STUDIES ON THE HUMAN TESTIS IN VITRO

Barbara Jane Beaton Simpson B.Sc.

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University of Edinburgh

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CONTENTS

		Page
DECLARATION		i
ACKNOWLEDGEMENTS		ii
ABSTRACT		iii
CHAPTER 1	Review of the Literature	1
1.1	HISTORICAL ASPECTS	2
1.2	SPERMATOGENESIS	5
1.3	THE SERTOLI CELL	11
1.3.1	The blood-testis barrier	11
1.3.2	Synthesis and secretion of proteins from the Sertoli cell	15
	(i) Androgen binding protein	15
	(ii) Inhibin	16
	(iii) Transferrin	17
	(iv) Plasminogen activator	18
	(v) Gamma glutamyl transpeptidase	19
	(vi) Lactate and pyruvate production by the Sertoli cell	20
1.3.3	Sertoli cell growth factors	21
	(i) Sertoli cell regulators of mitosis and meiosis	21
	(ii) Somatomedin-C/insulin-like growth factor	22
	(iii) Testicular interleukin-1	24
1.3.4	Sertoli cell androgen receptors	25
1.4	INTERSTITIAL TISSUE	27
1.4.1	The Leydig cell	28
1.4.2	Heterogeneity of adult Leydig cells	29
1.4.3	Testicular androgens	30
1.4.4	Biological action of androgens	31
1.4.5	Testicular androgen receptors	31
1.4.6	Extratesticular androgen action	32
1.4.7	Other Leydig cell products	32
	(i) Oestradiol	32
	(ii) Oxytocin and vasopressin	34
	(iii) Pro-opiomelanocortin-derived peptides	35
1.5	ENDOCRINE CONTROL OF THE TESTIS	36
1.5.1	Luteinizing hormone and the Leydig cell	36
1.5.2	Mechanism of action	36
1.5.3	Regulation of LH receptors	37
1.5.4	Leydig cell response to LH	38
1.5.5	Follicle-stimulating hormone	39
1.5.6	FSH and the Leydig cell	40
1.5.7	Mode of action	41
1.5.8	Control of FSH secretion	41
1.5.9	Source of inhibin	42
1.5.10	Mode of action	43
1.5.11	Testicular steroids and FSH secretion	43

Page

1.6	PARACRINE CONTROL OF THE TESTIS	45
1.6.1	Sertoli cell-Leydig cell interactions	45
	(i) Evidence for paracrine regulators of Leydig cell function	46
	(ii) Follicle-stimulating hormone	47
	(iii) Oestradiol	47
	(iv) Testicular-LHRH	47
	(v) Physiological role of testicular-LHRH	48
	(vi) Mode of action	48
	(vii) Sertoli cell/interstitial fluid stimulatory factor(s)	50
	(viii) Testicular opiates	52
1.6.2	Sertoli cell-germ cell interactions	54
	(i) Functional cycle of the Sertoli cell	54
	(ii) Germ cell effects	56
1.6.3	Sertoli-peritubular-Leydig interactions	58
1.6.4	Leydig cell-macrophage interactions	61
1.7	SCOPE OF THIS THESIS	63
CHAPTER 2	Materials and Methods	64
2.1	HUMAN TESTICULAR TISSUE, PLASMA AND INTERSTITIAL FLUID	65
2.1.1	No treatment	65
2.1.2	Radiation treated	65
2.1.3	Oestrogen treated	65
2.1.4	Post mortem	65
2.1.5	Plasma	65
2.1.6	Interstitial fluid	66
2.2	MORPHOMETRY AND STEREOLOGY	67
2.2.1	Histology	67
2.2.2	Photography	67
2.2.3	Random sampling	67
2.2.4	Standardization	67
2.2.5	Minimal sample size	67
2.2.6	Measurement of testicular volume	67
2.2.7	Section thickness	68
2.2.8	Determination of round spermatid and Leydig cell number	68
2.2.9	Seminiferous tubule length	69
2.2.10	Daily sperm production	70
2.2.11	Morphometric analysis of DSP	71
2.2.12	Quantitation of spermatogenesis by enumeration of mature spermatids	71
2.3	DISTRIBUTION OF TESTOSTERONE IN THE HUMAN TESTIS	72
2.3.1	Whole testis testosterone	72
2.3.2	Isolation of human seminiferous tubules	72
2.3.3	Extraction of whole testis and seminiferous tubule testosterone, dihydrotestosterone and oestradiol on celite columns	72
	(i) Whole testis	72
	(ii) Seminiferous tubules	73

Page

(iii) Preparation of celite columns 73
(iv) Elution of steroids on celite columns 73
2.3.4 Iodination of tracer for ^{125}I-testosterone assay 74
2.3.5 Testosterone standards 74
2.3.6 ^{125}I-testosterone assay 75
2.3.7 Measurement of circulating hormones 75
(i) Testosterone 75
(ii) LH and FSH 76
(iii) Oestradiol 76

2.4 LEYDIG CELL STUDIES IN VITRO 78
2.4.1 Patients 78
2.4.2 Animals 78
2.4.3 Percoll gradients 78
2.4.4 Preparation of human Leydig cells 78
2.4.5 Preparation of rat Leydig cells 79
2.4.6 Identification of Leydig cells 80
2.4.7 Leydig cell incubation 80
2.4.8 Measurement of Leydig cell testosterone 81
2.4.9 hCG binding 81

2.5 INHIBIN BIOACTIVITY IN HUMAN TESTICULAR EXTRACTS 83
2.5.1 Patients 83
2.5.2 Preparation of testicular extracts 83
2.5.3 In vitro inhibin bioassay 83
2.5.4 Bioassay of pituitary cell mitogenic activity 85

2.6 STATISTICS 86

CHAPTER 3 Relationship between Testosterone and Spermatogenesis in the Senescent Human Testis 87

3.1 Introduction 88

3.2 (i) Daily sperm production 89
(ii) Morphometric analysis of daily sperm production 93
(iii) Quantitation of spermatogenesis by enumeration of mature spermatids 93
(iv) Systemic hormone levels and their relationship to daily sperm production 93
(v) Intratesticular testosterone levels and their relationship to daily sperm production 101
(vi) Leydig cell number, intratesticular testosterone and daily sperm production 101
(vii) Re-evaluation of intratesticular levels of testosterone 101

3.3 Discussion 105

Page

CHAPTER 8 Concluding Remarks 191

REFERENCES 199

PUBLICATIONS 236

DECLARATION

Except where acknowledgement is made by reference, the studies described herein were performed by the author.

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ABSTRACT

The obligatory role of pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the control of testicular function is well established. However there is increasing experimental evidence, largely in the rat, that quantitatively normal spermatogenesis not only requires an adequate local supply of testosterone but also the complex interactions between the various cellular components within the seminiferous tubules and interstitial compartments of the testis. The critical role of paracrine mechanisms in the testis may be reflected by the defective spermatogenesis in idiopathic male infertility where systemic levels of LH, FSH and testosterone are normal or elevated. Thus to further our understanding of the hormonal control of spermatogenesis and to define possible aetiological mechanisms in the infertile man, the study of paracrine mechanisms in the human testis is of paramount importance.

The approach to study paracrine mechanisms in the human testis was to establish in vitro techniques whereby individual components of the testis were isolated and specific functional markers defined so that their subsequent interaction could be further studied in vitro. At the same time, the delineation of these 'local' parameters were related to the overall functional states of the testis as defined by circulating levels of LH, FSH, testosterone and the histological assessment of spermatogenesis.

Testes were obtained at orchidectomy for prostatic carcinoma. Methods were established to examine the effect of intratesticular levels of testosterone and systemic levels of LH, FSH and testosterone on quantitative measures of spermatogenesis. For this purpose a simple

technique which involved enumeration of spermatid nuclei in fixed testicular homogenates to determine daily sperm production was adopted. Daily sperm production in this group of ageing testes was generally lower than has been observed previously for younger men. Although intratesticular levels of testosterone varied widely there was no indication of an intratesticular deficiency of testosterone as a critical factor in subnormal spermatogenesis in the ageing testis.

Inhibin, a peptide marker of Sertoli cell function was measured in human testicular extracts by bio- and radioimmunoassay. The relationship observed between FSH and both inhibin bioactivity and immunoactivity imply that the role of inhibin in the testis may be somewhat different to the classical concept of FSH feedback.

A technique for the routine isolation of human Leydig cells was established. Human Leydig cells purified by Percoll density centrifugation were highly responsive to hCG, although sensitivity and receptor number were significantly lower compared to the rat. This system was used to test for the effects of putative paracrine factors on human Leydig cell function.

In conclusion, a number of in vitro techniques have been established and validated which provide a basis for future investigation of seminiferous tubule and Leydig cell function in the human testis.

CHAPTER 1

Review of the Literature

1.1 HISTORICAL ASPECTS

Since Neolithic times the importance of the male gonads in libido and potency has been recognized. Castration as a punishment for sexual offences was described as early as 2000 BC. Aristotle (400 BC) studied the effects of pre- and postpubertal castration in roosters and in man. Despite his observations and accurate illustrations of testicular anatomy, Aristotle maintained that the testes were not necessary for fertility. The Romans believed the testes to be witnesses of a man's virility, and hence our word "testis" comes from the Latin "testis" meaning witness or spectator.

With the development of the simple microscope, de Graaf (1668) described the testis as being composed of tubules. He also established that the production of the fertilizing portion of the semen originates in the testes. In 1677 Leeuwenhoek demonstrated the existence of "animacules" or spermatozoa, which he thought were young developing animals, in seminal fluid. By 1840 Koelliker concluded that the spermatozoa are not young developing animals, but are the product of cell development within the seminiferous tubules.

The "branched cells" of the testis which now bear his name were described by Sertoli in 1865. He opposed previous suggestions that the spermatozoa arose from his "branched cells" and described how the cells, which we now call round spermatids, metamorphose into spermatozoa. Sertoli also identified two types of spermatogonia and described three developmental stages of the spermatocytes.

From his studies on the transplantation of testes from roosters to ectopic sites in capons, Berthold (1849) concluded that a substance from the testis must be transmitted through the bloodstream to affect other tissues. This was the first description of the endocrine function of

any gland. Although he did not attribute any endocrine function to the cells named after him, Leydig (1850) first described the interstitial cells of the testis. An endocrine role for the Leydig cells was established in 1903 when Bouin and Ancell reported that in man and animals with cryptorchidism, in whom there is no spermatogenesis but the interstitial cells are intact, androgen-dependent functions, such as the development of secondary sexual characteristics, are maintained.

Attempts to isolate the male hormones began in 1927 when McGee prepared an extract from bull testes, which, when administered to capons, was capable of restoring their masculinity. In 1935 the male hormone, testosterone, was isolated from the testis by David and co-workers and Ruzicka & Wettstein.

Around this time the importance of the pituitary involvement in gonadal function was realized. Following the observations of Cushing (1910) and Aschner (1912), who removed the pituitary in dogs and showed that hypopituitarism led to genital hypoplasia, the idea that the functions of the gonads might be regulated by a factor from the anterior pituitary had been suggested. The two pituitary hormones which in the ovary stimulate follicular growth (follicle-stimulating hormone - FSH) and cause luteinization (luteinizing hormone - LH) were extracted from the anterior pituitary (Fevold et al. 1931) and the relation of FSH to spermatogenesis and LH to Leydig cell function was demonstrated by Greep and co-workers (Greep et al. 1936; Greep & Fevold, 1937). The precise role of FSH became less clear when it was shown that testosterone in the absence of the pituitary is capable of maintaining spermatogenesis (Walsh et al. 1934). The precise role of FSH in regulating spermatogenesis remains a contentious issue to this day.

The past 30-40 years have seen great advances in our understanding

of testicular structure and function. Detailed morphological studies in the 1950's and 1960's revealed the structure and organization of spermatogenesis in a number of species. Clermont (1963) and Heller & Clermont (1964) described the cycle of the human seminiferous epithelium and its organization into cell associations.

During the 1970's the emphasis was on the hormonal regulation of spermatogenesis. The development of a number of techniques which enabled the detection of sex steroids and pituitary hormones, and the identification of target cells within the testis for these hormones, subsequently led to the elucidation of molecular mechanisms for hormone action.

In the 1980's a number of factors produced locally within the testis, which are thought to mediate the interaction between the various cellular elements in response to gonadotrophin stimulation, have been identified. Most of the information on these intratesticular or paracrine factors is derived from studies in the rat. The importance of paracrine control of the human testis and possible dysfunction of these mechanisms in idiopathic male infertility are only just being realized, and, as such, the need to study paracrinology of the human testis is of paramount importance.

1.2 SPERMATOGENESIS

Spermatogenesis is the process by which the male gametes, the spermatozoa, are formed. This takes place within the seminiferous tubules where there is a close morphological relationship between the developing germ cells and the Sertoli cells (see 1.3.1). Cell processes from adjacent Sertoli cells extend laterally to envelop the developing germ cells (Fig. 1.1). Within this arrangement, the germ cells proceed through the developmental stages of spermatogenesis.

The spermatogonia are the stem cells from which all spermatozoa are derived, the spermatogonia themselves being descended from the primordial germ cells which reach and multiply in the genital cords in the developing testis. In the fully differentiated testis the spermatogonia are situated along the basement membrane of the seminiferous tubule. In man three basic types of spermatogonia can be distinguished - dark type A, pale type A and type B (Clermont, 1963). Dark type A spermatogonia divide to maintain the basic store of spermatogonia and also give rise to some pale type A cells which divide and differentiate into type B spermatogonia. Type B spermatogonia divide to produce the primary spermatocytes. The spermatocytes then go through a long prophase which shows characteristic configurations of the chromosomes (leptotene, zygotene, pachytene). This prophase is then followed by the subsequent steps of the first reduction division that yields the secondary spermatocytes containing the haploid number of chromosomes. Each secondary spermatocyte then undergoes the second reduction division to produce two round spermatids. These spermatids then go through a series of nuclear and cytoplasmic modifications to produce mature spermatozoa.

The seminiferous epithelium therefore is composed of four or five

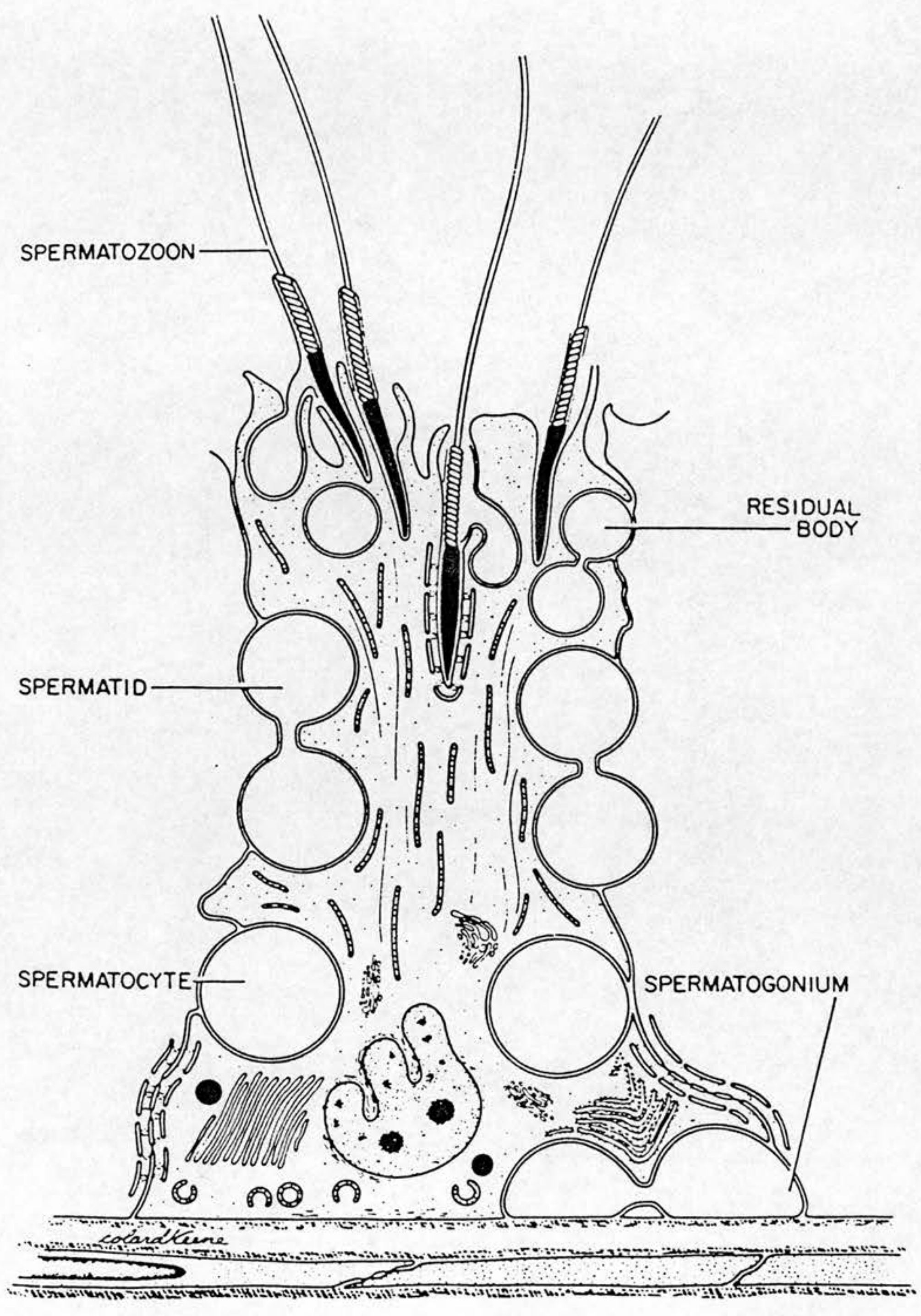


FIGURE 1.1

Diagramatic representation of the developing germ cells enveloped in the Sertoli cell cytoplasm.
From: Histology, M. Dym, p.979, Weiss, L. and Greep, R.O. (Eds.),McGraw-Hill, New York, 1977.

distinct generations of germ cells at different stages of development. Cytological studies have shown that at any point in the seminiferous tubule these generations of cells are not randomly arranged but form cellular associations of fixed composition, such that spermatids at a given stage of development are always associated with the same types of spermatogonia and spermatocytes. The activities of several generations of germ cells pass through a cycle, the length of which and the number of stages involved vary in different species (Clermont, 1972). In man, each cycle occupies about sixteen days (Heller & Clermont, 1964) and comprises six different cell associations or stages (Clermont, 1963; Fig. 1.2). In the rat, the cycle comprises fourteen different stages (I - XIV) lasting approximately twelve days (Le Blond & Clermont, 1952). These stages of the cycle of the seminiferous epithelium reflect the constant rate of germ cell development, the cyclic commitment of the cells to differentiation and the gradual displacement of successive generations of maturing cells towards the tubular lumen.

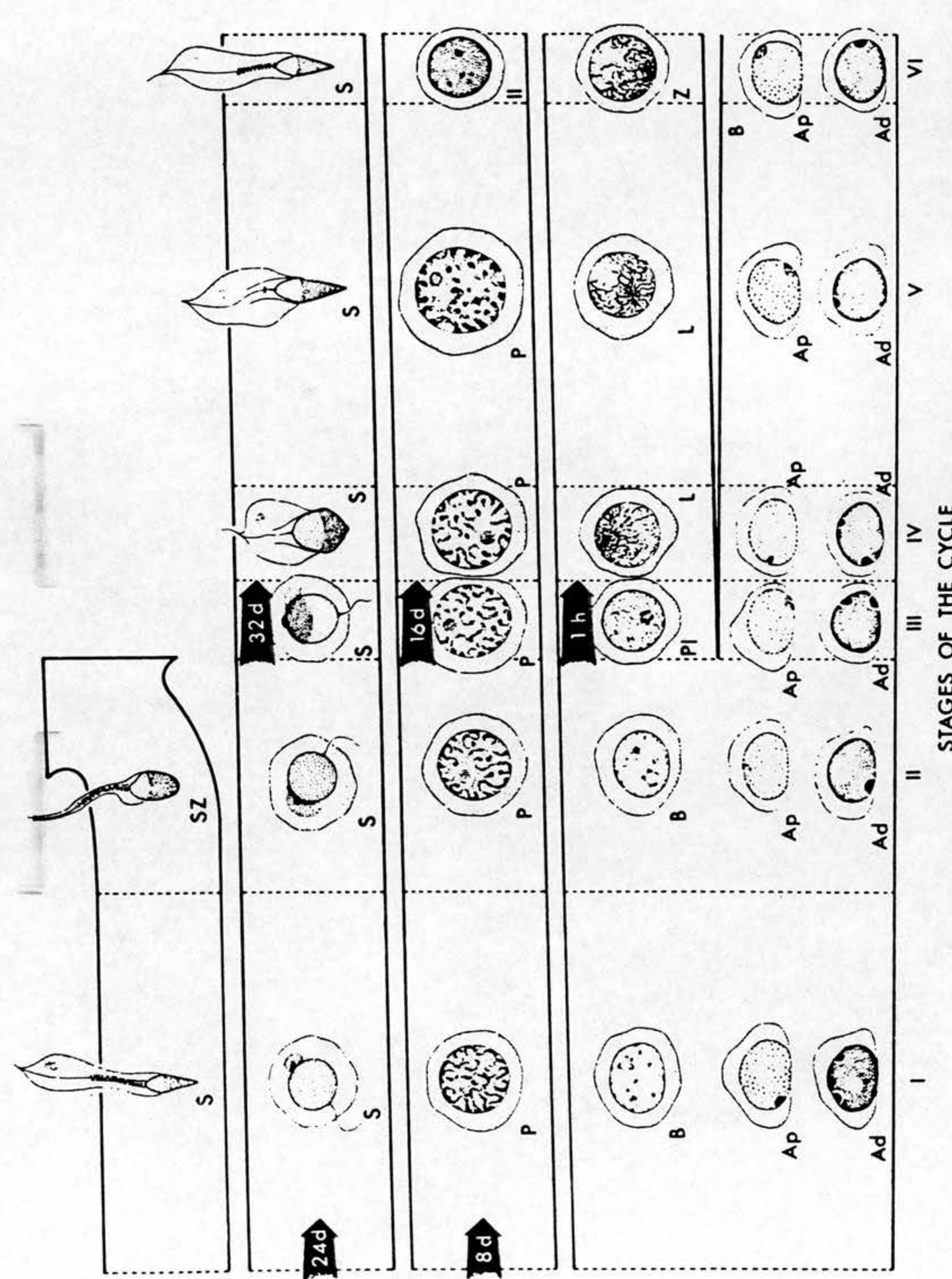
The functional requirements of each individual stage have been studied in the rat, aided by the spatial arrangement of each successive stage of the spermatogenic cycle along the length of the seminiferous tubule. This spatial arrangement is known as the wave of the seminiferous epithelium, and in most mammals, including the rat, each stage is distributed in sequence along the length of the seminiferous tubule (Perey et al. 1961). This has permitted the development of transillumination-assisted seminiferous tubule dissection in which pools of seminiferous tubules at one specific stage of the cycle can be isolated and their functional requirements investigated (Parvinen, 1982; Parvinen & Ruokonen, 1982). Such studies have not been possible in the human as different stages occupy helically running strip-shaped areas

FIGURE 1.2

Diagramatic illustration of the cellular composition of six stages (I - VI) of the cycle of the seminiferous epithelium in man. The stages correspond to cell associations which succeed one another in time in any given area of the seminiferous tubule according to the sequence I - VI. Following stage VI, stage I reappears and the sequence starts over again. The space allotted to each stage of the cycle is proportional to its relative duration. The arrows correspond to the most advanced labelled cells at various time intervals (indicated on the arrows) after an intratesticular injection of ^{3}H-thymidine.

Ad, Ap, B - dark type A, pale type A, type B spermatogonia; Pl, L, Z, P - preleptotene, leptotene, zygotene, pachytene primary spermatocytes; II - secondary spermatocytes; S - spermatids; SZ - spermatozoa.

From: Y. Clermont (1970), In: The Human Testis, E. Rosemberg & C.A. Paulsen (Eds.), Plenum Press.



STAGES OF THE CYCLE
I
II
III
IV
V
VI
SZ
24d
32d
8d
16d
1 h
S
P
B
Pl
L
Z
Ap
Ad

of the seminiferous epithelium (Schulze & Rehder, 1984). One area of the seminiferous tubule is occupied by up to three stages (Kerr & de Kretser, 1981), and as such, individual stages cannot be isolated. Nevertheless there is much evidence to suggest that the functional requirements of germinal cells in the human testis are analogous to those in the rat (Sharpe, 1986).

While the morphological and kinetic aspects of spermatogenesis have been studied extensively, we are now beginning to appreciate that the control of spermatogenesis involves local interaction between the various cell types within the testis. The problem that concerns us now is the identification of the factors involved in this process.

1.3 THE SERTOLI CELL

Since it was first described the Sertoli cell has been associated with the process of spermatogenesis, but its importance is only now being realized.

Primitive Sertoli cells, which differentiate from the mesenchymatous medullary gonadal blastema, form cell cords which embody the germ cells (Witschi, 1970; Jost et al. 1974). Mitosis of the Sertoli cell occurs only in the immature testis (Clermont & Perey, 1957; Steinberger & Steinberger, 1971), apparently independent of hormonal control. After puberty Sertoli cell number remains constant, and this fact has been employed in the use of the Sertoli cell as a constant reference in the quantitation of spermatogenesis (Rowley & Heller, 1971). An age-related decline in Sertoli cell number has however recently been observed (Johnson et al. 1984).

1.3.1 Blood-testis barrier

By virtue of its position in the seminiferous epithelium, the Sertoli cell is intimately involved in the process of spermatogenesis. The Sertoli cell maintains the integrity of the seminiferous epithelium by specialized contacts with adjacent germ cells (see Russell, 1980 for review). These specialized junctions also facilitate the movement of maturing germ cells from the basal aspects of the seminiferous epithelium to the tubular lumen (Russell, 1980).

In addition to the specialized Sertoli-germ cell contacts, adjacent Sertoli cells are connected by tight junctions in the basal aspect of the seminiferous epithelium. These tight junctions are the site of the blood-testis barrier which separates the seminiferous epithelium into basal and adluminal compartments (Fawcett, 1975) (Fig. 1.3). The basal compartment contains spermatogonia and early spermatocytes which share a

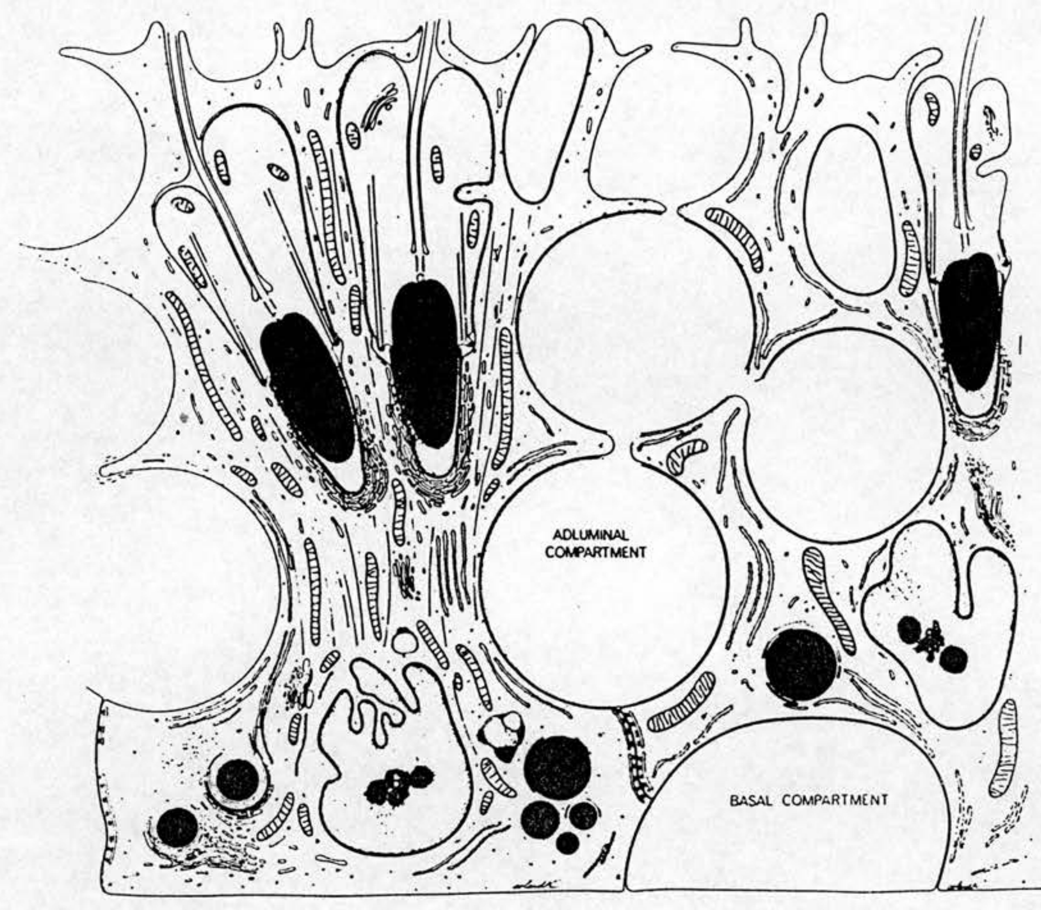


FIGURE 1.3

Illustration of Sertoli cell tight junctions which are the main component of the blood-testis barrier. These structures divide the seminiferous epithelium into a basal compartment which is occupied by the spermatogonia and preleptotene spermatocytes, and an adluminal compartment containing the postmeiotic germ cells.

From: D.W.Fawcett (1975), Handbook of Physiology, Section 7 Vol V.

position with the Sertoli cell on the basal lamina and are interposed between adjoining Sertoli cells in such a way that the Sertoli cells arch over these cells and meet above them. Internal to the tight junctions late spermatocytes and spermatids are situated in the adluminal compartment.

The first evidence for the existence of the blood-testis barrier came from the realization that seminiferous tubule fluid and rete testis fluid were very different in composition from blood plasma and testicular interstitial fluid (Setchell et al. 1969). The variation in the rate of entry of various radiolabelled markers into the seminiferous tubule from the blood added to the evidence for the existence of a barrier. Electron microscopic studies with horseradish peroxidase and lanthanum, both electron-dense intracellular markers, showed that cell-cell junctions within the seminiferous epithelium constituted a barrier to the passage of substances into the tubular fluid (Fawcett et al. 1970; Dym & Fawcett, 1970).

Tight junctions between Sertoli cells first appear at puberty with the development of the primary spermatocytes (Vitale et al. 1973). This event coincides with the completion of Sertoli cell proliferation and the secretion of seminiferous tubule fluid from these cells into the newly formed tubular lumen. Seminiferous tubule fluid contains high concentrations of potassium and bicarbonate and low levels of sodium and chloride, compared to blood plasma or interstitial fluid (Setchell & Waites, 1975). One of the main functions of the blood-testis barrier is to maintain the ionic composition of the seminiferous tubule fluid which creates a specific intratubular environment suitable for meiosis and maturation of the postmeiotic germ cells (Setchell, 1980).

Entry of substances into the seminiferous tubules depends mainly on their lipid solubility; in general entry is high where lipid solubility is high (Setchell & Waites, 1975). Testosterone enters seminiferous tubule fluid much more rapidly than its lipid solubility would suggest (Cooper & Waites, 1975; Setchell et al. 1978b). A specific saturable carrier mechanism involving facilitated diffusion is thought to be utilized in the transport of testosterone into the seminiferous tubules (Setchell et al. 1978b).

FSH gains access to the basal compartment of the seminiferous tubules by penetrating between the myoid cells at sites where cell-cell junctions are relatively open (Dym & Fawcett, 1970). FSH is unable to cross the blood-testis barrier and enter the adluminal compartment of the seminiferous epithelium (Setchell et al. 1976). Therefore not surprisingly all FSH-receptors are situated in the basal compartment of the seminiferous epithelium (Orth & Christensen, 1979).

In addition, haploid germ cells, which express surface autoantigens after meiosis, within the adluminal compartment of the seminiferous epithelium are protected by the blood-testis barrier which ensures that they are not recognized by the immune system (Millette & Bellvé, 1977; O'Rand & Romrell, 1977).

The integrity of the blood-testis barrier is maintained even when the spermatocytes enter meiotic prophase and move from the basal into the adluminal compartment. During this migration new tight junctions at the base of the spermatocytes are formed while the old adluminal junctions are still intact (Russell, 1977b).

From what we know about the blood-testis barrier it seems likely that its functional integrity is essential for normal spermatogenesis. Further study is required to see if specific abnormalities in the

structure and function of the blood-testis barrier are associated with abnormal spermatogenesis and impaired fertility.

1.3.2 Synthesis and secretion of proteins from the Sertoli cell

The tight junctional complexes which form the blood-testis barrier prevent the free access of compounds in blood plasma to the germ cells in the adluminal compartment. The Sertoli cell by the secretion of a number of serum- and testis-specific proteins, is responsible for regulating the environment in which the germ cells in the adluminal compartment divide and differentiate.

A number of Sertoli cell products have been identified and used to monitor the hormonal and cellular control of the Sertoli cells and the seminiferous epithelium in vitro. Attempts have also been made to measure specific Sertoli cell products in blood or semen to provide a means of assessing testicular function and dysfunction in vivo.

(i) Androgen binding protein

Androgen binding protein (ABP), the first Sertoli cell protein to be identified in the rat, is thought to maintain high concentrations of androgen within the seminiferous tubule and lumen of the caput epididymis and thus be important in spermatogenesis and sperm transport (Hansson & Tveter, 1971; Ritzén et al. 1971; French & Ritzén, 1973; Hagenas et al. 1975).

The purification and subsequent development of a radioimmunoassay for ABP showed that small amounts of this specific protein are secreted into the systemic circulation in rats (Gunsalus et al. 1978, 1980). This observation gave rise to the idea that by measuring ABP levels in blood, Sertoli cell function could be monitored, and as such the structure and function of ABP has been observed in a number of species.

Studies attempting to demonstrate the presence of ABP in the human

testis have been hampered by the presence of testosterone-oestrogen binding globulin (TeBG) from serum (Bardin et al. 1981). A number of studies have suggested the presence of a distinct human ABP which can be distinguished from TeBG by charge and binding kinetics (Hsu & Troen, 1978). Furthermore, human Sertoli cells cultured in vitro have been shown to secrete ABP which has physicochemical characteristics similar to rat ABP, but as yet, correlation of human ABP with other aspects of human testicular function is lacking.

(ii) Inhibin

Inhibin is a gonadal glycoprotein which feeds back to the anterior pituitary to regulate the secretion of FSH, and in the male is produced by the Sertoli cell (Steinberger & Steinberger, 1976). The existence of a testicular factor which in some way regulates the pituitary gland was suggested as early as 1923 when Mottram & Cramer observed morphological changes in the anterior pituitary gland of a male rat whose seminiferous epithelium was severely disrupted by radiation. Then in 1932 McCullagh, who showed that injection of a water soluble extract from bovine testes could inhibit the morphological changes in the pituitary cells following irradiation, named this hypothetical testicular factor inhibin. Since these early studies, suppressive effects of steroid-free testicular preparations from a number of species on the pituitary production and secretion of FSH have been described in a number of in vivo and in vitro systems.

Conflicting reports existed concerning the properties of inhibin and its purification until in 1985 the full sequence of two 32 kDa forms of porcine follicular fluid inhibin were published (Mason et al. 1985). The availablity of purified inhibin has enabled the development of radioimmunoassay techniques for its measurement, which has confirmed the

hormonal nature of this peptide in the female (Tsonis et al. 1987b). The development of more sensitive assay techniques for the detection of inhibin in the systemic circulation in the male could prove a useful tool in the diagnosis of infertility in men with disorders of the seminiferous epithelium. Furthermore, because of its ability to suppress the secretion of FSH, which theoretically could lead to a reduction in germ cell production, inhibin has often been regarded as a potential regulator of fertility in men. This avenue of potential male contraception must however await confirmation of the actual role of FSH in germ cell development in man.

(iii) Transferrin

Transferrin from the liver is found in the serum of all vertebrates, and until recently it was thought that the Sertoli cell served as an intermediate in the transport of iron from serum transferrin, across the blood-testis barrier to the germ cells in the adluminal compartment of the seminiferous epithelium. However testicular transferrin is secreted by the Sertoli cell (Skinner & Griswold, 1980), and it is now believed that the transport of iron to the developing germ cells is mediated by the shuttling of the iron between the two distinct forms of transferrin (Djakiew et al. 1986).

Diferric transferrin binds to its receptor on the basal plasma membrane of the Sertoli cell, after which is is internalized by receptor mediated endocytosis into the endocytic organelles (Morales & Clermont, 1986). Here, in an acidic atmosphere, iron is dissociated from serum transferrin and released into the Sertoli cell cytoplasm. Subsequently the transferrin molecule, still bound to its receptor, recycles to the cell surface as apotransferrin, and the iron, now bound to testicular transferrin is secreted from the apical pole of the Sertoli cells to the

developing germ cells and/or the lumen of the seminiferous tubule (Djakiew et al. 1986).

Secretion of transferrin by the rat Sertoli cell varies according to the stage of the spermatogenic cycle, secretion being highest in stages IX to XIV (Wright et al. 1983). Human Sertoli cells stimulated by FSH also secrete transferrin in culture (Lipshultz et al. 1982; Holmes et al. 1984). Cellular interaction between the Sertoli cells and the peritubular cells also appears to be involved in the control of transferrin secretion in both the rat and man (see 1.6.3).

The majority of the transferrin in human seminal plasma is thought to be of testicular origin and the use of seminal plasma transferrin as a marker of Sertoli cell function has been suggested (Holmes et al. 1982). Liu and co-workers (1986) confirmed that seminal plasma transferrin reflects Sertoli cell function, but that this is of limited clinical value as the range of transferrin in seminal plasma from normal men is wide and overlaps with values from men with genital tract obstruction or seminiferous tubule failure.

(iv) Plasminogen activator

Sertoli cells in culture secrete the protease plasminogen activator under the regulation of FSH (Lacroix et al. 1977; Lacroix & Fritz, 1982). Plasminogen, the substrate of plasminogen activator is synthesized in the liver. Diffusion of circulating plasminogen into the adluminal compartment of the seminiferous tubules has not been demonstrated, suggesting the possible local synthesis of plasminogen. Plasminogen can be detected in culture medium from rat seminiferous tubules, however its cellular source remains unknown (Saksela & Vihko, 1986).

The secretion of plasminogen activator by the Sertoli cells is highly stage-specific, with maximal secretion during stages VII and VIII

in the rat (Lacroix et al. 1981; Vihko et al. 1984; Vihko et al. 1985). At these stages in the spermatogenic cycle, preleptotene primary spermatocytes enter the adluminal compartment of the seminiferous epithelium prior to meiosis and concomitantly spermatids are released into the tubular lumen (Le Blond & Clermont, 1952). In the absence of these specific germ cells, the stage VII-VIII secretion of plasminogen activator is abolished indicating the role of plasminogen activator in the restructuring of the seminiferous epithelium during germ cell movement (Vihko et al. 1984).

(v) Gamma glutamyl transpeptidase

Gamma glutamyl transpeptidase (GGT) catalyses the transpeptidation reaction which appears to be involved in the transport of certain amino acids across the blood-testis barrier (Bustamante et al. 1982). Human Sertoli cells contain GGT activity similar to that of the rat (Lipshultz et al. 1982). In contrast to the rat, human Sertoli cell GGT is responsive to FSH. This response is hormone specific and dose-dependent (Lipshultz et al. 1982). In both the rat and the human GGT activity was thought to be correlated solely with the Sertoli cell and thus appeared to be an important marker of Sertoli cell function (Hodgen & Sherins, 1973; Krueger et al. 1974; Lipshultz et al. 1982; Tindall et al. 1983). More recently, studies in the rat have demonstrated that GGT activity is localized predominantly in the arterial and arteriolar endothelium (Niemi & Setchell, 1986). The specific activity of GGT increases coincident with puberty and has been linked to the cessation of Sertoli cell mitosis prior to the onset of spermatogenesis (Hodgen & Sherins, 1973; Lu & Steinberger, 1977). This pubertal increase in GGT activity could be associated with the increased volume of the vascular

endothelium which takes place around this time (Niemi & Setchell, 1986). GGT activity in testis homogenates and in homogenized cultured Sertoli cells (Lu & Steinberger, 1977) could be explained by the effects of contaminating endothelial cells (Niemi & Setchell, 1986). This new information casts doubt on the possibility of the use of GGT as a biological marker of Sertoli cell function.

(vi) Lactate and pyruvate production by the Sertoli cell

Glucose is essential for the maintenance of spermatogenesis in vivo (Mancini et al. 1960). Induction of acute hypoglycaemia results in germ cell degeneration (Zysk et al. 1975). The capacity of isolated germ cells to utilize glucose as an energy source however appears to be poor, as isolated pachytene spermatocytes and round spermatids do not survive in the presence of glucose alone (Jutte et al. 1981). The failure of the germ cells to utilize glucose directly appears to be due to a block in glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase (Cervenka et al. 1986). The maintenance of metabolic activity of germ cells, as indicated by oxygen consumption and protein synthesis, is dependent on exogenous lactate (Jutte et al. 1981). Lactate production by the Sertoli cell in co-culture with pachytene spermatocytes is able to maintain the metabolic activity and integrity of the germ cells, but only in the presence of glucose (Jutte et al. 1982).

The specific isoenzyme of lactate dehydrogenase (LDH-C4) is present in pachytene spermatocytes and round spermatids (Meistrich et al. 1977). Lactate from the Sertoli cells can be utilized by means of LDH-C4, which has been shown to preferentially catalyze the oxidation of lactate to pyruvate (Blanco et al. 1976). Lactate oxidation to pyruvate via the tricarboxylic acid cycle supplies the germ cells with energy in the form

of ATP.

LDH-C4 activity from the testis is present in seminal plasma (Clausen & Øvlison, 1965). The extracellular presence of cell specific intracellular enzymes is an indication of cell and/or membrane destruction. LDH-C4 activity in human seminal plasma has been shown to originate from germ cells which have degenerated before release into the semen, with increased LDH-C4/sperm ratio indicating dysfunction of the seminiferous epithelium (Eliasson & Virji, 1985). LDH-C4 in relation to sperm number is therefore an important marker, indicating the functional status of the seminiferous epithelium.

1.3.3 Sertoli cell growth factors

A number of Sertoli cell-derived growth factors appear to be involved in the paracrine regulation of the testis. Not surprisingly, spermatogenesis, the most proliferative cell process in the body, might be expected to be regulated by mitogenic factors of Sertoli cell origin. Increasing awareness that growth factors are regulators of cell metabolism and growth has prompted study of specific growth factor contribution to the spermatogenic process.

(i) Sertoli cell regulators of mitosis and meiosis

Seminiferous growth factor (SGF) is a polypeptide thought to be of Sertoli cell origin which is present in all mammals, including man (Feig et al. 1983). The action of SGF is to induce DNA synthesis and cell proliferation of prepubertal Sertoli cells, regulate the mitotic division of the spermatogonia in the basal compartment of the seminiferous epithelium and to induce the meiotic reduction divisions of the spermatocytes in the adluminal compartment of the seminiferous epithelium (Bellvé & Feig, 1984).

In addition to SGF, meiosis in the male is regulated by two Sertoli

cell products - meiosis-preventing substance (MPS) and meiosis-inducing substance (MIS). In the human MPS activity is found in the foetus and the prepubertal male, whereas little or no MPS activity is found in the adult testis (Grinsted & Byskov, 1981). Prior to puberty the germ cells appear to be prevented from entering meiosis by MPS (Byskov, 1978; Grinsted et al. 1979). MIS activity is found both in foetal and adult testes.

In the rat maximum MIS secretion is observed at the onset of meiosis in stages VII and VIII. It is also secreted in high amounts during the meiotic reduction divisions of stages XII and I (Parvinen, 1982). In contrast to the human, MPS is secreted at a constant rate in the testes of adult rats. The role of MPS in the adult rat seems to be the prevention of the spermatogonia from entering meiosis until maximum MIS secretion at the appropriate stage of the cycle.

(ii) Somatomedin-C/insulin-like growth factor

In both clinical (Kulin et al. 1981) and experimental (Swerdloff & Odell, 1977; Zipf et al. 1978) situations, growth hormone (GH) increases LH-stimulated testosterone production by the Leydig cells in a dose-dependent manner. Many of the physiological actions of GH are thought to be mediated by the somatomedins, a family of small polypeptide growth factors with insulin-like activity (Herrington et al. 1983). Somatomedin-C/insulin-like growth factor 1 (Sm-C/IGF 1) is the most directly GH dependent of these peptides and originates in a number of tissues, including ovarian granulosa cells (Adashi et al. 1985) and testicular Sertoli cells (Ritzén, 1983).

Sm-C/IGF 1 accumulates in the medium of cultured Sertoli cells from immature rats (Tres et al. 1986) and immature pigs (Benahmed et al. 1987). Recently immunoreactive Sm-C/IGF 1 has been reported to be

present in rat peritubular myoid cell-conditioned medium (Skinner & Fritz, 1986). In addition, both rat Sertoli and peritubular cells display Sm-C/IGF 1 immunoreactivity as determined by immunofluorescence and RIA (Tres et al. 1986). However the 1% peritubular cell contamination of rat Sertoli cell cultures is not sufficient to account for the amount of Sm-C/IGF 1-like activity detected in Sertoli cell culture medium (Smith et al. 1987).

Immunocytochemical studies have demonstrated that Sertoli cells show binding affinity for Sm-C/IGF 1 (Tres et al. 1986), and the mitogenic effects of IGF 1 on immature Sertoli cells in culture have been shown previously (Borland et al. 1984). In hypophysectomized adult rats however very little binding of Sm-C/IGF 1 to Sertoli cells is observed (Kasson & Hsueh, 1987). Sm-C/IGF 1 may therefore have autocrine effects on Sertoli cell proliferation in the prepubertal animal.

Binding of Sm-C/IGF 1 to pachytene spermatocytes has been observed in immunofluorescent studies of Sertoli-germ cell co-cultures (Tres et al. 1986). The significance of Sm-C/IGF 1 activity in Sertoli and germ cell function is unknown, but it has been suggested that late meiotic prophase spermatocytes may be stimulated by Sm-C/IGF 1 to progress into meiotic division (Tres et al. 1986).

The presence of receptors for Sm-C/IGF 1 on Leydig cells (Handelsman et al. 1985) adds this polypeptide to the increasing list of potential Sertoli cell regulators of Leydig cell function (see 1.6.1). The addition of Sm-C/IGF 1 to cultured porcine Leydig cells results in increased LH/hCG binding in addition to increased hCG stimulated testosterone production (Benahmed et al. 1987; Kasson & Hsueh, 1987). Sm-C/IGF 1 while having no effect on its own, augments the steroidogenic

action of LH/hCG. Although the mechanism by which Sm-C/IGF 1 exerts its effects on Leydig cell function remains unclear, this does not appear to be due to a Sm-C/IGF 1 related increase in Leydig cell growth (Benahmed et al. 1987).

The results suggest that Sm-C/IGF 1 derived from the Sertoli cell, possibly under the control of GH, augments the actions of LH on Leydig cell androgen biosynthesis, Sertoli cell function and ultimately spermatogenesis.

(iii) Testicular interleukin-1

Interleukin-1 (IL-1), which represents a family of polypeptides, was initially described as a macrophage-derived growth factor (Dinarello, 1985). Infections, immunological reactions, injury, toxins and inflammatory processes trigger IL-1 production. IL-1 is a potent mitogen not only of lymphocytes but also of a number of other cell types including fibroblasts, glial cells, osteoblasts, chondrocytes, synovial cells, mesangial kidney cells and epithelial cells (Dinarello, 1985). Significant amounts of IL-1 have been found in homogenates and cytosol preparations of rat testis (Khan et al. 1987). Testicular macrophages, which are predominantly confined to the interstitium (El-Demiry et al. 1986), appear not to be the major source of testicular IL-1, as interstitial cell homogenates show negligible activity in a murine thymocyte bioassay for IL-1 (Khan et al. 1987). Seminiferous tubules from cryptorchid rats, devoid of germ cells, retain IL-1 activity, pointing to the Sertoli cell as its most likely source. Whether IL-1 produced from these cells is related to the macrophage IL-1 family remains to be clarified.

IL-1 activity has also recently been demonstrated in the human testis (Arver & Söder, 1986). Relapse of childhood acute lymphoblastic

leukaemia often starts in the testis (Nesbit et al. 1980), and it has been suggested that the presence of IL-1 in the testis may provide a favourable environment for the rapid proliferation of lymphocytes in this disease (Khan et al. 1987). Purification of human testicular IL-1 is now underway to investigate whether this mitogenic factor is involved in the prolific germ cell activity during spermatogenesis (O. Söder, personal communication).

1.3.4 Sertoli cell androgen receptors

Androgens are known to be essential for quantitatively normal spermatogenesis to proceed (Steinberger, 1971). Although it is conceivable that androgens could act directly on the germ cells, conflicting evidence exists as to the presence of androgen receptors on these cells (Sanborn et al. 1975; Grootegoed et al. 1977; Wright & Frankel, 1980). The indirect action of androgens on spermatogenesis by influencing Sertoli cell function is well accepted. High affinity receptors specific for testosterone have been demonstrated in the Sertoli cell. The presence of cytoplasmic androgen receptors (Tindall et al. 1977) and the translocation of the hormone-receptor complex to nuclear binding sites (Mulder et al. 1976; Sanborn et al. 1977), chromatin acceptor sites for androgen receptor complexes (Tsai et al. 1977) and promotion of Sertoli cell secretory activity in the presence of testosterone (Louis & Fritz, 1979) indicate the Sertoli cell as an androgen target cell in the rat. Androgen receptors are however also present on peritubular cells (Verhoeven, 1980), and androgen effects on these cells are thought to modulate the function of the Sertoli cell (see 1.6.3).

Androgen receptors have been demonstrated in the seminiferous tubules of the human testis (Winters & Troen, 1984). To date there is

no report of localization of the androgen receptor to specific testicular cell types in man.

1.4 INTERSTITIAL TISSUE

The interstitial tissue, which gives support to the seminiferous tubules, is composed of loose connective tissue interspersed with Leydig cells, macrophages, fibroblasts, vascular and lymphatic vessels and unmyelinated nerve fibres (Fawcett et al. 1973). Copious amounts of interstitial fluid, formed from serum by capillary filtration, circulates in the interstitium (Setchell & Sharpe, 1981). This fluid is a means of communication between the avascular seminiferous tubules and the vascularized interstitial tissue.

The Leydig cell is the most common interstitial cell type. These cells occur singly or in clumps of varying sizes and are often associated with rich plexuses of vascular and lymphatic capillaries (Fawcett et al. 1973).

Macrophages are present in the testicular interstitium of a number of species including man (Kerr & de Kretser, 1981) and the rat (Christensen & Gillim, 1969). Testicular macrophages are observed in close association with the Leydig cells. In the rat, processes which extend from the Leydig cells have been shown to interdigitate and make contact with macrophage surface membranes (Miller et al. 1983). Although these specialized contacts are thought to be associated with the transport of waste materials and Leydig cell metabolites, it has been suggested that testicular macrophages are involved in the endocrine function of the testis (see 1.6.4).

Testicular fibroblasts of the human testis are thought to be Leydig cell precursors (Chemes et al. 1985). Following hCG stimulation these cells have been shown to differentiate into mature Leydig cells capable of secreting testosterone (Chemes et al. 1985).

1.4.1 The Leydig cell

In mammals there are two generations of Leydig cells. Foetal Leydig cells are of mesenchymal origin and are stimulated to develop by placental hCG (Clements et al. 1976). These cells possess receptors for LH, and under the influence of foetal pituitary LH they secrete testosterone (Huhtaniemi et al. 1977) for masculinization of the foetus (Roosen-Runge & Anderson, 1959). During early postnatal life foetal Leydig cells undergo involution. Although the ultimate fate of these cells is unknown, they are thought either to degenerate or to de-differentiate and become fibroblast-like mesenchymal cells which at puberty respond to LH and differentiate into the adult generation of Leydig cells (Chemes et al. 1985). This adult Leydig cell population although derived mainly from interstitial mesenchymal cells are also, to a lesser extent, derived from mitosis of fully differentiated Leydig cells (Mancini et al. 1963). Adult Leydig cells are rich in smooth endoplasmic reticulum, Golgi apparatus and mitochondria, the organelles involved in the synthesis of androgens and other Leydig cell products (Ewing & Zirkin, 1983).

The onset of puberty is associated with altered frequency and amplitude of LHRH secretion from the hypothalamus resulting in the increased secretion of LH from the pituitary. Proliferation and differentiation of adult Leydig cells has until recently been attributed to the prepubertal increase in LH levels (Christensen & Peacock, 1980). In the rat however conflicting evidence exists concerning the prepubertal rise in LH with some reports of increased plasma LH (de Jong & Sharpe, 1977) and others of no increase (Ketelslegers et al. 1978). There is however a much closer relationship between rising FSH levels and the differentiation of the adult generation of Leydig cells in the rat (Swerdloff et al. 1972). Furthermore FSH, acting via the Sertoli

cell, promotes rapid growth of Leydig cells in immature, hypophysectomized rats (Kerr & Sharpe, 1985a,b) (see 1.5.6).

The synthesis and secretion of androgens by the Leydig cell proceeds under the control of the trophic hormones from the anterior pituitary (see 1.5). In addition these cells receive other hormonal and paracrine input. Specific receptors for LHRH (Bourne et al. 1980; Sharpe & Fraser, 1980; Clayton & Catt, 1981; Huhtaniemi et al. 1985), prolactin (Huhtaniemi et al. 1981), insulin-like growth factor (Handelsman et al. 1985), vasopressin (Meiden & Hsueh, 1985), epidermal growth factor (Ascoli, 1981; Welsh & Hsueh, 1982) and catecholamines (Anakawe et al. 1985) have been localized on rat Leydig cell membranes. The physiological role of these potential regulators of Leydig cell function remains to be clarified.

1.4.2 Heterogeneity of adult Leydig cells

A number of studies have demonstrated the heterogeneous nature of the adult Leydig cell population. In the normal human testis, mature Leydig cells which are rich in smooth endoplasmic reticulum, Golgi apparatus and mitochondria are often observed adjacent to smaller cells with poorly defined smooth endoplasmic reticulum and numerous lipid droplets (Kerr & de Kretser, 1981).

The idea that there may be two distinct populations of adult rat Leydig cells came initially from a study by Janszen and co-workers (1976) who found two types of Leydig cells following isolation by density centrifugation. Both cell types produced testosterone basally and showed increased cAMP activity in response to LH. LH-stimulated testosterone production however was observed in the "denser" cells only (Janszen et al. 1976). Similar results have been obtained in a number

of studies (Payne et al. 1980a; Cooke et al. 1981; Kerr et al. 1985a). The lack of responsiveness of the "less dense" Leydig cells is not due to the number or affinity of LH-receptors between the two populations (Payne et al. 1980a). Furthermore pretreatment in vivo with LH increases testosterone production of the "less dense" cells in vitro to levels comparable to the more responsive "dense" cells (Payne et al. 1980b).

Cooke and colleagues (1981) suggested that the heterogeneity of the adult Leydig cell population in the rat may be indicative of a cycle of Leydig cell function. An attempt to correlate this proposed Leydig cell cycle to the spermatogenic cycle was made by Bergh (1982, 1983). He observed that Leydig cells adjacent to the most androgen dependent stages of the spermatogenic cycle (stages VII-VIII) are larger than other Leydig cells in the same testis. Moreover, this effect is abolished in the abdominal testis of unilaterally cryptorchid rats (Bergh & Damber, 1984). It appears therefore that the seminiferous tubules may secrete a factor(s) in a cyclical manner which locally influences Leydig cell morphology and possibly function, and may in part explain the heterogeneous nature of the Leydig cell population.

1.4.3 Testicular androgens

The Leydig cell is a highly differentiated cell whose primary function is the synthesis and secretion of androgens. The main secretory product of the Leydig cell is testosterone which is synthesized via biosynthetic pathways common to other steroid secreting tissues. Cholesterol is synthesized de novo in the Leydig cell smooth endoplasmic reticulum, while the mitochondria exhibit the side-chain cleavage enzymes which convert cholesterol to pregnenolone (van der Vusse et al. 1973). The enzymes involved in the conversion of

pregnenolone to testosterone are localized in the Leydig cell smooth endoplasmic reticulum (Murota et al. 1966). Dehydroepiandrosterone, androstenedione and dihydrotestosterone are also secreted by the Leydig cell (Hammond et al. 1977). Secreted by the testis in small amounts, dihydrotestosterone is derived mainly by reduction of testosterone by 5α-reductase at target sites in reproductive tissues.

1.4.4 Biological actions of androgens

The possibility that androgens might play a crucial role in human spermatogenesis was overlooked for a long time despite evidence for a profound effect of androgens on spermatogenesis in lower species. The reluctance to consider a role for androgens in human spermatogenesis resulted from studies in oligospermic and euspermic men in whom the peripheral administration of testosterone leads to an arrest of spermatogenesis and azoospermia (Heller et al. 1950). Peripheral administration of androgens at low doses is now known to suppress the release of pituitary LH via negative feedback mechanisms (Santen, 1981), and while still being able to support secondary sex characteristics and libido, reduces intratesticular testosterone to levels insufficient to support spermatogenesis (Steinberger, 1971).

1.4.5 Testicular androgen receptors

Testicular androgen activity is mediated via androgen receptors which have been localized in Sertoli cells (Mulder et al. 1976; Sanborn et al. 1977; Tindall et al. 1977). The action of androgens on spermatogenesis is thought to be mediated via Sertoli cell function (see 1.3.4).

Androgen receptors have also been detected in Percoll-purified Leydig cells from adult rats (Gulizia et al. 1983) and immature pigs (Isomaa et al. 1987) and also in an established Leydig cell tumour line

(Nakhla et al. 1984). The function of these receptors, which are specific for androgens, is unknown but it has been suggested that testicular androgens regulate their own production via an ultrashort negative feedback mechanism (Purvis & Hansson, 1978; Adashi & Hsueh, 1981).

1.4.6 Extratesticular androgen action

Circulating testosterone in the foetus is responsible for the development and maintenance of the Wolffian ducts and their derivatives, the epididymides, vasa deferentia, seminal vesicles and ejaculatory ducts (Jost et al. 1973). Differentiation of the male external genitalia and also the prostate are dependent on dihydrotestosterone, the 5α-reduced form of testosterone (Wilson et al. 1983).

In addition to the reproductive tissues, testosterone acts on a large number of body tissues including muscle, kidney, liver, adipose tissue, red blood cells, skin, hair, bone and also the immune and central nervous systems (for review, see Mooradian et al. 1987). Testosterone exerts its effects on these tissues through the androgen receptor both unchanged (e.g. testis, muscle, immune system) and via 5α-reductase (e.g. prostate, bone, skin, hair) and also in tissues with high aromatase activity as an oestrogen via the oestrogen receptor (e.g. adipose tissue, central nervous system).

1.4.7 Other Leydig cell products

(i) Oestradiol

In immature rats the Sertoli cell is the predominant source of oestradiol (Rommerts et al. 1978). These cells lose their aromatase activity during maturation after which they are unable to secrete oestradiol in response to FSH (Rommerts et al. 1982). LH-stimulation of isolated Leydig cells from adult rats increases their aromatase activity

(Valladares & Payne, 1979) suggesting that in the mature animal the Leydig cell is the main source of oestradiol. The human testis also secretes oestradiol, again the interstitial tissue being the site of aromatization (Payne et al. 1976).

In vivo administration of oestradiol to animals, including man, results in a decrease in circulating plasma levels of testosterone and inhibition of the testosterone response to LH stimulation (Jones et al. 1978; Saez et al. 1978). It has been argued that this anti-testicular effect of oestradiol is due to suppression of pituitary LH secretion (de Jong et al. 1975; Chowdhury et al. 1980). However the demonstration of decreased plasma testosterone without any change in the level of LH in oestrogen treated rats (Chowdhury et al. 1974; Sholiton et al. 1975) and also of high affinity cytoplasmic oestrogen receptors on Leydig cells (Brinkman et al. 1972) suggests that oestradiol might act directly on the Leydig cell to suppress testosterone production. Oestradiol exerts its inhibitory effect by blocking 17α-hydroxylase and 17,20 desmolase (Wang et al. 1980). The fact that desensitization of the Leydig cells following large doses of LH/hCG (see 1.5.3) is due partly to inhibition of these enzymes suggests that oestradiol may be a mediator in this process (Catt et al. 1980), but whether this effect is mediated via the oestrogen receptor or via functional modification of the steroidogenic enzymes is not clear. Evidence to suggest that oestrogenic effects are not mediated by the oestrogen receptor came from Damber and co-workers (1983) who were unable to inhibit this oestrogenic effect by tamoxifen, a potent anti-androgen which blocks the oestradiol receptor. In contrast Cigorraga and colleagues (1980) showed that tamoxifen is capable of inhibiting the decrease in responsiveness of the Leydig cells to LH following a large dose of LH (hCG). So, any role of

the Leydig cell oestrogen receptor in the gonadotrophin induced changes in testicular steroidogenesis remains to be clarified.

(ii) Oxytocin and vasopressin

Oxytocin of Leydig cell origin has been detected in the rat testis (Guldenaar & Pickering, 1985). It induces the contraction of the peritubular myoid cells which propel the mature spermatozoa towards the rete testis (Nicholson et al. 1986). Oxytocin has also been reported to inhibit testosterone production by long-term (3 day) cultures of testicular cells from hypophysectomized rats (Adashi et al. 1984). The significance of these results is questionable since the inhibition of testosterone production may be due to the effect of oxytocin on the myoid cells present in the testicular cultures (Nicholson et al. 1986). In short-term (24 h) culture of purified rat Leydig cells, no effect of oxytocin on testosterone production is observed (Sharpe & Cooper, 1987). Similarly intratesticular injection of oxytocin has no effect on interstitial fluid testosterone content (Sharpe & Cooper, 1987). It seems unlikely, therefore, that oxytocin is an autocrine regulator of testosterone production in the rat testis.

Vasopressin is also present in the rat testis although its source is unknown (Kasson et al. 1985; Kasson & Hsueh, 1986). Vasopressin has been reported to be a potent inhibitor of hCG-stimulated testosterone production by testicular cell cultures (Meidan et al. 1985). Data from long-term (2-3 day) cultures of purified Leydig cells show that in the presence of supraphysiological levels of hCG, vasopressin inhibits testosterone production (Sharpe & Cooper, 1987). During short-term (5 h) culture vasopressin stimulates basal testosterone production and has no effect on hCG-stimulated testosterone production (Sharpe & Cooper, 1987). The lack of an in vivo effect of vasopressin raises doubts as to

the significance of this peptide in the paracrine control of Leydig cell function (Sharpe & Cooper, 1987).

(iii) Pro-opiomelanocortin-derived peptides

Pro-opiomelanocortin (POMC)-derived peptides have been detected in the male reproductive tract of a number of species with β-endorphin, α-melanocyte-stimulating hormone and adrenocorticotrophic-like factors present specifically in Leydig cell cytoplasm (Tsong et al. 1982a). A growing number of studies suggest that POMC-derived products may have some local intratesticular effects (1.6.1 (viii)).

1.5 ENDOCRINE CONTROL OF THE TESTIS

The hormonal control of spermatogenesis involves the interaction of gonadal steroid hormones and the pituitary gonadotrophic hormones LH and FSH on specific cells of the testis. The involvement of the pituitary gonadotrophins in the normal functioning of the gonads was realized more than 50 years ago, but it is only in the last 15 years, with advances in the isolation and detection of both LH and FSH, that their basic mechanisms of actions have been elucidated.

1.5.1 Luteinizing hormone and the Leydig cell

The first demonstration of LH action on the Leydig cell was in 1936 when it was shown to maintain normal Leydig cell morphology in hypophysectomized rats (Greep et al. 1936). It was not until over 30 years later that binding of LH was shown to be restricted to the testicular interstitial cells of the rat (Mancini et al. 1967; de Kretser et al. 1971). Subsequent studies have demonstrated receptors for LH on the cell surface of Leydig cells in human foetal testes (Huhtaniemi et al. 1977) and adult human testes (Hsu et al. 1978).

LH is thought to exert its action on the activity of the enzymes involved in the synthesis of testosterone (Hall, 1970; Ewing & Brown, 1977). The endpoint of LH action appears to be the regulation of mitochondrial cholesterol side chain cleavage involving the cytochrome P450 system (Menon et al. 1967; van der Vusse et al. 1975). Furthermore, LH is thought to be involved in the conversion of cholesterol ester to free cholesterol (Moyle et al. 1973) and in the transport of the free cholesterol to the mitochondria (Hall et al. 1979).

1.5.2 Mechanism of action

The mechanism of LH action on the Leydig cell has been studied

extensively. Stimulation of androgen synthesis by LH is brought about mainly by changes in the production of cAMP, the subsequent activation of protein kinase and phosphorylation of specific proteins leading to the increase in steroidogenic enzyme activity (Purvis et al. 1981; Dufau et al. 1984).

Changes in endogenous levels of cAMP in the testis do not however completely support the idea that cAMP mediates the effects of LH on steroidogenesis (Cooke et al. 1977). Recent data indicate that a number of transducing systems including cAMP, protein kinase-C and fluxes of intracellular concentrations of calcium are required for the stimulatory action of LH on androgen production (Hall et al. 1981; Lin, 1985; Themmen et al. 1985).

LHRH has also been shown to stimulate androgen production without apparent changes in cAMP production (Sullivan & Cooke, 1984). The mechanism of action of LHRH on Leydig cell steroidogenesis is not clear but LHRH has been shown to increase intracellular calcium and to stimulate incorporation of labelled phosphate into phosphatidyl inositol (Molcho et al. 1984).

1.5.3 Regulation of LH receptors

The ability of peptide hormones to regulate the concentrations of their specific receptor sites in endocrine target cells has been demonstrated in numerous tissues. Initially receptors for insulin and growth hormone were shown to be inversely proportional to the concentrations of the homologous hormone in the peripheral circulation (Roth et al. 1975).

LH (hCG) negatively regulates the number of its own receptors on the Leydig cell plasma membrane. LH receptor number is decreased by exogenous gonadotrophins (Hsueh et al. 1977; Sharpe, 1976) and also by

elevation of endogenous LH levels following treatment with LHRH-A (Catt et al. 1979). A single supraphysiological dose of LH (hCG) results in a rapid (within 3-6 h) increase in receptor number (Hsueh et al. 1977; Sharpe, 1980; Huhtaniemi et al. 1981). Following the, as yet unexplained, initial transient up-regulation, high doses of LH induce a prominent reduction or down-regulation in LH receptor numbers, and an impaired responsiveness of the Leydig cell to subsequent hormonal stimulation (desensitization) (Nozu et al. 1981 a,b, 1982). Desensitization is the result of uncoupling of the receptor from adenylate cyclase and the internalization of the hormone-receptor complex. In the endosomal vesicles the hormone is dissociated from the receptor and degraded while the receptor is returned to the cell surface (Habberfield et al. 1986). This internalization and recycling process appears to be important in maintaining the ability of the Leydig cell to bind LH.

1.5.4 Leydig cell response to LH

Administration of LH (hCG) to men results in a biphasic response in which there is a modest (20-60%) acute increase in plasma testosterone levels within 6 h, followed by a 2- to 3-fold rise 2-3 days later (Saez & Forest, 1979; Smals et al. 1979; Padron et al. 1980; Martikainen et al. 1980; Nankin et al. 1980). A similar biphasic response is observed in the rat (Hsueh et al. 1976; Sharpe, 1976).

The mechanism of this biphasic steroid response is not completely understood although desensitization and down regulation of the LH receptor is thought to be involved (Padron et al. 1980). The secondary rise in testosterone production, the magnitude of which is determined by the dose of LH (hCG) administered, has been attributed to trophic effects of LH (hCG) on cytoplasmic organelles and possibly Leydig cell

number. Although data on the effects of a single injection of LH (hCG) is limited, chronic administration of hCG in man has been shown to result in an increase in Leydig cell smooth endoplasmic reticulum, mitochondria and Golgi apparatus, reflecting an increase in the capacity of these cells to secrete testosterone (de Kretser, 1967). Similarly in the rat, chronic stimulation with LH (hCG) increases cell size and number (Christensen & Peacock, 1980; Nussdorfer et al. 1980).

1.5.5 Follicle-stimulating hormone

The seminiferous epithelium is the principal site of action of FSH in the testis (Means & Vaitukaitis, 1972). Autoradiographic studies have shown binding of FSH primarily to the basal aspect of the Sertoli cell and also to the spermatogonial membrane (Orth & Christensen, 1977, 1978). Binding of FSH to receptors located on the plasma membrane of the Sertoli cell stimulates adenylate cyclase (Fakunding et al. 1975), activates cAMP-dependent protein kinase (Fakunding & Means, 1977) and stimulates RNA and protein synthesis (Means et al. 1976). In the rat, ABP has been used extensively as a marker for FSH action on the Sertoli cell (Ritzén et al. 1981).

The responsiveness of the Sertoli cells to FSH with respect to accumulation of cAMP declines significantly during sexual maturation (Steinberger et al. 1977). This diminished responsiveness of the Sertoli cells to FSH with age is not due to impaired hormone binding, as the binding of FSH actually increases during this period (Steinberger et al. 1977). The loss of cellular response to FSH is due to decreased production of cAMP secondary to increased phosphodiesterase activity. This lack of response can be reversed by the phosphodiesterase inhibitor 1-methyl-3-isobutyl-xanthine (MIX) (Fakunding et al. 1975; Means et al. 1976). The FSH-dependent functions of the Sertoli cell in the immature

animal are regulated by testosterone in the mature animal (Hansson et al. 1976).

The precise role of FSH became less clear when it was shown that in adult animals testosterone alone, when administered in high doses, is capable of maintaining or restoring complete spermatogenesis in the absence of FSH (Boccabella 1963; Buhl et al. 1982; Marshall et al. 1983). In mature hypophysectomized rats, in the presence of normal levels of testosterone, failure to restore or maintain normal germ cell number implies the need for other factors for quantitatively normal spermatogenesis (Chowdhury, 1979; Stevens & Steinberger, 1983). Similarly in hypogonadotrophic men, and men made hypogonadotrophic by the administration of exogenous testosterone, both FSH and LH are required for quantitatively normal spermatogenesis, although either FSH or LH alone is sufficient to stimulate spermatogenesis in the presence of testosterone (Bremner et al. 1984). These findings suggest that in addition to testosterone, FSH is also required for quantitatively normal spermatogenesis.

Although the adult seminiferous tubules have been shown to respond to FSH only minimally (Ritzén et al. 1981) the actions of FSH appear to be dictated locally by stage-dependent changes in the response to FSH (Parvinen et al. 1980). Significant variation occurs in both the binding of FSH and in the FSH-stimulated cAMP production depending on the stage of the cycle of the seminiferous epithelium. The number of FSH receptors and the amount of cAMP produced in response to FSH is highest at stage I, and lowest during stages VII-VIII (Parvinen et al. 1980).

1.5.6 FSH and the Leydig cell

In the testis, although receptors for FSH are found only on the

Sertoli cells and the spermatogonia (Means et al. 1976; Orth & Christensen, 1977), in vivo studies have suggested that FSH also regulates Leydig cell steroidogenesis. This information derives from studies in which administration of FSH to immature hypophysectomized rats results in increased Leydig cell LH receptor number (Odell et al. 1973; van Beurden et al. 1976; Chen et al. 1977) and increased capacity of the Leydig cell to bind LH and secrete testosterone (Odell & Swerdloff, 1975; Chen et al. 1976, 1977; Selin & Moger, 1977). Morphometric studies by Kerr and Sharpe (1985a, 1985b) have shown that these effects are due to FSH-induced differentiation of the adult population of Leydig cells.

1.5.7 Mode of action

The regulatory effect of FSH on Leydig cell steroidogenesis is not exerted directly on the Leydig cell but is mediated through the Sertoli cell. In Sertoli-Leydig cell co-cultures FSH enhances Leydig cell hCG binding, hCG-stimulated testosterone production and the development of organelles involved in steroidogenesis (Benahmed et al. 1986). Leydig cells cultured in conditioned medium from FSH-stimulated Sertoli cells in culture as compared to conditioned medium from non-stimulated Sertoli cells show an increase in their steroidogenic capacity (Benahmed et al. 1986). Since in vivo the Sertoli and Leydig cells are in different compartments of the testis and unable to communicate through cell contact, these results suggest that FSH regulates Leydig cell function by means of FSH-stimulated Sertoli cell factors (see 1.6.1).

1.5.8 Control of FSH secretion

In many physiological and pathological conditions the levels of the gonadotrophins LH and FSH change independently. In men suffering from germinal cell failure, without damage of the Leydig cells, the levels of

plasma FSH are often elevated whereas plasma LH levels are frequently normal (Franchimont et al. 1975). Since the synthesis of both LH and FSH is controlled by hypothalamic LHRH, there must be some factor which can regulate the release of FSH at the pituitary level.

Regulation of FSH secretion via a negative feedback mechanism by a water-soluble substance from the testis was postulated by McCullagh (1932). This substance he called inhibin. It has since been confirmed that water-soluble testicular extracts suppress FSH secretion without altering LH release in male animals (Baker et al. 1976; Keogh et al. 1976; Eddie et al. 1978).

1.5.9 Source of inhibin

Direct evidence that inhibin is produced by the Sertoli cell in the male came from in vitro studies in which medium from cultured rat Sertoli cells was shown to selectively suppress FSH secretion in cultures of pituitary cells (Steinberger & Steinberger, 1976; Bicsak et al. 1987).

Inhibin is present in abundance in seminal plasma in a number of species, including man (Franchimont et al. 1979). Seminal plasma inhibin is of prostatic origin (Becsak et al. 1984) and reports as to whether it is capable of suppressing FSH release from pituitary cells in culture are conflicting (Ramasharma et al. 1984; Sheth et al. 1984; de Jong & Robertson, 1985; Liu et al. 1985). The action of prostatic inhibin is at present unknown.

The presence of inhibin in rete testis fluid (Setchell & Jacks, 1974; Baker et al. 1976) may indicate a means by which this peptide gains entry to the peripheral circulation in order to mediate its effects on the anterior pituitary (Baker et al. 1976). Little is currently known about the mechanism(s) or route(s) by which inhibin is

secreted from the testis into the peripheral circulation although in sheep, the high concentration of inhibin in the ovarian vein (Tsonis et al. 1986) suggests that a vascular route may be important.

1.5.10 Mode of action

Since the structure of inhibin was defined less than 2 years ago (Mason et al. 1985), no report has yet appeared on the localization of receptors for inhibin. So although the anterior pituitary is generally accepted to be the site of inhibin action, direct action of inhibin on the hypothalamus has also been reported (Lumpkin et al. 1981).

Using a 32 kDa inhibin from porcine follicular fluid, Fukuda and co-workers (1987) have confirmed that inhibin acts to suppress FSH, but not LH or any other pituitary hormone. Suppression of basal FSH release is thought to be a consequence of reduced intracellular stores of FSH (Scott & Burger, 1981b; Fukuda et al. 1987). Cycloheximide, a potent inhibitor of protein synthesis, is capable of mimicking the actions of inhibin, suggesting that the difference in response of both LH and FSH to inhibin is due to differences between these hormones in susceptibility to biosynthetic inhibition (Fukuda et al. 1987). LHRH-stimulated release of both FSH and LH is suppressed by inhibin, and cycloheximide, indicating that inhibin acts on the gonadotrophs by inhibiting protein biosynthesis (Fukuda et al. 1987).

1.5.11 Testicular steroids and FSH secretion

Testicular steroids are known to be involved in the regulation of LH secretion (Santen, 1981), but their role in the secretion of FSH is not clearly defined. Testosterone in pharmacological doses has been shown to suppress FSH (Baker et al. 1976) whereas other androgens such as dihydrotestosterone and 17α-hydroxyprogesterone have no effect (Stewart-Bentley et al. 1974). Similarly oestrogens have been shown to

suppress FSH (Baker et al. 1976) but are required in doses which are large enough to produce gynecomastia in castrate men (Walsh et al. 1973). In rats both oestrone and oestradiol have been shown to suppress FSH (Swerdloff et al. 1973).

1.6 PARACRINE CONTROL OF THE TESTIS

The importance of the gonadotrophic hormones LH and FSH in the development and maintenance of testicular function cannot be over-emphasized. The gonadotrophins provide the basic stimuli on which normal testicular function depends. However, it is difficult to imagine that the maintenance of a highly complex structure such as the testis could be achieved by the gonadotrophins alone. Indeed the way in which the testis responds to these stimuli appears to be mediated by gonadal factors. The testis is composed of various somatic and germinal cells which interact in response to gonadotrophic stimulation by producing paracrine factors. These factors mediate the actions of the gonadotrophins according to local testicular requirements and, as such, co-ordinate the functions of the various testicular cell types.

1.6.1 Sertoli cell-Leydig cell interactions

The Sertoli and Leydig cells were for a long time considered to be completely independent cell types. Evidence of a close interdependence of the two cell types is now accumulating.

The concept that the Leydig cells, by providing high local concentrations of testosterone, regulate testicular function via the Sertoli cells is well-established (Steinberger, 1971; Ritzén et al. 1981; Sanborn et al. 1984). The maintenance of quantitatively normal spermatogenesis is completely dependent on adequate supplies of testosterone (Desjardins et al. 1973; Nieschlag et al. 1973), while the absolute amount of this hormone required remains unknown.

Results from several studies indicate the cyclical variation in the requirements of the Sertoli cell for testosterone (Dym et al. 1977; Russell & Clermont, 1977; Russell et al. 1981). In all cases gonadotrophic deprivation leads to the disappearance of post-meiotic

germ cells in stages VII and VIII in the rat. Recent studies involving the administration of the Leydig cell toxin ethane dimethanesulphonate (EDS) to adult rats have demonstrated disruption of post-meiotic germ cells in stages VII and VIII as a result of short-term testosterone deprivation (Kerr et al. 1985b, 1986; Bartlett et al. 1986). In view of this stage-specific dependence of spermatogenesis on testosterone, it is difficult to perceive how the testosterone supply could be regulated sufficiently by the episodic release of pituitary LH to meet the demands of individual stages of germ cell development. There now exists considerable evidence to suggest that tubular factors, in response to gonadotrophic stimulation, influence Leydig cell testosterone production.

(i) Evidence for paracrine regulators of Leydig cell function

Evidence for communication between the interstitial and tubular compartments of the testis came initially from a number of studies which demonstrated that tubular damage, as a result of anti-androgens (Aoki & Fawcett, 1978), cryptorchidism (Kerr et al. 1979a) vitamin A deficiency, hydroxyurea treatment, foetal irradiation (Rich et al. 1979) or efferent duct ligation (Risbridger et al. 1981), secondarily effects Leydig cell function. Aoki & Fawcett (1978) observed the involution of seminiferous tubules around intratesticular implants of the anti-androgen cyproterone acetate, accompanied by hypertrophy and hyperplasia of Leydig cells in the neighbouring interstitial tissue. These changes were not accompanied by significant elevation of gonadotrophin levels and did not occur in tubules distant to the implants or in the contralateral testis, suggesting the presence of some local, paracrine regulator of Leydig cell function.

(ii) Follicle-stimulating hormone

A growing number of studies have suggested that FSH, in addition to its stimulatory influence on the Sertoli cell, may also exert effects on Leydig cell steroidogenic function. FSH pre-treatment of hypophysectomized rats results in an increase in LH-stimulated testosterone secretion by the Leydig cells both in vivo (Odell & Swerdloff, 1975; Selin & Moger, 1977) and in vitro (Chen et al. 1976; van Beurden et al. 1976; Chen et al. 1977). In addition, FSH appears to stimulate LH receptor number (Chen et al. 1977). As binding of FSH to Sertoli cells (Orth & Christensen, 1977) but not to Leydig cells (de Kretser et al. 1971; Means & Vaitukaitis, 1972) is well-established, these observations suggest that the regulatory effect of FSH on Leydig cell steroidogenesis may result from an interaction between the Sertoli and Leydig cells via the production of a mediator by the Sertoli cell.

(iii) Oestradiol

Oestradiol was suggested as a possible local feedback messenger between the Sertoli and Leydig cells (Aoki & Fawcett, 1978). However, Sertoli cells are now known to lose their aromatase activity during maturation (Rommerts et al. 1982), after which they are unable to secrete oestradiol, effectively ruling out the possibility of oestradiol as a potential messenger between the tubular and interstitial cells.

(iv) Testicular-LHRH

The possibility that the regulatory effect between the seminiferous tubules and the Leydig cells is mediated, at least in part, by testicular-LHRH has been suggested as a result of a number of observations.

An LHRH-like peptide of testicular origin has been reported in the rat (Dutlow & Millar, 1981; Sharpe et al. 1981, 1982a; Bhasin et al. 1983; Nagendranath et al. 1983; Hedger et al. 1985). Reports of

LHRH-like activity in spent media from Sertoli cell cultures indicates the Sertoli cell as the site of its production (de Jong et al. 1979; Sharpe et al. 1981, 1982a; Nagendranath et al. 1983).

LHRH was found to have no direct effects on the Sertoli cell (Gore-Langton et al. 1981) in keeping with the finding that specific LHRH binding sites are present in Leydig but not Sertoli cells (Bourne et al. 1980; Sharpe & Fraser, 1980; Clayton & Catt, 1981; Huhtaniemi et al. 1985). These results suggest that the Sertoli cells produce an LHRH-like peptide in response to FSH which acts on the Leydig cells to locally regulate their function.

(v) Physiological role of testicular-LHRH

Most of the information available on the physiological effects of testicular-LHRH has been obtained from the use of agonistic analogues of hypothalamic LHRH. The suitability of these agonists, in the absence of purified testicular-LHRH, has been proved by the demonstration that LHRH-agonists prevent the gonadal actions of native LHRH in a dose-dependent manner (Hsueh et al. 1981).

Initial studies with long-term (3 or more days) exposure to LHRH-agonists reported a potent direct inhibitory effect on steroidogenesis both in vivo and in vitro (Bambino et al. 1980; Hsueh et al. 1981; Hsueh & Jones, 1981). Short-term incubations (of up to 24 hours) with LHRH agonist stimulates Leydig cell testosterone production. This stimulatory effect has been demonstrated in vivo, in both normal (Sharpe & Rommerts, 1983; Sharpe et al. 1983) and hypophysectomized rats (Sharpe et al. 1982; Sharpe & Harmar, 1983), and in vitro (Hunter et al. 1982; Sharpe & Cooper, 1982a, 1982b).

(vi) Mode of Action

The physiological role for testicular-LHRH seems to be as a

stimulatory regulator of intratesticular levels of testosterone. The method of action of testicular-LHRH is thought to be mediated by its effects on testicular blood flow and capillary permeability (Sharpe, 1984a). The action of LHRH is to stimulate testicular blood flow (Damber et al. 1984) and to reduce capillary permeability (Sharpe, 1984a). Receptor binding sites for testicular-LHRH are present only on the Leydig cells therefore LHRH stimulates the Leydig cells which in turn are thought to secrete a vasoactive factor(s), possibly β-endorphin which acts upon the testicular capillaries (Sharpe 1984a). By controlling capilliary permeability, testicular LHRH is in effect controlling the volume of interstitial fluid and ultimately the intratesticular level of testosterone. In addition, by controlling interstitial fluid volume, testicular-LHRH is directly involved in the passage of other hormones, in particular LH, into the intratesticular environment (Sharpe, 1984a).

So although testicular-LHRH clearly modulates testosterone production by the rat Leydig cells, whether it has a localized effect on the Leydig cells adjacent to particular androgen-dependent states (VII and VIII) of the cycle of the seminiferous epithelium has not been demonstrated. Immunohistochemistry has shown that tubular content of LHRH varies in different cross sections (Paull et al. 1981). These authors did not however relate LHRH content with the stage of the spermatogenic cycle. A recently reported Sertoli cell LHRH-peptidase may be involved in the modulation of testicular-LHRH secretion in response to local environmental requirements at different stages of the cycle (Hedger et al. 1986). Secretion of this peptidase into the interstitial fluid may be a means by which the Sertoli cell can confine testicular-LHRH to a site of action by preventing the accumulation of this peptide and its subsequent diffusion through the interstitial fluid.

(vii) Sertoli cell/interstitial fluid stimulatory factor(s)

Substantial evidence exists for the presence of a Sertoli cell factor, which is not testicular-LHRH, but which has local stimulatory effects within the testis (Grotjan & Heindel, 1982; Benahmed et al. 1984; Parvinen et al. 1984; Sharpe & Cooper, 1984; Janecki et al. 1985; Verhoeven & Cailleau, 1985). Seminiferous tubules at stages VII to VIII incubated with rat Leydig cells enhance testosterone production by the Leydig cells, while LHRH-antagonist is not capable of diminishing the seminiferous tubule effect (Parvinen et al. 1984).

Verhoeven & Cailleau (1985) reported a stimulatory factor from immature Sertoli cell culture media which was not inhibited by LHRH-antagonist. Maximally effective levels of this factor plus LHRH-agonist had additional effects, suggesting that they act via different receptor systems. The ability of both FSH and dibutyryl cAMP to stimulate the levels of this factor in spent media from immature Sertoli cell cultures undoubtedly points to the Sertoli cell as the source (Verhoeven & Cailleau, 1985).

Although the physiological significance of these in vitro effects are questionable, their potential importance is substantiated by the comparable action of a factor produced in vivo. Testicular interstitial fluid (IF) collected from normal adult rats is capable of enhancing Leydig cell testosterone production by 6-fold or more in response to maximally stimulating levels of hCG (Sharpe & Cooper, 1984; Sharpe, 1985).

This effect is not due to IF gonadotrophins as IF-factor activity is also present in IF from hypophysectomized rats (Sharpe & Cooper, 1984). In addition, antiserum to LH fails to diminish the stimulatory effect of the IF-factor (Sharpe & Cooper, 1984). The most important

determinant of the magnitude of the stimulatory effect of IF-factor is the ambient level of intratesticular testosterone (Sharpe & Cooper, 1984; Sharpe, 1985). Testicular damage which reduces intratesticular testosterone, for example cryptorchidism, results in increased stimulatory effects of the IF-factor (Sharpe & Cooper, 1984; Sharpe et al. 1986b). Testicular damage is often accompanied by changes in IF volume which may give erroneous estimates of IF-factor activity. IF volume is decreased in cryptorchid rats, however IF-factor activity remains elevated even after IF volume has returned to normal levels (Sharpe et al. 1986b). FSH is also elevated as a result of testicular damage, but this does not explain the increased level of IF-factor activity as in rats made unilaterally cryptorchid increased activity of the IF-factor is seen in the abdominal testis only and not in the contralateral scrotal testis (Sharpe et al. 1986b).

The Leydig cell toxin EDS provides a means by which the effect of intratesticular testosterone on IF-factor activity can be investigated (Sharpe et al. 1986a). Short-term deprivation of testosterone following Leydig cell destruction leads to an increase in the activity of the IF-factor. However at 3-7 weeks post EDS treatment when intratesticular levels of testosterone are back to normal, IF-factor activity is still elevated. This has been attributed by Sharpe et al. (1986) to disruption of spermatogenesis which still persists to some extent even after normal testosterone levels are restored (Bartlett et al. 1986). In EDS-treated rats intratesticular levels of testosterone are back to normal at around 3 weeks post-EDS, however Leydig cell number is still greatly reduced (Kerr et al. 1985b). During this period of reduced Leydig cell number the increased activity of the IF-factor may well be the mechanism by which intratesticular testosterone levels are returned

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to normal thus permitting restoration of spermatogenesis (Sharpe et al. 1986a).

Any communication between the Sertoli and Leydig cells must take place via the testicular interstitial fluid, and as such, both the interstitial fluid-factor and the Sertoli cell derived factor may be identical, or possibly members of a family of related peptides.

(viii) Testicular opiates

In addition to testosterone, other Leydig cell products are thought to have local effects on the seminiferous epithelium. Immunoreactive β-endorphin, a peptide which is derived from the processing of a precursor molecule pro-opiomelanocortin (POMC) (Krieger et al. 1980), has been reported in human semen (Sharp & Pekary, 1981) and in rat testis extracts (Sharp et al. 1980; Tsong et al. 1982a). Immunoreactive β-endorphin has been identified in the Leydig cells, but not in the Sertoli or germ cells, of a number of species (Tsong et al. 1982b). In hypophysectomized rats, in which plasma levels of β-endorphin are undetectable, this POMC-derived peptide is still detectable in the Leydig cells (Tsong et al. 1982a). In addition, POMC mRNA has been demonstrated in rat Leydig cells (Chen et al. 1984; Pintar et al. 1984). Opioid receptors have been identified in mature and immature rat Sertoli cells (Fabbri et al. 1985). These results raise the possibility that POMC is synthesized in the Leydig cells and that POMC-derived products may have some local intratesticular effects.

Several POMC-derived peptides, in addition to β-endorphin, such as α-endorphin, γ-endorphin, adrenocorticotrophin (ACTH) and α melanocyte stimulating hormone (α MSH) have been found in the testis (Margioris et al. 1983). α MSH is synthesized by the Leydig cells and has been shown to directly stimulate cAMP production by the Sertoli cells and to

modulate the response of these cells to FSH (Boitani et al. 1986). This action of α-MSH on the Sertoli cell may represent a paracrine regulatory mechanism within the testis.

β-endorphin-like immunoreactivity and ACTH are present in rat interstitial fluid, in concentrations which far exceed those of plasma, confirming local synthesis of these peptides (Valenca & Negro-Vilar, 1986). Synthesis of β-endorphin is modulated according to the local hormonal milieu. In hypophysectomized rats, administration of hCG results in an increased secretion of β-endorphin-like immunoreactivity into the interstitial fluid whereas LHRH-A appears to have inhibitory effects on the interstitial fluid levels of β-endorphin-like immunoreactivity (Valenca & Negro-Vilar, 1986). However in the short-term, administration of LHRH-A to intact animals results in elevated interstitial levels of β-endorphin-like immunoreactivity concomitant with decreased interstitial fluid volume (Valenca & Negro-Vilar, 1986). These authors suggest that in response to LH, testicular opiates may regulate interstitial fluid production, and in doing so, effectively alter intratesticular hormone concentrations.

Evidence of a paracrine effect of β-endorphin has been observed in immature rats. Administration of a β-endorphin antagonsit, naloxone, results in increased Sertoli cell division, testicular hypertrophy and increased ABP production, suggesting that β-endorphin might inhibit Sertoli cell function in immature rats (Gerendai et al. 1983). Adult Sertoli cell function, however, as indicated by ABP production is not affected by intratesticular treatment with opioid antagonists (Gerendai et al. 1984). In normal rats, naloxone administration results in decreased serum testosterone levels suggesting that endogenous testicular opiates may regulate testosterone production. It is not

clear whether these opiates regulate Leydig cell testosterone production directly, by an autocrine mechanism, or indirectly through another cell type, by a paracrine mechanism.

Thus there is evidence to suggest that POMC-derived peptides are present in the male reproductive tract, with β-endorphin and ACTH-like factors present specifically in Leydig cell cytoplasm. These peptides have been shown to exert local inhibitory effects on the Sertoli cells. However, little is known of the nature of receptors for POMC-derived peptides on Sertoli cells. Whether these peptides act directly on the Sertoli cells or via an intermediate factor(s) awaits identification and characterization of their receptors on the Sertoli cell.

1.6.2 Sertoli cell-germ cell interactions

The epithelium of the seminiferous tubule is unique in its organization consisting of a permanent population of Sertoli cells, and interspersed among these the germ cells in a continuously evolving state. The differentiation of the developing germ cells takes place in a highly organized manner at precise, unvarying time intervals. Each particular stage of the spermatogenic cycle appears to have different functional requirements. The Sertoli cell provides the specific milieu which is required by the germ cells in their different stages of development.

(i) Functional cycle of the Sertoli cell

Several observations suggest the existence of a functional cycle of the Sertoli cell, such that neighbouring Sertoli cells may be performing different functions depending on their germ cell association, despite being exposed to the same gonadotrophic stimulation. Sertoli cells undergo morphological and histochemical changes during the cycle of the seminiferous epithelium. Variations in cell volume (Cavicchia & Dym,

1977), nuclear morphology (Le Blond & Clermont, 1952), lipid morphology and position (Kerr & de Kretser, 1975) and some enzyme activities (Parvinen & Vanha-Pertulla, 1972; Hilscher et al. 1979) related to the cycle of the seminiferous epithelium have been reported.

More recently in vitro studies of stage-specific tubule segments have also demonstrated cyclical variations in Sertoli cell function (Parvinen, 1982). The number of Sertoli cell FSH-receptors varies during the cycle with preferential binding of FSH occurring between stages XII and V in the rat (Parvinen et al. 1980). The ability of FSH to stimulate cAMP production in the presence of a phosphodiesterase inhibitor, also varies in a cyclical manner with cAMP production being lowest during stages VII - VIII reflecting maximal activity of the phosphodiesterase inhibitor (Parvinen et al. 1980). This pattern of FSH induced cAMP production is thought to relate to the stage of spermatid development, indicated by the activity of the spermatid-specific enzymes protein carboxyl-methylase and manganese-dependent adenylyl cyclase (Cusan et al. 1981; Gordeladze et al. 1982). These enzymes are involved in the maturation of step 12 to 17 spermatids during stages XII to V in the rat (Cusan et al. 1981). Their activity is closely related to the rise in FSH-induced cAMP production which is thought to be important for spermatid maturation (Parvinen, 1982). These spermatid specific enzyme activities fall off in parallel with decreased production of cAMP prior to spermiation (Gravis, 1978). These data suggest that the action of FSH on the Sertoli cell is dictated locally by the stage of the cycle.

In addition, preferential action of testosterone occurs in the rat during stages VII to VIII (Parvinen, 1982). Decreased availability of testosterone leads to the degeneration of stage VIII spermatocytes and

spermatids. Androgen dependent germ cells at stages VII and VIII of the cycle are thought to communicate with adjacent Leydig cells to maximize the availability of testosterone (see 1.6.1).

The complex series of changes by which spermatogonia are transformed into spermatozoa can be divided into three phases, spermatocytosis, meiosis and spermiogenesis. During the first phase, spermatocytosis, the spermatogonia proliferate by mitotic division to replace themselves and to produce primary spermatocytes. These mitotic divisions occur at specific stages of the spermatogenic cycle, indicating the presence of a mitotic control mechanism. Likewise in the second phase, meiosis, the two successive reduction divisions which produce the secondary spermatocytes and the spermatids, are strictly limited to particular stages. The Sertoli cell products, seminiferous growth factor (SGF), meiosis-inducing substance (MIS) and meiosis-preventing substance (MPS) are thought to control germ cell mitotic and meiotic divisions (see 1.3.3 (i)). Other Sertoli cell products, somatomedin-C/IGF1-like factor, testicular interleukin-1 and possibly inhibin are also potential regulators of germ cell growth (see 1.3.3).

The culmination of the third phase of spermiogenesis, spermiation, results in the release of the mature spermatozoa into the tubular lumen. This event occurs concomitantly with the translocation of the preleptotene spermatocytes from the basal to the adluminal aspects of the seminiferous tubules. Both spermiation and translocation coincide with increased secretion of plasminogen activator from the Sertoli cell as described above (see 1.3.2 (iv)).

(ii) Germ cell effects

As spermatogenesis is dependent on an ordered sequence of changes

in Sertoli cell function, cell-to-cell contacts between the Sertoli cells and the germ cells could modulate the responsiveness of the Sertoli cell to FSH. In other words, do germ cells exert paracrine effects on the Sertoli cell? Germ cell removal from Sertoli cell enriched cultures induces a reduction of FSH-dependent ABP secretion, while replacing the removed germ cells promotes ABP secretion (Galdieri et al. 1984). Furthermore, only germ cells at certain stages of differentiation have this effect. Pachytene spermatocytes co-cultured with Sertoli cells show surface junctional structures which are thought to allow metabolic communication between the two cell types (Ziparo et al. 1980, 1982). Round and elongate spermatids, which do not form these junctional structures in vitro, have no effect on ABP production when co-cultured with Sertoli cells (Galdieri et al. 1984). Following sequential depletion of spermatogonia, spermatocytes and spermatids in rats by in vivo chronic gamma irradiation, there is a high degree of correlation between elongate spermatids and Sertoli cell activity as determined by ABP production (Pinon-Lataillade et al. 1986). The responsiveness of the Sertoli cell to FSH appears therefore to be modulated, at least in part, by their surface interactions with germ cells.

Depletion of pachytene spermatocytes by X-irradiation results in a reduction of plasminogen activator secretion during stages VII to VIII (Vihko et al. 1984; Parvinen et al. 1986). Moreover, plasminogen activator secretion by the Sertoli cell is stimulated by retinoic acid during stage VII (Vihko et al. 1987), but the receptor for retinoic acid is localized in the germ cells (Porter et al. 1985). The effects of retinoic acid therefore seem to be indirect and mediated by the germ cells.

Isolated Sertoli cells in culture respond to stimulation by FSH by exhibiting changes in cell shape which are thought to indicate inherent cyclicity of Sertoli cell function (Spruill et al. 1981). However the evidence presented here seems to suggest that the differentiation of germ cells is not entirely influenced by the metabolic responses of the Sertoli cells, but that it is a two-way process with Sertoli cell function being modulated by the germ cells.

1.6.3 Sertoli-peritubular-Leydig interactions

External to the basement membrane of each seminiferous tubule lies the peritubular tissue (Ross & Long, 1966; Bustos-Obregon & Holstein, 1973). This consists of approximately 3-5 alternating layers of collagen fibres and myoid cells separated by thin layers of microfibrils and basement membrane-like material, and is devoid of any blood vessels or nerves (de Kretser et al. 1975). Located between the seminiferous epithelium and the interstitium , the peritubular tissue is strategically placed so as to exert an influence on Sertoli cell and Leydig cell function.

The development of the highly organised peritubular tissue has been attributed to stimulation by the gonadotrophins. Patients with hypogonadotrophic hypogonadism show poorly organized peritubular tissue which subsequent to gonadotrophin therapy takes on a mature appearance (de Kretser & Burger, 1972). However neither LH nor FSH bind to isolated peritubular cells (Steinberger et al. 1975; Fritz et al. 1975), and it is likely that the effect of the gonadotrophins is exerted via androgen production by the Leydig cells (Sar et al. 1975; Skinner & Fritz, 1985a,b). This androgen mediated effect is supported by studies both in vivo and in vitro. Bressler & Ross (1972) observed myoid cell differentiation in testosterone treated hypophysectomized adult rats

given intratesticular implants of immature testicular tissue. In vitro, the anti-androgen cyproterone acetate prevents the development of rat myoid cells (Hovatta, 1972b).

Contractions of the peritubular myoid cells are responsible for the passage of mature spermatozoa along the lumen of the seminiferous tubules towards the epididymis (Ross & Long, 1966). Testicular oxytocin from the Leydig cells is thought to play a role in the regulation of seminiferous tubule contractions, although this remains to be substantiated (Nicholson et al. 1984; Guldenaar & Pickering, 1985).

In vivo the Sertoli cells are surrounded by the peritubular cells separated only by the basal lamina. Co-culture of peritubular and Sertoli cells results in the synthesis of laminin by the Sertoli cells, and fibronectin and type I collagen by the peritubular cells (Tung et al. 1984) which together lead to the formation of a basal lamina-like structure (Tung & Fritz, 1980). Monoculture of peritubular cells and Sertoli cells fail to synthesize their respective components of the basal lamina (Tung & Fritz, 1984). The role of the basal lamina is not clear but it is thought to promote histotypic expression of the Sertoli cells in vitro. Sertoli cells plated out on polystyrene culture dishes form monolayer cultures. If however they are plated on top of peritubular cells, the basal lamina thus formed influences the orientation and polarity of the Sertoli cells such that they remain columnar with abundant cytoplasmic processes and junctional complexes between adjacent cells (Tung & Fritz, 1984).

An additional cellular interaction which may have a regulatory role between the peritubular and Sertoli cells is that mediated by the secretion of paracrine factors. Co-culture with peritubular cells promotes the secretion of ABP and transferrin from rat Sertoli cells

both in the presence and absence of FSH (Tung & Fritz, 1980; Hutson & Stocco, 1981; Skinner & Fritz, 1985 a,b, 1986). In the early studies the effects of peritubular cells could not be mimicked by peritubular cell-conditioned medium. Rat peritubular and Sertoli cells cultured in parabiotic chambers demonstrated that peritubular cell secretions were unable to influence ABP production (Hutson, 1983). This would suggest that the action of the peritubular cells requires a specific cell-cell interaction. More recent studies demonstrate that peritubular cells produce a protein (P-Mod-S) which is capable of stimulating Sertoli cell production of ABP and transferrin in a dose-dependent manner (Skinner & Fritz, 1985a,b,1986). The discrepancy between these studies can be explained by the presence of contaminating peritubular cells in the Sertoli cell-enriched preparations of the earlier studies which may have obliterated the effects of the non-concentrated peritubular cell-conditioned medium (Skinner & Fritz, 1985b).

The availability of pure Sertoli cell-enriched preparations, contaminated by approximately 0.3% peritubular cells (Tung et al. 1984), made possible experiments which established that the maintenance of ABP production by the Sertoli cells is androgen-independent but dependent on the presence of peritubular cells. Androgen actions on the seminiferous tubule are therefore in part mediated via their actions on peritubular cells. Thus androgens produced by the Leydig cells stimulate the peritubular cells to produce the paracrine factor P-Mod-S which in turn modulates the functions of the Sertoli cells. Similar mechanisms are thought to exist in the human as co-culture of human peritubular and Sertoli cells results in increased production of transferrin by the latter (Holmes et al. 1984).

The difficulty in obtaining pure myoid cell preparations has

hindered the purification of myoid cell secretory products. Myoid cells are known to secrete a number of proteins (Kissinger et al. 1982), many of which are also secreted by the TR-M cell line, which is thought to be of myoid cell origin (Mather et al. 1982). It has been suggested that this cell line could be useful in the study of myoid cell function and the role they play in the regulation of testicular function (Mather et al. 1983).

1.6.4 Leydig cell - macrophage interactions

Testicular macrophages are situated in the interstitial tissue where they are closely associated with the Leydig cells (Fawcett et al. 1973; Miller et al. 1983). The regulatory effect of FSH on Leydig cell steroidogenesis is thought to result from interactions between the Sertoli and Leydig cells but recent observations suggest that testicular macrophages may also be involved.

Studies by Yee and Hutson have demonstrated high affinity binding sites for FSH on testicular macrophages (1985a), and that these cells respond specifically to FSH in vitro by increased lactate production (1983), and in vivo by increased incorporation of labelled amino acids into secreted products and an accumulation of cAMP (1985b). Furthermore, culture medium from testicular macrophages enhances rat Leydig cell steroidogenesis (Yee & Hutson, 1985c). Macrophages are active in the secretion of many products including prostaglandins and leukotrienes, in particular leukotriene B4 (LTB4) (Takemura & Werb, 1984). LTB4 has been implicated in the LH-dependent stimulation of Leydig cell testosterone production (Papadopoulos et al. 1986). These studies suggest that FSH-regulated secreted products from testicular macrophages may be involved in the paracrine control of Leydig cell function similar to the situation which exists in the ovary, in which

secretion of progesterone by the luteal cells is stimulated by secretory products from ovarian macrophages (Kirsch et al. 1981).

1.7 SCOPE OF THIS THESIS

From the time of the early microscopists, who demonstrated that the testis was divided into seminiferous tubule and interstitial compartments, the function and control of these compartments have been considered completely independently. The isolation of two gonadotrophic hormones from the anterior pituitary, LH which acts on the interstitial Leydig cells and FSH which acts on the Sertoli cells in the seminiferous tubule compartment, reinforced this concept of independent control.

Over the past five years there has been increasing experimental evidence, largely in the rat, to suggest that quantitatively normal spermatogenesis requires not only stimulation by the gonadotrophic hormones, but also the complex interactions between the various cellular components within the seminiferous tubules and interstitial compartments of the testis - the so-called paracrine control. Thus, in order to further our understanding of the paracrine control of testicular function in man, the studies described herein were directed towards establishing techniques whereby individual components of the testis could be studied in isolation and in combination and related to the overall functional state of the testis as defined by systemic hormone levels and histological assessment of spermatogenesis. These studies provide a basis for future investigation of seminiferous tubule and Leydig cell function in the human testis.

CHAPTER 2

Materials and Methods

2.1 HUMAN TESTICULAR TISSUE, PLASMA AND INTERSTITIAL FLUID

Testicular tissue for all of the studies described was obtained from men undergoing either total or subcapsular orchidectomy for prostatic carcinoma. All samples were collected from the operating theatre as soon as possible following orchidectomy and placed on ice. Patients ranged in age from 50 to 94 years (median 73 years) and were sub-divided into groups according to treatment regimen as follows:-

2.1.1 No treatment

Orchidectomy was the primary treatment for prostatic carcinoma in this group, other forms of treatment being contraindicated because of the risk of thromboembolism and cardiovascular complications or because of the advanced state of the disease.

2.1.2 Radiation treated

All patients who had received radiotherapy at some stage prior to orchidectomy were included in this group. In all cases radiotherapy was completed at least 2 years prior to orchidectomy.

2.1.3 Oestrogen treated

This group comprised patients who were receiving diethyl stilboestrol (DES; 3 mg daily) up to the time of orchidectomy.

2.1.4 Post mortem

Testes from one 17 year old man, killed in a road traffic accident, were obtained 12 h after death.

2.1.5 Plasma

Peripheral blood was obtained from the patients prior to anaesthesia. After centrifugation at 1000 x g for 10 min at 4°C, plasma samples were pipetted off and stored at -20°C prior to measurement of testosterone, LH, FSH and oestradiol.

2.1.6 Interstitial fluid

Interstitial fluid (IF) was collected from testes after total orchidectomy by the method described by Sharpe & Cooper (1983). A small incision was made in the caudal pole and the testis placed in a glass funnel. The IF was allowed to drain from the testis into a glass centrifuge tube for approximately 16 h, after which time it was centrifuged at 4°C for 10 min to remove any contaminating red blood cells. The interstitial fluid was then aspirated and incubated at room temperature for 30 min with charcoal (Norit A; 20 mg charcoal/ml IF), centrifuged for 10 min at 1000 x g and the supernatant extracted a second time using the same procedure. Following this double charcoal extraction, testicular IF levels of testosterone were undetectable. IF was stored at -40°C.

2.2 MORPHOMETRY AND STEREOLOGY

2.2.1 Histology

Testicular tissue of approximately 1 mm cubed was sampled at random from cranial, equatorial and caudal regions of the testis and fixed in 3% glutaraldehyde buffered with 0.2 M sodium cacodylate. Tissue blocks were post-fixed in 2% cacodylate-buffered osmium tetroxide for 2 h, stained with 1% uranyl acetate in 0.05 M maleate buffer, dehydrated in alcohols and embedded in Epon-araldite. Semithin 1 μm sections were cut with a Reichert OmU_3 ultramictrotome, stained with toluidine blue and examined with a Zeiss photomicroscope.

2.2.2 Photography

Photomicrographs were prepared using standard techniques and printed at a final magnification of 1200 x.

2.2.3 Random sampling

Careful attention was taken to ensure a systematic method of random sampling such that all morphometric analysis was performed on unbiased, representative samples.

2.2.4 Standardization

All micrographs were taken at the same magnification. In addition, a micrograph of a stage micrometer was taken with each film to correct for any minor inconsistencies in the microscope or in the film processing routine.

2.2.5 Minimal sample size

On average 4000 observations were made for each parameter measured for each individual patient (20 sections x 10 areas/section x 20 objects/area).

2.2.6 Measurement of testicular volume

The volume of a preweighted block of testicular tissue was

determined by fluid displacement. A beaker containing water was placed on a balance and weighed. A preweighed block of testicular tissue was suspended by a thin thread so that it was completely immersed in the water but did not touch the bottom of the beaker. This new weight (g) minus the weight of the beaker plus water was equal to the volume of the testicular tissue (cm^3). The specific gravity of testicular tissue was also determined by allowing the tissue to drop to the bottom of the beaker and this new weight measured. This weight, minus the weight of the beaker plus water, divided by the volume of the testicular tissue was equal to the specific gravity. The specific gravity of human testicular tissue approximates unity (1.03 ± 0.08, n=20), thus "g" and "cm^3" are interchangeable.

Tissue processing for light microscopy alters testicular volume by shrinkage (fixation and dehydration) and swelling (embedding). The extent of these effects were determined by measuring testicular volume before and after processing. Testicular tissue processed according to the protocol detailed above (2.2.1) was reduced in volume to 58.7% - 75% compared to fresh tissue. Measurements of numerical density (see 2.2.8) from semithin sections were corrected accordingly and expressed in terms of fresh intact tissue.

2.2.7 Section thickness

20 sections were re-embedded in Epon-araldite, cut on edge and their thickness determined with an eyepiece graticule. Mean section thickness did not deviate significantly from 1 μ (1 ± 0.3, $p > 0.05$).

2.2.8 Determination of round spermatid and Leydig cell number

The number of round spermatid and Leydig cell nuclei in individual testes was derived by the measurement of numerical density (Nv) using the Floderus equation $N_V = N_A/D+t-2h$ (Floderus, 1944), where N_A is

the number of nuclei counted in a known area, D is the mean caliper diameter of the nucleus of interest, t is the section thickness and h is the height of the smallest visible nuclear cross section.

Quantitation of N_A was carried out using a Zeiss photomicroscope with a Plan Apo 40 x oil objective. Round spermatid and Leydig cell nuclei were counted in random areas (i.e. including areas which did not contain the cell type of interest) using a squared lattice with an area of 44,100 μm^2. Approximately 300 reference areas, or 13.2 x 10^6 μm^2, were examined to determine N_A for each individual.

Measurement of average nuclear diameter (D) was carried out on photomicrographs, taken with a Zeiss photomicroscope with a Plan Apo 40 x oil objective, printed at a final magnification of 1200 x. Nuclear diameter was measured in mm in approximately 4000 cells (20 sections x 10 areas x 10 cells / area) per individual, converted to μ using a photomicrograph of a stage graticule taken at the same magnification and the mean taken as the actual nuclear diameter. Since the nuclei were often elongated in section, both the longest and shortest diameters were measured and the average of the two measurements considered as the actual diameter.

The height of the smallest visible cap section (h) was arbitrarily estimated to be one-third of the section thickness, or approximately 10% of the nuclear diameter (Mori & Christensen, 1980).

N_V gave the number of nuclei per μm^3. This was converted to number per cm^3 (1 cm^3 = 10^{12} μm^3) i.e. number per g of testis.

2.2.9 Seminiferous tubule length

Total seminiferous tubule length per testis was calculated from the formula:-

$$\text{Length} = \frac{\text{volume (of seminiferous tubules)}}{\text{area (of seminiferous tubule cross section)}}$$

Counts to determine volume density of seminiferous tubules were made with a Plan Apo x 10 objective and 10 x eyepieces, one of which contained a squared grid comprising 49 intersecting points. The volume density of seminiferous tubules was derived by dividing the number of points landing on seminiferous tubules by the total number of points observed (49 points/area x 10 areas/slide x 10 slides/individual = 4900 points).

The area of seminiferous tubule cross sections was derived by tracing the perimeter of approximately 100 transverse sections (5 transverse sections/slide x 20 slides) for each individual with an image analyser.

Seminiferous tubule length was expressed as m/testis.

2.2.10 Daily sperm production

Spermatogenesis was quantitated as daily sperm production (DSP) using a technique described by Johnson and co-workers (1981). Testicular tissue, of approximately 30-50 mg, was immersion fixed in 2% glutaraldehyde buffered with 0.2 M sodium cacodylate. Fixed tissue was stored at 4°C until homogenates were prepared by dicing glutaraldehyde-fixed tissue in 10 ml homogenizing fluid consisting of 150 mM NaCl, 0.05% (v/v) Triton X-100 and 3.8 mM NaN_3. Diced parenchyma was homogenized at room temperature for 6 min in a VirTis 23 macrohomogenizer (Techmation Ltd., U.K.). All spermatid nuclei were counted in a haemocytometer under phase contrast microscopy using the classification system of Clermont (1963) and Heller & Clermont (1964). DSP/g was calculated by dividing the number of spermatids in the

homogenate by the product of the weight of tissue homogenized by a time divisor of 23.9 days. The time divisor is designated to determine the number of days sperm production represented by the spermatids counted in the homogenate. This was assumed to be the sum of the duration of the stages of the spermatogenic cycle in which spermatids are present.

For each patient six blocks of tissue were selected at random from both left and right testes. DSP for each testis was expressed as X $\pm$ SD.

The precision of this technique was estimated by the coefficient of variation which was 8.1 $\pm$ 4.5% between spermatid counts within the same homogenate, and 14.8 $\pm$ 5.5% between different homogenates from the same testis.

2.2.11 Morphometric analysis of DSP

DSP was calculated in 7 individuals on 1μ semithin sections by dividing numerical density of round spermatid nuclei (see 2.2.8) by a time divisor of 8.9 days (Johnson et al. 1981). The correlation coefficient between DSP/g obtained using both homogenization and morphometric techniques was tested for significance.

2.2.12 Quantitation of spermatogenesis by enumeration of mature spermatids

Mature spermatids (Sc1, Sc2, Sd1, Sd2 in stages I, II, V & VI) were counted in approximately 100 transverse seminiferous tubule cross sections (20 slides x 5 cross sections/slide) per man. Seminiferous tubules cut obliquely were not enumerated. Mature spermatid nuclei were counted and the total number divided by the number of tubules in which they were observed.

2.3 DISTRIBUTION OF TESTOSTERONE IN THE HUMAN TESTIS

2.3.1 Whole testis testosterone

Testes were decapsulated and parenchymal weight determined. Six blocks of tissue (approximately 0.5 g) were sampled at random to determine whole testis testosterone. Each preweighed block of tissue was stored at -20°C prior to extraction of testosterone.

2.3.2 Isolation of human seminiferous tubules

Seminiferous tubules from representative cranial, equatorial and caudal regions were isolated from both right and left testes. Seminiferous tubule dissections were carried out on a trans-illuminated cooled stage through which ice-cold water was pumped continuously. Attached to the stage was a transparent 1 mm squared grid. Small blocks of testicular tissue, of approximately 1 g, were in turn placed in 5 cm plastic Petri dishes containing ice-cold medium 199 containing Hank's salts and 20 mM Hepes buffer (= m199H, Flow Laboratories, UK). Seminiferous tubules were teased apart with watchmakers forceps, and isolated tubules of varying lengths, with no adherent connective tissue or interstitial cells, were transferred to another Petri dish, again containing m199H. This medium was aspirated to leave only a covering on the surface of the Petri dish. Individual seminiferous tubules were aligned above the transparent grid and cut into segments of 0.5 cm. 20 x 0.5 cm aliquots of isolated seminiferous tubules were transferred to glass tubes containing 300 µl distilled water to cause lysis of the tissue, and stored at -20°C prior to extraction of tubule testosterone.

2.3.3 Extraction of whole testis and seminiferous tubule testosterone, dihydrotestosterone and oestradiol on celite columns

(i) Whole testis

Tritiated testosterone (1,2,6,7-^{3}H, 80-105 Ci/mmol, Amersham

International PLC), dihydrotestosterone (1,2-^{3}H (N), 55.5 Ci/mmol, New England Nuclear) and oestradiol (2,4,6,7-^{3}H, 100 Ci/mmol, Amersham International PLC), each at approximately 4000 counts per minute (cpm) in a volume of 100 μl were added to each preweighed testis block to determine percentage recovery. Each testis block was homogenized and centrifuged at 1500 x g for 10 min at 4°C. The resulting organic phase was pipetted off and the residue extracted a second time with 2 ml methanol. Both organic phases were pooled and dried down under nitrogen. The residue was reconstituted in 0.5 ml iso-octane (2,2,4-trimethylpentane).

(ii) Seminiferous tubules

After the addition of ^{3}H-testosteorne , ^{3}H-dihydrotestosterone and ^{3}H-oestradiol (each at 2000 cpm in 100 μl) to monitor for recovery, each aliquot of seminiferous tubules was sonicated for 30 sec using an MSE Soniprep 150 (MSE Instruments Ltd., UK). The resulting preparation was extracted with 3 ml diethyl ether and vortex mixed for 10 min. The aqueous phase was frozen in a solid CO_2-ethanol bath and the organic phase dried down under nitrogen and reconstituted in 0.5 ml iso-octane.

(iii) Preparation of celite columns

Celite 545 (Johns Manville Co., USA) was acid washed overnight with concentrated hydrochloric acid, rinsed with tap water until it reached neutral pH, and dried. Each time columns were prepared, celite was activated at 300°C for 12 h. Activated celite was mixed with ethylene glycol (1 g : 0.5 ml) and packed into glass columns. Each column was flushed through twice with iso-octane prior to addition of the samples.

(iv) Elution of steroids on celite columns

Whole testis and seminiferous tubule extracts in 0.5 ml iso-octane

were loaded onto individual celite columns and allowed to flow through completely. Each column was then washed with 2.5 ml iso-octane and the eluate discarded. The dihydrotestosterone fraction was eluted with 5 ml iso-octane. Next the testosterone fraction was eluted with 5 ml 20% benzene in iso-octane. Finally the oestradiol fraction was eluted with 6.5 ml 40% ethyl acetate iso-octane. Each fraction was dried down under nitrogen and reconstituted in phosphate gelatin buffered saline (PGBS, 0.1 M). Extracts of whole testis and seminiferous tubule dihydrotestosterone and oestradiol have as yet not been assayed, and will not be discussed further. Each testosterone fraction was assayed using an ^{125}I-testosterone assay.

2.3.4 Iodination of tracer for ^{125}I-testosterone assay

2 μg testosterone-3 carboxymethyl oxime-histamine were iodinated with 2 mCi Na-I-125 (Amersham International Ltd., UK) using chloramine-T (1 mg/ml). This reaction was stopped after 2 min by the addition of 10 μl sodium bisulphite (1 mg/ml) in 0.05 M phosphate buffered saline. The products of this reaction were extracted twice with ethyl acetate and separated by thin layer chromatography using a solvent mixture of chloroform:methanol:glacial acid (90:10:1 by volume). Two bands of iodinated material were obtained and the most active (lower) band was eluted in ethanol and stored at 4°C for up to 2 months.

2.3.5 Testosterone standards

Standards were prepared from a solution containing 1 μg testosterone in 1 ml ethanol. 640 μl of this solution were evaporated to dryness and the residue reconstituted in 100 ml PGBS to give a concentration of 640 pg/100 μl. Dilutions of this stock solution were made to produce standards ranging from 640-5 pg/100 μl. Results were calculated from the standard curve run with each assay using a log-logit transformation.

2.3.6 ^{125}I-testosterone assay

Testosterone was assayed by a double-antibody radioimmunoassay using ^{125}I-labelled testosterone-3-carboxymethyl oxime-histamine as tracer and an antiserum raised in the sheep against the same testosterone conjugate (Sharpe & Bartlett, 1985). Testosterone samples, standards and quality controls (100 μl in duplicate) were added to 500 μl PGBS, approximately 15,000 cpm ^{125}I-labelled testosterone tracer and 100 μl of a 1:350,000 dilution of the testosterone antiserum and incubated at room temperature for 4 h. Following incubation, 100 μl of a 1:1,000 dilution of normal sheep serum (Scottish Antibody Production Unit) and 100 μl of a 1:25 dilution of goat anti-sheep precipitating serum (Scottish Antibody Production Unit) were added and incubated for a further 16 h at 4°C. Each tube was then centrifuged at 1500 x g for 30 min at 4°C, the supernatant decanted, and the precipitate counted in a gamma counter (NE 1600, Nuclear Enterprises Ltd., U.K.). The results were computed by a Commodore 4032 micro-computer (CBM Computers Ltd., U.K.) linked to the NE 1600. The inter- and intra-assay coefficients of variation, calculated from quality controls run with each assay, were 12.3 $\pm$ 3.1% and 8.2 $\pm$ 3.0% respectively. The limits of detection of the assay were 0.2 and 3.2 ng/ml.

2.3.7 Measurement of circulating hormones

(i) Testosterone

Tritiated testosterone, approximately 1000 cpm in a volume of 20 μl, was added to 100 μl plasma in duplicate. This was extracted with 1.5 ml redistilled hexane-diethyl ether (4:1). After vortex mixing for 5 min the aqueous phase was frozen in a solid CO_2-ethanol bath. The organic phase was decanted, dried down under nitrogen and reconstituted in 200 μl PGBS. 100 ul was assayed using the ^{125}I-testosterone radioimmunoassay (2.3.6) and 50 μl was used to determine percentage

recovery of individual samples. Quality control plasma samples were extracted and assayed in exactly the same manner.

(ii) LH and FSH

Human plasma LH and FSH were measured using a double antibody radioimmunoassay (Hunter & Bennie, 1979), and the results expressed in terms of WHO standards 68/40 and 78/549 respectively. Briefly, 100 μl sample, 150 μl 1% BSA/PBS and 50 μl antiserum (LH: F87-2, 1:200,000, from the Chelsea Hospital for Women; FSH: M93/2, 1:250,000 from Dr. S. Lynch, Birmingham) were incubated for 24 h at 4°C. Following the addition of 50 μl tracer (both from the Chelsea Hospital for Women, LH: 18,000 cpm/tube; FSH 15,000 cpm/tube) the asssay was incubated for a further 24 h at 4°C before the addition of 100 μl donkey anti-rabbit serum (Scottish Antibody Production Unit, 1:32) and 100 μl normal immune rabbit serum (Scottish Antibody Production Unit, 1:200). Following incubation overnight at 4°C, 1 ml 0.9% saline was added to each tube and centrifuged for 30 min at 1500 x g at 4°C. The supernatant was tipped off and the remaining precipitate counted in an NE 1600 gamma counter. The results were computed by a Commodore 4032 micro-computer linked to the NE 1600.

(iii) Oestradiol

Oestradiol was extracted from 100 μl plasma with 1 ml diethyl ether. After mixing for 30 sec the aqueous phase was frozen in a solid CO_2-ethanol bath. The organic phase was decanted and dried down on a heating block. To each extract 200 μl antiserum (BW 26/9/80, Tenovus Institute for Cancer Research, 1:4,500,000) and 200 μl tracer (oestradiol-3-carboxymethyl ether-[^{125}I]-iodo-histamine, 15,000 cpm/200 μl) were added and incubated at room temperature for 2 h. Following incubation, 500 μl 1.25% dextran-coated charcoal

(Norit A charcoal, Sigma Ltd., UK; dextran T70, Pharmacia, UK) was added and each tube allowed to incubate for 15 min on ice. Each tube was then centrifuged for 10 min at 1500 x g at 4°C and the supernatant decanted and counted in an NE 1600 and the results calculated using a log spline curve fit. The intraassay coefficient of variation was $<$ 5%.

2.4 LEYDIG CELL STUDIES IN VITRO

2.4.1 Patients

Testicular tissue was obtained from men aged between 57 and 85 years who were undergoing orchidectomy for prostatic carcinoma. No patient had received any previous treatment, or had any known endocrine disease or any chronic systemic illness known to affect Leydig cell function.

2.4.2 Animals

Adult male Sprague Dawley rats (80-100 days), obtained from Charles River Ltd., UK were housed under conventional conditions and killed by dry ice-generated CO_2.

2.4.3 Percoll gradients

Stock Percoll (Pharmacia, UK) was mixed with 10x medium 199 with Hanks' salts (Flow Laboratories, UK) (9:1 v/v) to produce a solution of 90% Percoll with a density of 1.12 g/ml. This solution was diluted with appropriate quantities of medium 199 containing Hanks' salts and 20 mM Hepes buffer (= m199H, Flow Laboratories, UK) to produce solutions of Percoll with densities of 1.09, 1.07, 1.05 and 1.03 g/ml. Discontinuous gradients of Percoll consisting of successive 10 ml layers of each of the Percoll solutions, beginning with 1.09 g/ml and topped with 10 ml m199H alone were prepared and stored overnight at 4°C prior to use.

2.4.4 Preparation of human Leydig cells

Testes were weighed, decapsulated and cut into small pieces of about 5 mm cubed. Approximately 5-8 g of tissue was then placed into 50 ml conical glass flasks and washed briefly with 25 ml medium 199 containing Hanks's salts and 20 mM Hepes buffer (= m199H, Flow Laboratories, UK). This medium was then decanted and replaced by 8 ml m199H containing 0.5 mg/ml collagenase (Type I; Sigma UK) and 2.5 mg/ml

bovine serum albumin (BSA, Fraction V; Sigma, UK). The tissue was then incubated in a shaking water bath (180 cycles/min) for 10 min at 32°C. The contents of each flask were then transferred to measuring cylinders, diluted with 40 ml m199H and inverted 2 or 3 times. Undispersed tissue and tubule fragments were then allowed to settle and the supernatant was filtered through a double layer of gauze. The filtrate, containing isolated cells was then centrifuged at 200 x g for 5 min at 4°C and the precipitated cells resuspended in medium 199H containing 0.5 mg/ml BSA. The cell suspension obtained from the paired testes of each subject was then filtered once through gauze to remove cell aggregates before being loaded onto a discontinuous gradient of Percoll (Pharmacia, UK). The gradient was then centrifuged at 1800 x g for 20 min at 4°C. Three cell bands were formed at the interface between the successive layers of Percoll and these were termed band 1 (at the interface between 1.03 and 1.05), band 2 (between 1.05 and 1.07) and band 3 (between 1.07 and 1.09). The bands were aspirated, washed in m199H containing 0.5 mg/ml BSA and centrifuged at 250 x g for 5 min at 4°C. The precipitated cells were then resuspended in equal volumes of m199H containing 0.5 mg/ml BSA and medium 199 containing Earles' salts, 0.5 mg/ml BSA, L-glutamine (2 mM: Flow Laboratories, UK), penicillin (100 IU/ml; Flow Laboratories, UK) and streptomycin (100 μg/ml; Flow Laboratories, UK) (= m199E). Nucleated cells were then counted in a haemocytometer. In some experiments all 3 cell bands were studied but, in the majority of experiments, cells from bands 2 and 3 were combined prior to the final washing step described above.

2.4.5 Preparation of rat Leydig cells

Rat Leydig cells were isolated using a technique essentially identical to that described above for the preparation of human Leydig

cells, but with the following modifications:-

1. 10 testes were used for each Leydig cell preparation.
2. Groups of 5 decapsulated testes were placed in 50 ml conical flasks together with 8 ml m199H containing 0.25 mg/ml collagenase plus 2.5 mg/ml BSA and incubated for 7 mins in a shaking water bath at 32°C.
3. Leydig cells were obtained from band 3 only.

2.4.6 Identification of Leydig cells

Leydig cells were identified histochemically by staining for 3β-hydroxysteroid dehydrogenase (3β-HSD). Cell smears were prepared on glass slides, air dried and fixed for 10 min in acetone. Fixed cells were stained for 3β-HSD by incubating for 2 h at 32°C in a solution consisting of 0.1 M Tris-HCl (13%), N,N-dimethylformamide (5.4%), sodium cyanide (0.0003%) and anhydrous magnesium chloride (0.0004%) in distilled water, to which was added nitro-blue tetrazolium (0.82 mg/ml), dehydroepiandrosterone (0.27 mg/ml) and β-nicotinamide adenine dinucleotide (0.91 mg/ml). Following incubation the slides were rinsed with tap water, dried and mounted in glycerine jelly. The percentage of stained nucleated cells (Leydig cells) were determined by counting random fields of at least 250 cells by light microscopy.

2.4.7 Leydig cell incubation

Aliquots of 0.05×10^6 cells were incubated in a final volume of 0.25 ml m199E in plastic multiwell dishes (Nunc, Denmark) at 32°C under a humidified atmosphere of 95% air : 5% CO_2. Apart from triplicate wells which contained no hormone additions to determine basal testosterone production all other wells contained hCG (Chorulon, Intervet) at various concentrations but always including a dose (20,000 mIU/ml) that represented a 100- to 1000-fold excess above that needed to maximally stimulate testosterone production. All incubations were done

21°C in 63 x 11 mm polystyrene tubes in the presence of concentrations of ^{125}I-labelled hCG (NIAMDD hCG-CR121) ranging from 0.01 to 3.3 pM, to enable construction of saturation curves. Non-specific binding was determined by parallel incubation in the presence of a 1000-fold excess of unlabelled hCG. The ^{125}I-labelled hCG was prepared using lactoperoxidase and had a specific activity of 12 mCi/mg as determined by self-displacement. Cell preparations from one human and one group of five rats were compared in the same binding assay.

2.5 INHIBIN BIOACTIVITY IN HUMAN TESTICULAR EXTRACTS

2.5.1 Patients

Testes were obtained from 21 men aged between 60-89 years (median 74) who were undergoing orchidectomy for prostatic carcinoma. Fifteen patients received no previous treatment, while six men had received radiotherapy 2-3 years before orchidectomy. No patient had any endocrine disease or chronic illness known to affect testicular function. Patients receiving endocrine therapy were excluded from the study.

2.5.2 Preparation of testicular extracts

Testicular aqueous extracts were prepared from human, rat (adult male Sprague Dawley), ram, boar (from the local abattoir) and marmoset (adult from our own colony) as described previously by Au et al. (1983). Briefly, testicular tissue of known weight was homogenized on ice in 1:1 (w/v) sterile Dulbecco's phosphate buffered saline (DPBS, pH 7.4) containing penicillin (50 IU/ml) and streptomycin (50 µg/ml). Homogenates were centrifuged at 100,000 x g for 1 h at 4°C and the aqueous fraction aliquoted, snap frozen and stored at -20°C. Each sample was passed through C18 Sep-Pak cartridges (Waters Associates, Milford, MA, USA) to remove the steroids before bioassay.

2.5.3 In vitro inhibin bioassay

Inhibin was measured using a sheep pituitary cell bioassay as described previously (Tsonis et al. 1986). Heads from ewe lambs aged between 8-15 months were obtained from the local abattoir. Anterior pituitary glands were removed, chopped into blocks of approximately 4 mm^3 and washed 6 to 8 times in DPBS containing 7.5 mM glucose/l and 0.1% BSA. Pituitary blocks were placed in a 25 ml spinner flask with 10 ml 0.5% trypsin made up in DPBS + 0.1% BSA and stirred gently for 30

min at 37°C. The trypsin solution was removed and replaced with 10 ml Dulbecco's Modified Eagles's Medium (DMEM, Flow Laboratories, UK) supplemented with 10% lamb serum (Flow Laboratories), 2.5% foetal bovine serum (Flow Laboratories) 10 mM Na HCO_3/1, 2 mM glutamine/1 (Sigma, UK), penicillin (50 IU/ml) and streptomycin (50 μg/ml) and stirred gently for 30 min at 37°C. The supplemented DMEM was removed and replaced with 10 ml $DPBS^-$ (Ca^{2+} and Mg^{2+} free) supplemented with 2mM EDTA/1 and 0.1% BSA and stirred gently for 10 min at 37°C. This medium was centrifuged at 500 x g for 10 min at room temperature while the remaining tissue was washed 3-4 times with 10 ml DPBS and dispersed using a series of 3 Pasteur pipettes of decreasing bore (5-2 mm) which were sterile, siliconized and flame treated. The dispersed cells were made up to 20 ml with supplemented DMEM and centrifuged twice at 500 x g for 10 min at room temperature, the pellet being resuspended in 3-5 ml supplemented DMEM between spins. The precipitated cells were added to those isolated during the first spin, diluted to 20 ml with supplemented DMEM and counted in a haemocytometer. The average cell yield per pituitary was 30-50 x 10^6 cells. Pituitary cells were diluted to a final concentration of 180,000-200,000/50 μl with supplemented DMEM. 50 μl aliquots of cells were incubated in a final volume of 600 μl supplemented DMEM in plastic multiwell dishes (Nunc, Denmark) and cultured for 48 h in a humidified atmosphere of 95% air : 5% CO_2.

Following the initial 48 h culture period, the medium was aspirated and replaced with either test sample, ovine rete testis fluid inhibin standard (oRTF 1U/mg, kindly donated by Dr. J.K. Findlay (see Eddie et al. 1979), or ovine follicular fluid quality control (oFF; Tsonis et al. 1986) and cultured with anti-oestradiol and anti-progesterone serum (Tsonis et al. 1986, 1987c) for a further 48 h. Both the oRTF inhibin

standard and the oFF quality control were assayed in quadruplicate over a five-fold dose range to establish an FSH-inhibition curve. Testicular extracts were tested in duplicate at three dilutions using up to 50 µl/culture well. Ovine FSH in the culture medium was assayed by a specific double antibody radioimmunoassay using an NIH-oFSH-S14 standard as described by McNeilly and colleagues (1976). FSH dose-response curves were expressed as percent of control (no exposure to inhibin). Regression analysis was performed to determine the slope, index of precision (lamda) and significance of regression (Finney's g; Finney, 1964). Testicular extracts were compared to the oRTF standard and relative potencies calculated for samples which showed no significant departure from parallelism and linearity. The index of precision of the bioassay was 0.055 and the sensitivity was 0.05 - 0.1 U/ml relative to the oRTF standard.

2.5.4 Bioassay of pituitary cell mitogenic activity

Ovine pituitary cells were cultured for 48 h (2.5.3) after which the culture medium was removed and replaced with medium containing extracts of human, rat, ram, boar and marmoset testes. After a further 72 h of culture the medium was aspirated and the pituitary cells removed from the culture plates by the use of trypsin DPBS$^-$ containing 0.2% trypsin and 0.08% EDTA (250 µl) was added to each culture well for 20 min at room temperature. The trypsin was neutralized by the addition of 250 µl DPBS$^+$ containing 0.1% BSA. The cells were lifted from the culture plate by repeated, gentle aspiration using a Gilson pipette. Cell number was counted using a haemocytometer.

2.6 STATISTICS

As normal distribution of values could not be assumed, both parametric and non-parametric (in parentheses) analyses were applied to the results. Where appropriate analysis was by Student's t test (Mann-Whitney test), simple regression (Spearman's rank order correlation coefficient), multiple interactive regresssion and analysis of variance (Kruskal-Wallis one way analysis of variance). In no case did the significance of difference change according to which test was applied. All results are expressed in terms of parametric analyses, significance being determined by the t- statistic.

CHAPTER 3

Relationship between Testosterone and Spermatogenesis in the Senescent Human Testis

3.1 Introduction

The function of the mammalian testis is two-fold. Firstly there is the production of androgens by the Leydig cells under the influence of LH. Secondly there is the production of the male gametes, the spermatozoa, by the process of spermatogenesis. Maintenance of spermatogenesis requires a specific hormonal milieu, and androgens have become regarded as the essential hormones of this milieu (Desjardins et al. 1973; Nieschlag et al. 1973). In the past it has been accepted that high intratesticular levels of testosterone are an essential prerequisite of spermatogenesis, and a number of studies have reported the existence of paracrine factors which act to maintain high intratesticular levels of testosterone (Sharpe, 1984, 1986). Indeed declining levels of testosterone, as a result of a reduction in Leydig cell number, has been proposed as the reason for impaired spermatogenesis in elderly men (Neaves et al. 1984).

However a small number of observations have challenged this concept that high intratesticular levels of testosterone are essential for the production of spermatozoa. In hypophysectomized rats (Boccabella, 1963; Buhl et al. 1982) and stalk-sectioned monkeys (Marshall et al. 1983), the administration of exogenous testosterone resulting in intratesticular levels of testosterone which are 10-20% of normal is sufficient to maintain spermatogenesis, at least qualitatively. In intact adult rats, following the peripheral administration of testosterone proprionate which, by suppressing serum LH to undetectable levels, reduces intratesticular testosterone levels to 20% of normal, spermatogenesis is maintained quantitatively (Cunningham & Huckins, 1979). Similarly in rats made hypogonadotrophic by treatment with a luteinizing hormone-releasing hormone antagonist, subcutaneous implants

of testosterone maintained spermatogenesis quantitatively in the presence of intratesticular levels of testosterone which are 15% of normal (Rea et al. 1986). In this study, as in that by Cunningham & Huckins, while the administration of exogenous testosterone suppresses LH to undetectable levels, FSH is reduced only moderately. Thus it has been suggested that in the presence of FSH, high intratesticular levels of testosterone are not essential for spermatogenesis to proceed as normal.

The present study attempted to investigate the relationship between intratesticular levels of testosterone and spermatogenesis in the senescent human testis. Testes obtained from a group of men who had received orchidectomy as primary or secondary treatment for prostatic carcinoma provided an ideal source of tissue with varying degrees of hormonal and germ cell disruption.

3.2 (i) Daily sperm production

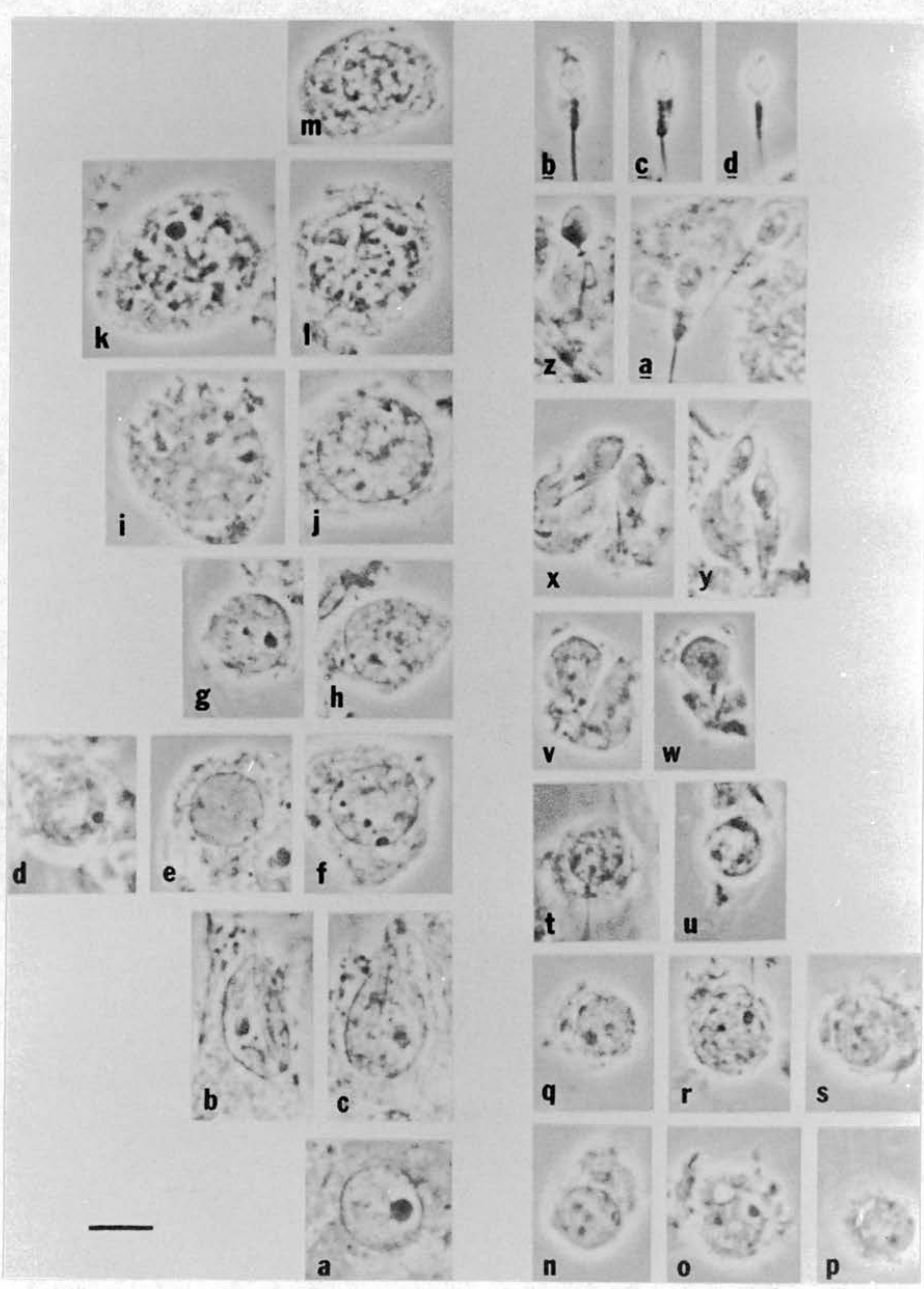
Daily sperm production (DSP) was quantitated as described previously (see 2.2.10). Briefly this involved homogenizing approximately 30-50 mg of fixed testicular tissue in 10 ml homogenizing fluid. Spermatid nuclei were examined under phase contrast microscopy, classified according to Figure 3.1 and enumerated.

DSP in 34 normal untreated males with prostatic carcinoma (60-90 years, median 75 years; $2.8 \pm 1.3 \times 10^6$/g) was lower than that reported for 28 healthy men aged between 20-48 years ($6.0 \pm 2.1 \times 10^6$/g; Johnson, 1986), but similar to that reported for 28 men aged between 50-90 years ($3.8 \pm 1.6 \times 10^6$/g; Johnson, 1986). No significance was attached to the difference in DSP observed between the young (20-48) and older (60-90) age groups as individual values for the younger men were not available (Fig. 3.2). Despite the apparent

FIGURE 3.1

Illustration from Johnson et al. (1981) of Leydig cell, Sertoli cell and germ cell nuclei in human testicular homogenates viewed by phase contrast microscopy.
a) A typical Leydig cell nucleus with euchromatic nucleoplasm and a single nucleolus.
b,c) Ovoid nuclei of Sertoli cells showing typical folding of the nuclear envelope, abundant euchromatin and a single nucleolus.
d-h) Spherical nuclei of spermatogonia with fine nucleoplasm and multiple nucleoli.
i-l) Nuclei of primary spermatocytes with conspicuous strands of chromatin and prominent single nucleoli.
m) A characteristic secondary spermatocyte nucleus which is smaller than that of primary spermatocytes, yet larger than those of round spermatids.
n-s) Spherical nuclei of Sa spermatids with distinctive nucleoli and visible acrosomal caps.
t-w) Elongating nuclei of Sb spermatids with less obvious acrosomal caps than Sa spermatids, but with distinctive flagellar development.
x,y) Elongated nuclei of Sc spermatids with dark staining nucleoplasm containing vacuoles.
z-d) Nuclei of Sd spermatids with condensed nucleoplasm which appears highly refractile under phase contrast.

Bar length = 7.7 μm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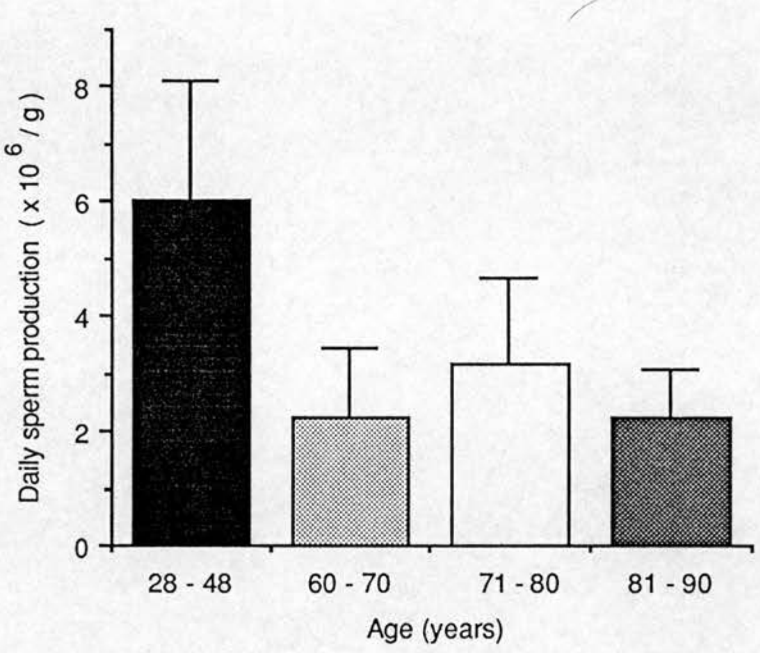


FIGURE 3.2

Age related change in daily sperm production in 28 healthy men (28 - 48 years, from Johnson et al. 1981) and in 34 men with prostatic carcinoma (60 - 90 years). Values are mean ± SD. There was no significant difference between daily sperm production in men from 60 - 90 years.

decrease in DSP in the older men, a number of these men had DSP levels within the range of the younger men. Furthermore, there was no significant age related decrease in DSP between 60-90 years (Fig. 3.2).

(ii) Morphometric analysis of daily sperm production

In order to validate the homogenization approach to the quantitation of DSP, morphometric analysis of this parameter was carried out in 7 men (see 2.2.11). Parameters of round spermatic nuclei and DSP are shown in Table 3.1. A highly significant correlation was observed between estimates of DSP determined by enumeration of spermatid nuclei in fixed testicular homogenates and by morphometric analysis ($R = 0.97$, $P < 0.001$, Fig. 3.3).

(iii) Quantitation of spermatogenesis by enumeration of mature spermatids

Enumeration of mature spermatids (Sc1, Sc2, Sd1, Sd2 in stages I, II, V & VI) in 1 μ histological sections is a means by which testicular biopsies can be evaluated. This technique was used to evaluate spermatogenesis in 22 men and compared to DSP determined by counting spermatid nuclei in testicular homogenates (Fig. 3.4). A high correlation between the two techniques was found ($R = 0.86$, $P < 0.001$) providing further validation for the homogenization approach to the quantification of spermatogenesis in the human testis.

(iv) Systemic hormone levels and their relationship to daily sperm production

In 21 patients who underwent orchidectomy as primary treatment for prostatic carcinoma, blood samples were obtained prior to anaesthesia and the endocrine status of these men compared to that of 100 healthy young men aged 18-50 years (normal data by kind permission of F.C.W. Wu). Plasma testosterone did not decline significantly as a function

Table 3.1. Testicular parenchymal weight, parameters of round spermatid nuclei and daily sperm production in 7 men based on morphometric analysis

Patients	1	2	3	4	5	6	7
Parenchymal wt (g)	7.7	17.1	24.4	17.1	29.5	20.2	15.6
Round spermatid nuclear diam (μ)	7.4	7.3	7.5	6.9	7.2	7.2	6.8
Round spermatid number/g (x 10^6)	9.5	18.0	15.9	45.0	28.4	38.2	25.7
Daily sperm production/g (x 10^6)	1.1	3.1	1.8	5.1	3.2	4.3	2.9

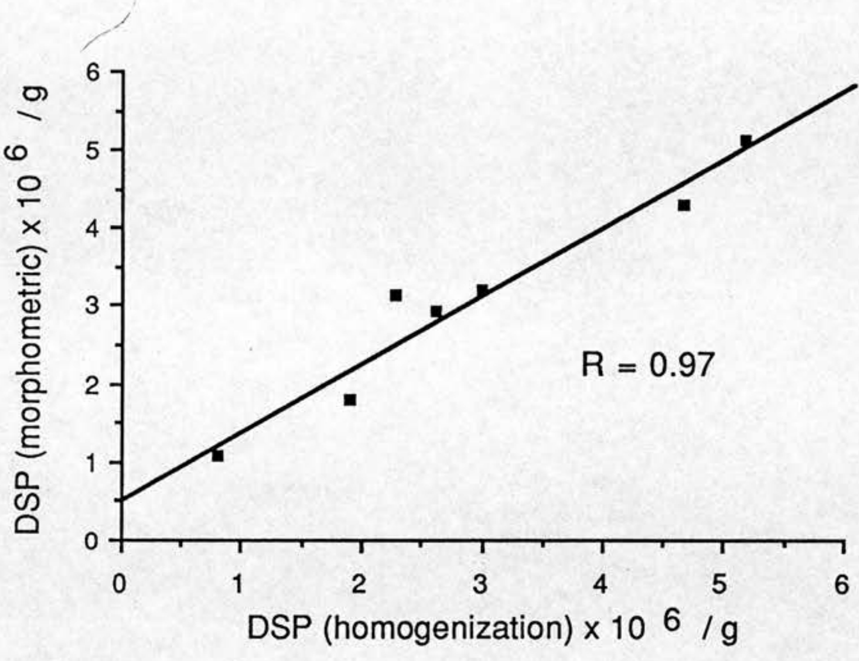


FIGURE 3.3

Correlation between estimates of daily sperm production (DSP) in 7 men derived by enumeration of spermatid nuclei in homogenates of glutaraldehyde-fixed human testes and by morphometric analysis ($R = 0.97$, $P<0.001$).

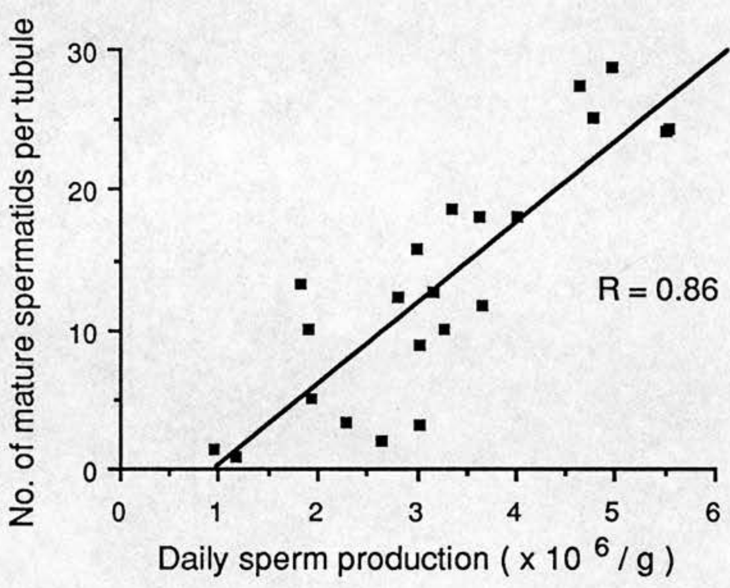


FIGURE 3.4

Correlation between the number of mature spermatids per tubule cross section and daily sperm production in 22 patients ($R = 0.86$, $P<0.001$).

of age (Fig. 3.5). Although the mean testosterone level of 6.0 $\pm$ 1.2 in the 18-50 age group was greater than that in each of the older age groups (60-70, 3.1 $\pm$ 1.3; 71-80, 3.5 $\pm$ 2.2; 81-90, 2.5 $\pm$ 1.0), this was not statistically significant.

LH was significantly elevated in the 60-70 (12.0 $\pm$ 5.4), 71-80 (9.5 $\pm$ 7.5) and 81-90 (12.3 $\pm$ 3.9) age groups compared to the 18-50 group (5.6 $\pm$ 1.8) ($P < 0.001$). LH did not however rise significantly between 60 and 90 years. Similarly, FSH was significantly increased at each of the older age groups (13.7$\pm$ 9.6, 8.9 $\pm$ 7.1, 6.5 $\pm$ 3.4) compared to the 18-50 group (3.2 $\pm$ 1.5) ($P < 0.001$), but did not increase as a function of age between 60-90 years.

The distribution of circulating testosterone, LH and FSH in these patients, who were divided into those with normal levels of FSH (n=14) and those with high FSH (n=7), plus a further 7 patients who had received radiation treatment prior to orchidectomy, was observed (Fig. 3.6). Significant correlations were observed between plasma testosterone and LH ($R = -0.47$, $P < 0.05$) and between LH and FSH ($R = 0.73$, $P < 0.001$).

Levels of spermatogenesis were also correlated to circulating hormone levels in each of the different patient groups (Fig. 3.7). DSP was reduced in the high FSH and radiation treated groups and this was reflected in the significant negative correlations observed between DSP and both FSH ($R = -0.74$, $P < 0.001$) and LH ($R = -0.57$, $P < 0.01$). A weak positive correlation which just reached significance ($R = 0.40$, $P < 0.05$) was also observed between DSP and the wide range of circulating levels of testosterone.

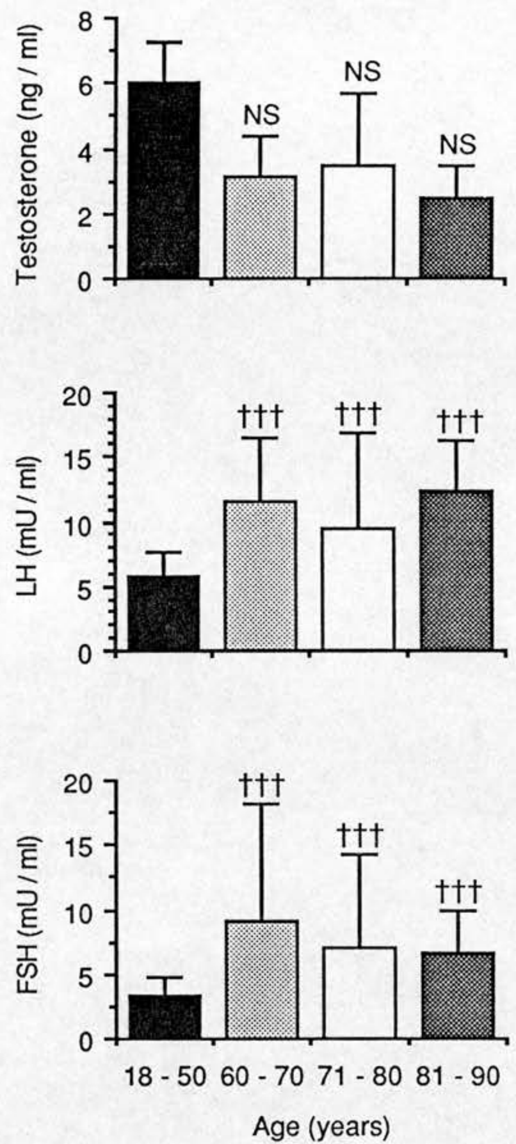


FIGURE 3.5

Age related change in circulating levels of testosterone, LH and FSH. Data was obtained from 100 healthy men (18 - 50 years), and from 21 men with prostatic carcinoma (60 - 90 years). While circulating testosterone was not significantly different between any of the age groups, both LH and FSH were significantly increased in the older men compared to the younger group (†††, $P<0.001$). Values are mean ± SD.

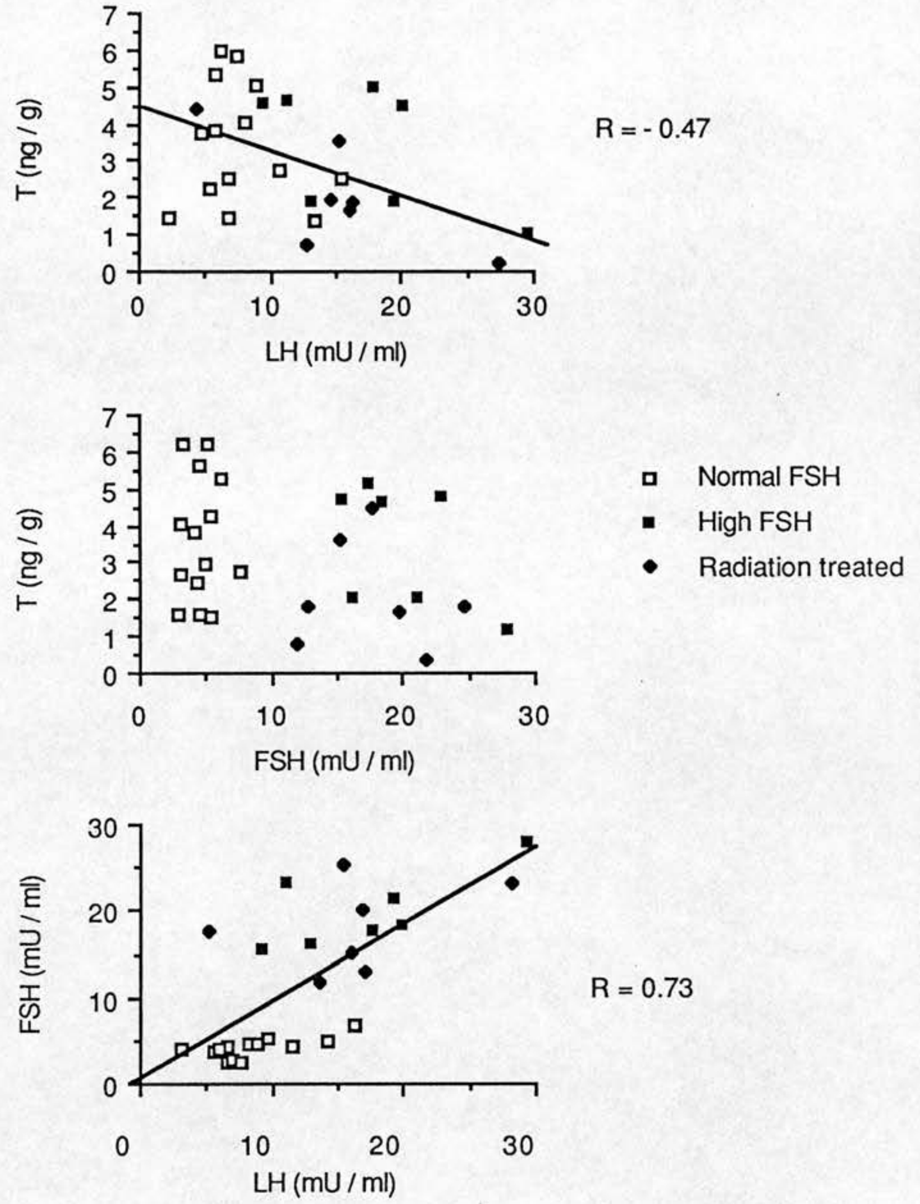


FIGURE 3.6

Distribution of circulating testosterone, LH and FSH. Patients were grouped according to FSH (untreated with normal FSH, n = 14 ; untreated with high FSH, n = 7), and treatment (radiation treated, n = 7). Significant correlations were observed between plasma testosterone and LH ($R = -0.47$, $P<0.05$) and between LH and FSH ($R = 0.73$, $P<0.001$).

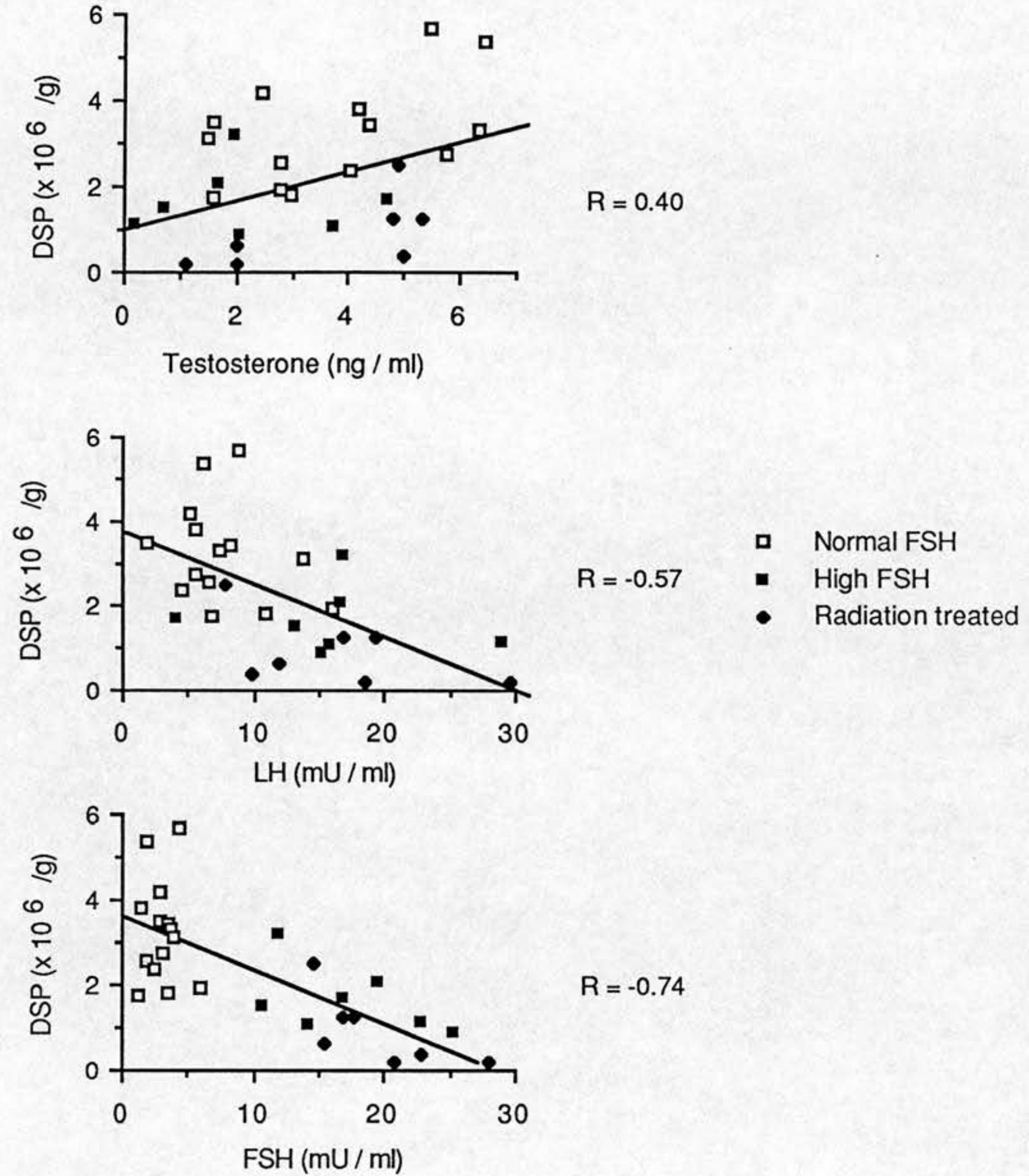


FIGURE 3.7

Correlation between daily sperm production (DSP) and circulating levels of testosterone (R = 0.40, P<0.05), LH (R = -0.57, P<0.01) and FSH (R = -0.74, P<0.001).

(v) Intratesticular testosterone levels and their relationship to daily sperm production

Figure 3.8 shows the distribution of DSP in relation to circulating and intratesticular hormone levels in four groups of patients. As in the previous graph, three of these groups comprised of 21 untreated or radiotherapy treated patients, with an additional group of 9 men who had been treated with diethylstilboestrol (DES) up to the time of orchidectomy. Despite the wide variation in DSP, gonadotrophins, and circulating levels of testosterone in all four groups, excluding the DES treated men, there was no apparent change in testicular levels of testosterone, either in the whole testis or in the seminiferous tubules. Interestingly, despite the very low levels of both circulating and intratesticular testosterone levels in the DES treated group, spermatogenesis in these men was not completely arrested with DSP levels of $0.5 \pm 0.4 \times 10^6$/g.

Surprisingly when DSP was correlated to testicular levels of testosterone in 39 individuals, high levels of intratesticular testosterone were associated with low levels of DSP (Fig. 3.9).

(vi) Leydig cell number, intratesticular testosterone and daily sperm production

Total Leydig cell number was determined in 14 patients in the untreated group. While there was a wide variation in total Leydig cell number, no correlation was observed between daily sperm production and testicular or tubular testosterone (Fig. 3.10).

(vii) Re-evaluation of intratesticular levels of testosterone

In assessing post-isolation changes in intratesticular testosterone following orchidectomy, some patients showed a significant increase in testosterone levels at each of the time intervals, while others showed

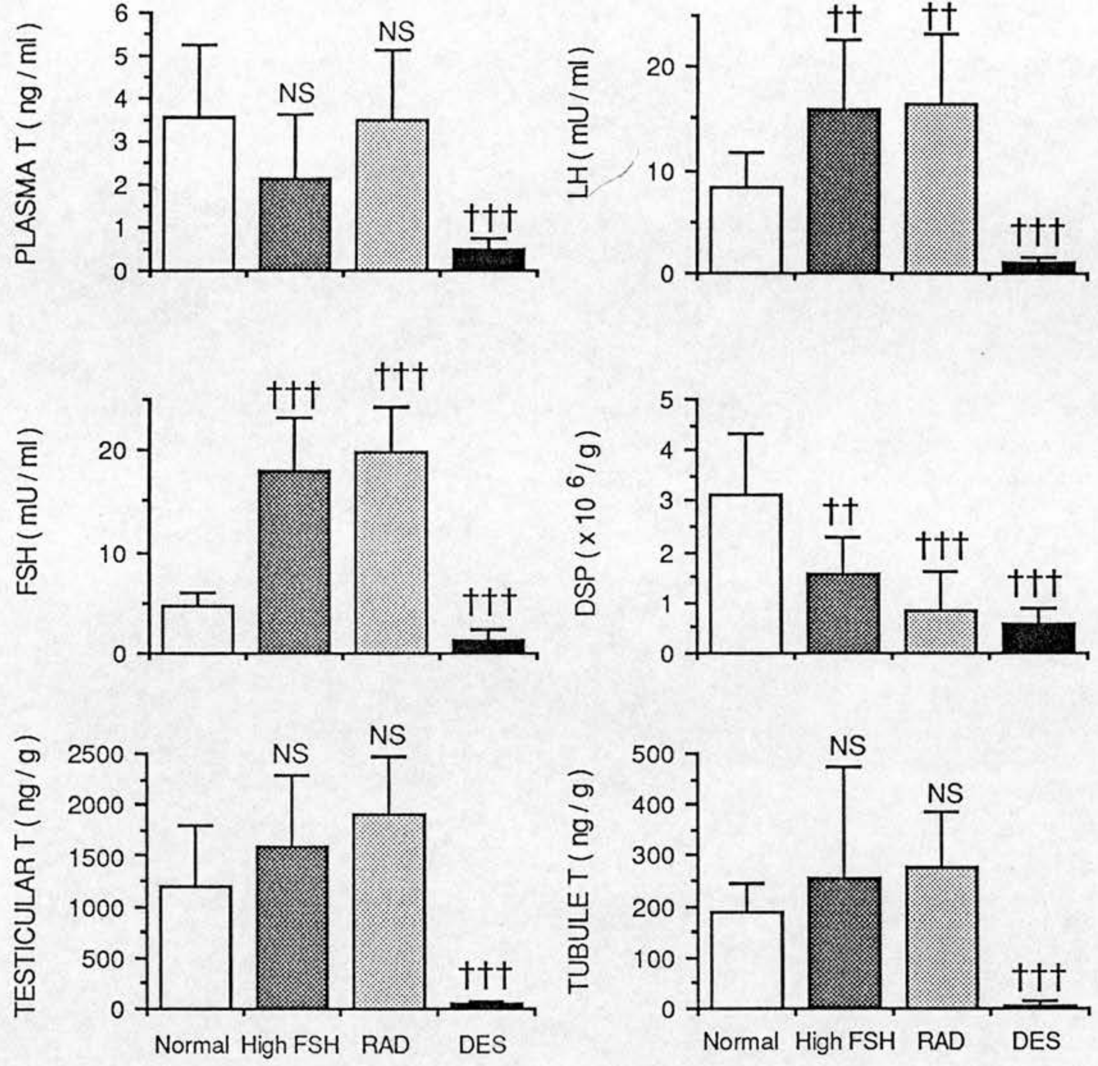


FIGURE 3.8

Distribution of plasma testosterone, LH, FSH, daily sperm production, testicular and tubular levels of testosterone in four groups of men with prostatic carcinoma - untreated normal FSH (n = 14), untreated high FSH (n = 7), radiation treated (n = 7) and diethylstilboestrol (DES) treated (n = 9). All parameters were significantly decreased in the DES treated group compared to normal (†††, P< 0.001). LH (††, P<0.01) and FSH (†††, P<0.001) were significantly elevated in both the high FSH and radiation treated groups compared to normal, while plasma and intratesticular measures of testosterone were not changed significantly.

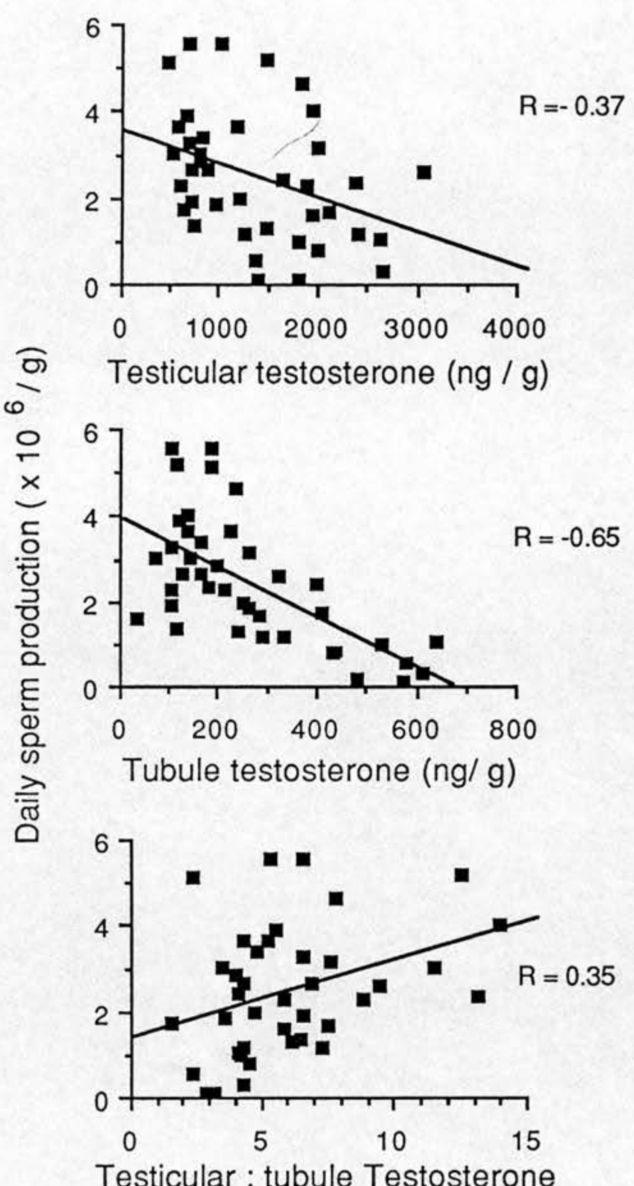


FIGURE 3.9

Correlations between daily sperm production and testicular testosterone (R = -0.37, P<0.05), tubular testosterone (R = -0.65, P<0.001) and the ratio between testicular and tubular testosterone (R = 0.35, P<0.05) in 39 patients. Thus daily sperm production appears to be higher in situations of low intratesticular levels of testosterone.

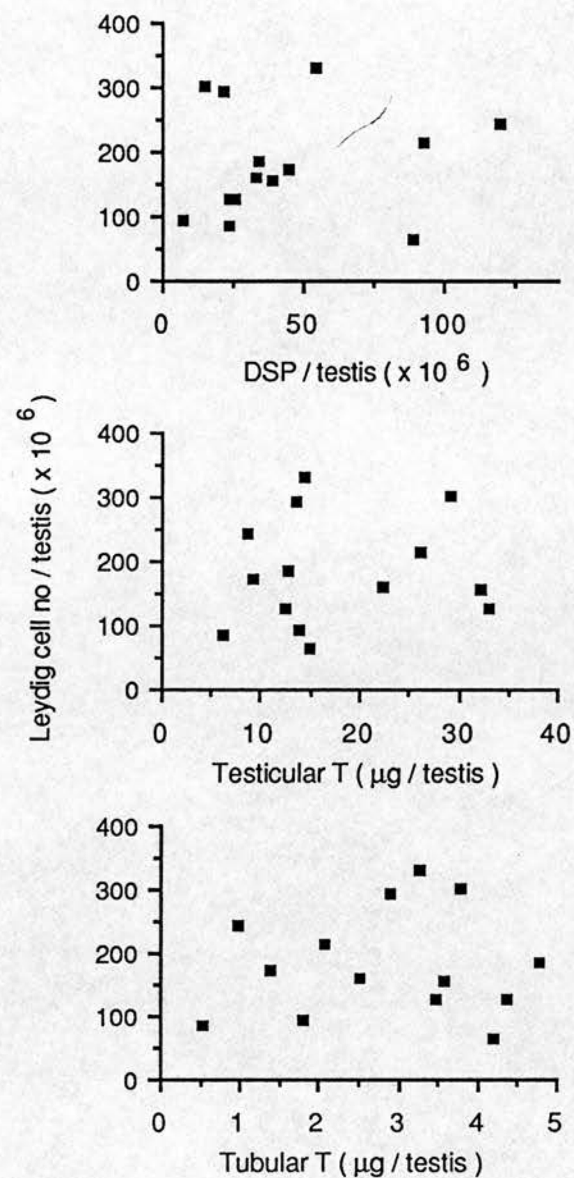


FIGURE 3.10

Lack of significant correlation between Leydig cell number and daily sperm production (DSP), total testicular and tubular testosterone.

a significant decline (Fig. 3.11). However no overall significant difference existed when testosterone levels from 6 patients were expressed as mean ± SD of percentage of initial levels.

3.3 Discussion

With the uncertainty as to the level of testosterone required to maintain spermatogenesis, this study set out to determine whether declining levels of spermatogenesis in elderly men were associated with reduced intratesticular levels of testosterone. Methods first described to quantitate intratesticular distribution of testosterone in the rat (Sharpe & Bartlett, 1985) (see 2.3), and a new approach to quantitate spermatogenesis in the human testis (Johnson et al. 1981) were adopted in these studies. This novel approach to the quantitation of spermatogenesis involves homogenizing small (approximately 10-50 mg) pieces of fixed testicular tissue and then enumerating spermatid number under phase contrast microscopy (see 2.2.10). Spermatogenesis quantitated by this method is expressed as daily sperm production (DSP). This technique was validated by a classical morphometric technique (see 2.2.11), and a high degree of correlation was observed between DSP determined by each of the two methods.

Testicular biopsy has for over 40 years been used to differentiate between obstructive and non-obstructive azoospermia, and also as a tool to assess spermatogenic disruption in cases of oligospermia. While microscopic observation of the histologic features provides much information, the need for quantitative analysis of testicular biopsies has long been realized. In 1981 Silber & Rodriguez-Rigau described a simple method for quantitating testicular biopsies by counting easily recognizable darkly staining mature spermatids in stages I, II, V and V1.

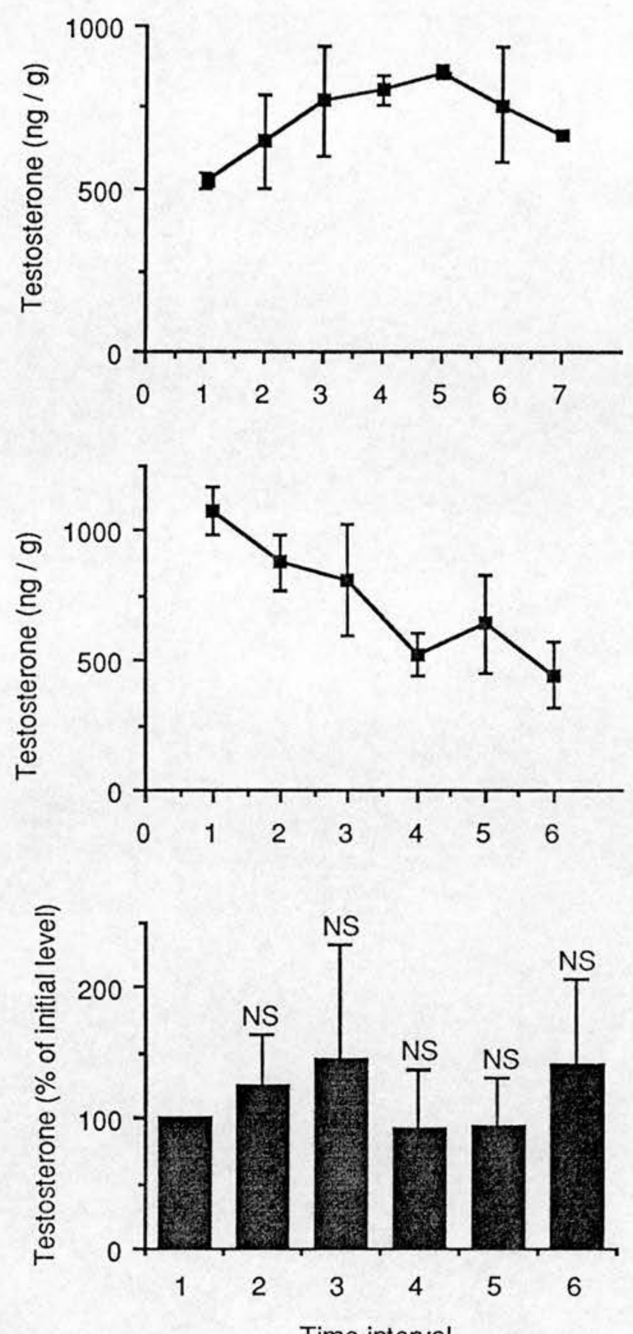


FIGURE 3.11

Changes in intratesticular testosterone following orchidectomy. Time intervals 1-7 represent tissue obtained during orchidectomy (1), and at 5 min (2), 1h (3), 2h (4), 3h (5), 4h (6) and 12h (7) after orchidectomy. Some patients showed a significant increase in intratesticular levels of testosterone (top) at each of the intervals following orchidectomy, while others showed a significant decline (middle). Each value is the mean ± SD of three testicular samples obtained at the same time from different areas of the same testis. No overall significant difference existed when testosterone levels from 6 patients were expressed as mean ± SD of percentage of initial levels (bottom).

Except in cases of obstruction, the results obtained by this method have been shown to correlate well with sperm in the ejaculate. In the absence of proper validation of this technique, in the present study DSP was correlated to mature sperm number in histological sections in 22 men, and a high degree of concordance was observed between the two. Comparing both techniques for the quantification of spermatogenesis, the homogenization approach is much more simple and the results are available much faster. While the homogenization technique requires the use of a specific homogenizer (VirTis 23 macrohomogenizer) and also a phase contrast microscope, it is possible that the adoption of this approach in the clinical situation would provide a convenient method to quantitatively assess testicular biopsies without laborious histological preparation.

While circulating levels of testosterone were on the whole reduced in this group of men, this was not a significant function of age. Indeed as has been reported previously (Harman & Tsitouras) a number of these men into their 7th, 8th and 9th decades had circulating levels of testosterone which were in the range of normal young men. Plasma levels of both LH and FSH were significantly increased in these men compared to younger men, although neither LH nor FSH appeared to increase as a function of age between 60-90 years. The highest serum levels of LH and, in particular, FSH have been shown to occur in men with the lowest levels of DSP.

Declining levels of spermatogenesis in elderly men have been attributed to reduced numbers of Leydig cells resulting in inadequate levels of intratesticular testosterone (Neaves et al. 1984). Contrary to this, the results of the present study indicate high intratesticular levels of testosterone in men with low levels of DSP. Interestingly,

high intratesticular levels of testosterone are not reflected in the peripheral circulation in these men, in whom circulating testosterone was low to normal. Therefore despite the capacity of the Leydig cells to produce testosterone, this did not appear to gain entry to the circulation. Testosterone from the interstitial tissue must pass through the walls of the testicular capillaries in order to enter the peripheral circulation. Unlike capillaries in other endocrine tissues, testicular capillaries are unfenestrated, yet despite this, transport though the capillary wall is thought to be passive (Setchell et al. 1978b). A frequent observation in the testes of old men is the thickened peritubular tissue surrounding the seminiferous tubules, and indeed of fibrosis of the testicular tissue to a greater or lesser extent. While there are no reports of this in the literature, thickening of the vasculature may provide a barrier to the passive diffusion of testosterone from the interstitial tissue to the circulation.

Increased testicular levels of testosterone in these men may be as a result of Leydig cell stimulation by elevated levels of LH. In rats the administration of hCG results in Leydig cell hypertrophy and hyperplasia (Christensen & Peacock, 1980). The physiological implications of this for the present study are such that chronically elevated LH may induce an increase in Leydig cell number and/or volume which would enable the Leydig cell population to produce excessive amounts of testosterone. However evidence of Leydig cell hyperplasia was not observed in this study, pointing against a compensatory mechanism of elevated LH.

In order that increased intratesticular levels of testosterone associated with suppressed spermatogenesis were not merely a reflection

of decreased testicular volume, and hence elevated Leydig cell number per gram of tissue, Leydig cell number was determined in 14 patients who had not received any treatment prior to orchidectomy. While there was a wide spread in Leydig cell number, no direct relationship was found between Leydig cell number and DSP or intratesticular measures of testosterone, confirming the results of Neaves and colleagues (1987). High intratesticular levels of testosterone could therefore be the result of a block in the steroidogenic pathway in these men. Under normal circumstances, intratesticular testosterone is convered to dihydrotestosterone by 5α-reductase, and to oestradiol by aromatase. A block in this pathway could result in accumulating levels of biologically inactive testosterone.

These studies therefore appear to support increasing evidence in the rat that high intratesticular levels of testosterone may not be essential in the maintenance of spermatogenesis (Cunningham & Huckins, 1979; Rea et al. 1986). Such studies are however completely dependent on the accurate measurement of intratesticular levels of testosterone, and doubts about the accuracy of current techniques have emerged recently. In a study by Maddocks and colleagues (1986), in which push-pull canulae were used to collect interstitial fluid from anaesthetized rats, the levels of testosterone measured in this fluid were much lower than those reported previously. These results have raised the possibility that conventional techniques to isolate the testes prior to testosterone measurement may yield spuriously high testosterone levels due to post-isolation synthesis of this steroid (Sharpe, 1987). Indeed by comparing testosterone levels in right and left testes from the same rat isolated in different ways, post-isolation synthesis of testosterone was significant, and on average resulted in a

75% overestimate of testosterone levels (Sharpe et al. 1987).

Attempts were made to determine post-isolation synthesis of testosterone in human testes. Biopsies were obtained during operation prior to orchidectomy and placed immediately in liquid nitrogen. Following orchidectomy testes were placed on ice and samples obtained at various time intervals afterwards were also snap frozen in liquid nitrogen. While post-isolation synthesis of testosterone was significant in some cases, in others testosterone levels fell following orchidectomy. This difference could possibly reflect differing functional capacity of the Leydig cells in different men, such that those with healthy Leydig cells would continue to produce testosterone following isolation, while less healthy Leydig cells would not. Overall there was no significant effect of post-isolation synthesis of testosterone. Therefore the effect of this on the results of the present study are unclear.

In conclusion, the response of the human testis to ageing is highly variable. While age is a significant correlate of DSP, Leydig cell number, testosterone and gonadotrophin levels, its predictive value for individuals is weak. The lack of any significant relationship between Leydig cell number and testosterone in the decline of DSP suggests that more significant overriding relationships must exist. Indeed the importance of high intratesticular levels of testosterone in the maintenance of spermatogenesis in both rat and man is now being questioned (Rommerts, 1987), and it must be considered that in both species the Leydig cells may produce testosterone in excess of that required to maintain spermatogenesis.

CHAPTER 4

Isolation of Human Leydig Cells and Analysis of their Function in vitro

4.1 Introduction

The effects of LH and human chorionic gonadotrophin (hCG) on rat Leydig cell LH/hCG receptor number and steroidogenesis in vitro have been widely investigated (Catt et al. 1980). The limited information available from in vitro studies in the human has suggested that human Leydig cells may be inherently less responsive to hCG than are rat Leydig cells (Huhtaniemi et al. 1982). Furthermore, the response of the human testis to hCG in vivo is characterized by a rapid but modest (20-60%) increase in serum levels of testosterone within 6 h of intramuscular injection (Saez & Forest, 1979; Martikainen et al. 1980; Padron et al. 1980). In contrast, in the rat, serum testosterone levels are increased by 10- to 20-fold over the same time period after hCG injection (Hsueh et al. 1976; Chasalow et al. 1979). This difference, which has not yet been explained satisfactorily, has led to the belief that the function of human Leydig cells may be very different from that in the rat.

The different approaches employed to study Leydig cell function in vitro, in both the rat and man, has made it difficult to compare results between the two species. The introduction of density centrifugation techniques has permitted the isolation of rat Leydig cell populations which are highly responsive to hCG (Sharpe & Fraser, 1983). In the present study, such techniques were used to develop a procedure for the isolation of human Leydig cells. Testicular tissue was obtained from ten men aged between 57-85 years who were undergoing orchidectomy for prostatic carcinoma. Leydig cells were isolated as described in Section 2.4, and compared to rat Leydig cells in terms of fractionation characteristics, cell yield and responsiveness to hCG in vitro.

4.2 (i) Pattern of Leydig cell fractionation on gradients of Percoll

When loaded onto discontinuous Percoll gradients, both rat and human testicular cells fractionated into three bands, corresponding to the interfaces between successive layers of Percoll of different densities (Fig. 4.1). In the human, band 1 occurred at the interface between 1.03 and 1.05 and was the most intense cell band as 95-97% of the added cells migrated to this band. Band 2 occurred at the interface between 1.05 and 1.07 and 2-4% of cells migrated to this band. Band 3 occurred at the interface between 1.07 and 1.09 and only 0.7-1.5% of cells migrated to this band. In the rat, although cell bands 1-3 formed in exactly the same positions as in the human, a different fractionation pattern of the added testicular cells was observed with 58-65% migrating to band 1, 29-33% to band 2 and 6-9% to band 3.

In both the human and the rat band 1 contained 12-28% Leydig cells as determined by 3β-HSD staining, and 77-95% (human) and 38-46% (rat) of all Leydig cells were retained in this band. In the human, band 2 cells showed the highest percentage of 3β-HSD positive cells (48-70%) with 4-18% of all Leydig cells being retained in this band. In the rat only 30-45% of band 2 cells stained positively for 3β-HSD and 37-40% of all Leydig cells were retained in this band. Band 3 was particularly enriched in Leydig cells (75-90%) in the rat with 15-22% of all Leydig cells migrating to this band while in the human 30-56% of band 3 cells stained positively for 3β-HSD and only 1-6% of all Leydig cells migrated to this band.

After 20 h incubation, band 1 cells from both the rat and man showed only a 1.5- to 2.5-fold response to hCG and made very low amounts of testostrone (Fig. 4.1). In the rat, Leydig cells from band 3 always secreted more testosterone and were more responsive to hCG (8.5-fold

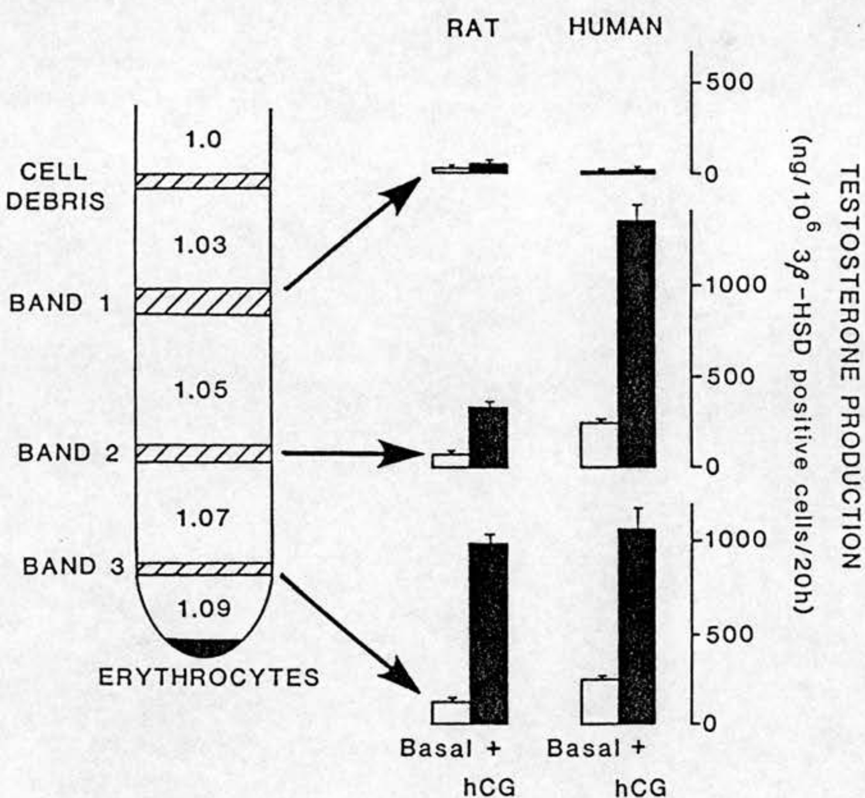


FIGURE 4.1

Left hand panel - Schematic representation of the pattern of fractionation of rat and human testicular cells on discontinuous gradients of Percoll using successive layers of Percoll of density 1.09, 1.07, 1.05, 1.03 and 1.0 g/ml. Right hand panel - Basal and maximal hCG (20 IU / ml) - stimulated testosterone production (ng/10^6 3ß-HSD positive cells per 20h) for representative rat and human testicular cells obtained from cell bands 1 - 3. Values are mean ± SD of triplicate incubations.

stimulation) than were cells from band 2 but in the human, cells from bands 2 and 3 were generally comparable, both in terms of the amounts of testosterone secreted and the magnitude of their response (4.5- to 13-fold) to hCG (Fig. 4.1 and Table 4.1). In view of this finding coupled with the high yield of Leydig cells in band 2, for most subsequent studies human Leydig cells from bands 2 and 3 were combined.

(ii) Leydig cell yield

For the six subjects from whom Leydig cells were prepared the total yield of 3β-HSD positive cells for bands 1, 2 and 3 were 10.6-16.6, 0.7-5.7 and 0.3-1.9 x 10^6, respectively, while the total Leydig cell yield (i.e. bands 1,2 & 3) ranged from 11.6 - 24.2 x 10^6. From morphometric studies (Neaves et al. 1984) aged human testes contain approximately 200 x 10^6 Leydig cells which means that in the current studies approximately 6-12% of the total number of Leydig cells were isolated, while the number of highly responsive Leydig cells (i.e. bands 2 & 3) constituted only 0.5-4% of the total number present in the testes. These values differ somewhat from those obtained for the rat in that 10.6-22.1, 11.2-19.0 and 6.3-7.2 x 10^6 Leydig cells were isolated in bands 1, 2 and 3 respectively, from a total of 10 testes in several experiments. As adult rat testes contain approximately 45 x 10^6 Leydig cells (Mori & Christensen, 1980) this means that 6.2-10.7% of all Leydig cells are recovered on the Percoll gradient with the highly responsive cells (i.e. those in band 3) constituting 1.4-1.6% of the total present in the testes.

In two experiments an attempt was made to increase Leydig cell yield further by re-digesting testicular tissue with collagenase after the initial isolation procedure. Cells from this second dispersion fractionated identically to those from the first dispersion although the

Table 4.1 Basal and hCG (20 IU/ml)-stimulated testosterone production by Percoll-purified human Leydig cells obtained from cell bands 2 and 3.

Incubation additions	Testosterone secretion (ng/10^6 3β-HSD positive cells/20h)		
	Band 2	Band 3	P
Basal	234 ± 6	246 ± 12	N.S.
hCG	1342 ± 98	1066 ± 110	N.S.
Basal	136 ± 14	182 ± 7	$P < 0.02$
hCG	642 ± 52	875 ± 46	$P < 0.02$
Basal	81 ± 3	101 ± 38	N.S.
hCG	1052 ± 107	1008 ± 39	N.S.

Values are the mean ± S.D. of triplicate incubations, using cells prepared from 3 different men.
P values refer to comparison of respective values for bands 2 and 3.

total number of Leydig cells obtained in bands 2 and 3 was only 40-50% of that obtained in the first dispersion. Somewhat surprisingly, both band 2 and 3 Leydig cells from the second dispersion produced significantly more testosterone than did cells from the first dispersion, and this was equally evident under both basal and hCG-stimulated conditions (Table 4.2).

(iii) Variation in Leydig cell responsiveness between men and its relationship to hormone levels and testicular morphology

Basal testosterone production and the maximal response to hCG was determined for Percoll-purified Leydig cells from a total of 10 men. The percentage of 3β-HSD positive cells was determined for only 6 of these men and the results are shown in Table 4.3; for the remaining 4 men the magnitude of the Leydig cell response to hCG was similar to that shown in Table 4.3 except that in 3 of the 4 men the amounts of testosterone produced per 10^6 nucleated cells was low as shown for patient 1.

At 5 h basal testosterone values ranged from 6-63 ng/10^6 3β-HSD positive cells, and by 20 h this parameter was increased to reach 44-234 ng/10^6 3β-HSD positive cells, values which were comparable to those obtained for Leydig cells from young adult rats (Table 4.3).

At 5 h human Leydig cells responded to hCG by increasing testosterone production between 4- and 12-fold. The response to hCG at 20 h varied from 5- to 17-fold (Table 4.3). This range and the absolute amounts of testosterone produced (up to 1342 ng/10^6 3β-HSD positive cells) were in general very similar to the values found for rats (up to 1463 ng/10^6 3β-HSD positive cells), although patient 1 (basal value = 44 ng, hCG stimulated = 171 ng) had clearly lower testosterone responses in vitro. As mentioned above, low testosterone values (basal = 4-12

Table 4.2 Basal and hCG (20 IU/ml)-stimulated testosterone production (ng/10^6 3β-HSD positive cells per 20h) by cell bands 2 and 3 from Percoll gradients. Results are shown for two men using cells obtained from two successive collagenase dispersions.

	Percoll	Basal Conditions		hCG-stimulated	
Patient	cell band	Dispersion 1	Dispersion 2	Dispersion 1	Dispersion 2
1	2	81 ± 3	130 ± 7 ***	1052 ± 107	1998 ± 260 **
	3	101 ± 38	192 ± 11 *	1009 ± 39	1645 ± 78 **
2	2	135 ± 1	109 ± 9 **	984 ± 64	620 ± 7 ***
	3	71 ± 8	356 ± 26 ***	581 ± 79	1891 ± 137 ***

Values are the mean ± S.D. of triplicate measurements.

* $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$, in comparison with respective values for dispersion 1.

Table 4.3 Summary of basal and hCG (20 IU/ml)-stimulated testosterone production by Percoll-purified human Leydig cells (bands 2 + 3) from 6 men in relation to age, mean testis weight and the plasma T, LH and FSH levels.

Patient no.	Testis wt. (g)	Age (yr)	Plasma hormone level: T (ng/ml)	LH (mIU/ml)	FSH (mIU/ml)	Testosterone production (ng/10^6 3β-HSD positive cells): 5h incubation Basal	5h incubation hCG	20h incubation Basal	20h incubation hCG
1	17.5	76	2.1	11.0	14.0	6 ± 1	45 ± 6	44 ± 0	171 ± 8
2	15.4	73	2.5	10.4	5.3	63 ± 15	361 ± 62	103 ± 36	782 ± 230
3	17.0	79	5.5	3.9	12.1	28 ± 3	346 ± 35	90 ± 26	1028 ± 83
4	15.7	71	2.3	3.7	3.6	-	-	234 ± 10	1342 ± 170
5	33.3	68	2.5	15.6	10.6	-	-	80 ± 9	1330 ± 181
6	27.5	77	-	-	-	-	-	160 ± 27	764 ± 135

Values for testosterone production are the mean $\pm$ S.D. of triplicate incubations. At both 5 and 20h in all men, hCG-stimulated testosterone production was significantly increased ($P < 0.001$) above basal values.
For reference purposes, values obtained at 20h for 6 successive preparations of Percoll-purified Leydig cells from the rat gave the following values (mean $\pm$ S.D.) – Basal 139 ± 52 (range 86 - 235), hCG 1036 ± 329 (range 698 - 1463).

$ng/10^6$ nucleated cells; hCG = 24-42 $ng/10^6$ nucleated cells) were also found at 20 h in 3 other men for whom the percentage of Leydig cells was not determined. Men with poorly responsive Leydig cells in vitro could not be distinguished from those whose Leydig cells made large amounts of testosterone on the basis of age, testicular weight or the plasma levels of LH, FSH or testosterone (Table 4.3).

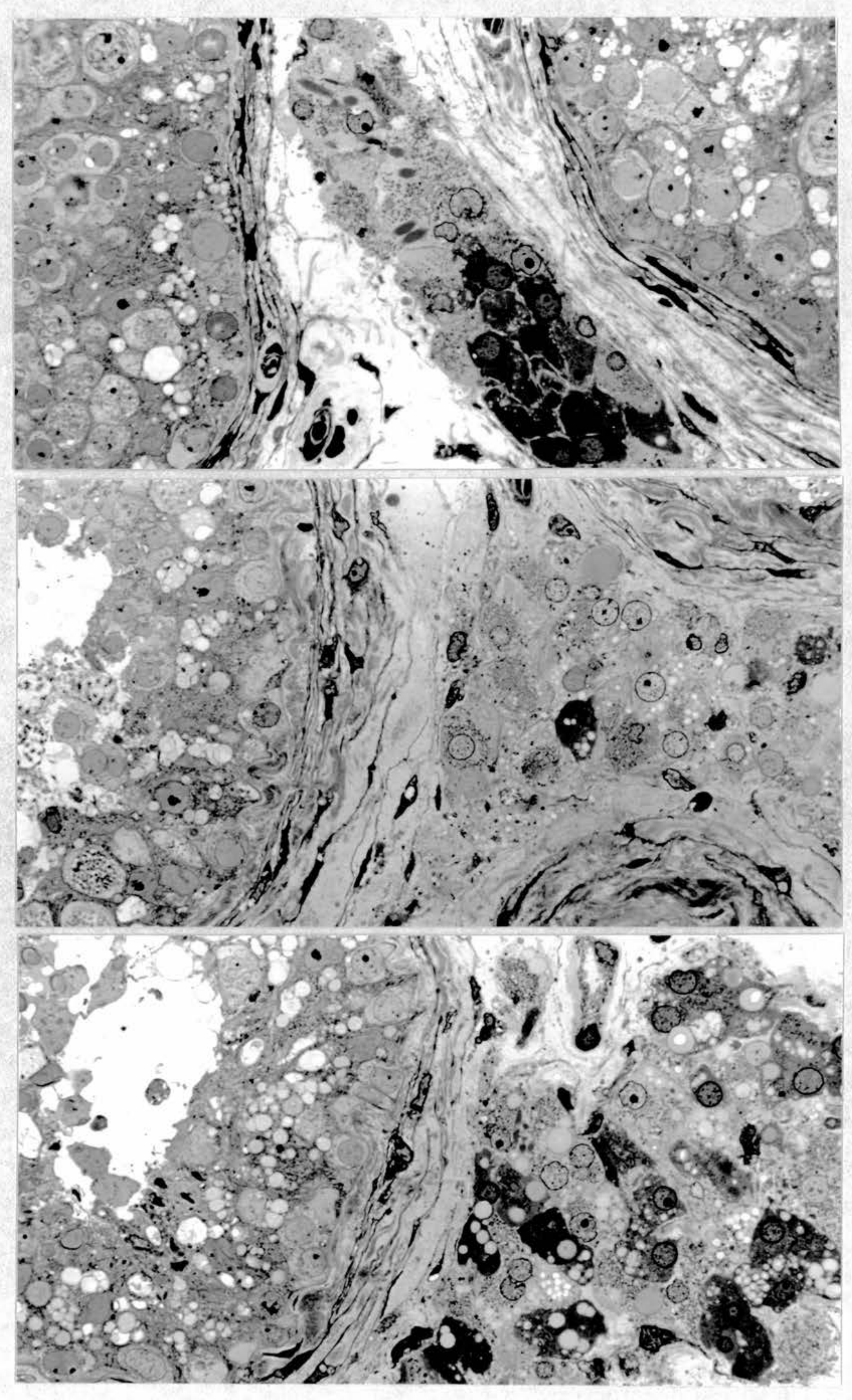
Testicular morphology was examined in all patients in order to ascertain whether the differences between men in the capacity of their Leydig cells to make testosterone in vitro could be related in any way to morphological criteria. In all patients testicular morphology showed the Leydig cell population to be heterogenous with small and large Leydig cell clusters consisting of either pale or darkly staining cells or a mixture of both. Although it is emphasised that no quantitative measurements were made on these sections, it appeared that highly responsive Leydig cell preparations were isolated from testes which showed clusters of light and darkly staining Leydig cells (Fig. 4.2). In contrast to this, unresponsive preparations were isolated from testes which appeared to have low numbers of darkly staining cells which contained large accumulations of lipid (Fig. 4.2).

(iv) Dose response to hCG and Leydig cell hCG-receptors

The sensitivity of human Leydig cells to hCG was assessed from detailed dose response curves (Fig. 4.3). That shown for rat Leydig cells is typical of that found routinely in our laboratory and showed that the dose response curve covered doses of hCG ranging from 0.03 to 0.5 mU/ml; addition of higher concentrations of hCG did not alter the maximum response. In contrast, for three representative does response curves from human Leydig cells there was a large shift to the right. Thus, the dose-response to hCG covered the range 0.4 to 100 mU/ml,

FIGURE 4.2

Representative light micrographs of Leydig cells which were highly responsive (top) and less responsive (middle and bottom) to hCG. Highly responsive Leydig cells were isolated from testes which contained a mixture of dark and pale staining Leydig cells. Less responsive Leydig cells were isolated from testes which contained a higher proportion of pale staining cells and any dark staining cells, when present, exhibited large lipid accumulations. Magnification x 650.



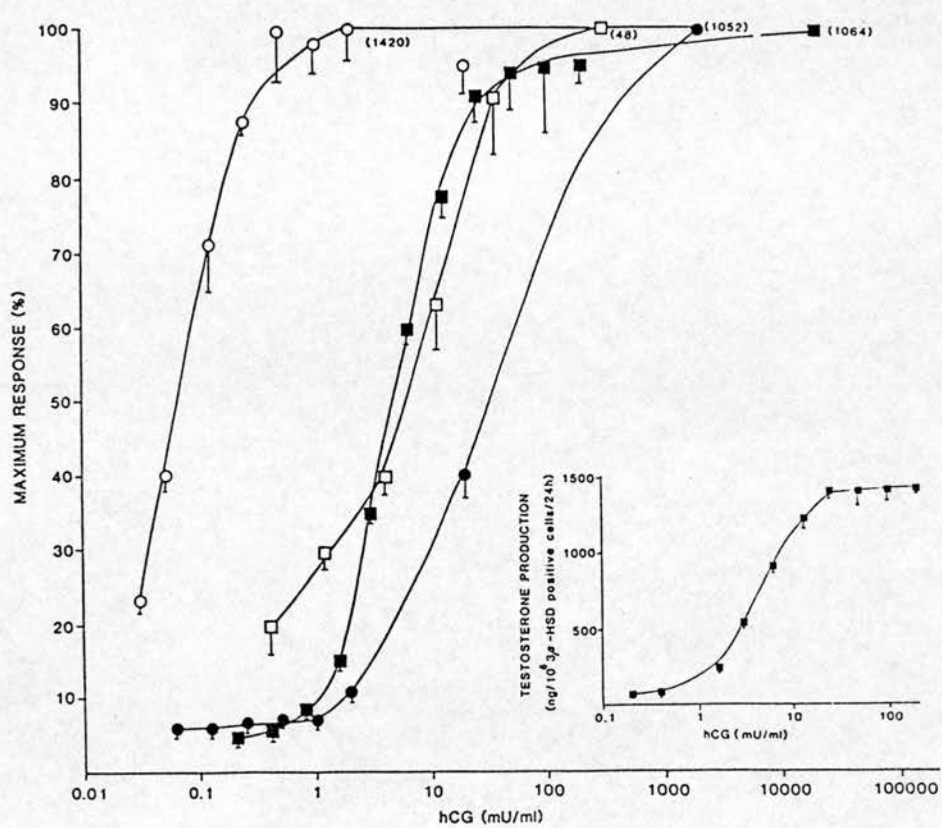


FIGURE 4.3

Comparison of the dose response curves to hCG for Percoll - purified rat and human Leydig cells. Values were obtained from a representative rat Leydig cell preparation (○) and from three human Leydig cell preparations which showed 17-fold (■), 13-fold (●) or 6-fold (□) responses to maximally stimulating levels of hCG. As the maximal testosterone responses for these cell preparations differed, each dose response to hCG has been expressed as a percentage of the maximal response i.e. to 20 IU hCG/ml. The latter values, expressed as ng/10^6 3ß-HSD positive cells, are shown in parentheses. Inset shows the full dose response curve for the most responsive human Leydig cell preparation. Each point is the mean ± SD of triplicate incubations.

indicating a 10- to 100-fold reduction in sensitivity compared to rat Leydig cells (Fig. 4.3).

In view of this difference in sensitivity, the number and affinity of Leydig cell LH (hCG)-receptors was assessed from Scatchard analysis of ^{125}-hCG binding. From this study, purified human Leydig cells contained approximately 5-fold fewer LH (HCG)-receptors than did purified rat Leydig cells, while the affinity of receptors (Kd$\sim$ 10^{-10}M) was comparable for both cell preparations (Fig. 4.4).

4.3 Discussion

In this study a method for the isolation of highly responsive human Leydig cells was established. In terms of testosterone output and responsiveness to hCG, human Leydig cells were in general comparable to those of the rat, suggesting that the function of human Leydig cells may be more similar to those of the rat than has been thought previously.

Leydig cells from both the human and the rat separated into three bands when run on discontinuous Percoll gradients. In both species, band 1 contained 12-28% Leydig cells as identified by 3β-HSD staining, but whilst band 3 was the most Leydig cell-enriched fraction in rat cell preparations (75-90% Leydig cells), in the human band 2 (48-70% Leydig cells) was consistently more Leydig cell-enriched than was band 3 (30-56% Leydig cells). However, in contrast to the rat, human Leydig cells from bands 2 and 3 were very similar in terms of their response to hCG and the amount of testosterone secreted per 10^6 Leydig cells. The reason for the difference in separation characteristics of rat and human Leydig cells is not known, but may be due to differences in cytoplasmic content of lipid or organelles. Human Leydig cells usually contain large numbers of lipid droplets (Christensen, 1975), whereas normal adult rat Leydig cells rarely contain such inclusions (Clegg, 1961).

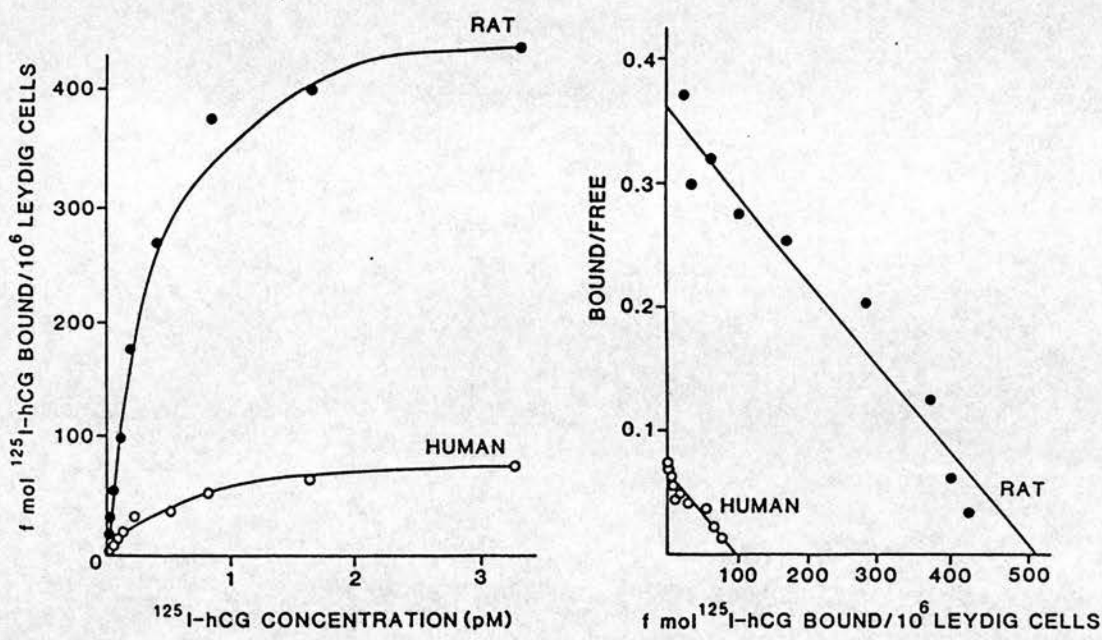


FIGURE 4.4

Left hand panel - Specific binding of ^{125}I-hCG to human and rat Leydig cells with increasing concentrations of ^{125}I-hCG. Right hand panel - Scatchard plot of the same data.

The presence of lipid droplets would be expected to decrease the buoyant density of Leydig cells with the result that they would fractionate higher up the gradient during separation on Percoll. Repeated attempts to examine the morphology of the 3 bands obtained following Percoll separation of human testicular cells were however unsuccessful.

With the exception of their somewhat different pattern of fractionation on Percoll, human Leydig cells were in most instances very similar to those from the rat, both in terms of the amount of testosterone produced per 10^6 cells and in terms of the magnitude of their response to hCG. This finding contrasts with what has been generally accepted until now, namely that human Leydig cells are poorly responsive in vitro and in vivo when compared to the rat (Saez & Forest, 1979; Smals et al. 1979; Martikainen et al. 1980; Nankin et al. 1980; Padron et al. 1980; Huhtaniemi et al. 1982). Previous studies in vitro have generally used either testis pieces (Nieschlag et al. 1979) or crude cell preparations, the latter being prepared using higher concentrations of collagenase and longer dispersion periods, both of which will severely reduce the responsiveness of rat Leydig cells (R.M. Sharpe, personal communication). It is therefore likely that the combination of gentler preparative conditions coupled with Leydig cell purification account for the differences between the present and previous findings. There remains the possibility that human Leydig cells from bands 2 and 3 after Percoll separation represent a selected sub-population of highly responsive cells, and indeed the same may be true for rat Leydig cells isolated on Percoll. The obvious heterogeneity of Leydig cells in fixed tissue from human testes (see Fig. 4.2 and below) could be taken as evidence in favour of this possibility, although this would fail to explain why some of the

purified Leydig cell preparations from patients in our study made small amounts of testosterone despite fractionating identically to highly responsive cell preparations. This variation could not be linked to any of the systemic endocrine parameters or to testicular weight, confirming the findings of Huhtaniemi et al. (1982) and Nieschlag et al. (1979). However, qualitative morphological assessment of fixed tissue suggested that there may have been inherent differences in the Leydig cells, such that poorly responsive cell preparations derived from testes in which the ratio of darkly staining to lightly staining Leydig cells was low in comparison to testes generating highly responsive Leydig cells (see Fig. 4.2). These 'dark' and 'pale' Leydig cells are a well-recognized phenomenon in the human testis, and Christensen and Gillim (1969) have suggested that the different staining intensities represent artifacts resulting from immersion fixation. A more recent study (Schulze, 1984) suggests that this is not the case, since dark and pale Leydig cells were observed also in perfusion-fixed human testes. Detailed morphology of the cells in bands 2 and 3 will be required to determine whether the dark and pale cells predominate in particular bands, and whether there is any difference in steroidogenic capacity between the two cell types. In fixed tissue, morphometric studies will be necessary to confirm the impression that there may be a relationship between the relative number of dark or pale Leydig cells and the subsequent steroidogenic capacity of purified Leydig cells in vitro.

Although the magnitude of response to hCG of most human Leydig cell preparations was comparable to that for the rat, human Leydig cells were 10- to 100-fold less sensitive to hCG stimulation. This difference in sensitivity may have been due to the 80% fewer LH-receptors on human, as opposed to rat Leydig cells. These results are comparable to findings

of previous studies using testicular homogenates (Huhtaniemi et al. 1982), or isolated human Leydig cells (Davies et al. 1979), and lessens the possibility that this difference results from the artifactual removal of LH-receptors during collagenase digestion, an event known to occur during the isolation of human luteal cells (Bramley et al. 1985). In this respect Huhtaniemi et al. (1982) reported that prolonged (30 min) collagenase dispersion of human testicular tissue had no significant effect on LH-receptor number.

The testes used in this, and most previous studies, were obtained from ageing men who generally have raised serum levels of LH (Harman & Tsitouras, 1980; Takahashi et al. 1983). This might account for the decreased LH-receptor number, as it is well established that in both rats (Catt et al. 1980) and men (Sharpe et al. 1980), systemic administration of LH or hCG will lead to the down-regulation of Leydig cell LH-receptors. The latter change is not usually associated with any diminution of the testosterone response to excess LH or hCG in vitro but does result in a clear shift to the right of the dose response curve to hCG (Catt et al., 1980; Sharpe & McNeilly, 1980), a change also evident in the present studies. Contrary to this reasoning, in a study by Leinonen et al. (1982) no correlation was found between serum LH levels and the number of LH binding sites in testes obtained from men with prostatic carcinoma. In addition, our limited number of dose response curves to hCG would not support such a relationship, as the least sensitive human Leydig cell preparation (Patient 1, Fig. 4.3) derived from a patient with normal serum levels of LH (Table 4.3).

The response of both the human and rat testis to hCG in vivo is characterized by a biphasic increase in testosterone production (Hsueh et al. 1976; Chasalow et al. 1979; Saez & Forest, 1979; Smals et al.

1979; Martikainen et al. 1980; Padron et al. 1980). Testosterone levels reach a peak within a few hours of intramuscular injection of hCG after which they return to normal prior to the delayed response 2-3 days later. The delayed response is of the same magnitude in both man and the rat (2- to 3-fold increase), and is thought to be due to hCG-induced hypertrophy and hyperplasia of the Leydig cells. The acute response however, seen within 6 h of hCG administration, differs greatly between man and the rat. Martikainen et al. (1982) showed by measuring spermatic vein and systemic steroid concentrations, that the human testis was able to respond to hCG within 30 min although the magnitude of testosterone rise at this time interval was under 2-fold. In contrast, in the rat, serum testosterone levels are increased by 10- to 20-fold within 6 h of hCG injection (Hsueh et al. 1976; Sharpe, 1976). This difference in the acute response in vivo contrasts with the similarity in response of purified Leydig cells from the rat and man in vitro. This discrepancy between in vitro and in vivo findings is not fully understood but may be related to differences between man and rat in terms of the relative number of Leydig cells to blood volume.

From the morphometric analysis of Mori and Christensen (1980) it can be calculated that in a normal rat there are approximately 3×10^6 Leydig cells per ml of blood, while in aged men there are only approximately 0.056×10^6 Leydig cells per ml blood (using the morphometric data of Neaves et al. 1984). Therefore since the rat has approximately 50-fold more Leydig cells per ml of blood than does the human, it is not surprising that the in vivo response to hCG, as measured by peripheral testosterone concentration, in the rat is much greater than in man despite the fact that in vitro the respone to hCG is similar in both species on a per-cell basis. For the human male to

maintain testosterone in serum at a level comparable to that in the rat, this would necessitate either increasing Leydig cell mass 50-fold or retarding clearance of testosterone. This may perhaps explain why man, but not the rat, has a serum testosterone-binding globulin (TeBG). TeBG has been found in the blood of several species (Corvol & Bardin, 1973; Bardin et al. 1981) and while its role has never been fully understood it is clear that it retards the metabolic clearance of testosterone. This suggestion is speculative, but it is of interest that animals such as the ram do have a serum testosterone-binding globulin (Corvol & Bardin, 1973; Bardin et al. 1971), but of lower affinity than in man (i.e. less effective in prolonging the half life of testosterone) and, from morphometric data (Hochereau-de Reviers et al. 1985), these animals have an estimated Leydig cell to blood volume ratio of 0.4×10^6 cells/ml, a figure which is intermediate between that of the rat and man.

These results suggest that the in vitro testosterone response to hCG of human Leydig cells may be more similar to that of the rat than has been thought previously, which could mean that the extensive findings from in vitro studies of rat Leydig cells may have direct relevance to the human. The establishment of this technique for the isolation of human Leydig cells has enabled the study of putative paracrine control mechanisms in the human testis (Chapter 5), and more importantly, raises several exciting possibilities for future research.

CHAPTER 5

Paracrine Control of the Human Leydig Cell

5.1 Introduction

In the rat there is now a substantial body of evidence to suggest that Leydig cell function is under the dual control of both the endocrine system, by LH from the anterior pituitary, and the paracrine system, by a number of factors produced locally within the testis. In vitro studies have shown the effects of putative paracrine factors such as testicular LHRH, oxytocin, vasopressin, opiates and a factor(s) present in Sertoli cell/seminiferous tubule conditioned medium and testicular interstitial fluid on Leydig cell testosterone production. However the techniques applied to these studies, such as the stage dissection of seminiferous tubules and the isolation of both Sertoli and Leydig cells, have been limited to experimental animals, and similar studies on the human testis are lacking.

The opportunity to study the effect of putative paracrine factors on human Leydig cell function has now arisen with the development of a reliable method for the isolation of human Leydig cells. In this study the effects of LHRH-A, vasopressin, human transforming growth factor β (TGFβ) and interstitial fluid factor on human Leydig cell function were investigated by the measurement of testosterone production.

5.2 (i) Effects of putative paracrine hormones on Leydig cell function

The effects of luteinizing hormone releasing hormone-agonist (LHRH-A), vasopressin and transforming growth factor β (TGFβ) on basal and hCG (20 IU/ml)-stimulated testosterone production by Percoll-purified human Leydig cells were studied at 5,20 and 48 h of incubation.

LHRH-A ([D-Ser(Bu^t)^6] des Gly NH_2^{10} LHRH-ethylamide; Hoechst A.G., Frankfurt, F.R.G.; 10^{-6}M) had no significant effect on basal or hCG-stimulated testosterone production at 5,20 or 48 h in

Leydig cell preparations from 9 individual men (Fig. 5.1). In 2 of these preparations the addition of LHRH-A ([D-Ala^6 Nα MeLeu^7 Pro^9 NEt] LHRH, Dr. R. Milton, Dept. of Chemical Pathology, University of Cape Town, South Africa; 10^{-5}M) produced identical results (Fig. 5.2).

The addition of vasopressin (Sigma, UK; 10^{-6}M) to each of 7 Leydig cell preparations failed to alter basal or hCG-stimulated testosterone production at 5,20 or 48 h (Fig. 5.3). Similar results were obtained following the addition of TGFβ (Dr. A. Roberts, NIH; 10^{-8}M) to each of 4 Leydig cell preparations (Fig. 5.4).

(ii) <u>Effects of testicular interstitial fluid and serum on Leydig cell testosterone secretion in vitro</u>

The effect of charcoal-extracted testicular interstitial fluid (IF) and serum from both the rat and man on rat and human Leydig cell steroidogenesis during 20 h of incubation was tested in the presence of hCG (20 IU/ml) at a dose in excess of that required to maximally stimulate testosterone production.

Using Percoll-purifed preparations of rat Leydig cells, the addition of rat IF consistently enhanced Leydig cell testosterone production in response to maximally stimulating levels of hCG ($P < 0.001$), whereas human IF was without effect (Fig. 5.5). When added to one preparation of human Leydig cells, rat IF was shown to stimulate testosterone production in a dose-dependent manner (Fig. 5.6), but this result was not consistent. Fig. 5.7 shows the reverse effect of rat IF on human Leydig cell testosterone production. In this case the highest dose of rat IF (50 μl) did not significantly increase testosterone levels above those obtained with hCG alone, but did at a dose of 25 μl ($P < 0.05$) and also 12.5 μl ($P < 0.001$). The dose-dependent stimulatory

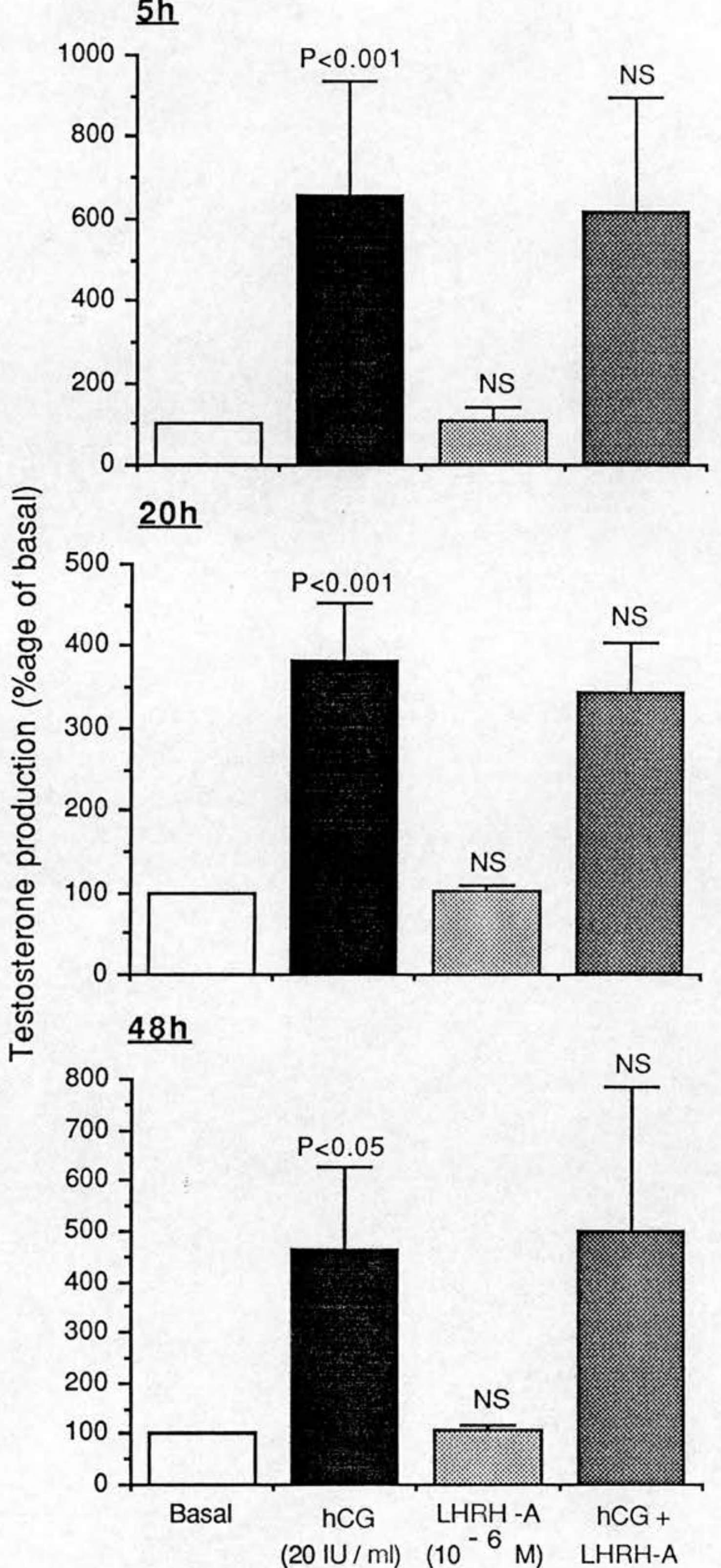


FIGURE 5.1

Effect of LHRH-A ([D-Ser(Bu^{t} $)^{6}$] des Gly NH_2^{10} LHRH-ethylamide on human Leydig cells in vitro. Each value is the mean ± SD of 9 Leydig cell preparations, and is expressed as percentage of basal production. hCG - stimulated testosterone production was significantly increased compared to basal levels, P<0.001, P<0.05. LHRH-A did not stimulate testosterone production above basal or hCG - stimulated levels.

Basal

hCG (20 IU / ml)

LHRH-A 1 ([D-Ser (Bu^t) ^{6}] des Gly NH$_2$^{10} LHRH-ethylamide) (10 $^{-6}$ M)

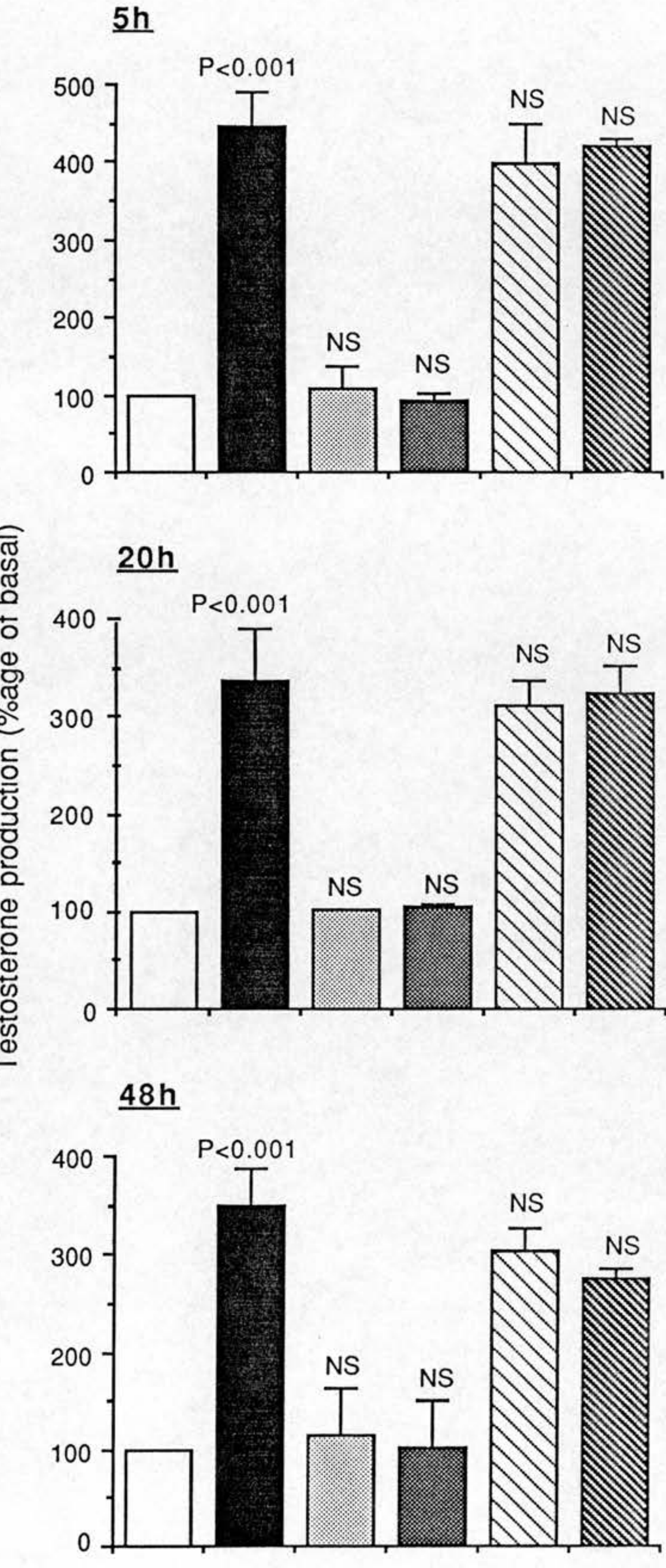
LHRH-A 2 [D-Ala^6 N$^\alpha$ Me Leu^7 Pro^9 N Et LHRH] (10 $^{-5}$ M)

hCG + LHRH-A 1

hCG + LHRH-A 2

FIGURE 5.2

Comparison between the effects of two LHRH - agonists on the production of testosterone by human Leydig cells in vitro. Each value is the mean ± SD of 2 Leydig cell preparations, and is expressed as percentage of basal production. hCG - stimulated testosterone production was significantly increased compared to basal levels, P<0.001. Neither of the two LHRH - agonists stimulated testosterone production above basal or hCG - stimulated levels.


5h
P<0.001
NS
NS
NS
NS
500
400
300
200
100
0
20h
P<0.001
NS
NS
NS
NS
400
300
200
100
0
48h
P<0.001
NS
NS
NS
NS
400
300
200
100
0
Testosterone production (%age of basal)

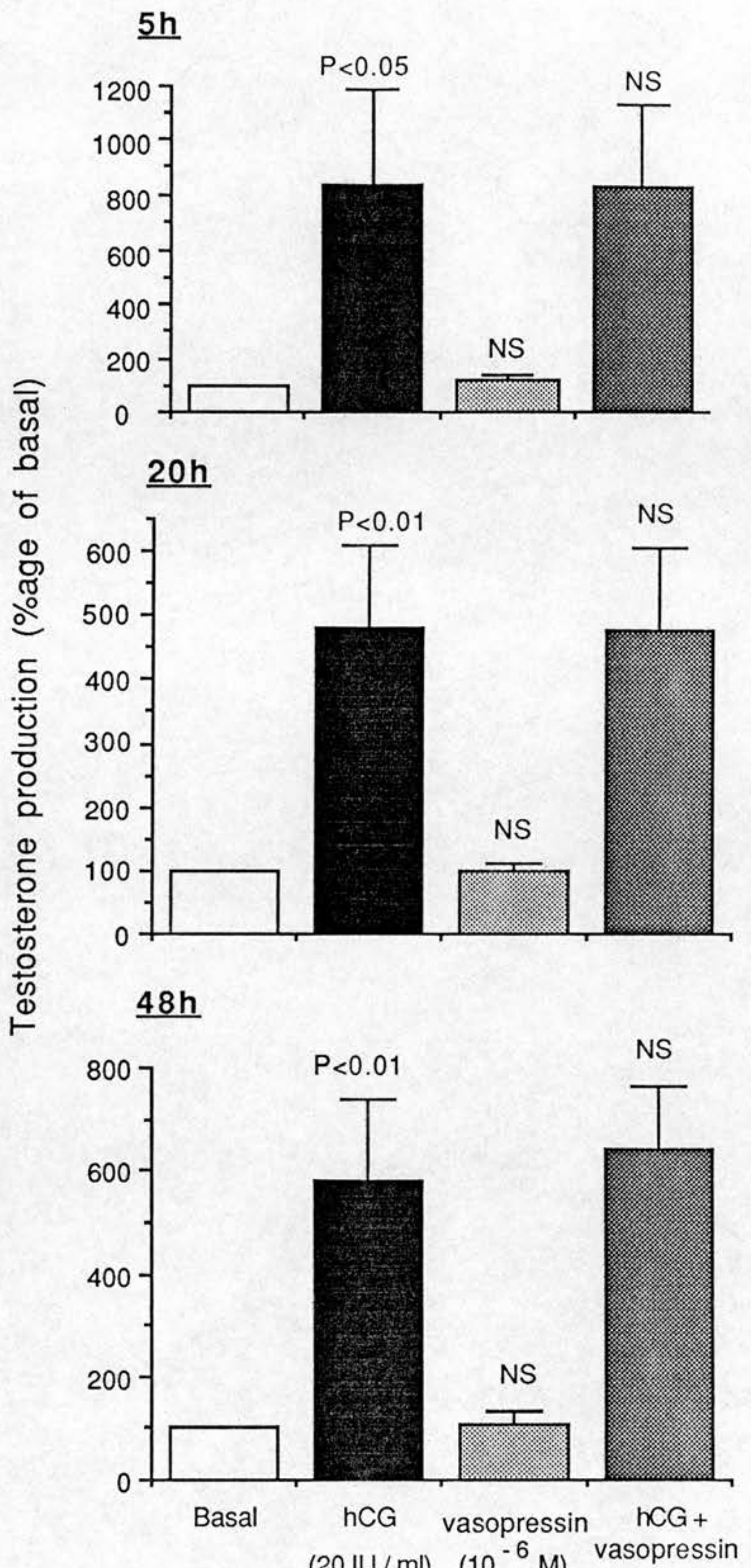


FIGURE 5.3

Effect of vasopressin on human Leydig cells in vitro. Each value is the mean ± SD of 7 Leydig cell preparations, and is expressed as percentage of basal production. hCG - stimulated testosterone production was significantly increased compared to basal levels, $P<0.05$, $P<0.01$. Vasopressin did not stimulate testosterone production above basal or hCG - stimulated levels.

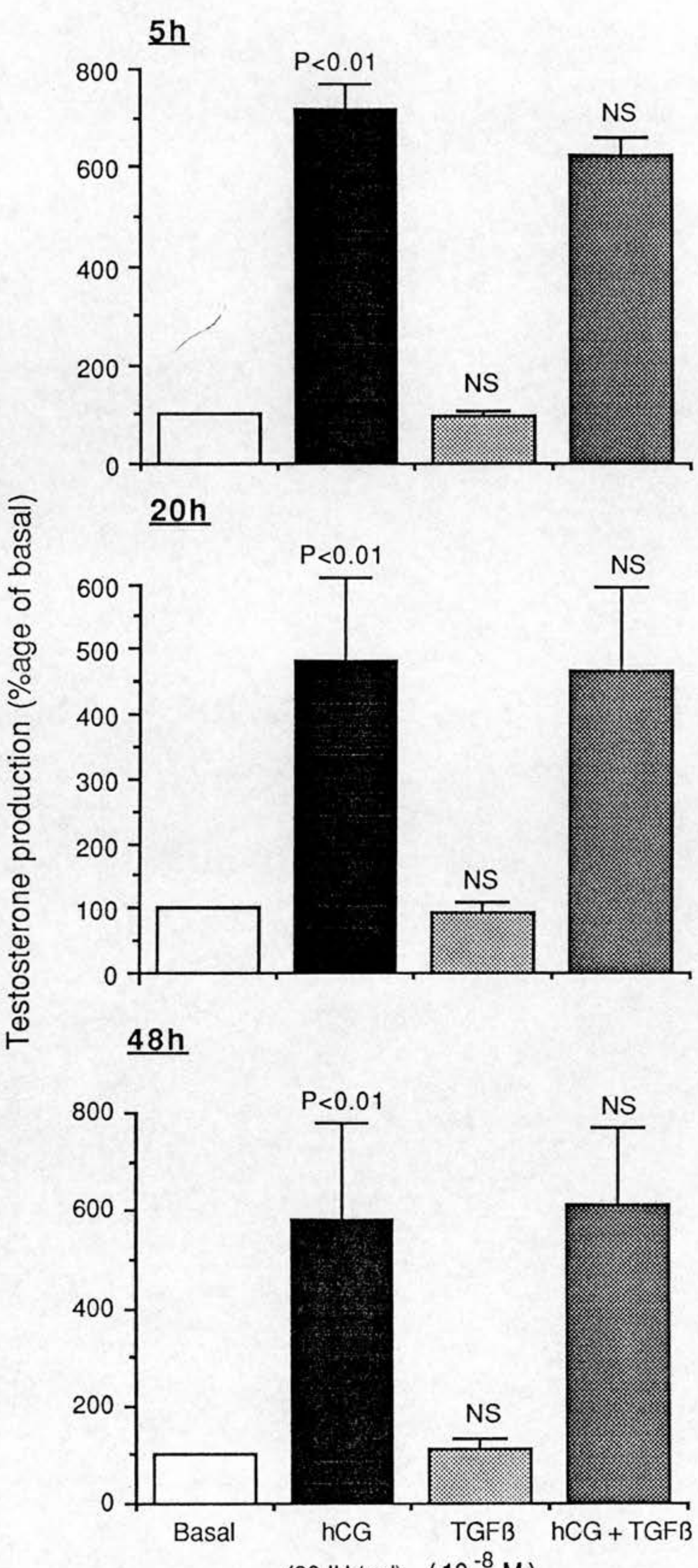


FIGURE 5.4

Effect of TGFß on human Leydig cells in vitro. Each value is the mean ± SD of 4 Leydig cell preparations, and is expressed as percentage of basal production. hCG - stimulated testosterone production was significantly increased compared to basal levels, P<0.01. TGFß did not stimulate testosterone production above basal or hCG - stimulated levels.

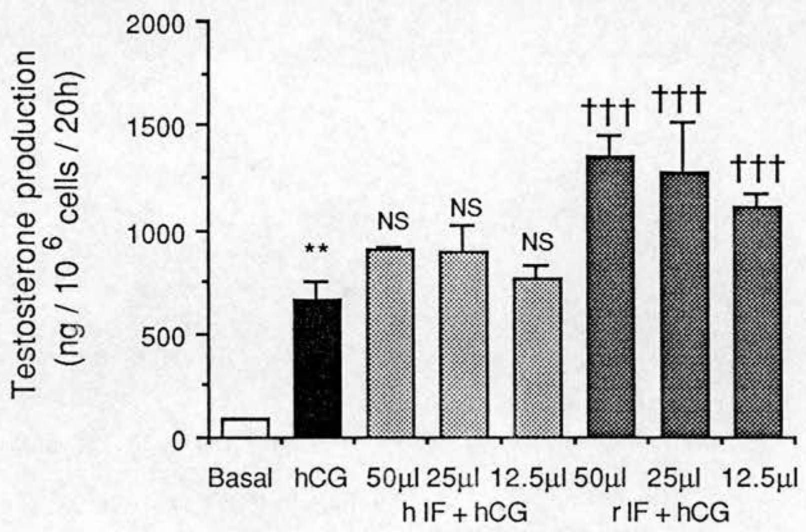


FIGURE 5.5

Effect of human and rat testicular interstitial fluid (IF) on rat Leydig cell testosterone production in vitro. hCG (20 IU / ml) - stimulated testosterone production was significantly increased compared to basal levels ** P<0.01. Rat IF significantly increased Leydig cell testosterone production above the level obtained with a maximally stimulating dose of hCG ††† P<0.001. Human IF did not significantly enhance hCG - stimulated testosterone production (NS). Values are the mean ± SD of triplicate incubations.

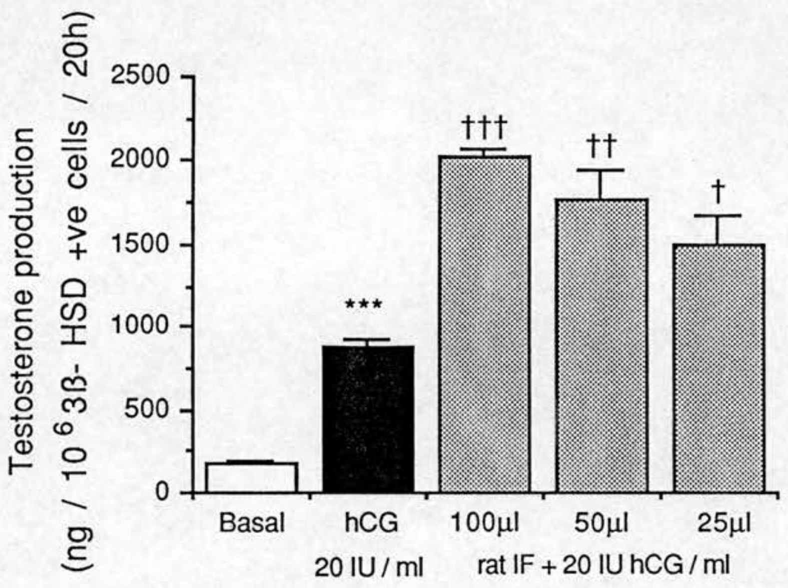


FIGURE 5.6

Stimulatory effect of rat interstitial fluid (IF) on human Leydig cell testosterone production in vitro. hCG - stimulated testosterone production was significantly increased compared to basal levels,*** P<0.001. Testosterone production was increased significantly in the presence of IF compared to hCG - stimulated testosterone production alone, ††† P<0.001, †† P<0.01, † P<0.05. Values are the mean ± SD of triplicate incubations.

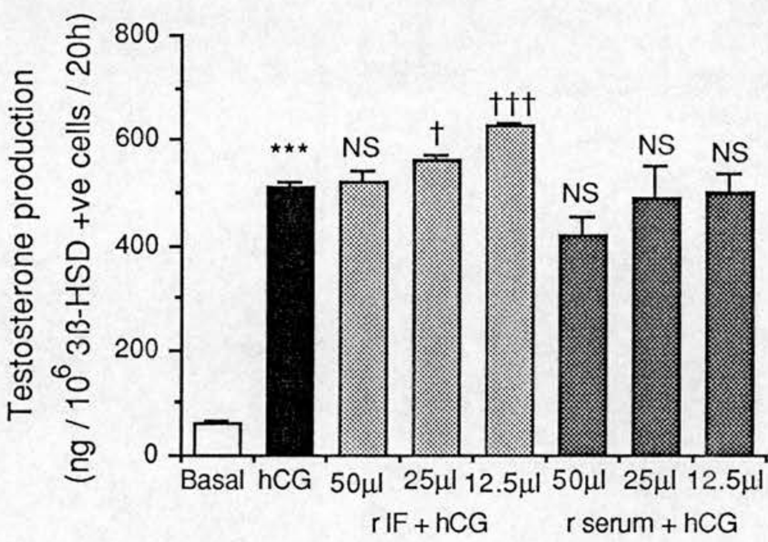


FIGURE 5.7

Effect of rat interstitial fluid (IF) and rat serum on human Leydig cell testosterone production in vitro. hCG (20 IU / ml)-stimulated testosterone production was significantly increased compared to basal levels,*** P<0.001. Testosterone production was increased significantly with decreasing amounts of interstitial fluid, † P<0.05, ††† P<0.001, but was not changed significantly with the addition of rat serum (NS).

effect of human IF on testosterone production by human Leydig cells was observed only once (Fig. 5.8). On this occasion, the IF used was obtained from a man who had been receiving the anti-androgen cyproterone acetate as treatment for prostatic carcinoma.

In another human Leydig cell preparation (Fig. 5.9) 50 µl of human IF significantly decreased testosterone production compared to hCG-stimulated testosterone production alone ($P < 0.01$), while 25 µl and 12.5 µl were without effect (NS). Rat IF added to the same Leydig cell preparation was without effect at 50 µl and 25 µl (NS), but at 12.5 µl testosterone production was significantly increased ($P < 0.05$). Controls in the form of human and rat serum were mainly without effect (NS), although at 12.5 µl rat serum did significantly enhance testosterone production.

In an identical experiment with another human Leydig cell preparation (Fig. 5.10) both human and rat IF did not significantly increase testosterone production above levels obtained with a maximally stimulating dose of hCG. Likewise rat serum controls were without effect, however human serum significantly enhanced testosterone production ($P < 0.05$, $P < 0.01$).

Figure 5.11 shows data pooled from seven individual human Leydig cell preparations indicating that there is no consistent effect of human or rat IF on testosterone production. Likewise, serum controls did not significantly enhance testosterone production. The large errors associated with human IF derive from one preparation (Fig. 5.8) where the addition of human IF increased testosterone production from 19.3- to 28.3-fold that of basal production.

5.3 Discussion

In the rat, the presence of Leydig cell receptors for an LHRH-like

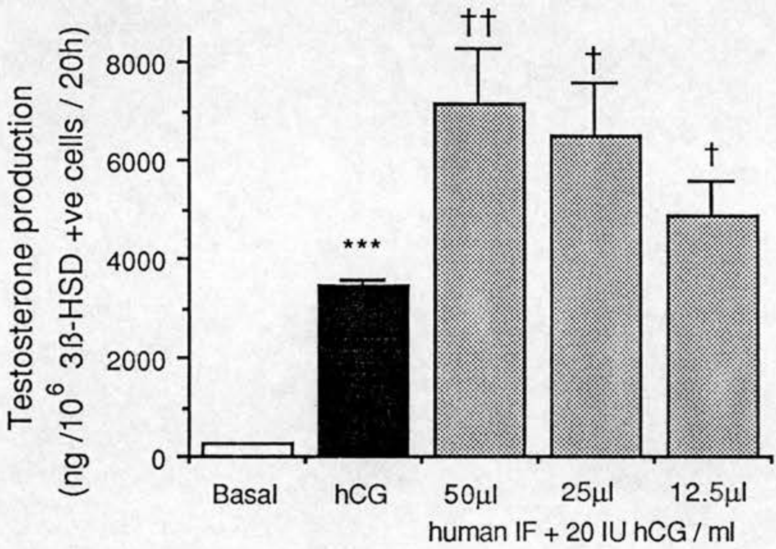


FIGURE 5.8

Stimulatory effect of human testicular interstitial fluid (IF) on human Leydig cell testosterone production in vitro. hCG (20 IU / ml) - stimulated testosterone production was significantly increased compared to basal levels,*** P<0.001. Testosterone production was increased significantly in the presence of IF compared to hCG-stimulated testosterone production alone, †† P<0.01, † P<0.05. Values are the mean ± SD of triplicate incubations.

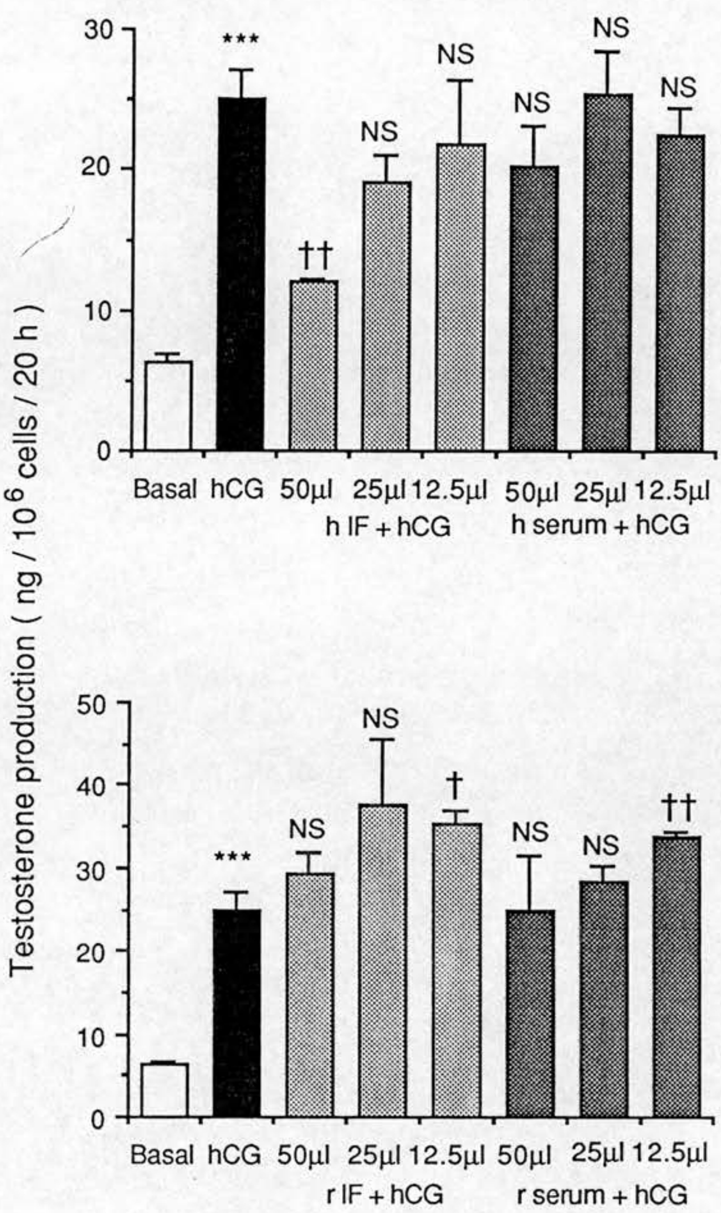


FIGURE 5.9

Effect of human and rat interstitial fluid (IF) and serum on human Leydig cell testosterone production in vitro. hCG (20 IU / ml)-stimulated testosterone production was significantly increased compared to basal levels, *** $P<0.001$ The addition of 50µl human IF significantly decreased testosterone production, †† $P<0.01$. Rat IF stimulated testosterone production at a concentration of 12.5µl, † $P<0.05$, but was without effect at higher concentrations (NS). Rat serum significantly increased testosterone production at a concentration of 12.5µl, †† $P<0.01$.

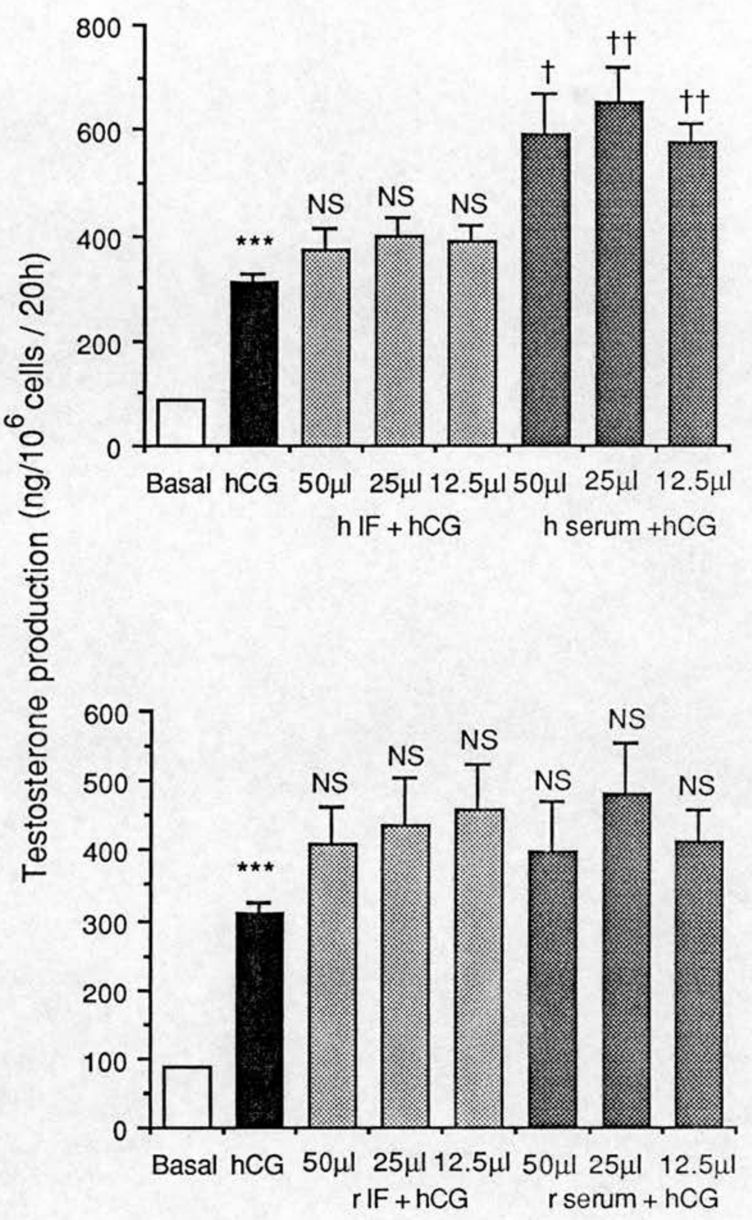


FIGURE 5.10

Effect of human and rat interstitial fluid (IF) and serum on human Leydig cell testosterone production in vitro. hCG (20 IU / ml)-stimulated testosterone production was significantly increased compared to basal levels, *** $P<0.001$. Human serum significantly increased testosterone production at 20 h of incubation, † $P<0.05$, †† $P<0.01$, whereas human and rat IF had no significant effect.

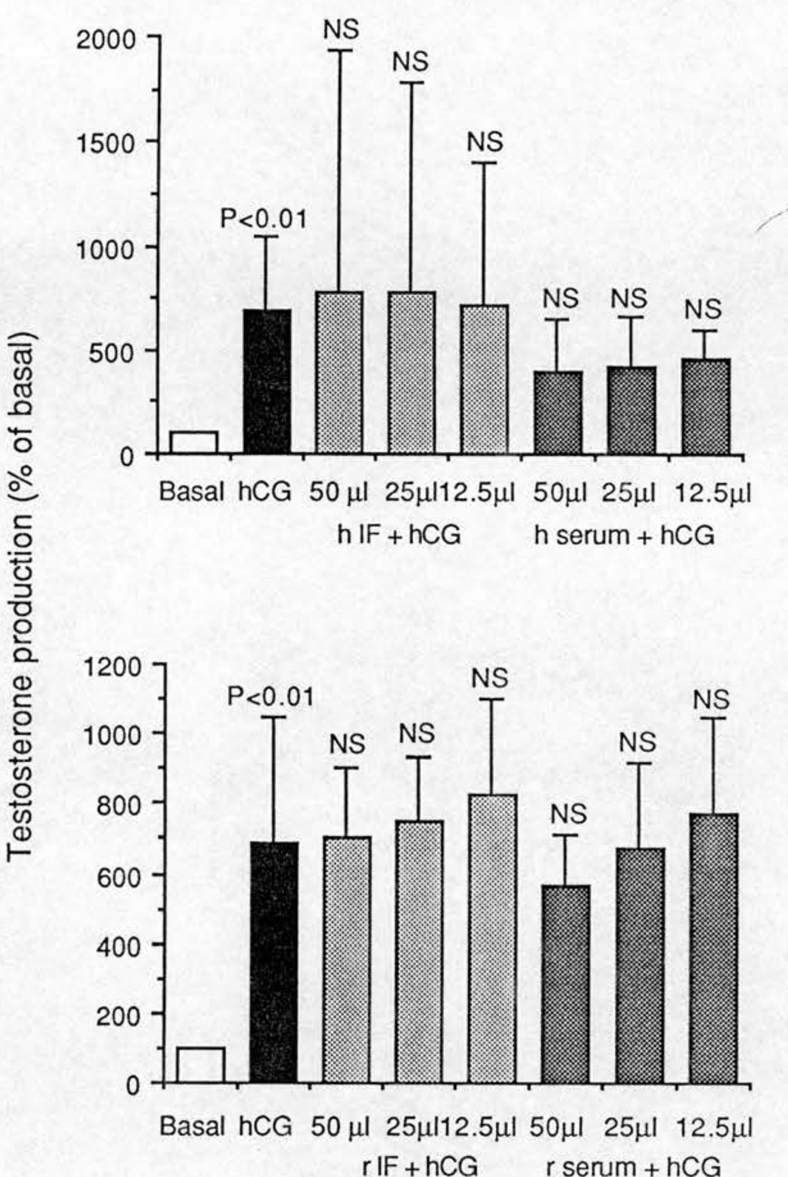


FIGURE 5.11

Effect of human and rat interstitial fluid (IF) and serum on human Leydig cell testosterone production in vitro. Each value is the mean ± SD of 7 Leydig cell preparations, and is expressed as percentage of basal production. hCG (20 IU / ml)-stimulated testosterone production was significantly increased compared to basal levels, $P<0.001$. Increasing concentrations of IF and serum from both species had no significant effect on testosterone production (NS).

peptide (Bourne et al. 1980; Sharpe & Fraser, 1980; Clayton & Catt, 1981; Huhtaniemi et al. 1985) of Sertoli cell origin (de Jong et al. 1979; Sharpe et al. 1981, 1982a; Nagendranath et al. 1983) has led to the idea that the regulatory effect between the Sertoli cell and the Leydig cell is mediated, at least in part, by this peptide. This idea is backed up by experimental evidence using analogues of hypothalamic LHRH. Short-term (20 h) incubations with LHRH-agonist stimulates testosterone production. This effect has been demonstrated in vivo, in both normal (Sharpe & Rommerts, 1983; Sharpe et al. 1983), and hypophysectomized rats (Sharpe et al. 1982; Sharpe & Harmar, 1983), and in vitro (Hunter et al. 1982; Sharpe & Cooper, 1982a, 1982b).

In the present study, LHRH-agonist ([D-Ser$(Bu^t)^6$] des Gly NH_2^{10} LHRH-ethylamide) had no effect on basal or hCG-stimulated testosterone production by human Leydig cells at a dose known to stimulate testosterone production in the rat (Fig. 5.1). In order that this negative effect was not merely a reflection of enzymatic degradation of the agonist, a second LHRH-agonist (D-Ala^6 N^{α}MeLeu^7 Pro^9 N ethylamide), substituted in the 6, 7 and 10 positions resulting in greater resistance to enzyme degradation and increased binding affinity (Karten & Rivier, 1986) was tested, and it too was without effect (Fig. 5.2). This lack of a direct gonadal effect of LHRH-agonist may reflect the uncertainty about the presence of receptors for this peptide in the human testis. The absence of receptors for LHRH was reported by Clayton & Huhtaniemi (1982) in the testes of both the human and the monkey, yet despite this Popkin et al. (1985) observed that testicular receptors for LHRH did exist in the human, but that they were of much lower affinity (100-1000 fold lower) than those found in the rat. Similarly mice do not possess specific binding sites for LHRH and

subsequently LHRH-agonist has no effect on hCG binding, testosterone or cAMP production (Hunter et al. 1982). If LHRH-like peptide does indeed exist in the human testis it may be quite different structurally from the LHRH-agonists used, whereas in the rat testicular-LHRH and the LHRH-agonist [D-Ser$(Bu^t)^6$] des Gly $NH_2{}^{10}$ ethylamide do not differ in size or structure (Sharpe & Fraser, 1980).

Chronic administration of LHRH-agonist in man (Linde et al. 1981; Schurmeyer et al. 1984) as well as in the rat (Bambino et al. 1980; Hsueh et al. 1981; Hsueh & Jones, 1981) results in a direct inhibitory effect on steroidogenesis both in vivo and in vitro. While it is clear that by binding to specific Leydig cell receptors, LHRH-agonist directly inhibits steroidogenesis in the rat, pituitary desensitization appears to be the mode of action in man. Thus in response to hCG, serum levels of testosterone in man are unchanged before and after the administration of LHRH-agonist (Evans et al. 1984; Heber et al. 1984; Schaison et al. 1984; Rajfer et al. 1987).

Significant species differences therefore appear to exist between the rat, man, monkey and mouse in their response to LHRH-agonist. While in the rat a direct gonadal effect appears to predominate, the hypothalamic-pituitary axis is the primary, if not the only, site of action of LHRH-agonist in man.

Vasopressin is another peptide which is present in the rat testis (Kasson et al. 1985) and is reported to act through specific high affinity receptors on the Leydig cells (Meidan et al. 1985; Kasson & Hsueh, 1986). In long-term cultures (3 days) of mixed testicular cells (Meidan et al. 1985; Kasson & Hsueh, 1986) and purified Leydig cell preparations (Sharpe & Cooper, 1987), vasopressin inhibits Leydig cell testosterone production. However, during short-term culture (5 h)

vasopressin stimulates basal testosterone production but has no significant effect on hCG-stimulated testosterone production (Sharpe & Cooper, 1987). This stimulatory effect is dose-dependent and of small magnitude (3-fold).

In the present study, vasopressin (10^{-6}M) did not significantly alter basal or hCG-stimulated testosterone production by human Leydig cells at 5, 20 or 48 h (Fig. 5.3). This may be due to the lack of vasopressin-like peptide or receptors for such a peptide in the human testis. If such a peptide does exist, structural differences between the native peptide and the vasopressin used in the present study may be the reason for the observed lack of effect. Indeed the physiological significance of the effects of vasopressin in the rat testis are questionable since the levels of interstitial fluid testosterone remain unchanged following intratesticular injection of vasopressin in vivo (Sharpe & Cooper, 1987).

Dimers of the β-subunit of inhibin, which have been shown to be potent stimulators of pituitary FSH synthesis and secretion in vitro (Ying et al. 1986), show significant structural homology with TGFβ (Derynck et al. 1985; Mason et al. 1985). This structural homology raises the possibility that dimers of the β-subunit of inhibin may play a role as autocrine and/or paracrine modulators of gonadal function, analogous to the effects of TGFβ reported in other tissues (Brown & Blakeley, 1984).

When added to short- and long-term cultures of human Leydig cells (Fig. 5.4), TGFβ (10^{-8}M) had no significant effect on testosterone production. Likewise in the rat, TGFβ has no consistent effect on Leydig cell testosterone production (R.M. Sharpe, personal communication). These results indicate that β-dimer forms of inhibin

are unlikely to have any effect on the Leydig cell. Studies in the rat have shown that TGFβ can enhance FSH-stimulated LH receptor induction and secretion of oestradiol and progesterone by granulosa cells in a dose-dependent manner (Ying et al. 1986; Dodson & Schomberg, 1987). If β-dimer subunits of inhibin do exist as independent entities, they may exert paracrine effects on the Sertoli cell. Moreover, in vivo, these peptides may be released into the peripheral circulation to increase pituitary FSH synthesis and secretion.

Any factor produced within the seminiferous tubules which has an effect on Leydig cell steroidogeneisis must pass into the testicular interstitial fluid (IF). A factor present in rat IF has been shown to enhance Leydig cell testosterone production in the presence of maximally stimulating levels of hCG (Sharpe & Cooper, 1984). This factor, which is not LHRH (see 1.6.1 (vii)), appears to be similar to a Sertoli cell factor observed in a number of studies (Grotjan & Heindel, 1982; Benahmed et al. 1984; Parvinen et al. 1984; Janecki et al. 1985; Verhoeven & Cailleau, 1985).

Testicular IF contains a number of factors present in serum (Sharpe, 1979) from which it is formed (Setchell & Sharpe, 1981). The addition of serum to cell cultures is known to result in changes in their structure and function, so as a control for these non-specific serum effects, in the present study where possible, both IF and serum from the same individual or animal were added to the Leydig cell preparations.

The addition of both human and rat IF and serum to human Leydig cells produced inconsistent results (Figs. 5.6 - 5.11). In different Leydig cell preparations, human and rat IF either stimulated testosterone production in a dose-dependent manner (Figs. 5.6 & 5.8),

or in a reverse dose-dependent manner (Figs. 5.7 & 5.9), or was without effect compared to maximally stimulating levels of hCG (Fig. 5.10). Furthermore, the stimulatory effects of both human (Fig. 5.10) and rat serum (Fig. 5.9) on Leydig cell testosterone production are difficult to interpret.

Although the variable results obtained with human and rat IF on Leydig cells of the opposite species may be explained simply by species differences, the lack of any consistent effect of human IF on human Leydig cell testosterone production is in direct contradiction to the results obtained in a recent study by Verhoeven & Cailleau (1987). In this study spent media from human seminiferous tubule cultures was reported to contain a Leydig cell stimulatory factor similar to the IF factor(s) described in the rat. This factor derived from two young patients (25 and 37 years) with Leydig cell tumours. These tumours synthesize androgens and oestrogens directly and indirectly by aromatization of androgens (Bercovici et al. 1987). These patients as a rule have suppressed gonadotrophin levels, impaired spermatogenesis and are infertile. Analogous to this is the situation in the present study, in which IF obtained from a patient who had received the gestagenic anti-androgen cyproterone acetate, as primary treatment for prostatic carcinoma, significantly enhanced hCG-stimulated testosterone production by human Leydig cells (Fig. 5.8). This patient had undetectable levels of gonadotrophins, and severely disrupted spermatogenesis.

Similarly, in the rat, situations which lead to reduced intratesticular testosterone levels and subsequently suppressed spermatogenesis, for example depletion of Leydig cells by EDS (Sharpe et al. 1986a) or short-term cryptorchidism (Sharpe et al. 1986b), also results in increased IF-factor activity. Following short-term

morphological damage to the seminiferous epithelium when testosterone and LH levels are back to normal, IF factor activity remains elevated, the activity being highest in animals with the most severe disruption of spermatogenesis (Sharpe et al. 1986a, 1986b).

In situations of acute severe spermatogenic disruption IF-factor activity appears therefore to be elevated. This however fails to explain why IF obtained from normal adult rats is capable of stimulating Leydig cell testosterone production. Human IF was obtained from men who underwent orchidectomy as primary treatment for prostatic carcinoma. These men, as a function of age, have chronically disrupted spermatogenesis, and elevated gonadotrophin levels. Raised serum levels of FSH are usually associated with Sertoli cell malfunction (de Kretser & Kerr, 1983) and secretory functions of the Sertoli cell decline when there is impairment of spermatogenesis (Au et al. 1983; de Kretser & Kerr, 1983; Jégou et al. 1984). Furthermore Sertoli cell number is known to decrease in the human testis with age (Johnson et al. 1984). A combination of reduced Sertoli cell number, and a reduction in the function of the remaining Sertoli cells, may be the cause of reduced production of IF-factor by the human Sertoli cells in this group. At the present time therefore, we can only speculate as to the role of IF-factor in the paracrine control of the human testis.

Despite the clear cut effects of a number of putative paracrine factors on Leydig cell function in the rat, similar effects on human Leydig cell function were not observed. Essentially, the dose of LHRH-A, vasopressin and TGFβ used in the present study was the same as that used in the rat studies. Dose-response experiments would have to be performed before it could be established that these factors have no effect on human Leydig cell function. If indeed these putative

paracrine factors exist in the human testis, they may exert their effects on other cell types, the peritubular cells or macrophages for example. Furthermore, apart from LHRH which has been shown consistently to have effects in vivo, it remains to be established whether the other factors described above have any physiological significance.

CHAPTER 6

Inhibin Bioactivity and Immunoactivity in Human Testicular Extracts

6.1 Introduction

Inhibin is a peptide produced by the gonads which acts on the anterior pituitary to selectively inhibit the synthesis and secretion of follicle-stimulating hormone (FSH) (de Jong, 1979; Baker et al. 1983). Within the last 2 years the purification, nucleotide sequencing and gene cloning of bovine, porcine and human inhibin have been achieved (Miyamoto et al. 1985; Robertson et al. 1985; Forage et al. 1986; Fukuda et al. 1986; Mason et al. 1986). However, studies to define a physiological role for inhibin are still scarce, particularly in the male.

In the male rat, testicular inhibin is produced by the Sertoli cell (Steinberger & Steinberger, 1976; Bicsak et al. 1987). Spermatogenic disruption results in a decrease in inhibin with an accompanying increase in levels of plasma FSH (Au et al. 1983, 1984, 1987). Following hypophysectomy, rat testicular inhibin is decreased and can be restored by exogenous FSH (Au et al. 1985). These data provide convincing experimental evidence for the role of inhibin within the pituitary FSH-testicular feedback loop in the male rat.

In infertile men, atrophy of seminiferous tubules is usually associated with a selective increase in circulating FSH (de Kretser et al. 1974) and this has been attributed to decreased inhibin feedback. Human seminal plasma inhibin levels have been correlated with FSH and sperm count in normal and infertile men (Scott & Burger, 1981a). Human seminal plasma inhibin is however distinct from testicular inhibin, being of prostatic rather than gonadal origin (Beksac et al. 1984). Furthermore, there is conflicting evidence as to whether seminal plasma inhibin is able to suppress pituitary FSH secretion in vitro (Ramasharma et al. 1984; Sheth et al. 1984; de Jong & Robertson, 1985; Liu et al.

1985).

At present, direct evidence for the existence of testicular inhibin in the human male is lacking. The aim of this study was to quantify levels of inhibin in human testes by means of a sensitive bioassay and to relate inhibin bioactivity to circulating FSH and spermatogenesis. In addition, following the development of a radioimmunoassay for the detection of inhibin in the human (McLachlan et al. 1986), by kind permission of Professor D.M. de Kretser, a comparison was made between testicular inhibin immunoactivity and bioactivity.

6.2 (i) Testicular inhibin bioactivity, gonadotrophins, steroids and spermatogenesis

Inhibin bioactivity was detectable in testicular extracts from all 21 patients using a sheep pituitary cell bioassay (see 2.5.3). In the ten men from whom both testes were available, there was no significant difference in inhibin bioactivity (4.5 $\pm$ 1.7 U/ml left and 4.5 $\pm$ 1.9 U/ml right) or daily sperm production (DSP) (1.7 $\pm$ 0.9 x 10^6/g left and 1.6 $\pm$ 1.1 x 10^6/g right) between left and right testes. In these cases, results were expressed as the average of the two testes.

The mean values of testicular inhibin, DSP, plasma FSH, LH, testosterone and oestradiol concentrations in the whole group and in the untreated and post-radiotherapy sub-groups are shown in Table 6.1 The group as a whole had low DSP, low normal testosterone and minimally increased FSH and LH with reference to young adults, although the range of individual values was very widely distributed. Patients who received radiotherapy previously had significantly lower inhibin bioactivity ($P < 0.05$) and DSP ($P < 0.01$) but higher circulating FSH ($P < 0.001$) and LH ($P < 0.01$) compared with the untreated group while testosterone and oestradiol remained unchanged (Fig. 6.1).

Table 6.1. Levels of testicular inhibin bioactivity, daily sperm production (DSP), FSH, LH, testosterone and oestradiol in 21 patients (whole group) consisting of untreated (n=16) and post-radiotherapy (N=5) sub-groups, and both single-factor and multiple interactive regression analyses between inhibin and the other variables. Values are means ± S.D.

	Whole group	Untreated	Radiation treated	Simple R	Multiple R
Inhibin (U/ml)	4.4 ± 1.3	4.8 ± 1.1	3.2 ± 1.4		
DSP (x 10^6/g)	2.0 - 1.3	2.5 ± 1.2	0.8 ± 0.7	0.25	0.25
FSH (mU/ml)	11.9 ± 8.3	8.4 ± 6.7	20.7 ± 4.4	-0.36	0.36
LH (mU/ml)	11.8 ± 6.2	9.5 ± 4.5	17.6 ± 6.4	-0.15	0.39
Testosterone (ng/ml)	3.2 ± 1.6	3.1 ± 1.5	3.3 ± 1.7	0.23	0.47*
Oestradiol (pM/l)	96.6 ± 28.1	93.5 ± 30.2	104.0 ± 18.1	0.11	0.48*

*$P < 0.05$

For reference purposes, corresponding values for normal fertile males under 50 years are:

DSP, 5.9 ± 0.8 x 10^6/g; FSH, 1.5 - 10 mU/ml; LH, 1.5 - 10 mU/ml Testosterone 3 - 10 ng/ml, Oestradiol 200 pM/l

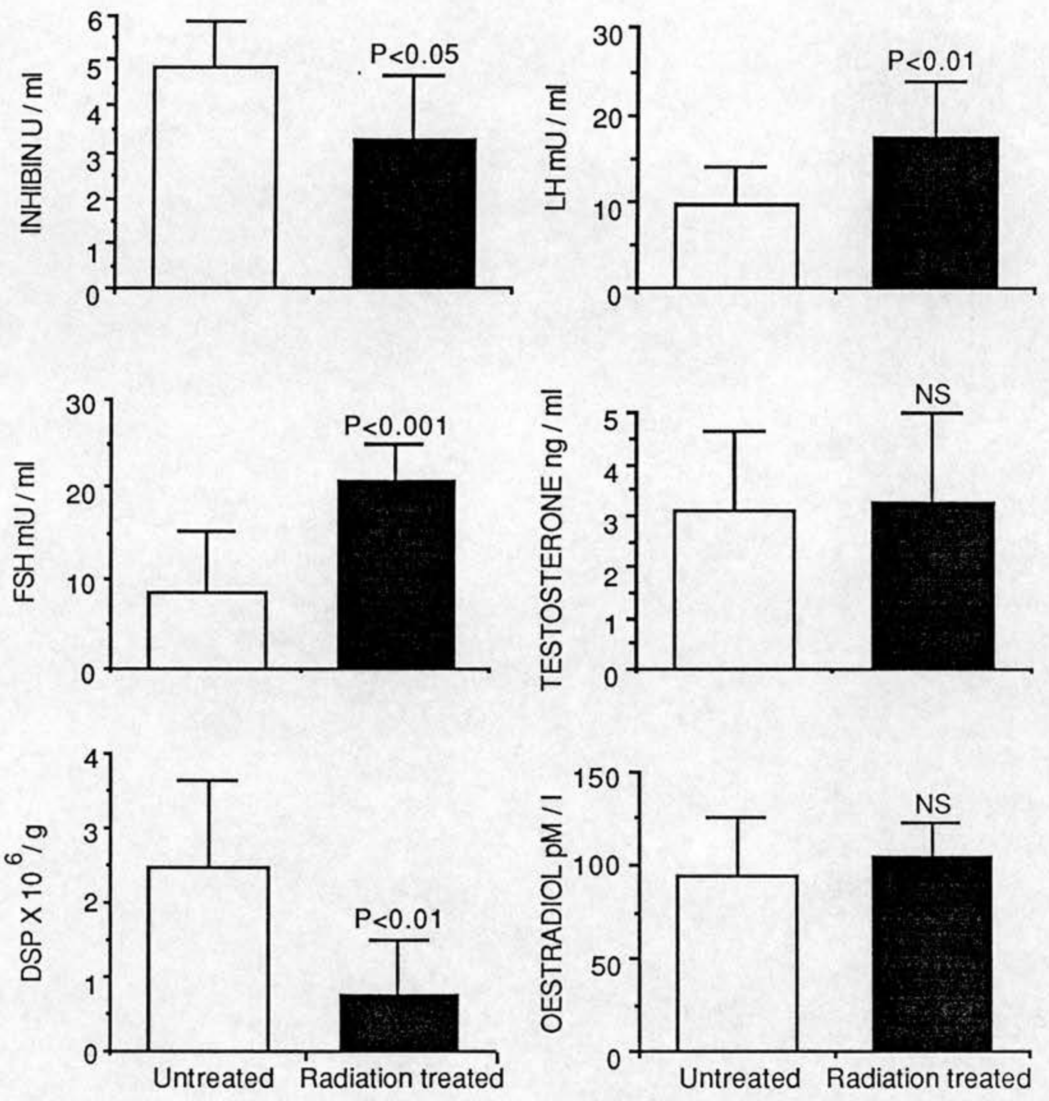


FIGURE 6.1

Comparison between the levels of inhibin, FSH, daily sperm production (DSP), LH, testosterone and oestradiol in both the untreated and radiation treated groups. Inhibin and DSP were significantly reduced (P<0,05, P<0.01 respectively), FSH and LH significantly increased (P<0.001, P<0.01 respectively), and testosterone and oestradiol unchanged (NS) in the radiation treated as compared to the untreated group. Values are mean ± SD from 15 patients in the untreated group and 6 patients in the radiation treated group.

The distribution of these variables against inhibin in individual patients are shown in Figure 6.2. As expected, there was a highly significant inverse relationship between DSP and FSH in the overall group ($R = -0.78$, $P < 0.001$). However, the apparent negative correlation between inhibin and FSH did not reach statistical significance in this group ($R = -0.36$). Single-factor regression analysis also failed to elicit any significant relationships between inhibin and DSP, LH, testosterone or oestradiol (Table 6.1). These variables, when analysed by multiple interactive regression, did become significantly correlated with inhibin ($R = 0.48$, $P < 0.05$).

(ii) Comparison between testicular inhibin immunoactivity and bioactivity

In 17 patients, in whom inhibin bioactivity had been measured previously, testicular inhibin immunoactivity was measured by radioimmunoassay using an antiserum raised against purified bovine follicular fluid inhibin as a primary immunogen boosted with 31 kDa bovine inhibin. Results were expressed in terms of a human IVF plasma pool assayed against a partially purified human follicular fluid standard (D.M. de Kretser, personal communication). Testicular inhibin bioactivity (4.8 ± 1.1 U/ml - untreated group; 3.2 ± 1.4 U/ml - radiation treated group) was significantly lower than immunoactivity (155.6 ± 75 U/ml - untreated group; 392.8 ± 397.4 U/ml - radiation treated group). Furthermore, single-factor regression analysis showed the lack of correlation between inhibin bioactivity and immunoactivity in this group of men (Fig. 6.3). A positive correlation was observed between inhibin immunoactivity and FSH ($R = 0.62$, $P < 0.001$) and also between inhibin immunoactivity and LH ($R = 0.74$, $P < 0.001$), in a situation where LH and FSH were positively correlated ($R = 0.81$, $P <$

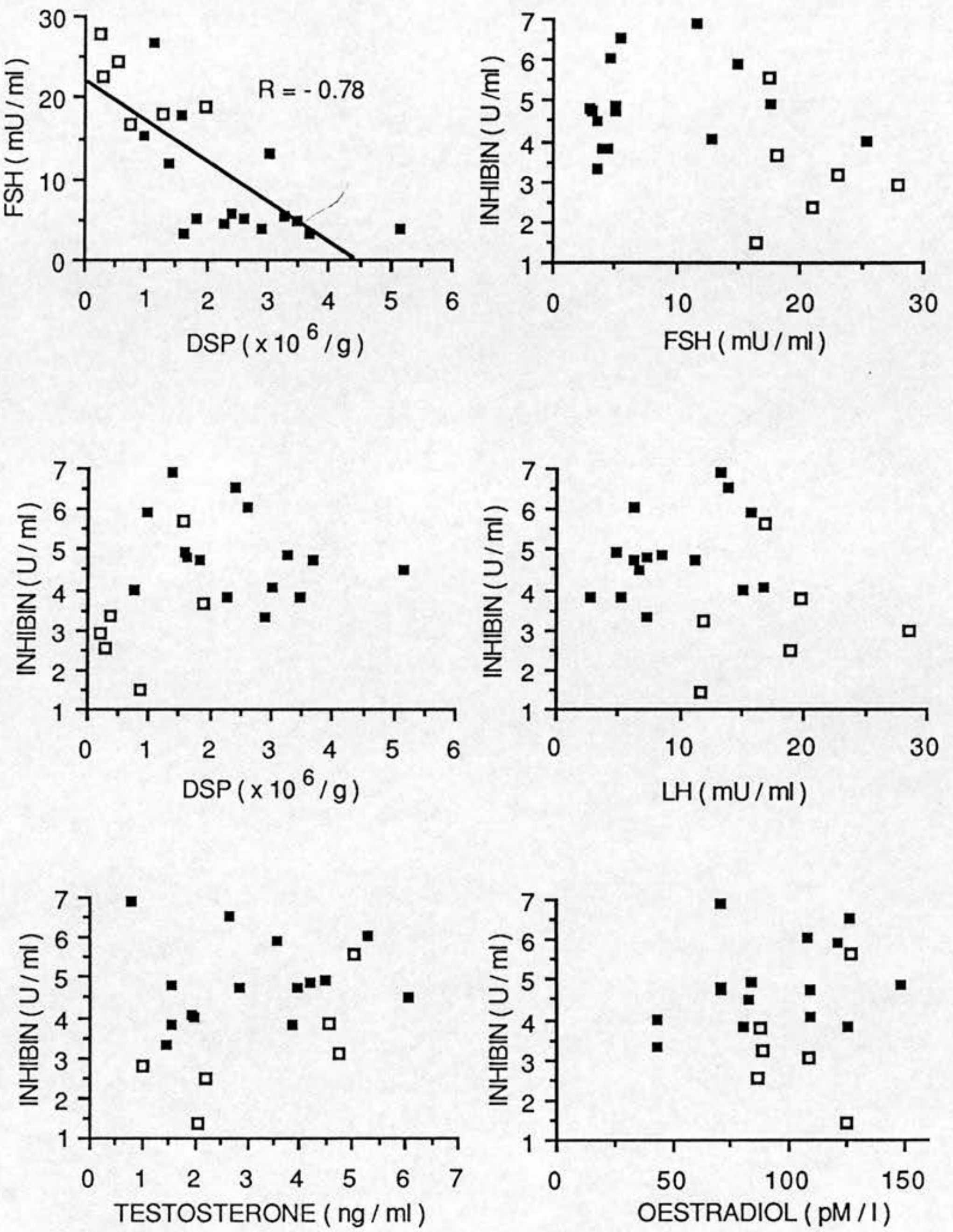


FIGURE 6.2

Single-factor regression analyses showing a highly significant inverse relationship between daily sperm production and FSH ($R = -0.78$, $P<0.001$), but a lack of significance between inhibin bioactivity and daily sperm production (DSP), FSH, LH, testosterone and oestradiol. The closed squares represent 15 patients in the untreated group, and the open squares, 6 patients in the post-radiotherapy group.

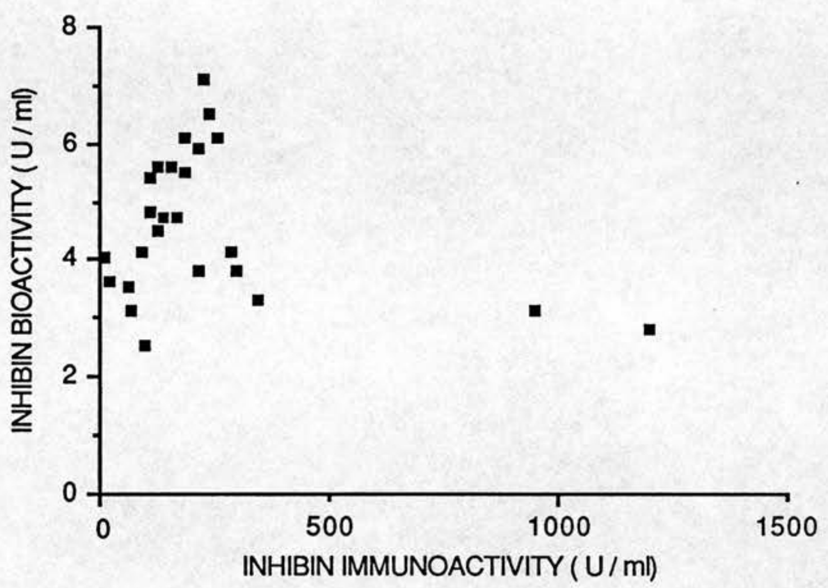


FIGURE 6.3

Regression analysis showing the lack of significant correlation between inhibin bioactivity and immunoactivity in testicular extracts from 17 patients (including values for both left and right testes in eight patients).

0.001) (Fig. 6.4). No significant relationship existed between inhibin immunoactivity and either DSP, testosterone or oestradiol (Fig. 6.4).

(iii) Relationship between inhibin immunoactivity and FSH in the peripheral circulation

Previous studies have failed to detect inhibin bioactivity in spermatic vein blood in men undergoing embolization treatment for variocoeles or in the peripheral circulation of two normal males who received FSH infusion (Metrodin, Serono; 25 IU 3 hourly for 5 days) (F.C.W. Wu & C.G. Tsonis, unpublished observations). With the availability of a radioimmunoassay capable of detecting plasma levels of inhibin immunoactivity in males, the relationship between FSH and plasma levels of inhibin can now be investigated.

Plasma from the two males who received infusion of FSH for 5 days was assayed for inhibin immunoactivity, FSH, LH and testosterone. While FSH levels rose significantly during FSH infusion, plasma levels of inhibin immunoactivity, and also LH and testosterone, were not altered significantly (Fig. 6.5).

6.3 Discussion

The presence of FSH-suppressing bioactivity in pooled human testicular extracts has been reported previously (Krishnan et al. 1982), but this is the first attempt to quantify levels of inhibin in the human testis and to relate this to direct and indirect measures of spermatogenesis. Despite the high average age of the patients (60 - 89 years, median 74) and the low DSP in some cases, inhibin bioactivity was detectable in all samples. The mean level of inhibin in the human testis of 4.38 U/ml ($\sim$90 U/testis) is, on a per testis basis, approximately half that reported by Au et al. (1984) in testicular extracts from normal adult male rats (120 U/ml; $\sim$180 U/testis) using a

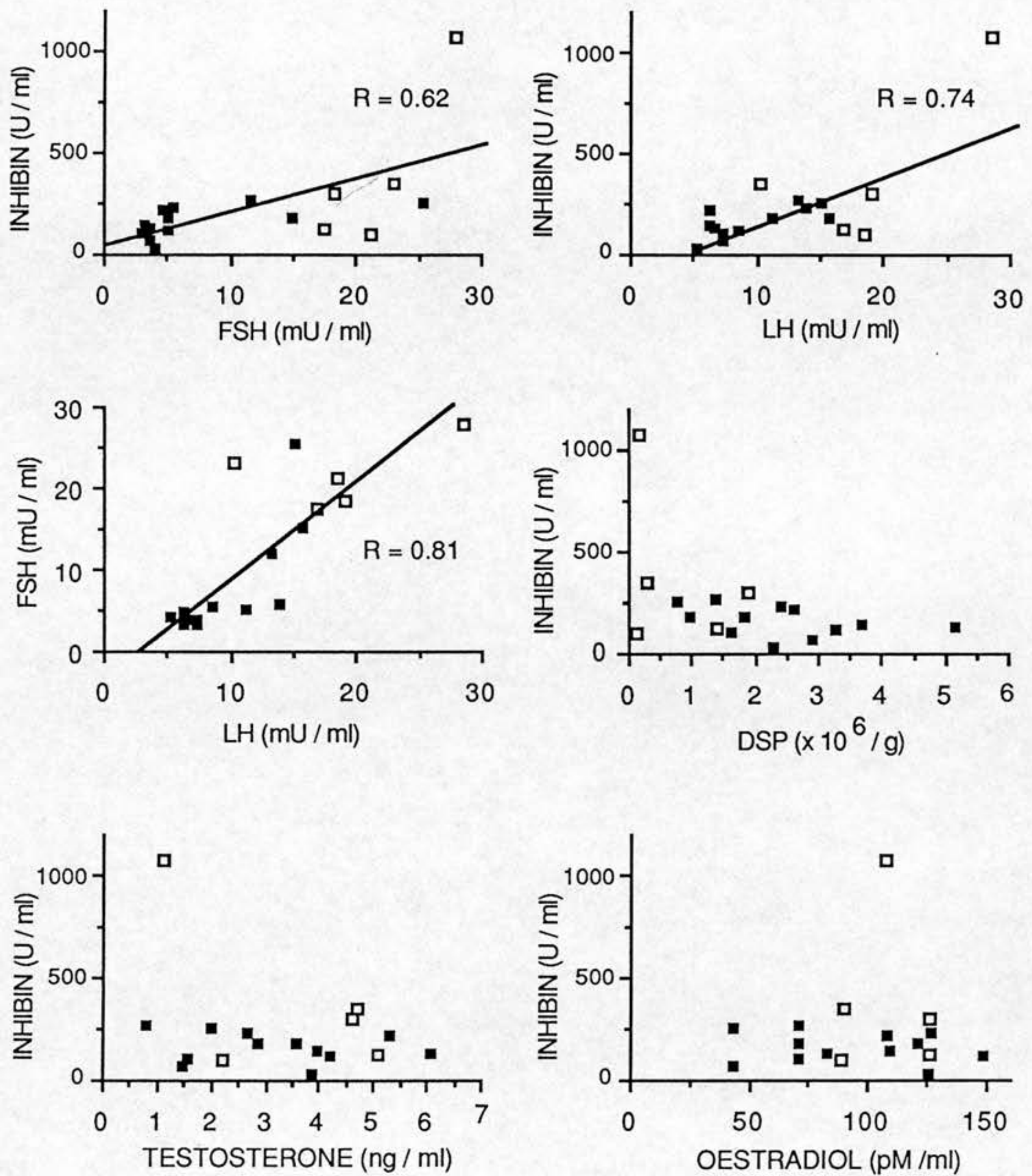


FIGURE 6.4

Single-factor regression analyses showing highly significant correlations between inhibin immunoactivity and FSH (R = 0.62, P<0.001) and LH (R = 0.74, P<0.001), and also between LH and FSH (R = 0.81, P<0.001). Inhibin immunoactivity was not significantly correlated to DSP, testosterone or oestradiol. The closed squares represent 12 patients in the untreated group, and the open squares, 5 patients in the post-radiotherapy group.

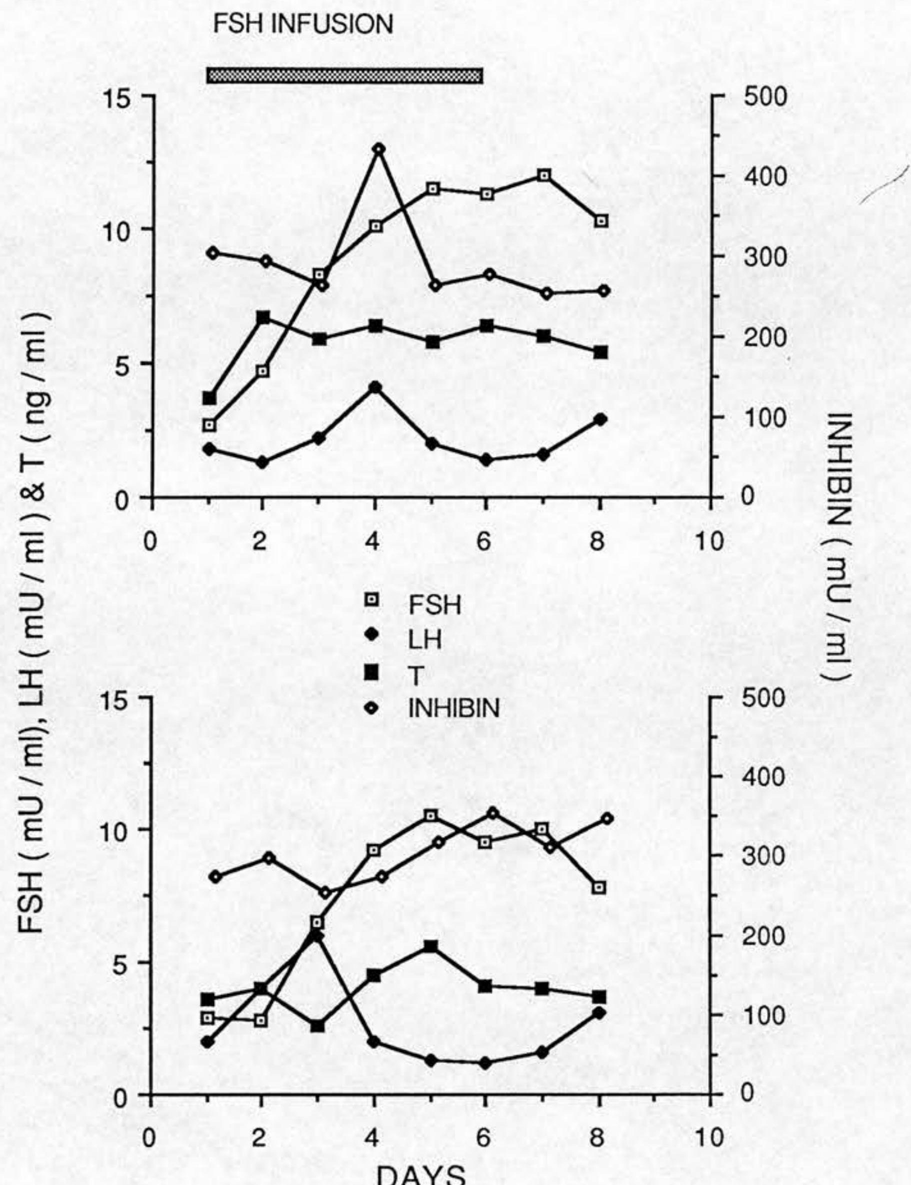


FIGURE 6.5

Effect of FSH infusion (Metrodin, 25 IU 3 hourly) in two normal males on plasma levels of inhibin, FSH, LH and testosterone. While FSH rose significantly during Metrodin infusion, plasma levels of inhibin were not significantly altered. Likewise, LH and testosterone remained unchanged.

rat pituitary cell bioassay but the same inhibin reference standard (ovine rete testis fluid, 1U/mg) as in the present study. This may be due to differences in the sheep and rat inhibin bioassays or may reflect the decreased number of Sertoli cells seen in the ageing human testis (Johnson et al. 1984). Furthermore, inhibin bioactivity observed in the human female during both spontaneous and gonadotrophin-stimulated cycles seem to be considerably higher than that observed in human testicular extracts. Thus the concentrations of inhibin bioactivity in follicular fluid at mid-cycle in women receiving human menopausal gonadotrophin and/or clomiphene were 200-300 U/ml (Baird et al. 1987) and in peripheral plasma, in both spontaneous and stimulated cycles, 1-8 U/ml (Tsonis et al. 1987b, Tsonis et al. 1987c). In contrast, inhibin was undectable in the spermatic vein of normal adult rats (Au et al. 1984). Similarly, we were unable to detect inhibin bioactivity in spermatic vein blood in 12 men undergoing embolization treatment for varicocoeles or in the peripheral plasma of two normal males who received FSH infusion for 5 days (F.C.W. Wu & C.G. Tsonis, unpublished observations). Taken together, these results suggest that inhibin production in the human male may be substantially lower than in the female.

In patients who received radiotherapy, testicular inhibin bioactivity was significantly lower than in those not previously treated (Table 6.1), although in no instance was inhibin undetectable. In the former group, DSP was profoundly reduced and FSH, though grossly elevated, was still below the castrate range, while testosterone and oestradiol were normal. These findings are compatible with the concept that inhibin is important in the feedback control of FSH, and implicate a relative deficiency of inhibin as the underlying mechanism for the

predominant FSH rise frequently observed in infertile men with spermatogenic failure. Scott & Burger (1981a) reported the absence of seminal plasma inhibin in eight azoospermic men with increased FSH, but their finding that vasectomized men also had inhibin activity in seminal plasma makes it unlikely that they were detecting a hormone of gonadal origin as in the present study. Although a role for prostatic inhibin in the control of FSH secretion has been postulated (Ramasharma et al. 1984; Sheth et al. 1984), a more important point is the possibility that pituitary FSH-suppressing activity in vitro may not be specific to inhibin as it is currently defined.

If inhibin does play a physiological role as a modulator of FSH secretion, one might expect to find a negative correlation between inhibin and FSH and a positive one between inhibin and DSP in a situation where a significant inverse relationship between FSH and DSP exists (Fig. 6.2). In the 21 patients studied, the relationships between these three variables were in the directions anticipated but there was no significant correlation between testicular inhibin bioactivity and circulating FSH or DSP (Fig. 6.2). One possible explanation for this may be that gonadal inhibin concentrations are not representative of inhibin in the peripheral circulation. Studies on rat Sertoli cell cultures show that FSH stimulates both intracellular and secreted levels of inhibin in a dose-dependent manner, with approximately 1/2 - 2/3 of the inhibin being secreted (Bicsak et al. 1987). Little is currently known about the mechanism(s) or route(s) by which inhibin is secreted from the testis into the peripheral circulation although, in sheep, the high concentration of inhibin in the ovarian vein (Tsonis et al. 1986) and ovarian lymph (Findlay et al. 1986) indicate that both vascular and lymphatic routes may be

important. However as yet, it has not been possible in the male to determine the exact relationship between circulating inhibin and FSH.

An interesting analogy is our finding that testicular testosterone concentrations from a similar group of men bore no significant correlation to either circulating testosterone or LH (Simpson & Wu, 1986). One interpretation of these findings for both testosterone and inhibin is the compensatory response of the Leydig and Sertoli cells to germ cell loss or degeneration, so that the gonadal production of these hormones is stimulated by the elevated levels of LH and FSH respectively. However, these changes in gonadal inhibin and testosterone are not reflected by levels of these hormones in the peripheral circulation. This type of chronic adaptation in the pituitary-testicular axis of the ageing human male is in distinct contrast to that observed during acute experimental disruption of spermatogenesis in young adult rats (Au et al. 1983, 1984, 1987), where an inverse relationship between testicular inhibin bioactivity and circulating FSH was consistently observed.

Following spermatogenic disruption in rats, the decline in testicular inhibin does not occur immediately, but is delayed to coincide with a reduction in testicular weight, an event known to reflect germ cell loss. Restoration of normal spermatogenesis following heat damage (Au et al. 1987) results in a return to normal FSH levels associated with a return to normal levels of testicular inhibin. The possibility of an interaction between different Sertoli cell/germ cell associations to regulate the production of inhibin has been suggested previously. The involvement of the spermatids in the regulation of inhibin production has been suggested by a number of authors

(Franchimont et al. 1975; Davies et al. 1978; Dandekar et al. 1984). In the present study however no significant relationship between testicular inhibin bioactivity and spermatid number (expressed as DSP) was observed (Fig. 6.2). Other studies have suggested the interaction between spermatocytes and Sertoli cells in the regulation of inhibin production, as FSH levels remain within the normal range despite the depletion of more advanced germ cells by vitamin A deficiency (Krueger et al. 1974) or hydroxyurea treatment (Mecklenburg et al. 1975). More recent studies have shown that "crude" germ cells co-cultured with Sertoli cells have no signficant effect on inhibin production (Steinberger, 1980). It would appear therefore that, as in the control of ABP secretion by the Sertoli cell (see 1.6.2 (ii)), particular Sertoli cell-germ cell associations may be required to regulate inhibin production.

Our finding that DSP and FSH were the two parameters with the highest correlation to inhibin is compatible with the notion that the latter is an index of Sertoli cell function. However, the fact that the correlations did not reach significance until testosterone was included implies that the interaction between FSH, germ cells, Leydig cells and Sertoli cells in the production of inhibin is more complicated than we had previously envisaged. In keeping with this are the findings that androgens stimulate inhibin production by rat testes in vivo and in vitro (Steinberger, 1981; Verhoeven & Franchimont, 1983), and also by human granulosa-lutein cells in vitro (Tsonis et al. 1987a).

In 1985 the full sequence of 32 kDa forms of inhibin in bovine and porcine follicular fluid were published (Ling et al. 1985; Mason et al. 1985; Robertson et al. 1985). This smallest active form of inhibin was shown to comprise of an α (18 kDa) and a β chain (13 kDa) interlinked by

a disulphide bridge. This 32 kDa form of inhibin is derived from a 58 kDa form by proteolytic processing of the α subunit (Robertson et al. 1985; Forage et al. 1986; Leversha et al. 1987), a process which has been shown to occur in plasma (McLachlan et al. 1986). However, there is little information on the molecular form or heterogeneity of the inhibin molecule within the testis or in the circulation. Our reliance on in vitro bioassays for the detection of testicular inhibin has the disadvantage that other intragonadal factors may interfere with the end point of FSH suppression in cultured pituitary cells. Recent work has demonstrated the existence of a homodimer of the inhibin β-subunit in porcine follicular fluid which is capable of stimulating FSH release in vitro (Ling et al. 1986; Vale et al. 1986). A significant structural homology has also been described between the β-subunit of inhibin and human transforming growth factor-β which can stimulate and inhibit cell growth (Mason et al. 1985), suggesting that the inhibin subunit may possess paracrine activities. The mitogenic activity of sheep pituitary cells in culture has been shown to be stimulated by conditioned media from cultured chicken ovarian thecal cells (Tsonis et al. 1987d) and by a factor from human testicular extracts (Chapter 7), both of which also possess FSH-suppressing activity. These findings highlight the non-specificity and potential pitfalls of in vitro bioassays, and therefore, with the development of specific radioimmunoassays for inhibin, testicular inhibin immunoactivity was measured in 17 patients in whom inhibin bioactivity had been measured previously.

Although testicular inhibin immuno- and bioactivity were expressed in terms of different standard preparations, and as such cannot be compared directly, nevertheless, the two were not significantly correlated (Fig. 6.3). Contrary to the accepted dogma that inhibin

selectively inhibits the synthesis and secretion of FSH from the anterior pituitary, a highly significant positive correlation was observed between inhibin immunoactivity and circulating levels of FSH (Fig. 6.4). The differences observed in the relationships between inhibin immuno- and bioactivity and FSH may be explained, at least in part, by the demonstration of inhibin-related factors which are capable of stimulating pituitary cell release of FSH in vitro (Ling et al. 1986; Vale et al. 1986). By increasing the release of pituitary FSH, these factors would result in inhibin activity measured by bioassay being underestimated. The radioimmunoassay used in the present study did not however cross-react with these FSH-releasing peptides, or indeed, TGFβ, MIS or any other potentially cross-reacting peptide (D.M. de Kretser, personal communication). In addition, the presence of a potent mitogenic factor in human testicular extracts, which stimulated the division of ovine pituitary cells in vitro, thus inhibiting the production of FSH from these cells, may also confound the inhibin bioactivity measurements (see Chapter 7). In view of the difficulties inherent to the inhibin bioassay, the significance of the positive correlation between inhibin immunoactivity and FSH must be further considered.

While at present there is no information as to the relationship between testicular levels of inhibin and those in the peripheral circulation, if, in the light of the present results, testicular inhibin is representative of levels in plasma, a positive correlation between FSH and plasma inhibin might be expected to exist. While bioassay techniques proved too insensitive to measure inhibin in the peripheral circulation of two males who received FSH infusion for five days, inhibin immunoactivity was measureable in these samples. Despite the 5-fold increase in circulating levels of FSH in these men,

no consistent rise in plasma inhibin was observed (Fig. 6.5). Studies in vitro have shown conclusively that FSH causes a dose-dependent increase in immature rat Sertoli cell inhibin production, while comparison between secreted and intracellular levels of inhibin reveal that approximately 1/2 - 2/3 of the inhibin is secreted (Bicsak et al. 1987). The discrepancy between testicular and plasma levels of inhibin in relation to FSH may indicate that in man also, Sertoli cell inhibin is stored prior to release into the circulation. This is in contrast to the situation in the female where, following FSH-stimulation, granulosa cells do not contain measurable intracellular levels of inhibin (Bicsak et al. 1986). The fact that Sertoli cell inhibin is stored while granulosa cell inhibin is not, suggests that there may be differences in inhibin processing between the two cell types.

The indication of the present results that inhibin alone is not the negative feedback regulator of FSH production is in keeping with studies in vitro, and also with recent data in the human female and other species. In the sheep it has been shown that during the follicular phase of the oestrous cycle, the secretion of inhibin from the ovary declines concomitantly with a fall in levels of FSH as oestradiol increases (Tsonis et al. 1986). Similarly, during the menstrual cycle in women, inhibin production by the granulosa cell is stimulated by FSH, while inhibition of FSH is mainly by the interaction of both inhibin and oestradiol (Tsonis et al. 1987b). Testosterone has also been shown to stimulate the production of inhibin by both the granulosa cells prior to luteinization and by the corpus luteum (Tsonis et al. 1987a). Therefore the role of LH and steroid hormones in the negative feedback control of FSH secretion in the female must be considered, and the function of

inhibin in this process re-investigated.

In the male, investigation of the extent to which steroids are involved in the control of FSH release is made difficult in view of the non-cyclical nature of steroid secretion. Conflicting evidence exists as to whether androgens are effective in stimulating Sertoli cell inhibin production in vitro (Verhoeven & Franchimont, 1983; Bicsak et al. 1987). In the present study no significant relationship existed between testicular inhibin immunoactivity and peripheral levels of testosterone or oestradiol (Fig. 6.4) or intratesticular levels of testosterone.

In conclusion, this is the first report of the presence of inhibin immunoactivity in the human testis. Comparison between inhibin bioactivity and immunoactivity revealed the non-specificity and potential pitfalls of in vitro inhibin bioassay. The highly significant positive correlation between testicular inhibin immunoactivity and circulating levels of FSH suggest that in the male, as in the female, the concept of FSH-stimulated inhibin production in the negative feedback control of FSH must be re-examined. The development of radioimmunoassay techniques, capable of detecting inhibin levels in the peripheral circulation, will assist greatly in this task. Recent findings have however led to the speculation that the production of inhibin may be under complex paracrine/autocrine control, and that inhibin might also subserve a paracrine function(s) within the testis in addition to its better recognized systemic role of FSH regulation. While the importance of inhibin in the negative feedback control of FSH should not be underestimated, the mechanism of this control appears to be much more complex than thought previously. Future studies must take

into account the interaction between inhibin and the steroid hormones in this process, in particular the modulatory effects of steroids on the production and processing of gonadal inhibin.

CHAPTER 7

Mitogenic Activity in Testicular Extracts

7.1 Introduction

During the course of a routine bioassay to determine whether inhibin was present in the pre-ovulatory follicle of the domestic hen, Tsonis and colleagues (1987d) observed that conditioned medium from chicken thecal/stromal cells, but not granulosa cells, stimulated the proliferation of ovine pituitary cells in culture (Tsonis et al. 1987d). While the identity, cellular origin and physiological significance of the thecal/stromal factor remains to be established, it has been suggested that this factor may be involved in the division of thecal layer fibroblasts or in the stimulation of granulosa cells during folliculogenesis (Tsonis et al. 1987d). This increasing awareness that cell metabolism and cell division may be regulated by growth factor has also stimulated much interest in their possible role in spermatogenesis.

When measuring inhibin bioactivity in human testicular extracts during the present study, it was observed that these extracts also stimulated the proliferation of ovine pituitary cells in culture. A number of Sertoli cell-derived growth factors are thought to be involved in autocrine and/or paracrine regulation of testicular function (see 1.3.3). The mitogenic activity in human testicular extracts was therefore further investigated in relation to a number of aspects of testicular function. Preliminary findings are reported in this chapter.

7.2 (i) Presence of mitogenic activity in human testicular extracts

Mitogenic activity was determined in testicular extracts in a group of 17 men (61-86 years, median 74) who had underwent orchidectomy for prostatic carcinoma. This group consisted of 9 previously untreated, 5 radiation treated, 2 diethylstilboestrol (DES) treated men and 1 man treated with cyproterone acetate. A human testicular extract pool was

also prepared from 21 men in whom testicular inhibin had been measured previously (Chapter 6). Mitogenic activity of human testicular extracts was determined using the same conditions as for the ovine pituitary cell inhibin bioassay (see 2.5.3), and the same time-course as employed previously to detect mitogenic activity in chicken thecal/stromal cells (Tsonis et al. 1987d). Following pre-incubation for 48 h, ovine pituitary cells were incubated with testicular extract, or culture medium alone (control), for a further 48-72 h, before being lifted from the culture plates by the addition of trypsin (see 2.5.4). Pituitary cell number was then counted in a haemocytometer.

Following the initial observation that human testicular extracts had potent mitogenic effects on ovine pituitary cells in culture, the effect of a human testicular extract pool (HTE) on ovine pituitary cell number was observed. The addition of HTE from 6.25 µl to a maximum of 25 µl ($P < 0.001$), produced a dose-dependent increase in pituitary cell number while the addition of higher doses reduced the viability of these cells in culture (Fig. 7.1). In spite of the increased number of pituitary cells, however, no accompanying increase in FSH, LH or prolactin was observed (Fig. 7.2). Indeed the total release of FSH was suppressed in a dose-dependent manner, while LH and prolactin were not significantly changed. In contrast, when expressed as release per final pituitary cell number (percentage of control), both LH and prolactin were also suppressed in a dose-dependent manner, although FSH was suppressed to an even greater extent (Fig. 7.2).

When mitogenic activity in individual testicular extracts from 16 patients was correlated to FSH release in the same ovine pituitary cell culture, a significant negative correlation ($R = -0.51$, $P < 0.05$) was observed (Fig. 7.3). Thus if the presence of mitogenic activity is associated with a reduction in the release of FSH from pituitary cell,

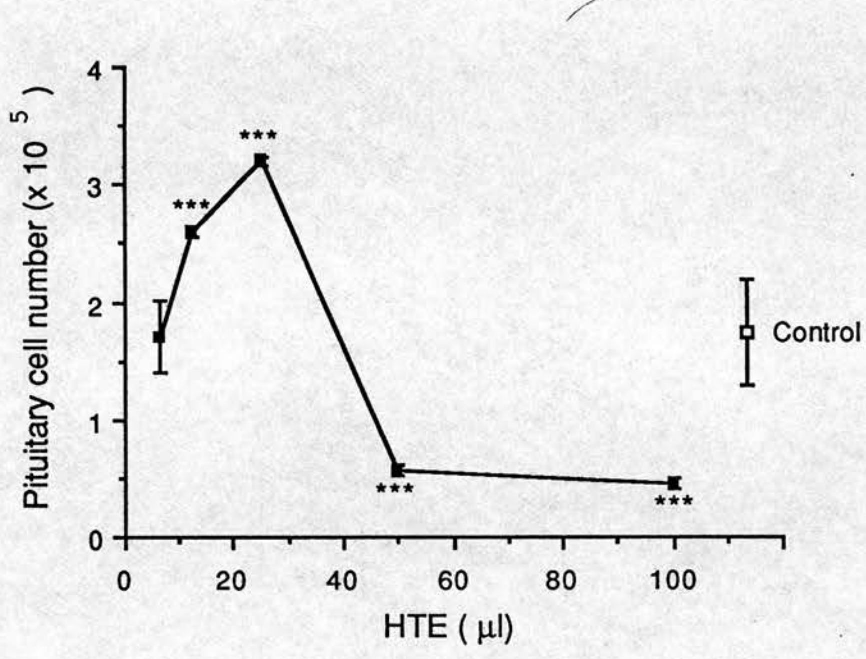


FIGURE 7.1

Increase in cultured ovine pituitary cell number with increasing levels of a human testicular extract pool (HTE) up to a dose of 25 µl compared to control (***, $P<0.001$). The addition of higher doses reduced the viability of these cells in culture (***, $P<0.001$). Values are the mean ± SD of triplicate incubations.

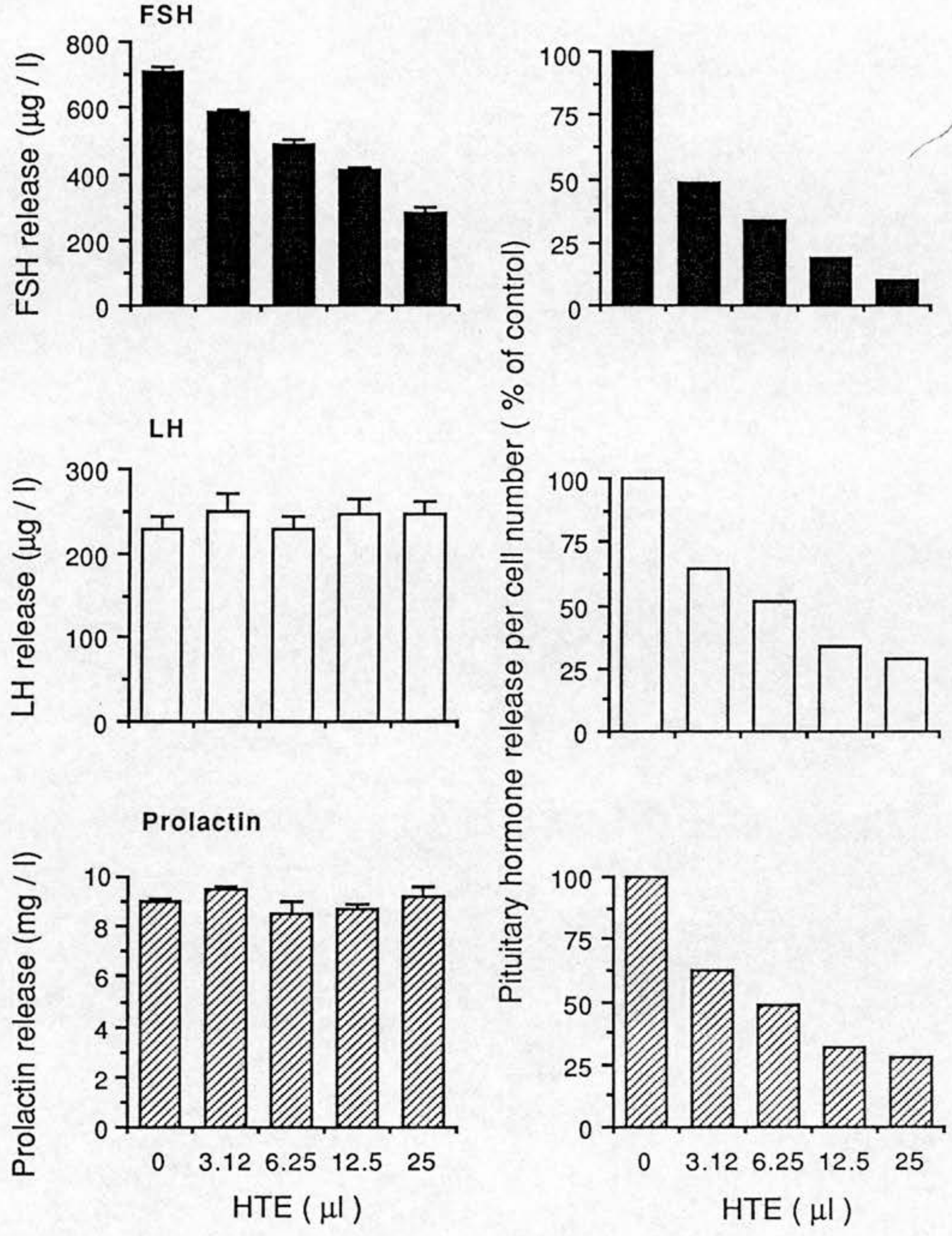
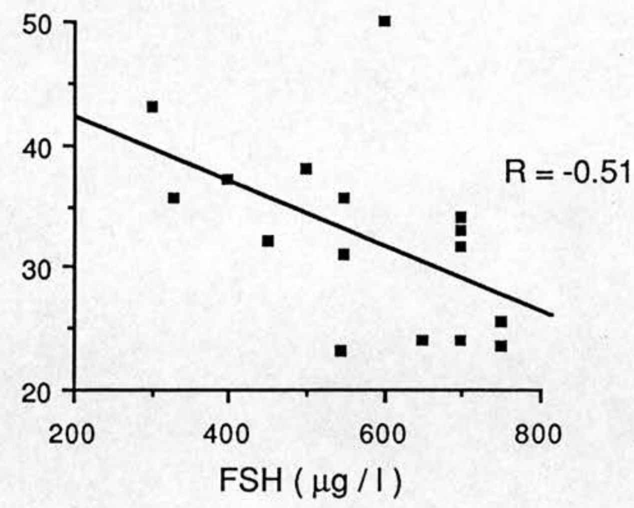
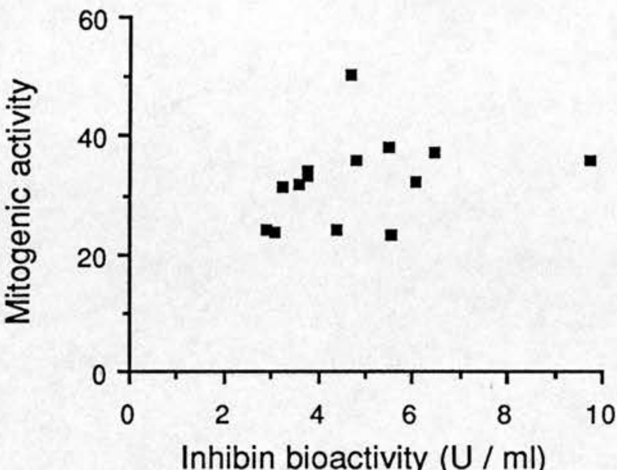


FIGURE 7.2

Histograms showing the effect of increasing concentrations of a human testicular extract pool (HTE) on the total release of FSH, LH and prolactin from cultured ovine pituitary cells (left panel), and the release expressed as percentage of control per final pituitary cell number (right panel).





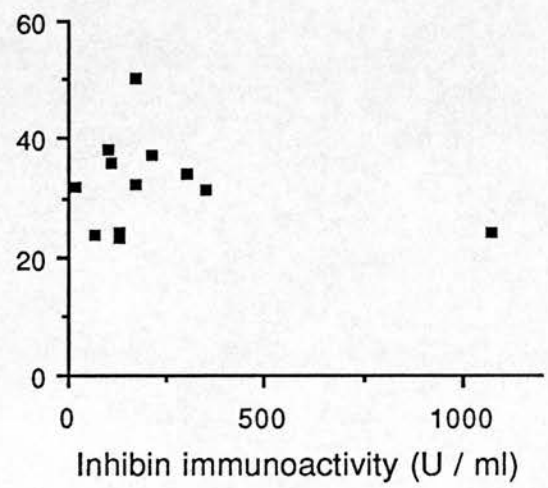


FIGURE 7.3

The effect of increased mitogenic activity in individual testicular extracts on the release of FSH from ovine pituitary cells in culture ($R = -0.51$, $P<0.05$) ($n = 16$), and on testicular inhibin bioactivity ($n = 14$) and immunoactivity ($n = 13$).

inhibin bioactivity measured in the same assay system might be expected to be overestimated. However inhibin bioactivity measured in 14 of these patients just failed to show the expected positive correlation with mitogenic activity (Fig. 7.3). Inhibin immunoactivity measured in 13 of these patients failed to show any significant relationship with mitogenic activity. These observations suggest that the HTE mitogenic activity is associated with an apparent suppressive effect on FSH release independent of inhibin bioactivity.

To assess the individual variability in mitogenic activity, testicular extracts from 17 men were examined at a maximum dose of 25 µl and at a 1:4 dilution. The results were divided into four groups according to treatment regimen, and are shown in Figure 7.4. In all cases, except for one man who had been treated with cyproterone acetate, the addition of testicular extracts stimulated the division of pituitary cells in culture. There was no significant difference in the response of the pituitary cells to testicular extracts from untreated or radiation treated patients although the hormonal and germ cell status of these patients were very different (see Fig. 6.1). Interestingly at a dose of 25 µl, testicular extracts from two men treated with DES did not stimulate pituitary cell division to the same extent as did the 1:4 dilution (Fig. 7.4). While the intratesticular hormonal milieu, and also the state of the seminiferous epithelium was significantly different between the treatment groups, no correlation was observed between either daily sperm production, testicular levels of testosterone or gonadotrophin levels and mitogenic activity (Fig. 7.5) - mitogenic activity being an arbitrary figure calculated from final pituitary cell number minus control.

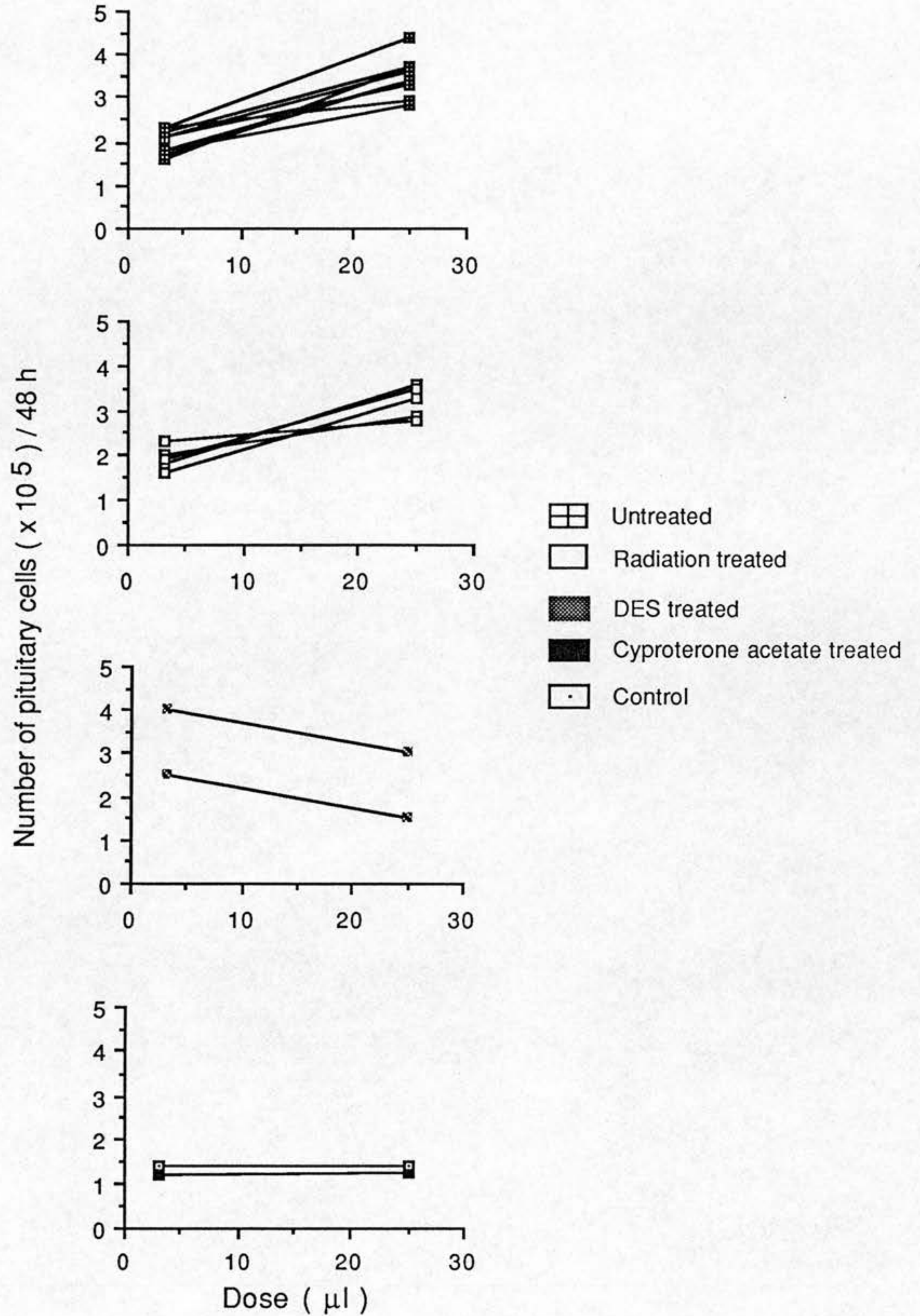
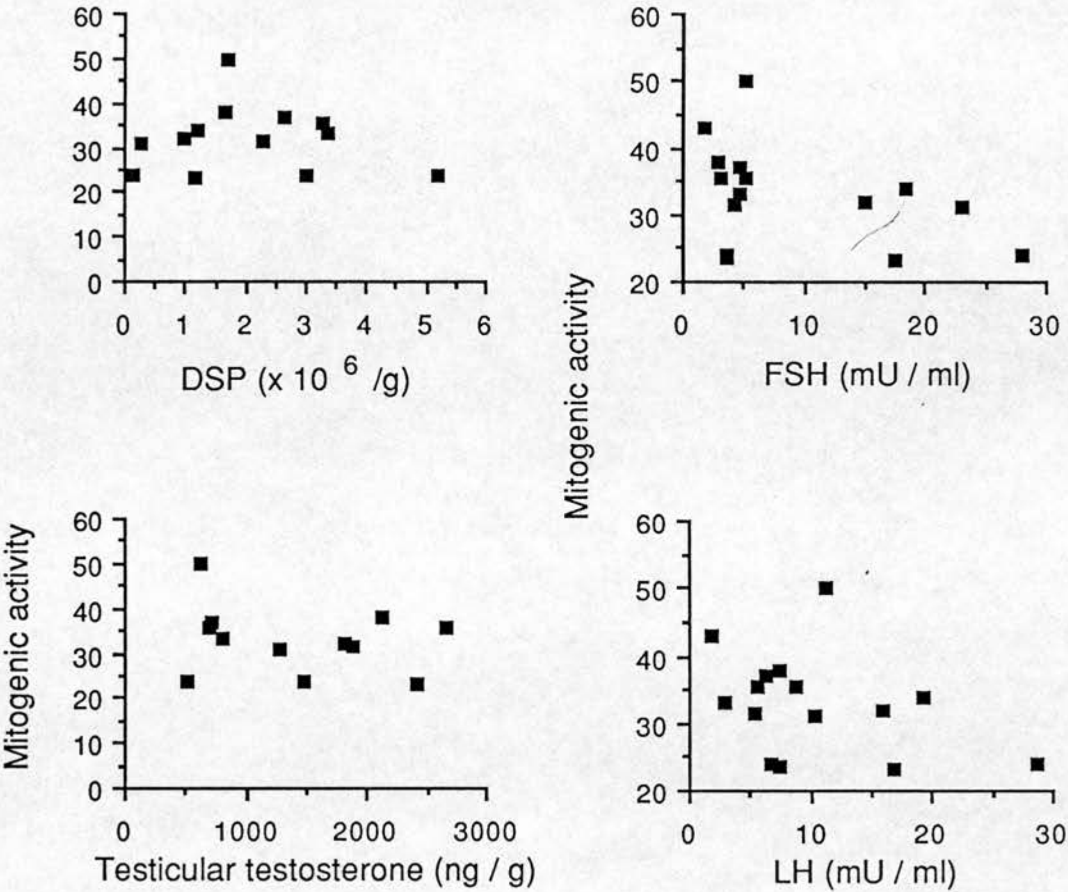


FIGURE 7.4

Effect of testicular extracts at two doses from 16 individual men on cultured ovine pituitary cell number. The results are divided into four groups according to treatment.Values are expressed as the mean of duplicate incubations.



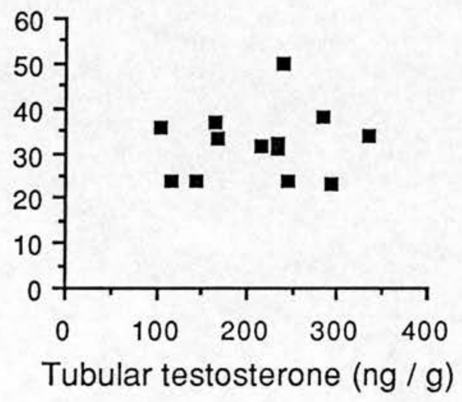


FIGURE 7.5

No significant correlation was observed between mitogenic activity (an arbitrary figure calculated from final pituitary cell number minus control) and daily sperm production (DSP), testicular testosterone, tubular testosterone, FSH or LH.

7.2 (ii) Presence of testicular mitogenic activity in other species

In addition to HTE, the mitogenic activity of testicular extracts from a number of species was observed. When added to ovine pituitary cells in culture, testicular extracts from rat, boar and marmoset did not stimulate pituitary cell number above control levels, while ram testicular extract contained a mitogenic factor which appeared to be even more potent than that present in the HTE pool (Fig. 7.6).

7.2 (iii) Effect of human testicular extract on rat Leydig cell function

In order to look at a more physiological situation, the effect of HTE and rat testicular extracts were observed in cultures of rat Leydig cells (Fig. 7.7). All additions of both rat and human testicular extracts significantly depressed Leydig cell number compared to control levels ($P < 0.001$).

7.3 Discussion

While determining the presence of inhibin bioactivity in human testicular extracts it was observed that these steroid free extracts contain a factor which has potent mitogenic effects on ovine pituitary cells in culture. The addition of a human testicular extract pool to the pituitary cells results in a 2- to 3-fold increase in pituitary cell number above control by 48 - 72 h of incubation. Similarly it has been shown that chicken thecal/stromal cell conditioned media (ThCM), but not granulosa cell conditioned media (GCCM), stimulates the proliferation of ovine pituitary cells during inhibin bioassay (Tsonis et al. 1987d). The addition of GCCM to ovine pituitary cells causes a dose-dependent suppression of FSH while not affecting the release of LH or prolactin. This effect has been attributed to the presence of inhibin from the granulosa cells, the secretion of which increases during follicular

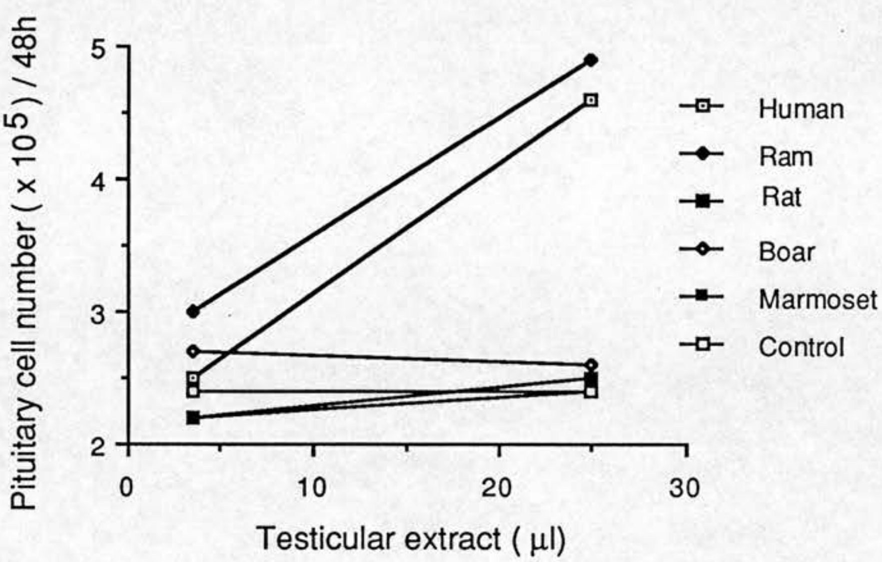


FIGURE 7.6

Effect of testicular extracts from human, ram, rat, boar and marmoset on cultured ovine pituitary cell number. Extracts from human and ram testes significantly increased pituitary number above control levels, while those from rat, boar and marmoset were without any significant effect. Values are expressed as the mean of triplicate incubations.

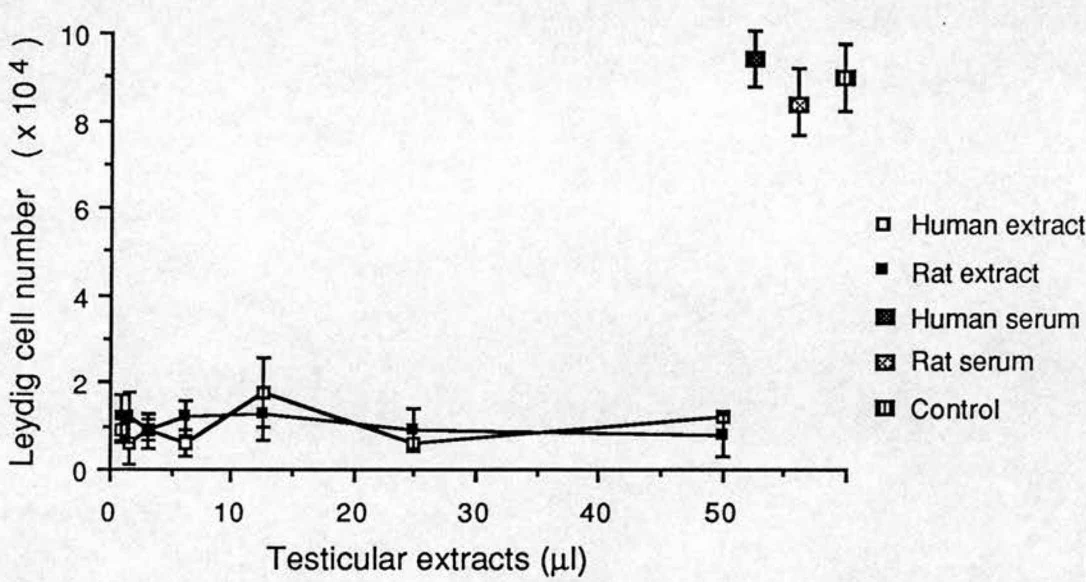


FIGURE 7.7

Effect of rat and human testicular extract pools and serum controls on rat Leydig cell number in vitro. At all doses studied, both rat and human testicular extracts suppressed Leydig cell number to values significantly below control and serum levels ($P<0.001$).

development (Tsonis et al. 1987d). While stimulating a 4- to 5-fold increase in total pituitary cell number during 96 h of incubation, ThCM, which should not contain inhibin activity, also suppresses FSH release in a dose-dependent manner while not affecting the release of LH or prolactin. Tsonis and colleagues suggest that this inhibin-like activity of the ThCM is due either to contamination of the preparation by granulosa cells, or to the presence of the mitogenic activity since a previous study (Yoshimura et al. 1974), which followed the life stage and secretory cycle of the anterior pituitary gonadotrophs, suggested that following pituitary cell division the release of FSH is suppressed.

Considering that bioassay of inhibin depends upon the extent to which FSH release is suppressed in pituitary cell cultures, then the presence of any factor which might alter the synthesis or release of FSH would bias inhibin bioactivity measured in this system. Following the addition of HTE, ovine pituitary cell proliferation was determined by counting total cell number, as suitable facilities for distinguishing between gonadotrophs, lactotrophs, somatotrophs and other pituitary cell types were not available. Therefore in this study, as in that by Tsonis et al., there is no way of knowing whether the mitogenic factor induced the gonadotrophs to divide. Pituitary cell proliferation may have been the result of division of primitive stem cells, of non-secretory chromophobe cells, of one particular type of pituitary cell or of all pituitary cell types. Considering the extent of pituitary cell proliferation (2- to 3-fold in 48 - 72 h) in view of the lengthy pituitary cell cycle (A.M. McNichol, personal communication), it would appear that the mitogenic factor was inducing all pituitary cell types to proliferate.

On the assumption that all pituitary cell types were dividing equally, the total output of FSH, LH and prolactin were expressed in terms of release per final pituitary cell number. While FSH release was suppressed due at least in part to the presence of inhibin, the release of LH and prolactin were also suppressed, although to a lesser extent than FSH. This indicates that the mitogenic factor may indeed affect peptide hormone release from a number of pituitary cells, including the gonadotrophs. Furthermore when mitogenic activity of individual testicular extracts was correlated to the release of pituitary cell FSH, a significant negative correlation was observed, indicating that increased mitogenic activity is associated with reduced output of FSH. Also the lack of any significant relationship between inhibin immunoactivty and mitogenic activity illustrates that the release of FSH is suppressed to a greater extent than by inhibin alone. Despite this, inhibin bioactivity just failed to show a positive correlation with mitogenic activity. If therefore the assumption that following the addition of HTE all pituitary cells divide equally, then it would appear that the newly formed pituitary cell population has reduced capacity to secrete its peptide hormones. Reduced production of FSH would result in overestimation of inhibin bioactivity in human testicular extracts.

Spermatogenesis is the most proliferative cell process in the male, and as such, has prompted study of specific growth factor contribution to this process. A number of Sertoli cell-derived growth factors are thought to be involved in autocrine and/or paracrine regulation of testicular function. Although the cellular origin of the mitogenic factor remains unknown, it is likely to derive from the Sertoli cell, and as such investigation of the physiological role of the mitogenic factor in germ cell development and function is therefore of great

interest. At the time of this study however, the techniques for the isolation and co-culture of Sertoli cells and germ cells were not set up in our laboratory. Instead however, with the availability of techniques for in vitro study of Leydig cell function, an attempt was made to study the effect of HTE on rat Leydig cell steroidogenic capacity in vitro. At all doses, HTE and also steroid free testicular extracts from rats, severely reduced the viability of the Leydig cells in vitro compared to control.

Studies to identify this factor are in the preliminary stages, but it is unlikely to be fibroblast growth factor, which has been shown previously to have only minimal mitogenic activity on pituitary cells in culture (Baird et al. 1986), or either epidermal growth factor or transforming growth factor β, which show only limited mitogenic activity in the present system (Tsonis et al. 1987d). Likewise, it appears not to be interleukin-1α or β which do not stimulate the division of ovine pituitary cells in culture (G. Duff & J. Symons, personal communication). While this rules out a number of possibilities, it is feasible that this mitogenic factor is one of many other well-documented growth factors of Sertoli cell origin, for example, seminiferous growth factor (Feig et al. 1983), meiosis-inducing substance (Grinsted & Byskov, 1981) or insulin-like growth factor (Tres et al. 1986).

Since the purification of this mitogenic factor requires a constant supply of material, and as human testicular tissue is not available on demand, the presence of this factor was investigated in other species. Testicular extracts from ram, rat, boar and marmoset were added to the ovine pituitary cell bioassay and their effects on cell number investigated. Testicular extracts from rat, boar and marmoset did not

stimulate cell proliferation above control levels. Ram testicular extract however contained a mitogenic factor with similar potency to that present in the human.

In trying to explain these species differences it was noted that the ram testes were obtained during mid-June, a time of great germ cell proliferation, and increased gonadotrophin levels in these seasonal breeding animals. Similarly in the elderly patients in this study, gonadotrophin levels were also elevated as a result of suppressed spermatogenesis, while in the rat, boar and marmoset, spermatogenesis and gonadotrophin levels were normal. It is possible therefore that in situations of suppressed spermatogenesis, under the appropriate steroidal and/or gonadotrophic milieu, this factor is produced, possibly by the Sertoli cell which acts on the germ cells to stimulate their development and function. In senescence however this putative mechanism appears not to function efficiently, as despite the presence of the mitogenic factor, germ cell development is chronically suppressed. Thus while the intratesticular hormonal and gonadotrophic environment and germ cell status were very different between individual patients, no correlation was observed with mitogenic activity. If indeed this factor is derived from the Sertoli cell, which decline in number with age (Johnson et al. 1984), a combination of reduced Sertoli cell number, and a reduction in the function of the remaining Sertoli cells, may result in insufficient production of this mitogenic factor to induce germ cell proliferation.

The serendipitous observation that the addition of human testicular extracts to ovine pituitary cell cultures results in great proliferation of these cells, throws into doubt the validity of the sheep pituitary cell bioassay for the detection of inhibin in these extracts. As yet it

is not possible to say whether the gonadotrophs, and in particular the synthesis and secretion of FSH are affected by this mitogenic activity. If as seems likely the gonadotrophs are induced to divide, any subsequent alteration in FSH synthesis or release would bias inhibin bioactivity measured in this system. From the present results, increasing mitogenic activity appears to be associated with suppression of pituitary cell FSH release, resulting in overestimation of inhibin bioactivity. Indeed this may explain the lack of correlation between inhibin bioactivity and immunoactivity observed in these testicular extracts. Until histochemical or monoclonal antibody techniques are applied to determine the extent of acidophil, basophil and chromophobe proliferation, then we can only speculate as to the implications of the mitogenic factor in the in vitro bioassay of testicular inhibin.

The presence of this putative mitogenic factor in testicular extracts implies a gonadal role, possibly in germ cell proliferation during episodes of high gonadotrophic stimulation. Studies are now underway to determine the presence of this factor in human foetal testes, and future studies might also investigate its presence in the rat during puberty and also following experimental disruption of the seminiferous epithelium.

To conclude, a factor of testicular origin has been found to have potent mitogenic effects on ovine pituitary cells in culture. While this is unlikely to be of any physiological significance, it has important implications for detection of testicular inhibin in the human using pituitary cell bioassay techniques. Studies to identify and to determine a possible gonadal role for this factor are continuing.

CHAPTER 8

Concluding Remarks

8.1 Concluding remarks

Fertility in the male has been shown conclusively to be dependent on the pituitary gonadotrophins, LH and FSH, which regulate testicular development and maintain its function. In addition to endocrine control by the gonadotrophins, there is increasing evidence of paracrine involvement in the control of testicular function, paracrine control being a form of regulation whereby one cell type in a gland or organ selectively influences the activity of an adjacent cell type through the synthesis and release of factors which pass through interstitial spaces to act on neighbouring target cells. In the rat, an increasing number of paracrine factors are being identified which modulate the testicular actions of the pituitary gonadotrophins according to local conditions and requirements.

In a recent World Health Organization (WHO) survey of 6682 infertile males from 25 countries (Diczfalusy, 1986), while testicular dysfunction, including primary idiopathic testicular dysfunction and abnormal sperm morphology and motility, accounted for approximately 21% of all infertility, a further 42.8% appeared to have no demonstrable cause as investigated by conventional semen analysis and endocrine tests. This high percentage of apparent idiopathic infertility or subfertility in man may reflect disturbance of local intratesticular paracrine regulation.

Studies on the paracrinology of testicular function have almost exclusively been limited to the rat, and similar in vitro studies in the human are lacking. However since the process of spermatogenesis through its testosterone dependent stages of mitosis, meiosis, translocation and spermiation is basically similar in both the rat and man, it seems likely that paracrine mechanisms in the human testis would not be

appreciably different from those in the rat. With this in mind, the present study was aimed towards establishing techniques whereby individual components of the human testis could be studied in isolation, and related to the overall functional state of the testis as defined by systemic hormone levels and histological assessment of spermatogenesis.

Testes from elderly men with prostatic carcinoma were utilized to establish a basis for the in vitro study of testicular paracrinology in the human. The lack of investigation of this nature in the human previously has undoubtedly arisen from the shortage of available material. While tissue was obtained from elderly men, who in general are known to have decreased levels of spermatogenesis and circulating testosterone, and also decreased Sertoli and Leydig cell number, concomitant with increased gonadotrophin levels, a number of these men had quite normal levels of these parameters, despite their age and prostatic illness. Indeed the ability of Leydig cells from these patients to produce testosterone in response to hCG was in many cases similar to that in young adult rats. Furthermore, patients treated with either radiotherapy, synthetic oestrogens or anti-androgens provided a suitable model of chronically suppressed testicular function. These patients therefore provided a suitable model to study paracrine control in the human testis.

Better known for its systemic endocrine role, testosterone is also the most important paracrine hormone in the testis. The main thrust of this study revolved around the importance of testosterone in the maintenance of spermatogenesis. While spermatogenesis is completely dependent on an adequate supply of this hormone, the question of how much testosterone is required locally to maintain spermatogenesis has been fraught with controversy over the years. Initial studies set out

to determine the distribution of testosterone within the human testis, and whether a lack of available intratesticular testosterone was related to a decline in spermatogenesis. While the decline in peripheral levels of testosterone commonly seen with advancing age has fuelled the idea that reduced Leydig cell function in elderly men is the cause of reduced sperm production, the results of the present study do not confirm this. On the contrary, men with high levels of daily sperm production, in the range of normal healthy young men, appeared to have lower levels of intratesticular testosterone compared to those with low levels of daily sperm production, who had surprisingly high intratesticular levels of testosterone. At face value these results would suggest that the original hypothesis of depressed levels of spermatogenesis associated with reduced available testosterone is not substantiated. However towards the end of this study it became apparent from studies in the rat that the methods employed to measure absolute amounts of testosterone within the testis may yield spuriously high results. This is due to continued Leydig cell function following isolation of the testes. In view of the apparent methodological difficulties, the validity of the techniques used in the present study were re-evaluated. While the post-isolation synthesis of testosterone in the human was not consistently observed, in a number of cases testosterone production did continue significantly after testicular isolation. This variability may reflect the functional capacity of the Leydig cells in different individuals and may also explain why the original validation of the techniques did not pick up this problem. As such, before any conclusions can be drawn as to the minimum amount of testosterone required to maintain quantitatively normal spermatogenesis, intratesticular concentrations of testosterone in both the rat and man

must be re-evaluated taking special precautions to minimize the post-isolation synthesis of testosterone.

The most widely studied paracrine mechanism in the rat is that of the interaction between the Sertoli and Leydig cells in the maintenance of high intratesticular concentrations of testosterone. A number of factors thought to be produced by the Sertoli cell have been shown to increase Leydig cell production of testosterone in vitro. The ability to perform this type of study in the rat is due to the ease with which Leydig cells can be isolated and their function studied in vitro. Previous in vitro human Leydig cell studies have generally involved the use of testis pieces or crude cell preparations, both of which are unsuitable for investigation of the paracrine control of Leydig cell function. In the present study, density centrifugation techniques were adopted for the isolation of highly responsive human Leydig cells, permitting further investigation of their function in vitro.

The effect of a number of putative paracrine factors which have been shown to have stimulatory effects on rat Leydig cell function in vitro were studied in 5 - 48 h incubations of human Leydig cells. The addition of LHRH-agonist, testicular interstitial fluid, vasopressin and TGFβ at concentrations known to stimulate testosterone production in the rat, had no significant effect on human Leydig cell testosterone production. In addition to possible species differences, this lack of effect may be accounted for in a number of ways. Firstly there is the possibility that human Leydig cells isolated on Percoll may represent a sub-population of cells which are highly responsive to hCG, but which are not representative of the whole Leydig cell population. This seems unlikely however as poorly responsive Leydig cells in vitro fractionate on Percoll in exactly the same manner as do highly responsive cells.

Secondly there is the possibility that reduced responsiveness of the human Leydig cells to these putative paracrine factors may reflect the advanced age of the sample population. While this is indeed a possibility, again it seems an unlikely explanation as at least some of the men studied had peripheral hormone levels and quantitative measures of spermatogenesis in the range of healthy young men. From these studies therefore it would seem that the fine regulation of Leydig cell steroidogenesis via paracrine mechanisms may not be required for the maintenance of spermatogenesis in man. Indeed the importance of these putative paracrine mechanisms in the in vivo situation in the rat have been questioned recently in view of evidence that in this species spermatogenesis can be maintained in the presence of testosterone levels which are approximately 10 - 20% of available intratesticular levels.

In the light of these results it seemed appropriate to move on to study human Sertoli cell function, utilizing the new techniques available for Sertoli cell isolation and co-culture with germ cells. At this point however the policy for the treatment of men with prostatic carcinoma changed, and the number having primary orchidectomy dropped considerably. With the increasing use of LHRH-agonists as first line treatment, orchidectomy will no longer be the choice of treatment for this condition. As such, the future of in vitro studies involving human testicular material looks bleak.

While studies in vitro will inevitably further our understanding of testicular physiology and pathophysiology, the assessment and treatment of malfunction of testicular paracrine mechanisms in the clinical situation is a long way off. One way forward in this area appears to be by non-invasive techniques through the identification of markers which

can be measured and related to testicular function. This approach has already been adopted to some extent. For example, transferrin in seminal plasma is thought to be of Sertoli cell origin, and measurement of this transferrin has been suggested as a suitable marker of Sertoli cell function. Unfortunately this has turned out to be of little clinical value as the range of seminal transferrin levels overlaps between normal men and those with seminiferous tubule malfunction.

Now that the technology is available to detect circulating inhibin in man, inhibin too may be a possible marker of Sertoli cell function. The highly significant positive correlation between testicular inhibin immunoactivity and circulating levels of FSH, found in the present study, suggest that the concept of FSH-stimulated inhibin production in the negative feedback control of FSH must be re-examined. These findings have led to the speculation that the production of inhibin may be under complex paracrine/autocrine control, and that inhibin might also serve a paracrine function within the testis in addition to its better recognized role of FSH regulation. So before inhibin can be used as a suitable marker of testicular function, further investigation is required to clarify its role in man.

So at the present time while there is a lack of potential markers of paracrine events within the testis, the results of a recent study provide hope that such a marker will be found. The isoenzyme lactate dehydrogenase - C4 in semen, when expressed in relation to the number of ejaculated sperm, is a good indicator of the efficiency of spermatogenesis in that individual. While this enzyme does not reflect any testicular paracrine event, it demonstrates that in the near future markers of testicular paracrine events may be detected in blood or semen. As such it is this approach, in conjunction with studies in

vitro, that we must now undoubtedly pursue.

Advances in our knowledge of the paracrine control of the testis should have major consequences in our understanding of and ability to treat idiopathic infertility in men. The studies reported herein provide a basic starting point for future investigations of paracrine control in the human testis.

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