%)	0.301	0.159
Concentration of motile cells (10^6/ml)	0.077	0.401
Uncorrected ATP (μM)	0.019	0.476
Corrected ATP (μM)	-0.074	0.405
ATP/10^6 cells (pM)	0.116	0.353
ATP/10^6 motile cells (pM)	-0.145	0.318
Following albumin column separation :		
Sperm concentration (10^6/ml)	0.027	0.466
Motility (%)	0.370	0.107
Concentration of motile cells (10^6/ml)	0.193	0.264
ATP concentration (nM)	0.655	0.008
ATP/10^6 cells (pM)	0.241	0.214
ATP/10^6 motile cells (pM)	0.171	0.289

Table 3.9

Multiple regression analysis of various measurements on cryostored semen samples; Mean values per donor related to pregnancies per cycle per cent for donors achieving pregnancies only (*n* =13)

Parameter entered	*r*	% Variance explained	*R*
Sperm concentration (10^6/ml)	-0.18[a]	3.43	0.185
Motility (%)	0.30[a]	10.03	0.317
ATP corrected for turbidity (μM)	-0.07[a]	10.33	0.321
Sperm concentration following column separation (10^6/ml)	0.03[a]	35.45	0.595
Motility following column separation (%)	0.37[a]	36.63	0.605
ATP following column separation (μM)	0.65[b]	65.47	0.809

[a] *P* = not significant

[b] *P* < 0.02

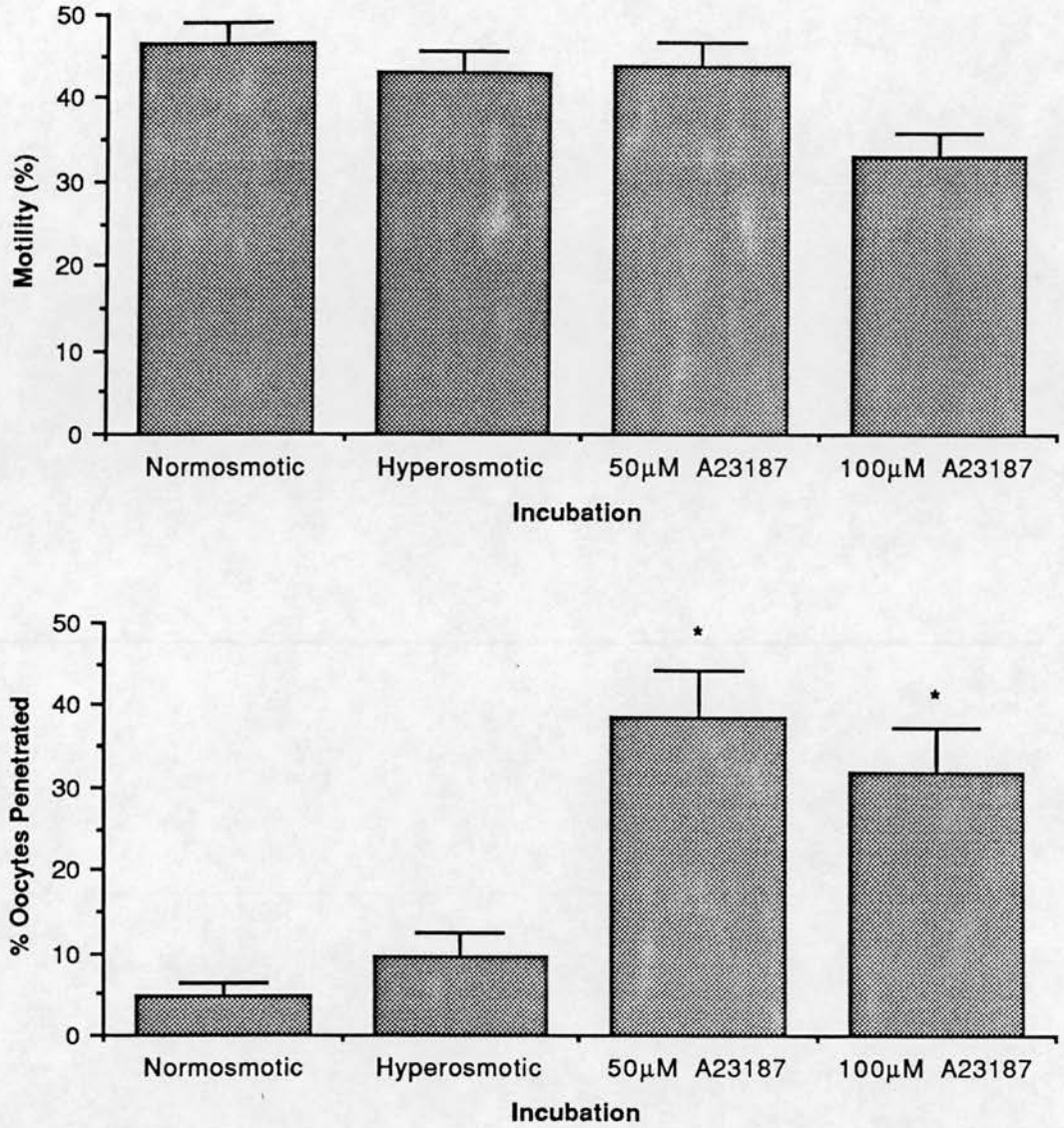


Figure 3.1

Effects of different incubation conditions employed in the zona-free hamster oöcyte penetration test on sperm motility and egg penetration

Effect of normosmotic, hyperosmotic, 50µM and 100µM A23187 media, (for details see text) on the motility of spermatozoa and the % of oöcytes penetrated. (* = $P < 0.001$, paired t-test with Normosmotic control). The capacity of A23187 to maximise sperm oöcyte fusion, while causing only minimal alteration in motility is clearly seen.

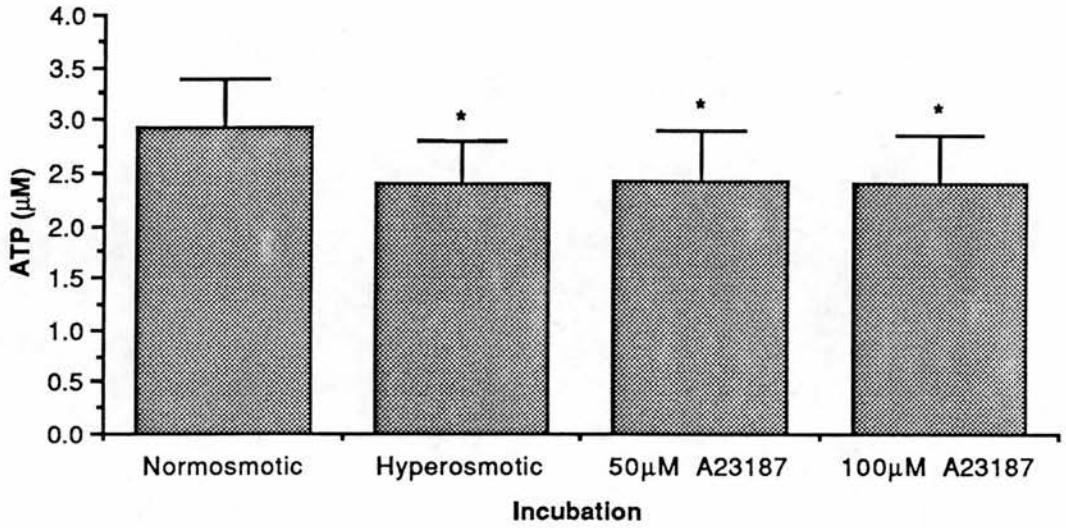
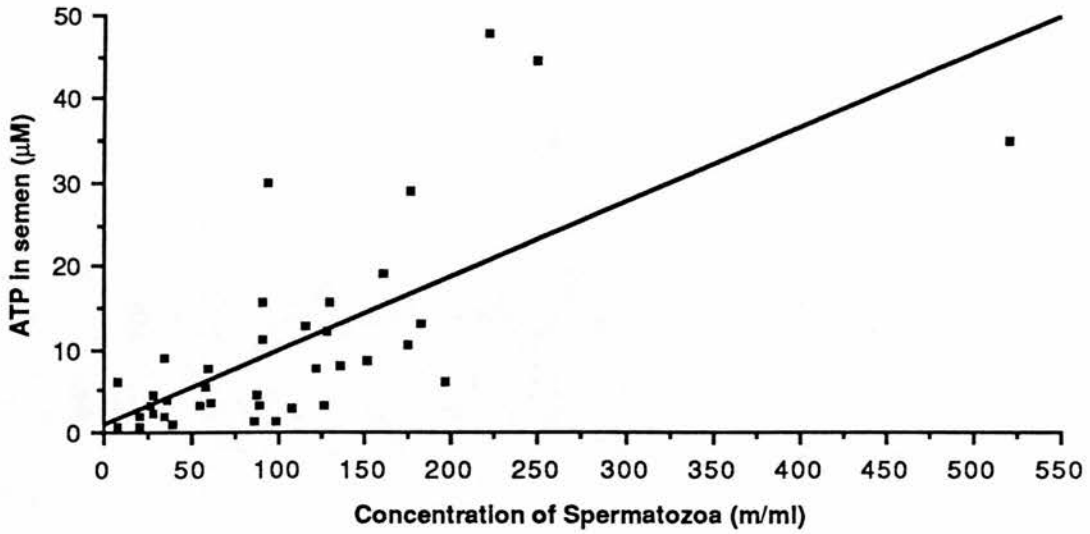


Figure 3.2

Effects of different incubation conditions employed in the zona-free hamster oöcyte penetration test on cellular ATP levels

The effect of the four different incubation conditions employed during the performance of the zona-free hamster oöcyte penetration test (Normosmotic, hyperosmotic, 50µM and 100µM A23187, for details see text) on the concentrations of ATP in the washed sperm suspensions. (* = $P <$ 0.02, paired t-test with Normosmotic control).



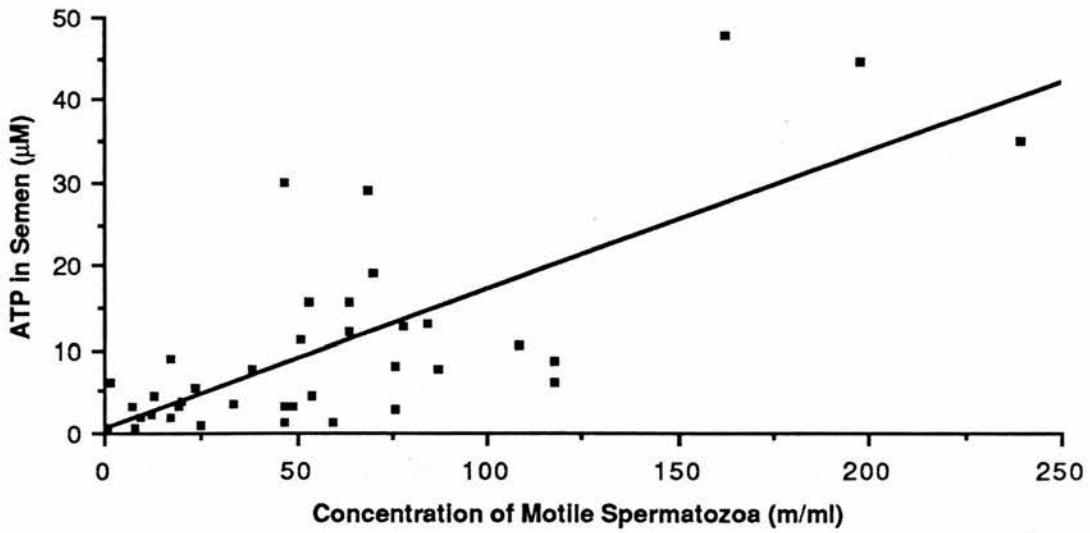


Figure 3.3

Relationships between ATP in semen and sperm concentration and motile sperm concentration

ATP concentrations in the semen of a group of male patients attending an infertility clinic, whose partners were normal. Upper graph shows the relationship between ATP in semen (µM) and sperm concentration (10^6/ml) (r =0.697, P<0.001, n = 38).

Lower graph shows the relationship between ATP in semen (µM) and motile sperm concentration (10^6/ml) (r =0.745, P<0.001, n = 38).

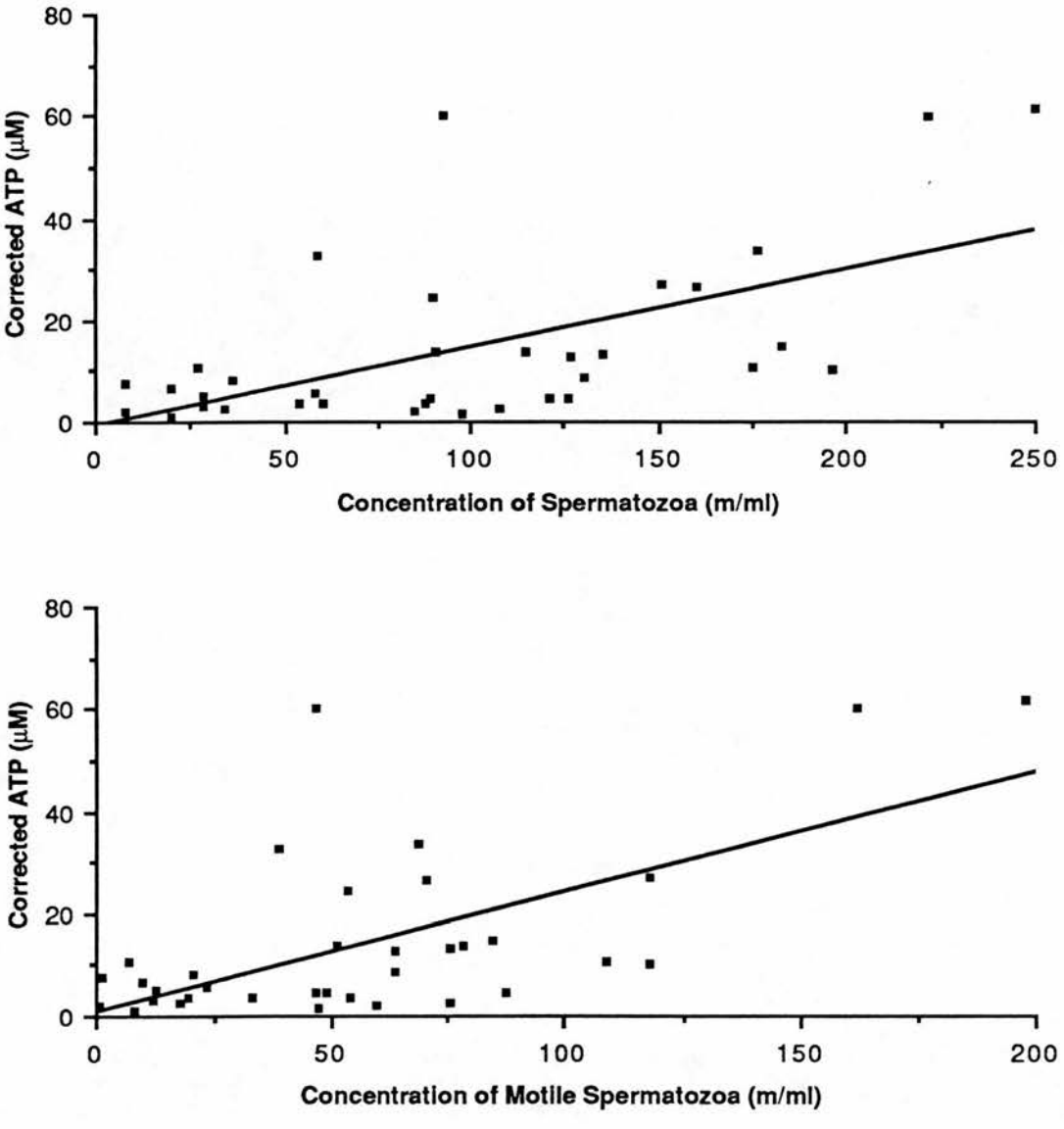


Figure 3.4

Relationships between ATP in semen, corrected for turbidity, and sperm concentration and motile sperm concentration

ATP concentrations in the semen of a group of male patients attending an infertility clinic, whose partners were normal. Upper graph shows the relationship between ATP in semen, corrected for turbidity (µM) and sperm concentration (10^6/ml) (r =0.59, P <0.001, n = 36). Lower graph shows the relationship between ATP in semen corrected for turbidity (µM) and motile sperm concentration (10^6/ml) (r =0.623, P <0.001, n = 36).

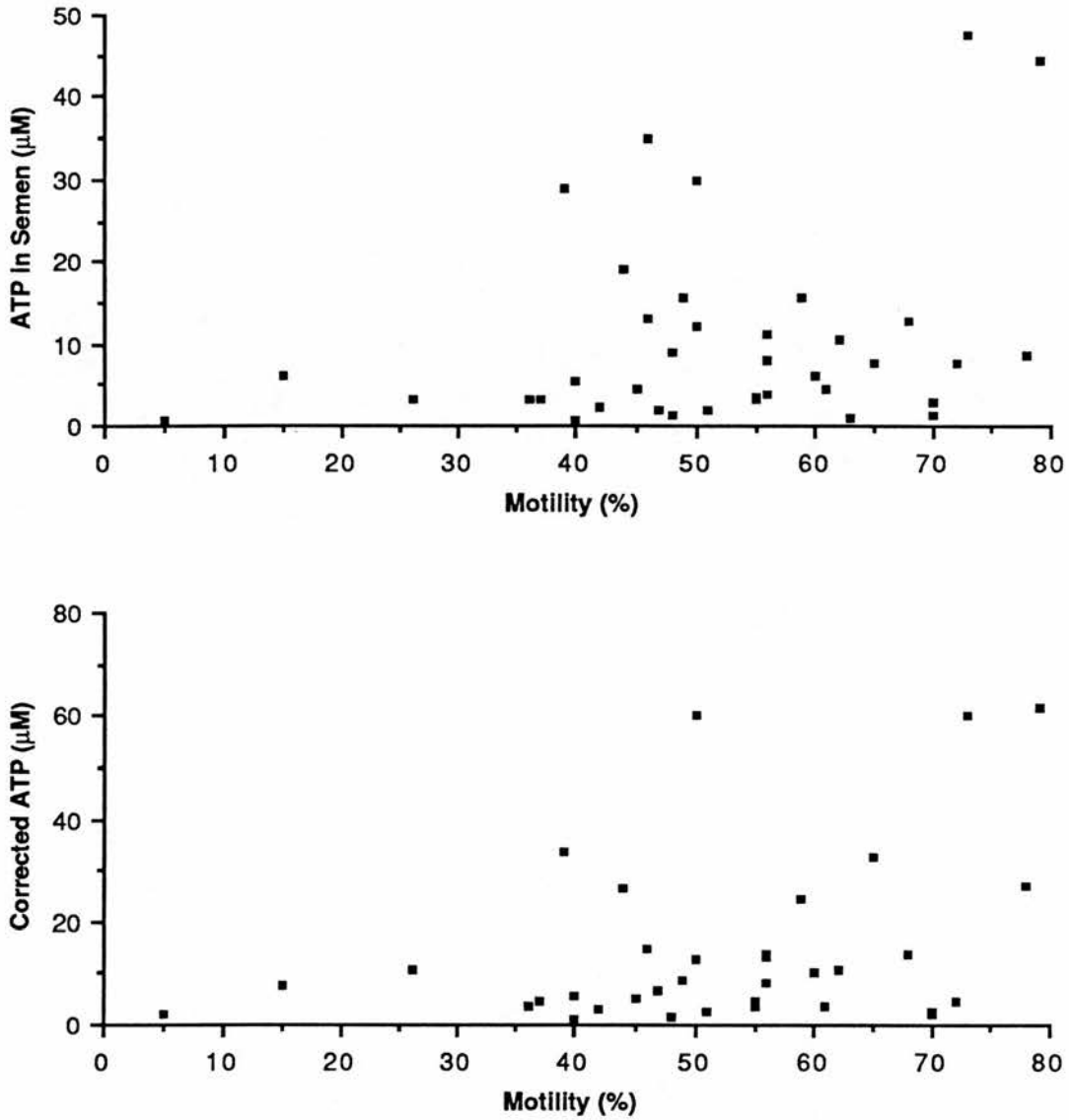


Figure 3.5

Relationships between ATP in semen and sperm motility

ATP concentrations in the semen of a group of male patients attending an infertility clinic, whose partners were normal. Upper graph shows the relationship between ATP in semen, (µM) and sperm motility (%) (r =0.282, P = NS, n = 38). Lower graph shows the relationship between ATP in semen corrected for turbidity (µM) and sperm motility (%) (r =0.359, P = NS, n = 38).

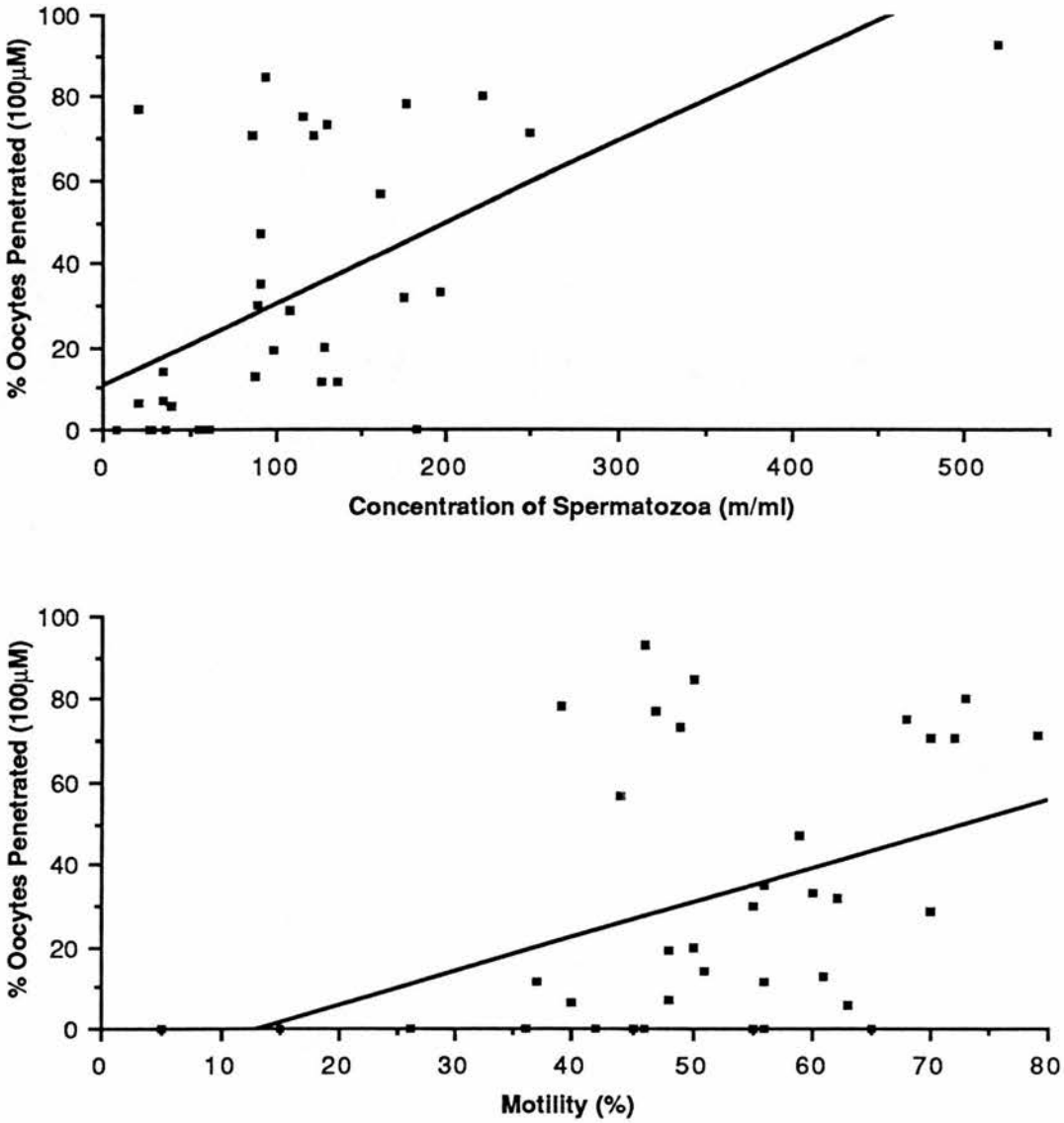
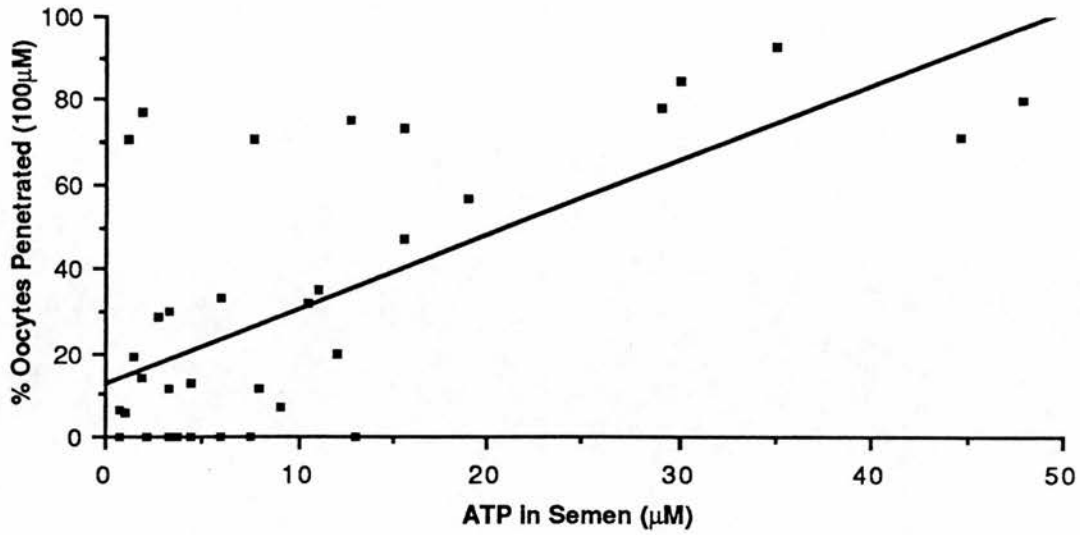


Figure 3.6

Relationships between hamster oöcyte penetration and sperm concentration and motility

In the semen of a group of male patients attending an infertility clinic, whose partners were normal. Upper graph shows the relationship between sperm density (10^6/ml) and % hamster oöcyte penetration in the presence of 100µM A23187 (r =0.534, P < 0.01, n = 35). Lower graph shows the relationship between sperm motility (%) and % hamster oöcyte penetration in the presence of 100µM A23187 (r =0.403, P < 0.05, n = 36).



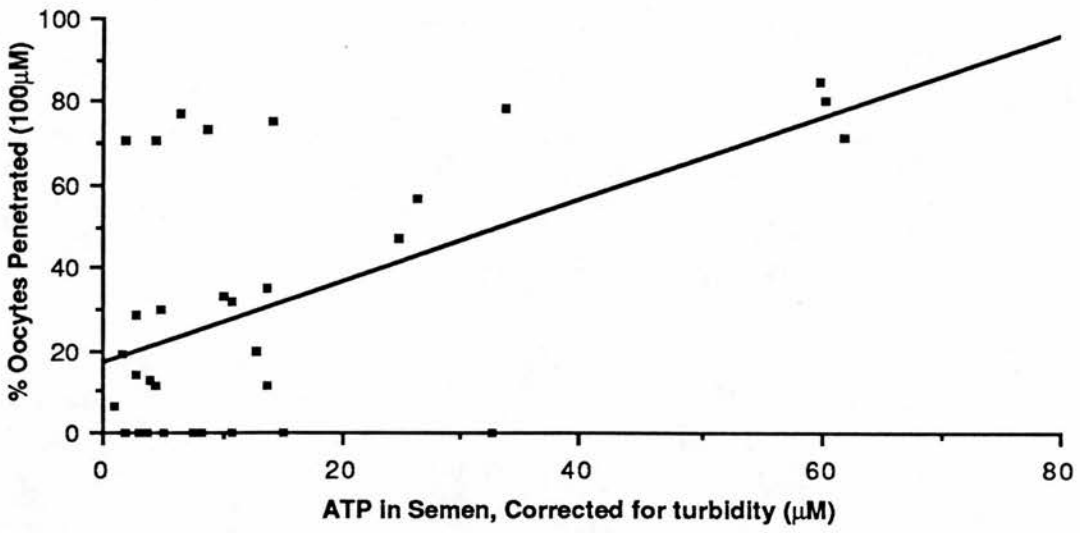


Figure 3.7

Relationships between ATP in semen and hamster oöcyte penetration

In the semen of a group of male patients attending an infertility clinic, whose partners were normal. Upper graph shows the relationship between ATP in semen (µM) and % hamster oöcyte penetration in the presence of 100µM A23187 (r =0.674, P < 0.001, n = 36). Lower graph shows the relationship between ATP in semen, corrected for turbidity (µM) and % hamster oöcyte penetration in the presence of 100µM A23187 (r =0.551, P < 0.01, n = 34).

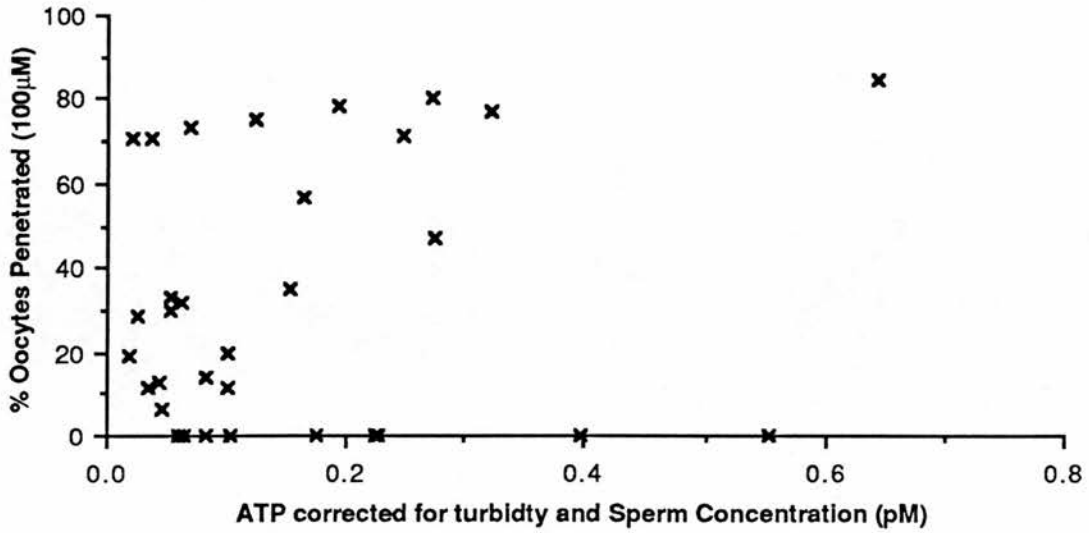


Figure 3.8

Relationships between ATP in semen expressed "per cell" and hamster oöcyte penetration testing

ATP concentrations in the semen of a group of male patients attending an infertility clinic, whose partners were normal. The graph shows the lack of any relationship between relationship between ATP in semen, corrected for turbidity and sperm concentration (pM ATP/10^6 cells) and % hamster oöcyte penetration in the presence of 100µM A23187 (r =0.196, P = NS, n = 36).

CHAPTER FOUR

THE USE OF *IN-VITRO* TESTS OF SPERM FUNCTION IN ASSESSING THE FERTILIZING ABILITY OF HUMAN SPERMATOZOA

4.1 Introduction

The central importance of the conventional criteria of semen quality in the clinical evaluation of the potentially infertile male has been outlined in Chapters One and Two. It was emphasized that these criteria, namely semen volume, sperm number, motility and normal morphology, are of a purely descriptive nature (World Health Organization, 1980; 1987), and are of limited value in assessing the ability of a given ejaculate to achieve fertilization *in-vivo* (Smith et al, 1977; Van Zyl et al, 1977; Glass and Ericsson, 1979; Åbyholm, 1980; Aitken et al, 1984a; Aitken, 1985). In an attempt to circumvent this problem, assays have been developed which examine the functional properties of the spermatozoa within a given ejaculate, rather than simply describing their quantity, shape and activity. For example, techniques have been described for quantitative analysis of the detailed movement characteristics of human spermatozoa (Overstreet et al, 1979; David et al 1981; Katz and Overstreet, 1981; Katz et al, 1981; Serres et al, 1984), while the zona-free hamster oöcyte penetration test has been frequently employed to assess the ability of human spermatozoa to capacitate, acrosome react and fuse with the vitelline membrane of the oöcyte (Barros et al, 1979; Rogers et al, 1979; 1983; Binor et al, 1980; Matthews et al, 1980; Overstreet et al, 1980; Hall, 1981; Karp et al, 1981; Tyler et al, 1981; Aitken et al, 1982a; b; c; 1983a; b; c; 1984a; b; Martin et al, 1982; Stenchever et al, 1982; Templeton et al, 1982; Wickings et al, 1983; Aitken and Elton, 1984; 1986; Blasco, 1984; Yanagimachi, 1984; Aitken , 1985; Rogers, 1985; Shalgi et al, 1985). In addition, the possible value of ATP estimation in assessing the fertilizing ability of human spermatozoa *in-vivo* and *in-vitro* has been suggested (Comhaire et al, 1983) and this has been discussed extensively in Chapter Three. These tests of sperm function

have been widely applied to normal fertile men (Aitken et al, 1982a), to couples with unexplained infertility (Aitken et al, 1982b; 1984a) and to couples with oligozoospermia (Aitken et al, 1982c; 1984b; Aitken, 1985) and the results of prospective clinical studies (Aitken et al, 1984a; Aitken, 1985) indicate that they may be of significant value in predicting the fertilizing ability of human semen *in-vivo* .

However, the results of such prospective studies are inevitably confounded by the existence of significant inter-ejaculate variability in the conventional criteria of semen quality (Poland et al, 1986) and in the functional competence of the spermatozoa (Rogers et al, 1983). Hence the properties of a sample analysed at the beginning of such a prospective study may not reflect the attributes of the actual sample which successfully initiated a pregnancy. Clearly, an accurate assessment of the relationship between the results of sperm function tests *in-vitro* and fertility *in-vivo* can only be obtained if these determinations are made on the same ejaculate.

In addition, the possible confounding effect of undiagnosed pathology occurring in the female partner must be taken into account. However carefully such patients are evaluated (see Chapter One), the possibility of undetectable pathology causing subfertility cannot be excluded (Foss and Hull, 1986) and if present would erroneously result in an ejaculate being classified as infertile. Whilst it is not possible to circumvent this problem completely, the confounding effect of undiagnosed female pathology can be minimised by maximising the number of female recipients exposed to an ejaculate.

Such an analysis can only be carried out within the context of an AID (artificial insemination by donor) service. In this situation, a cryostored sample of human semen may be subjected to a number of laboratory tests and then individual aliquots of the same sample can be inseminated into several independent recipients, each of whom has ben carefully screened to eliminate any detectable pathology. This design uniquely permits an accurate evaluation of the diagnostic value of *in-vitro* tests, while, as far as possible, avoiding the confounding effects of variations in the fertility of the female partner.

4.2 Materials and Methods

Patients

As in Chapter Three, semen samples were obtained by masturbation from a a panel of healthy semen donors who had been proven to be normospermic within limits previously defined for our local population (Chapter Two; Aitken et al, 1982a; Templeton et al, 1982) (sperm concentration $\geq 20 \times 10^6$/ml, motility $\geq$ 40% motile spermatozoa, morphology $\geq$ 40% normal forms). These semen samples were used for therapeutic donor insemination and were frozen and stored according to the techniques described by Jackson and Richardson (1977) and described in detail in Chapter Three. The male partners of couples undergoing treatment by therapeutic donor insemination normally suffered from azoospermia or oligozoospermia (sperm concentration $< 20 \times 10^6$/ml while their female partners had been demonstrated to be normal within limits previously defined (Templeton et al, 1982; Aitken et al, 1984a), and thus had normal findings on routine history and examination (see Chapter One), regular menstrual cycles within the limits 28 $\pm$ 4 days, ovulatory levels of

progesterone in the luteal phase of the cycle (documented in at least two cycles) and normal pelvic anatomy with bilateral tubal patency demonstrated at diagnostic laparoscopy and dye hydrotubation (Templeton and Kerr, 1977). Intracervical insemination was performed once in each cycle of treatment with 0.8ml of thawed cryostored semen and was timed to coincide with ovulation, which was determined with the use of daily plasma luteinising hormone radioimmunoassays (Djahanbakhch et al, 1981). Each cycle of treatment was ovulatory, as judged by luteal phase progesterone estimations. An ejaculate was regarded as successful if a pregnancy was achieved and unsuccessful if the ejaculate failed to produce a pregnancy after four or more inseminations into different recipients.

Laboratory evaluation of semen

Aliquots of the cryostored semen samples were removed from the liquid nitrogen storage container and allowed to come to room temperature. They were then warmed in an incubator at 37°C in an atmosphere of 5% CO_2 in air until the sample reached 37°C. The analyses performed on these sample fell into the four major areas : (i) Sperm concentration and motility, (ii) Measurement of ATP, (iii) Assessment of sperm movement characteristics, and (iv) Zona-free hamster oöcyte penetration. Sperm concentration, motility and the measurement of ATP could be performed directly on the thawed semen sample, however, in order to assess both the movement characteristics and zona-free hamster oöcyte penetration it was necessary to was the spermatozoa free of the cryoprotective medium. This was achieved using a modification of the procedure described by Ericsson et al (1973), described in detail in Chapter Three, which entailed the mixing of the cryostored semen with BWW medium (Biggers et al, 1971) and layering this mixture over columns of human serum albumin in BWW.

Collection of the lower fractions of these columns after a period of incubation resulted in a preparation of spermatozoa free from the cryoprotective buffer.

Sperm concentration and motility : Assessments were made of sperm concentration (10^6/ml) and motility (%) on both the thawed semen samples and the spermatozoa separated on the albumin columns using standard laboratory techniques (World Health Organization, 1980; 1987) as outlined in Chapter Two.

ATP measurement : ATP was measured using a bioluminescent assay as described in Chapter Three. Measurements were performed on both the thawed semen sample and the spermatozoa separated on albumin columns, and in the case of the former, turbidity was corrected for by the use of an internal standard solution of ATP.

Sperm movement characteristics : These were studied according to the general procedure of Overstreet et al (1979) and the detailed methodology of Aitken et al (1982a). In outline, a 10µl drop of the suspension of spermatozoa which had been isolated on the albumin columns was placed on a microscope slide under a glass cover slip, having a chamber depth of 21µm and was examined at x 250 under dark field illumination, while a heated microscope stage was used to keep the sample at 37°C throughout. Samples were photographed using a Polaroid® camera attachment and a time-exposure system set to allow an accurate exposure time of 1 second. The tracks left by the spermatozoa (Figure 4.1, p 161) were then analysed following examination of the photographs under a low-power (x6) magnification system and the following parameters of sperm

movement were measured : (i) mean velocity (μm/sec), (ii) mean amplitude of lateral head displacement (μm), (iii) mean frequency of head rotation (/sec), (iv) yawing spermatozoa (%), (v) rolling spermatozoa (%), (vi) spermatozoa with a minimal (<4.5μm) amplitude of lateral head displacement (%) (Overstreet et al, 1979). All the above measurements were repeated on the subpopulation of progressively motile spermatozoa, characterised by a linear velocity of progression in excess of 25μm/sec (Overstreet et al, 1979).

Zona-free hamster oöcyte penetration testing : This was performed according to the detailed methodology of Aitken et al (1984b) and as described in detail in Chapter Three. In outline, the spermatozoa which had been isolated on albumin columns were resuspended at a concentration of 10 x 10^6/ml in medium BWW containing a 100μM aqueous suspension of the Ca^{++}, Mg^{++} salt of the divalent cation ionophore A23187 (Calbiochem, Bishops Stortford, England). The spermatozoa were incubated under these conditions for 3 hours, after which they were centrifuged and resuspended at a concentration of 10 x 10^6/ml in normal medium BWW prior to the introduction of zona-free hamster oöcytes. The incubations were continued for a further 3 hours, at which time the oöcytes were examined for the presence of decondensing sperm heads with an attached or closely associated tail (Figure 4.2, p 162). The use of A23187 in this modified zona-free hamster oöcyte penetration test aims to circumvent the problem of inter-individual variation in capacitation time (Perreault and Rogers, 1982) by enabling the spermatozoa to capacitate and acrosome react simultaneously, thus maximising the ability of the assay to examine the full functional competence of the gametes (Aitken et al, 1984b). The details of

the effects of A23187 on sperm physiology will be explored in detail in subsequent Chapters of this thesis.

Statistical Analysis

Comparisons between groups were performed with the use of the *t*-test, once the normal distribution of the data had been checked and the equality of variances assessed by the variance ratio; only differences with $P < 0.05$ were considered significant. Where unequal variances were found, the comparison was made with the use of the t* statistic, which employs separate rather than pooled variance estimates. Discrimination between groups employed a multivariate discriminant analysis following Rao's method, with the aid of the Statistical Package for the Social Sciences (SPSS) (Nie et al, 1975).

4.3 Results

A total of 48 ejaculates was analysed, produced by a panel of 21 semen donors. Of these 48 ejaculates, 27 were classified as successful, in that they produced one or more pregnancies following insemination at an average rate (± SEM) of 3.1 ± 0.3 inseminations per pregnancy. In contrast, the remaining 21 ejaculates were considered unsuccessful since they failed to produce a pregnancy despite having been used in at least four accurately timed cycles of insemination into at least four different recipients (mean ± SEM number of inseminations for each sample in this group being 5.3 ± 0.3). Five donors produced both successful and unsuccessful ejaculates. It is clear that any ejaculate whose use results in the initiation of a pregnancy can be accurately classified as successful, however, the classification of an ejaculate as unsuccessful following four or more inseminations requires that

an ejaculate have a pregnancy rate per cycle > 25% in order to be classified as successful. A pregnancy rate per cycle of this order is rather higher than that normally reported for the use of cryostored semen (Thorneycroft et al, 1984) and may be attributed to the accuracy with which inseminations were timed in the present study. Following the analyses set out above, 25 measured or derived variables were available for each ejaculate, and these are listed in Table 4.1 (p 148), divided into the four groups of analyses performed, namely sperm concentration and motility, ATP measurements, movement characteristics and the outcome of zona-free hamster oöcyte penetration testing.

Direct comparisons between the successful and unsuccessful groups were made in respect of the mean value of each variable measured using the *t* - test or t^*-statistic. These comparisons are set out in Table 4.2 (p 149) for sperm concentration and motility, Table 4.3 (p 150) for ATP measurements, Tables 4.4 and 4.5 (pp 151-152) for the movement characteristics and Table 4.6 (p 153) for the outcome of the zona-free hamster oöcyte penetration test. It can be seen that statistically significant differences were found in the case of only three of the 25 variables measured, namely the concentration of motile cells in the original cryostored ejaculate ($t = 2.20$, $P = 0.033$), the % of zona-free hamster oöcytes penetrated ($t^* = 2.14$, $P = 0.040$) and the mean number of spermatozoa per hamster oöcyte ($t^* = 2.30$, $P = 0.028$) (Figure 4.3, p 163). No significant differences were observed between the successful and unsuccessful groups in respect of any of the other variables measured (Tables 4.2 - 4.6, pp 149-153).

Multivariate discriminant analyses (Nie et al, 1975) were then employed to to determine whether the data generated by a combination of variables

could discriminate between the successful and unsuccessful ejaculates. When sperm concentration, motility and motile sperm concentration alone were employed, it was not possible to discriminate between these two groups of ejaculates (P = 0.2621). However, if , in addition to these parameters, the movement characteristics of the spermatozoa were included in the analysis, then significant discrimination between the groups was achieved (P = 0.0489).

The variables selected as being of value in this discrimination are listed in Table 4.7 (p 154), along with their standardised discriminant function coefficients. It can be seen that the concentration of motile spermatozoa and their velocity of forward progression were both positively related with fertility, while a low amplitude of lateral head displacement was strongly negatively related to fertility, as was the frequency of rotation, proportion of rolling and proportion of yawing spermatozoa in the sub-population of progressively motile spermatozoa (i.e. those traveling at velocities > 25μm/sec). The ability of these six variables to distinguish fertile from infertile ejaculates is shown in Table 4.8 (p 155), which indicates that the accuracy of this particular set of discriminating variables was approximately equal for successful and unsuccessful samples, and that the overall accuracy of the classification was 72.92%.

If, instead of sperm movement characteristics, the results of the zona-free hamster oöcyte penetration test were employed in the discriminant analysis (in addition to sperm concentration and motility) then it was once more possible to achieve significant discrimination between the two groups of ejaculates (P = 0.0319). The variables selected as being of value in this discrimination are listed in Table 4.9 (p 156), along with their standardised

discriminant function coefficients. This shows that again the concentration of motile spermatozoa in the cryostored sample was positively related to fertility, as was the percentage of motile spermatozoa following incubation with A23187 and the result of the penetration assay expressed as mean number of spermatozoa per oöcyte. The overall ability of the discriminant function based upon these three variables to distinguish successful from unsuccessful samples was 75.00% (Table 4.10, p 157).

In contrast, when the ATP measurements were entered into the discriminant analysis in addition to sperm concentration and motility, significant discrimination between successful and unsuccessful ejaculates was not obtained ($P = 0.0813$). Furthermore, when ATP measurements were included alongside the movement characteristics of the spermatozoa or the results of the zona-free hamster oöcyte penetration test in the analyses described above, none of the ATP values was selected as being helpful in discriminating between the successful and unsuccessful ejaculates.

Finally, the concentration of spermatozoa and their motility, the movement characteristics of the spermatozoa, the results of zona-free hamster oöcyte penetration testing and the measurements of ATP were all included in the discriminant analysis. The variables selected as being of value in the discrimination are listed in Table 4.11 (p 158), together with their standardised discriminant function coefficients. All of these variables were positively related to fertility, except the frequency of head rotation recorded for the progressively motile spermatozoa, and none of the ATP measurements was selected as being of value in the discrimination. The discriminant function based upon these six variables was highly significant ($P = 0.0191$) and was 81.25% accurate overall at classifying the samples

into the fertile and infertile groups, being 77.8% and 85.7% accurate for the successful and unsuccessful ejaculates respectively (Table 4.12, p 159).

4.4 Discussion

The experimental design employed in this study recognises the significant variation in sperm quality which exists between successive ejaculates from the same individual (Rogers et al, 1983; Poland et al, 1986). As a result of such variability, not all ejaculates produced by a given individual will be capable of establishing a pregnancy and so the relationship between semen quality and fertility can only be accurately determined on an ejaculate-to-ejaculate basis. The use of an AID service has provided a unique opportunity to investigate the fertilizing potential of individual ejaculates *in-vivo* and hence provide a means of establishing the predictive value of *in-vitro* tests of sperm function.

In evaluating the data generated by this study, it should be emphasized that such an experimental design inevitably involves the use of cryostored spermatozoa. However, the agreement observed between the discriminating variables selected in this study and those identified in prospective studies on fresh semen (Aitken et al, 1984a; Aitken, 1985) suggests that the findings should apply equally well to non-cryostored cells. It should also be noted that such a design gives a relative rather than an absolute indication of the fertilizing potential of a sample, since a single ejaculate can be used for only a limited number of inseminations. These reservations apart, the use of cryostored samples in an AID setting has generated unique data on the relative fertilizing potential of individual ejaculates, with a minimum of interference due to differences in the fertility

of female partners (Foss and Hull, 1986). As a result of these analyses a series of criteria have been defined which define the fertilizing potential of a semen sample with great accuracy.

Of the characteristics of sperm movement identified in the discriminant analyses (Tables 4.7 and 4.11, pp 154-158) the mean velocity of the spermatozoa was consistently selected as an important discriminating variable, and was positively related to fertility. In contrast, several aspects of sperm movement recorded in the progressively motile subpopulation, particularly the frequency of sperm head rotation (Table 4.11, p 158), were found to be negatively related to fertility. This finding suggests that there is an optimal velocity of forward progression which spermatozoa must exhibit if they are to successfully ascend the female reproductive tract and fertilize the oöcyte. This finding supports the findings of previous *in-vitro* studies (Cohen et al, 1982; Aitken et al, 1983a) and the prospective analysis by Aitken et al (1984a) who found that amongst couples with unexplained infertility, the mean velocity of the progressively motile subpopulation of spermatozoa was negatively correlated with fertility, while the mean velocity of the total population of spermatozoa was positively related to the achievement of pregnancy.

In endeavouring to understand why there exists an optimal velocity of forward progression for the achievement of fertility *in-vivo* it is interesting to note that we have observed a negative correlation between the velocity of spermatozoa and the intracellular concentration of ATP (r = -0.361, P = 0.01, n =48). It is therefore possible that in rapidly moving cells the utilization of ATP by the dynein ATPase of the flagellum exceeds the ability of the spermatozoa to generate ATP by such processes as glycolysis.

During the 6 to 12 hour period required for significant numbers of human spermatozoa to ascent the female reproductive tract (Templeton and Mortimer, 1980; Mortimer and Templeton 1982; Mortimer, 1983) a progressive reduction in the intracellular levels of ATP might then influence the functional competence of the spermatozoa through an effect on ATP dependent cellular processes such as calcium ion extrusion (Borle, 1981). The fact that ATP levels were not identified as being of value in discriminating between successful and unsuccessful ejaculates mat be due to the fact that such measurements, performed on ejaculated or freshly isolated cells, do not reflect the long-term fate of the nucleotide *in-vivo* .

A second aspect of sperm movement which was found to be of significant value in discriminating the successful from the unsuccessful specimens was the amplitude of lateral sperm head displacement. The significance of this movement characteristic lies in its association with the capacity of human spermatozoa for penetrating cervical mucus (Aitken et al, 1985). This criterion appears to be positively correlated with the success of mucus penetration because it reflects the amplitude of the flagellar beating envelope (David et al, 1981), which in turn determines the forward thrust which can be generated by the spermatozoa at the cervical mucus interface. Samples with a minimal amplitude of sperm head displacement display a correspondingly diminished capacity for cervical mucus penetration *in-vivo* (Aitken et al, 1985; 1986), reduced fertility *in-vitro* (Feneux et al, 1985) and in human *in-vitro* fertilization (Jeulin et al, 1986). Given the relationships that exist between the amplitude of lateral sperm head displacement and both cervical mucus penetration and human *in-vitro* fertilization, one might expect a strong relationship between the ability of spermatozoa to penetrate cervical mucus and their performance in human *in-vitro* fertilization. Such a

relationship has been demonstrated by Hull et al (1984) in a study of patients undergoing *in-vitro* fertilization. Amongst their control patients, 55% of human oöcytes collected were fertilized, in contrast to only 16% of oöcytes fertilized amongst patients with poor results of *in-vivo* testing of cervical mucus penetration by spermatozoa. Interestingly, amongst a group of patients with unexplained infertility and good penetration of cervical mucus by spermatozoa, only 36% of human oöcytes were fertilized *in-vitro* , significantly lower than the control group, emphasizing the need to apply a variety of tests in the assessment of sperm function.

The last of the three groups of variables selected as being of value in discriminating between successful and unsuccessful ejaculates *in-vivo* was the outcome of the zona-free hamster oöcyte penetration test. Previous studies (Templeton et al, 1982; Aitken et al, 1984a; Schats et al, 1984; Aitken, 1985) have demonstrated the usefulness of this bioassay in assessing the fertility of an individual, and the ability of his spermatozoa to penetrate cervical mucus and ascend the female reproductive tract. These studies have normally expressed the outcome of the test in terms of the percentage of oöcytes penetrated, and in the present study a significant difference was observed for this criterion of semen quality between the two groups of ejaculates (Table 4.6, p 153). A significant difference between these groups was also observed in respect of the mean number of spermatozoa penetrating each oöcyte and in multivariate discriminant analysis it was this latter variable which was selected as being of greatest value. The reason for this is that when penetration rates are high, as in the present study, the mean number of spermatozoa per oöcyte, rather than the percentage of oöcytes penetrated yields more information on the relative penetrating potential of independent samples (Aitken and Elton, 1984).

These data are also of significance in that they constitute the first demonstration that hamster oöcyte penetration tests incorporating the ionophore A23187 generate information which relates to the fertilizing potential of the spermatozoa *in-vivo* . This is of biological significance because this test system specifically measures the ability of human spermatozoa to generate a fusogenic equatorial segment in response to the calcium influx induced by the ionophore (this will be discussed at length in Chapters Five and Six). Such a test does not include an assessment of the ability of human spermatozoa to capacitate *in-vitro* , but this did not apparently curtail the ability of the system to determine the functional competence of the spermatozoa *in-vivo* . These findings are of practical significance, since the capacitation of human spermatozoa requires prolonged incubation times and proceeds at different rates in different individuals (Perreault and Rogers, 1982). In contrast, the A23187-stimulated system provides for a rapid assessment of sperm function in which inter-individual differences are eliminated through the ability of the ionophore to synchronise the timing of the acrosome reaction.

Whilst the successful prediction of *in-vivo* fertilizing potential in 81.25% of cases is significant, it must be appreciated that the technique of multivariate discriminant analysis produces discriminating coefficients that best fit the particular data set and the accuracy of such predictions will require prospective studies for their validation. Such studies are currently under-way. An example is given in Table 4.13 (p 160) of how the information generated by multivariate discriminant analyses, such as have been performed in the present study, could be applied in clinical practice. In Tables 4.7, 4.9 and 4.11(pp 154, 156 and 158), the so-called Standardized Discriminant Function Coefficients for the measured variables have been

listed and as has been stated, the size of these values is a measure of the extent of the relationship between the variable (for example percentage motility) and the phenomenon under classification (in this case fertility), while the sign of the Standardized Discriminant Function Coefficient is an indication of the direction of the relationship. Having performed such an analysis, it is possible to use the information generated to predict the classification of an unknown sample. To do this one measures the same parameters on the unknown sample as were measured during the original discriminant analysis and multiplies the values obtained for these parameters by the Unstandardized Discriminant Function Coefficient for each variable. These products are then summated, together with a constant to yield a discriminant score, which is then compared to a critical score. If the score for the unknown sample is greater than this critical value, the sample is classified as successful, if less, unsuccessful. The example given in Table 4.13 (p 160) is based upon the discriminant analysis set out in Tables 4.9 and 4.10 (pp 156-157) and for which the Unstandardized Discriminant Function Coefficients of the measured variables are seen in the table, together with the constant of -3.96113 and the critical score of 0.13971. Examples are given of two hypothetical ejaculates, the first having a concentration of motile spermatozoa of 12 x 10^6/ml, a post capacitation motility of 50% and a penetration score of 4.00 spermatozoa/hamster oöcyte, yielding a score of 0.24807, which is greater than the critical score of 0.13971, and thus the sample would be predicted to be successful. The second ejaculate has values of 9 x 10^6/ml. 40% and 1.00 for the same variables, yielding a score of -0.8899, and would thus be predicted to be unsuccessful.

In conclusion, the conventional criteria of semen quality are simple to measure, but provide information of poor diagnostic value (Smith et al, 1977; Glass and Ericsson, 1979; Aitken et al, 1984a; Aitken, 1985). Examination of sperm movement characteristics or zona-free hamster oöcyte penetration are more complex and costly investigations to perform but its is nevertheless clear that they are of value in providing the clinician with an accurate assessment of the likely fertilizing ability of an ejaculate *in-vivo* . This information alone is of value in helping patients decide between several of the therapeutic options which arise in their management. Moreover, as it is becoming clear that a substantial proportion of infertility is due to defective sperm function (Aitken et al, 1982b; c; 1984a) then the ability to detect the defects involved has important implications for research aimed at uncovering the biochemical basis of defective sperm function as well as evaluating the efficacy of proposed treatments for male infertility.

Table 4.1

Comparisons between a group of successful and unsuccessful ejaculates used in an AID programme : Measurements performed upon each ejaculate.

Conventional Semen Characteristics	Concentration of spermatozoa (10^6/ml) Motility of spermatozoa (%) Concentration of motile spermatozoa (10^6/ml)
ATP Measurements	ATP concentration (μM) ATP concentration corrected for turbidity (μM) ATP corrected for sperm concentration (pM/10^6 sperm) ATP corrected for motile sperm concentration (pM/10^6 motile sperm) [Above measurements repeated on spermatozoa following separation on albumin columns]
Movement Characteristics	Mean Velocity (μm/sec) Mean amplitude of lateral head displacement (μm) Mean frequency of head rotation (/sec) Yawing spermatozoa (%) Rolling spermatozoa (%) Spermatozoa with a minimal (< 4.5μm) amplitude of lateral head displacement [Above measurements repeated for progressively motile sperm population = velocity > 25 μm/sec]
Zona-free hamster Oöcyte penetration	Motility of spermatozoa following incubation with A23187 Zona-free oöcytes penetrated (%) Mean number of sperm / oöcyte

Table 4.2

Comparisons between a group of successful and unsuccessful ejaculates used in an AID programme : Sperm concentration and Motility.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Comparison[c] *P*
Sperm concentration (10^6/ml)	50.22 ± 5.03	44.67 ± 8.21	0.549
Motility (%)	32.22 ± 2.29	27.62 ± 2.38	0.176
Concentration of motile cells (10^6/ml)	15.24 ± 1.49	10.52 ± 1.49	0.033[d]

[a] $n = 27$, mean ± SEM, [b] $n = 21$, mean ± SEM,
[c] Comparisons based on t-test or t^*-statistic,
[d] Statistically significant, $P < 0.05$

Table 4.3

Comparisons between a group of successful and unsuccessful ejaculates used in an AID programme : ATP Measurements.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Comparison[c] *P*
ATP Concentration (μM)	3.40 ± 0.40	2.69 ± 0.42	0.232
ATP corrected for turbidity (μM)	5.01 ± 0.75	5.64 ± 1.32	0.684
ATP corrected for sperm concentration ($pM/10^6$ cells)	111.66 ±17.13	146.63 ±29.30	0.310
ATP corrected for motile sperm concentration ($pM/10^6$ cells)	378.91 ±59.13	602.99 ±141.34	0.155
ATP Concentration following column separation (μM)	0.36 ± 0.05	0.28 ± 0.07	0.325
ATP following column separation corrected for sperm concentration ($pM/10^6$ cells)	20.60 ± 2.83	21.95 ± 4.19	0.784
ATP following column separation corrected for motile sperm concentration ($pM/10^6$ cells)	37.48 ± 5.40	41.46 ± 8.61	0.685

[a] $n = 27$, mean ± SEM, [b] $n = 21$, mean ± SEM,

[c] Comparisons based on t-test or t^*-statistic,

Table 4.4

Comparisons between a group of successful and unsuccessful ejaculates used in an AID programme : Sperm Movement Characteristics, entire sperm population.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Comparison[c] *P*
Mean Velocity (μm/sec)	48.65 ± 1.28	50.64 ± 2.60	0.448
Mean amplitude of lateral head displacement (μm)	7.08 ± 0.25	7.07 ± 0.34	0.967
Mean frequency of head rotation (/sec)	11.24 ± 0.34	12.01 ± 0.59	0.226
Yawing Spermatozoa (%)	2.69 ± 0.75	2.00 ± 0.82	0.555
Rolling Spermatozoa (%)	68.27 ± 2.51	73.00 ± 4.02	0.299
Spermatozoa with a minimal (< 4.5μm) amplitude of lateral head displacement (%)	29.04 ± 2.81	25.67 ± 4.11	0.490

[a] $n = 27$, mean ± SEM, [b] $n = 21$, mean ± SEM,
[c] Comparisons based on t-test or t^*-statistic,

Table 4.5

Comparisons between a group of successful and unsuccessful ejaculates used in an AID programme : Sperm Movement Characteristics, progressive (velocity > 25μm/sec) sperm population.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Comparison[c] *P*
Progressive (> 25μm/sec) ; Mean velocity (μm/sec)	48.98 ± 1.25	51.15 ± 2.48	0.391
Progressive (> 25μm/sec) ; Mean amplitude of lateral head displacement (μm)	6.99 ± 0.25	7.19 ± 0.34	0.622
Progressive (> 25μm/sec) ; mean frequency of head rotation (/sec)	11.24 ± 0.35	12.15 ± 0.58	0.161
Progressive (> 25μm/sec) ; yawing (%)	3.08 ± 0.79	2.33 ± 0.83	0.544
Progressive (> 25μm/sec) ; rolling (%)	67.77 ± 2.49	73.33 ± 4.04	0.223
Progressive (> 25μm/sec) ; spermatozoa with a minimal (< 4.5μm) amplitude of lateral head displacement	29.15 ± 2.88	25.00 ± 4.05	0.400

[a] n = 27, mean ± SEM, [b] n = 21, mean ± SEM,
[c] Comparisons based on t-test or t^*-statistic,

Table 4.6

Comparisons between a group of successful and unsuccessful ejaculates used in an AID programme : Zona-free hamster oöcyte penetration testing using A23187.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Comparison[c] *P*
Motility of spermatozoa following incubation with A23187 (%)	46.30 ± 2.20	39.62 ± 2.74	0.061
% of zona-free hamster oöcytes penetrated	82.42 ± 4.52	63.16 ± 7.78	0.040[d]
Mean number of sperm per oöcyte	5.12 ± 1.12	2.34 ± 0.44	0.028[d]

[a] $n = 27$, mean ± SEM, [b] $n = 21$, mean ± SEM,
[c] Comparisons based on t-test or t^*-statistic,
[d] Statistically significant, $P < 0.05$

Table 4.7

Discrimination between a group of successful and unsuccessful ejaculates used in an AID programme : multivariate discriminant analysis based upon sperm concentration and motility and sperm movement characteristics.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Standardised discriminant function coefficient
Concentration of motile spermatozoa (10^6/ml)	15.24 ± 1.49	10.51 ± 1.50[c]	0.56024
Mean velocity (μm/sec)	48.65 ± 1.28	50.64 ± 2.60	0.64552
Amplitude of lateral head displacement < 4.5μm (%)	29.04 ± 2.81	25.67 ± 4.11	-4.50812
Frequency of head rotation of progressive (>25 μm/sec) spermatozoa (/sec)	11.24 ± 0.35	12.15 ± 0.58	-1.02452
Percentage of progressive (>25 μm/sec) rolling spermatozoa	67.77 ± 2.49	73.33 ± 4.04	-4.39228
Percentage of progressive (>25 μm/sec) yawing spermatozoa	3.08 ± 0.79	2.33 ± 0.83	-0.51156

[a] n = 27, mean ± SEM, [b] n = 21, mean ± SEM,
[c] Statistically significant, $P < 0.05$

Table 4.8

Classification of successful and unsuccessful ejaculates, based upon the variables listed in Table 4.7.

Actual Group Membership	N	Predicted Group Membership[a,b] *n* (%)	
		Successful	Unsuccessful
Successful	27	20 (74.1)	7 (25.9)
Unsuccessful	21	6 (28.8)	15 (71.4)

[a]Overall percentage of grouped cases correctly classified = **72.92%**

[b]Significance of discriminant function $P = 0.0489$

Table 4.9

Discrimination between a group of successful and unsuccessful ejaculates used in an AID programme : multivariate discriminant analysis based upon sperm concentration and motility and results of zona-free hamster oöcyte penetration testing.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Standardised discriminant function coefficient
Concentration of motile spermatozoa (10^6/ml)	15.24 ± 1.49	10.51 ± 1.50[c]	0.54776
Motility of spermatozoa following a 3-hour incubationwith A23187 (%)	46.30 ± 2.20	39.62 ± 2.74	0.66146
Mean number of spermatozoa per oöcyte	5.12 ± 1.12	2.34 ± 0.44	0.55898

[a] n = 27, mean ± SEM, [b] n = 21, mean ± SEM,
[c] Statistically significant, $P < 0.05$

Table 4.10

Classification of successful and unsuccessful ejaculates, based upon the variables listed in table 4.9.

Actual Group Membership	**N**	**Predicted Group Membership[a,b]** *n* (%)	
		Successful	Unsuccessful
Successful	27	20 (74.1)	7 (25.9)
Unsuccessful	21	5 (23.8)	16 (76.2)

[a]Overall percentage of grouped cases correctly classified = **75.00%**

[b]Significance of discriminant function P = 0.0319

Table 4.11

Discrimination between a group of successful and unsuccessful ejaculates used in an AID programme : multivariate discriminant analysis based upon all measured variables.

Parameter	Successful Ejaculates[a]	Unsuccessful Ejaculates[b]	Standardised discriminant function coefficient
Concentration of motile spermatozoa (10^6/ml)	15.24 ± 1.49	10.51 ± 1.50[c]	0.45806
Motility of spermatozoa following a 3-hour incubation with A23187 (%)	46.30 ± 2.20	39.62 ± 2.74	0.78173
Mean number of spermatozoa per oöcyte	5.12 ± 1.12	2.34 ± 0.44	0.54942
Mean velocity (µm/sec)	48.65 ± 1.28	50.64 ± 2.60	0.88963
Percentage of spermatozoa with yawing movement	2.69 ± 0.75	2.00 ± 0.82	0.50769
Frequency of head rotation of progressive (>25µm/sec) spermatozoa (/sec)	11.24 ± 0.35	12.15 ± 0.58	-1.16133

[a] $n = 27$, mean ± SEM, [b] $n = 21$, mean ± SEM,

[c] Statistically significant, $P < 0.05$

Table 4.12

Classification of successful and unsuccessful ejaculates, based upon the variables listed in table 4.11.

Actual Group Membership	N	Predicted Group Membership[a,b] *n* (%)	
		Successful	Unsuccessful
Successful	27	21 (77.8)	6 (22.2)
Unsuccessful	21	3 (14.3)	18 (85.7)

[a]Overall percentage of grouped cases correctly classified = **81.25%**

[b]Significance of discriminant function $P = 0.0191$

Table 4.13

Practical application of the information from multivariate discriminant analysis in predicting the fertilizing ability of an ejaculate - illustrative calculation on two ejaculates

Variables :	Concentration of motile spermatozoa (10^6/ml)		Motility following 3h incubation with A23187 (%)		Mean No of spermatozoa per oöcyte		Sum
Sample 1 :	12.0		50.0		4.00		
Unstandardized discriminant function coefficient	0.07863		0.05626		0.11316		
Product	0.94356	+	2.813	+	0.45264	=	4.2092
					Constant		-3.96113
			Discriminant Score				**0.24807[a]**
Sample 2 :	9.0		40.0		1.00		
Unstandardized discriminant function coefficient	0.07863		0.05626		0.11316		
Product	0.70767+		2.2504+		0.11316	=	3.07123
					Constant		-3.96113
			Discriminant Score				**-0.8899[a]**

[a]The critical value of the discriminant score in this discriminant analysis is 0.13971. Thus Sample 1 would be predicted to be fertile, while Sample 2 would be predicted to be infertile.

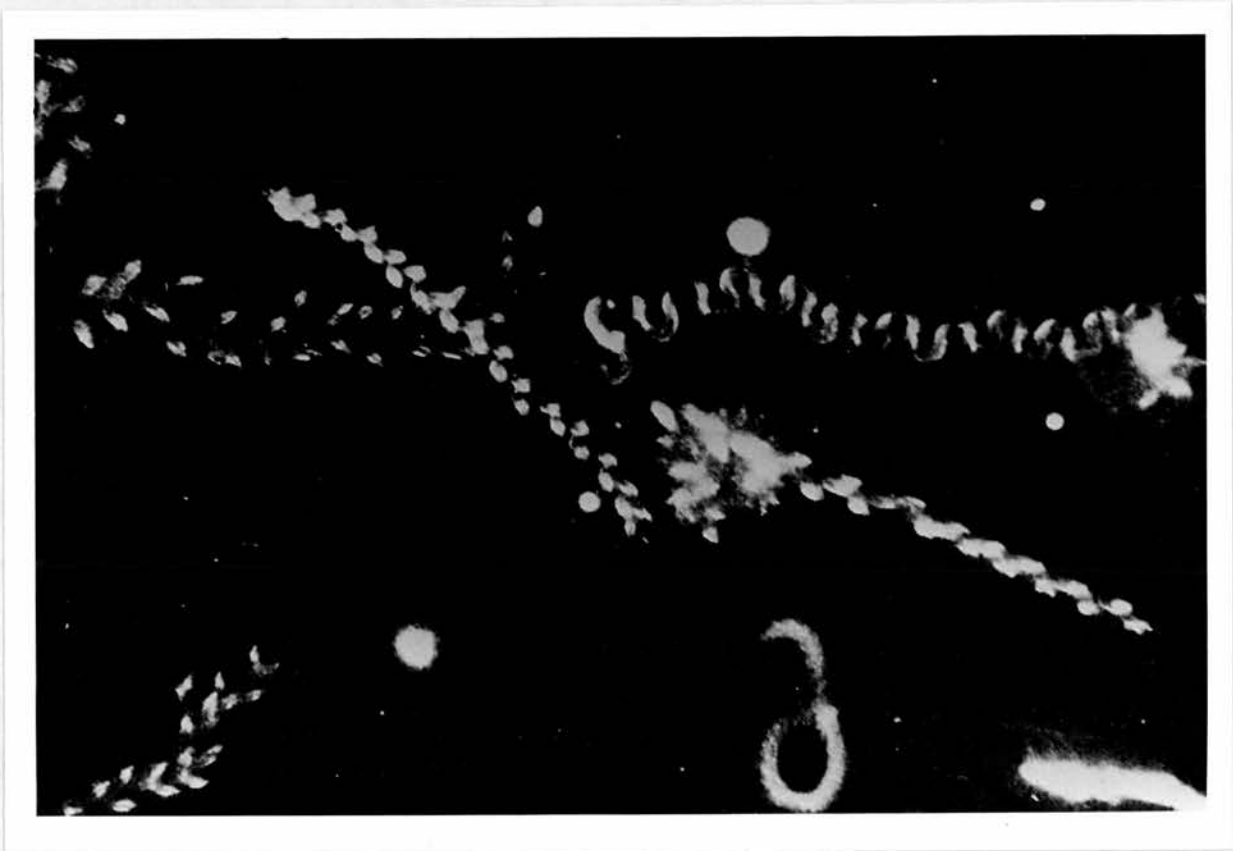


Figure 4.1

Sperm Movement Characteristics

Human spermatozoa were examined under dark field illumination, with a time exposure of one second. The above photograph is an example of the tracks obtained using this technique, which were then analysed as described in the text.

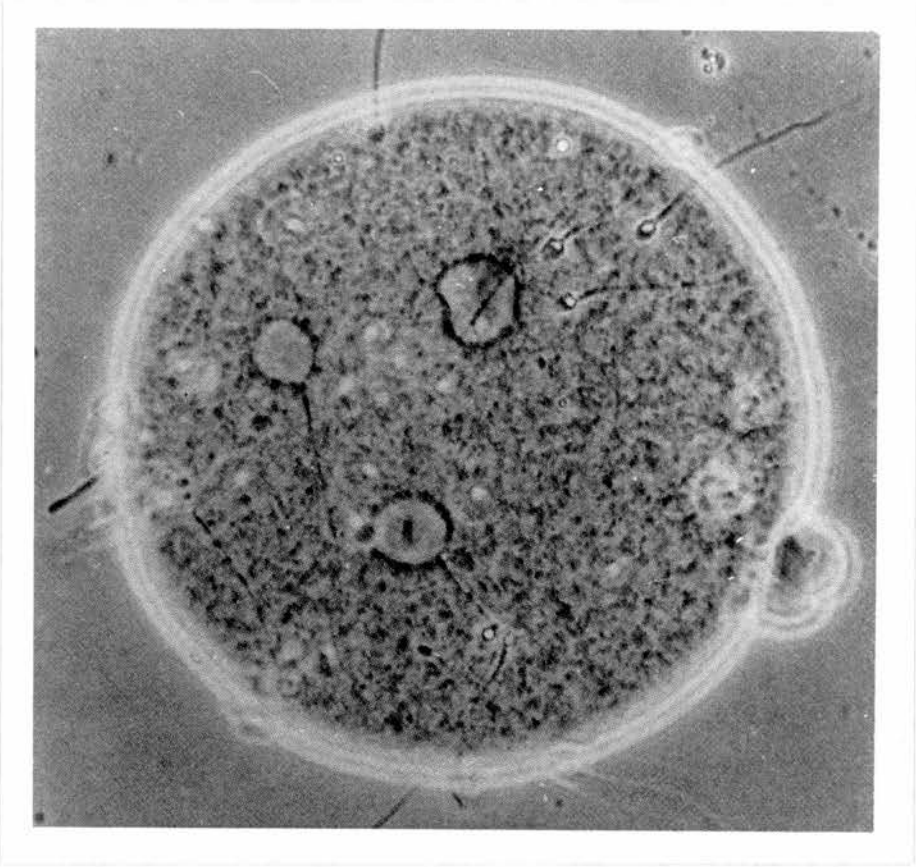


Figure 4.2

Zona Free Hamster Oöcyte Penetration Test

Example of a zona-free hamster oöcyte which has been co-incubated with human spermatozoa as described in the text.

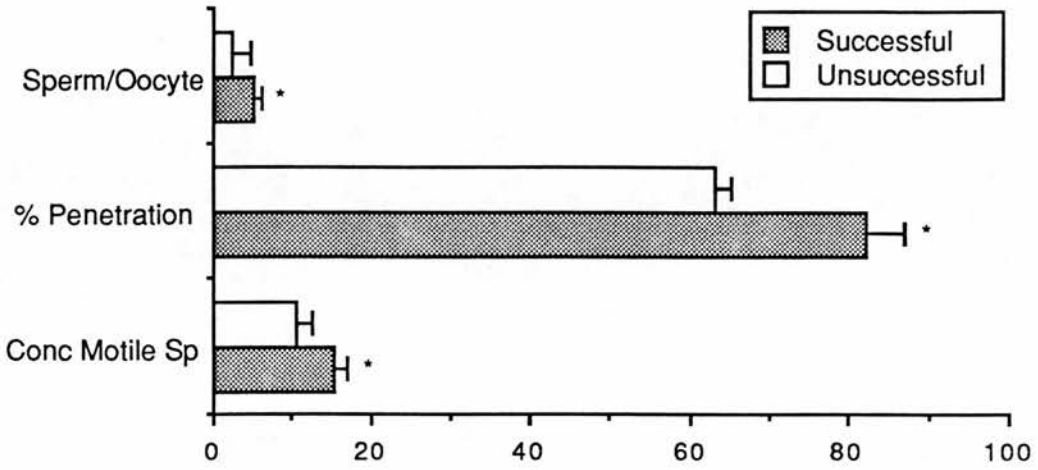


Figure 4.3

Comparisons between successful and unsuccessful ejaculates used in an AID programme.

This figure shows the values (mean ± SEM) of the three variables for which statistically significant differences were noted.

a. Concentration of motile spermatozoa (10^6/ml)

b. % of zona-free hamster oöcytes penetrated

c. Mean number of sperm per oöcyte

* = $P < 0.05$

CHAPTER FIVE

THE MEASUREMENT OF INTRACELLULAR CALCIUM IN HUMAN SPERMATOZOA

5.1 Introduction

The fundamental importance of intracellular calcium in cell function is well recognized (Borle, 1981; Campbell, 1982; Bronner and Peterlik, 1984; Marmé, 1985), and the possible role of intracellular calcium as a key regulator of human sperm function has begun to be appreciated (Yanagimachi and Usui, 1974; Singh et al, 1978; Peterson et al, 1979a; b; Garbers and Kopf, 1980; Triana et al, 1980; Fraser, 1982; Shackmann and Shapiro, 1982; Yangimachi, 1982; Hong et al, 1984; Rufo et al, 1984). Yanagimachi and Usui (1974) originally demonstrated that extracellular Ca^{2+} was required for the successful completion of capacitation and for the the acrosome reaction in guinea pig spermatozoa. Since then, much effort has been devoted to unraveling the mechanisms by which the sperm cell regulates its intracellular Ca^{2+} and by which this intracellular Ca^{2+} influences cell function. Fraser (1982) concluded that mouse spermatozoa require calcium for at least part of the process of capacitation, as well as for the acrosome reaction, and Singh and colleagues (Singh et al, 1978) have suggested that an influx of calcium into guinea-pig spermatozoa is an important component of capacitation. This latter finding was confirmed by Triana et al (1980) for the spermatozoa of the bull, rabbit and hamster while Breitbart and co-workers have demonstrated the existence of a Ca^{2+}-ATPase in the plasma membranes of ram and bovine spermatozoa (Breitbart and Rubinstein, 1983; Breitbart et al, 1983; 1984). Much of this detailed work has been undertaken on non-human gametes (Singh et al, 1978; Peterson et al, 1979a; Triana et al, 1980; Fraser, 1982; Breitbart and Rubinstein, 1983; Breitbart et al, 1983; 1984; Rufo et al, 1984; Murphy et al, 1986; Robertson and Watson, 1986; Roldan et al, 1986), and the direct

measurement of free intracellular calcium concentration ($[Ca^{2+}]_i$) has not yet been reported for the spermatozoa of any species.

From the point of view of the clinician involved in the diagnosis and treatment of potentially subfertile males, it has become apparent that defective sperm function may be a major causative factor in human infertility, this information having been derived from work involving interspecific *in-vitro* fertilization experiments on semen from couples with idiopathic infertility and oligozoospermia (Aitken et al, 1982b; 1984a; 1985; Aitken, 1985) and it has been demonstrated in Chapters Three and Four that techniques are now available which will permit the accurate diagnosis of human sperm dysfunction. Elucidation of the fundamental cellular lesions in such defective spermatozoa is the logical prelude to more precise diagnosis and to the evolution of therapeutic strategies aimed at their correction. In this regard, a recent study by Hardisty et al (1983) has demonstrated that defective platelet function may be associated with impaired responsiveness of the platelets to changes in cytoplasmic free calcium, and evidence is available to suggests that abnormalities of cellular function regulated by intracellular calcium may also be important in mediating defective sperm function (Aitken et al, 1984b). A study was therefore undertaken to establish techniques which would permit the measurement of free intracellular calcium in the human spermatozoon.

Established techniques for the measurement of intracellular calcium involving photoproteins (such as aequorin, derived from the jelly-fish *Aequorea forskalea* , and obelin, derived from the hydrozoa *Obelia lucefera* and *Obelia geniculata*) (Campbell et al, 1979; Campbell, 1982), metallochromic indicators (such as murexide, antipyrylazo III and arsenazo

III) (Scarpa, 1979; Williamson et al, 1983; Yingst and Hoffman, 1984; Wiener and Scarpa, 1985) and microelectrodes (Simon et al, 1978; Ammann et al, 1979; Alvarez-Leefmans et al, 1981) are not readily applicable to spermatozoa. Measurement of the uptake of $^{45}Ca^{2+}$ is a widely used technique for studies on a wide range of cell types (Burgess et al, 1983; Balla et al, 1985) and for studies on spermatozoa in particular (Babcock et al, 1975; Peterson et al, 1979a; b; Singh et al, 1980; 1983; Breitbart and Rubinstein, 1983; Byrd et al, 1983; Breitbart et al, 1984; Rufu et al, 1984; Robertson and Watson, 1986) however it suffers from the problem of being non-specific with respect both to the state of the calcium measured (free or bound), and to the intracellular location of the calcium measured (cytoplasmic, intramitochondrial, intranuclear or membrane bound). Tsien and colleagues (Tsien 1980; Tsien et al, 1982a; Grynkiewicz et al, 1985) have developed a series of fluorescent calcium indicators, prominent amongst which have been quin-2 and fura-2, and which have made possible the direct measurement of intracellular free calcium (Pozzan et al, 1981; Tsien, 1981; Tsien et al, 1982a; b; Poenie 1985; Rink and Pozzan, 1985). Quin-2 is available as a membrane permeant tetra-acetoxymethyl ester (quin-2-AM) in which form it readily crosses the plasma membrane and enters the cytoplasm. Once within the cytoplasm, the ester is hydrolysed by intracellular esterases to form the parent free acid (quin-2), which is membrane impermeant, and is thus trapped within the cytoplasm (Tsien, 1981). The fluorescence of quin-2 increases 5-fold in going from the calcium-free to the calcium-saturated form, and its fluorescence may thus be used as an indicator of the level of free calcium within the cytoplasm.

Fura-2 functions in a manner very similar to quin-2, but has enhanced quantum efficiency and photochemical stability, yielding up to 30-fold

brighter fluorescence and major changes in wavelength as well as fluorescence intensity upon binding Ca^{2+} (Grynkiewicz et al, 1985), making it the preferred dye for measurements upon single cells (Poenie et al, 1985; Tsien et al, 1985; Williams et al, 1985). These indicators have now been used for the direct measurement of free cytoplasmic calcium in a number of cell types, including lymphocytes (Tsien, 1981; Tsien et al, 1982a;) oöcytes (Poenie et al, 1985), platelets (Hardisty et al, 1983; Yamanishi et al, 1983), pancreatic acinar cells (Ochs et al, 1983), adrenal medullary cells (Knight and Kesteven, 1983), pituitary cells (Albert and Tashjian, 1984), hepatocytes (Charest et al, 1983), muscle cells (Williams et al, 1985) and nerve terminals (Nachshen, 1984).

In the present study, the value and feasibility of using $^{45}Ca^{2+}$, quin-2 and fura-2 to investigate the role of intracellular calcium in human sperm function has been examined. The results emphasize the value of quin-2 for such studies, since with the aid of this technique, the first measurements of cytoplasmic free calcium in human spermatozoa have been obtained.

5.2 Materials and Methods

Cell Preparation and Handling

Semen samples were obtained by masturbation from a panel of semen donors, were allowed to liquefy prior to analysis and were analysed within 2 hours of production. A routine semen analysis was first performed, as outlined in Chapter Two (World Health Organization, 1980; 1987) in order to determine semen volume (ml), concentration of spermatozoa (10^6/ml), and motility of spermatozoa (% motile spermatozoa). The sample was then transferred to a sterile centrifuge tube and diluted with modified BWW

medium (Biggers et al, 1971) containing 20mM Hepes buffer and 3mg/ml human serum albumin. Normal medium BWW also contained 1.7mM ionic calcium, although for certain experiments, medium containing no added calcium (NAC) was employed. The free calcium concentration of this latter medium was approximately 10μM, as measured with a calibrated Orion calcium-sensitive electrode (q.v.). The sample was centrifuged at 500 x g for 5 minutes, following which the supernatant was discarded, and the sample washed a further two times in fresh 5ml volumes of medium BWW. The washed sample was finally resuspended in medium BWW at a concentration of 20 x 10^6 spermatozoa/ml. and was maintained in an incubator at 37°C in an atmosphere of 5% CO_2 in air.

Measurement of Free Calcium Concentration in Solutions

This was performed using an Orion Research (Cambridge, Massachusetts, USA) calcium electrode (model 93-20) together with a single junction reference electrode (model 90-01) and a digital pH/mV meter (model 701A). This electrode was of a type containing a liquid internal filling solution in contact with a gelled organophilic membrane containing a calcium-selective ion exchanger, allowing a potential to develop across the membrane, dependent upon the level of free calcium ions in solution. The electrode assembly was suspended in the solution to be measured, which was kept constantly agitated with the aid of a magnetic strirrer. The system was calibrated daily using a range of free calcium concentrations from 10^{-2} to 10^{-7}M Ca^{2+} (10mM to 0.1μM) made up in double distilled water. Typical calibration curves are shown in Figure 5.1 (p 188), from which it can be seen that this electrode produced a linear response for free calcium concentrations in excess of 4μM, but that below this accurate measurement was difficult.

Measurement of $^{45}Ca^{2+}$ Uptake

This was performed essentially as described by Babcock et al (1975, 1976), modified as follows. Washed spermatozoa were suspended in BWW medium at a concentration of 20 x 10^6/ml, and $^{45}Ca^{2+}$ was added to this cell suspension at an activity of 10μCi/ml, equivalent to an additional 19nM Ca^{2+}. At various time points, 50μl aliquots of this cell suspension were transferred to the wells of a millititer filtration plate, having a pore size of 2μm, in a vacuum assembly (Millipore UK Ltd., Harrow, England). The cells were filtered on to the membrane and were rapidly washed with 3 x 250μl volumes of ice cold 0.9% saline containing 10mM unlabeled Ca^{2+}. This entire washing procedure took approximately 30 seconds and was found to yield reproducible count rates. The filter discs were then punched out into vials containing a toluene based scintillation fluid, for counting in a ß-counter. Measurements were normally performed in replicates of four, and the results were expressed as counts per minute per 10^6 cells.

Measurement of Adenosine Triphosphate

This was performed using a bioluminescent assay, exactly according to the protocol described in Chapter Three.

Loading of Spermatozoa with Quin-2

Stock solutions of quin-2-AM and quin-2 were made up in dry dimethylsulphoxide (DMSO) and were kept dark and desiccated at -20°C between experiments. Washed spermatozoa, at a concentration of 20 x 10^6/ml, were suspended in normal medium BWW containing 1.7mM Ca^{2+}, and quin-2-AM was added to a concentration of 50μM, the final concentration of DMSO not exceeding 0.5% v/v; DMSO alone was added to

the control incubations. These cell suspensions were incubated at 37°C for 20 minutes in an atmosphere of 5% CO_2 in air, and were then diluted 1:10 with fresh medium BWW. The incubation was normally continued for a further 100 minutes, and at the end of this time the cells were pelleted by centrifugation at 500 x g for 5 minutes and then washed twice to minimize the carryover of extracellular quin. The sperm pellet was finally resuspended to the original volume in medium BWW and incubated at 37°C in an atmosphere of 5% CO_2 in air prior to use.

Measurement of Quin-2 Fluorescence

Measurements were performed initially using either an Aminco-Bowman or a Baird spectrofluorimeter with a single cell sample chamber thermo-stated to 37°C. Latterly, measurements were performed using a Perkin-Elmer LS5 spectrofluorimeter, with a thermo-stated 4 cell sample holder. For the examination of fluorescence spectra, the excitation monochromator was set at 339nm, and the emission monochromator was scanned over the range 400-550nm. The measurement of intracellular calcium ($[Ca^{2+}]_i$) depended upon fluorescence readings with the excitation and emission monochromators set to 339 and 492nm, respectively. Prior to each experiment, the emission spectra of the quin-2-AM and of the spermatozoa incubated with quin-2-AM and DMSO were examined, to confirm successful uptake and de-esterification of the quin-2-AM by the spermatozoa. At the conclusion of each experiment, the system was calibrated according to the general procedure described by Tsien et al (1982a; b). Triton-X-100 was added to a concentration of 0.5% v/v, followed by EGTA to a concentration of 10mM, and the readings obtained were recorded as F_{max} and F_{min}, respectively. Calculation of intracellular calcium was based upon the equation : $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$, where F = fluorescence

(arbitrary units); F_{max} = maximal fluorescence, following addition of triton to release the intracellular quin-2 into the medium; F_{min} = minimal fluorescence, following addition of EGTA in Tris base, to strip all calcium from the quin-2; and K_d = a dissociation constant of 115nM.

Time Course of Quin-2 Entry and De-esterification

The uptake and de-esterification of quin-2 by cells can be readily monitored owing to the fact that the ester of quin-2 (quin-2-AM) has a characteristic emission spectrum, with a fluorescence maximum at 430nm. As the quin-2-AM is hydrolysed to the free acid, this disappears to be replaced by the emission spectrum characteristic of the free acid, having maximal fluorescence at 492nm (Figure 5.2, p 189) Sperm cells were loaded with quin-2 according to the protocol outlined above, and at various time points during the procedure, aliquots of the cell suspension were removed, and the cells were washed twice by centrifugation at 500 x g before finally being lysed with triton-X-100, to release the intracellular quin-2 into solution. The lysed cells were then removed from the solution by centrifugation, and the supernatant, containing the quin-2, was used to assess the hydrolysed state of the intracellular quin-2 by examination of its emission spectrum.

Intracellular Concentration of Quin-2

A calibration curve was prepared for quin-2 free acid, by dilution in BWW medium. This was found to be linear over the range in which measurements were made (1-12μM). Samples of spermatozoa were loaded with quin-2 according to the above protocol, and loading was confirmed to be complete in every case by the shift of the emission spectrum. The cells were then lysed by the addition of triton-X-100, releasing the trapped quin-2 free acid into the medium, and a series of fluorescence measurements were obtained

of the previously intracellular quin-2 free acid, exposed to 1.7mM Ca^{2+}, the differences in which were due only to the varying concentrations of quin-2 present. It was therefore possible, using the estimate of Ford and Harrison (1983) for the water space of human spermatozoa, to calculate the intracellular concentration of quin-2 free acid.

Loading of Spermatozoa with Fura-2

Stock solutions of fura-2-AM and fura-2 were made up in dry dimethylsulphoxide (DMSO) and were kept dark and desiccated at -20°C under nitrogen between experiments. Washed spermatozoa, at a concentration of 20 x 10^6/ml, were suspended in normal medium BWW containing 1.7mM Ca^{2+}, and fura-2-AM was added to a concentration of 1μM, the final concentration of DMSO not exceeding 0.5% v/v; DMSO alone was added to the control incubations. These cell suspensions were incubated at 37°C for 120 minutes in an atmosphere of 5% CO_2 in air, and at the end of this time the cells were pelleted by centrifugation at 500 x g for 5 minutes and then washed twice to minimize the carryover of extracellular fura-2. The sperm pellet was finally resuspended in 1ml of medium BWW and incubated at 37°C in an atmosphere of 5% CO_2 in air prior to use.

Measurement of Fura-2 Fluorescence

Measurements were performed in essentially in the same manner as for quin-2. For the examination of fluorescence spectra, the excitation monochromator was set to either 340nm or 380nm, and the emission monochromator was scanned over the range 400 - 600nm. The measurement of intracellular calcium ($[Ca^{2+}]_i$) depended upon fluorescence readings with the excitation monochromator set alternately to either 340 or 380nm and the emission monochromator set to 500nm. At the

conclusion of each experiment, the system was calibrated according to the general procedure described by Tsien et al (1982a; b). Triton-X-100 was added to a concentration of 0.5% v/v, followed by EGTA to a concentration of 10mM, and the readings obtained were recorded as F^{340}, F^{380}, F^{340}_{max}, F^{380}_{max}, F^{340}_{min} and F^{380}_{min}, respectively. For each pair of measurements, the ratio of F^{340}/F^{380} was calculated, and calculation of intracellular calcium was based upon the equation : $[Ca^{2+}]_i = K_d (R - R_{min}) / (R_{max} - R)$, where R = 340/380 ratio at resting fluorescence (arbitrary units); R_{max} = 340/380 ratio at maximal fluorescence, following addition of triton to release the intracellular fura-2 into the medium; R_{min} = 340/380 ratio at minimal fluorescence, following addition of EGTA in Tris base, to strip all calcium from the fura-2; and K_d = a dissociation constant of 200nM (Poenie et al, 1985).

Materials

Chemicals were obtained from the Sigma chemical Co (St. Louis, MO), except as stated below. Carbonylcyanide-M-chlorophenyl hydrazone (CCCP), carbonyl- cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone were made up as stock solutions in dimethylsulphoxide (DMSO) and stored at -20°C. Quin-2-tetra-acetoxymethyl ester and quin-2 free acid were also made up as stock solutions in DMSO and were stored dark and desiccated at -20°C. Fura-2 and fura-2-AM were obtained from Calbiochem (Bishops Stortford, England) and were also made up as stock solutions in DMSO and stored dark and desiccated under nitrogen at -20°C between experiments. FCCP was obtained from the Aldrich Chemical Co Ltd (Dorset, England); $^{45}Ca^{2+}$ was from Amersham International (Buckinghamshire, England). Millititer filtration plates were obtained from Millipore UK Ltd (Harrow, England).

Statistical Analysis

Statistical comparisons were performed using the Student *t* test or the Mann-Whitney U test as appropriate. Probabilities with $P < 0.05$ were regarded as significant.

5.3 Results

Measurement of Free Calcium In Solutions

Standard medium BWW (see Appendix 1) was found to contain 1.7mM Ca^{2+}, while the same medium, made up without the addition of $CaCl_2$ (no added calcium, NAC), was found to contain approximately 10μM Ca^{2+}. A group of 43 semen samples, was collected, the spermatozoa pelleted by centrifugation at 500 x g, and the level of free ionic calcium contained in the supernatant seminal plasma was measured. This was found to be only 0.12 ± 0.0062mM (mean ± SEM, n = 43) although seminal plasma contains between 5 and 6mM calcium (Arver and Sjöberg, 1983).

Uptake of $^{45}Ca^{2+}$

When washed ejaculated human spermatozoa were incubated in the presence of $^{45}Ca^{2+}$, the cells readily accumulated the isotope, as shown by the rise in cpm/10^6 cells over a 60 minute period. This experiment was initially performed in complete medium BWW, containing 1.7mM Ca^{2+}. Under these circumstances, the cell associated calcium rose from 29.4 ± 23.4 to 301 ± 37.45 cpm/10^6 cells (mean ± range, n=2) over a 60 minute period. This experiment was repeated in BWW containing no added calcium, in order to determine the extent to which the Ca^{2+} content of the conventional medium (1.7mM) had reduced the number of counts recorded.

Without added calcium, the BWW contained approximately 10μM unlabeled Ca^{2+} (see above) and 19nM $^{45}Ca^{2+}$. Immediately after human spermatozoa had been suspended in this calcium-deficient medium, the cell-associated $^{45}Ca^{2+}$ was 363 ± 62.7 cpm/10^6 cells (mean ± SEM, n = 23), rising after 60 minutes to 832.8 ± 76.4 cpm/10^6 cells ($P < 0.001$) (Figure 5.3, p 190). In a separate series of experiments washed spermatozoa were incubated in medium BWW with no added calcium, to which 10μCi/ml $^{45}Ca^{2+}$ had been added, and the incubation was continued for up to 8 hours. It was observed that the cell-associated calcium immediately following addition of the isotope was 272.77 ± 97.1cpm/10^6 cells, rising to 571.55 ± 32.8 cpm/10^6 cells after 60 minutes, and to 693.57 ± 204.0 cpm/10^6 cells after 120 minutes, following which it did not rise significantly, being only 888.02 ± 208.0 cpm/10^6 cells after 8 hours of incubation (Figure 5.4, p 191).

It was therefore apparent that much more $^{45}Ca^{2+}$ became associated with a given number of spermatozoa when the incubations were performed in BWW containing a reduced amount of unlabeled Ca^{2+}. While the total amount of cell-associated Ca^{2+} will be substantially reduced under these circumstances, the proportion of cell associated Ca^{2+} that is $^{45}Ca^{2+}$ is much greater, and this experimental situation was thought to be more favourable for the examination of calcium fluxes.

Effect of Mitochondrial Uncouplers on $^{45}Ca^{2+}$ Uptake

Known inhibitors of mitochondrial electron transport were added to suspensions of spermatozoa immediately prior to the addition of isotope, and aliquots were removed for the determination of isotopic uptake immediately, and thereafter at 30, 60, and 120 minutes. CCCP (20μM),

rotenone (5μM), and FCCP (5μM) all caused significant suppression of the observed accumulation of $^{45}Ca^{2+}$. In experiments using CCCP and rotenone, control count rates rose from 421.1 ± 81.4 to 841.6 ± 77.9 cpm/10^6 cells over a 60 minute incubation period, and this was suppressed at 60 minutes to 379.6 ± 29.4 cpm/10^6 cells by CCCP and to 348.9 ± 31.2 cpm/10^6 cells by rotenone (both $P < 0.001$) (Figure 5.5, p 192). Simultaneous measurements of adenosine triphosphate suggested that rotenone, but not CCCP had caused a reduction in intracellular ATP concentrations over the same period of time (Figure 5.6, p 193), although the changes noted did not reach statistical significance. In a separate series of experiments using FCCP, control count rates rose from 231.4 ± 37.0 to 1010.0 ± 190.5 cpm/10^6 cells over the same time period, and this was suppressed to 545.7 ± 64.3 cpm/10^6 cells by FCCP ($P < 0.01$) (Figure 5.7, p 194). Expression of these results as a percentage of control uptake at each time point revealed that the suppression of $^{45}Ca^{2+}$ uptake was generally maximal at 30 minutes and declined thereafter (Figure 5.8, p 195). These experiments indicated that washed ejaculated human spermatozoa were permeable to $^{45}Ca^{2+}$, but that a significant proportion of the cell-associated calcium measured by this technique was intramitochondrial. In an attempt to measure the extramitochondrial free calcium, a series of experiments was next performed using the fluorescent calcium indicator, quin-2.

Uptake and De-Esterification of Quin-2-AM

When ejaculated human spermatozoa were incubated with quin-2-AM according to the experimental procedure described above, both the uptake and intracellular de-esterification of the quin-2-AM to produce trapped intracellular quin-2 free acid could be observed. Figure 5.9 (p 196) shows

the typical emission spectra obtained during such an experiment. Spectrum (i) is that of quin-2-AM in BWW, showing the characteristic fluorescence maximum at 435nm, spectrum (ii) is that of washed human spermatozoa which have been incubated with quin-2-AM, according to the protocol described above, spectrum (iii) is that of the control population of spermatozoa which have been incubated with DMSO alone, to show cell autofluorescence (which was considerable at wavelengths below 450nm), and spectrum (iv) is spectrum (ii) less spectrum (iii), that is, the spectrum of cells loaded with quin-2, corrected for cell autofluorescence. It can be seen that this derived spectrum has a fluorescence maximum at 492nm, thus demonstrating that the spermatozoa have succeeded in accumulating quin-2-AM, de-esterifying it to quin-2 free acid, and have retained this latter molecule within the cell.

Time Course of Quin-2 Uptake and De-Esterification

Washed ejaculated human spermatozoa were incubated with 50μM quin-2-AM, and aliquots were periodically removed for assessment of the esterification status of the intracellular quin-2, as described above. The results of such an experiment are shown in Figure 5.10 (p 197), which shows a series of emission spectra of intracellular quin-2 obtained at 15, 75, 105, 135 and 240 minutes following the addition of quin-2-AM. With the passage of time, the spectrum gradually shifts from that of quin-2-AM to that of quin-2 free acid, and uptake and de-esterification of quin-2-AM was usually seen to be complete at 120 minutes following the addition of quin-2-AM.

It is possible that extracellular hydrolysis of quin-2-AM may result from the activity of extracellular esterases present in seminal plasma, and carried

over during the washing of spermatozoa prior to loading. Such hydrolysed quin-2 should be membrane impermeant and thus should be discarded when the cells are washed prior to lysis in the above experiment. However a small carry-over, and thus a contribution of extracellular esterases to the above data, cannot be completely excluded.

Intracellular Concentration of Quin-2

Ford and Harrison (1983) have estimated the mean water space of human spermatozoa to be 2.21 ± 0.106µl/10^8 spermatozoa. Assuming that quin-2 free acid will distribute equally throughout the aqueous compartments of the spermatozoon, the intracellular concentration of quin-2 free acid was calculated to be 15.24mM (SEM = 2.67, n = 16). This is equivalent to 0.34nmol quin-2 free acid per 10^6 spermatozoa. It is of interest that an intracellular quin-2 concentration of 15mM is nearly ten fold higher than that cited in previously published studies (Tsien et al, 1982a; Knight and Kesteven, 1983; Ochs et al, 1983). Determination of the intracellular concentration of quin-2 is dependent upon a number of assumptions, the most important of which being the presumed volume of distribution of the trapped quin-2. In practice, this is an unknown quantity, and available methodologies for estimating intracellular quin-2 concentrations tend to assume that the indicator will be uniformly distributed throughout the cell (Knight and Kesteven, 1983). Tsien et al (1982a) have demonstrated that this is not so, and have indicated that the final intracellular quin-2 concentration may be several hundred times that of the ester present in the cell suspension during loading (in the present case 50µM). Unless the volume of distribution of intracellular quin-2 is known, the assumption that it will distribute throughout the cell will tend to underestimate the cytoplasmic concentration, just as our assumption that quin-2 distributes throughout the

water space of the spermatozoon will tend to overestimate this figure, given that quin-2 does not appear to be excluded from the nucleus (Tsien et al, 1982a). In the light of this, it is probably more helpful to express the cell-associated quin-2 as nmol quin-2/10^6 spermatozoa, and in this regard, our figure of 0.34nmol quin-2/10^6 spermatozoa is in close agreement with recently published figures for human neutrophils of 0.22 - 0.90nmol quin-2/10^6 cells (Lew et al, 1984).

The loading of spermatozoa with quin-2 according to the protocol described above did not appear to affect cellular function, as reflected in the percentage of motile cells, which was 46.2 ± 2.6 in quin-2 loaded cells, compared to 42.6 ± 3.3 in control cells (both mean ± SEM, n = 5).

Resting $[Ca^{2+}]_i$ Levels in Human Spermatozoa

Tsien et al (1982a; b) have described in detail a methodology for calculating the free intracellular calcium content of a given cell, according to its level of quin-2 fluorescence, utilizing the equation of $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$, in which $[Ca^{2+}]_i$ = free intracellular calcium, K_d = the dissociation constant of quin-2 for Ca^{2+}, F = fluorescence signal of quin-2 loaded cells, F_{max} = maximal fluorescence, obtained by the addition of triton-X-100 and F_{min} = minimal fluorescence, obtained by the addition of excess EGTA. The K_d of quin for Ca^{2+} will depend to some extent upon the pH and ionic environments in which the indicator is located, and this information is not at present available for human spermatozoa, and will, in any case, presumably be different for the BWW medium in which F_{min} and F_{max} are determined. Nevertheless, this method of calculating $[Ca^{2+}]_i$ has been widely used, generally employing the value of K_d cited by Tsien et al (1982a; b) of 115nM.

Applying this methodology to human spermatozoa, the free intracellular calcium of washed ejaculated spermatozoa was estimated to be 146nM, (SEM = 19.9, n = 24).

Uptake and De-Esterification of Fura-2

It was also possible to load fura-2 into human spermatozoa, and to use this indicator to measure intracellular calcium, however, this approach is significantly less straightforward than with quin-2. Fura-2 differs from quin-2, in that changes in the amount of calcium bound to the indicator result in changes in the fluorescence maximum as well as in fluorescence intensity. Figure 5.11 (p 198) shows the fluorescence spectra of fura-2 free acid, using excitation wavelengths of 340nm (upper panel) and 380nm (lower panel), and demonstrates the effect of the addition of calcium. Without calcium (in the presence of EGTA), it can be seen that the emission spectrum at 340nm excitation has a maximum at around 470nm, and when calcium is added (to a free calcium concentration of 1.7mM) there is a shift in this maximum to around 490nm, as well as an increase in fluorescence. When the excitation wavelength is 380nm, it is seen that an addition of calcium results in a decrease in fluorescence. Thus the ratio of fluorescence, measured simultaneously with excitation/emission wavelengths of 340/500nm and 380/500nm, is a very sensitive indicator of the level of free calcium. However, realistic measurements of this ratio require highly sophisticated equipment, capable of measuring at two wavelength pairs simultaneously. Unlike quin-2, there is no major change in the shape of the spectrum of fura-2 in going from the penta-acetoxymethylester (fura-2-AM) to the free acid of fura-2, and Figure 5.12 (p 199) shows the spectra of both compounds, exposed to 1.7mM Ca^{2+}.

Therefore monitoring the successful uptake and de-esterification of fura-2 is not readily possible as is the case for quin-2.

When cells were examined during loading with fura, and the spectrum of the intracellular dye evaluated, it was observed that fura-2 entered the cells rapidly. After 90 minutes of incubation, significant amounts of fura-2 were trapped within the cells, as shown by the characteristic spectra (Figure 5.13, p 200) seen in the supernatants of lysed cell preparations. For this reason, the protocol employed to load cells with fura-2 was similar to that for quin-2, requiring a 120 minute incubation with fura-2-AM. This resulted in easily detectable loading, and Figure 5.14 (p 201) shows typical spectra which were obtained during the loading of human spermatozoa with fura-2. This demonstrates the spectra of (i) loaded cells, (ii) control cells and (iii) the corrected spectrum after taking cellular autofluorescence into account. When measurements of resting intracellular calcium were made, using the procedures discussed above, a value of 406.5 ± 42.6nM was obtained (mean ± SEM, n = 3). Clearly this is higher than that obtained using quin-2, however, simultaneous measurements using quin-2 and fura-2 on the same cell populations were not undertaken in the present study.

5.4 Discussion

The importance of intracellular calcium in the regulation of cell function is firmly established, and much attention is currently being paid to understanding the mechanisms by which cells maintain their internal calcium homeostasis. Understanding these basic mechanisms of cellular function is the essential background against which to study the aetiology of cell dysfunction, and this has important ramifications in elucidating the

causes of male infertility. It is well established that a proportion of male infertility is the result of spermatozoal dysfunction (Aitken et al, 1982a; b; 1984a) and there is evidence to suggest that some of this dysfunction may involve an impaired response to changes in intracellular calcium (Aitken et al,1984b).

The finding that human seminal plasma contains only 0.12 ± 0.0062mM Ca^{2+} (mean ± SEM) is in agreement with other recently published data (Arver and Sjöberg, 1983; Ford and Harrison, 1984). Arver and Sjöberg (1983) found the mean level of free calcium in human seminal plasma to be 0.17 ± 0.05mM (± SD), despite a total calcium concentration of 5.7 ± 2.5mM (mean ± SD). Similarly, Ford and Harrison (1984) found the mean free calcium content of human semen to be 0.24 ± 0.022mM (± SEM), and the total calcium content to be 11.04 ± 0.41mM. Interestingly, this latter group of workers found that while the level of total calcium in semen varied significantly between semen donors, the level of free calcium did not, and they demonstrated that the difference between the total and free calcium was accounted for by the chelating activity of citrate, present in semen at a concentration of 27.2 ± 1.62mM. This low level of free calcium in semen contrasts strikingly with the level of free calcium in the medium normally employed in the present studies for the incubation of spermatozoa, namely 1.7mM, however, studies on the free calcium concentration of human cervical mucus, *in-situ* , have shown that it contains approximately 1.60mM Ca^{2+}, and that this value falls slightly to 1.01mM, after sexual arousal (Levin and Wagner, 1985). Studies on human tubal fluid (Borland et al, 1980) suggest that this contains 1.13 ± 0.24mM calcium. Evidence exists to suggest that, in human and ram, any effects of Ca^{2+} in seminal plasma upon the spermatozoa within the ejaculate may be mediated through a

regulator protein present in seminal plasma, calsemin (Bradley and Forrester, 1982).

When human spermatozoa were incubated in the presence of $^{45}Ca^{2+}$, the amount of isotopic calcium associated with each cell rose, and continued to do so over a 2-hour period. Assuming that normal washed ejaculated human spermatozoa are capable of maintaining ionic equilibrium, this increase in cell-associated counts indicates that exchange of isotopic for non-isotopic calcium is occurring. The effect of mitochondrial electron transport inhibitors upon this increase in cell-associated $^{45}Ca^{2+}$ serves to illustrate some of the difficulties encountered in interpreting data obtained with this isotope. Rotenone, CCCP and FCCP all inhibited the exchange of labeled for unlabeled calcium (as has been previously reported by others) (Singh et al, 1978; Peterson et al, 1979b), by a mechanism independent of any effect on the capacity of the cell to generate ATP, presumably by interfering with the passage of calcium into the mitochondrial pool. The fact that $^{45}Ca^{2+}$uptake was reduced in the presence of mitochondrial inhibitors strongly suggests that the plasma membrane of human spermatozoa is permeable to calcium, rather than impermeable, as has been suggested (Peterson and Freund, 1976; Peterson et al, 1979a). Further evidence for the existence of extramitochondrial calcium pools in the human spermatozoon was obtained in the present study, since significant $^{45}Ca^{2+}$ accumulation continued to occur, even in the presence of mitochondrial inhibitors (Figures 5.4, 5.5, p 191-192).

The elucidation of the number and nature of these various intracellular and extracellular pools of calcium, and of the factors controlling the exchange of calcium with each pool will ultimately be of great interest. However, of more

immediate interest is the measurement of free cytoplasmic ionised calcium, since it is this parameter that will exert an effect on calcium-dependent cellular processes. It was clear from the data that such measurements were not possible with $^{45}Ca^{2+}$, as a substantial proportion of the cell associated calcium measured with this technique was non-cytoplasmic. For this reason, an alternative technique for the measurement of free intracellular calcium was employed. The recent development by Tsien (1981) of the fluorescent calcium indicator quin-2 (2-[[2-[bis (carboxymethyl) amino] -5-methyl-phenoxy] methyl]-6-methoxy-8-[bis (carboxymethyl) amino] quinoline) has made possible the direct measurement of free intracellular calcium. The general principles of this technique have recently been reviewed by Rink and Pozzan (1985).

The present study has demonstrated that it is readily possible to obtain uptake and de-esterification of the tetra-acetoxymethyl ester of quin-2 (quin-2-AM) by washed ejaculated human spermatozoa. It would appear from the data generated in this study that the rate-limiting step in this process is de-esterification, rather than uptake (Figure 5.10, p 197) and that the intracellular concentration of quin-2 is high. The exact intracellular location of the quin-2 remains the subject of speculation at present; however, data from other cell types (Tsien et al, 1982a) would seem to indicate that the majority of the trapped quin-2 behaves as if contained within the cytoplasm and nucleus, and is not significantly trapped within organelles such as mitochondria. The "cytoplasmic" space of spermatozoa is proportionately rather small, and it would be of great interest to establish whether, for instance, quin-2 enters the acrosomal space of human spermatozoa. It is possible that these large quantities of quin-2 will affect cellular function, both directly by buffering calcium, and as a result of the presence of the

breakdown products of quin-2-AM (protons, acetate, and formaldehyde). As has been stated, intracellular quin-2 was without effect on overall percentage motility; however, more detailed studies (employing techniques such as those described in detail in Chapter Four) of the movement characteristics and fertilizing ability of spermatozoa loaded with quin-2 will be required to further evaluate this possibility. Measurement of the resting level of free intracellular calcium in human spermatozoa according to the method proposed by Tsien et al (1982a) yielded a value of 146 ± 19.9nM, well within the physiological range for other cell types. The assumptions underlying these observations have been very fully discussed elsewhere (Rink and Pozzan, 1985). Other workers have now published data on the free calcium content of the spermatozoa of several other species measured using quin-2. Simpson and White (1986) have suggested that the resting free calcium content of ejaculated ram sperm is 193.8 ± 8nM, and that of epididymal boar sperm 175 ± 8nM (both mean ± SEM, n = 3). Similarly, Vijayaraghavan and Hoskins (1986) have reported that the intracellular calcium concentration of bovine epididymal sperm is 44.8 ± 3.2nM (n = 14), while Mahanes et al (1986) found the intracellular free calcium of ejaculated rabbit sperm to be 144 ± 14nM, with no evidence of a change in this value following capacitation.

It was also possible to demonstrate the uptake of fura-2 into human spermatozoa, and to obtain measurements of intracellular calcium using this technique. Although the procedure for loading fura-2 into human spermatozoa is no different from that for quin-2, the practical measurement of intracellular calcium is substantially more complex, necessitating the simultaneous monitoring of fluorescence at 340/500nm and 380/500nm. In order that this be done accurately, sophisticated dual beam

spectrofluorimeters are required, and this seems likely to limit the widespread use of fura-2, despite its undoubted advantages for making single cell measurements (Poenie et al, 1985; Tsien et al, 1985; Williams et al, 1985). For this reason, all subsequent measurements in the current study have been made using quin-2.

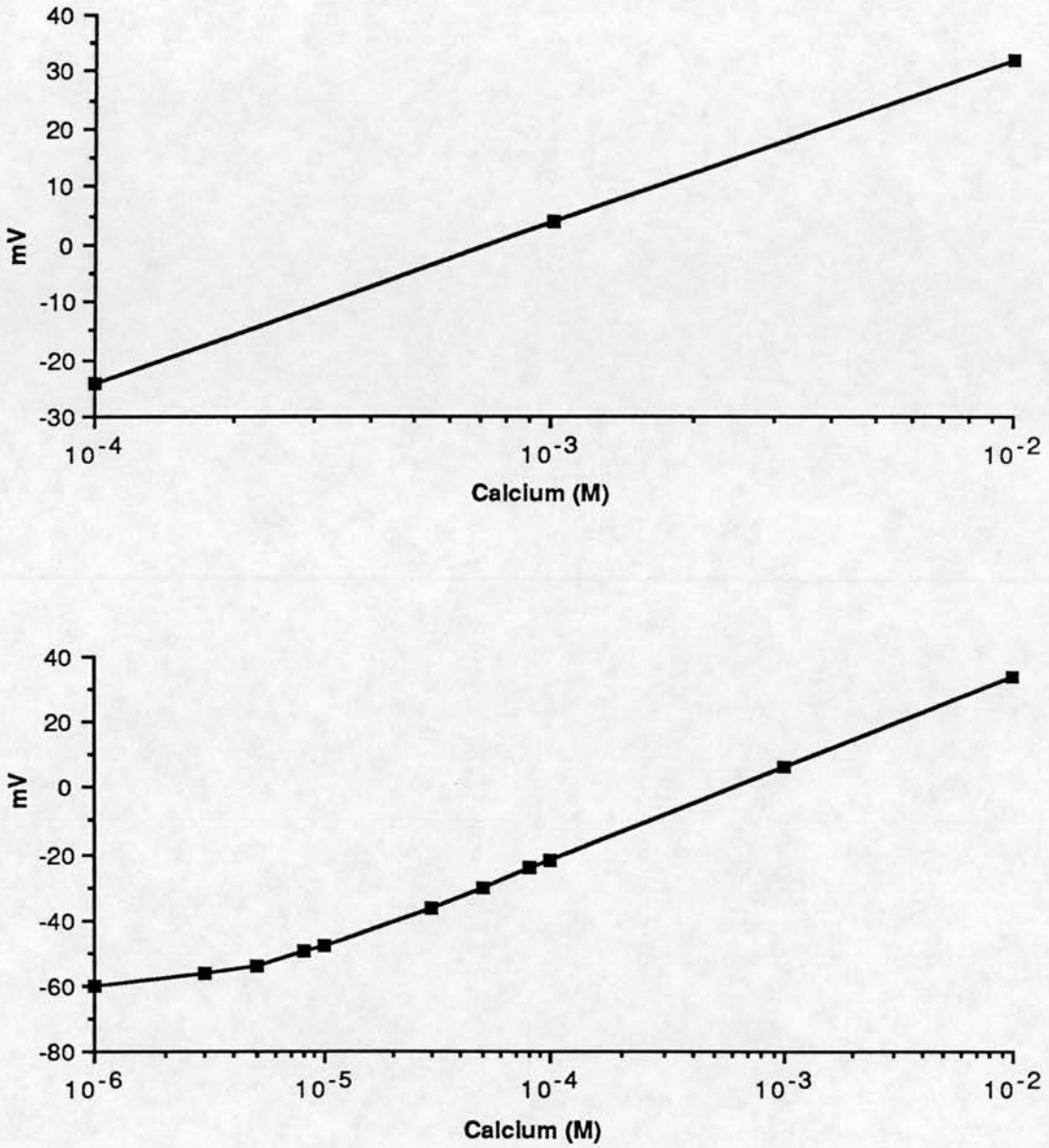


Figure 5.1

Calibration of a Calcium Sensitive Macroelectrode

The electrode yielded a perfectly linear response over the range 4μM to 10mM Ca^{2+} , thus permitting accurate measurements of free calcium in solution over this range. At levels of free calcium below 4μM, accurate measurement was not possible.

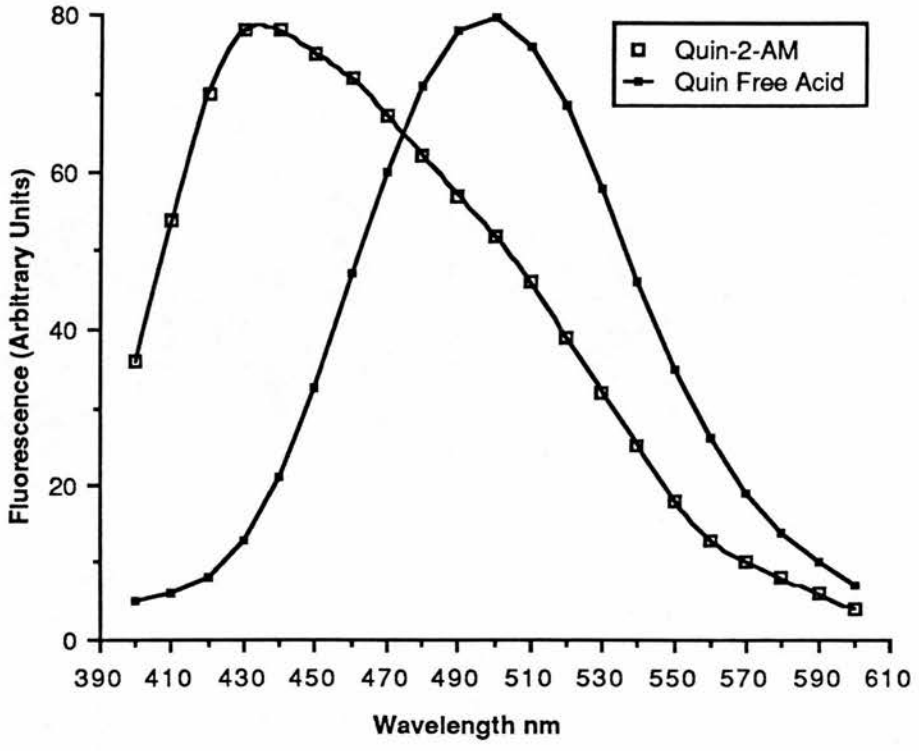


Figure 5.2

Fluorescence Emission Spectra of Quin-2-AM and Quin-2 Free Acid

Quin-2-AM and quin-2 free acid were made up in normal medium BWW, containing 1.7mM Ca^{2+}. Excitation was at 339nm, and emission spectra were scanned over the range 400 - 600nm.

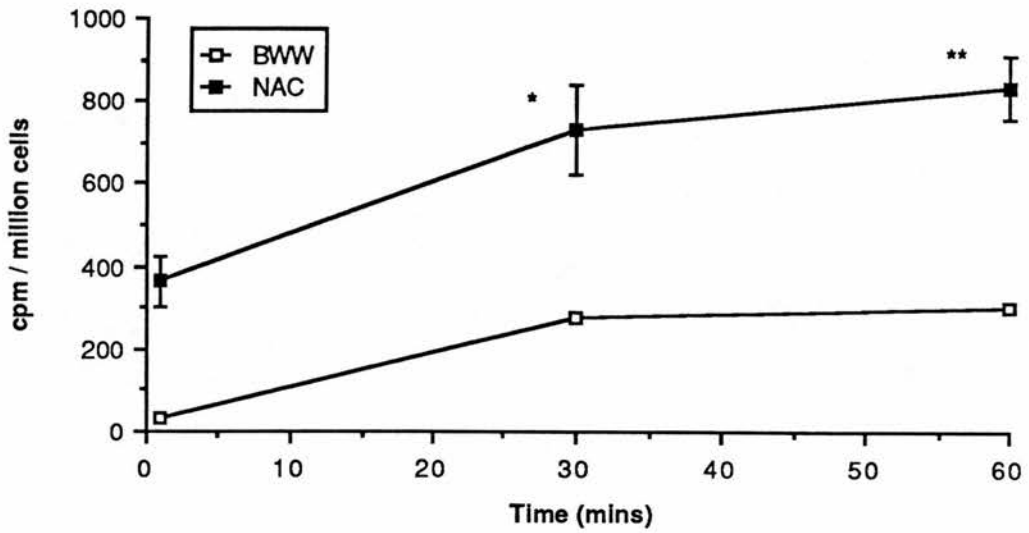


Figure 5.3

Uptake of $^{45}Ca^{2+}$ by washed ejaculated human spermatozoa over one hour

Spermatozoa were incubated with 10μCi/ml $^{45}Ca^{2+}$ in medium BWW containing either 1.7mM unlabeled Ca^{2+} [BWW, open symbols] (mean of two determinations), or 10μM unlabeled Ca^{2+} [NAC, solid symbols] (mean ± SEM, n = 23, differences from t = 0, *P < 0.01, **P < 0.001).

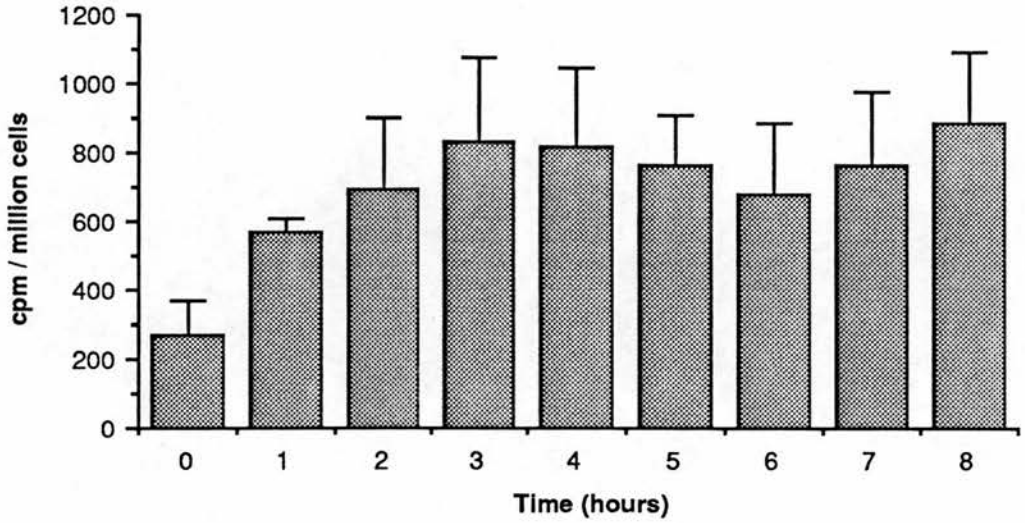


Figure 5.4

Uptake of $^{45}Ca^{2+}$ by washed ejaculated human spermatozoa over eight hours

Spermatozoa were incubated with 10μCi/ml $^{45}Ca^{2+}$ in medium BWW containing 10μM unlabeled Ca^{2+} (mean ± SEM, n = 4). Overall, no significant increase in cell associated calcium occurred after 60-120 minutes incubation.

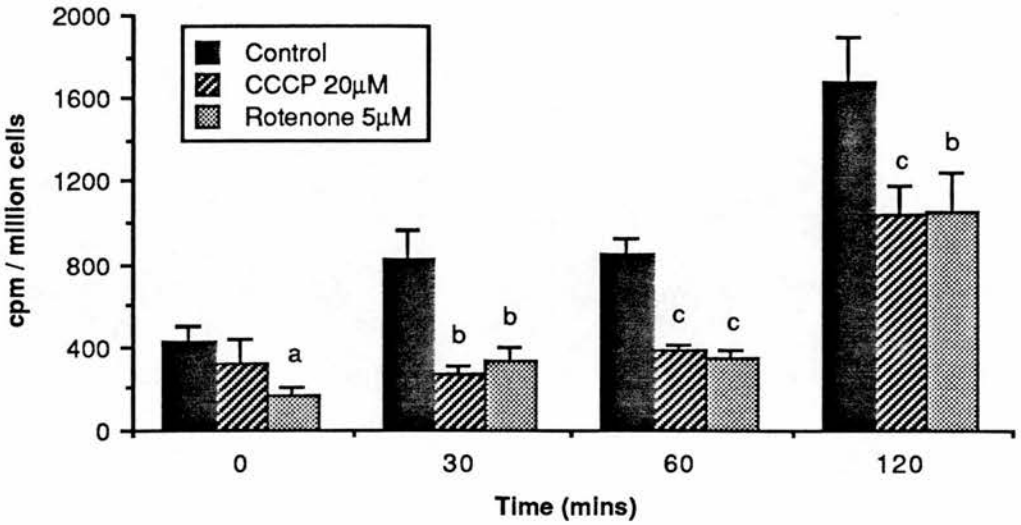


Figure 5.5

Effect of Inhibitors of Mitochondrial Electron Transport on $^{45}Ca^{2+}$ Uptake by Washed Ejaculated Human Spermatozoa : CCCP and Rotenone

CCCP (20μM) and Rotenone (5μM) were added immediately prior to the addition of 10μCi/ml $^{45}Ca^{2+}$ and cells removed for counting immediately and after 30, 60 and 120 minutes. Results are mean ± SEM. Differences from control, a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$.

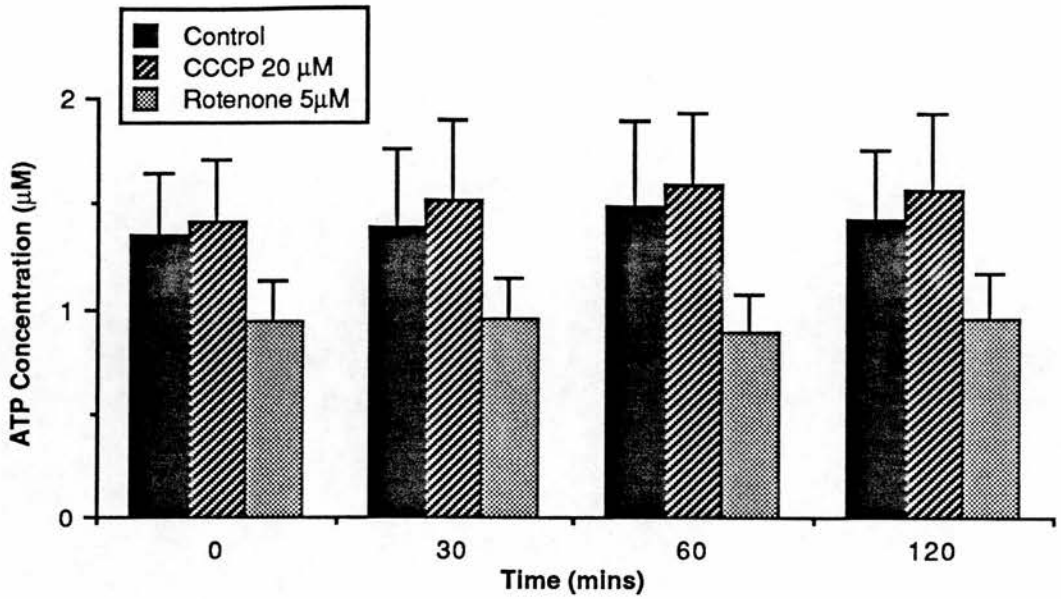


Figure 5.6

Effect of Inhibitors of Mitochondrial Electron Transport on ATP Concentrations in Washed Ejaculated Human Spermatozoa : CCCP and Rotenone

CCCP (20µM) and Rotenone (5µM) were added as in figure 5.5, and ATP concentrations determined on the cell suspensions as described in Chapter Three. Results are mean ± SEM.

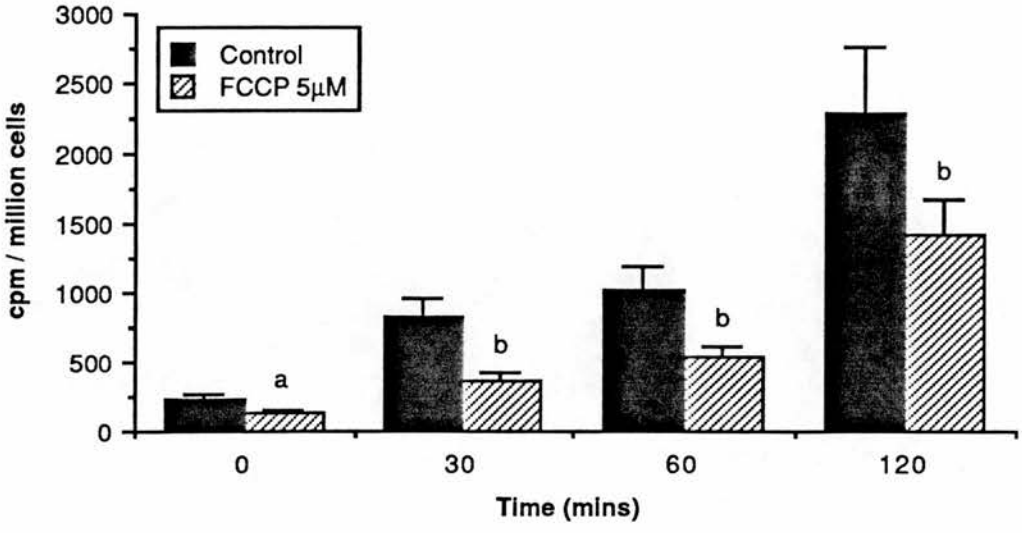


Figure 5.7

Effect of Inhibitors of Mitochondrial Electron Transport on $^{45}Ca^{2+}$ Uptake by Washed Ejaculated Human Spermatozoa : FCCP

FCCP (5μM) was added immediately prior to the addition of 10μCi/ml $^{45}Ca^{2+}$ and cells removed for counting immediately and after 30, 60 and 120 minutes. Results are mean ± SEM. Differences from control, a = $P < 0.02$; b = $P < 0.01$.

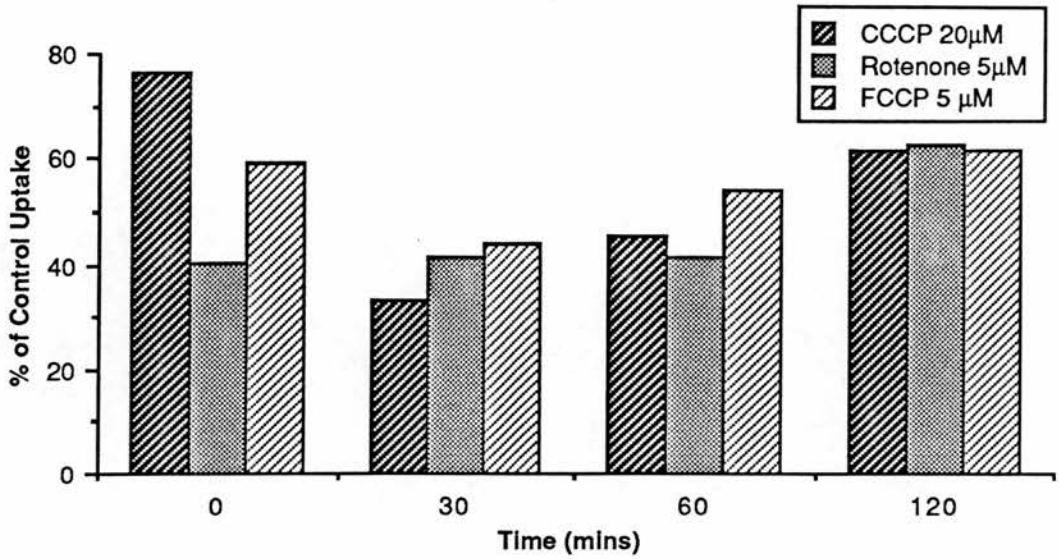


Figure 5.8

Effect of Inhibitors of Mitochondrial Electron Transport on $^{45}Ca^{2+}$ Uptake by Washed Ejaculated Human Spermatozoa : Percentage inhibition

CCCP (20μM), Rotenone (5μM) and FCCP (5μM) were added immediately prior to the addition of 10μCi/ml $^{45}Ca^{2+}$. Results are expressed as percentage of control uptake (cpm/10^6 cells) at the same time point. It is seen that suppression of uptake was generally maximal at 30 minutes and declines thereafter.

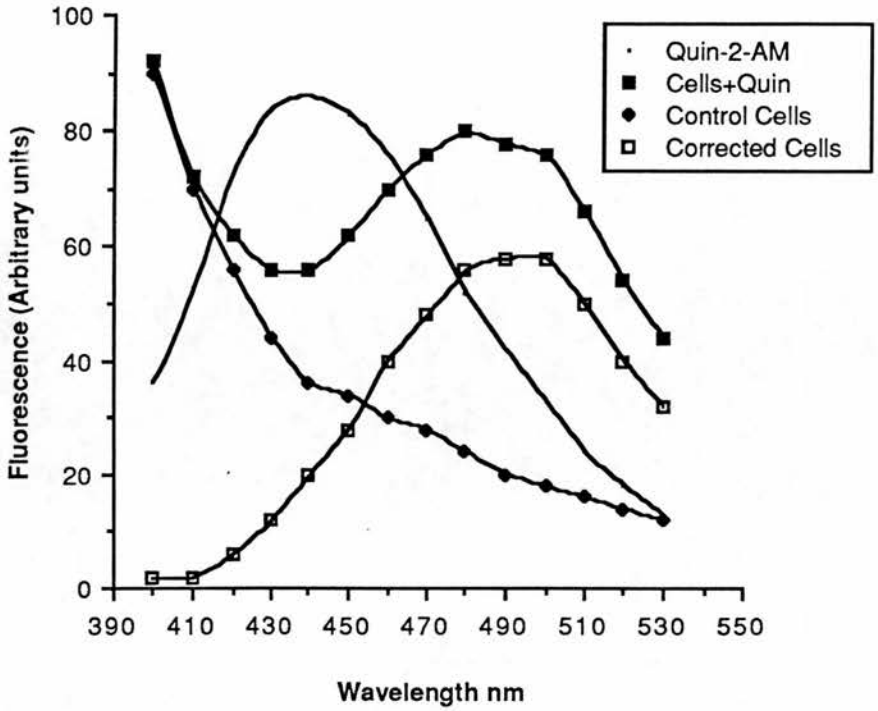


Figure 5.9

Loading of Quin-2-AM into Human Spermatozoa : Fluorescence Spectra

Emission spectra obtained during uptake and de-esterification of quin-2-AM by human spermatozoa. Excitation wavelength = 339nm, emission wavelengths = 400 - 530nm.

i) Quin-2-AM = spectrum of quin-2-AM in BWW containing 1.7mM Ca^{2+}.

ii) Cells + Quin = spectrum of spermatozoa that have been loaded with quin-2, according to the protocol described in the text.

iii) Control cells = cells incubated with DMSO alone (cell autofluorescence).

iv) Corrected Cells = spectrum of cells loaded with quin-2, corrected for cell autofluorescence, showing the characteristic spectrum of quin-2 free acid.

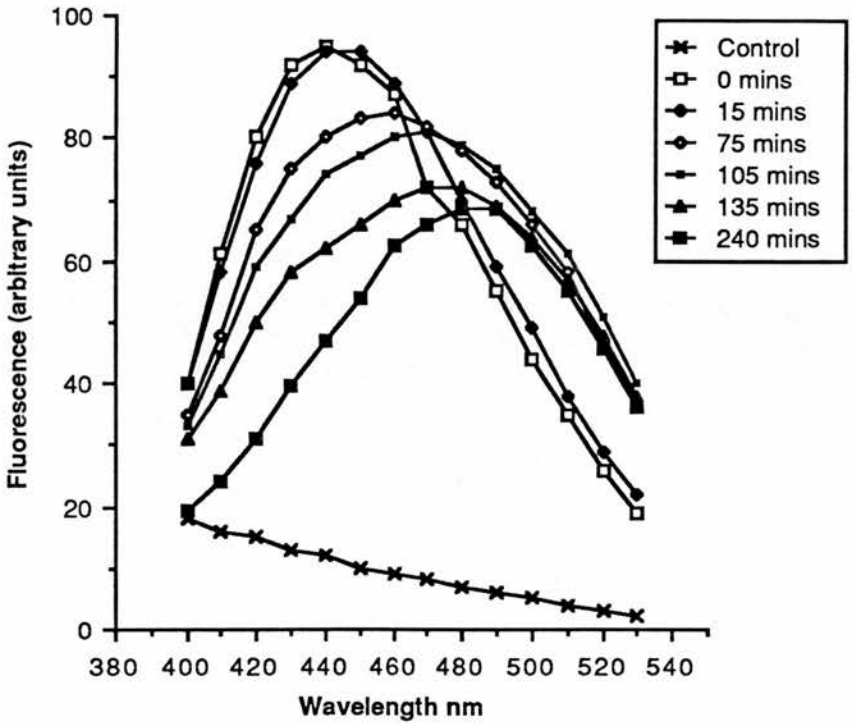


Figure 5.10

Loading of Quin-2-AM into Human Spermatozoa : Time course of uptake

Emission spectra obtained during uptake and de-esterification of quin-2-AM by human spermatozoa. Excitation wavelength = 339nm, emission wavelengths = 400 - 530nm.

Cells were incubated with 50μM quin-2-AM. At various times, cells were removed, washed, lysed and the supernatant was examined as above.

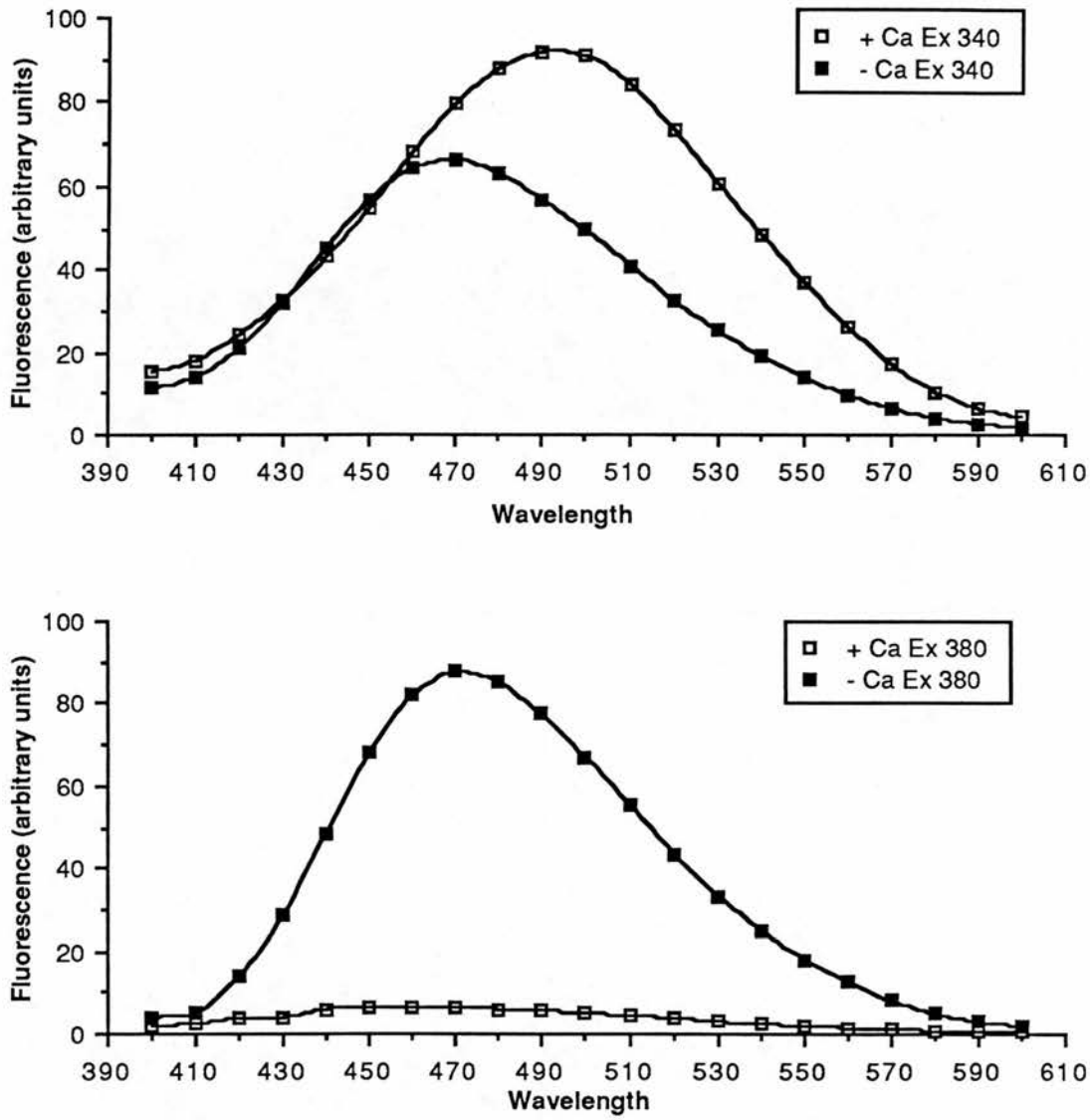


Figure 5.11

Fura-2 in the measurement of intracellular calcium : Effect of calcium concentration on fluorescence

This figure demonstrates the principal of measurement using fura-2. The upper panel shows the emission spectra of fura-2 free acid, over the range 400-600nm emission with excitation at 340nm, in the presence (+) and absence (-) of calcium. The lower panel shows the same, but with excitation at 380nm. A change in fluorescence maximum as well as in intensity is seen.

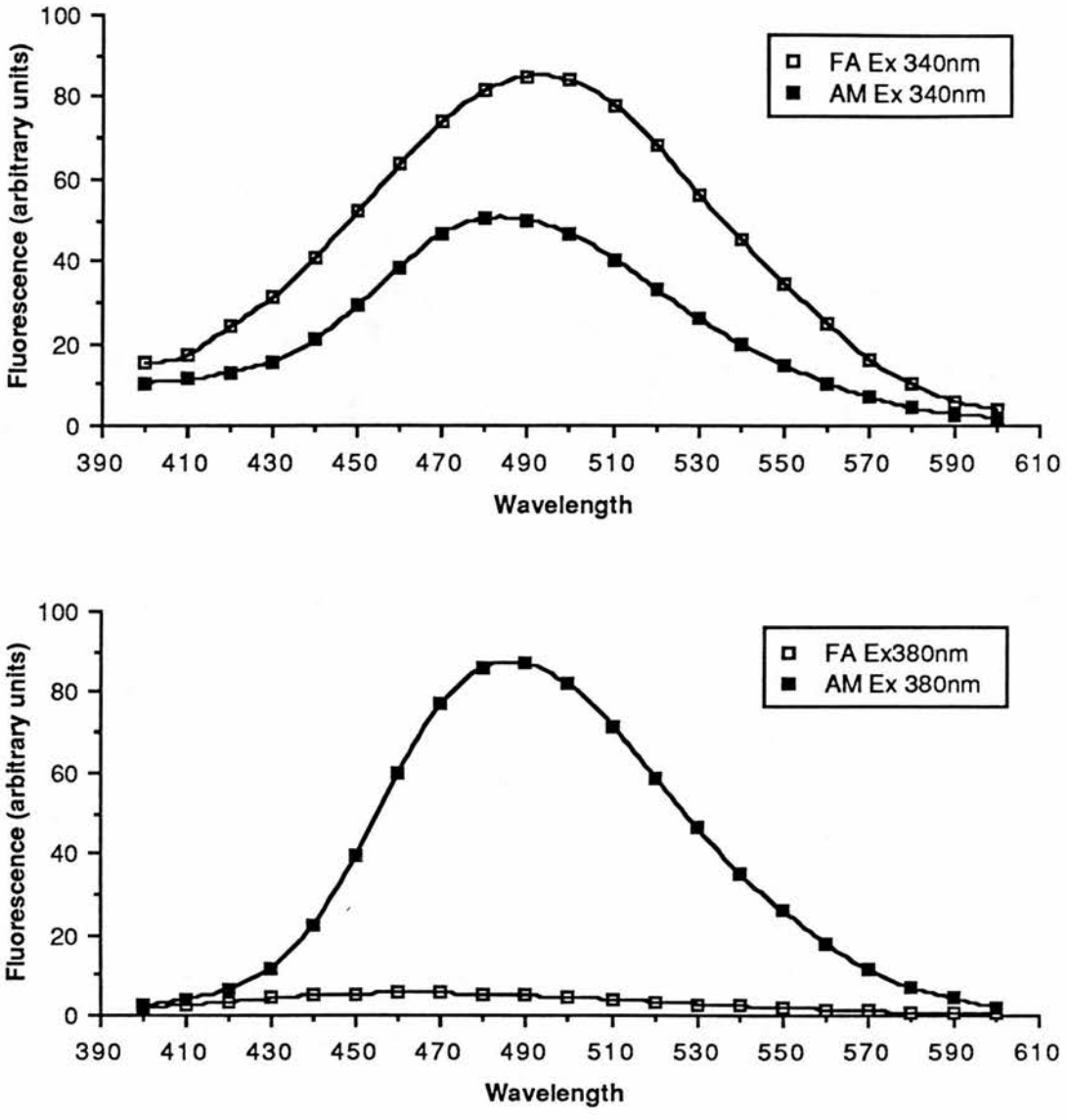


Figure 5.12

Fura-2 in the measurement of intracellular calcium : Changes in spectra with de-esterification

Spectra of fura-2-AM and fura-2 free acid, with excitation at 340nm (upper panel) and 380nm (lower panel). In contrast to quin-2, there is little change in the fluorescence maximum of fura-2 in going from the esterified to the de-esterified state.

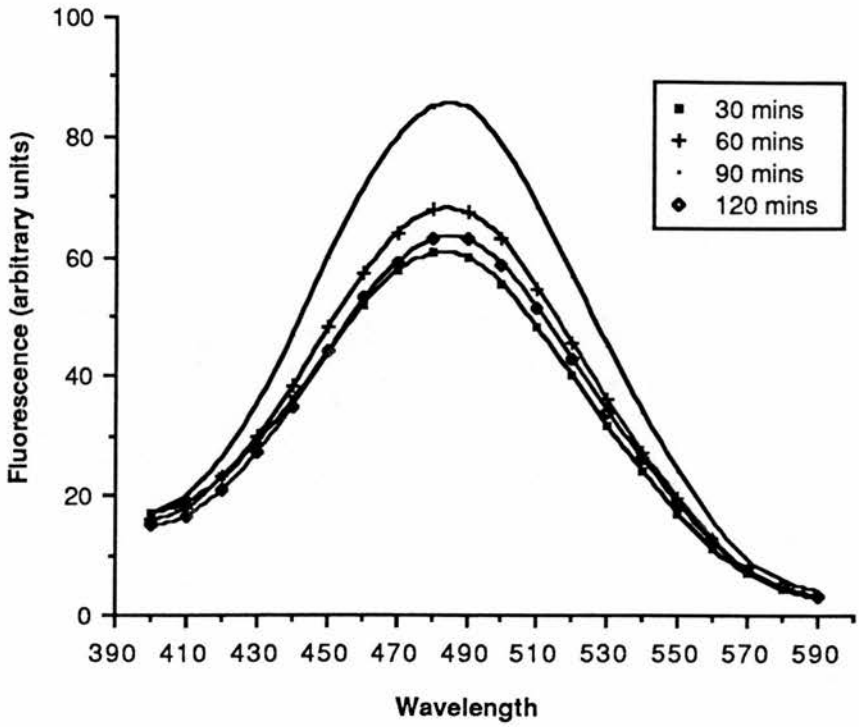


Figure 5.13

Fura-2 in the measurement of intracellular calcium : Time course of uptake

Spectra of the supernatants of lysed human spermatozoa during loading with 1μm fura-2-AM. Fura-2 is rapidly trapped within the cells.

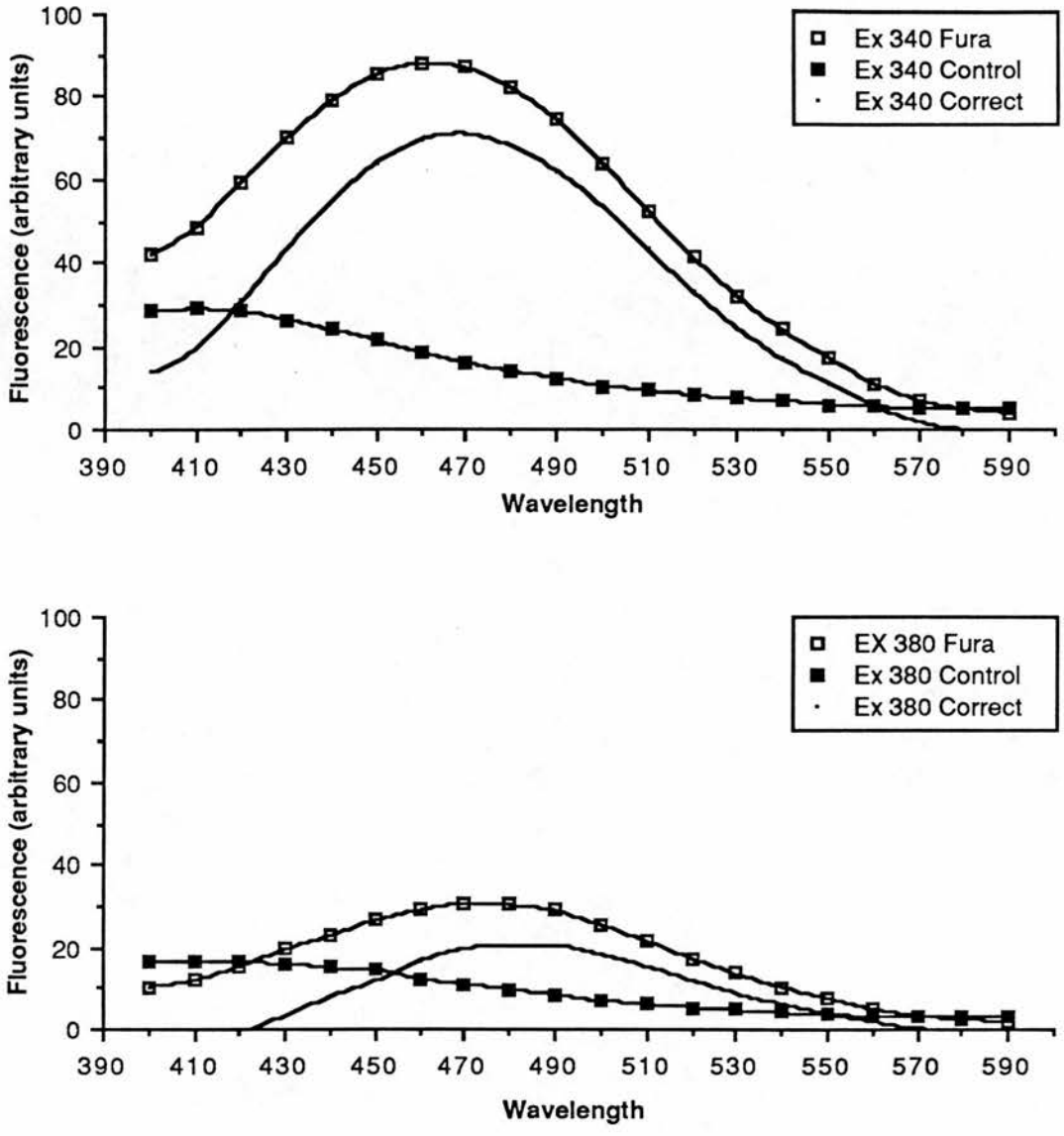


Figure 5.14

Fura-2 in the measurement of intracellular calcium : Demonstration of uptake

Spectra obtained during the loading of fura-2 into human spermatozoa, upper panel, excitation = 340nm, lower panel, excitation = 380nm.

i) Fura = spectrum of cells incubated with 1μM fura-2-AM and washed.

ii) Control = spectrum of cells incubated with DMSO alone.

iii) Corrected = spectrum of loaded cells, corrected for autofluorescence.

CHAPTER SIX

THE REGULATION OF INTRACELLULAR CALCIUM BY HUMAN SPERMATOZOA AND ITS MANIPULATION DURING *IN-VITRO* TESTING OF SPERM FUNCTION

6.1 Introduction

The importance of extracellular calcium in the normal functioning of the spermatozoa of a variety of animal species is now well established (Yanagimachi and Usui, 1974; Peterson and Freund, 1976; Singh et al, 1978; 1983; Peterson et al, 1979a; b; Garbers and Kopf, 1980; Triana et al, 1980; Fraser, 1982; Shackmann and Shapiro, 1982; Yanagimachi, 1982; Breitbart et al, 1984; Hong et al, 1984; Rufo et al, 1984). It has been suggested that extracellular calcium is required for the completion of the acrosome reaction of guinea pig (Yanagimachi and Usui, 1974), hamster (Yanagimachi, 1982), and mouse spermatozoa (Fraser, 1982), and that calcium may also be important in normal capacitation, with an influx of calcium being an important component of capacitation (Singh et al, 1978; Triana et al, 1980). Comparatively little data is available concerning the calcium metabolism of human spermatozoa.

The data presented in the previous chapter indicate that human spermatozoa maintain a free intracellular calcium concentration of the order of 100 - 200nM in the face of an extracellular calcium concentration of 1 - 2mM. The mechanisms by which human spermatozoa seek to maintain calcium homeostasis, and by which changes in intracellular calcium might influence cell function are not clear. The majority of available experimental evidence supports the view that, under resting conditions, calcium enters the cell passively, flowing down a concentration gradient, and the spermatozoon maintains its low level of free intracellular calcium by actively extruding calcium across the plasma membrane and by sequestering it in cellular organelles such as the nucleus and mitochondrion (Borle, 1981; Campbell, 1982). From the point of view of the entry of calcium into the cell, doubt has been expressed concerning the permeability of the plasma

membrane of human spermatozoa to calcium (Peterson and Freund, 1976; Peterson et al, 1979a), and the possibility of the existence of voltage sensitive calcium channels in the sperm plasma membrane is also the subject of debate (Singh et al, 1983; Roldan et al, 1986). From the point of view of calcium efflux from the cell, a number of groups have shown that bull and ram sperm plasma membranes contain a Ca-ATPase (Breitbart and Rubinstein, 1983; Breitbart et al, 1983; 1984), however, this finding has not been confirmed by other workers (Rufo et al, 1984) and while spermatozoa have been shown to contain the calcium regulating protein calmodulin (Pariset et al, 1983; Norland et al, 1985) it is not clear whether drugs which interfere with the function of calmodulin affect calcium homeostasis directly or non-specifically (Peterson et al, 1983; Wasco and Orr, 1984; Nagae and Srivastava, 1986).

In terms of the diagnosis of sperm dysfunction, data presented in Chapter Four demonstrates how the pharmacological manipulation of the calcium balance of the human spermatozoon with the antibiotic A23187 can be used to advantage in the context of the zona-free hamster oöcyte penetration test. The practical advantages of performing the zona-free hamster oöcyte penetration test in the presence of A23187 have been mentioned previously, in that this methodology circumvents the problems of inter-ejaculate and inter-individual variability in the capacitation time of spermatozoa (Perreault and Rogers, 1982). In order to fuse with zona-free hamster oöcytes, human sperm must first capacitate and then undergo the acrosome reaction, while the plasma membrane overlying the equatorial region of the sperm head must acquire fusogenic properties. In circumstances where the the results of zona-free hamster oöcyte penetration testing are abnormal, it is not clear which of the above

processes is defective. Assessment of the ability of human spermatozoa to undergo capacitation is a particularly difficult problem, because, although the process of capacitation is thought to involve the entry of calcium into the cell (Singh et al, 1978; Triana et al, 1980), the mechanisms involved in this putative calcium entry *in-vivo* are at present unknown. In an *in-vitro* context, the mechanisms facilitating capacitation during the pre-incubation involved in the performance of the hamster egg penetration test are obscure, and the biological relevance of capacitation under such circumstances is questionable (Aitken et al, 1984b). In practical terms, the problem of variability in capacitation times can be overcome by the use of the divalent cation ionophore A23187, which is presumed to permit an influx of calcium into the cell and thereby facilitates rapid and simultaneous occurrence of capacitation and acrosome reaction in those cells capable of this. The diagnostic value of the results obtained from the hamster oöcyte penetration test performed under such circumstances have been described in Chapter Four.

A23187 is a carboxylic acid antibiotic produced by *Streptomyces chartreusensis* , and whose capacity to function as a divalent cation ionophore is well described (Reed and Lardy, 1972; Wong et al, 1973; Babcock et al, 1976), although there is evidence to suggest that, in addition to its ability to allow divalent cations across biological membranes, it may have other important effects, including the disruption of membrane structure by means of modifying protein-lipid interactions (Klausner et al, 1979). Similarly, ionomycin, produced by *Streptomyces conglobatus* , also functions as a divalent cation ionophore, and has a narrower range of cation selectivity than A23187, being more specific for calcium (Liu and Hermann, 1978; Kauffman et al, 1980). It is of note that A23187 exhibits

fluorescence at the wavelengths used for measuring intracellular calcium by means of quin-2, whereas ionomycin and the halogenated derivative of A23187, Bromo-A23187 (Deber et al, 1985) do not demonstrate this interfering autofluorescence.

When normospermic semen samples were incubated in the presence of 50 or 100μm $Ca^{2+}Mg^{2+}$ A23187, there were marked increases in the percentage of zona-free hamster oöcytes penetrated by such spermatozoa, together with increases in the degree of polyspermy, despite the absence of any effect of these doses of ionophore on overall motility or on the forward velocity or amplitude of lateral sperm head displacement (Figure 6.1, p 219) (Aitken et al, 1984b). Lower doses of A23187 produced smaller increases in penetration rate and higher doses produced a fall in penetration rate as well as a fall in overall motility and in the amplitude of lateral sperm head displacement. In contrast to the general stimulation of sperm function seen in normospermic donors, no overall increase in egg penetration was observed amongst a group of oligozoospermic samples when treated with A23187, however 65.5 - 68.6% of oligozoospermic samples scored 0% penetration in the conventional assay, and this was reduced to 48.6 - 50.0% of samples scoring zero% penetration in the presence of 50 - 100μm A23187 (Aitken et al, 1984b).

A study was therefore undertaken to investigate the regulation of intracellular calcium by human spermatozoa, and to examine the effects of divalent cation ionophores, such as A23187 and ionomycin upon the level of free intracellular calcium.

6.2 Materials and Methods

Cell Preparation and Handling

Semen samples were obtained from normal donors, and the spermatozoa were washed and resuspended in medium BWW, normally containing 1.7mM Ca^{2+}, as described in Chapter Five. For some experiments, cells were suspended in medium which contained no added calcium (NAC), and which, as was discussed above, contained approximately 10μm free calcium. In addition, calcium deficient BWW was made up with the addition of 100μm EGTA to chelate any remaining free calcium, and thus produce a "calcium free" medium BWW.

Loading of Spermatozoa with Quin-2 and Measurement of $[Ca^{2+}]_i$

The loading of spermatozoa with quin-2 was performed and the measurement of intracellular calcium undertaken according to the detailed protocols discussed in Chapter Five. All measurements were performed in a Baird or Perkin Elmer LS-5 spectrofluorimeter, thermostated to 37°C, unless otherwise required by the experiment. In the case of time course studies, samples of quin-loaded sperm were maintained at 37°C for the duration of the experiment, by holding the cuvettes containing the spermatozoa in the water bath used to thermostat the spectrofluorimeter and on all occasions, cells and solutions were mixed prior to measuring fluorescence. At the end of each experiment, the system was calibrated as described in Chapter Five, by the addition of Triton-X-100 followed by EGTA.

Materials

Chemicals were obtained from the Sigma Chemical Co (St Louis, MO, USA) except as stated below. Quin-2-AM was made up as a stock solution in DMSO and was stored dark and desiccated at -20°C between experiments. A23187 ($Ca^{2+}Mg^{2+}$ salt) , ionomycin (Ca^{2+} salt) and 1-oleoyl, 2-acetyl glycerol (OAG) were obtained from Calbiochem (Bishops Stortford, England) and were similarly made up as stock solutions IN DMSO and were stored dark and desiccated at 4°C between experiments. Rotenone was also made up as a stock solution in DMSO and stored at -20°C. Verapamil hydrochloride and diltiazem hydrochloride were made up as stock solutions in ethanol and were stored at -20°C.

Statistical Analysis

Statistical comparisons were performed using the Student t test, Mann Whitney U test, or analysis of variance, as appropriate. Probabilities with $P < 0.05$ were regarded as significant.

6.3 Results

Effect of Extracellular Calcium on $[Ca^{2+}]_i$

Washed spermatozoa were loaded with quin-2, and were then resuspended in either normal medium BWW (containing 1.7mM Ca^{2+}), medium BWW with no added calcium (NAC) (containing 10μm Ca^{2+}) or medium BWW containing no added calcium to which 100μm EGTA had been added (calcium free BWW). After allowing a period of equilibration, intracellular calcium was measured according to the procedures described in Chapter Five, but modified in the case of spermatozoa suspended in calcium-free BWW to include the addition of 1.7mM Ca^{2+} following cell

lysis, in order to obtain a value for F_{max}, followed by the addition of excess EGTA to obtain F_{min}. This was not necessary in the case of spermatozoa in NAC medium, as quin-2 fluorescence is saturated at calcium concentrations of 10μm.

Extracellular calcium was found to have a significant effect on intracellular calcium. In normal medium BWW, intracellular calcium was 206.4 ± 44.6nM (mean ± SEM, n = 3), however when extracellular calcium was reduced to 10μm, intracellular calcium fell to 54.3 ± 17.7nM, and in the complete absence of extracellular calcium, intracellular free calcium was only 9.8 ± 2.9nM (Figure 6.2, p 220). All values were statistically significantly different from each other ($P < 0.05$, n = 3, Mann Whitney U Test).

Absence of Voltage Sensitive Calcium Channels

Having established that human spermatozoa were permeable to calcium, it was of interest to establish whether or not calcium was entering human spermatozoa via voltage sensitive calcium channels. In order to study this, spermatozoa loaded with quin-2 were resuspended in calcium deficient medium BWW (NAC), resulting in a fall in intracellular calcium from around 200nM to 50nM. When the level of fluorescence of a suspension of quin-loaded sperm in NAC medium was continuously monitored, a constant level of fluorescence was observed. When calcium was added to the solution to a concentration of 1.7mM, a gradual increase in fluorescence was seen, reaching a plateau after about 4 minutes, and presumably reflecting the passage of calcium through the plasma membrane into the extramitochondrial space (Top panel, Figure 6.3, p 221).

The addition of agents known to block the entry of calcium through voltage sensitive calcium channels, verapamil and diltiazem (both at doses of 10μm), did not affect the resting level of intracellular calcium or the subsequent passage of calcium into the cells when the extracellular calcium concentration was increased (Figure 6.3, p 221, Lower panels). When these changes in fluorescence were expressed as a percentage increase in fluorescence over the resting level, no statistically significant differences were observed (control = 136.5 ± 4.6, mean ± SEM, n = 5; verapamil = 128.0 ± 4.7, mean ± SEM, n = 4; diltiazem = 139.1 ± 13.4, mean ± SEM, n = 5).

Effect of Rotenone on $[Ca^{2+}]_i$

When rotenone at a dose of 10μm was added to quin-loaded spermatozoa, and their level of free intracellular calcium measured after 10 minutes incubation, it was seen that there was no significant change in intracellular calcium levels, with control cells having levels of 160.3 ± 19.6nM, and rotenone treated cells having levels of 191.9 ± 39.0nM (mean ± SEM, n = 4, P = 0.171, not significant, Mann Whitney U). (Figure 6.4, p 222). This was in contrast to the effect of rotenone on cell-associated $^{45}Ca^{2+}$, where an immediate suppression of cell associated calcium was seen (Chapter Five). It was not possible to examine the effects of FCCP and CCCP on $[Ca^{2+}]_i$ because these agents cause quenching of quin-2 fluorescence.

Effect of Temperature on $[Ca^{2+}]_i$

Spermatozoa loaded with quin-2 were placed in the measuring chamber of a spectrofluorimeter, thermostated with a water jacket, the temperature of which was constantly monitored with a thermocouple. The fluorescence signal was continually monitored as the temperature of a suspension of

quin loaded cells was gradually lowered from 37°C to 15 °C over a period of 15 minutes, and then was gradually re-warmed to 37°C. A typical tracing from such an experiment is shown in Figure 6.5 (p 223), revealing that as the temperature of the sample fell, there was a corresponding rise in quin-2 fluorescence, presumably reflecting a rise in intracellular calcium, which proved to be reversible on rewarming the sample.

Effect of Divalent Cation Ionophores on $[Ca^{2+}]_i$

Given the clinical value of the zona-free hamster oöcyte penetration test performed in the presence of A23187, it was of interest to study the effects of divalent cation ionophores on the intracellular calcium concentrations of human spermatozoa.

Studies by Aitken and colleagues (Aitken et al, 1984b) have indicated that the stimulatory effects of A23187 on human sperm function are apparent after only 30 minutes of incubation, and for this reason a dose response study of the acute effects of both A23187 (as its calcium/magnesium salt) and ionomycin (as its calcium salt) was undertaken. A23187 was added to suspensions of quin-loaded spermatozoa at doses of 10, 25, 50 and 100μm, and ionomycin at doses of 0.25, 0.5, 1 and 10μm, and after a short incubation of 10 minutes, intracellular calcium concentrations were measured. The autofluorescence of A23187 was taken into account by measuring $[Ca^{2+}]_i$ at each dose level, including control, on separate cuvettes of cells. This ensured that the ionophores were present throughout the measurement of F, F_{max} and F_{min}, thus ensuring that the fluorescence of the ionophore was a constant factor, and so did not influence the measurement of $[Ca^{2+}]_i$.

A group of four semen samples from normal donors was studied, and the resting intracellular calcium concentration of these samples was found to be 162.3 ± 24.32nM (mean ± SEM, n = 4). None of the doses of A23187 studied produced any significant change in intracellular calcium concentrations in this short term study, with the calcium concentration of cells treated with 100μm A23187 for ten minutes being only 154.6 ± 57.9nM. In contrast, all of the doses of ionomycin studied produced significant increases in intracellular calcium within ten minutes, with 0.25μm ionomycin raising the level of free calcium to 206.3 ± 29.9nM, 0.5μm to 226.2 ± 37.9, 1μm to 255.0 ± 49.5 and 10μm ionomycin raising intracellular free calcium to 600.3 ± 146.3nM. All of these differences were significantly higher than the control level ($P < 0.05$). (Figure 6.6, p 224)

A time course study was next undertaken, to examine the effect of 100μm A23187 and 10μm ionomycin on free intracellular calcium over the period of a two hour incubation. Quin-loaded spermatozoa were treated with the appropriate reagent at time 0, and fluorescence was measured at 0, 5, 15, 30, 60, and 120 minutes, at the end of which time each cuvette of cells was calibrated and intracellular calcium concentrations were calculated. The control population of cells showed no change in free intracellular calcium over this time period, having a baseline value of 72.9 ± 15.1nM (mean ± SEM, n = 3), rising to 102.1 ± 24.2nM after 120 minutes. Cells treated with 100μm A23187 showed a gradual rise in intracellular calcium over two hours, from 74.0 ± 26.3nM at the start of the experiment , rising to 152.8 ± 49.7nM after 60 minutes, and to 282.7 ± 14.4 after two hours (significantly greater than control, $P < 0.05$, Mann Whitney U). In contrast, cells treated with 10μm ionomycin showed an immediate and sustained increase in intracellular free calcium, with values of 181.8 ± 44nM being achieved

immediately upon addition of ionophore, and rising to 2338.3 ± 759.5nM after 60 minutes and 1420.5 ± 556.7nM after 120 minutes. (all values significantly greater than control, $P < 0.05$, Mann Whitney U). (Figure 6.7, p 225). A shorter time course study was also undertaken using doses of 50μm A23187 and 1μm ionomycin. This also revealed significant increases in intracellular free calcium after 60 minutes of incubation, with levels rising to three times the control values (Figure 6.8, p 226).

Effect of 1-oleoyl, 2-acetyl glycerol on $[Ca^{2+}]_i$

Stimulation of human sperm function can be achieved with compounds other than A23187, one such compound being 1-oleoyl, 2-acetyl glycerol (OAG) (Aitken R J and Clarkson J S, unpublished observations). It was thus of interest to examine whether or not such stimulation was mediated through an increase in intracellular free calcium.

An acute dose response study was undertaken of the effects of varying doses of OAG, added to human spermatozoa in differing external calcium concentrations. Spermatozoa were loaded with quin-2 and suspended in either calcium free medium BWW (containing 100μm EGTA), NAC medium, containing 10μm Ca^{2+}, or normal medium BWW, following which OAG was added to concentration of 25, 50, 100, or 200 μg/ml, DMSO alone to control incubations, and intracellular calcium was measured after 10 minutes. The results are shown in Figures 6.9, 6.10 and 6.11 (pp 227-229), which show that in addition to the previously documented effects on intracellular calcium of varying extracellular calcium concentrations, there were no significant changes in intracellular calcium in response to any of the doses of OAG studied. It is seen that there is a suggestion of a rise in $[Ca^{2+}]_i$ in response to the higher doses of OAG, however none of these values achieved

statistical significance (n = 3, $P > 0.05$ on Mann Whitney U, analysis of variance and paired t testing).

When 50μg/ml OAG was added to spermatozoa in the presence of 1.7mM extracellular calcium, and intracellular calcium measured at 0, 5, 15, 30, 60 and 120 minutes after addition, it was again seen that intracellular calcium was higher at every time point in the OAG treated samples, but that the differences were again non significant. (Figure 6.12, p 230) Both of the above results may simply reflect the small sample size used in the present study, however it was clear that OAG did not result in increases in intracellular calcium of the same order of magnitude as those induced by the divalent cation ionophores.

6.4 Discussion

The available experimental evidence is consistent with the view that the human spermatozoon is permeable to calcium and that calcium flows into the cell down a concentration gradient. The cell then seeks to maintain a low intracellular calcium by sequestering calcium into the mitochondrion and by actively extruding Ca^{2+} across the plasma membrane (Breitbart and Rubinstein, 1983; Breitbart et al, 1983; Peterson et al, 1979a; b). The present study supports the view that calcium enters the cell passively and that such entry is not through voltage-sensitive calcium channels, since the ingress of calcium was not inhibited by agents agents known to affect such channels, such as verapamil and diltiazem. However, these studies have involved only washed ejaculated spermatozoa, and further work will be required to investigate whether or not human spermatozoa develop calcium channels during the process of capacitation, which may subsequently be

involved in calcium entry during the acrosome reaction. However, other workers have published data which suggests that the plasma membranes of spermatozoa may not contain voltage sensitive calcium channels. Singh and colleagues (1983), using $^{45}Ca^{2+}$, have observed that the calcium channel antagonists D600 and TMB-8 inhibit the net uptake of Ca^{2+} by sperm suspensions, but have provided evidence to suggest that this effect may be mediated through some non-specific action of the drugs concerned rather than by a specific effect on voltage sensitive calcium channels (Singh et al, 1983). Roldan et al (1986) have demonstrated that drugs which block voltage sensitive calcium channels (verapamil, nifedipine and nimodipine) do not block the occurrence of the acrosome reaction in guinea pig or golden hamster spermatozoa incubated in capacitating calcium containing media. Their results suggest that the entry of calcium into the spermatozoa of these species, which is thought to be an important component of the acrosome reaction, is not through voltage sensitive calcium channels.

The fact that changes in extracellular calcium concentrations have a profound effect on intracellular calcium concentrations is also in keeping with the view that calcium enters the cell down a concentration gradient, and is actively extruded against such a gradient. A reduction in extracellular calcium levels would reduce the driving force for the passive influx of Ca^{2+}, and would diminish the energy requirement for calcium extrusion.

The evidence presented in Chapter Five demonstrated that a substantial proportion of cell-associated calcium is mitochondrial, since treatment with drugs which block mitochondrial electron transport cause a suppression in cell-associated $^{45}Ca^{2+}$. Evidence from other cell types (Borle, 1981) would suggest that mitochondrial sequestration of calcium is an important means

whereby the cell reduces the level of free calcium in the cytoplasm, with as much as 40% of total cell calcium being sequestered in this way. It has been observed in the present study that inhibitors of mitochondrial electron transport, while reducing the total cell-associated $^{45}Ca^{2+}$, do not affect ATP production consistently, and indeed, in the case of rotenone, do not cause any change in free intracellular calcium levels. This is in keeping with the general observation that mitochondria can continue to sequester calcium in the absence of ATP and of respiratory substrate (Borle, 1981).

The fact that a reduction in temperature is accompanied by a reversible rise in intracellular calcium would suggest that membrane-bound ATPases are likely to be an important component of the cellular mechanisms regulating calcium homeostasis. Bolanos et al (1983) and Johnson et al (1984) have reported that human spermatozoa stored for periods of time at 2 - 5°C in the presence of TES-Tris/egg yolk buffer have a significantly enhanced capacity to penetrate zona-free hamster oöcytes when compared with fresh semen. These authors speculated that this result may have been due to an effect of the buffer employed. The present study suggests an alternative hypothesis, namely that the storage of spermatozoa at these low temperatures will result in the accumulation of intracellular calcium as a consequence of reduced activity of membrane ATPases involved in the process of calcium extrusion. The work of Holt and North (1985) has demonstrated that ram sperm plasma membranes contain a calcium-stimulated ATPase, the activity of which is dependant upon temperature. Reduction in the activity of such an enzyme in human spermatozoa may lead to the accumulation of of intracellular calcium, which may be involved in triggering mechanisms leading to capacitation and the acrosome reaction (Singh et al, 1978; Triana et al, 1980; Yanagimachi and Usui, 1974; Fraser, 1982). Robertson

and Watson (1986) have shown that ram spermatozoa do in fact accumulate calcium during cooling and that the ability of the spermatozoa to restore a normal level of cell-associated calcium following cooling depends upon the rate and extent of cooling. This work suggests that the damage sustained by human spermatozoa which are cryostored for therapeutic purposes may in part be due to impairment of the ability of the cell to maintain its calcium homeostasis, and that manipulation of the calcium environment in which cryostorage takes place may improve cryosurvival and result in better sperm function following cryostorage.

The suggestion that a calcium influx is an important component of capacitation and the acrosome reaction (Singh et al, 1978; Triana et al, 1980; Yanagimachi and Usui, 1974; Fraser, 1982) is supported by our observation that agents known to stimulate sperm function *in-vitro* are associated with an increase in levels of free intracellular calcium. A23187, at doses which result in dramatic increases in sperm-oöcyte fusion, causes a two to three fold increase in $[Ca^{2+}]_i$ over a period of one to two hours. Ionomycin in contrast produces increases in $[Ca^{2+}]_i$ which are more rapid in onset and greater in magnitude. Detailed studies on the effect of ionomycin on sperm function *in-vitro* remain to be undertaken. The increase in $[Ca^{2+}]_i$ produced by A23187 is relatively slow in onset, taking at least 60 minutes to become significant, in contrast to the stimulating effect of the drug on sperm function, which has reached maximal levels by 30 minutes of exposure (Aitken et al, 1984b). This raises the possibility that the changes in $[Ca^{2+}]_i$ measured by quin-2 are an innaccurate reflection of changes which may be taking place in a number of different cellular calcium pools, with the quin fluorescence signal providing an average value for the whole cell. Single cell studies of changes in free calcium in sub-cellular compartments are

now possible using fura-2, and work with this indicator may clarify this problem in future. An alternative explanation for the discrepancy between the time course of the effects of A23187 on sperm function and on intracellular calcium may be that A23187 is having effects on cellular processes other than calcium homeostasis. In this regard, it is of note that other drugs capable of stimulating sperm function, such as OAG, do not appear to cause changes in intracellular calcium of the same order of magnitude as the calcium ionophores. Clearly many biochemical events are involved in the processes leading up to the development by the spermatozoon of the capacity to fuse with the oölemma, and it would be surprising if all changes in sperm function were to be explicable on the basis of changes in one intracellular cation. Of the many aspects of cell biology which could be affected by A23187, in addition to intracellular calcium homeostasis, control of intracellular pH is one important possibility and this will be investigated further in the following chapter.

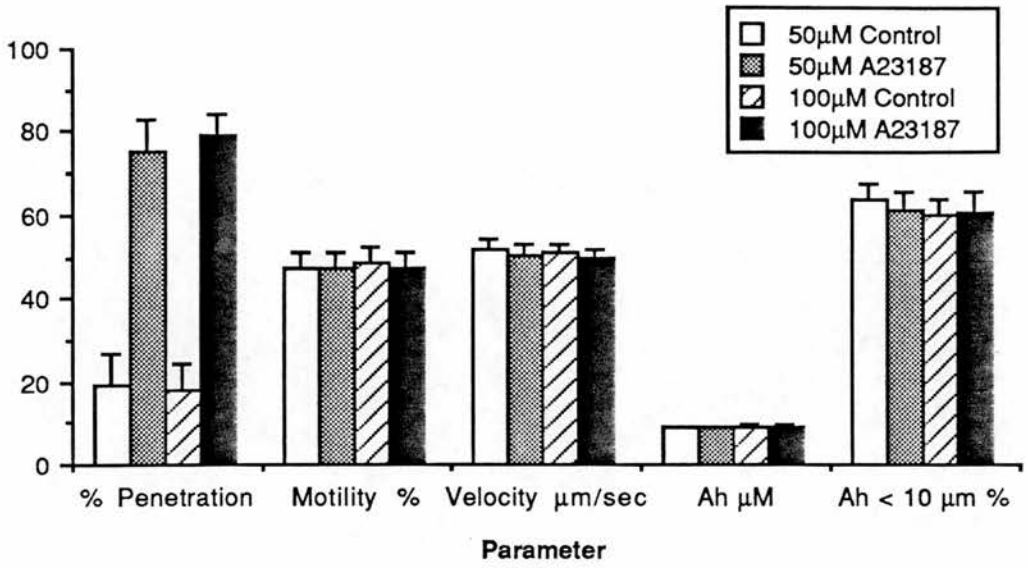


Figure 6.1

Effect of 50μM and 100μM A23187 on normal human spermatozoa

Spermatozoa from normospermic donors were incubated with 50 or 100μm $Ca^{2+}Mg^{2+}$ A23187. Large increases in % zona-free hamster oöcyte penetration (P < 0.01, Wilcoxon matched pairs signed ranks test) and in polyspermy (not shown) were seen , but no effect was observed on the velocity of forward progression (velocity μm/sec), the mean amplitude of lateral head displacement (Ah μm) or the percentage of cells with an amplitude of lateral head displacement below 10μm (Ah < 10 μm %).

[Data from Aitken et al, 1984b]

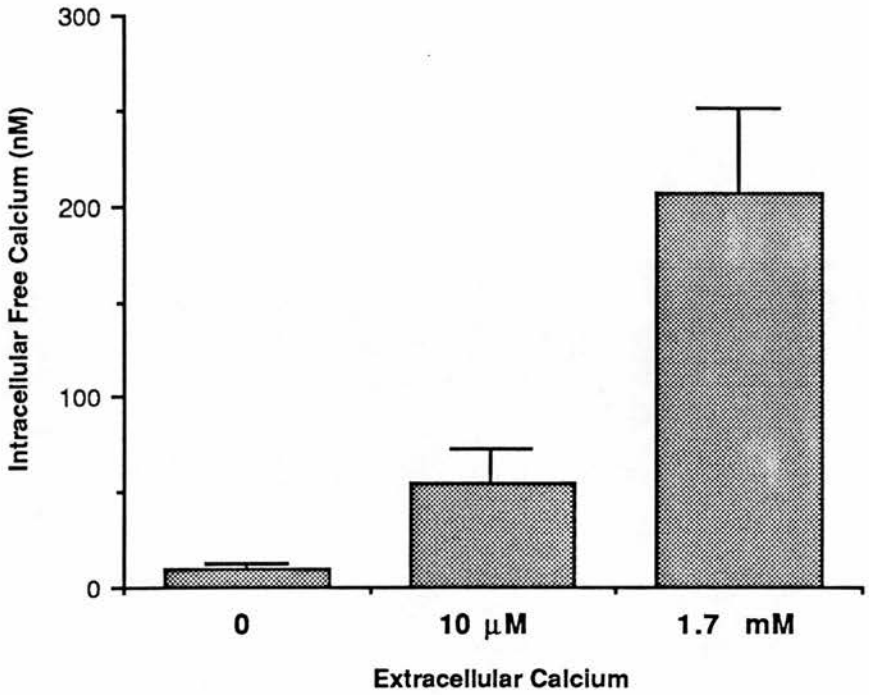


Figure 6.2

Effect of Extracellular Calcium on Intracellular Calcium

Washed ejaculated spermatozoa were suspended in medium BWW containing either no calcium (100μm EGTA), 10μm calcium (NAC) or 1.7mM calcium (normal BWW), and their level of free intracellular calcium measured with quin-2. All values were statistically significantly different from each other ($P < 0.05$, n = 3, Mann Whitney U Test).

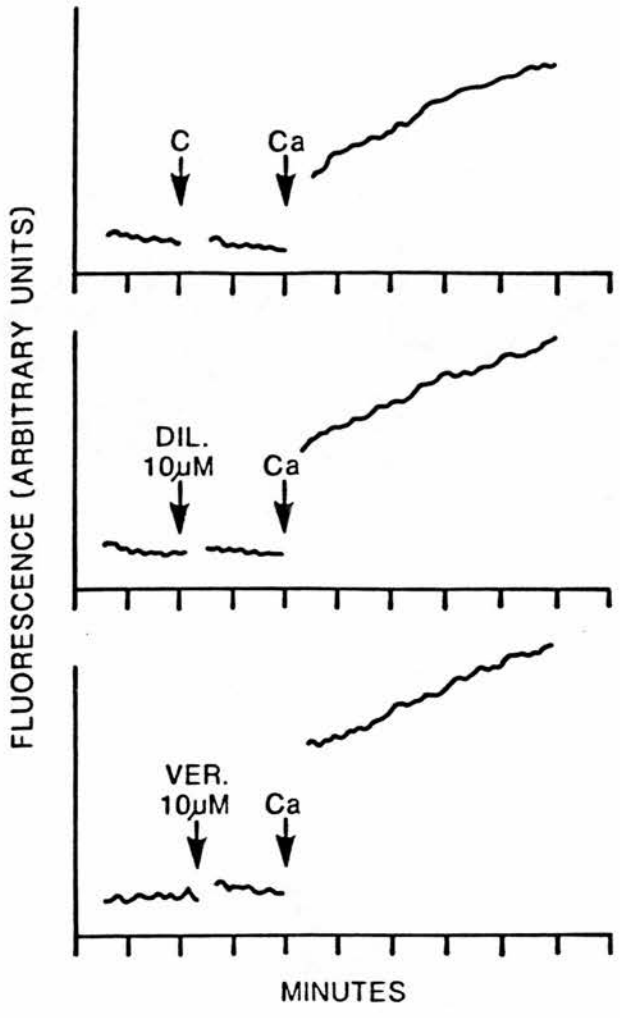


Figure 6.3

Absence of Voltage Sensitive Calcium Channels

Influx of calcium into human spermatozoa measured by quin-2 fluorescence. Spermatozoa were washed into medium containing 10μm Ca^{2+}. Diltiazem (10μm) (Dil), verapamil (10μm) (VER), or control (C) were added followed by 1.7mM Ca^{2+} (Ca).

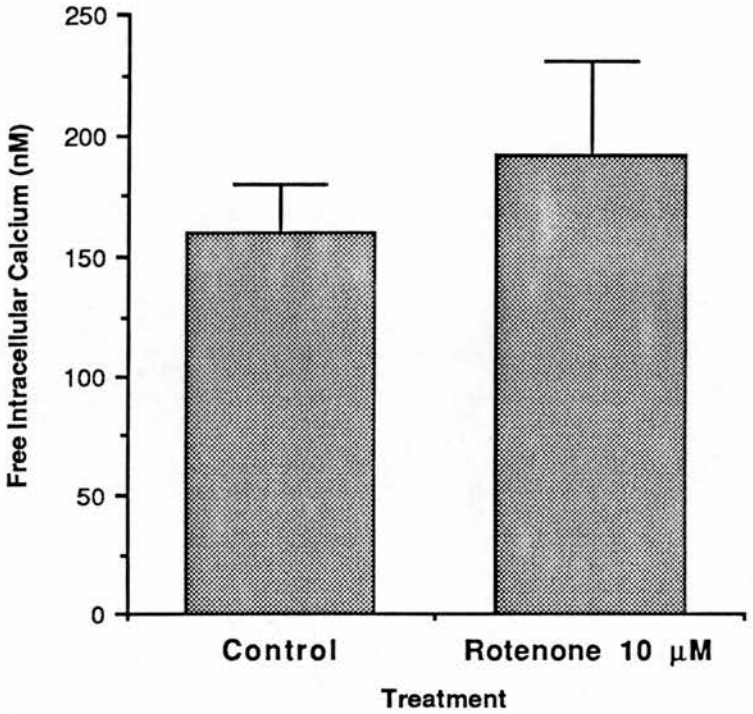


Figure 6.4

Effect of 10μm Rotenone on Intracellular Calcium

Spermatozoa loaded with quin-2 were treated with 10μm rotenone for 10 minutes, following which intracellular calcium was measured. Results are mean ± SEM, n = 4, difference not significant, P = 0.171, Mann Whitney U.

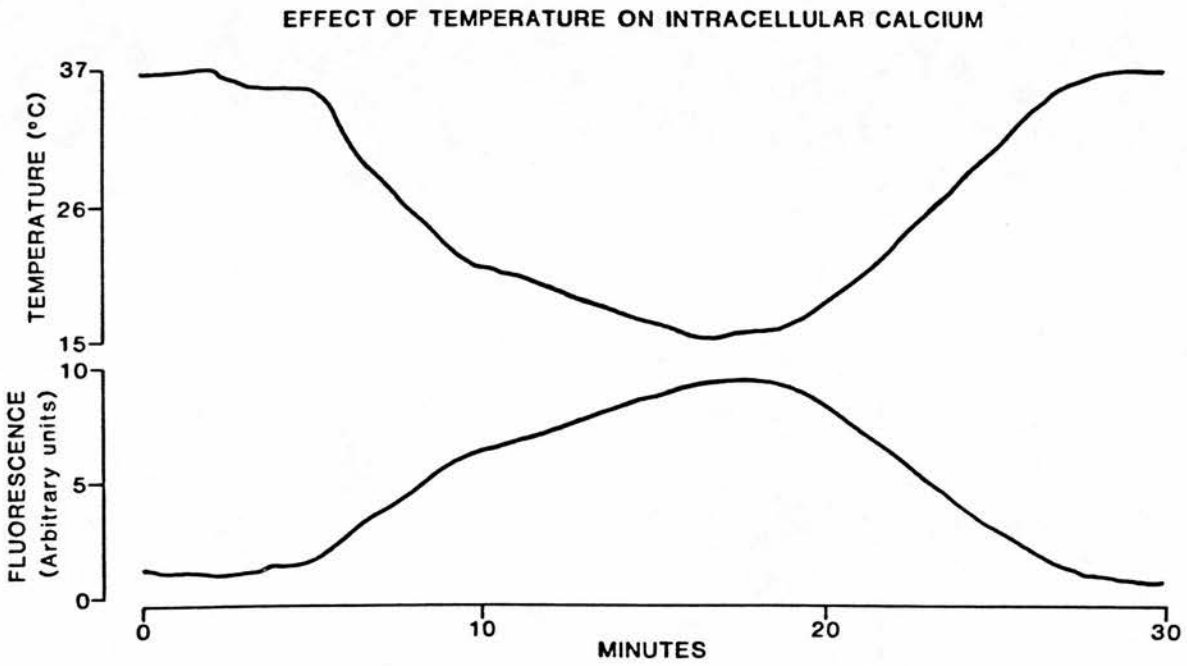


Figure 6.5

Effect of temperature on intracellular calcium in human spermatozoa

Effect of temperature on intracellular calcium in human spermatozoa measured by quin-2 fluorescence. Quin-loaded spermatozoa were cooled and warmed gradually in a thermostated spectrofluorimeter, while temperature and fluorescence intensity were constantly monitored.

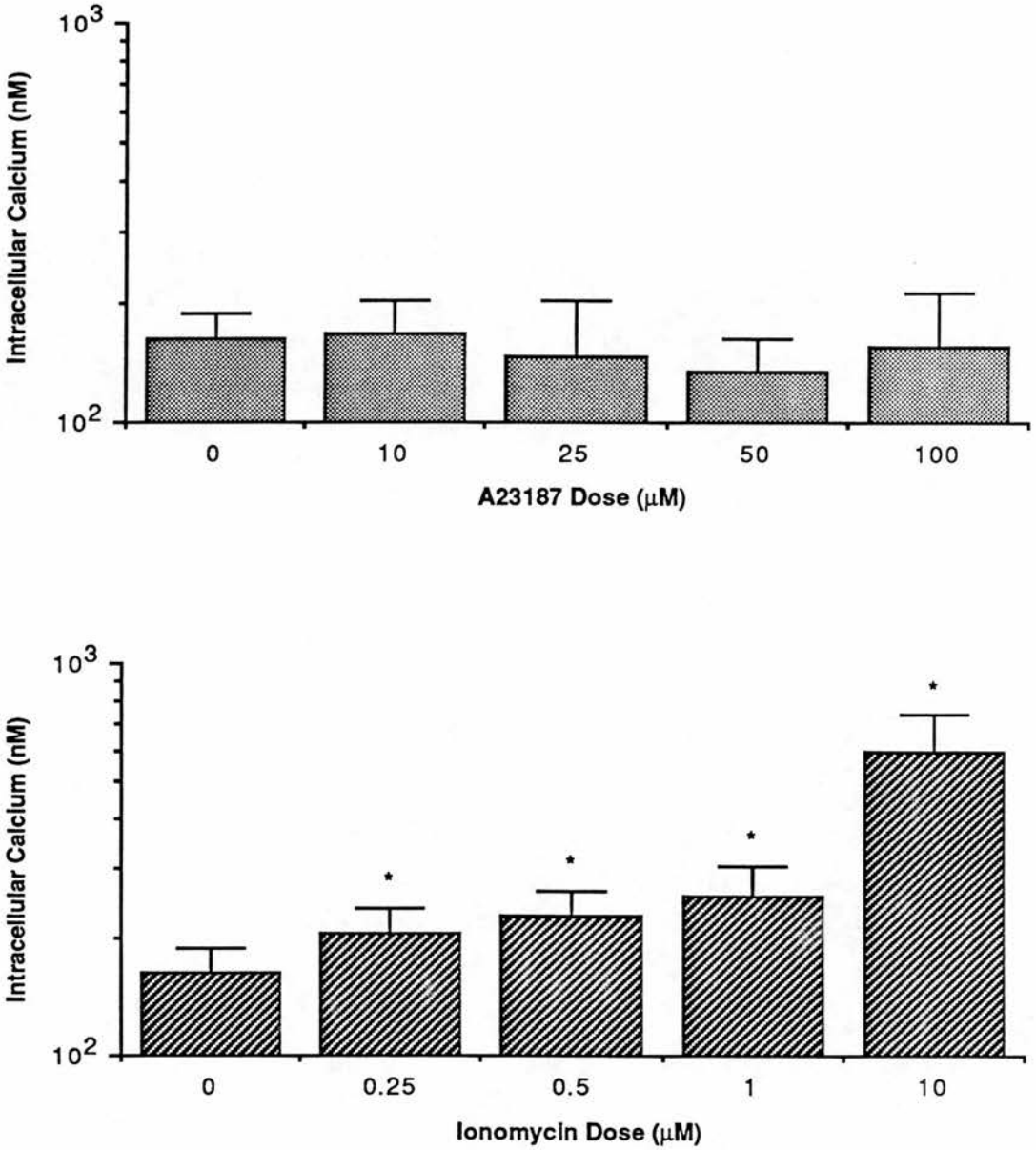


Figure 6.6

Acute Dose Response Study of Effects of A23187 and Ionomycin on Intracellular Calcium In Human Spermatozoa

Samples of human spermatozoa loaded with quin-2 were treated with divalent cation ionophores at various doses, and intracellular calcium measured after 10 minutes incubation. Results are mean ± SEM, n = 4, * = $P < 0.05$, Paired t-test.

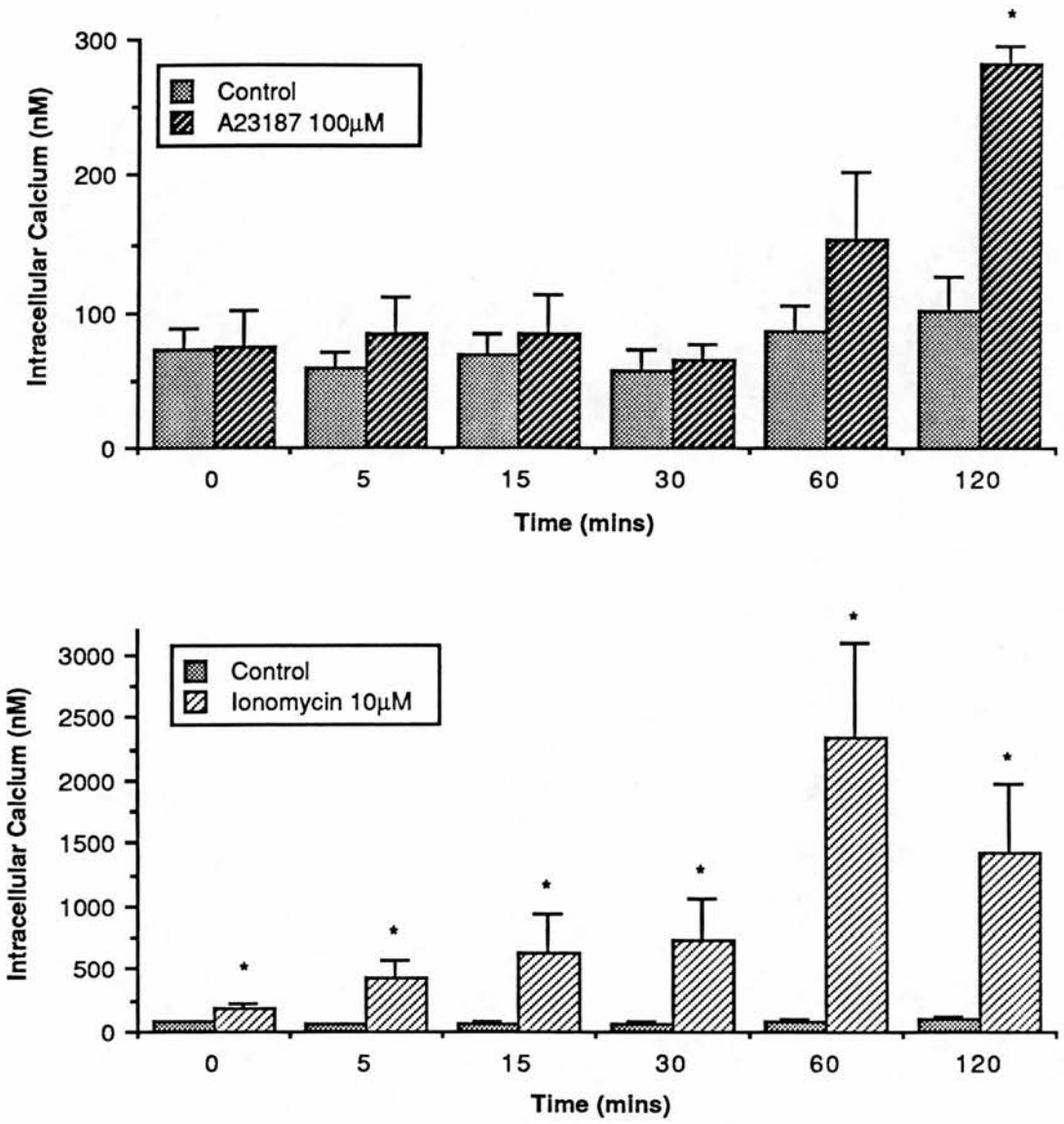


Figure 6.7

Time Course Study of the Effects of 100µM A23187 and 10µM Ionomycin on Intracellular Calcium In Human Spermatozoa

Samples of quin-2 loaded human spermatozoa were incubated with either DMSO (control), 100µM $Ca^{2+}Mg^{2+}$ A23187, or 10µm Ionomycin, and intracellular calcium measured at various time points. Results are mean ± SEM, n = 3, * = $P < 0.05$, Mann Whitney U test.

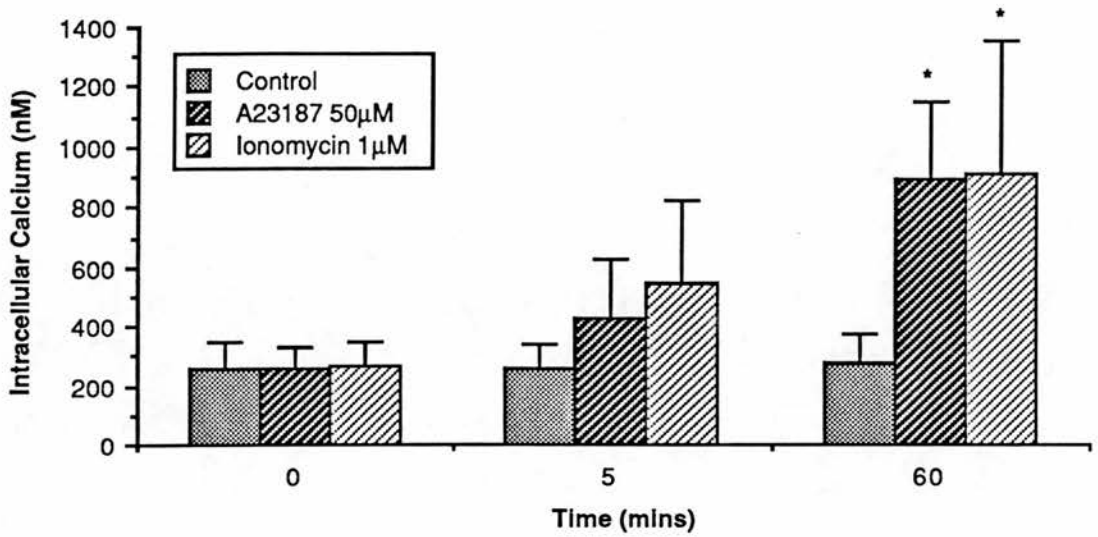


Figure 6.8

Time Course Study of the Effects of 50μM A23187 and 1μM Ionomycin on Intracellular Calcium In Human Spermatozoa

Samples of quin-2 loaded human spermatozoa were incubated with either DMSO (control), 50μM $Ca^{2+}Mg^{2+}$ A23187, or 1μm Ionomycin, and intracellular calcium measured at various time points. Results are mean ± SEM, n = 3, * = $P < 0.05$, Mann Whitney U test.

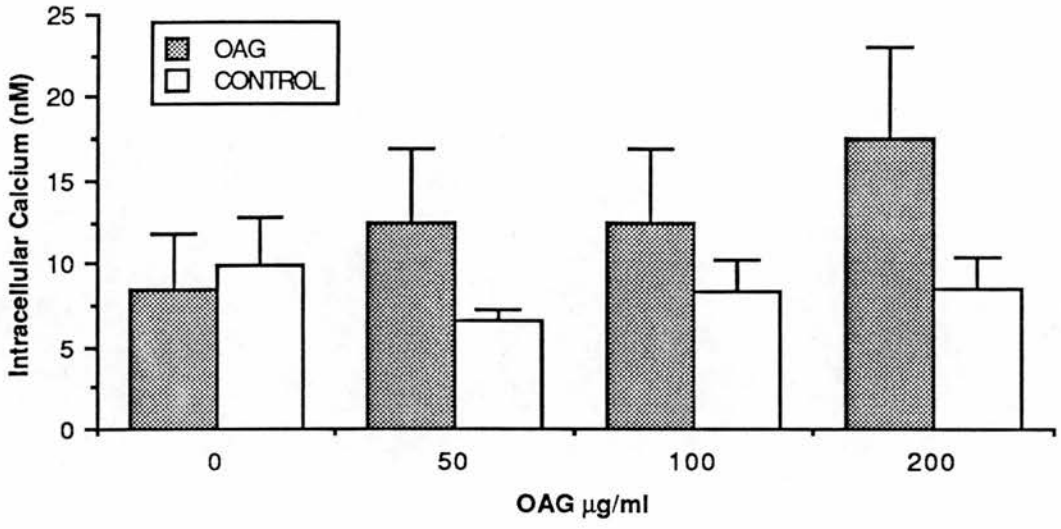


Figure 6.9

Effect of 1-oleoyl, 2-acetyl glycerol on Intracellular Calcium in calcium-free medium

1-oleoyl, 2-acetyl glycerol at various dose levels was added to suspensions of quin-loaded spermatozoa in calcium free medium BWW (containing 100µm EGTA), and intracellular calcium was measured after 10 minutes incubation. DMSO alone was added to control incubations. Results are mean ± SEM, n = 3. Differences were not significant.

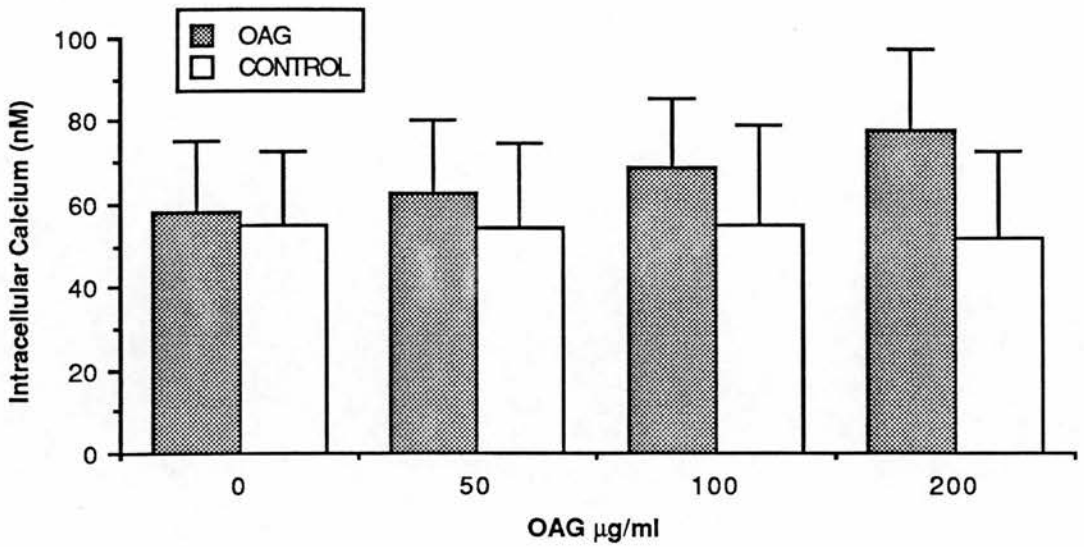


Figure 6.10

Effect of 1-oleoyl, 2-acetyl glycerol on Intracellular Calcium in calcium deficient medium

1-oleoyl, 2-acetyl glycerol at various dose levels was added to suspensions of quin-loaded spermatozoa in calcium deficient medium BWW (containing 10µm Ca^{2+}), and intracellular calcium was measured after 10 minutes incubation. DMSO alone was added to control incubations. Results are mean ± SEM, n = 3. Differences were not significant.

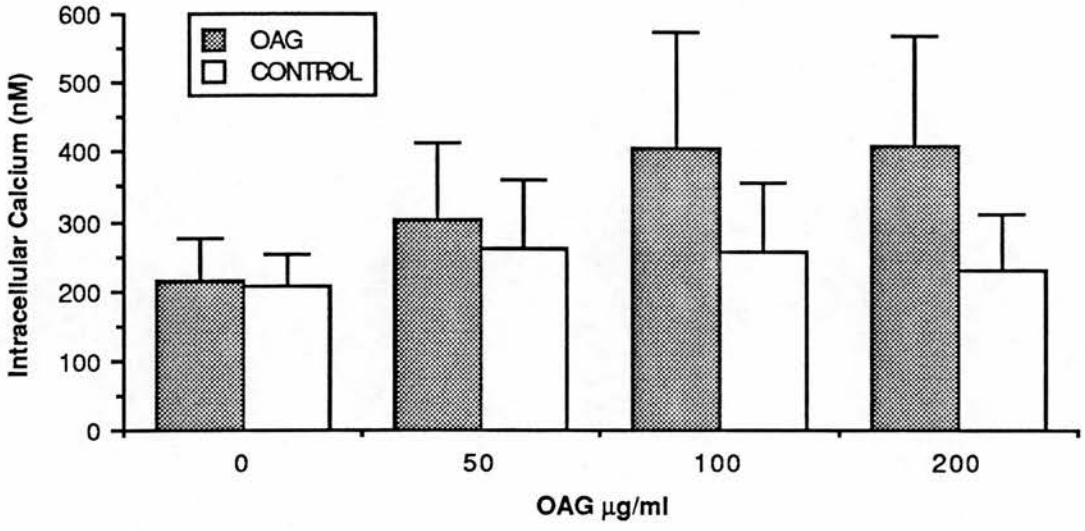


Figure 6.11

Effect of 1-oleoyl, 2-acetyl glycerol on Intracellular Calcium in normal medium

1-oleoyl, 2-acetyl glycerol at various dose levels was added to suspensions of quin-loaded spermatozoa in normal medium BWW (containing 1.7mM Ca^{2+}), and intracellular calcium was measured after 10 minutes incubation. DMSO alone was added to control incubations. Results are mean ± SEM, n = 3. Differences were not significant.

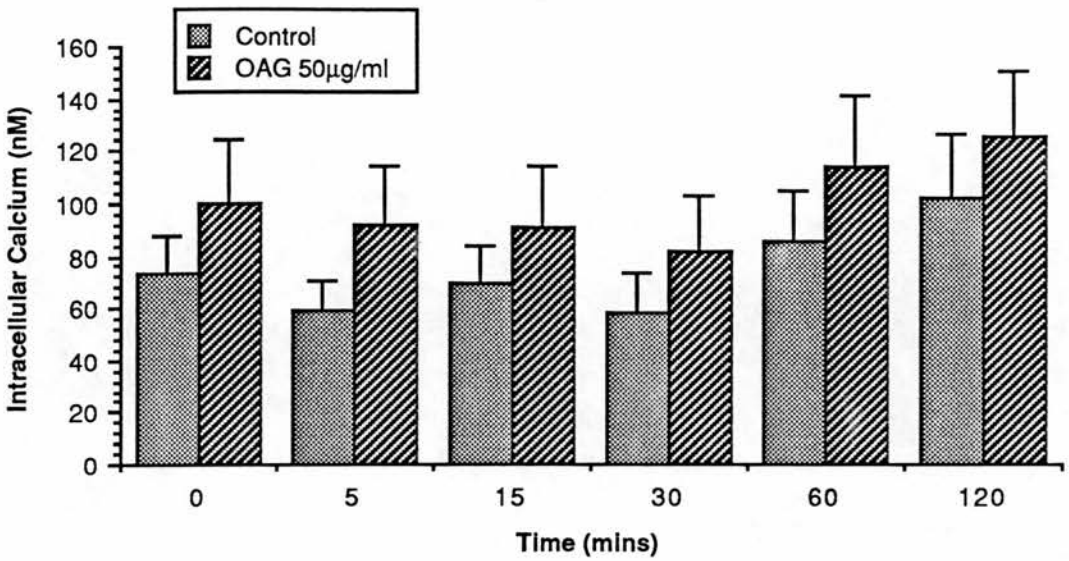


Figure 6.12

Effect of 1-oleoyl, 2-acetyl glycerol on Intracellular Calcium : Time course study

1-oleoyl, 2-acetyl glycerol at a dose of 50 µg/ml was added to suspensions of quin-loaded spermatozoa in normal medium BWW (containing 1.7mM Ca^{2+}), and intracellular calcium was measured immediately and after 5, 15, 30, 60 and 120 minutes incubation. DMSO alone was added to control incubations. Results are mean ± SEM, n = 3. Differences were not significant.

CHAPTER SEVEN

THE MEASUREMENT OF INTRACELLULAR pH IN HUMAN SPERMATOZOA AND ITS MANIPULATION DURING *IN-VITRO* TESTING OF SPERM FUNCTION

7.1 Introduction

Just as intracellular calcium has become recognised as an important intracellular regulator, so the importance of cytoplasmic pH regulation in the control of cell function is unquestionable, in view of the marked pH sensitivity of virtually all intracellular processes (Grinstein et al, 1984b). And just as calcium homeostasis is maintained in large part by the existence of calcium extrusion mechanisms, so the tendency of cells to become acidified by passive fluxes of H^+ and HCO_3^-, and by metabolic acid production, must be counteracted by regulatory acid-extruding mechanisms. It has been suggested that, at least in mammalian cells, two regulatory systems may exist, a coupled counter transport of extracellular sodium ions (Na_o^+) for intracellular protons (H_i^+), and a similar counter transport of chloride (Cl^-) for bicarbonate (HCO_3^-) (Grinstein et al, 1984a,b). Comparatively little published information is available concerning the effects of pH on sperm function and no direct measurements of intracellular pH in human spermatozoa have yet been published.

Murphy and Yanagimachi (1984) have examined the effect of extracellular pH upon the motility and acrosome reaction of guinea pig spermatozoa, and have observed that, while spermatozoa rapidly underwent the acrosome reaction and remained vigorously motile in media with pH levels of 7.5 or 8.2, with lower levels of extracellular pH, both motility and the occurrence of the acrosome reaction were reduced. They speculated that this inhibitory effect of low levels of extracellular pH may be mediated through an inhibition of the passage of calcium ions through the sperm plasma membrane. However, evidence from other groups (Vijayaraghavan et al, 1985) suggests that changes in intracellular pH alone may be important in

regulating motility including events such as the initiation of motility in epididymal spermatozoa.

Whilst relatively little is known about the role of intracellular pH in the functioning of mammalian spermatozoa, a large body of information has been accumulated on the role of intracellular pH in controlling the function of sea-urchin spermatozoa and oöcytes (Hansbrough and Garbers, 1981; Schackmann et al, 1981; Christen et al, 1982; Payan et al, 1983; Bibring et al, 1984). Christen et al (1982) found that the intracellular pH of the spermatozoa of the sea urchin *Strongylocentrotus purpuratus* was acidic with respect to the extracellular environment under normal conditions and was significantly influenced by the pH of the extracellular environment, being 5.7 in medium of pH 5.0, and rising to 7.4 in medium of pH 8.0 (the pH of normal sea-water). In addition, they found that motility and respiration were increased when intracellular pH was elevated, while other workers (Schackmann et al, 1981) have found that an increase in intracellular pH accompanies the acrosome reaction of sea urchin spermatozoa. It seems likely that sodium is significantly involved in the control of intracellular pH in sea urchin spermatozoa (Hansbrough and Garbers, 1981; Bibring et al, 1984).

A number of approaches to the measurement of intracellular pH in mammalian cells have been employed, and have suggested that resting intracellular pH in such cells is in the region of 7.0 - 7.4 (Thomas et al, 1979; Rogers et al, 1983; Grinstein and Furuya, 1984; Grinstein et al, 1984a; b). Thomas and colleagues reported the use of 6-carboxyfluorescein diacetate, a colourless neutral diacetate compound which diffuses into the cell, where intracellular enzymes release the strong chromophore, fluorescein, by

hydrolytic removal of the acetate groups (Thomas et al, 1979). Since the spectrum of fluorescein is highly pH dependant, it was possible to employ changes in the spectrum of the intracellular fluorescein to monitor the internal pH of Ehrlich ascites tumour cells, a value of pH 7.1 - 7.4 being obtained. Geisow (1984) has also used the pH dependence of fluorescein isothyocyanate (FITC) delivered by pinocytosis, to study the pH of macrophage lysozomes.

The technique developed by Tsien and colleagues (Tsien, 1980; 1981; Tsien et al, 1982a; b) of employing membrane permeant acetoxymethyl esters as a means of loading indicator compounds into cells has also been applied to pH indicators. Grinstein et al (1984a) have described the use of 5,6-dicarboxyfluorescein acetoxymethyl ester, and Rogers et al (1983) the use of the acetoxymethyl ester of quene-1 in the measurement of intracellular pH in thymic lymphocytes, with values of 7.01 $\pm$ 0.06 and 7.15 being reported for pH_i when pH_e = 7.3. More recently, Grinstein et al (1984b) and Grinstein and Furuya (1984) have reported the use of bis(carboxyethyl)carboxyfluorescein (BCECF), an indicator originally described by Rink et al (1982), for the measurement of intracellular pH, and available as an acetoxymethyl ester (BCECF-AM). Using this technique, values of 7.25 -7.27 were obtained for the resting intracellular pH of thymocytes (pH_e = 7.3) (Grinstein et al, 1984b).

A study was therefore conducted to determine the intracellular pH of human ejaculated spermatozoa, and to determine the effect of extracellular pH and, in particular, of agents which activate human spermatozoa, upon intracellular pH.

7.2 Materials and Methods

Cell Preparation and Handling

Semen samples were obtained from a panel of normal semen donors and were subjected to routine semen analysis as described in Chapter Two, before being washed three times by centrifugation in medium BWW as described in Chapter Five. The spermatozoa were finally resuspended in medium BWW to a concentration of 20 x 10^6/ml prior to being loaded with the pH indicator.

Loading of Spermatozoa with BCECF-AM

2,7-Biscarboxyethyl-5(6)-carboxyfluorescein-tetraacetoxymethyl ester (BCECF-AM) was obtained from HSC Research Development Corporation, Toronto, Ontario, Canada, and was made up as a stock solution in dry dimethylsulphoxide (DMSO) at a concentration of 0.5 µg/µl, and was kept dark and desiccated at - 20°C between experiments. Washed spermatozoa at a concentration of 20 x 10^6/ml were suspended in normal medium BWW, buffered with bicarbonate and HEPES, and BCECF-AM was added to a concentration of 5 µg/ml (6.2 µM) the final concentration of DMSO not exceeding 1% v/v. Control cell suspensions were treated with DMSO alone. These cell suspensions were incubated for 30 minutes at 37°C in 5% CO_2 in air, following which the cells were pelleted by centrifugation at 500 x g for five minutes, and were washed twice in fresh medium BWW to minimise the carryover of extracellular BCECF-AM. Cells were finally resuspended at a concentration of 2 x 10^6 spermatozoa/ml.

Measurement of BCECF Fluorescence

Measurements were performed using a Perkin-Elmer LS-5 spectrofluorimeter with the sample chamber thermostated to 37°C. For the examination of fluorescence spectra, the excitation monochromator was set to 500nm and the emission monochromator was scanned over the range 520 - 600nm. Measurement of intracellular pH depended upon fluorescence readings with the excitation monochromator set to 500nm and the emission monochromator set to 530nm. The fluorescence of BCECF loaded cells was measured and, at the conclusion of each experiment, the system was calibrated by the addition of triton-X-100 to release the trapped intracellular BCECF into solution. The pH of the extracellular solution was then titrated with the addition of 2% (v/v) of a 3.2% solution of hydrochloric acid in two stages, allowing the construction of a calibration curve of pH against fluorescence, from which the value of intracellular pH (pH_i) could be calculated.

7.3 Results

Uptake of BCECF-AM

The emission spectrum of BCECF-AM (0.1 µg/ml in medium BWW) is shown in Figure 7.1 (p 245), demonstrating that it has an emission maxima at around 530nm when excitation is at 500nm. In an identical manner to the loading of cells with quin-2 , the hydrophobic membrane permeant ester BCECF-AM is cleaved within the cell to yield the non-permeant free acid BCECF, however unlike quin-2 no change in the fluorescence spectrum of BCECF-AM occurs during de-esterification making direct monitoring of the uptake and de-esterification of the indicator impossible. . When washed ejaculated human spermatozoa were loaded with BCECF according to the

protocol outlined above, the spectra shown in Figure 7.2 (p 246) were obtained. It was observed that the spectrum of BCECF loaded spermatozoa is identical to that of BCECF, suggesting that successful uptake had occurred, and that cellular autofluorescence was negligible at these wavelengths. When BCECF-AM was scanned repetitively, it was observed that there was virtually no fall in the level of the fluorescence signal, suggesting that photobleaching was much less of a problem than with quin-2.

Measurement of Intracellular pH

This was performed according to the protocol outlined above, the system being calibrated by releasing the intracellular dye into solution using triton-X-100, and titrating this released dye to three measured pH levels with hydrochloric acid. A typical calibration curve is shown in Figure 7.3 (p 247), from which it can be seen that the relationship between pH and fluorescence was linear over the range of pH values in question. The value of pH_i was estimated from curves such as these by calculating the value of pH corresponding to the level of fluorescence of the intracellular dye prior to its release with triton-X-100. The effect of red shift known to occur under intracellular conditions was not corrected for in this series of experiments as in practice it tends to be constant (Grinstein et al, 1984a). It was found that the resting intracellular pH of washed ejaculated human spermatozoa was 7.02 ± 0.033 (mean $\pm$ SEM, $n = 6$) pH units.

Effect of extracellular pH upon Intracellular pH

Spermatozoa loaded with BCECF were suspended in either medium BWW, medium BWW without HEPES buffer, or medium BWW without hepes and with the addition of 105mg $NaHCO_3$/25ml. This resulted in media with pH

values of 7.64, 8.42, and 8.50 respectively. After 10 minutes of incubation in such media, the intracellular pH of the spermatozoa was measured and was found to be 6.8 in medium of pH 7.64, 7.12 in medium of pH 8.42, and 7.31 in medium of pH 8.5. These results are shown graphically in Figure 7.4 (p 248), illustrating an apparent dependence of intracellular pH upon extracellular pH. In addition, it was observed that different batches of medium BWW tended to have slightly varying pH values, over the range 7.72 - 7.77. The resting intracellular pH values obtained for spermatozoa suspended in these different batches of media also showed apparent variation with extracellular pH with intracellular pH being 6.992 in medium of pH 7.72 and 7.056 in medium of pH 7.77. (Figure 7.5, p 249)

Effect of Divalent Cation Ionophores upon Intracellular pH

Given the profound effects of the divalent cation ionophore A23187 upon sperm function (see Chapter 4) and upon intracellular calcium (see Chapter 6), it was of interest to examine the effects of this agent upon intracellular pH. Spermatozoa loaded with BCECF were incubated for 10 minutes in the presence of either 100 μM $Ca^{2+}Mg^{2+}$ A23187 or DMSO control, following which intracellular pH was measured. It was observed that A23187 treatment resulted in a significant increase in intracellular pH from 7.026 ± 0.033 to 7.378 ± 0.076 (mean ± SEM, n = 6, $P < 0.05$, Wilcoxon matched pairs signed ranks test) (Figure 7.6, p 250).

A preliminary series of experiments was undertaken to compare the effects of A23187 (100 μM) and ionomycin (1 μM) upon intracellular pH. The results are shown graphically in Figure 7.7 (p 251), suggesting that while A23187 produces an increase in intracellular pH, ionomycin fails to do so. The effects of these ionophores were studied over a 10 minute period in

spermatozoa loaded with BCECF, during which time the addition of 100 μM A23187 resulted in an increase in intracellular pH from 7.019 ± 0.019 to 7.254 ± 0.099 (n = 3), while ionomycin produced almost no change, intracellular pH being 7.066 ± 0.034 (n = 2) after treatment. Due to the small numbers however, these results are not statistically significant, and further studies are required to clarify this result.

7.4 Discussion

A role for intracellular pH (pH_i) in the control of eukaryotic cell function has been suggested in a variety of cell types. Until recently, the measurement of intracellular pH was a complex undertaking (Thomas et al, 1979; Geisow, 1984) however, the development of membrane permeant fluorescein derivatives has made possible the direct measurement of cytoplasmic pH and measurements have now been made on a variety of cell types. Rogers et al (1983) have measured intracellular pH in mouse thymocytes and in rat basophil leukaemic cells, and have found resting values of 7.1 - 7.3 in single cells, and of 7.15 ± 0.04 in cell suspensions. Similarly, Grinstein et al (1984b), studying the proposition that cellular alkalinization is associated with cellular proliferation, measured pH_i in human and rat thymic lymphocytes and obtained values of 7.25 - 7.27.

The role of pH in spermatozoal function has not been extensively studied. The importance of extracellular pH in the maintenance of motility and the completion of the acrosome reaction by guinea pig spermatozoa was studied by Murphy and Yanagimachi (1984) who found that low levels of extracellular pH (6.1) inhibited both motility and the acrosome reaction.

These workers postulated that the sperm plasma membrane is relatively freely permeable to H^+ , so that pH_i (intracellular pH) will change in response to changes in pH_e (extracellular pH), and given that dynein ATPase, which is essential for sperm motility, has an alkaline pH optimum, then sperm motility will be adversely affected by the fall in intracellular pH which would result from a fall in extracellular pH. This suggestion was supported by the work of Vijayaraghavan et al (1985) who have measured intracellular pH in bovine epididymal spermatozoa using a null point technique with the indicator carboxyfluorescein diacetate, examining the proposition that the initiation of motility in epididymal spermatozoa is associated with cellular alkalinization. They found the pH_i of caput epididymal spermatozoa to be 5.84 ± 0.1 whilst that of caudal spermatozoa was 6.27 ± 0.05. It is known that epididymal pH is generally low, being 6.0 in the bull (Salisbury, 1962) and 6.6 in the rat (Levine and Kelly, 1978), and the initiation of motility which follows the dilution of epididymal spermatozoa into seminal plasma or balanced salt solutions with higher pH levels may be a consequence of an egress of protons. Similarly, Makler et al (1981) have shown that the motility of ejaculated human spermatozoa is reversibly inhibited by low levels of extracellular pH. The inhibition of the acrosome reaction observed by Murphy and Yanagimachi (1984) in medium of low pH was felt by them to be due to inhibition of calcium influx, since spermatozoa incubated in capacitating conditions, but at low pH, did not acrosome react when exposed to medium of normal pH. Although data on mammalian spermatozoa is limited, substantial amounts of data are available concerning the pH_i of sea urchin spermatozoa, which suggest that intracellular pH is closely related to extracellular pH, that decreased values of pH_i are associated with inhibition of respiration and motility (Christen et al, 1982), that the acrosome reaction is associated with cellular

alkalinization (Schackmann et al, 1981) and that the maintenance of intracellular pH is in part controlled by a sodium-dependent H^+ efflux (Bibring et al, 1984). Clearly, sea-urchin spermatozoa have to solve very different problems with respect to their ionic environment, when compared to mammalian spermatozoa, however, there would appear to be some fundamental similarities with respect to the control of pH_i.

The present study is the first to report upon the intracellular pH of ejaculated human spermatozoa. The use of an acetoxymethyl ester of a fluorescein derivative (2,7-Biscarboxyethyl-5(6)-carboxyfluorescein-tetraacetoxymethyl ester)(BCECF-AM) previously described by other workers (Grinstein and Furuya, 1984; Grinstein et al, 1984b; Rink et al, 1982) was shown to be effective in washed ejaculated human spermatozoa, with successful uptake and de-esterification being achieved. (Figure 7.2, p 246) Spermatozoa remained normally motile after intracellular loading with BCECF, suggesting that the presence of the indicator was not having a major effect on cell function, although the intracellular concentration of indicator was not determined in the present study. In common with other cell types, human spermatozoa appear to maintain their intracellular pH at a level which is acidic, relative to their extracellular environment, with a value of 7.02 ± 0.033 (mean ± SEM, n = 6) pH units being obtained for resting pH_i when pH_e was in the range 7.72 - 7.77. This suggests that H^+ and $HCO3^-$ are not passively distributed across the cell membrane, and that regulatory mechanisms may exist. Evidence from other cell types indicates that one such regulatory mechanism is an amiloride sensitive Na^+/H^+ antiport, and Grinstein et al (1984a) have shown that in thymic lymphocytes, intracellular alkalinization is associated with acidification of the surrounding medium and with an increase in cellular Na^+ content. This process was inhibited by

amiloride and similar results have been obtained for human neutrophils (Grinstein and Furuya, 1984) in which it was observed that factors involved in the control of cell function (such as chemotaxis) caused activation of the Na^+/H^+ exchange.

Evidence from studies on the sea-urchin, *Strongylocentrotus purpuratus* , suggests that such mechanisms may also exist in mature sperm cells. Bibring et al (1984) have suggested that the intracellular production of H^+ as a result of respiration is balanced by a sodium-dependant H^+ removal which regulates pH_i, and Hansbrough and Garbers (1981) have shown that monensin, which causes a Na^+ influx across the cell membrane (Sandeaux et al, 1982), induces a proton efflux and stimulates sperm motility. Similar effects were observed in response to speract, a low molecular weight peptide associated with sea-urchin eggs, and involved in the physiological activation of spermatozoa, and these effects upon H^+ and Na^+ were not blocked by amiloride or sodium channel blockers. (Hansbrough and Garbers, 1981)

Evidence obtained in the present study supports the view that the intracellular pH of ejaculated human spermatozoa is related to the extracellular pH (Figures 7.4 and 7.5, p 248-249) and this relationship has previously been demonstrated for sea-urchin spermatozoa (Christen et al, 1982). A rise in extracellular pH of 0.05 pH units from 7.72 to 7.77, resulted in a similar rise in intracellular pH of 0.064 pH units from 6.992 to 7.056, and this relative inability of human spermatozoa to maintain control of pH_i in the face of relatively small changes in pH_e is surprising. One possible explanation is that the change in pH_i is not real but apparent, and is an artefact arising from the fact that measurements were made upon cell

suspensions rather than individual cells. A cell suspension is a heterogeneous population of cells with varying degrees of functional competence, from cells which are functionally normal to cells which are non-viable. It is possible that BCECF enters cells which are incapable of maintaining pH homeostasis, but which retain sufficient integrity to de-esterify and trap the indicator, thereby biasing the measurement obtained from the population as a whole. The ideal solution to this problem would be to perform measurements on single cells, and this approach has been described in other cell types (Rogers et al, 1983), however extremely sophisticated equipment with the ability to accumulate photons from a single cell is required, and this would present major problems in working with a motile cell type such as the spermatozoon. An alternative solution would be the separation of the initial heterogeneous population into populations of more consistent functional quality, by means such as density gradient centrifugation, however this introduces another variable and would make comparisons, for example between patients, difficult.

The ability of the divalent cation ionophore A23187 to cause cellular alkalinisation from pH 7.026 ± 0.033 to 7.378 ± 0.076 (mean ± SEM, n = 6), a change of approximately 0.35 pH units (Figure 7.6, p 250) was noted in the present study. In addition to causing cellular alkalinization, data presented in Chapters 4 and 6 indicated that doses of A23187 of the order of 100 μM were capable of causing an increase in intracellular calcium concentration, and of stimulating sperm function. It is possible that the ability of A23187 to stimulate human sperm function is related in part to its ability to cause simultaneous elevation of Ca^{2+}_i and of pH_i. As was noted above, Hansbrough and Garbers (1981) have shown that speract, a low molecular weight peptide associated with sea-urchin eggs and involved in the

physiological activation of sea-urchin spermatozoa induces a proton efflux and stimulates sperm motility, and Decker et al (1976) have shown that A23187 causes acrosome reaction in the sperm of the sea-urchin *Arbacia punctulata* at alkaline pH. The mechanisms by which A23187 elevates intracellular pH require further investigation but may be related to the tendency of media containing $Ca^{2+}Mg^{2+}$ A23187 to be slightly alkaline.

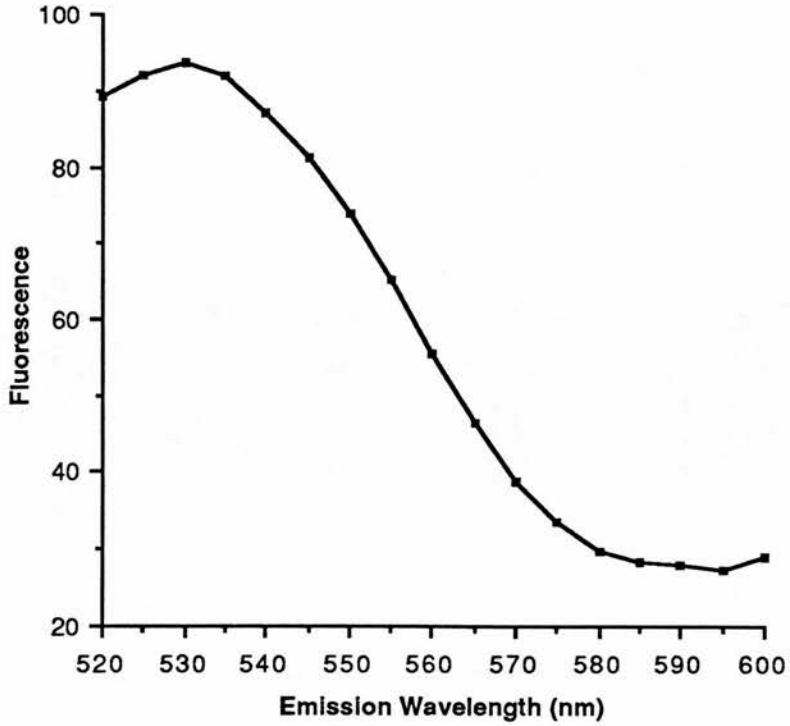


Figure 7.1

Emission Spectrum of BCECF-AM

Emission spectrum of BCECF-AM (0.1 μg/ml in medium BWW) over the range 520-600nm, with excitation at 500nm. An emission maxima is seen at 530nm.

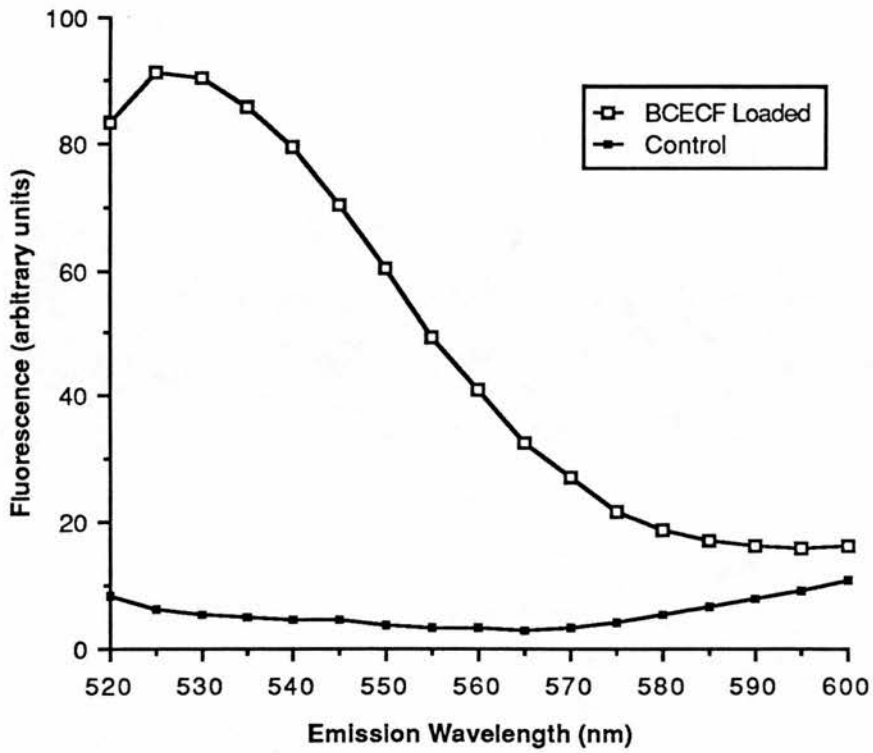


Figure 7.2

Emission Spectra of Control and BCECF Loaded Human Spermatozoa

Washed ejaculated human spermatozoa were loaded with BCECF according to the protocol described in the text, and control cells were incubated with DMSO alone. The cells were washed free of indicator and their emission spectra studied (Ex = 500nm, Em = 520 - 600nm). Uptake of BCECF is shown by the emission maxima at 530nm, control cell autofluorescence was negligible over these wavelengths.

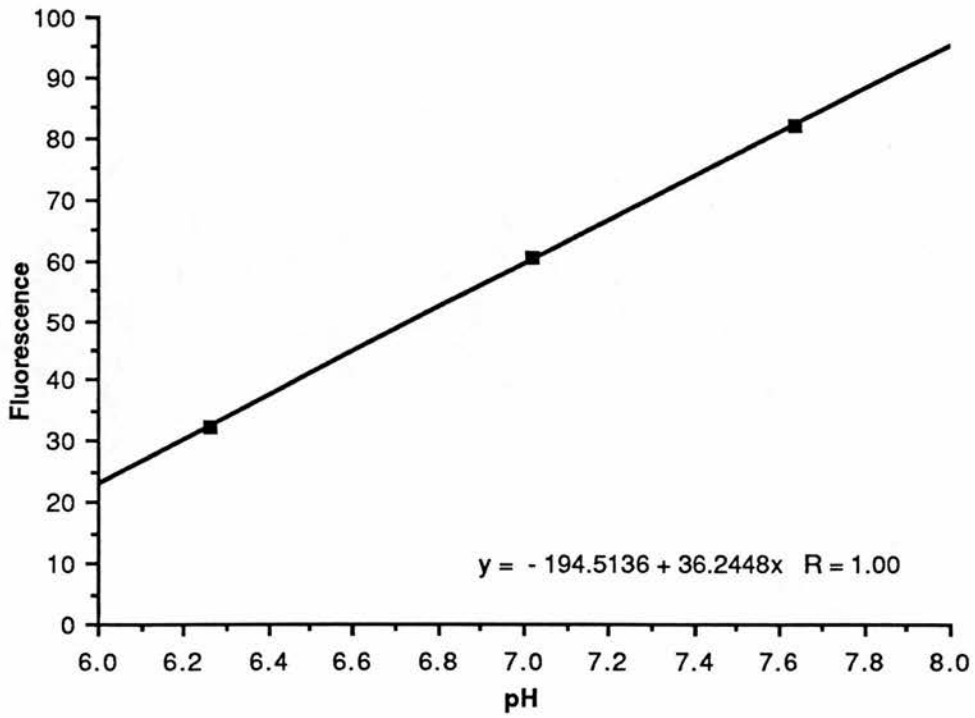


Figure 7.3

Calibration Curve for BCECF

Intracellular BCECF was released from spermatozoa into the surrounding media by the addition of triton-X-100. The fluorescence signal was measured at this pH, and following the addition of two concentrations of HCl. The pH of the media was measured at each calibration point using an Orion pH meter, and fluorescence plotted against pH. This enabled the value of pH_i to be estimated from the previously measured level of fluorescence of intracellular dye.

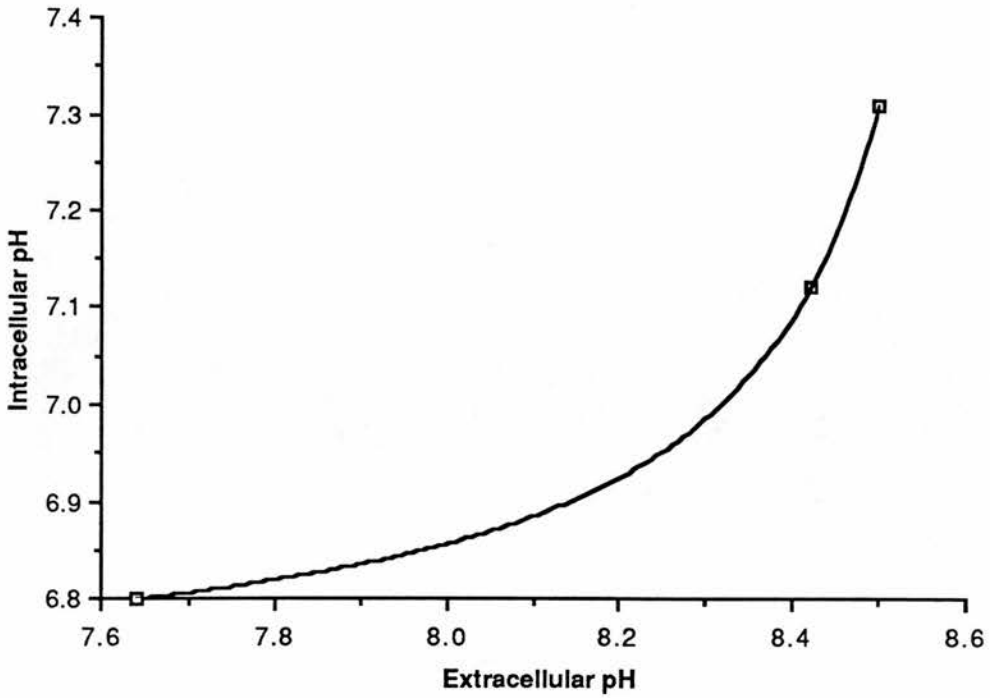


Figure 7.4

Effect of Extracellular pH upon Intracellular pH of Human Spermatozoa

Human spermatozoa were suspended in medium BWW, the pH of which was altered by the omission of HEPES buffer or the addition of bicarbonate. The intracellular pH was measured by the use of BCECF. It was observed that intracellular pH was markedly influenced by extracellular pH.

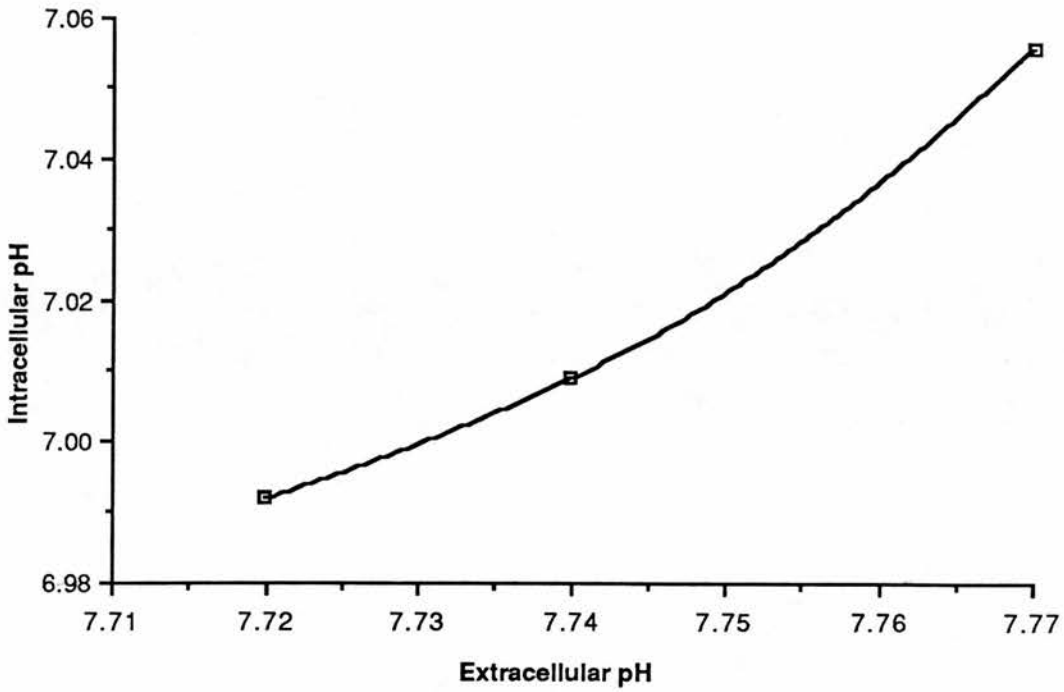


Figure 7.5

Effect of Extracellular pH Upon Intracellular pH

Variations in the pH of the medium BWW from 7.72 to 7.77 resulted in similar changes in the intracellular pH of the spermatozoa suspended in such media, from around 6.99 to 7.06.

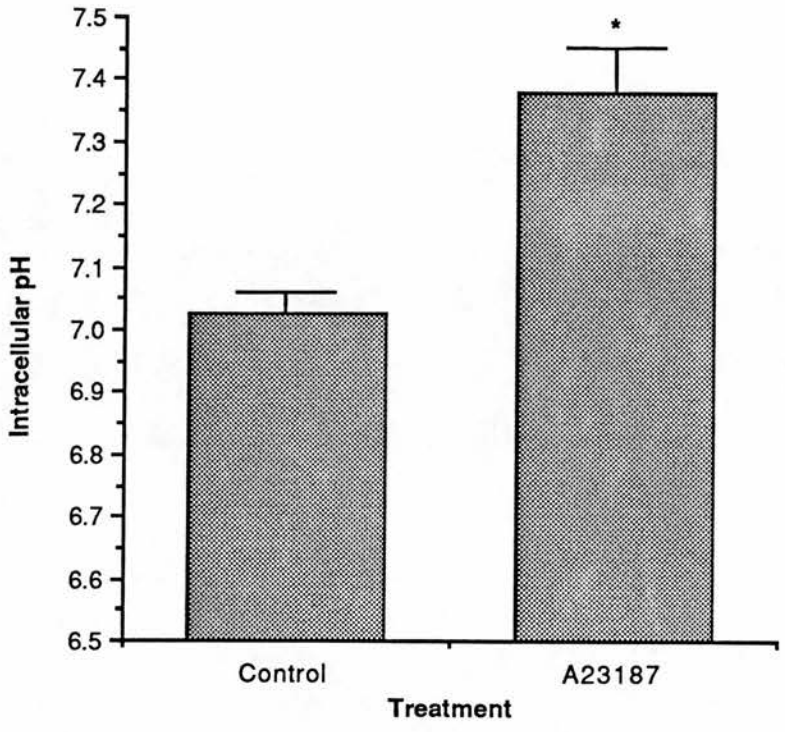


Figure 7.6

Effect of A23187 upon intracellular pH

The effect of 100 μM A23187 upon intracellular pH was studied in spermatozoa loaded with BCECF. Over a 10 minute period, the addition of 100 μM A23187 resulted in an increase in intracellular pH from 7.026 ± 0.033 to 7.378 ± 0.076 (n = 6, mean ± SEM, $P < 0.05$, Wilcoxon matched pairs signed ranks test).

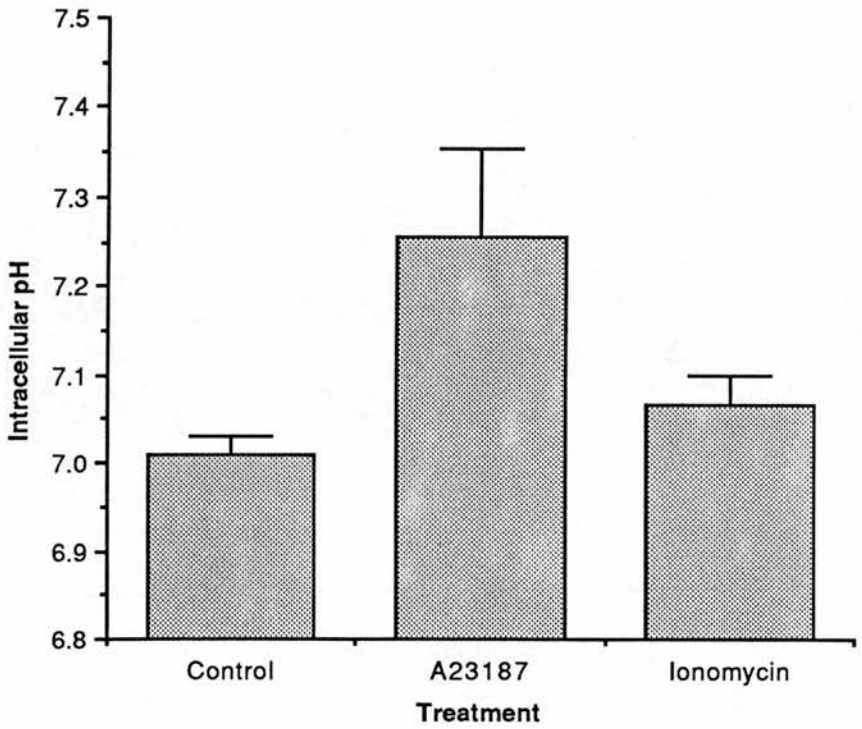


Figure 7.7

Effects of Different Cation Ionophores upon Intracellular pH

The effects of 100 μM A23187 and 1 μM Ionomycin upon intracellular pH were studied in spermatozoa loaded with BCECF. Over a 10 minute period, the addition of 100 μM A23187 resulted in an increase in intracellular pH from 7.019 ± 0.019 to 7.254 ± 0.099 (n = 3), while ionomycin produced almost no change (pH = 7.066 ± 0.034) (n = 2).

CHAPTER EIGHT

SUMMARY AND CONCLUSIONS

8.1 Introduction

8.2 Conventional Criteria of Semen Quality

8.3 *In-vitro* Tests of Sperm Function

8.4 Intracellular Free Calcium and pH

8.5 Conclusions

8.1 Introduction

Infertility, the failure of a couple to conceive after twelve months of normal unprotected intercourse, is a common clinical problem the existence of which has been recognised for almost four thousand years. Contemporary estimates suggest that one couple in six will be affected at some stage of their reproductive lives. The suffering and distress which this causes can only be guessed at.

The aetiology of much of this infertility is poorly understood. Studies sponsored by the World Health Organization (Cates et al, 1985; Diczfaluzy, 1986) have suggested that abnormalities will be detectable within the female partner in 52% of couples, and within the male partner in 43% of couples, while studies in the U K have suggested that defective sperm function is the commonest single defined abnormality detected during the investigation of infertility, occurring in 24% of couples, and that in addition, no apparent abnormality will be found in 28% of couples (Hull et al, 1985). If we accept, therefore, that the contribution of the male partner to the causation of infertility is a significant one, then the problems which confront the clinician in managing an infertile couple are firstly establishing whether or not there is anything wrong with the male partner, secondly, establishing what is wrong, and thirdly being able to intervene to correct any abnormality detected.

The conventional approach to the clinical evaluation of the male partner of an infertile couple was outlined in Chapter One, in which it was established that the taking of a history, the performance of a clinical examination and the undertaking of basic investigations will result in the diagnosis of a small

number of conditions which are known to be responsible for male infertility. These include problems such as obstructive azoospermia, the immotile cilia syndrome, and hypogonadotrophic hypogonadism. In a much larger number of cases, either no abnormality will be detected, or an abnormality will be detected, the significance of which is unclear in any individual case. Amongst the latter would be included the diagnosis of varicocele, of accessory gland infection and the detection of abnormalities of semen quality short of azoospermia or severe oligozoospermia. In such cases, it is difficult for the clinician to provide his patient with a clear statement of whether or not a problem exists that is preventing the initiation of pregnancy. Without the means of accurate diagnosis, it is difficult to provide logical therapy, and difficult to evaluate the effectiveness of therapeutic intervention.

8.2 Conventional Criteria of Semen Quality

The contribution of the male partner to the initiation of pregnancy consists in the production and delivery of gametes, and uniquely, these gametes are naturally produced in a readily available suspension suitable for clinical evaluation. For this reason, the conventional semen analysis has evolved as the principal investigation of the male partner, the techniques for undertaking this analysis are well described (World Health Organization, 1987) and various criteria of normality have been suggested. As was outlined in Chapter Two, the conventional criteria of semen quality are descriptive, providing information on the volume of semen, its physical characteristics and on the concentration, motility and morphology of spermatozoa, together with any additional cellular elements. The definition of "normality" in semen analysis has traditionally been approached by

evaluating the semen quality of a group of men of proven fertility, and such a study of a group of recently fertile men in Edinburgh has been presented in Chapter Two , in which it was shown that the 5th and 95th percentiles of semen volume were 0.9 and 6.1 ml, of sperm concentration 23 and 260 x 10^6/ml and of motility 36 and 71%. It is hard to see how values in excess of the 95th percentile can be associated with infertility, and thus the 5th percentiles are normally cited as the lowest acceptable levels of "normality", rounded for convenience to values of 20 x 10^6/ml for sperm density and 40% for motility and morphology. The small discrepancy between the values cited in the present study and those promulgated by the World Health Organization emphasizes the need for individual laboratories to establish their own normal ranges.

Whichever criteria of normality are used, it is evident that a significant proportion of men with normal semen quality are infertile, and that a significant proportion of men with abnormal semen quality are fertile (Macleod and Gold, 1951; Nelson and Bunge, 1974; Rehan et al, 1975; Smith and Steinberger, 1977; Åbyholm, 1981). Anywhere between 5 and 15% of fertile men are found to suffer from oligozoospermia. The question confronting the clinical andrologist is whether or not a given patient is producing gametes capable of establishing a pregnancy *in-vivo* , and questions relating to the cause of defective gamete production and the prospects for therapeutic intervention are secondary to this. Recent studies have clearly demonstrated the inability of the conventional criteria of semen quality to distinguish between those men presenting to an infertility clinic who will succeed in their attempts to initiate a pregnancy and those who will fail (Aitken et al, 1984a; Aitken, 1985). These studies have suggested that

in-vitro tests of human sperm function may be of value in providing the patient with a more accurate diagnosis.

8.3 *In-vitro* Tests of Sperm Function

In-vitro tests of sperm function which have been advocated include the measurement of semen adenosine triphosphate (ATP) concentrations (Calamera et al, 1979; Schirren et al, 1979; Caldini et al, 1982; Orlando et al, 1982; Comhaire et al, 1983), the study of the movement characteristics of spermatozoa by time exposure photomicrography (Overstreet et al, 1979; Katz et al, 1980; Katz and Overstreet, 1981;Schats et al, 1984; Aitken et al, 1985; 1986) and the ability of spermatozoa to fuse with zona-free hamster oöcytes (Yanagimachi et al, 1976; Aitken et al 1982a; b; c; Rogers, 1985).

Adenosine triphosphate is an important component of the subcellular biochemical economy of the spermatozoon, being concerned with motility and the maintenance of ionic gradients amongst many other functions, however it is only one component among many, and it would have been surprising if variation in cell function could be explained on the basis of variations in the level of one nucleotide. It was demonstrated that the concentration of ATP in an ejaculate was positively correlated with both the concentration of spermatozoa and the motile sperm concentration of that ejaculate, and that there was also a positive correlation between the concentration of ATP in an ejaculate and the outcome of zona-free hamster oöcyte penetration testing. However, more detailed analysis demonstrated that this latter relationship was a reflection of the relationships between ATP concentration and sperm number, and that there was no relationship between ATP "per cell" and the outcome of the oöcyte penetration test. The

design of the zona-free hamster oöcyte penetration test employed in this study involved the incubation of spermatozoa in normosmotic medium, hyperosmotic medium or in the presence of the divalent cation ionophore A23187. It was of interest that the various relationships which were observed with the outcome of this bioassay were all with the A23187 stimulated incubations, supporting the view that this modification of the assay maximises the ability of the zona-free hamster oöcyte penetration test to examine the full functional competence of the male gamete.

The ability of *in-vitro* tests of sperm function to distinguish accurately between ejaculates which were capable of establishing a pregnancy *in-vivo* and those which were not was carefully examined in a retrospective study of semen used in a therapeutic donor insemination service. Prospective studies of *in-vitro* tests of sperm function (Aitken et al, 1984a; Aitken 1985) have suggested that they are of significant clinical value. However, such studies are confounded by the existence of significant inter-ejaculate variability in both the conventional criteria of semen quality and in the functional competence of the spermatozoa. An accurate assessment of the relationship between the results of sperm function tests and *in-vivo* fertility requires that these measurements are made on the same ejaculate. In addition, in a prospective study of infertile patients, the possibility of undiagnosed female pathology contributing to the infertility cannot be excluded, the effect of which can be minimised by maximising the number of female recipients exposed to an ejaculate. Such a study can only be carried out within the context of a therapeutic donor insemination service.

A study was therefore performed to examine the diagnostic value of adenosine triphosphate measurements, sperm movement characteristics

and the zona-free hamster oöcyte penetration test in distinguishing between a group of ejaculates which had succeeded in producing pregnancies and a second group that had failed to do so despite multiple inseminations. It was demonstrated that neither the conventional criteria of semen quality nor semen ATP concentrations were capable of distinguishing between the successful and unsuccessful ejaculates. However, when the conventional semen analysis was supplemented with sperm movement characteristics, an accuracy of classification of 72.92% was obtained and when the conventional semen analysis was supplemented with the zona-free hamster oöcyte penetration test, an accuracy of classification of 75.00% was obtained. When all three groups of tests were employed, an accuracy of classification of 81.25% was obtained. This clearly demonstrated the clinical value of *in-vitro* tests of sperm function in the evaluation of male infertility. The zona-free hamster oöcyte penetration test would appear to evaluate the ability of spermatozoa to capacitate, acrosome react, generate a fusogenic equatorial segment and fuse with the vitelline membrane of the oöcyte, while sperm movement characteristics appear to provide information on the ability of spermatozoa to penetrate the barriers presented by cervical mucus and the zona-pellucida.

Whilst the zona-free hamster oöcyte penetration test is a complex and time consuming assay, the evaluation of sperm movement characteristics is susceptible to automation, and several computer based image analysis systems are now available for this purpose. This technology appears to represent a significant advance in the accurate diagnosis of male infertility.

8.4 Intracellular Free Calcium and pH

The design of the zona-free hamster oöcyte penetration test employed in the above studies incorporated the incubation of the spermatozoa with the divalent cation ionophore A23187. This modification of the assay circumvents the problems of inter-ejaculate and inter-individual variability in *in-vitro* capacitation times which are known to occur. Whilst having demonstrated, for the first time, the diagnostic value of this modification of the assay, questions arose regarding the effects of A23187 upon the physiology of the sperm cell and regarding the possible light these observations shed upon the fundamental sub-cellular lesions present in the spermatozoa of infertile men. For this reason, studies were undertaken to examine the regulation of intracellular free calcium and pH by human spermatozoa.

Studies using $^{45}Ca^{2+}$ demonstrated that the plasma membrane of the human spermatozoon is permeable to calcium, and that a significant proportion of the calcium entering the sperm cell is sequestered within the mitochondrion. Studies using the intracellular calcium indicator quin-2 (Tsien et al, 1982a) allowed the first measurements of free intracellular calcium in human spermatozoa, suggesting that the intracellular level of ionised calcium is of the order of 1 - 200nM in the face of an extracellular concentration of 1 - 2mM. Evidence was obtained to support the view that this extracellular calcium enters the cell passively, rather than through voltage sensitive calcium channels, and that the spermatozoon then actively extrudes this calcium. Thus the level of free intracellular calcium was found to be significantly affected by the level of extracellular calcium, and by temperature.

The antibiotic A23187, which has been shown to stimulate sperm function, caused a significant increase in intracellular calcium, as did ionomycin, a related divalent cation ionophore. However, other agents known to stimulate sperm function appeared to do so by mechanisms independent of intracellular calcium. For example, 1-oleoyl, 2-acetyl glycerol (OAG), an agent known to activate protein kinase C, stimulates sperm function, but does not cause any significant changes in intracellular free calcium.

Measurements of intracellular pH were obtained with the use of the indicator BCECF, and it was shown that the intracellular pH of the human spermatozoon was slightly lower than that of the extracellular environment, and appeared to be profoundly affected by extracellular pH. A23187 appeared to cause a significant rise in intracellular pH. These observations would be consistent with the view that the natural tendency of cells to become acidified is counteracted by the presence of regulatory acid extruding mechanisms.

8.5 Conclusions

> "Oh, the powers of nature. She knows what we need, and the doctors know nothing."
>
> Benvenuto Cellini
> Autobiography, 1558 - 66

Despite the size of the problem of male infertility, there are few, if any, effective therapeutic strategies available to the clinical andrologist. The principal problem confronting the clinical andrologist is one of diagnosis. It is clear that the conventional approaches to the diagnosis of male infertility are inadequate, and that new tests of sperm function can go some considerable way to providing the clinician with a diagnosis, and so the patient with some sort of prognosis.

The lack of effective treatment reflects our lack of fundamental knowledge concerning the abnormalities of cell biology which affect the spermatozoa of infertile men. The present work has shown how the manipulation of the cell biology of the spermatozoon can be used to diagnostic advantage. More detailed studies of cell biology should provide us with an understanding of the causes of male infertility, upon which knowledge can be built an effective therapeutic armamentarium.

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APPENDIX

APPENDIX 1

Composition of Medium BWW (Biggers et al, 1971)

Component	Mol Wt	g/l	mM	mOsm
NaCl	58.4	5.540	94.59	189.19
KCl	74.6	0.356	4.78	9.56
$CaCl_2.5H_2O$	308.3	0.527	1.71	5.13
KH_2PO_4	136.1	0.162	1.19	2.38
$MgSO_4.7H_2O$	246.5	0.294	1.19	2.38
$NaHCO_3$	84.0	2.106	25.07	50.14
NaPyruvate	110.0	0.028	0.25	0.50
NaLactate	112.1	2.416	21.58	43.10
Glucose	180.2	1.0	5.50	5.56
Crystalline Serum Albumin		1.0		
Antibiotic Stock[1]		1.0 ml		
Distilled H_2O		1000.0 ml		
Total				**308.0**

[1] 100000 IU penicillin/ml, 50 mg streptomycin/ml