Novel Approaches to

Male Hormonal Contraception

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Page Number

Table of Contents

Abstract of Thesis

The principle of male hormonal contraception has been investigated for the past 20 years. This thesis reviews what is known already from the literature and presents the results of three new clinical trials investigating different aspects of this approach.

These trials were undertaken by myself as a member of the Contraceptive Development Network research group at the University of Edinburgh.

MO15: A clinical trial of male hormonal contraception with a treatment period of 48 weeks. Subjects (n=29) were randomised to receive either 2 MENT Ac implants or 600mg per 12 weeks testosterone pellets in conjunction with 2 etonogestrel implants and outcome measures of sperm count, and reproductive hormone data were collected to assess contraceptive effects. Haematology and biochemistry parameters, prostate volume, PSA, bone density and blood pressure measurements were recorded to assess safety and the effects on androgen dependent tissues. The spermatogenic suppression achieved was similar and effective in both groups. Thereafter, the MENT group failed to maintain suppression and 6 men nnoted loss of libido due to a decline in the release rate of the MENT implants. No adverse effects of MENT on the prostate or bone mass were demonstrated. A small but significant increase in systolic blood pressure was observed in the MENT group and the implications of this remain to be further qualified. In the testosterone group profound and consistent spermatogenic suppression was demonstrated and azoospermia was achieved in all men. An increase in haemoglobin and prostate size and reduction in HDL-C were noted. MENT with progestogen can achieve rapid

suppression of spermatogenesis similar to testosterone, but this promising result was not sustained due to a reduction in the MENT release from the implants. This dose of testosterone, compared with previous studies using lower dose with a higher dose of etonogestrel, had non-reproductive side effects without any increase in spermatogenic suppression.

Conclusion: These data indicate the importance of the doses of progestogen and testosterone for optimum spermatogenic suppression while minimizing side effects.

M016: A randomised controlled trial investigating the effects of gonadotrophin withdrawal and progestogen administration on hormone production, metabolism and action in the human testis. Thirty subjects were randomised to no treatment or gonadotrophin suppression by GnRH antagonist with testosterone (CT) +/- additional administration of the progestogen desogestrel (CTD) for 4 weeks before testicular biopsy. Gene expression was quantified by PCR. Both treatment groups showed similar suppression of gonadotrophins and sperm production and markedly reduced expression of steroidogenic enzymes. Addition of progestogen resulted in expression of 5α-reductase type 1 compared with both controls and the CT group. Inhibin-α and the spermatocytes marker acrosin-binding protein were significantly lower in the CTD but not CT groups, compared with controls, but did not differ between treated groups. Men who showed greater falls in sperm production also showed reduced expression of these three genes but not of the spermatid marker protamine 1.

Conclusion: These data provide evidence for direct progestogenic effects on the testis and highlight steroid 5α-reduction and disruption of spermiation as important components of the testicular response to gonadotrophin withdrawal.

M017: Investigation of testicular function in normal men and those receiving a male hormonal contraceptive regimen. 20 subjects were recruited and hourly blood samples were taken over 24 hours for measurement of testosterone, inhibin B, LH, FSH and cortisol. Urinary excretion of testosterone and the testicular steroid epitestosterone was also measured. In the controls, a diurnal variation in serum testosterone and LH but not FSH was detected. The treated group had similar testosterone concentrations but showed no diurnal variation. Periodicity was detected in inhibin B concentrations in 5 controls and in 9 of the treated group. Urinary testosterone excretion did not show a diurnal variation in either group, but this was apparent for epitestosterone with a morning peak in both groups despite the markedly lower excretion in the treated men.

Conclusion: The diurnal variation of testosterone in normal men is due to change in secretion rather than clearance and is largely LH driven. An endogenous rhythm in both testicular steroidogenesis (epitestosterone) and Sertoli cell function (Inhibin B) is also present.

Declaration and Acknowledgements

The contents of this thesis have not been submitted elsewhere for any degree, diploma or professional qualification.

This thesis has been composed by myself, and I have been responsible throughout for patient recruitment, clinical management and laboratory studies unless otherwise acknowledged.

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Publications list

Chapters 2, 3 and 4 have been peer reviewed and published as the following journal articles:

Walton MJ, Bayne RAL, Wallace I, Baird DT, Anderson RA. *Direct effect of progestogen on gene expression in the testis during gonadotrophin withdrawal and early suppression of spermatogenesis*. Journal of Clinical Endocrinology and Metabolism (2006) 91(7): 2526-2533.

Walton MJ, Anderson RA, Kicman AT, Elton RA, Ossowska K, Baird DT. *A diurnal variation in testicular hormone production is maintained following gonadotrophin suppression in normal men*. Clinical Endocrinology (2007) 66(1): 123-9.

Walton MJ, Kumar N, Baird DT, Ludlow H, Anderson RA. *7α-methyl-19-nortestosterone (MENT) vs. testosterone in combination with etonogestrel implants for spermatogenic suppression in normal men*. Journal of Andrology (2007) in press

Abbreviations

The following abbreviations have been used throughout the text:

GnRH	gonadotrophin releasing hormone
hCG	human choriogonadotrophin
SHBG	sex hormone binding globulin
FSH	follicle stimulating hormone
LH	luteinising hormone
T	testosterone
DHT	dihydrotestosterone
PR	production rate
MCR	metabolic clearance rate
DEXA	duel energy xray absorptiometry
MRI	magnetic resonance imaging
AR	androgen receptor
HDL-C	high density lipoprotein
LDL	low density lipoprotein
EPO	erythropoietin
HH	hypogonadotrophic hypogonadism
TU	testosterone undecanoate
TE	testosterone enanthate
AMH	anti Mullerian hormone
iTT	intratesticular testosterone

DMPA	depot medroxyprogesterone acetate
LNG	levonorgestrel
NET	norethisterone
CPA	cyproterone acetate
MENT	7α-methyl-19-nortestosterone
MHC	male hormonal contraception
WHO	World Health Organisation
CONRAD	Contraceptive Research and Development Program
SERMS	selective oestrogen receptor modulators
SARMS	selective androgen receptor modulators
NB-DNJ	N-butyldeoxynojirimycin
SMA	styrene maleic anhydride
IVD	intra-vas device
NSV	no scalpel vasectomy
BMI	body mass index
SEM	standard error of the mean
PCR	polymerase chain reaction
SRD5A1	5α-reductase type 1
CYP17A1	17α hydroxylase
HSD3B2	3β hydroxysteroid dehydrogenase
ACRBP	acrosin binding protein
MAGEA4	melanoma antigen family A4
PRM1	protamine 1

Chapter 1: Introduction

The development of new commercially–viable male hormonal contraceptives has been described for at least 20 years as being "5 years away". The mainstay of development has largely been undertaken by the public sector, with the pharmaceutical companies only participating in significant clinical trials in recent years. However, they have now realised that these products would have commercial viability and significant commercial trials of potential methods are now underway. The background science requires a close understanding of the process and control of spermatogenesis, regulation of testosterone secretion and its many functions, and the influence on the human body of the administration of exogenous hormones. These aspects will be discussed in detail in this introduction along with the previous work in the field of male hormonal contraceptive development and a brief mention of potential non-hormonal targets.

Chapter 1.1: Physiology of Spermatogenesis

1.1A: Process of Spermatogenesis

Spermatogenesis is the process whereby immature germ cells undergo divisions, differentiation and meiosis to become spermatozoa (see figure 1); this takes place in the seminiferous tubules of the testis in close conjunction with the Sertoli cells, the somatic cells of the seminiferous epithelium. The Sertoli cells are complex in structure with numerous cytoplasmic processes encircling the developing germ cells. They rest on the basement membrane and are responsible for the maintenance of the blood-testis barrier [1]. The developing germ cells are in intimate relationships with the Sertoli cells and multiple germ cell types are associated with any one Sertoli cell. The arrangements of the germ cells around Sertoli cells are not random; they are arranged according to specific cellular associations [2]. These are responsible for the creating the cycle of the seminiferous epithelium, different arrangements of cellular associations constituting a specific stage [3]. The different stages/phases of spermatogenesis are highly ordered and occur over a specific length of time. In different species varying numbers of recognizable cellular associations have been identified within the testis, in the rat there are 14 but in the human there are 6 [4, 5].

Spermatogonia are the least differentiated germ cells in the testis and they are found on the basement membrane between the Sertoli cells. They have been classified into three subgroups: A spermatogonia, B spermatogonia and in some mammalian species Intermediate spermatogonia have also been identified (not present in humans). A new model of spermatogonial proliferation in the human testis has recently been proposed with supporting data from non-human primates [6].

Classically, the spermatogonial stem cells are believed to be a variant of the type A spermatogonia [2]. The type A spermatogonia undergo a succession of mitoses to become type B spermatogonia, which can be distinguished by their smaller nuclei and the gathering of chromatin crusts around the nuclear membrane [5]. There are a variable number of generations of both type A and type B spermatogonia depending on the species in question. In a given species the numbers are fixed, for example in man there are two type A and one type B [7], but in the monkey there are one/two type A and four type B [5]. The last mitosis of the type B spermatogonia forms pre-leptotene spermatocytes, which have Sertoli cell cytoplasmic projections interposed between themselves and the basement membrane. The pre-leptotene spermatocytes are identified as being the generation at which the majority of DNA is synthesised for the forthcoming meiotic stages [2]. The synthesis of DNA ceases as the spermatocytes enter the leptotene stage, identified by the presence of chromosomal filaments, and then the homologous chromosomes pair off during the zygotene phase. The ensuing pachytene stage, when the chromosomes thicken, is known as the stage at which the spermatocytes are most vulnerable to damage [5]. The swift diplotene stage results in partial separation of the chromosome pairs, which is then completed during the first meiotic division to become secondary spermatocytes containing a single set of chromosomes. The second meiotic division follows, when the secondary spermatocytes become the haploid round spermatids, which appear very similar but with smaller nuclei. The whole cluster of spermatids is linked syncytially by thin cytoplasmic bridges [8].

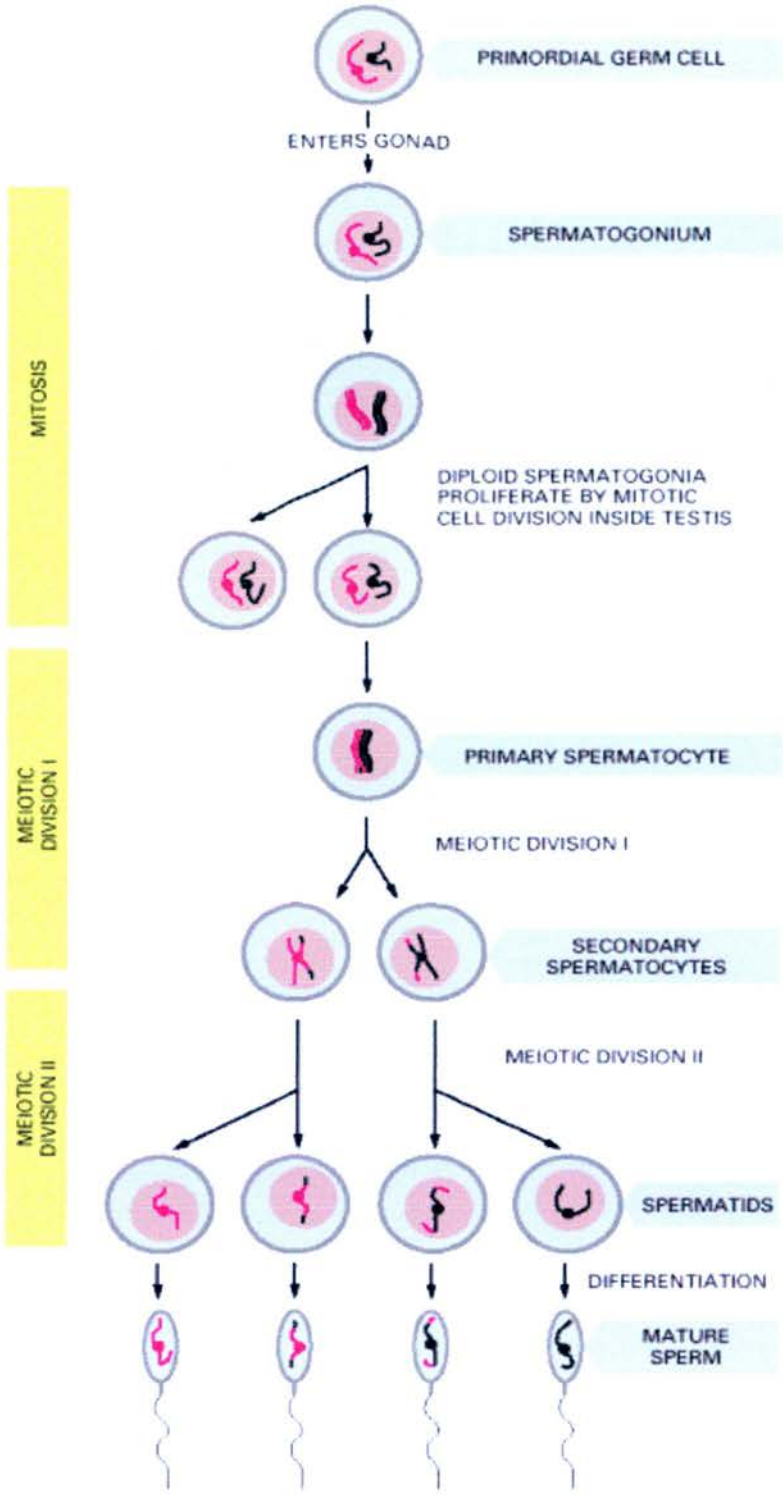


Figure 1: Spermatogenesis in the human male.

Molecular Biology of the Cell 4th Edition. Alberts et al. Garland Science 2002, New York and London

The final phase of spermatogenesis after meiosis is complete is known as spermiogenesis, which comprises several distinct processes which transform the spermatocytes into spermatozoa: formation of the acrosome and development of the tail: condensation and reshaping of the nucleus; and removal of virtually all cytoplasm. When all these processes are complete and all the characteristic features of spermatozoa are present, it is released from the Sertoli cell into the seminiferous tubule lumen [5]. The spermatozoa are then free to progress through the rete testis to the ducts of the epididymis.

1.1B: Role of Gonadotrophins

The testis has the dual purposes of both hormone production and spermatogenesis, dependent on the secretion of the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH) (see figure 2). The secretion of gonadotrophins is under the control of GnRH from the hypothalamus, with inhibitory feedback via steroid and peptide hormones from the testis. The pituitary control of spermatogenesis was first proposed in the 1920's by observing the effects of hypophysectomy in the rat, followed by the reintroduction of pituitary extracts [9]. It was later realised that two pituitary hormones were involved with differing effects on Sertoli cells and on testosterone production [10]. Luteinising hormone stimulates the production of testosterone (T) from the Leydig cells of the testis while FSH acts on receptors in the Sertoli cells. Therefore, suppression of these hormones should result in arrest of spermatogenesis, and is the basis of the hormonal approach to male contraception.

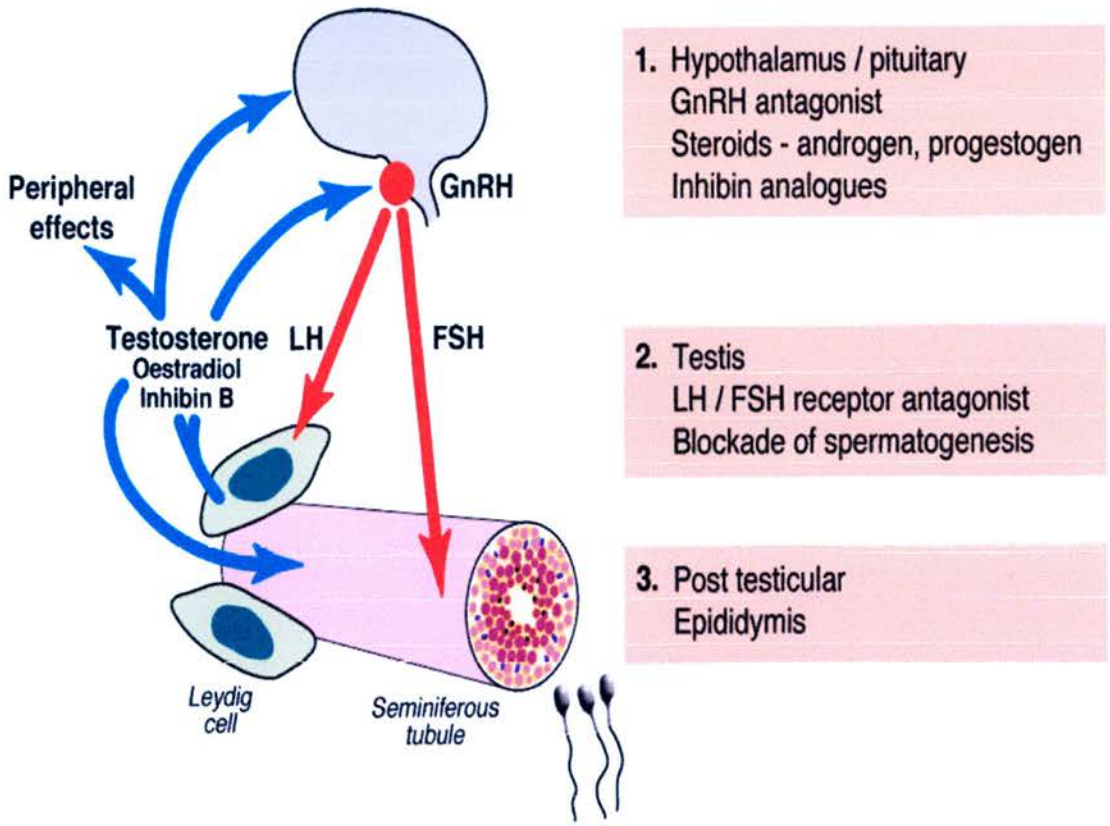


Figure 2: Endocrine control of testosterone secretion with potential targets for male hormonal contraception identified.

Image courtesy of Professor RA Anderson, University of Edinburgh

When gonadotrophins are withdrawn in the adult male sperm production ceases. However, the interaction between the effects of the two gonadotrophins is poorly understood. Gonadotrophin withdrawal may result from congenital GnRH deficiency, GnRH analogue administration, spontaneous mutation in the GnRH receptor, hypophysectomy, or suppression of gonadotrophins by exogenous androgens in a contraceptive regime. The exact roles for testosterone and FSH in spermatogenic regulation have also yet to be defined despite being extensively investigated. It has been established that FSH is vital for normal development of the fetal testis [11] but its role in the adult testis is less clear. Mice knockout models for both the FSH receptor and the FSH-β subunit have been produced: both phenotypes developed to sexual maturity and were fertile with qualitatively normal spermatogenesis although their testicular size was significantly reduced [12-14]. Men with inactivating FSH receptor mutations demonstrate a similar picture with variable reduction in sperm concentrations and reduced testes volumes but preserved fertility [15, 16]. However, two individuals with inactivating FSH-β subunit mutations were both azoospermic [17, 18]. Experimental work in humans has demonstrated that although the presence of FSH is not necessary for spermatogenesis to proceed, it is important for quantitatively normal sperm production. Thus spermatogenesis is maintained in men rendered FSH deficient due to hCG administration, and administration of hCG to men on a high dose testosterone regime resulted in restoration in spermatogenesis to 50% of baseline levels, but the addition of FSH restored this to normal quantitative levels [19]. These data demonstrate that a potential contraceptive regime aimed at reducing FSH secretion or action alone would not be successful.

Inactivating mutations of the human LH receptor affect male sexual differentiation [20], but the range of effects is wide and dependent on the extent of receptor inactivation. Testosterone production levels can be absent or variably low and the phenotype extends from mild undervirilization to a complete lack of masculinization [16]. A case report of a single male with total inactivation of the LH β subunit has been described and he was phenotypically different to that seen with the LH receptor mutation- being normally masculinized at birth but unable to progress through puberty [21]. This observed difference is due to his responsiveness to intrauterine hCG stimulating testicular testosterone secretion, as opposed to those subjects with the LH receptor deficiency [20].

Activating mutations cause precocious puberty due to increased androgen production during foetal life and the postnatal period, the degree of abnormalities seen depends on the extent of the LH receptor mutation [22].

1.1C: Role of Testosterone

Testosterone (T) is produced by the interstitial Leydig cells of the testis. Intratesticular concentrations of testosterone are generally 100 times that found in the peripheral circulation. In humans they measure approximately 400-600ng/g versus 6ng/ml in serum [23, 24]. The relationship between intratesticular testosterone concentrations and spermatogenesis has been a subject of much debate with the exact concentration of testosterone required to support spermatogenesis being difficult to quantify [25]. In Leydig-cell depleted rats administered testosterone, it was possible

for approximately 30% of normal testosterone concentrations to support spermatogenesis [26] and no Leydig cell products other than testosterone appeared to play a role in spermatogenic maintenance [27]. In a study on rats where FSH secretion was supported, spermatogenic initiation and maintenance was achieved in the presence of 10% of normal testosterone levels [28]. The testicular androgen receptor (AR) has been identified on Sertoli cells, peritubular cells and Leydig cells, but not germ cells [29]; therefore it is assumed that the stimulatory effect of testosterone acts on spermatogenesis via the Sertoli cell. Normal intratesticular testosterone levels are far higher than that required to fully saturate the androgen receptor, but when intratesticular levels are experimentally reduced in monkeys by 15-30% (still much higher than levels in the serum) significant impairment of spermatogenesis results [30]. In gonadotrophin-suppressed humans administered exogenous testosterone +/- progestogen a more profound suppression of intratesticular T concentrations was seen with their intratesticular levels measuring 2% of that of controls. This reduction resulted in gross disruption of both spermatogonial maturation and spermiation [31]. The reasons for the dependence of spermatogenesis on supraphysiological levels of T are not clear; nor is there any consistency between species of the degree of spermatogenic suppression achieved by reducing the intratesticular T levels.

Testosterone is metabolised within the testis by type 1 or 2 isoenzymes of 5α-reductase to dihydrotestosterone (DHT), a more potent androgen that has a higher affinity for the androgen receptor [32, 33]. In locations with a high concentration of T such as the testis, it is T that forms stable complexes with the AR [32]. However,

in areas of lower T concentration and high 5α-reductase activity such as the prostate, it is DHT that is the active androgen. In gonadotrophin-suppressed men receiving a hormonal contraceptive regime of T +/- progestogen, despite the significant intratesticular T suppression, DHT levels in the testis remained unaffected [31]. The significance of this to male hormonal contraceptive development is as yet unclear, but it may be that DHT levels may maintain some degree of spermatogenesis even if intratesticular T levels are markedly affected. This theory is supported by data from a contraceptive study investigating the DHT levels in men who received exogenous T and became azoospermic or oligozoospermic. Serum and seminal plasma DHT levels were higher in the men who did not achieve full spermatogenic suppression, which implies a degree of up-regulation in oligozoospermia [34, 35]. There is further evidence of the role of 5α-reduced androgens in spermatogenesis from work in rats. Adult male rats were given oestradiol and testosterone implants for nine weeks to inhibit spermatogenesis. Subsequently they were administered increasing doses of testosterone (implants of four different sizes 3, 6, 10 and 24cm) +/- a 5α-reductase inhibitor, and hourly production rates of round spermatids were assessed using stereological techniques. It was found that giving a 5α-reductase inhibitor significantly suppressed the hourly round spermatid production at the two lower testosterone doses. Therefore it seems that 5α-reduced androgens do have a role in restoration of spermatogenesis [36].

1.1D: Endocrine Control of Spermatogenesis

Spermatogenesis in humans and other species relies on the presence of both FSH and T but their respective functions in this process are not well defined. Initiation of some of the stages such as sperm release and germ cell survival require input from both T and FSH [37-39], whereas some stages appear to be dependent upon stimulation from only one. According to some studies in primates testosterone is responsible for stimulating the production of a quantitatively normal number of type-A spermatogonia [40], and FSH (with testosterone) for the creation of a normal number of type-B spermatogonia. However, in other studies FSH also appeared responsible for production of type-A spermatogonia [41, 42]. FSH alone was sufficient to reinitiate spermatogenesis in men during gonadotrophin suppression induced by exogenous testosterone administration [43]. A complementary study administering LH alone to men similarly suppressed also demonstrated a return to pre-treatment sperm concentrations [44]. Both hormones are therefore able to initiate and maintain normal spermatogenesis by acting via the Sertoli cell. These findings suggest that testosterone and FSH act in a truly synergistic way via different mechanisms, and are both equally important factors for normal spermatogenesis to occur.

Chapter 1.2: Testosterone Production and Metabolism

1.2A: Control of Testosterone Production

Testosterone is essential in the male for the development and maintenance of normal reproductive tissues namely the testis, prostate, epididymis, seminal vesicles and penis. It is also plays an important role in peripheral functions such as muscle mass/strength, and hair growth. In order to maintain normal androgen levels in the body the production rates of testosterone must be compatible with the rates of excretion. Over 95% of testosterone is produced by the Leydig cells of the testis which produce approximately 6-7 mg/day [45], the remainder of production is contributed by the adrenal cortex. Testosterone is produced from cholesterol via the $\triangle 5$ and $\triangle 4$ pathways, in humans the $\triangle 5$ pathway is the pre-dominant mechanism with dehydroepiandrosterone (DHEA) as the first C_{19} intermediate (see figure 3) [46]. The enzyme 3β-hydroxysteroid dehydrogenase (3βHSD) is responsible for the essential step of converting $\triangle 5$-3β-hydroxysteroids to $\triangle 4$-3-ketosteroids and the final stage in testosterone biosynthesis is the reduction of the 17-keto-group by 17β-hydroxysteroid dehydrogenase (17βHSD) [22].

LH is the main hormone involved in regulation of Leydig cell number and function and it exerts its action via the LH receptor [47]. Human choriogonadotrophin (hCG) can also activate this receptor because it has a very similar structure to LH. This similarity has been exploited effectively in both basic science and clinical studies to investigate the mechanisms of LH receptor stimulation. LH and hCG initially produce a stimulatory effect on testosterone secretion this response is more

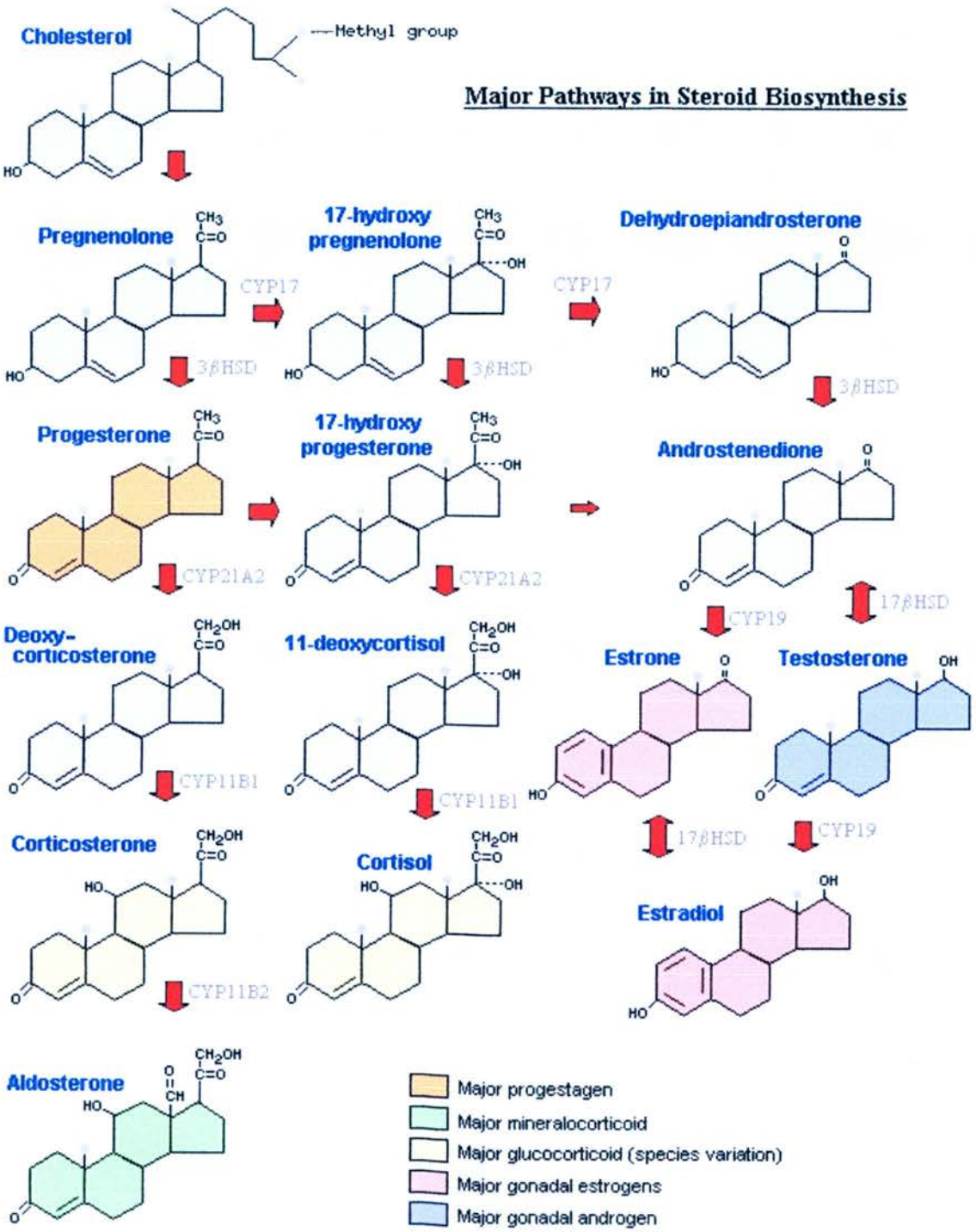


Figure 3: Significant pathways in steroid production

pronounced in rats than in man and manifests as a rapid rise in plasma T levels [48]. It is not currently clear why the response in man is not as marked [49].

Although LH is the dominant controlling force in T secretion other hormones have been shown to have an effect on Leydig cells, namely thyroid hormone and the glucocorticoids. Glucocorticoids have been demonstrated to have an inhibitory effect on T production by interfering with steroidogenesis and inducing Leydig cell apoptosis in rats [50]. Thyroid hormone appears to have a contrary effect and has been shown to both stimulate steroid production [51] and result in more rapid Leydig cell differentiation [52].

Locally produced or paracrine factors have also been demonstrated to play a part in the control of testosterone production. Over the years many differing studies have identified factors including IGF-1, TGFβ, PDGF, Inhibin, interleukin, and TNFα amongst others as having a role to play. In many publications both inhibitory and stimulatory effects have been shown for these and other factors and the true degree to which they contribute is hard to fathom as it is difficult to meaningfully assess them all in isolation [48, 53, 54]. The extent of their contribution seems to be far outweighed by the role that LH plays.

1.2B: 24-Hour Testosterone Production

Serum testosterone levels in young healthy men are not constant throughout any given 24-hour period. It is known that in young men testosterone exhibits diurnal variation with maximal testosterone levels in the early hours of the morning and minimal in the late afternoon/early evening [55]. This rhythmicity may remain in very healthy older men, but can become blunted by the aging process and this decline is accompanied by a decrease in mean serum T levels [56, 57]. When free T or non-sex-hormone-binding globulin bound, (non-SHBG-T) is measured then the differences between young and old men appear even more pronounced than when only total- T is assessed [58]. The cause for diurnal variation of serum T levels remains unknown, it may be related to pineal gland melatonin secretion [59] or to some as yet unidentified regulatory pathway possibly at testis level. There is currently a high level of interest in the fall in mean serum testosterone levels seen in the aging male, and much speculation as the existence of the "andropause." It is clear that a reduction in testosterone levels progressively above the age of 55 is seen, and this may contribute to many of the features of male aging including loss of body hair and musculature. Currently, androgen replacement in elderly men remains a contentious issue and should be reserved for those with T levels clearly below the normal range for young men and unequivocal symptomatology.

The serum concentration of testosterone (T) is dependent upon a balance between both T production rate (PR) and the metabolic clearance rate (MCR). Most of the studies that have been undertaken in this field have been to establish normal T

production rates, which it is possible to assess using a variety of methods, the commonest being isotope dilution and mass spectrometry but testicular blood flow studies and labelled T and urine collections have also been implemented. Reviewing the data available, estimates of T production rates in healthy young men have ranged from 3.23mg/day to 7mg/day [60]. A study by Vierhapper et al in 1997 using isotope dilution and mass spectrometry estimated T production rates to be 3.7 +/-2.2 mg/day in healthy men aged 19-32 years [61]. This figure is lower than would be expected from other data. An explanation for this may be that the high levels of labelled exogenous testosterone used in this study partially suppressed endogenous production. A recent similar study reported the 24-hour T production rate in young Caucasian males as 9.11+/- 1.11 mg/day and the PR in middle-aged men was significantly less at 3.88 +/- 0.27 mg/day [62]. Quantification of 24-hour T production rates and serum T levels are highly relevant for male contraceptive development as it is extremely important to avoid both supraphysiological and subphysiological doses of testosterone if at all possible as they may have unwanted effects.

1.2C: Peripheral actions of Testosterone

a. Testosterone and Bone

Osteoblasts and osteoclasts are responsible respectively for production and reabsorption of bone and an equilibrium between both these activities is essential to maintain normal bone mass. Both androgens and oestrogens are necessary for normal skeletal development and maintenance in men and women. The relative importance of both of these sex steroids has been called into question more recently in the male, with it now becoming apparent that oestrogen has a more significant role to play than had been previously thought. It had long been assumed that androgens played the dominant role in male bone physiology, with the evidence for this arising from the fact that hypogonadism is associated with osteopenia/osteoporosis [63, 64] and also from animal data [65, 66]. However, the conversion of testosterone to estradiol by aromatisation is a crucial process, men with oestrogen receptor mutations or a deficiency of aromatase demonstrate severe deficiencies in skeletal development [67, 68]. There is now known to be a correlation between endogenous estradiol levels and both bone mineral density and bone loss [69-71]. Bone density is determined by peak bone mass achieved during development, and subsequent bone resorption. Although both sex steroids are essential for the attainment of peak bone mass the 25% higher bone density exhibited in men compared with women is attributed to their higher testosterone levels [72]. In normal men bone density begins a slow decline at the ages of 30-35 years, which has been attributed to a fall in estradiol as opposed to testosterone [72].

In hypogonadal men the effects of androgen replacement have been evaluated. In a multicentre trial investigating the use of differing transdermal testosterone preparations in 227 hypogonadal men, significant increases in hip and spinal bone density were seen as assessed by dual-energy x-ray absorptiometry (DEXA) [73]. A study by Zitzmann et al in 2002 involving 521 men compared healthy controls with both newly-diagnosed hypogonadal men and men receiving testosterone replacement and monitored bone density by phalangeal quantitative ultrasound. They suggested that the bone density of androgen-deficient men can be greatly improved by testosterone replacement but may not reach that attained by healthy controls [74]. This outcome is supported by a similar smaller trial (n= 53) using DEXA for analysis [75].

The potent synthetic androgen 7 α-methyl-19-nortestosterone (MENT) is resistant to 5α-reductase but is aromatisable. Therefore it should be prostate-sparing but still support other androgen-dependent tissues. It has been tested as an androgen replacement in 16 hypogonadal men and demonstrated evidence of prostate sparing [76]. But whilst sexual function and erythropoiesis were maintained, a reduction in bone mass was seen over the 24 week treatment period in conjunction with a reduction in serum markers of bone formation. It may well be that the MENT metabolites are less effective in their activation of estrogen receptors than natural estradiol [76].

b. Testosterone and Skeletal muscle

Testosterone has a direct anabolic role to play in maintaining muscle size and strength. It is known that healthy hypogonadal men have a lower fat-free mass and higher fat mass when compared to age-matched eugonadal controls and that administration of testosterone can significantly improve this situation [77-79]. These studies all demonstrated that physiological replacement of testosterone increased fat-free mass, muscle size and strength. Similarly Mauras et al in 1998 reported that causing experimental suppression of testosterone in healthy young men by administration of a GnRH agonist resulted in an increase in fat mass, a significant decrease in fat-free mass and a reduction in muscle protein synthesis [80].

A study by Bhasin et al in 2001 further examined this area in order to establish whether these effects are dose-responsive. They enrolled 61 healthy eugonadal men aged 18-35 years and assessed a broad range of androgen-dependent functions including body composition, muscle size and strength, plasma lipids and haemoglobin. All subjects received monthly GnRH agonist injections and were randomised into one of 5 groups to receive weekly injections of 25, 50, 125, 300 or 600mg of testosterone enanthate for 20 weeks. They found that all the assessed outcome measures above had a linear relationship to testosterone dose, but sexual function and cognition did not vary significantly across the dose range. Therefore, the anabolic function of GnRH treated healthy young men can be predicted by testosterone dosage [81, 82]. A further recent study by the same group has shown that older men are just as responsive to the anabolic effects of graded testosterone doses as their younger counterparts [83].

But what are the effects of supraphysiological levels? For many years debate has continued regarding the effects of exogenous testosterone on healthy individuals resulting in supraphysiological levels; particularly with reference to abuse of anabolic steroids by athletes and body-builders. In recent years improved imaging techniques (magnetic resonance imaging and more refined body composition analysis) have made smaller changes in muscle size and fat-free mass more accurately detectable in order to establish how effective these compounds are. Another investigation by Bhasin et al in 1996 focused on the effects of supraphysiological doses on muscle strength and body composition. Forty-three normal men were recruited and assigned to one of 4 groups: placebo and no exercise, testosterone and no exercise, placebo plus exercise and testosterone plus exercise. Subjects received either placebo or 600mg testosterone enanthate weekly for 10 weeks. Men in the exercise groups underwent standardised weight lifting training 3 times a week. Muscle size was assessed by MRI, and strength of the arms and legs by bench presses and squat exercises. Men in the testosterone alone group demonstrated a mean increase in triceps area of 13.2% +/- 3.3. The greatest overall changes were seen in the T + exercise group with a mean increase in fat free mass of 9.5% (+/-1%) and increase in both triceps and quadriceps areas of 14% [84]. A study by Sinha-Hikim et al in 2002 investigated the mechanism behind the testosterone-induced increase in muscle size: to establish whether it was due to muscle hypertrophy or hyperplasia. Muscle biopsies were obtained from 39 men before and after 20 weeks of treatment with GnRH agonist and different doses of weekly testosterone enanthate (25, 50, 125, 300 or 600mg). The results demonstrated

that testosterone-induced increases in muscle volume were due to muscle fibre hypertrophy, rather than an increase in fibre numbers, and that the proportion of type 1 to 2 fibres remained unaltered. These changes were seen in a dose-dependent manner with both the 300 and 600mg doses showing profound effect [85].

c. Testosterone and cardiovascular disease

After many years of research the relationship between testosterone and cardiovascular disease is still not clear. In animal models in investigations of the effects of testosterone on atherosclerosis results have proved conflicting. Studies of castrated rabbits given androgen replacement alone or in conjunction with oestrogen have shown either no change or a beneficial effect of testosterone on the size of diet-induced atherosclerotic plaques in male animals, but a detrimental effect in female rabbits [86-88]. Adverse effects of testosterone administration were also seen in female ovariectomised cynomologus monkeys [89], implying a sex specific effect of exogenous testosterone on atherosclerosis.

From the animal models it may be inferred that low endogenous testosterone in men and hyperandrogenism in women (e.g polycystic ovarian syndrome) are both states more likely to promote the acceleration of atherosclerosis. This impression has not however been confirmed by prospective studies [90, 91] but appears to be the true in cross-sectional studies [92-94]. Hypoandrogenism in men and the converse state in women are known to be associated with obesity, insulin resistance, raised triglycerides and LDL cholesterol, and lowered HDL cholesterol but the

relationships cannot be straightforward as the correlation with atherosclerosis is unproven [95-97].

In humans, knowledge of the effects of exogenous hormones is highly relevant for both male contraceptive development and for testosterone replacement in the aging male, and again the data is highly inconsistent. Exogenous androgens appear to have a beneficial effect on some cardiac risk factors and on others a detrimental one. I will take for examples the differences in effect on HDL-C and lipoprotein (a).

A low HDL-C level is seen as an important risk factor for cardiovascular disease because HDL-C exhibits several actions that appear to be anti-atherogenic and therefore a drug that may reduce atherosclerosis by raising HDL levels would potentially have a significant impact [98]. In hypogonadal men testosterone replacement has been demonstrated to mildly reduce HDL-C levels but have no significant effect on total cholesterol, LDLs or triglycerides [77, 99]. In eugonadal men administered exogenous supraphysiological doses of testosterone +/- progesterone for male hormonal contraceptive trials have also demonstrated a decrease in HDL-C [100-102]; further emphasising the importance of avoiding supraphysiological doses.

Lipoprotein (a) levels vary greatly between individuals (0-300mg/dl), but it has been seen in prospective population studies and case-control trials that a high Lp (a) is a risk factor for cardiovascular disease [103, 104]. There is evidence that administration of exogenous testosterone reduces Lp (a) levels and this may have a

beneficial cardiac effect. Administration of supraphysiological doses of testosterone enanthate has been found to decrease Lp (a) levels by up to 59% [105, 106], and men whose own testosterone was suppressed by the GnRH antagonist cetrorelix exhibited an elevation in Lp (a) levels by 40-60% [107]. It is not possible to take the effect on these risk factors in isolation, the effects must be considered as part of the complex system of cardiovascular risk factors and the implications for the development of male hormonal contraception are still unknown and may be further clarified when large-scale hormonal contraceptive trials in men are undertaken.

d. Testosterone and erythropoiesis

The first studies demonstrating a relationship between testosterone and erythropoiesis were reported in the 1940s when healthy rats were shown to develop a significant increase in bone marrow activity and haemoglobin concentration when administered testosterone [108]. These findings were supported by a trial that showed anaemia seen in orchidectomised rats could be reversed by testosterone administration [109]. More recently trials showing that testosterone deficiency causes anaemia in man have also reported [110, 111].

The effects of testosterone on erythropoiesis are mediated via a number of processes:

- Stimulation of erythropoietin (EPO)
- Stimulation of bone marrow erythroid colonies
- Increased iron incorporation in red blood cells
- Increased haemoglobin synthesis
- Enhanced red cell glycolysis
- Increased red blood cell 2,3-diphosphoglycerate levels [112]

These effects can be demonstrated in a number of clinical trials of androgen replacement in hypogonadal men. Untreated hypogonadal men exhibit anaemia due to low concentrations of both erythrocytes and haemoglobin, which can be improved by androgen replacement therapy, a wide range of therapies have been investigated in this regard. A study of 227 hypogonadal men who used transdermal testosterone gel in two doses (100 or 50mg/day) showed significant rises in their blood cell mass in a dose-dependent manner [79]. The injectable preparation testosterone enanthate (TE) was studied in 60 men rendered hypogonadal by administration of a GnRH agonist where it was found to produce a dose-dependent response [81]. When the effectiveness of TE was compared to that of the non-scrotal transdermal testosterone patch in 66 hypogonadal men, both were found to significantly raise haematocrit but the incidence of abnormally high elevations was higher in the TE group (43.8%) that the patch users (15.4%) [113]. This is likely to be due to the wide fluctuation in serum testosterone concentration seen with short acting injectable preparations such as TE. The longer acting testosterone undecanoate (TU) results in a more stable serum concentration and has also been investigated as a replacement T preparation in

hypogonadal men. It has been demonstrated to produce significant stimulation of erythropoiesis but a lower incidence of polycythemia [114]. Testosterone pellets are the most long lasting form of testosterone replacement currently in use and they have also been shown to produce a suitable rise in haematocrit in a dose-dependent manner [115]. The synthetic androgen 7α-methyl-19-nortestosterone also demonstrated an ability to support normal erythropoiesis when its efficacy as androgen replacement was tested [76]. The maintenance of a normal haematocrit is highly important in relation to both androgen replacement therapy and the development of a marketable male hormonal contraceptive. Some men receiving testosterone replacement do develop polycythemia [116, 117] and this has been associated with an increased risk of cerebrovascular disease [118] although the relationship with cardiovascular disease is not yet clear. It is important that any delivery methods of testosterone used for both T replacement and a potential male hormonal contraceptive maintain as steady serum T concentrations as possible.

e. Testosterone and the prostate

The prostate gland is androgen dependent and requires the presence of testosterone for its growth and development at puberty, without it the gland will not grow to adult size (approx 20 grams). Within the prostate gland testosterone is irreversibly converted to 5α-dihydrotestosterone (DHT) by 5α-reductases, of which there are two isoforms. Type 1 5α-reductase is only present in low levels in the prostate, but is the pre-dominant form in the skin and liver [119]. The predominant isoform in the prostate is type 2 5α-reductase and once converted by this DHT controls prostate cellular proliferation via the androgen receptor. The intra-prostatic levels of DHT

remain constant throughout a 24-hour period, thus avoiding the peaks and troughs of testosterone in the circulation [55]. This constant hormone environment is critical to maintain a balance of prostate growth and regression [120] and to control androgen receptor function [121]. During exogenous hormone administration for male hormonal contraceptive trials no significant change in prostate size has been observed by transrectal ultrasound scanning [122] Although the question of: "how does several years of exogenous androgen administration affect one's prostate cancer risk?" currently remains unanswered and will stay so until long-term clinical trials or indeed post-licencing assessments are made. The prostate cancer prevention trial (PCPT) evaluated the effect of finasteride 5mg daily compared with placebo on the incidence of development of prostate cancer. The trial was stopped early due to premature attainment of the primary endpoint: a 25% reduction in the prevalence of prostate cancer was observed in the finasteride treated group (prostate cancer diagnosed in 18.4% of finasteride-treated subjects compared to 24.4% of placebo). However, in the finasteride-treated group the detected prostate cancers showed more aggressive Gleason grades [123]. While this trial confirmed its primary hypothesis that chronic suppression of intra-prostatic DHT reduces the incidence of prostate cancer the implications of the secondary findings are less clear. Most evidence points to the likely explanation of these findings as histological artefact because it is known that histological assessment of the prostate is altered by finasteride treatment [124, 125]. With the recent arrival of the synthetic androgen 17α-methyl-19-nortestosterone (MENT), which is resistant to 5α reductase, it is thought that it's prostate-sparing action makes it an excellent candidate for a male hormonal

contraceptive, with a probable reduction in safety concerns, and possible health benefits if a chemoprevention effect on prostate cancer can be proven.

f. Testosterone and Behaviour

Sexual behaviour: The normal physiological range for testosterone in men is far higher than that required to maintain normal sexual functioning, with the critical level appearing to be around 3ng/ml [126]. Studies of hypogonadal men and the testosterone replacement therapy that they receive have produced a very convincing case for the effect of testosterone on sexual arousal/desire and behaviour [127-129]. In male hormonal contraceptive trials administering supraphysiological doses of testosterone, significant increases in arousal and desire have been reported but no corresponding alteration in behaviour has been observed [130, 131]. There appears to only be a beneficial effect on sexual behaviour when administering T to those men with significantly low levels, i.e giving T replacement to eugonadal men with sexual problems has not demonstrated any beneficial alteration in behaviour [132].

Aggression: There are data from many studies linking high endogenous T levels with increased rates of aggressive behaviour. Many of these studies have been carried out looking at the hormonal levels in violent male offenders, which is probably not relevant to a target population for male hormonal contraception [133-135]. Looking at the evidence from hypogonadal men taking exogenous testosterone replacement and those men receiving testosterone as part of a male hormonal contraceptive trial it is possible to gather data on the effects of exogenous T administration, and in both these applications no effect on aggression has been observed [100, 129, 136, 137].

Mood: The investigation of the role of testosterone in maintaining mood was prompted by the observation that hypogonadal men given androgen replacement reported a reduced incidence of low mood and emotional lability. In recent years, further studies into this area have produced conflicting data with most studies failing to show a link between endogenous T levels and depression [138, 139], but some showing correlation between high endogenous T levels and emotional well-being [140, 141]. There is some strong data relating to the effects of administering exogenous testosterone in elderly men with low/borderline T levels and in healthy men without hypogonadism. In these groups a significant reduction in negative mood scores and a significant improvement in sense of well-being were recorded on T administration [138, 140]. A recent study further assessing the effects of exogenous testosterone administration in eugonadal men has reported detectable but minor mood changes, however they concluded that in the dose range used for male hormonal contraception these were unlikely to be significant [137].

Cognitive function: It is widely accepted that hormones play a significant role in sex-specific cognitive abilities with women outperforming men at verbal skills, and males excelling at non-verbal skills such as spatial awareness and mathematical reasoning. The role of testosterone in spatial awareness in men has been confirmed by studies on subjects with idiopathic or acquired hypogonadotrophic hypogonadism [142, 143]. Men with life-long testosterone deficiency perform more poorly in these tests than men with late-onset testosterone deficiency and normal controls. These findings imply that the hormonal status during development has life-long

implications for intellectual function. When healthy aging men were administered testosterone to improve sexual function it was noted that they showed an improved ability to complete a visual-spatial task compared to a placebo group [144].

Chapter 1.3: Inhibin B

1.3A: Testicular Production

Inhibins are glycoproteins of gonadal origin that exert inhibitory effects on gonadotrophin secretion. There are at least two biologically active forms Inhibin A and B, these are heterodimers with each consisting of an α subunit and either β_A (inhibin A) or β_B (inhibin B). In women both inhibin A and B are important and are produced by the granulosa and theca cells of the ovary. The physiologically relevant form in men is inhibin B produced by the testis [145].

Inhibin subunits can be located in the testis from fetal life. By the second trimester both α and β_B subunits are expressed in Sertoli and Leydig cells, β_A can also be isolated in Leydig cells and β_B in developing gonocytes [146]. The β_A subunit is predominantly used for production of the homodimer activin A, hence inhibin A synthesis remains low and is undetectable in the serum in the adult male [145, 147]. Following puberty the Sertoli cells continue to express only the α subunit and the β_B subunit is located predominantly in the germ cells and to a lesser extent in the Leydig cells [148]. Confirmation of this has been established by administering FSH to healthy men [149]. This does not lead to any significant increase in the circulating levels of inhibin B however levels of pro-α C rise, suggesting that Sertoli cell production of the α subunit is stimulated but that the release of the β_B subunit involves a more complicated process [150].

1.3B: Control of Secretion

Inhibin B production is partly under gonadotrophin control. This has been demonstrated by studies involving the administration of GnRH to men with hypogonadotrophic hypogonadism [151, 152]. Serum inhibin B concentrations were seen to rise from approx 25-30% of normal into the normal range. Pre-treatment inhibin B concentrations in these studies were highly variable and correlated with testicular volume (Sertoli cell number). Men in these trials with higher pre-treatment inhibin B concentrations were more likely to become fertile due to GnRH administration.

Further evidence comes from male hormonal contraception trials. Men who were treated with weekly exogenous testosterone injections (200mg testosterone enanthate TE i.m) for up to 18 months. During the treatment phase FSH and LH became rapidly suppressed and this trend was followed by significant spermatogenic suppression and a fall in inhibin B levels to approx 30% of their pre-treatment values [153]. A similar decline in inhibin B concentrations was reported in healthy men receiving a combination of levonorgestrel and TE [147]. So, it may be extrapolated from these results that approx 70% of inhibin B is gonadotrophin-dependent. However, suppression of inhibin B is not essential for effective gonadotrophic and spermatogenic suppression in male hormonal contraceptive trials. No suppression of inhibin B was seen in a trial using oral desogestrel and testosterone pellets [154], or in a study of cyproterone acetate with TE [155] despite these trials reporting significant spermatogenic suppression. The differences in degrees of inhibin B suppression in these different contraceptive trials may reflect differences in the way

that the various hormones used exert their effects i.e: they may act on different stages of spermatogenesis. The 25-30% of Inhibin B that appears to be gonadotrophin-independent remains present except in rare circumstances. Inhibin B can be totally undetectable in patients with a significant testicular abnormality [145], or following testicular irradiation [156]. In patients administered chemotherapy the fall in inhibin B levels can be used to illustrate progressive testicular damage [157].

1.3C: Inhibin B and spermatogenesis

Inhibin B levels demonstrate a positive correlation with sperm concentration in the ejaculate, this has been demonstrated in a variety of population groups from those in the general population [158], men of known fertility [159] and also infertile men [160]. The spermatids appear to have defined roles in the mechanism of inhibin B secretion in the testis [150]. Men proven to have Sertoli-cell only syndrome or spermatogenic arrest at a pre-spermatid phase demonstrate levels of inhibin B that are very low and may be undetectable. Whereas men with obstructive azoospermia and spermatidic arrest exhibit normal Inhibin B concentrations [148, 161]. However, it is also possible for a small minority of men with Sertoli-cell only syndrome to produce some inhibin B, and the origin of that remains unknown [162]. Sertoli cells also release inhibin B into the seminiferous tubules allowing for quantification of the level present in seminal plasma. Seminal plasma inhibin B concentrations are can be correlated to sperm count in fertile and infertile men and in men following vasectomy and are significantly reduced in azoospermic men regardless of the cause of their reduced sperm count [159, 163]. Confusingly, inhibin B can also be undetectable in seminal fluid in some men with normal spermatogenesis and normal

serum levels, and the reasons for this are not clear [164]. In the previously mentioned male contraceptive trial administering oral desogestrel with testosterone pellets, spermatogenic suppression was not accompanied by a fall in serum inhibin B levels. However, profound suppression of seminal inhibin B was seen [154].

Chapter 1.4: Anti-Mullerian Hormone

1.4A: Role of AMH

Anti-Mullerian hormone (AMH) is a growth inhibitory glycoprotein related to TGFβs, inhibins, and activins [165], its existence was first proposed by the French scientist Alfred Jost in the 1940s. Sertoli cells produce AMH in the fetal testis and until puberty at high levels, then at low levels thereafter [166, 167]. AMH is one of the earliest Sertoli cell-specific proteins expressed by the gonad [168]. Serum AMH levels therefore provide a useful marker of Sertoli cell maturation. As the blood-testis barrier becomes established AMH secretion switches from the basal to the ad-luminal compartment and therefore seminal-plasma levels rise above serum levels [169, 170].

The main role of AMH is to induce Mullerian duct regression. However, as production continues after this task has been performed, AMH must have further functions. Roles proposed include control of germ cell differentiation [171, 172], induction of the abdominal phase of testicular descent [173, 174], and suppression of lung maturation [175, 176]. AMH acts via two known receptor types. The dominant receptor is receptor type II which is present on cell membranes [177]. This receptor is responsible for ligand binding and then recruits receptor type I to transduce the signal. The intercellular transduction pathways after receptor type I vary according to the target cell [178]. AMH is known to inhibit differentiation of precursors into Leydig cells and expression of steroidogenic enzymes in primary Leydig cells [179] and Leydig cell derived lines [180] via the type II receptor. Male mice with AMH receptor II mutations exhibit Leydig cell hyperplasia [181]. There are three factors

that influence Sertoli cell AMH production at puberty. The dominant controlling factor is the rise in androgen levels but FSH production and the maturation of germ cells also have a role to play.

1.4B: Regulation of AMH production.

Androgen regulation- the decrease in serum AMH levels commences at stage 2 to 3 of puberty [182] coincident with a rise in intratesticular T concentration, before a rise in serum T levels is seen [183]. Serum T rise and AMH fall is seen in normal puberty and in both central and gonadotrophin independent precocious puberty. These observations suggest that it is T as opposed to gonadotrophins that is responsible for AMH down regulation [182]. This is further supported by the fact that males in which deficient androgen production is seen show abnormally high serum AMH [184, 185]. The decline in serum AMH reflects iTT concentrations not serum T. Males with hypogonadotrophic hypogonadism administered hCG develop a rise in iTT levels and decrease in serum AMH. Conversely, those HH subjects receiving T replacement directly are seen to have a rise in serum T levels predominantly and therefore a less significant decrease in AMH production [186].

FSH regulation- Serum AMH is markedly decreased in FSH deficient prepubertal mice, this correlates with reduced testicular volume and Sertoli cell number. Administration of FSH to these animals results in a significant increase in AMH levels [187]. In situations where the inhibitory effect of androgens is absent (such as androgen insensitivity or androgen synthesis defects), and FSH is elevated, the serum AMH recorded during puberty is often very high [184]. The effects of FSH can be

illustrated by a study involving patients with untreated congenital hypogonadotrophic hypogonadism. These subjects were administered recombinant FSH alone for one month and then in conjunction with hCG for a further two months. AMH and inhibin B levels were seen to rise following FSH administration. However, in contrast to the FSH alone, the combined FSH and hCG stimulation of the testis induced a dramatic drop in AMH levels and a lesser but significant fall in circulating inhibin B levels [188].

Germ cell regulation- AMH decrease and meiotic entry of germ cells are both androgen-dependent processes. Experimental observations in $XXsxr^b$ male mice demonstrate that meiotic germ cells have a role in the inhibition of AMH expression. $XXsxr^b$ mice are a natural model for XX males and are normally virilised [189]. They carry a translocated fragment of the Y chromosome containing the SRY gene that induces testicular development leading to normal androgen levels. As they carry two XX genes and lack Y genes essential for spermatogenesis, the germ cells cannot enter meiosis in these animals. In $XXsxr^b$ mice at puberty although a decline in AMH expression is seen this is not as pronounced as in controls due to the lack of meiotic cells [190].

1.4C: Clinical Applications of AMH

AMH can readily be identified in the serum in infants, throughout childhood and adults. In contrast to the high levels found in male infants, AMH is present at much lower levels in females and does not become detectable until the second decade. Therefore AMH provides a specific and sensitive marker for testicular tissue pre-puberty. This makes it a useful tool for the evaluation of gonadal disorders such as intersex states where it has been used to confirm the presence of testicular tissue [191-193]. It has also been used to confirm the complete removal of testicular tissue postoperatively in patients undergoing orchidectomy for gender reassignment or malignant potential. AMH has also been used as a tumour marker in some patients with gonadal tumours such as AMH-secreting granulosa or sex-cord tumours [194, 195].

Chapter 1.5: Current Male Hormonal Contraceptive Agents

1.5A: Introduction

Of the various solutions to the issue of the current lack of male contraceptives, the hormonal approach is the nearest to commercial reality. There have been no new male contraceptive methods developed in the last hundred years, with the currently available methods of condoms and vasectomy changing little. Spermatogenesis is dependent upon the secretion of the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH). The secretion of gonadotrophins is under the control of gonadotrophin releasing hormone (GnRH) from the hypothalamus, with inhibitory feedback via steroid and peptide hormones from the testis. Luteinising hormone stimulates the production of testosterone from the Leydig cells of the testis, which in conjunction with FSH acting on receptors in the Sertoli cells stimulates spermatogenesis. Therefore, suppression of both of these hormones is necessary to result in arrest of spermatogenesis, and this is basis of the hormonal approach to male contraception. It has not so far been possible to suppress spermatogenesis without also affecting testosterone secretion; therefore "add-back" androgens have been an essential component of all trial regimes of male hormonal contraceptives not based on administration of testosterone itself.

The development of this approach has been based in the public sector, with particular support from the World Heath Organisation (WHO) and Contraceptive Research and Development Program (CONRAD). A major barrier has been the lack of development of new testosterone preparations with most men currently requiring testosterone replacement receiving treatments that have not changed in decades.

This is now changing, largely stimulated by the potential for large-scale androgen replacement in ageing men, and several new preparations are emerging. This contrasts with the wide range of progestogens available, in several formulations including oral, long-acting injectables and implants. Although many studies investigating this area have been performed in the last few years, there has been only limited exploration of individual regimens, with little dose-response data and no clear comparison of different progestogens. Despite this, there has been much progress and it is now clear that effective hormonal male contraception can become a reality with dialogue between industry and the regulatory authorities as to how a novel medicine where drugs are taken by one person to have an effect on another (i.e. prevention of pregnancy) can be developed.

Currently both androgen alone and androgen+adjunct regimes are under investigation. The androgen only regimes consistently demonstrate a greater degree of spermatogenic suppression in Asian as opposed to Caucasian populations [196]. The reasons for this are as yet unclear but may be related to a variety of factors including ethnic differences in testosterone metabolism by 5α-reductase activity [197], differences in hypothalamic/pituitary feedback sensitivity to testosterone, and in rates of germ cell apoptosis [198]. Androgen production rates vary between Caucasian and Chinese populations and these appear to be environmental and dietary related rather than predominantly genetic differences [199]. Testosterone action via the androgen receptor varies according to the number of CAG repeats in the common polyglutamine sequence on exon 1 of the gene encoding the androgen receptor, resulting in varied transactivational activity. The number of CAG repeats is also

known to show ethnic variation [200]. As well as known differences in the efficacy of regimes between different ethnic populations there is also the problem of inter-individual variation within a population in the degree of response to hormonal methods. The reasons for this have yet to be fully defined, the degree of LH suppression achieved can be indicative of the likely degree of spermatogenic suppression, but there also appear to be gonadotrophin-independent mechanisms involved [201]. In recent years it has been accepted that although the gold-standard of spermatogenic suppression to aim for is azoospermia, this degree of suppression in not uniformly achieved and suppression to $\leq$ 1 million/ml would provide an effective method of contraception comparable with existing methods [202]. The addition of another substance to testosterone is important for improving efficacy, particularly in Caucasian populations, and enables the dose of the testosterone to be reduced, thus avoiding the potential problem of supraphysiological levels. Additional agents may also accelerate the suppression of spermatogenesis and improve the level of suppression achieved. The adjunct agents that have so far demonstrated greatest potential are progesterone derivatives and GnRH antagonists.

1.5B: Background

Although the principle of suppression of spermatogenesis by administration of exogenous hormones was first established in the early 20th century [203] it was the landmark studies undertaken by the World Health Organisation in the 1980s and early 90s that first truly raised the possibility of this theory developing practical applicability. These two large multicentre studies proved that exogenous administration of testosterone in the form of weekly intramuscular testosterone enanthate (TE) injections (200mg) could result in both a significant rate of azoospermia and acceptable contraceptive efficacy. The first study used the threshold of azoospermia as the cut off to enter the efficacy phase and once subjects had reached this level, attained by 70% of men (137 subjects), they used no other form of contraception for a period of 12 months. This resulted in only one pregnancy [204]. The significant proportion of participants that failed to fully suppress spermatogenesis led onto exploration of the contraceptive efficacy of oligozoospermia. In a second study, subjects were allowed to enter the efficacy phase in the subsequent trial once their sperm concentration had fallen below 3×10^6 /ml. Inadequate suppression of spermatogenesis to preclude entry to the efficacy phase of that trial occurred in only 2.2% of the 357 participants (8 subjects) while 268 men became azoospermic (77%), a similar proportion to that demonstrated in the previous study. There were no pregnancies demonstrated in the 230 person–years of contraceptive exposure in the azoospermia group and four pregnancies occurred during the 49.5 person-years of exposure in the oligozoospermia group. This data results in an overall pregnancy rate of 1.4/ 100 person-years (C.I 0.4-3.7) [205, 206]. These studies demonstrated that hormonal suppression of spermatogenesis to levels

sufficient for contraceptive efficacy was a possibility but the TE preparation used was not without its drawbacks, particularly frequent administration by injection, a significant proportion of men remaining inadequately suppressed, and the side effects from the high dose of testosterone. While TE was not proposed as a potential suitable preparation, these studies highlighted the need for improved methods for administering testosterone.

1.5C: Testosterone preparations

Testosterone preparations administered via a variety of routes are now available, but not all of these have proved themselves to be both effective and acceptable as potential male contraceptives. Testosterone is rapidly broken down by the liver in first-pass metabolism and is therefore unsuitable to be administered orally. There is only one orally active testosterone preparation- testosterone undecanoate and this has a short duration of action, necessitating administration 8-hourly. This results in extremely variable plasma concentrations [207]. Despite this, oral TU has been explored as a possible male hormonal contraceptive both alone and in combination with oral cyproterone acetate (CPA) but the results were not encouraging due to the frequency of drug administration, wide variation in plasma concentrations, and poor suppression of spermatogenesis and gonadotrophins achieved [208, 209]. Other oral formulations, which result in absorption across the oral mucosa have been investigated [210] but their development has not progressed. Recently a buccal adhesive testosterone preparation has been developed and is now commercially available, requiring 12 hourly administrations. This adheres to the gum surface and slowly delivers testosterone directly into the systemic circulation thereby avoiding first-pass metabolism. In studies involving hypogonadal men this preparation is well tolerated and provides good androgen replacement [211, 212], but its potential as a component of a male contraceptive is as yet unexplored.

Transdermal testosterone gels and patches would appear to be advantageous routes of application to potential users. Unfortunately although appearing to be well tolerated and effective for hormonal replacement in hypogonadal men [79, 213]

patches have not fared well as experimental contraceptive agents either alone or in combination with gestogens, demonstrating generally disappointing efficacy [214, 215]. It seems that the dose of testosterone released by the patches is not sufficient to suppress spermatogenesis to the same degree seen with other delivery routes such as long acting injectables or pellets as gonadotrophin suppression is incomplete. It is also likely that the frequency of skin irritation seen with the patches may reduce compliance. Testosterone gel is associated with far less skin irritation [79] but its efficacy as a potential contraceptive agent has yet to be investigated. Both the buccal and gel preparations appear to have significant advantages over the earlier self-administered testosterone formulations. If this is translated into improved efficacy in a contraceptive formulation with for example an oral gestogen, then the 'male pill', or at least a comparable self-administered contraceptive, may become a reality.

a. Injectable Androgens

Testosterone enanthate (TE) administration results in effective spermatogenic suppression but its short duration of action and therefore frequent administration make it an impractical contraceptive agent. The duration of action of injectable testosterone esters is related to the hydrophobicity of the side chain, and a range of preparations have been developed. The most promising at present is a formulation of TU, which has been developed by both European and Chinese pharmaceutical companies. The Chinese formulation in tea seed oil maintained testosterone concentrations in hypogonadal men above the lower normal limit for adult men for 50-60 days following a single administration of 500mg i.m [216, 217]. A subsequent study investigating dose-finding in normal Chinese men as a potential contraceptive

showed that 500mg every 4 weeks resulted in 11 out of 12 men becoming azoospermic. All 12 men receiving the higher dose of 1000mg/4 weeks achieved azoospermia but there was some accumulation of the testosterone [196]. A large multi-centre contraceptive efficacy study investigating the same product in China was reported last year. Volunteers initially received a loading dose of 1000mg TU i.m and then monthly maintenance of 500mg, only 9 of the 308 men recruited failed to achieve azoospermia or severe oligozoospermia (< 3 x 10^6/ml) during the suppression phase and 6 exhibited secondary failure causing sperm rebound and one pregnancy. There were no pregnancies seen in the men who became adequately suppressed, therefore the overall efficacy was 94.8% with no serious adverse effects reported [218]. In light of these favourable results a larger, 2 -year Phase III trial is currently underway.

Trials in Caucasian men using the European formulation of TU in castor oil have also been undertaken. This preparation has proved itself to provide adequate androgen replacement in hypogonadal men [114] and has also come under investigation as a male contraceptive both used alone and in combination with gestogens [102]. When given as a 6-weekly injection in combination with daily oral levonorgestrel it produced a comparable rate of spermatogenic suppression to that achieved by weekly TE injections with two thirds of volunteers becoming azoospermic [219]. This preparation will soon become commercially available.

Testosterone decanoate differs from TU by one carbon atom in the ester side chain. There are no published data on its effectiveness as an androgen substitute in

hypogonadal men but it has been investigated in healthy men as a contraceptive agent given 400mg i.m every four weeks in combination with 2 etonogestrel implants. 16 out of 20 subjects had become azoospermic by 12 weeks [220]. It remains to be seen whether increasing the time period between injections of TD would be possible, and further studies using this preparation are in progress.

b. Implantable Androgens

Pellets of fused crystalline testosterone have been available for many years for androgen replacement in hypogonadal men. They are inserted into the anterior abdominal wall using a trochar under local anaesthetic and completely dissolve. They have almost complete bioavailability of testosterone and exhibit near zero-order release resulting in very stable serum concentrations thus avoiding the peaks and troughs associated with short-acting injectables [221]. They have demonstrated good acceptability and reproducibility in providing androgen replacement to hypogonadal men with a dose of 4 x 200mg pellets lasting for approximately 5 months [222]. Studies investigating their contraceptive potential have shown that using testosterone pellets on their own allows for a significant androgen dose reduction whilst achieving the same degree of suppression of spermatogenesis and gonadotrophins as exhibited by TE. Approximately 70% of subjects reached azoospermia, but because the dose of androgen is lower the side effect prevalence is lessened [223, 224]. They do have the drawbacks of requiring minor surgery for their insertion and there is a risk of extrusion, of approximately 5%.

Testosterone pellets have also been used in combination with progestogens (see below). In many of our own studies, we have administered the pellets in a regimen of 400mg 12 weekly, ie approximately half the replacement dose for hypogonadal men but administered twice as often, giving the same overall dose. While this results in a fall in serum testosterone following initial administration, concentrations remain within the normal range and are not associated with either symptoms or laboratory evidence of hypogonadism. Importantly, this regimen avoids supraphysiological testosterone concentrations, which are clearly associated with adverse effects and may also enhance the depletion of testosterone within the testis. Testosterone pellets remain the closest to an ideal method of administration for contraception because of their pharmacokinetics: this is supported by the excellent rates of spermatogenic suppression achieved. An improved formulation allowing easier administration would be a major step forward in this field.

Synthetic androgens have in the past been associated with occasional liver damage [225]. However there is a resurgence of interest following from the establishment of the potential for oestrogens with selective actions in different tissues, i.e. selective oestrogen receptor modulators (SERMS). The androgen equivalents, selective androgen receptor modulators (SARMS), are in development [226, 227]. While not a true SARM, the synthetic implantable androgen MENT (7α-methyl-19-nortestosterone) shows some degree of tissue-specific action and has potential for both androgen replacement and male contraception [228, 229]. MENT is 10 times more potent at direct androgen-receptor mediated effects eg gonadotrophin suppression than natural testosterone [230], but it is resistant to 5α-reductase. 5α-

reductase normally acts as a testosterone amplifier by converting testosterone to the more potent androgen dihydrotestosterone. Thus MENT will have relatively lower potency in tissue in which testosterone is normally 5α-reduced, such as the prostate. MENT is however aromatised, which is of particular importance in the maintenance of bone mass in men as in women. It should therefore provide advantageous androgen replacement, being less stimulating to the prostate whilst maintaining other androgen dependent tissues. In the context of contraception, this might confer non-contraceptive health benefits. MENT acetate has been developed for use as a long acting subdermal implant. It has been shown in this form to maintain sexual functioning and mood in hypogonadal men [231] and to suppress gonadotrophins in normal men in a dose-dependent manner [232]. However over a 6-month period of administration in hypogonadal men a dose of 2 implants which maintained other androgen-dependent functions did not maintain lumbar spine bone mass [76]. This may reflect the importance of estrogens in maintaining bone mass in men. MENT is a substrate for aromatase [233], but the low dose of MENT required for androgen activity may result in too low a concentration of the aromatase product locally to support normal bone metabolism. This has implications for the future development of other synthetic androgens which are metabolised to estrogens in a different ratio to natural testosterone. A study investigating the effectiveness of MENT acetate as a contraceptive agent was recently reported. Thirty-five Caucasian men were recruited and assigned to receive either 1,2 or 4 MENT acetate implants. Dose related spermatogenic suppression was seen, with poor rates of suppression in the two lower dose groups but 8 out of 12 men in the 4-implant group becoming azoospermic. Reversible raised haematocrit and haemoglobin concentrations and transient lipid

changes associated with androgen administration were noted [234]. The value of MENT acetate as a contraceptive in conjunction with an additional agent remains to be investigated.

1.5D: Adjunctive Agents

Testosterone only regimes for male contraception have had variable degrees of experimental success, and on the whole have been found to result in a higher rate of azoospermia in Chinese as opposed to Caucasian populations. Currently it is only the long acting testosterone injectables and testosterone pellets that appear to be viable contraceptive components and further work is needed to develop self-administered forms of androgens that result in competitive rates of azoospermia. The use of adjunctive agents such as progestogens and GnRH antagonists may result in higher incidences of azoospermia, and these agents are also available in a variety of delivery methods.

a. Progestogens

Desogestrel and Etonogestrel

Desogestrel is a highly selective and potent progestogen that is converted by the liver to the active metabolite etonogestrel [235]. Desogestrel has been widely used as a constituent of the combined oral contraceptive in women and is now available on its own as Cerazette®. Etonogestrel is available as a subdermal implant (Implanon®) [236] that releases approximately 50μg/day of etonogestrel initially and provides contraception for three years in women [237]. Both of these preparations have been tested as experimental male hormonal contraceptives with encouraging results. Desogestrel given in conjunction with TE resulted in good suppression of spermatogenesis in two different studies. The dose combinations of 150μg/day desogestrel and 100mg TE i.m weekly [238], and 300μg/day desogestrel and 50mg

TE i.m weekly induced azoospermia in all Caucasian subjects in their respective groups (although n=8 only). Desogestrel (150μg or 300μg daily) has also been tested in combination with testosterone pellets (400mg 12 weekly s.c) in Chinese and Caucasian subjects. All subjects in the higher dose group became azoospermic (n=28) as did 22/31 in the lower dose group [239]. Similar results were obtained in a study involving African men in Cape Town and Nigeria [240].

Etonogestrel implants combined with testosterone pellets may make a favourable combination for long-acting contraception. Initial results were obtained in a study that combined one or two Implanon implants with 400mg of testosterone pellets 12 weekly (n=28). In the higher dose group 13 out of 14 subjects achieved a sperm concentration below 0.1 x 10^6 /ml and nine men in each group became azoospermic. The pattern of spermatogenic suppression was less consistent in the one implant group with 5 subjects developing a degree of partial recovery during the second half of the treatment phase [241]. The performance of this combination over a longer treatment period is described in chapter two.

Levonorgestrel

The androgenic progestogen levonorgestrel (LNG) has been tested both in its oral form and as an implant. Most results however have arisen from trials using it in combination with TE [242]. In doses of 125μg/day in conjunction with 100mg TE weekly LNG was demonstrated to result in profound spermatogenic suppression. This LNG dose showed reduced side effects of weight gain and suppression of HDL-C compared to higher dose groups (250 and 500μg) with no loss of spermatogenic

suppression [243] in Caucasian subjects. Impl`ntable LNG has also been investigated in combination with TE injections and transdermal testosterone patches. Spermatogenic suppression in the patch group was disappointing with only 35% achieving azoospermia versus 93% in the injection group (n= 20 and 14 respectively) [244]. A trial of the combination of LNG implants in conjunction with long-acting TU injections in China resulted in 6 out of 16 subjects becoming azoospermic but the doses of both drugs used were probably suboptimal [245].

Depot medroxyprogesterone acetate

DMPA was initially trialled as a male contraceptive in conjunction with short acting injectable TE and demonstrated encouraging rates of spermatogenic suppression in a number of studies [246-248]. Subsequently a single dose of 300mg DMPA with 800mg testosterone pellets was shown to be highly effective with 9 out of 10 men becoming azoospermic [249]. The effectiveness of this combination was recently confirmed in a contraceptive efficacy study administering 300mg DMPA every 3 months and 800mg testosterone pellets initially every 6 months, with this interval later being reduced to 4 monthly. Spermatogenic suppression was excellent with 53 out of 55 Caucasian subjects suppressing down to below 1 x 10^6 /ml to enter the 12 month efficacy phase. There were no pregnancies reported in 35.5 person-years of exposure and no serious adverse events, however the discontinuation rate was disappointingly high (n=27) [250]. DMPA has also been investigated in conjunction with the long-acting injectable TU. In a study based in Indonesia where 500mg TU in tea seed oil was given every 6 weeks in combination with 250 mg DMPA 12 weekly rapid and complete suppression of spermatogenesis was achieved [251]. In

China a similar study randomised 30 men to receive 1000mg of TU 8 weekly either alone or with 150 mg/300mg DMPA also administered at 8 weekly intervals. Consistent azoospermia or severe oligozoospermia was seen in all participants during the 24-week treatment period, with the exception of two men in the TU alone group who exhibited a rebound in sperm concentrations [252]

Norethisterone

NET was one of the first progestogens to be developed, it is reduced to 5α-NET and aromatised to ethinyl estradiol. Because of low receptor affinity 5α-NET also demonstrates anti-androgenic properties [253] in the prostate, therefore the overall effects of NET are potentially complex. It is available both as an injectable depot preparation, norethisterone enanthate (NETE), and as the oral product norethisterone acetate (NETA). Early studies paired NETA with percutaneous testosterone gel and produced encouraging results with all 5 Caucasian subjects becoming azoospermic [254]. The depot preparation NETE allows for long injection intervals similar to the long-acting TU and it has now been investigated in combination with this androgen. Subjects were administered 1000mg TU and 200mg NETE 6-weekly and 13 out of 14 demonstrated azoospermia during the 24 week study [102]. A follow-up study also included a higher NETE dose group (400mg), a daily oral NETA group and an initial testosterone-free interval of 2 weeks. There was no improvement in the high levels of spermatogenic suppression achieved in the previous trial but an increase in likely gestogen-related side effects such as nocturnal sweating [255]. This combination currently offers the significant potential advantage of the two components being administered in a single injection. As with other apparently

highly effective combinations however larger studies are needed to establish more accurately the true prevalence of azoospermia, i.e. whether a sufficiently large proportion of men do not suppress to azoospermia to undermine its advantages.

Cyproterone Acetate

Cyproterone acetate is an orally active progestogen with antiandrogenic properties. Initial studies in combination with TE injections showed favourable results with good spermatogenic suppression [256, 257] but the antiandrogenic effects were responsible for a decrease in haematocrit and haemoglobin and the short acting injections are again impractical. It is likely that the antiandrogenic effect of CPA also contributed to the highly effective spermatogenic suppression. Subsequently the effects of CPA in conjunction with TU on Caucasian subjects have been evaluated. In this study an initially higher dose of CPA (20mg/day) was given during the suppression phase and then a lower maintenance dose (2mg/day) or TU alone continued. There appeared to be no advantage is continuing CPA administration, as the spermatogenic suppression was maintained just as well by the TU alone. This study proposes a new potential role for the use of CPA during only the suppression phase, thus avoiding the problems of long term administration [258].

New progestogens

The commonly investigated progestogens such as desogestrel, norethisterone, and levonorgestrel often demonstrate unwanted effects such as weight gain and alter serum lipoproteins during male contraceptive trials [239, 243, 255]. There is commercial interest in developing progestogens with limited androgenicity for

female contraception but it is unclear whether these would have additional value in the context of administration to men. One of the new progestogens developed is dienogest, which has less antiandrogenic properties than CPA. It has suppressive effect on gonadotrophins and testosterone in men but there are as yet no reported contraceptive trials [259].

b. GnRH Analogues

GnRH agonists have established a wide range of roles in women's health, and in some aspects of male health such as the treatment of prostate cancer. They have been investigated both alone and in combination with androgen in potential contraceptive regimens but disappointingly reduced the spermatogenic suppression achieved with androgen alone [260, 261] and are therefore no longer considered as potential male contraceptive agents. The mechanism for this is inadequately maintained gonadotrophin suppression [262]. GnRH antagonists, on the other hand, have shown considerably more promise when used in conjunction with testosterone. In two early studies using the prototype GnRH antagonist Nal-Glu in conjunction with TE, azoospermia was achieved in 14 out of 16 participants [263, 264]. Further investigation of GnRH antagonist preparations has been hampered by their high cost, the need for frequent injections and the prevalence of histamine-like reactions. One avenue for reducing these problems is their use only in the suppression phase of treatment, with testosterone alone maintaining spermatogenic suppression thereafter [265]. GnRH antagonists are becoming more widely available and are now part of routine drug administration protocols in assisted conception. This generation of antagonists has been demonstrated to show highly effective spermatogenic

suppression in combination with an androgen [266]. New preparations with a longer duration of action are becoming available: these include abarelix and tevarelix [267, 268] and acyline [269]. However, a recent study demonstrated no acceleration of spermatogenic suppression or improvement in the rate of severe oligozoospermia by the addition of acyline to a testosterone and progestogen regimen [270]. Oral nonpeptide GnRH antagonists are also being developed, and show good in vivo suppression of the reproductive axis in non-human primates [271]. It is hoped that these or similar new GnRH antagonists will be highly effective male contraceptive agents but further data is needed.

1.5E: Conclusion

A great deal of progress has been made in recent years towards a viable male hormonal contraceptive but the ideal preparation is not yet clear (see table 1). Indeed, it is likely that for maximum involvement of men a range of self-administered daily regimens and long acting formulations will be required. Suppression to azoospermia requires complete suppression of FSH and LH but is not achieved in all subjects with many regimens thus far investigated. Current research efforts in this field consist of many small studies resulting in fragmented data, with few consistent explorations of combinations as highlighted by a recent Cochrane review [272]. These studies are also currently hampered by poor testosterone preparations but with the recent development of new longer acting TU this situation may improve. Other new preparations such as buccal testosterone and testosterone gel have yet to be investigated as contraceptive agents. We have used testosterone pellets extensively in our own contraceptive studies in combination with desogestrel and etonogestrel, as discussed above. These studies and those of other investigators [201, 224, 250] clearly show the advantages of a testosterone preparation with relatively stable pharmacokinetics thus avoiding the supraphysiological peaks present with even longer acting injectable esters such as testosterone undecanoate and decanoate. This, in addition to the dose-sparing achieved with these preparations, is likely to be the basis for the high prevalence of azoospermia achieved in these studies and illustrates the need for a preparation with the same pharmacokinetics but improved administration. Of the several progestogens under exploration only one currently has the potential for a single combined product. An additional benefit is that

progestogens appear to enhance spermatogenic suppression independent of the degree of gonadotrophin suppression [201].

For the hormonal approach there is still concern regarding the lack of data available on the long-term effects especially upon androgen dependent tissues such as the prostate, and on lipid metabolism. These questions will need to be addressed by larger, longer-term studies but the data described above clearly show that a viable commercial product is a real possibility.

Androgens	Comment	References
Testosterone Enanthate	Previous data available for comparison, impractical as frequent administration required	[205, 206]
Testosterone Decanoate/Undecanoate	Longer duration of action, but supraphysiological testosterone peaks remain	[102, 196, 218-220]
Testosterone Pellets	Long acting with stable plasma concentrations but require surgical insertion, and risk of expulsion	[223, 224, 239, 240]
MENT	Implant formulation at present. Possible health benefits from tissue selectivity: prostate sparing	[232, 234]
Trandermal testosterone	Poor spermatogenic suppression, associated with subphysiological testosterone concentrations. Compliance may be an issue. New self-administered preparations (gel, buccal tablet) no yet investigated.	[214, 215]
Progestogens		
Levonorgestrel	Androgenic progestin Oral and implant preparations available	[242-244]
Norethisterone	Oral or injectable, injectable may be combined with TU. Complex metabolism.	[102, 255]
Desogestrel	Oral preparation. Potent and highly selective	[238-240]
Etonogestrel	Active metabolite of desogestrel, implant and oral formulations. Implants give long duration of action but readily reversible.	[220, 241, 273, 274]
DMPA	Very effective but accumulation may present a problem for dosing regimen	[247-250]
Cyproterone Acetate	Orally active, antiandrogenic activity may enhance spermatogenic suppression.	[209, 256-258]
Other agents		
GnRH antagonists Cetrorelix Acyline Tevarelix	Expensive, require frequent administration. Longer duration preparations becoming available. Only cetrorelix investigated in this context so far.	[266, 268, 269]

Table 1. Products investigated as potential male hormonal contraceptives

Chapter 1.6: Non Hormonal methods

There is a broad range of non-hormonal methods of contraception being developed for men but few have yet to reach the clinical trial stage. The basis for this approach is the identification of targets that are specific to the tissues involved in male fertility ie within the testis, epididymis and vas deferens and the process of spermatogenesis. The main potential advantage of this approach is that testicular hormone production would be unaffected, avoiding many of the problems of MHC, and there may also be a more rapid onset of action. There presently seem to be more potential targets than research groups able to investigate them.

However, further targets are currently being identified by the technique of phenotype-driven mutagenesis. A large research programme is underway for the identification and production of mutant mouse models of infertility in an unbiased manner. The method of this programme is whole genome random mutagenesis with a phenotype screen for breeding failure as the only phenotype [275]. Successful reports from this programme could initiate investigation of completely new future contraceptive targets. A brief introduction to some of the most promising targets under current investigation is presented below.

1.6A: Testicular targets

Alkylated imino sugars are monosaccharide analogues that inhibit enzymes involved in glycoconjugate biosynthesis and catabolism. These compounds have been developed for the treatment of glycosphingolipid lysosomal storage diseases. One of these compounds, NB-DNJ (N-butyldeoxynojirimycin), is now in clinical use for

type 1 Gaucher disease. During preclinical studies it was noticed that male mice became reversibly infertile following 3 weeks administration of this prototype drug whereas female mice were unaffected [276]. The reproductive effects of NB-DNJ and another agent NB-DGJ have been further investigated in male mice. Although sperm morphology and physiology are altered the sperm remains genetically intact [277] which is an important safety consideration. However, the positive findings in mice have yet to be demonstrated in the human male. A recent study investigating the agent miglustat in men found no effect on sperm concentration, motility and morphology after six weeks of treatment [278].

1.6B: Immunocontraception

There is a large variety of potential targets in the male for immunocontraception, including FSH and GnRH, sperm-specific antigens and epididymal proteins. Immunisation against FSH in monkeys has not proved to be very encouraging, demonstrating variable efficacy with some individuals becoming inconsistently azoospermic and others having a sperm concentration that remained in the normal range [279, 280].

A recent more promising study on non-human primates investigated the use of immunisation against the protease Eppin as a non-hormonal contraceptive in the male. Eppin is expressed only in the testis, epididymis and by sperm. Seven of nine Bonnet monkeys immunised against Eppin demonstrated high antibody titres and all of these seven monkeys failed to impregnate known fertile females. Sperm production was not affected, the mechanism of action believed to be a specific

impairment of sperm motility. However, only five out of the seven monkeys regained their fertility following cessation of immunisation, leading to concerns about the reversibility of this approach to contraceptive development [281].

1.6C: Sperm Targets

A family of ion channel proteins unique to mature sperm have been identified, known as CatSpers (cation channel of sperm). CatSper 1 initiates calcium influx into the principal piece of the sperm tail, which is required for sperm motility [282]. Male mice with significant mutations in the CatSper 1 gene are infertile but appear to be otherwise normal [283]. CatSper 2 appears to have a similar function and other family members (CatSper 3 and 4) have been identified and are under further investigation [284]. A Catsper specific calcium channel blocker would have great potential for an effective contraceptive agent.

1.6D: Vas Deferens targets

A variety of vas occlusives have been investigated with the hope of producing a readily reversible male contraceptive. Traditional vasectomy is a highly effective form of contraception but its reversibility is not intended or guaranteed, nor is that of the slightly less invasive no-scalpel vasectomy technique. No-scalpel vasectomy is a new adaptation that utilises a specially designed pointed forceps to effect skin puncture and fix the vas. A number of biomedical devices have been developed in the hope that they can provide a reversible method including wires, injectable plugs, insertable plugs, a t-shaped control valve, and other intraluminal devices [285]. One of the most promising of these agents is RISUG, a co-polymer of styrene maleic

anhydride (SMA) in a solvent vehicle of DMSO, which has undergone extensive testing in man. While not preventing sperm production, it appears to destroy sperm as they pass, causing 'necrozoospermia' rather than azoospermia. During a phase II trial twenty-five men in India had this agent injected into the vas lumen bilaterally and all showed the absence of viable sperm, in most within 2 months of administration [286]. Previous studies have confirmed non-toxicity in monkeys [287] and reversibility has now been demonstrated in male rats [288] and also in primates [289]. A phase III clinical trial in man is currently ongoing [285]. The Intra-Vas device (formerly known as the Shug), an insertable plug has also entered clinical trials. A recent report from China compared the effectiveness of an intra-vas device (IVD) with no-scalpel vasectomy (NSV). Although the IVD group had a slightly reduced rate of contraceptive success (94.3% compared with 98.6% in the NSV group at 12 months), they reported a higher level of patient satisfaction and a reduced post-operative recovery time [290].

Conclusion

This is a brief description of a few of the large number of potential avenues for development of non-hormonal contraceptives, but many of those described show promise. There are distinct advantages to a non-hormonal method and it is to be expected that further advances in this field will allow clinical trials to be undertaken.

Chapter 2 Study M015: A randomised, single centre, open-label study comparing the effects of MENT AC implants or testosterone pellets in combination with etonogestrel implants on spermatogenesis and androgen-dependent tissues in normal men.

2.1 Introduction

Male hormonal contraception is based upon regression of spermatogenesis following suppression of gonadotrophins. The resulting profound reduction in testicular testosterone production requires the administration of an androgen to prevent hypogonadism, the androgen also effecting or contributing to gonadotrophin suppression. Administration of testosterone alone provides effective reversible contraception, as demonstrated in two studies which proved the validity of the concept on which hormonal male contraception is based [204, 206]. However the ideal of azoospermia was achieved in only 68% of Caucasian men and the dose of testosterone was so large as to produce unwanted side effects including changes in lipid metabolism, stimulation of the bone marrow, weight gain, and potential adverse effects on the prostate gland. Administration of a progestogen or gonadotrophin releasing hormone antagonist with testosterone has subsequently been demonstrated in many studies to increase spermatogenic suppression while allowing a reduction in the dose of testosterone towards that of physiological replacement [291, 292].

An alternative approach is the use of a synthetic androgen. Testosterone is a pro-hormone in many tissues, being converted by 5α-reductase to the more potent androgen dihydrotestosterone (DHT) e.g. in the prostate or by aromatase to estradiol e.g. in bone. A degree of tissue selectivity can thus be conferred by altered susceptibility to conversion by these enzymes. This is exemplified by 7α-methyl-19-

nortestosterone (MENT), a potent synthetic androgen that is resistant to 5α-reduction but can be converted by aromatase to an active estrogen [233, 293]. Theoretically, this will result in relative sparing of the prostate from androgenic stimulation while maintaining other physiological androgen-dependent functions including sexual behaviour, muscle and bone anabolism, and hypothalamic/pituitary feedback. MENT may therefore have advantages over testosterone as androgen replacement including as the androgenic component of a hormonal male contraceptive [229]. MENT implants (containing the acetate) induce dose-dependent suppression of the hypothalamo-pituitary-testicular axis in normal men [232, 234] demonstrating potential value in a male contraceptive regimen. Data to support the relative sparing of the prostate by MENT have been obtained in non-human primates [294] and hypogonadal men [76]. However while MENT maintains sexual behaviour and haematopoiesis in hypogonadal men [231], it may be less effective at maintaining bone mass [76].

MENT implants have recently been demonstrated to result in spermatogenic suppression in normal men, with 8 out of 12 achieving azoospermia at a dose of 4 implants [234]. There are no data on administration in combination with a progestogen.

Progestogens investigated in this regard include levonorgestrel [219, 242], cyproterone acetate [257, 258], medroxyprogesterone acetate [249, 250], norethisterone enanthate [255, 295] and desogestrel [101, 154, 238] although there are limited dose-relationship data and comparisons of different progestogens

available [272]. Etonogestrel is the active metabolite of desogestrel and is formulated as a long-acting implant for female contraception (Implanon®, N.V Organon, Oss, The Netherlands). We have previously demonstrated that etonogestrel implants with testosterone pellets result in dose-dependent suppression of spermatogenesis with reduced non-reproductive effects such as alterations in lipoprotein metabolism and weight gain [241, 273]. The present study was designed to investigate the effectiveness of MENT as the androgen component of a prototype male contraceptive regimen including effects on non-reproductive tissues particularly the prostate and bone, and compare it with a testosterone-based regimen. The testosterone regimen was chosen to investigate whether an increase in the testosterone dose with a sub-maximal etonogestrel dose would result in more complete suppression of spermatogenesis and whether this would be associated with increased non-reproductive side effects.

2.2 Methods

Subjects

Twenty-nine healthy Caucasian men with a mean age of 34.1 years (range = 23-50) were recruited from the same general population as previous studies [239, 241, 273]. Inclusion criteria included good mental and physical health including no previous history of depressive/aggressive disorders, body mass index (BMI) between 18-32 kg/m^2, normal pre-treatment FSH, LH and testosterone concentrations and standard biochemical and haematological parameters, and normal physical examination. Two pre-treatment semen samples were required with sperm concentration $>20x10^6/ml$ and motility and morphology within normal limits for the local population (morphology >30% normal, motility >25% class A or >50% class A+B). All participants provided written informed consent and were requested to continue their current contraception throughout the study. The trial received ethical approval from the Lothian Regional Ethics Committee and was performed according to GCP standards.

Study design and medication

The study was a randomised, open-label trial investigating MENT implants or testosterone pellets in combination with etonogestrel implants, both groups scheduled for treatment for a 48 week period. Following two screening visits subjects were randomised using sealed envelopes generated by a third party. The MENT group were administered 2 implants each containing 135 mg MENT acetate formulated as in previous studies to each release 400-500μg of MENT/day [76, 234] s.c with local anaesthesia into the medial aspect of one arm, and 2 etonogestrel

implants 4cm long, each containing 68mg of etonogestrel, Implanon®, N.V Organon) into the opposite upper arm. The testosterone group were administered 600 mg testosterone pellets (3x200mg, N.V Organon) s.c. with local anaesthesia into the anterior abdominal wall repeated at weeks 12 , 24 and 36. This group also received 2 etonogestrel implants at the same time as first administration of testosterone. The etonogestrel and MENT implants were removed at the end of the treatment period at which time the subjects entered the recovery phase; the testosterone pellets dissolve completely and do not require removal.

During treatment and recovery periods subjects were reviewed at 4–week intervals for venesection and semen analysis, and assessment of adverse events or other health issues. The number of episodes of sexual activity (sexual intercourse and masturbation) over the preceding two weeks were recorded by interview at 12-week intervals and at 16 weeks of the recovery phase at which times physical examination was also performed, and testicular volume was determined using a Prader orchidometer. Blood pressure was measured at every visit in the sitting position after 5 minutes rest using an automated device, and was required to remain < 140 mmHg systolic or < 90 mmHg diastolic for the duration of the study. All subjects were followed up until two semen samples had been submitted with sperm concentrations above 20 $x10^6$/ml.

Transrectal ultrasound using a biplanar probe (8 Mhz, Eccoccee, Toshiba, Stirling, UK) was used to measure total prostate volume pre-treatment, at 24 and 48 weeks treatment, and at 16 weeks recovery. Volume was calculated as 3.14/6 x AP

measurement x Transverse measurement x Longitudinal measurement. Bone mineral density at the lumbar spine and hip was determined at the same time points. All recordings were made using the same instrument: QDR-4500A (Hologic Inc, Bedford, MA) with standardization of the machine to correct for changes over time.

Assays

Blood samples were obtained at every visit, separated by centrifugation and serum stored at -20^0C until hormone assay. Testosterone, FSH, LH and estradiol by time-resolved immunofluorometric assay (DELFIA, Wallac). Assay sensitivity was 0.3 nmol/l for testosterone, 0.05IU/l for FSH and LH and 50pmol/L for estradiol. The intra-assay coefficients of variation (CVs) were <7%, inter-assay CVs were <5% for FSH and LH <10% for testosterone and estradiol. Inhibin B was assayed as previously described [159, 296] with an assay sensitivity of 7.8pg/ml. MENT in serum and that remaining in the implants following removal was measured by radioimmunoassay as described previously [297, 298] with intra-assay CV was 3.8-7.9% and the inter-assay CV was 8.0-12.3%. Due to cross-reactivity with testosterone and other serum factors in the MENT assay a mean value of 0.39 ± 0.01 nmol/l is seen in the samples prior to MENT treatment. Samples were analysed for biochemical and haematological parameters (including cholesterol and HDL-C) by autoanalyser at 12 weekly intervals.

Semen Analysis

Semen samples were submitted following 3-7 days abstinence. World Health Organisation methodology was used to assess sperm concentration at all assessments [299]. Azoospermia was confirmed by thorough examination of the re-suspended pellet following centrifugation at 3660g for 15 minutes.

Data Analysis

All results are presented as mean ± SEM. Serum hormone and biochemical data were log transformed before analysis by ANOVA for repeated measurements, and sperm concentrations were cube root transformed before ANOVA with Tukey's post hoc test. Proportions of men achieving thresholds for spermatogenic suppression to azoospermia and $<1x10^6$/ml were analysed by Fisher's exact test. For all comparisons, a P of <0.05 was considered significant. Sexual activity data was analysed by non-parametric testing

2.3 Results

Subjects, adverse events and withdrawals

29 subjects were recruited and randomly assigned to the two treatment groups. An additional 14 men were excluded during screening and 8 failed to complete the screening process. There were no significant differences in pre-treatment variables between the two groups (table1). Three subjects in the MENT group chose to leave the study after 8 weeks of treatment, two because of symptoms of low libido and erectile dysfunction and one for personal reasons unrelated to the study. Two subjects in the testosterone group withdrew after 24 weeks of treatment, one for personal reasons and the other for symptoms of labile mood, sleep disturbance and nocturia. Adverse events experienced included reduced libido and erectile function in 4 additional subjects in the MENT group who completed treatment. In the testosterone group, single pellets were extruded in 2 subjects: both occurred one week prior to scheduled re-administration and were therefore not replaced. One subject in the testosterone group reported increased libido and acne.

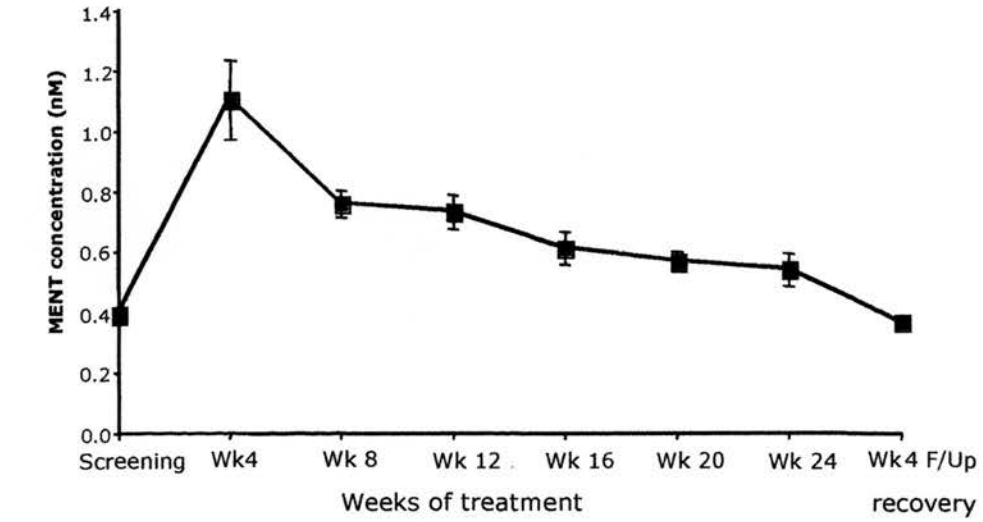
Due to the high incidence of reports of low libido and early withdrawal from the study in the MENT group it was decided in consultation with the study Data Monitoring and Safety Committee to shorten the MENT treatment period to 24 weeks whereas men in the testosterone group completed 48 weeks treatment.

	MENT Group	Testosterone Group
Age (years)	34.9 ± 1.5	32.2 ± 1.2
BMI (kg/m^2)	25.6 ± 0.8	24.7 ± 0.7
LH (IU/l)	3.3 ± 0.4	3.7 ± 0.3
FSH (IU/l)	2.0 ± 0.4	2.3 ± 0.4
Testosterone (nmol/l)	17.6 ± 2.7	21.4 ± 2.5
Sperm conc ($x10^6$/ml)	55.1 ± 9.1	58.7 ± 14.0

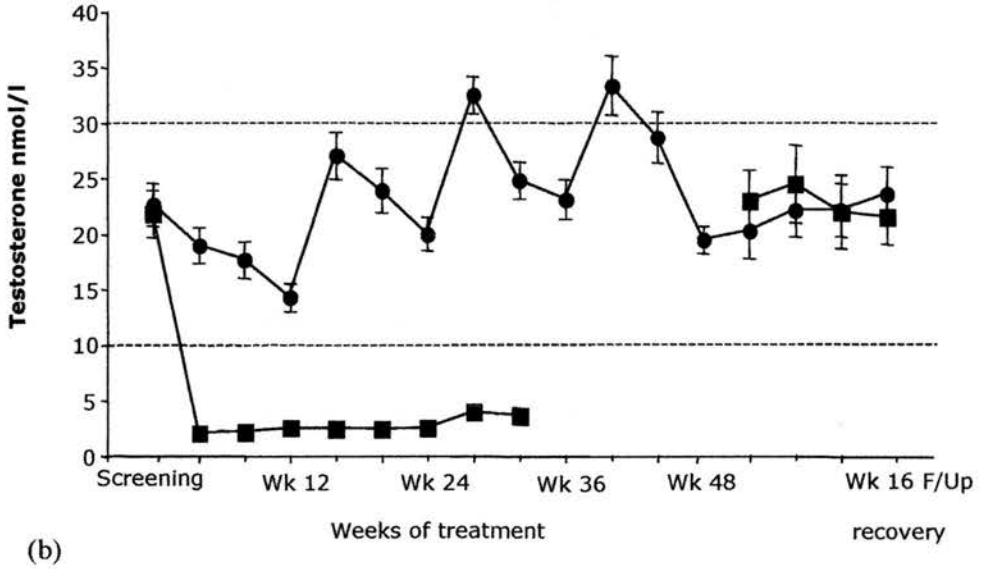
Table 1. Pre-treatment values for all subjects, mean ± SEM. MENT group n=13, testosterone group n=16.

MENT and Testosterone concentrations

Serum MENT concentrations in that group demonstrated an initial peak at four weeks of 1.1nM ± 0.1 nmol/L followed by a gradual decline to 0.54 ± 0.05 nmol/L by week 24 (figure 1a). Analysis of MENT remaining in the implants after removal demonstrated that the implants had released 31 ± 2% of original MENT content over 24 weeks, giving a calculated average release rate of 117 ± 6µg/day/implant. Implants removed after shorter insertion periods (51-60 days, n=3) showed a higher calculated release rate at 329 ± 6µg/day/implant. The two men who withdrew from the study because of reduced libido had similar serum MENT concentrations to the rest of the group, as did the other 4 who had reduced libido but continued. Testosterone concentrations in this group fell rapidly to 2.0 ± 0.4 nmol/L at 4 weeks ($P<0.0001$ vs. pre-treatment, figure 1b), and remained very low for the duration of treatment with no significant changes from values at 4 weeks.. Following removal of MENT and etonogestrel implants, testosterone concentrations rapidly returned to pre-treatment values.



(a)



(b)

Figure 1: Serum concentrations of (a) MENT, (b) testosterone during treatment and recovery phases. MENT group (square symbols) n=13, testosterone group (round symbols) n=19, mean ± sem. The MENT group received drug treatment for 24 weeks, the testosterone group for 48 weeks. Testosterone was administered at weeks 0, 12, 24 and 36. In (b) the normal range is indicated by the broken lines.

Serum testosterone concentrations in the testosterone group were significantly greater than in the MENT group at all time points during treatment ($P<0.0001$, figure 1b). There was some fluctuation in keeping with the schedule of testosterone administration at 12-week intervals with troughs prior to re-administration and peaks 4 weeks later. Trough and peak concentrations rose during the study, indicating some accumulation. After an initial fall over the initial 12 weeks of treatment ($P<0.001$) testosterone concentrations were similar to pre-treatment at 24 weeks but at 28 and 40 weeks were significantly higher than pre-treatment (both $P<0.001$). However average testosterone concentrations over both the first and second 24 week periods were not significantly different to pre-treatment (22.6 ± 1.9 vs. 20.2 ± 1.4 vs. 26.9 ± 1.5 nmol/L pre-treatment, 4-24 weeks and 28-48 weeks respectively).

Sperm concentrations

Both groups showed profound suppression of spermatogenesis during the initial months of the study. At 12 weeks of treatment 8 of 10 subjects (80%) in the MENT group and 13 of 16 (81%) in the testosterone group demonstrated spermatogenic suppression to $<1x10^6$/ml with 3 and 11 subjects respectively being azoospermic. However spermatogenic suppression with continuing treatment was inconsistent in the MENT group (figure 2a). Only 4 men maintained suppression at $<1x10^6$/ml until 24 weeks whereas partial recovery was seen in the remaining 6. In the 2 men who did not achieve suppression to $<1x10^6$/ml sperm concentrations were $>20x10^6$/ml at 24 weeks. Overall the mean sperm concentration rose to 11.5±5.2 $x10^6$/ml at that time.

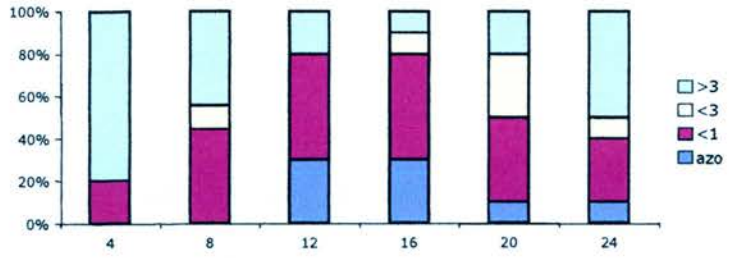


Figure 2a . (1) MENT Group

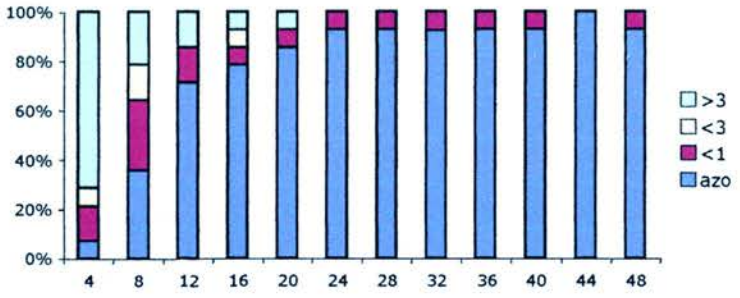


Figure 2a. (2) Testosterone Group

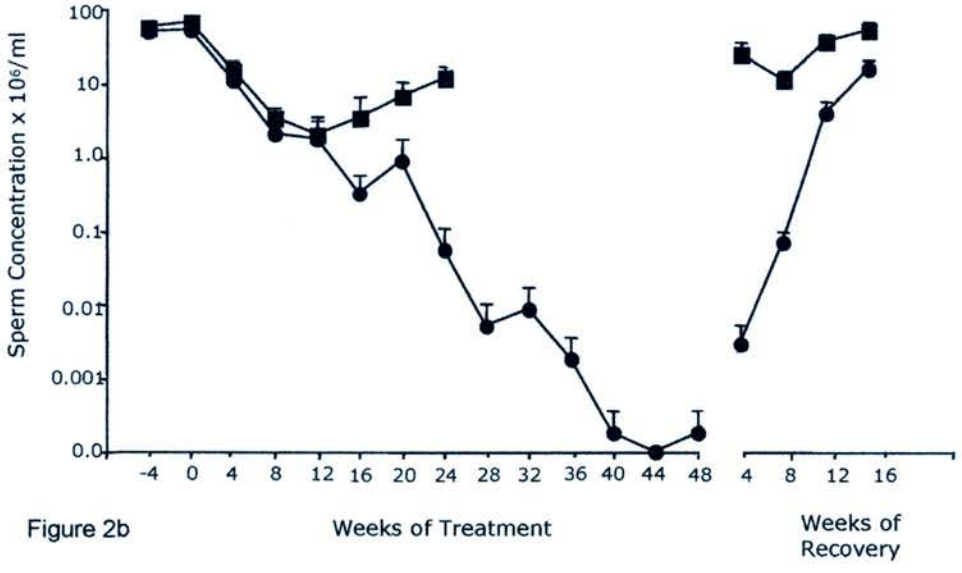


Figure 2b

Figure 2: (a) Sperm concentrations during treatment phases expressed as % of group achieving significant levels of spermatogenic suppression (>3x10^6/ml,<3x10^6/ml,<1x10^6/ml and azoospermia), shown as (1) MENT group and (2) T group. (b) Sperm concentrations during treatment and recovery phases. MENT group (square symbols) n=13, testosterone group (round symbols) n=19, mean ± sem. The MENT group received drug treatment for 24 weeks, the testosterone group for 48 weeks.

In contrast the testosterone group continued to demonstrate profound and maintained suppression of spermatogenesis (figure 2a). At 24 weeks of treatment 14 out of 16 subjects (88%) were azoospermic, and by the end of the treatment period all subjects demonstrated azoospermia (n=14). One of the two men not azoospermic at 24 weeks withdrew from the study for personal reasons unrelated to the study, and the other became azoospermic at week 44 of treatment. The range of time to achieve azoospermia was therefore 4 - 44 weeks, with a median time of 12 weeks. Once men in the testosterone group had become azoospermic, none showed significant resumption of spermatogenesis during treatment: spermatozoa were detected only in the centrifuged pellet of the ejaculate in one individual on one occasion.

Recovery was rapid following implant removal in the MENT group with 9 out of 10 subjects achieving sperm concentration $>20x10^6$/ml within 16 weeks of follow-up. Slower recovery was seen in the testosterone group, with a median duration of 28 weeks (figure 2b). One subject continued to show sperm concentrations of 10-$15x10^6$/ml after over 1 year of follow-up, with normal total sperm number, sperm motility and morphology, and gonadotropin concentrations. His mean pre-treatment sperm concentration was $31x10^6$/ml. A second subject underwent vasectomy before his sperm concentration had recovered to $>20x10^6$/ml, 64 weeks after removal of etonogestrel implants.

Other reproductive hormones

Initial suppression of both FSH and LH was rapid and profound in both treatment groups (figure 3). In the MENT group mean FSH was 0.18 ± 0.03 IU/l after

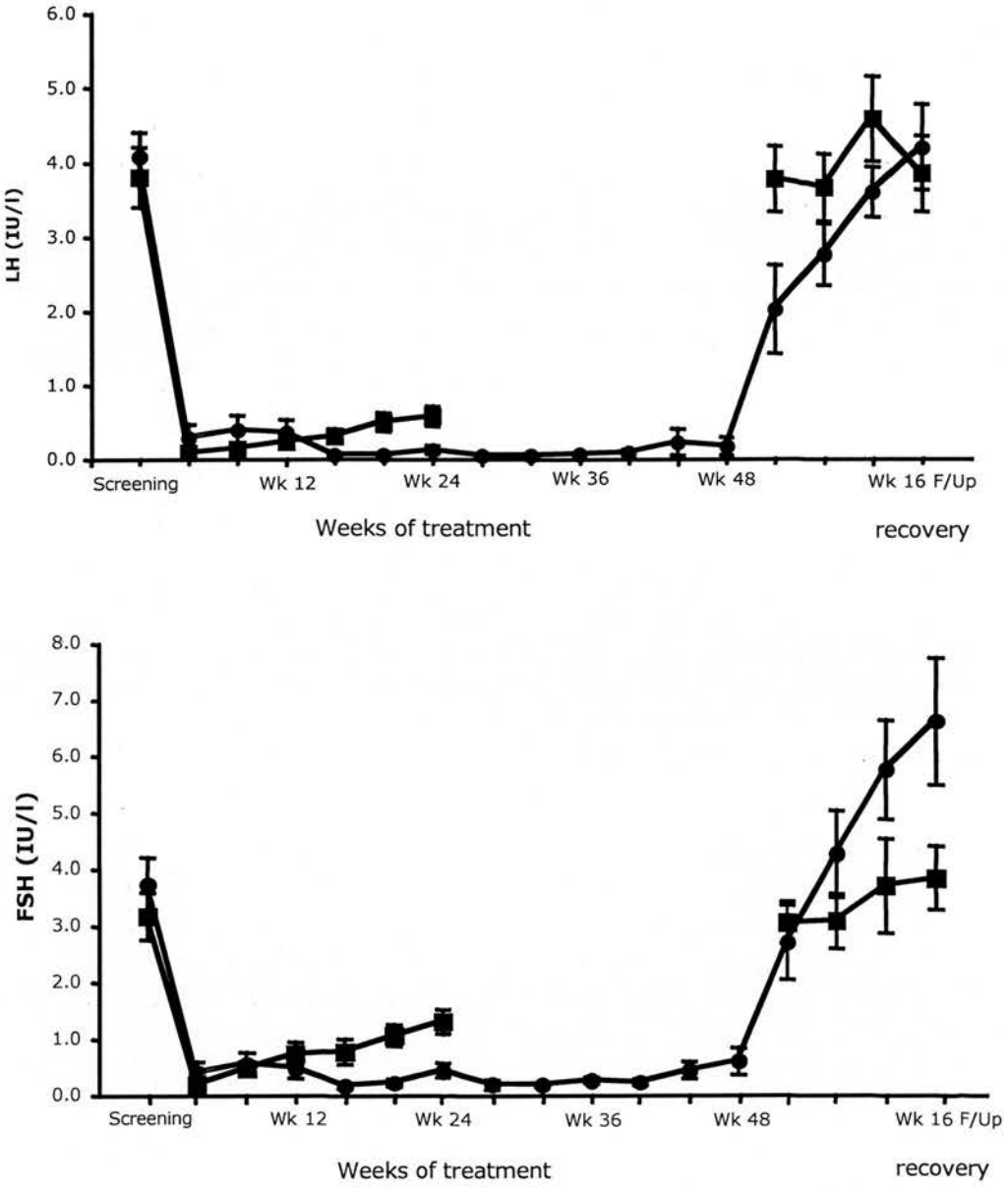
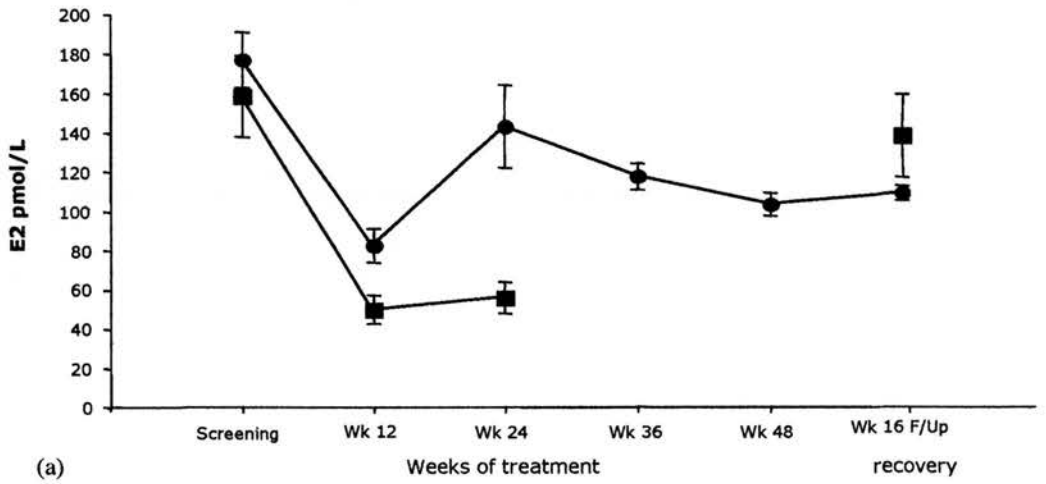


Figure 3: Serum concentrations of FSH and LH during treatment and recovery phases. MENT group (square symbols) n=13, testosterone group (round symbols) n=19, mean ± sem .

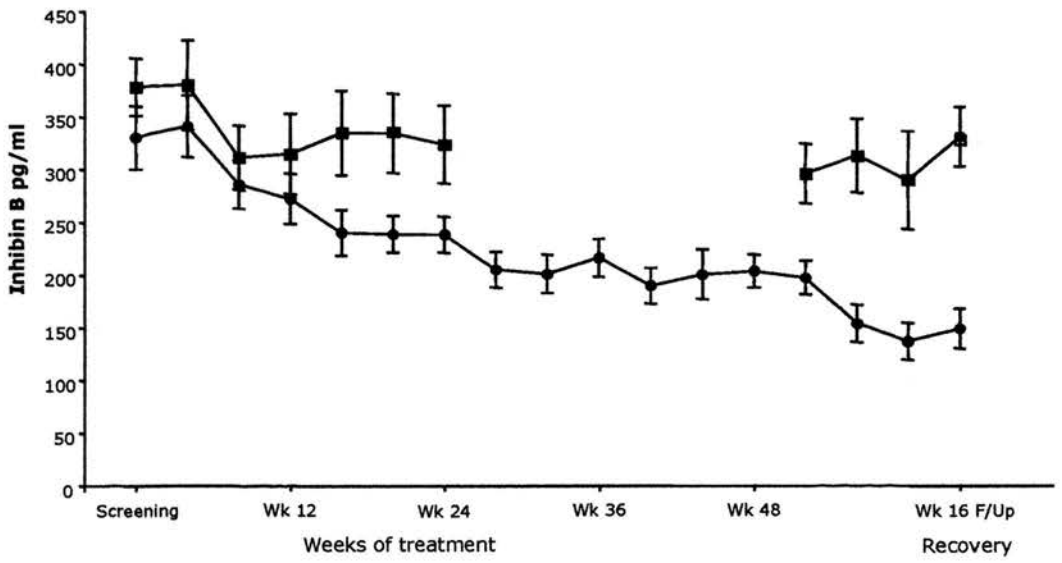
four weeks of treatment. However, progressive partial escape from suppression was evident with a rise to 1.29 ± 0.21 IU/l at 24 weeks (P=0.04, ANOVA of treatment values). Concentrations were similar to pre-treatment in the recovery phase. In the testosterone group the initial profound suppression was well maintained throughout the treatment period (0.43 ± 0.13 IU/l at week 24 and 0.59 ± 0.24 IU/l at week 48, figure 3). Minor rises were seen at times of trough testosterone concentrations that did not reach statistical significance. In the recovery phase there was an overshoot to above pre-treatment concentrations (6.6 ± 1.1 IU/l at 16 weeks post-treatment vs 3.7 ± 0.5 IU/l pre-treatment, P<0.01).

LH was profoundly suppressed in both treatment groups over the initial 12 weeks of treatment (figure 3). In the MENT group there was a small rise during continuing treatment but this did not reach statistical significance (0.23 ± 0.09 IU/L at 12 weeks, 0.57 ± 0.13 at 24 weeks, figure 1). In the testosterone group the suppression was more complete and consistent for the duration of treatment (0.12 ± 0.05 IU/L at 24 weeks; 0.16 ± 0.12 at 48 weeks).

Estradiol concentrations demonstrated a marked fall in the MENT group at 12 and 24 weeks treatment (P<0.001, figure 4a). In the testosterone group the results paralleled the testosterone concentrations with a small fall seen at 12 weeks (P<0.001), recovering by 24 weeks (ns vs pre-treatment, P<0.01 vs 12 weeks). There was a significant difference in estradiol concentrations (P=0.01) between the two groups at 24 weeks.



(a)



(b)

Figure 4: Serum concentrations of (a) estradiol and (b) Inhibin B during treatment and recovery phases. MENT group (square symbols) n=13, testosterone group (round symbols) n=19, mean ± sem .

Inhibin B concentrations showed a significant decline during treatment in both groups, but differed between groups (P=0.01, figure 4b). In the testosterone group, inhibin B concentrations declined during the first 24 weeks of treatment, with little fall thereafter. There was however a significant further fall during the recovery phase (P<0.0001 vs week 48), with a nadir at 12 weeks recovery. There was an inverse relation between inhibin B and FSH during the recovery phase (P=0.004 at 16 weeks). In the MENT group, inhibin B concentrations were only significantly lower than pre-treatment at 8 weeks (P=0.001).

Haematology and Lipids

Haemoglobin concentrations were significantly increased in the MENT group at 12 weeks but this did not persist at 24 weeks (table 2). A similar pattern was also seen in haematocrit but this did not reach statistical significance. In the testosterone group a slower progressive rise in haemoglobin concentration (P=0.0006) was observed which only became significantly greater than pre-treatment at 48 weeks, and returned to pre-treatment values during the recovery period. There was also a significant overall rise in haematocrit (P=0.009) in the testosterone group although none of the individual treatment time points were significantly different from pre-treatment (table 2).

There were significant falls in HDL-C concentrations in both groups during treatment with return to pre-treatment values in the recovery period (table 2). In both groups HDL-C was significantly lower than pre-treatment at all time points during

the treatment phase. Cholesterol concentrations in the MENT group but not the testosterone group also showed a gradual fall during treatment at 24 but not 12 weeks, returning to pre-treatment concentrations during recovery (table 2). There were no significant changes in triglyceride or LDL-C concentrations in either group. Neither group demonstrated any significant changes in any biochemical variables throughout the study.

	Pre-treatment	12 weeks	24 weeks	36 weeks	48 weeks	Recovery
Haemoglobin (g/l)						
T Group	152 ± 2.5	149 ± 2.2	153 ± 2.1	154 ± 2.5	157 ±	154 ± 2.5
MENT Group	149 ± 2.9	154 ±3.3*	148 ± 2.4	-	2.5**	148 ± 2.0
					-	
Haematocrit						
T Group	0.45 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.46 ± 0.01	0.46 ±	0.4 ± 0.01
MENT Group	0.44 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	-	0.01	0.43±0.01
					-	
HDL-C (nmol/l)						
T Group	1.4 ± 0.1	1.2 ± 0.1*	1.2±0.1**	1.2 ±	1.1 ±	1.3 ± 0.1
MENT Group	1.4 ± 0.1	1.2±0.2**	1.2±0.1**	0.01**	0.1**	1.3 ± 0.1
				-	-	
Cholesterol (nmol/l)						
T Group	4.8 ± 0.2	4.4 ± 0.2	4.6 ± 0.2	4.5 ± 0.2	4.7 ± 0.2	5.0 ± 0.2
MENT Group	4.9 ± 0.2	4.6 ± 0.2	4.4 ± 0.2*	-	-	4.9 ± 0.2
LDL-C (nmol/l)						
T Group	2.8 ± 0.2	2.6 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	3.0 ± 0.2	3.1 ± 0.2
MENT Group	2.9 ± 0.2	2.9 ± 0.2	2.5 ± 0.2	-	-	2.9 ± 0.2
Triglycerides						
(nmol/l)	1.3 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	1.1 ± 0.1	1.5 ± 0.2	1.4 ± 0.2
T Group	1.5 ± 0.2	1.3 ± 0.2	1.7 ± 0.2	-	-	1.5 ± 0.2
MENT Group						

Table 2. Serum values of haematology, lipid and PSA results as mean ± sem. MENT group n=13, testosterone group n=16. * $p<0.05$, ** $P<0.01$ vs pre-treatment

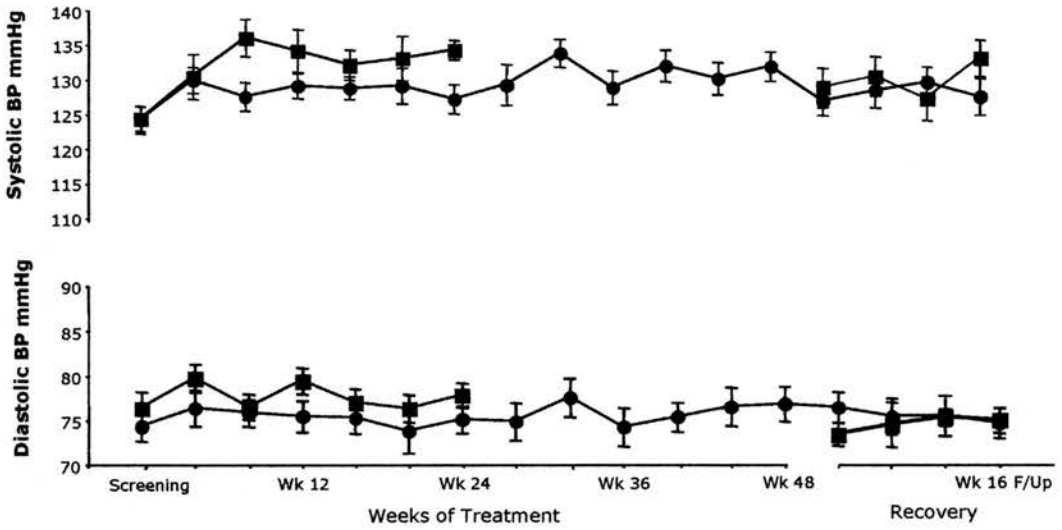


Figure 5: Systolic and diastolic blood pressure during treatment and recovery phases. MENT group (square symbols) n=13, testosterone group (round symbols) n=19, mean ± sem. The MENT group received drug treatment for 24 weeks, the testosterone group for 48 weeks.

Blood pressure

A significant rise in systolic blood pressure was observed in the MENT group throughout the treatment period (P=0.02, figure 5) with no significant change in diastolic blood pressure. Systolic blood pressure was not significantly different to pre-treatment during the recovery phase. There were no significant changes observed in the testosterone group in either systolic or diastolic blood pressure during the treatment period.

Testes and Prostate

Both treatment groups showed decreases in testicular volume during the study period. This fall was only transient in the MENT group, falling to 71% of pre-treatment volume after 12 weeks of treatment ($p<0.01$) but was not significantly different to pre-treatment at 24 weeks. In the testosterone group mean testis volume decreased significantly by week 12 ($p<0.01$) falling to a nadir of 67% at 48 weeks ($p<0.001$).

There was no significant change in prostate volume over the course of the study in the MENT group (table 3). However, in the testosterone group a significant increase in prostate volume was seen at the end of the treatment period (P=0.007) and it remained slightly elevated after 16 weeks in the recovery phase (P<0.05 vs pre-treatment, not significant vs week 48). Serum prostate specific antigen (PSA) concentration demonstrated a significant fall in the MENT group (table 3) at both 12 and 24 weeks of treatment but was unchanged in the testosterone group.

Body Composition, Sexual Behaviour and Bone Mineral Density.

There was no change in weight in the MENT group, but an increase in the testosterone group by the end of the treatment period (P=0.02), which persisted into the recovery period (table 3). There were no significant differences in sexual activity in either the MENT or testosterone treatment groups (table 3). Bone mineral density remained unchanged in both groups at both hip and spine throughout the treatment period (table 3).

	Pre-Treatment	12 weeks	24 weeks	36 weeks	48 weeks	Recovery
Weight (kgs)						
T Group	80.8 ± 3.8	80.6 ± 3.4	82.2 ± 3.5	83.6 ± 4.1	84.2 ± 3.9*	84.1 ± 3.8
MENT Group	80.5 ± 3.0	83.0 ± 3.6	81.5 ± 3.7	-	-	81.3 ± 3.3
Prostate vol (ml)						
T Group	14.7 ± 1.3	-	16.2 ± 0.9	-	17.4±0.9**	16.9 ± 1.2
MENT Group	16.3 ± 1.4	-	15.8 ± 2.3	-	-	16.4 ± 1.4
PSA (ng/ml)						
T Group	0.8 ± 0.1	0.87±0.1	0.84 ± 0.1	0.73 ± 0.1	0.72 ± 0.1	0.68 ± 0.1
MENT Group	1.1 ± 0.2	0.77 ± 0.1**	0.74±0.1**	-	-	0.80 ± 0.1
Testis vol (ml)						
T Group	18.6 ± 0.9	13.7±0.9**	12.4±0.6**	12.6±1.2**	11.5±1.1**	19.0 ± 0.9
MENT Group	18.1 ± 0.9	12.9 ± 1.0**	15.4 ± 1.1	-	-	18.6 ± 1.1
Sexual activity						
T Group	7.1 ± 0.9	8.7 ± 1.5	7.8± 0.9	7.6 ± 1.8	7.4 ± 1.9	7.1 ± 1.2
MENT Group	6.9 ± 0.9	5.9 ± 1.3	6.4 ± 1.3	-	-	7.9 ± 2.1
Spine BMD (g/m^2)						
T Group	1.03 ± 0.02	-	1.03 ± 0.03	-	1.08 ± 0.02	1.01± 0.06
MENT Group	1.05 ± 0.04	-	1.04 ± 0.04	-	-	1.01± 0.04
Total Hip BMD	1.08 ± 0.02	-	1.08 ± 0.02	-	1.10 ± 0.03	
T Group	1.01 ± 0.05	-	1.01 ± 0.05	-	-	
MENT Group						1.08± 0.03
						1.01± 0.05

Table 3. Mean ± sem. MENT group n=13, testosterone group n=16. * p<0.05, ** P<0.01 vs pre-treatment

2.4 Discussion

It is now established that administration of sex steroids can suppress spermatogenesis sufficiently and reversibly to allow development as a hormonal contraceptive for men [291, 292, 300]. What is less clear is how to optimise the rate, extent and inter-individual consistency of spermatogenic suppression while minimising the potentially adverse non-reproductive effects of treatment, which largely reflect administration of supraphysiological doses of testosterone. Administration of a progestogen allows considerable dose-sparing of testosterone, and previous studies using long-duration formulations of both testosterone and progestogens suggest that this approach may also allows a reduction in dose while maintaining efficacy [241, 249, 273].

The synthetic androgen MENT may offer advantages over testosterone for replacement therapy both in hypogonadal men and as a component of a hormonal male contraceptive [231, 234]. The potency may allow alternative formulations with an increased dosage interval, and the restricted metabolism may provide relative sparing to the prostate [293]. For application in male contraception, efficacy in gonadotrophin and spermatogenic suppression are essential and have been demonstrated by MENT implants alone [232, 234]. However as with testosterone alone, the dose of MENT required for adequate suppression was associated with undesirable non-reproductive effects indicating supraphysiological androgen action, i.e. increases in hemoglobin and hematocrit,. Administration with a progestogen allows a lowering of the dose of androgen towards physiological replacement. We

have previously demonstrated that two MENT implants formulated as in the present study appeared to provide replacement in hypogonadal men for 24 weeks [76]. Similarly, the dose of etonogestrel used here when combined with testosterone resulted in marked but not maximal suppression of spermatogenesis [241]. We therefore investigated this dose of etonogestrel with MENT in comparison with testosterone.

There was rapid spermatogenic suppression during the initial 12 weeks of treatment in both groups, with approximately 80% of both groups achieving suppression to <1 $x10^6$/ml at that time. This is comparable to the most effective regimens previously reported [255, 301] indicating the potential value of MENT in male contraception. Thereafter however suppression in the MENT group was inconsistent and some subjects complained of reduced interest in sex. Subsequent measurement of serum MENT showed similar concentrations to those previously reported using this formulation [76] although the assay has a high background for measurements in serum reducing its accuracy. Determination of MENT remaining in the implant after removal at the end of the study demonstrated that the release rate was low, although in those men in whom the implants were removed early the initial release rate was as expected. It therefore appears that a greater sustained release rate than achieved here is necessary for continuing spermatogenic suppression and support of sexual interest in normal men.

The inconsistent suppression and variable recovery of spermatogenesis in the MENT group was accompanied by an increase in FSH with no significant change in LH or

testosterone concentrations during the second half of the treatment period. Both gonadotropins are required for normal spermatogenesis [43, 44],and recent data from analysis of testicular biopsies following gonadotropins suppression and selective replacement with hCG or FSH has indicated specific sites of action in stimulating spermatogenesis [302]. Measurement of intratesticular testosterone concentrations and of the specific testicular androgen epitestosterone during administration of male contraceptive regimens has shown that testicular steroidogenesis is incompletely suppressed [31, 201, 303]. It appears that the rise in FSH seen here with low but detectable LH concentrations is sufficient to support normal spermatogenesis in some men, emphasising the need for maximal suppression of both gonadotropins and thus depletion of intratesticular testosterone for optimal spermatogenic suppression.

It is of note however that both men who withdrew from the study because of reduced sexual interest and function did so after only 8 weeks treatment, thus even the peak serum MENT concentrations were insufficient in these normal men. This contrasts with other evidence that the MENT dose was not insufficient, as haemoglobin concentration was in fact increased at 12 weeks and HDL-C was reduced, whereas in the testosterone group both these biochemical variables were unchanged, with no evidence of inadequate behavioural support. This discrepancy may indicate that a relatively higher dose of MENT is required for behavioural support in normal men compared to hypogonadal men [76, 231] and also compared to trophic effects on the bone marrow and liver, which may reflect its restricted metabolism.

Spermatogenic suppression continued in the testosterone group beyond 12 weeks treatment, with all men eventually becoming azoospermic. However this required more than 24 weeks treatment, and overall was very similar to previous data using the same dose of etonogestrel but with a lower dose of testosterone [220]. This contrasts with improved suppression using the lower dose of testosterone but higher dose of etonogestrel [273]. In these earlier studies there was no evidence of inadequate androgen replacement with these combinations and there were only minimal non-reproductive side effects on lipoprotein metabolism and weight. Together these data illustrate dosage effects for both the testosterone and progestogen component and demonstrate the importance of the progestogen dose compared to the testosterone dose in maximising spermatogenic suppression.

Disadvantageous effects of this increased dose of testosterone were exemplified by the progressive increase in haemoglobin and decrease in HDL-C concentrations, which were not seen in our previous studies using a lower dose of testosterone (400mg/12 weeks) despite average testosterone concentrations being similar to pre-treatment over the second 24 weeks of treatment. Further evidence for the dose of testosterone being supraphysiological is the small but significant increase in the size of the prostate. These results are in keeping with the physiological replacement dose of testosterone being nearer 5 than 7 mg/day [303] and highlight the importance of measuring the response of androgen-dependent variables such as haemoglobin concentration and haematocrit to determine the optimum replacement dose rather than solely serum testosterone which will be overestimate pre-treatment if only morning sampling is used [291].

The major potential advantage of MENT is that it is resistant to 5α-reduction and may therefore relatively spare the prostate. This is supported by data from non-human primates [294] and hypogonadal men [76]. The present data also support this, with a fall in serum PSA with a non-significant fall in prostate volume in the MENT group, The lack of change in prostate volume may reflect the relatively young age of the study population and the short duration of treatment, and both results are very similar to those previously reported in similarly-aged hypogonadal men [76]. This interpretation must be tempered however by the above reservations regarding the overall adequacy of the dose of MENT administered.

Maintenance of bone mass in men is dependent on serum testosterone and the local conversion by the enzyme aromatase to estradiol [304]. MENT is also a substrate for aromatase, but in hypogonadal men 2 MENT implants did not appear sufficient to maintain lumbar spine bone mass [76]. The present data do not show any evidence for loss of bone mass in the MENT group over this relatively short duration of treatment. This may reflect differences between normal and hypogonadal men, or the additional administration of the progestogen.

There was a small but significant elevation of systolic blood pressure in the MENT group. There was no change in diastolic pressure, and no change in the testosterone group. A similar finding was reported in a previous study investigating MENT alone [234]. The observed increase in systolic blood pressure may reflect increased arterial stiffness. Arterial stiffness is inversely related to testosterone concentrations in older

men [305], and systolic blood pressure is increasingly recognised to be a strong cardiovascular risk factor [306]. Large artery stiffness was increased by induced hypogonadism in men with prostate cancer [307] and recent data from the Framingham Heart study suggest that endogenous estradiol may be vasculoprotective [308]. During MENT administration both testosterone and estradiol concentrations are low. Although MENT is aromatised to an active estrogen [233], the low serum concentrations both of MENT, and therefore of potential active metabolites may contribute to increased arterial stiffness and a rise in systolic blood pressure.

2.5: Conclusion

In conclusion this study demonstrates that the combination of MENT and etonogestrel results in rapid and profound spermatogenic suppression. Formulation of MENT to give consistent release at a dose similar to that in the initial weeks of this study may be a promising approach for hormonal male contraception although non-reproductive effects were detected. The testosterone group also showed rapid spermatogenic suppression, but it is clear that even with a highly effective testosterone/progestogen combination it is of greater value to alter the dose of progestogen than that of the testosterone component to optimise the rate and extent of suppression and minimise non-reproductive androgenic effects.

Chapter 3 Study M016: Investigation of the effects of gonadotrophin withdrawal and progestogen administration on hormone production, metabolism and action in the human testis.

3.1: Introduction

Testicular function is dependent on trophic support from the gonadotrophins LH and FSH. LH stimulates steroidogenesis, particularly of testosterone from the Leydig cells, which together with FSH is necessary for normal spermatogenesis. The effects of testosterone and FSH are mediated through the Sertoli and peritubular cells as these cells but not germ cells express receptors for these hormones [29, 309-312]. Recently the demonstration that Sertoli cell specific knockout of the androgen receptor results in spermatogenic arrest at the spermatocyte/spermatid stage has provided clear evidence that androgen action via the Sertoli cell is required for normal spermatogenesis [313]. Selective withdrawal and replacement of LH (and thus intratesticular testosterone) and FSH in men have shown that both are required for quantitatively as well as qualitatively normal spermatogenesis [43, 44], but the pathways through which testosterone and FSH support spermatogenesis remain uncertain.

Understanding of gonadotrophin-dependent pathways regulating spermatogenesis is also important for understanding testicular mechanisms to enhance development of novel male contraception. Gonadotrophin suppression by the administration of testosterone alone, or with a progestogen or gonadotrophin hormone releasing hormone (GnRH) antagonist results in suppression of spermatogenesis to azoospermia in most men [291, 300]. There is however variation in the degree of

suppression between individuals. Some will become azoospermic with regimens that result in incomplete suppression of spermatogenesis in others, thus studies of the most effective regimens yet investigated generally show a small proportion of men who do not become azoospermic [239, 250, 255]. The basis for inter-individual variation in the response to contraceptive regimens is unclear but knowledge of this would allow more effective patient selection and drug administration. One potential mechanism for this is variation in activity of the enzyme 5α-reductase, which converts testosterone to the more potent androgen dihydrotestosterone (DHT) [34]. This possibility is supported by rodent data, which demonstrate a requirement for higher concentrations of testosterone to support spermatogenesis in the presence of a 5α-reductase inhibitor [36]. Two clinical studies involving administration of a 5α-reductase inhibitor did not however provide any positive evidence in support of this [224, 314], although the isoenzyme predominantly expressed in the testis, type 1 5α-reductase, is inhibited to a lesser extent by the drug used (finasteride) compared to the type 2 isoenzyme. Alternative potential mechanisms include differences in the number of CAG repeats in the androgen receptor and polymorphisms in androgen-related genes, although the data are inconsistent [200, 315, 316].

The addition of a progestogen to a testosterone-based regimen increases spermatogenic suppression while allowing a reduction in the dose of testosterone, which may have safety advantages [101, 249]. Progestogens increase the speed or degree of gonadotrophin suppression enhancing spermatogenic suppression, but there is also indirect evidence that progestogens may also suppress spermatogenesis by gonadotrophin-independent direct effects on the testis [201]. In keeping with this,

expression of both nuclear and novel membrane progesterone receptors has been demonstrated in the human testis [317] and progestogens have been demonstrated to have direct inhibitory effects on Leydig cell function in a murine cell line [318].

In this study we have investigated changes in gene expression in the human testis in response to gonadotrophin withdrawal by administration of a GnRH antagonist with add-back testosterone to prevent hypogonadism. The study was also designed to investigate potential testicular effects of addition of a progestogen, and to allow correlation of the degree of spermatogenic suppression with changes in gene expression. Genes involved in steroidogenesis and steroid metabolism and those expressed by Sertoli cells or germ cells at different stages of maturation were chosen to investigate the various testicular compartments. Sertoli cell genes included the androgen and FSH receptors directly relevant to Sertoli cell regulation, the FSH-regulated gene inhibin α (although also expressed in Leydig cells) and human homologues of the *Pem* gene (PEPP1 and PEPP2) identified in the mouse as highly androgen regulated [319, 320]. Germ cell genes were chosen to represent those expressed at different stages of spermatogenesis, i.e. by spermatogonia and primary spermatocytes (melanoma antigen family A4, MAGE A4) [321, 322], spermatocytes (acrosin binding protein, ACRBP) [323, 324] and round spermatids (protamine 1, PRM1) [325, 326].

3.2: Materials and Methods

Patient recruitment

Thirty healthy men requesting vasectomy were recruited. Inclusion criteria included age (18-50 years), no past significant medical problems, normal biochemical and haematological parameters, normal andrological examination, sperm concentration >20 $x10^6$/ml and normal plasma LH, FSH and testosterone concentrations. All subjects provided written informed consent. The study had ethical approval from the Lothian Regional Ethics Committee and was performed according to Good Clinical Practice guidelines.

Study design and drug treatment

The study was a randomised controlled trial of the effects of cetrorelix and testosterone with or without desogestrel. Following satisfactory completion of screening examination and investigations, thirty subjects were randomly allocated to one of three treatment groups by third-party randomisation using sealed envelopes in blocks of 10. Ten subjects were allocated to the control group and had a testis biopsy at the time of vasectomy without study drug administration. The other 20 subjects received both cetrorelix and testosterone injections for a four-week period prior to testis biopsy at the time of their pre-planned vasectomy with half (10) given the progestogen desogestrel in addition (CT and CTD groups respectively). Cetrorelix (Cetrotide®, Serono Europe Ltd, London, UK) 3mg s.c. was administered twice each week. Testosterone was administered as testosterone enanthate (Cambridge Laboratories, Wallsend, UK), 200mg im on the first day of administration of cetrorelix, and repeated 14 days later. The 10 men in the CTD group also took

desogestrel 300μg orally (Cerazette®, 4 x 75μg, Organon NV, Oss, The Netherlands) each day for the 28-day duration of the treatment period.

Biopsy

Testis biopsy was carried out under local anaesthetic at the time of vasectomy. The procedure was performed through a separate incision to that for the vasectomy. The lower pole of one testis was biopsied using two passes of a 14-gauge needle (Tru-Cut®, Allegiance Healthcare Corporation, USA) using a previously reported technique [327]. Tissue samples were immediately frozen in a dry-ice ethanol bath and stored at –80 ^{0}C prior to analysis. Due to the nature of the biopsy, specimens were not suitable for histological examination. Subjects were required to apply pressure to the scrotum for 30 minutes following the procedure and were given 2 prophylactic doses of ciprofloxacin 500mg. All subjects were reviewed 24 hours and 2 weeks following the procedure.

Isolation of RNA and synthesis of cDNA

Total RNA was extracted using the Qiagen RNeasy Mini kit. High RNA quality (RIN >6.5) was confirmed by running 1μl of a 1/10 dilution of each on RNA 6000 Nanochips in the Agilent 2100 Bioanalyzer (Agilent Technologies, South Queensferry, UK). One sample from a man in the CTD group and one from the control group were found to be of inadequate quality and was not analysed further. First strand cDNA (+/- Reverse Transcriptase) was synthesised from 3μg of total RNA as described previously [328].

Real Time Quantitative PCR

Quantitative real time RT-PCR was performed using the Lightcycler (Roche Diagnostics, East Sussex, UK) as described previously [329]. Reverse transcribed RNA samples were diluted 1/25 in nuclease-free water (Promega Ltd., Southampton, UK). 1 µl diluted first-strand cDNA was added to a final volume of 10 µl containing 50µg/ml BSA and 0.5 µM each of forward and reverse primer in 1 x Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Paisley, UK). Amplification was continued for 45 cycles with signal acquisition after each round of extension. Following amplification, continuous melt curve analysis was performed to ensure product accuracy and samples were analysed by agarose gel electrophoresis (data not shown) to confirm product size. Primers for each gene are given in Table 1 and were either previously published as indicated or designed using online Primer3 software.

Standard curves for each PCR were derived by making a series of dilutions (1/10 to 1/10000) of first-strand cDNA from one of the control group of samples, as previously described [328]. The dilutions yielded a straight line for each product. The slopes of these curves are a measure of the efficiency of the PCR ($E=10^{1/-slope}$), which gave an amplification rate of 1.6 to 1.9- fold per cycle for each product. For each experiment, amplification reactions were performed in duplicate for every cDNA sample used. Calculations for each mRNA concentration were made relative to RPL32 from the same sample to allow comparisons between biopsies. RPL32, which encodes a ribosomal protein, proved to be the most consistent reference gene for these samples although GAPD and B2M [330] yielded similar results. Allowances for differences in amplification rate for each product were achieved by

Gene	Forward Primer	Reverse Primer	Product size (bp)	Accession Number	Reference
CYP17	TCACAATGAGAAGGAGTGGCAC	AGCTTACTGACGGTGAGATGAG	94	NM_000102	[331]
3bHSD	GCGGCTAATGGGTGGAATCTA	CATTGTTGTTCAGGGCCTCAT	127	NM_000198	[333]
5aR1	TGAATACGTAACTGCAGCCAACT	TCTTCAAATTCCGGAGGTACCA	165	NM_001047	
AR	CTCTCTCAAGAGTTTGGATGGCT	CACTTGCACAGAGATGATCTCTG	342	NM_000044	[334]
INHa	TGAGGGCCCTGTTCTTGGATG	CTGGCGGCTGCGTGTATGCTG	278	M13144	
AMH	GCTGCCTTGCCCTCTCTAC	GAACCTCAGCGAGGGTGTT	117	NP000470	[332]
FSHR	CTTTTGCAGCTGCCCTCTTTC	TTGGCGATCCTGGTGTCACTA	205	NM_000145	
PEPP1	TGGAGGAGCTGGAAAGTGTT	CTGGGTCAGCACGTAGTTC	183	NM_139282	[320]
PEPP2	GAGCAGTTCCCCAGTGAGTT	ATGCCCTCTGATGTCTCCTC	121	NM_032498	[320]
MAGEA4	ACAGAGGAGCACCAAGGAGA	GGACCAGAGGAGAGGAGGAG	233	NM_002362	
PRM1	GCCAGGTACAGATGCTGTCGCAG	TTAGTGTCTTCTACATCTCGGTCTG	153	Z46940	[325]
ACRBP	CCACATGGACTTCTGGTGTG	CTGCTGGCTTTTGAAGGAAC	172	NM_032489	
RPL32	CATCTCCTTCTCGGCATCA	AACCCTGTTGTCAATGCCTC	152	NM_000994	[330]
GAPD	GACATCAAGAAGGTGGTGAAGC	GTCCACCACCCTGTTGCTGTAG	212	NM_002046	
B2M	ACTGAATTCACCCCCACTGA	CCTCCATGATGCTGCTTACA	113	NM_004048	[330]

Table 1 Real time RT-PCR Primers

determining the actual amount of amplification required to yield a signal for each target [331, 332].

Semen Analysis

Sperm concentration in the ejaculate was measured in all subjects on one occasion prior to inclusion in the study and again on the day of vasectomy, in both cases following 3-7 days abstinence. Concentration was assessed using World Health Organisation methodology [299]. Azoospermia was confirmed by thorough examination of the pellet following centrifugation of the whole semen sample.

Hormone Assay

Blood samples were obtained pre-treatment and on the day of vasectomy for all subjects. An additional sample was taken from the men in the two treatment groups after 14 days drug administration, immediately prior to second injection of testosterone enanthate. Following centrifugation, serum was stored at -20^0C until assay. Testosterone was measured by radioimmunoassay [335] and FSH and LH by time-resolved immunofluorometric in-house assay [273]. Assay sensitivity was 0.3 nmol/l for testosterone, 0.03 IU/l for FSH and 0.15 IU/l for LH. The intra-assay coefficients of variation (CVs) were <10% in each case, and all samples were analysed in single assays.

Statistical Analysis

Results are presented as mean ± SEM. Hormone and sperm data were compared using paired or unpaired t-tests as appropriate after cube-root and log transformation

respectively. Treatment effects on gene expression data were initially compared by ANOVA (3 groups) or unpaired t test (2 groups). Where ANOVA suggested significant treatment group effects, this was further investigated by unpaired t tests. For all comparisons a P-value of <0.05 was considered significant.

3.3: Results

Subjects, withdrawals, adverse events

Thirty men (mean age 38 years, range 30-47 years) were recruited to the study. There were no significant differences between treatment groups in age or pre-treatment sperm and reproductive hormone concentrations (table 2). One subject randomised to the control group withdrew from the study for personal reasons, thus 29 men underwent testis biopsy (figure 1). There were no significant adverse events. Two subjects complained of itching/redness at the site of cetrorelix injections and one subject in the CTD group reported mood swings and hot flushes during the treatment period.

	Control (n=9)	CT group (n=10)	CTD group (n=10)
Age (years)	39.8 ± 1.75	35.8 ± 1.16	39.8 ± 1.21
Sperm concentration (mill/ml)	66.6 ± 18.5	47.5 ± 8.2	59.9 ±13.8
LH (IU/l)	4.8 ± 0.65	4.5 ± 0.38	4.1 ± 0.46
FSH (IU/l)	4.6 ± 0.83	4.5 ± 0.5	5.1 ± 0.91
Testosterone (nmol/l)	18.2 ± 1.8	19.8 ± 2.3	20.7 ± 1.9

Table 2. Pre-treatment hormone data presented as mean ± SEM

Hormones

Gonadotrophin concentrations were markedly suppressed in all men in both treatment regimens at 14 days and then after 28 days, at the time of testicular biopsy ($P<0.001$, figure 2a and b). There were no differences in the concentrations of either LH or FSH between the CT and CTD groups at either time point. FSH was suppressed to the limit of detection or close to it in all men. LH was suppressed to

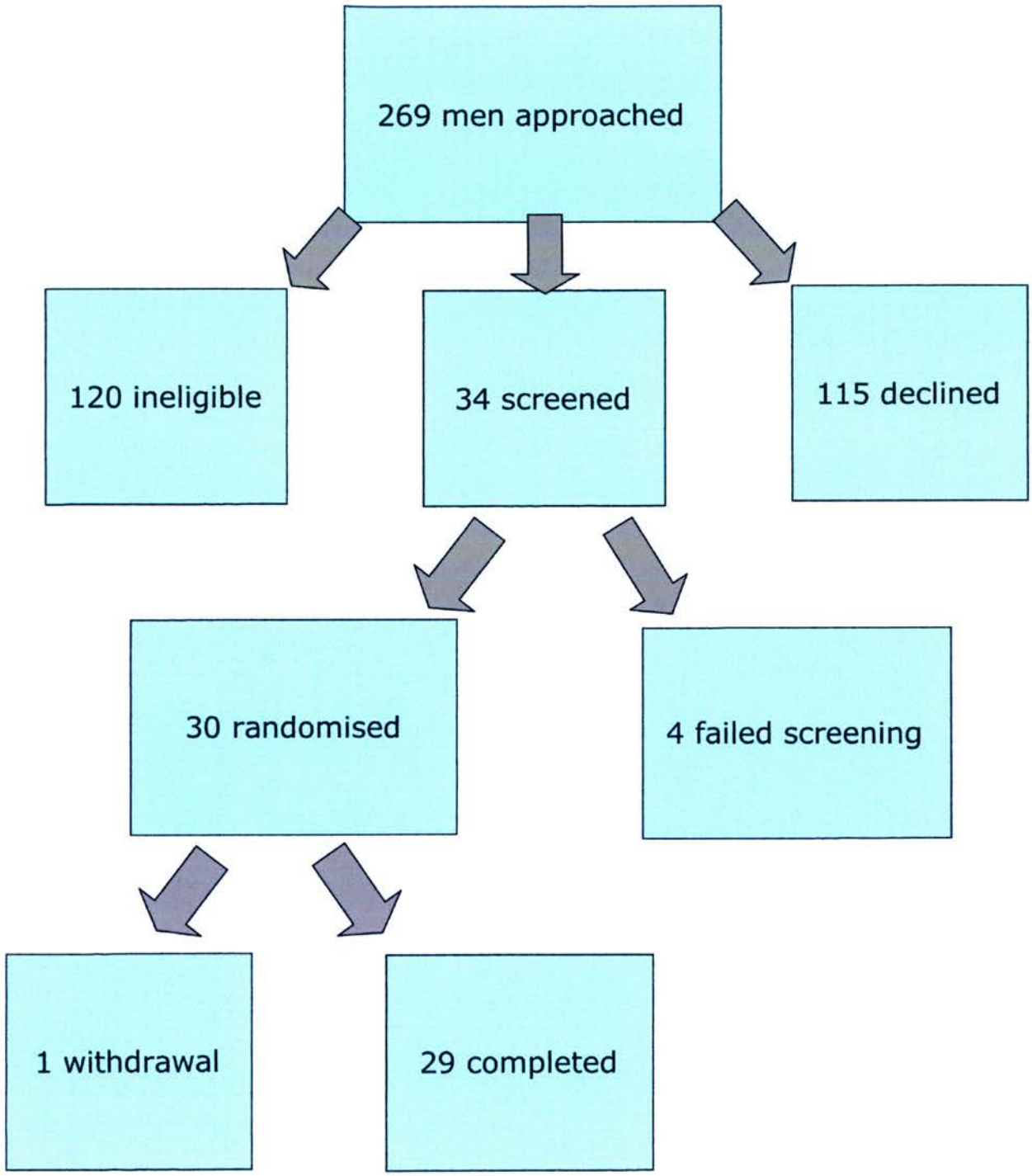
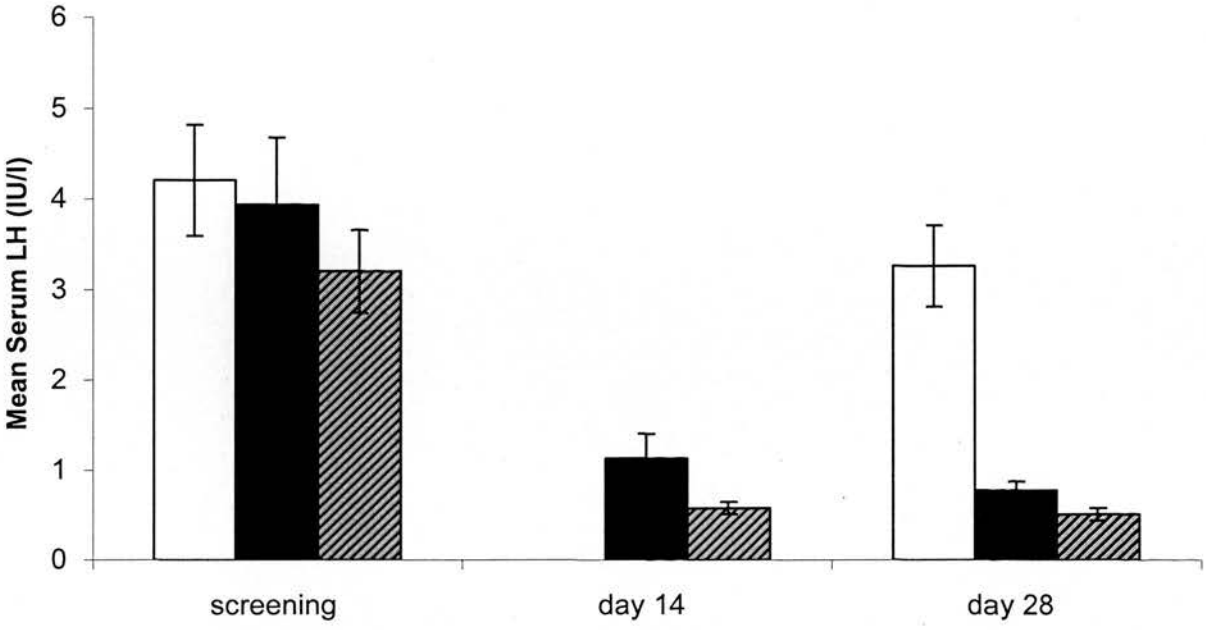
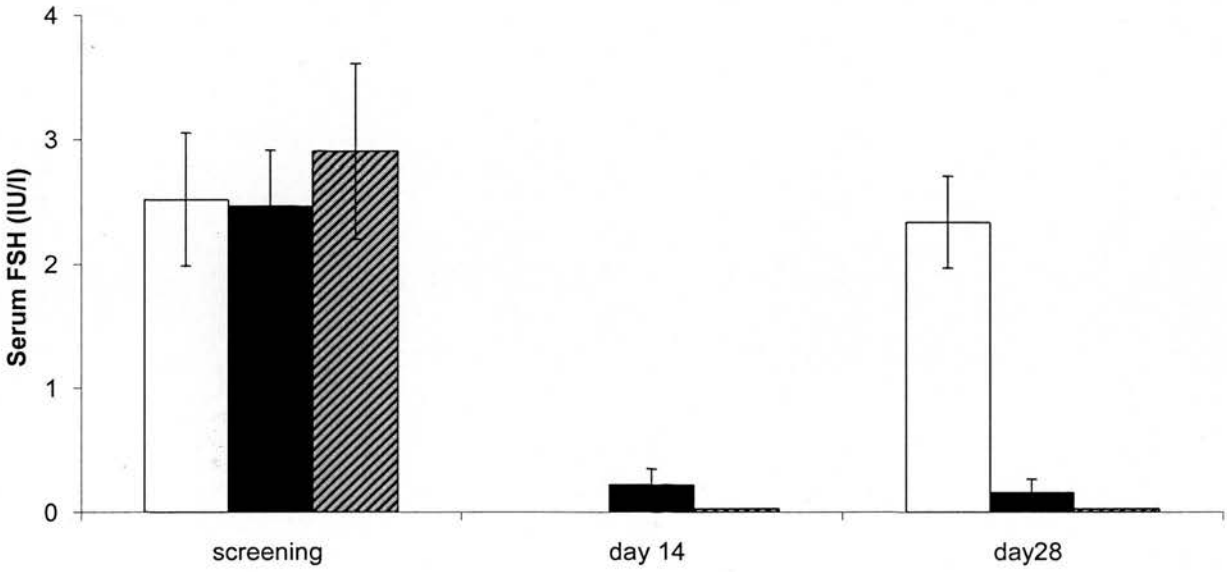


Figure 1. Study treatment pathway. Four subjects failed screening: 1 for high blood pressure, 1 for oligozoospermia, 2 for hypercholesterolemia.



(a)



(b)

Figure 2. Serum concentrations of LH (a) and FSH (b) in untreated controls (*white bars*) and men receiving cetrorelix and testosterone (CT group *black bars*) or cetrorelix, testosterone and desogestrel (CTD group *grey bars*). Results as means ± SEM, n = 8-10 per group.

0.52±0.09 IU/L and 0.51±0.07 IU/L in the CT and CTD groups respectively at the time of biopsy.

Mean testosterone concentrations remained in the normal range in the two treatment groups although were slightly reduced at 28 days (figure 3) in keeping with the administration schedule, as this was the point of trough concentration after the second injection. There were no significant differences in testosterone concentrations between the 3 groups either pre-treatment or at the time of testis biopsy.

Sperm Concentrations

Pre-treatment sperm concentrations were normal in all subjects. Profound suppression of sperm concentration was seen in both treatment groups within the 28-day treatment period (figure 4). Mean concentrations fell to 17.8±9.2 $x10^6$/ml in the CT group, and to 8.1±4.1 $x10^6$/ml in the CTD group compared to 47.5±8.2 and 59.9±13.8 $x10^6$/ml in the two groups pre-treatment. However, there was some inter-individual variability with 3 subjects in the CT group and 2 in the CTD group maintaining sperm concentrations in the normal range (>20 $x10^6$/ml) at day 28, whereas sperm concentrations were less than 5 $x10^6$/ml in all others, and one man in each group had become azoospermic. This allowed classification of 'suppressors' vs 'non-suppressors', the former having sperm concentrations <5 $x10^6$/ml after 4 weeks treatment, the latter having normal sperm concentrations at that time.

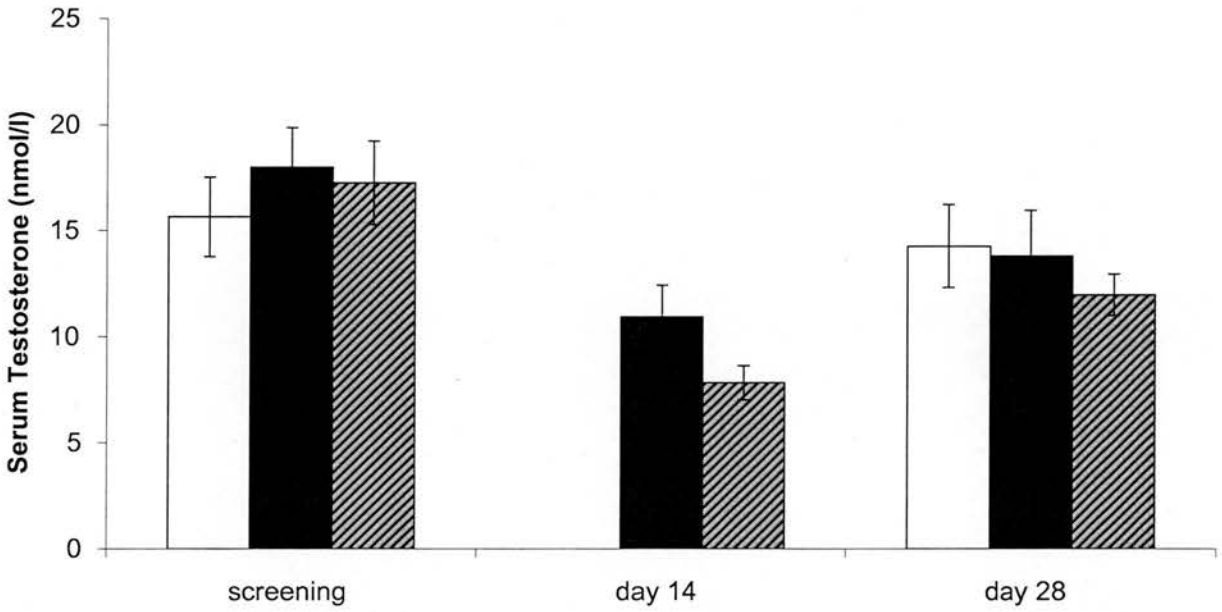


Figure 3. Serum concentration of testosterone in untreated controls (*white bars*) and men receiving cetrorelix and testosterone (CT group *black bars*) or cetrorelix, testosterone and desogestrel (CTD group *grey bars*). Results as means ± SEM, n = 8-10 per group.

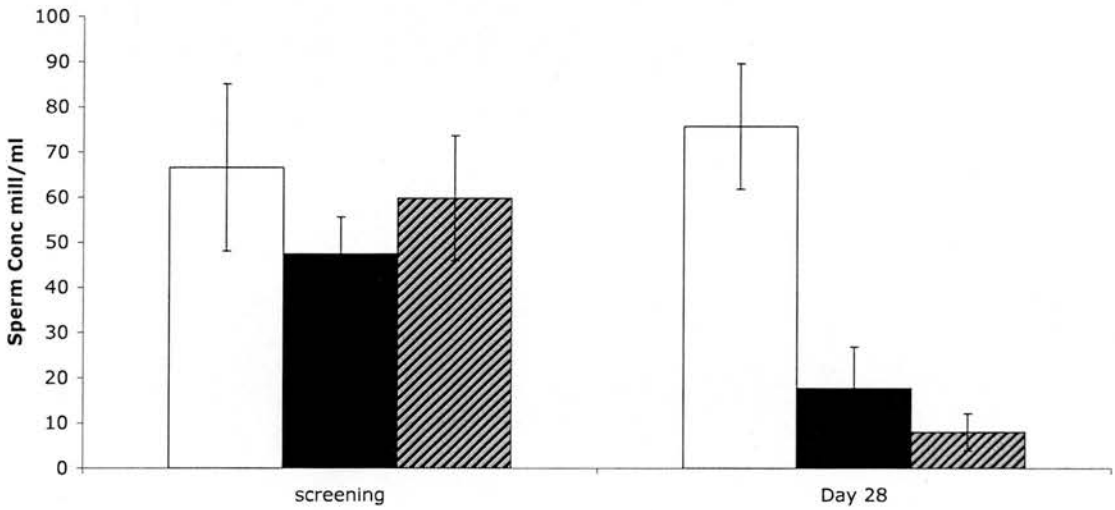


Figure 4. Sperm concentrations in untreated controls (*white bars*) and men receiving cetrorelix and testosterone (CT group *black bars*) or cetrorelix, testosterone and desogestrel (CTD group *grey bars*). Results as means ± SEM, n = 8-10 per group.

Testicular Biopsy Results

Testicular tissue specimens were analysed by treatment vs. control and by treatment group, and also by the degree of spermatogenic suppression. Candidate genes were divided into three groups on the basis of their testicular compartment, ie steroidogenic/steroid metabolism genes expressed in Leydig cells, Sertoli cell markers, and those specific to developing germ cells. The overall results are summarised in table 3.

Genes	*Controls*	*CT*	*CTD*
CYP17	27.37 ± 4.82	0.93 ± 0.21	0.9 ± 0.22
5aR1	0.3 ± 0.04	0.23 ± 0.02	0.17 ± 0.02
3bHSD	0.2 ± 0.03	0.05 ± 0.01	0.04 ± 0.01
AMH	0.01± 0.0	0.004 ± 0.0	0.004 ± 0.0
INHa	38.55 ± 9.77	18.97 ± 3.5	16.05 ± 3.37
AR	1.11 ± 0.13	1.22 ± 0.11	1.06 ± 0.12
FSHR	0.22 ± 0.03	0.18 ± 0.02	0.24 ± 0.06
hPEPP1	1.09 ± 0.17	0.82 ± 0.09	1.02 ± 0.07
hPEPP2	16.39 ± 2.9	12.20 ± 1.68	10.67 ± 1.16
MAGEA4	0.47 ± 0.1	0.35 ± 0.08	0.25 ± 0.05
PRM1	2388.7 ± 381.87	2422.01 ± 387.88	1818.86 ± 452.22
ACRBP	133.37 ± 10.96	105.95 ± 13.4	81.19 ± 12.81

Table 3. Mean gene expression of the three groups as a % relative to RLP32 ± SEM

Steroidogenic Genes

The two genes expressing the steroidogenic enzymes steroid 17-alpha-hydroxylase/17,20 lyase (CYP17A1) and 3βhydroxysteroid dehydrogenase (HSD3B2) showed markedly reduced expression in both treatment groups (both $p<0.0001$), to approximately 3% and 25% of the control group respectively (figure 5). This effect was similar in the two treatment groups. Expression of 5α-reductase type 1 (SRD5A1) was also reduced in both treatment groups overall compared to controls ($P=0.007$, figure 5), but analysis by group demonstrated that this was more

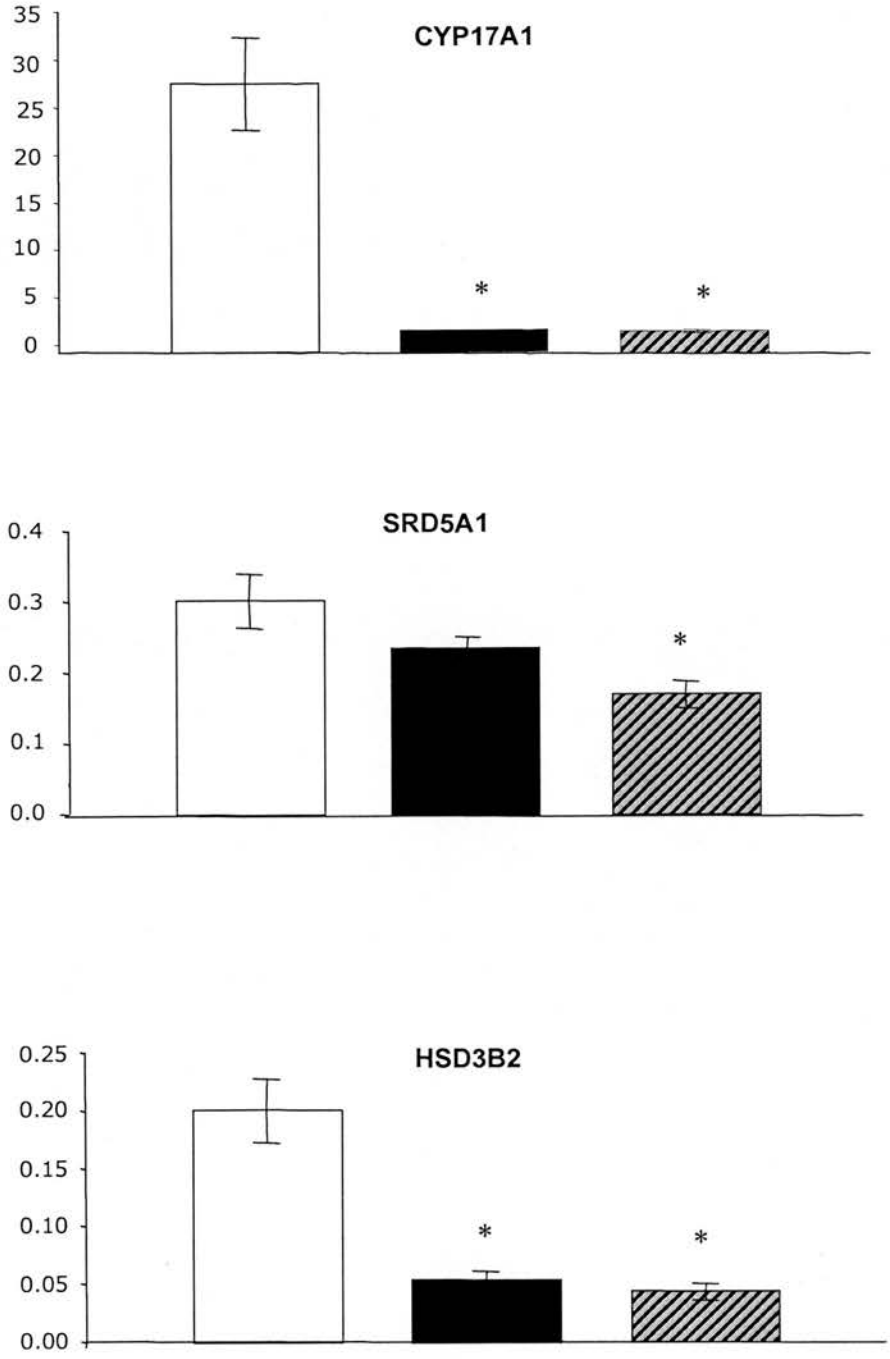


Figure 5. Expression of steroidogenic genes CYP17A1, HSD3B2 and SRD5A1 in controls (*white bars*) and men in CT (*black bars*) and CTD groups (*striped bars*). Results are means ± SEM, n = 8-10 per group. Gene expression relative to RLP32.
* P<0.001vs controls.

in the CTD group (57% of control, P=0.0065) whereas gene expression in the CT group was not significantly lower than controls (77%, P=0.1). There was also a significant difference in SRD5A1 gene expression between the CTD and CT groups (P=0.02).

Sertoli Cell Genes

Expression of inhibin α subunit (INHA) was reduced in both treatment groups together (P=0.007, figure 6) but this was more marked in the CTD group (42% of control, P=0.037), with the difference between the control and CT group not quite reaching statistical significance (49% of control, P=0.056). There was no significant difference between the CT and CTD treatment groups. There were no differences in expression of the genes encoding the androgen receptor (AR), FSH receptor (FSHR) or antimüllerian hormone (AMH) between the treatment and control groups. Expression of hPEPP1 was also unchanged, whereas there was a reduction in hPEPP2 expression in the two treatment groups combined (P=0.05) which did not reach statistical significance in either group separately (74%, P=0.2 and 65%, P=0.08 of control in the CT and CTD groups respectively).

Germ Cell Genes

Three genes specific to germ cells were investigated (figure 7). Expression of MAGEA4 was reduced particularly in the CTD group (to 53% of control), but this did not reach statistical significance (P=0.06) because of high inter-individual variability. MAGEA4 expression was not reduced in the CT group (75% of control, P=0.04). Expression of PRM1 was similar in the two treatment groups to control.

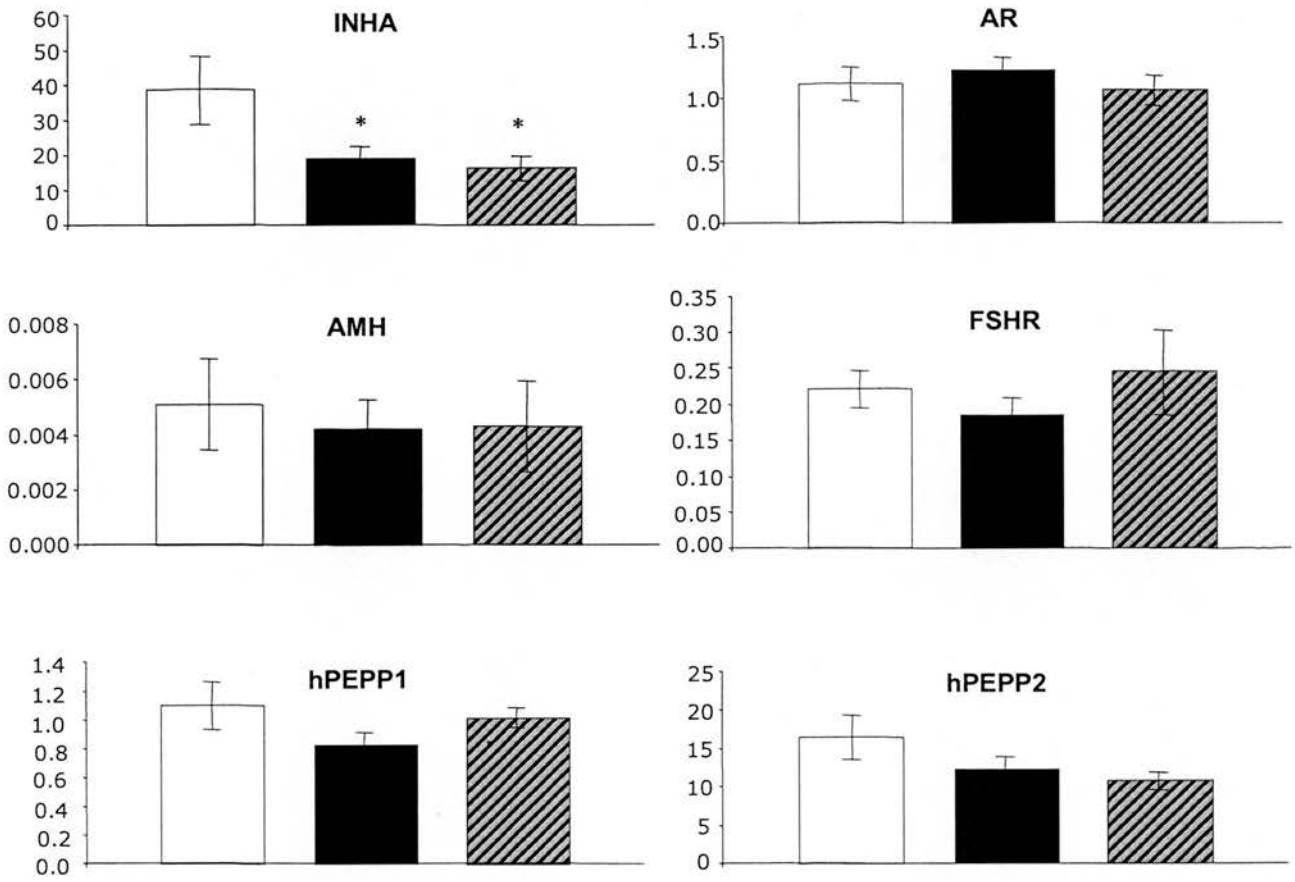


Figure 6. Expression of Sertoli cell genes INHA, AR, AMH, FSHR, hPEPP1 and hPEPP2 in controls (*white bars*) and men in CT (*black bars*) and CTD groups (*striped bars*). Results are means ± SEM, n = 8-10 per group. Gene expression relative to RLP32. * $P<0.05$ vs controls.

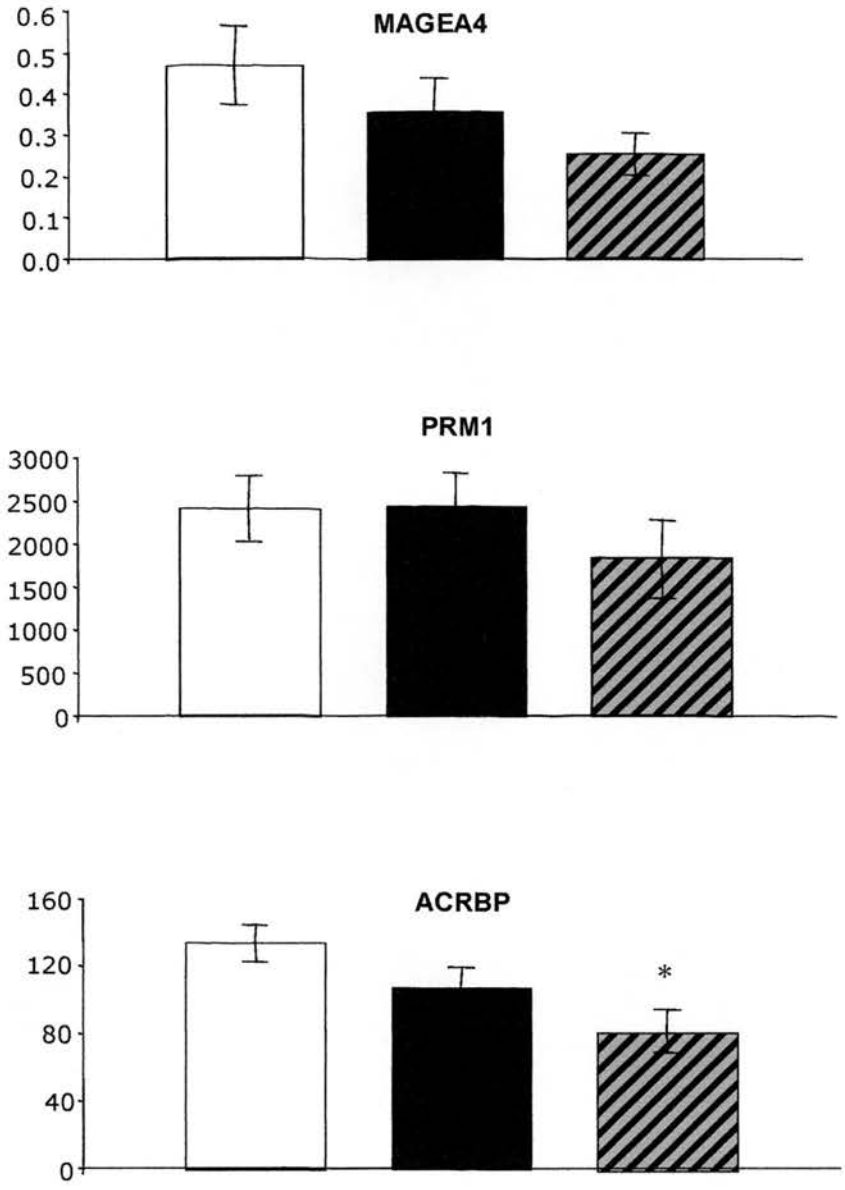


Figure 7. Expression of germ cell genes MAGEA4, PRM1 and ACRBP in controls (*white bars*) and men in CT (*black bars*) and CTD groups (*striped bars*). Results are means ± SEM, n = 8-10 per group. Gene expression relative to RLP32. * P<0.01 vs controls

Expression of ACRBP was reduced (P=0.02). Analysis by treatment group showed that this was confined to the CTD group (61% of control, P=0.008) with no difference between the CT and control groups (79% of control, P=0.1). Expression in the CT and CTD groups was not significantly different.

Gene expression analysed by sperm suppression.

Subjects were classified according to the degree of spermatogenic suppression independent of treatment group, with most men (n=14) showing marked spermatogenic suppression (sperm concentration $<5x10^6$/ml) even within the short treatment interval whereas others (n=5) maintained sperm concentrations within the normal range. There were no differences in sperm, LH or FSH concentrations pre-treatment, between the two groups, nor in LH and FSH concentrations after treatment (table 4).

	Control (n=9)	Suppressors (n=14)	Non-suppressors (n=5)
Sperm Concentration pre-treatment ($x10^6$/ml)	66 ± 16	50 ± 7	64 ± 24
Sperm concentration at biopsy (x 10^6/ml)	75.7 ± 14	1.4 ± 0.4	48 ± 9
LH pre-treatment (IU/L)	4.21 ± 0.61	3.46 ± 0.54	3.92 ± 0.68
LH at biopsy (IU/l)	3.54 ± 0.45	0.53 ± 0.06[a]	0.47 ± 0.14[a]
FSH pre-treatment (IU/L)	2.52 ± 0.37	2.64 ± 0.49	2.84 ± 0.8
FSH at biopsy (IU/l)	2.34 ± 0.37	0.05 ± 0.02[b]	0.03 ± 0[b]

Table 4. Reproductive parameters by spermatogenic suppression. [b] P<0.01, [c] P< 0.001 *vs.* controls.

Both groups showed markedly reduced expression of CYP17A1 and HSD3B2 compared to controls (both P<0.0001) with no difference between suppressors and non-suppressors. However expression of SRD5A1 did differ between groups, with a significant difference between controls and suppressors (62% of control, P=0.004) but not between controls and non-suppressors (81% of control, P=0.3, table 5) . It would therefore be expected that there would be differences within treatment groups by spermatogenic suppression. This was confirmed to be the case in the CT group (suppressors 0.20±0.01 vs 0.30±0.01 in non-suppressors, P=0.002) whereas there was no difference in the CTD group (0.17±0.03 vs 0.16±0.03 respectively).

Among the Sertoli cell genes, the reduction in INHA expression was only significant in the suppressors (42% of control, P=0.01; 55% of control in non-suppressors, P=0.2). hEPP2 expression was slightly reduced with borderline statistical significance in the suppressors (73% of control, p=0.05) but not in the non-suppressors (79% of control, P=0.4). As with the analysis by treatment group, expression of other Sertoli cell genes investigated did not differ between controls and either group (table 5).

Markers of spermatogenesis also showed differential changes in suppressors compared to non-suppressors. MAGEA4 expression was only reduced in suppressors (55% of control, P=0.02; 93% in non-suppressors, P=0.8). ACRBP expression showed similar changes, reduced to 65% of control in suppressors

(P=0.003) but similar to controls in the non-suppressors (89% of control, P=0.5). PRM1 expression did not differ from controls in either group (table 5).

Gene Expression	Controls (n=9)	Suppressors (n=14)	Non-suppressors (n=5)
Steroidogenesis			
CYP17A1	27.4 ± 4.8	0.88 ± 0.18[c]	1.01 ± 0.31[c]
HSD3B2	0.20 ± 0.03	0.05 ± 0.01[c]	0.05 ± 0.01[c]
SRD5A1	0.30 ± 0.04	0.19 ± 0.01[b]	0.24 ± 0.04
Sertoli cell			
INHA	38.6 ± 9.8	16.2 ± 2.4[b]	21.4 ± 6.5
FSHR	0.22 ± 0.03	0.22 ± 0.04	0.19 ± 0.04
AR	1.11 ± 0.13	1.07 ± 0.08	1.36 ± 0.20
AMH	0.005 ± 0.002	0.004 ± 0.001	0.005 ± 0.001
hPEPP1	1.09 ± 0.17	0.97 ± 0.07	0.75 ± 0.11
hPEPP2	16.4 ± 2.9	11.0 ± 1.1a	12.9 ± 2.4
Spermatogenesis			
MAGEA4	0.47 ± 0.10	0.26 ± 0.04[a]	0.43 ± 0.16
ACRBP	133 ± 11	86 ± 9[b]	118 ± 26
PRM1	2388 ± 381	1985 ± 310	2811 ± 685

Table 5. Gene expression analyzed by sperm suppression, values are gene expression relative to RLP32 (mean ± SEM). [a] P<0.05, [b] P<0.01, [c] P<0.001 vs. controls

3.4: Discussion

In this study the effects on testicular gene expression of induced hypogonadotrophism in normal men for four weeks by administering a GnRH antagonist plus testosterone are investigated. An additional group were administered desogestrel to investigate the effects of testicular progesterone receptor activation in addition to gonadotrophin withdrawal. GnRH antagonists may be of particular use in the suppression phase of male contraceptive regimens [265], increasing the speed and consistency of the response. While the most marked effects were on steroidogenic enzyme expression, there was evidence of a selective reduction in the testosterone metabolising enzyme SRD5A1 and of the spermatocyte-specific gene ACRBP but not of genes expressed at earlier stages of spermatogenesis in those men who were given desogestrel. The group not receiving desogestrel showed little or no differences in expression of these genes compared to the control group despite profound suppression of gonadotrophins and of spermatogenesis. Greater suppression of spermatogenesis after this short duration of gonadotrophin withdrawal was also associated with reduced expression of SRD5A1 and ACRBP, but also of INHA, expressed by Sertoli and Leydig cells [148, 159, 336] and of MAGEA4, which is expressed by spermatogonia and primary spermatocytes [321]. PRM1, which is expressed at later stages of spermatogenesis [325, 326], was not affected despite the dramatic reduction in sperm concentration in the ejaculate, to azoospermia in two men. This is consistent with data suggesting that gonadotrophin withdrawal results in a defect in spermiation in humans [31, 39]. These data therefore provide direct evidence that progestogens have specific intratesticular effects independent of gonadotrophin suppression, which may contribute to the

enhanced suppression of spermatogenesis demonstrated in trials of hormonal male contraceptive regimens [273, 274, 300, 301]. They also illustrate the value of this approach to the study of spermatogenic suppression in response to potential contraceptive regimens and other manipulations of testicular function.

Percutaneous biopsies of the testis using either a tru-cut device as here, or a fine 21-gauge needle are regularly performed in the management of infertility. They are used to retrieve sperm from patients with obstructive azoospermia for intracytoplasmic sperm injection (ICSI) and for diagnostic purposes [327, 337-339]. There were no serious complications during this study, supporting the use of this relatively non-invasive technique for research purposes. However the nature of the biopsy and its size made it unsuitable for histology or the measurements of gene products such as testosterone or inhibin.

Gonadotrophins, Testosterone Levels, and Sperm Concentration

The treatment regimen resulted in suppression of gonadotrophins while maintaining normal peripheral testosterone concentrations. The profound and similar gonadotrophin suppression between those men receiving cetrorelix with testosterone and those additionally receiving desogestrel allows the identification of testicular effects of desogestrel although it is possible that there was a slightly greater suppression of gonadotrophin secretion in the CTD group that we were unable to detect. Detailed analysis of the relationships between gonadotrophins and spermatogenic suppression has demonstrated that LH but not FSH concentrations predict ongoing low rates of spermatogenesis [201] and that progestogens, including

desogestrel as used here, have additional gonadotrophin-independent effects. Spermatogenesis was also rapidly suppressed in both treatment groups, also to a similar degree in the two treatment groups. There are limited data on the use of GnRH antagonists to suppress spermatogenesis, but these results, albeit of only short treatment duration, provide support to suggestions that they are highly effective at inducing gonadotrophic and spermatogenic suppression [264-266, 340]. While most men showed profound suppression of spermatogenesis after only 4 weeks of treatment, some 25% still had sperm concentrations in the normal range. It is likely that these men would have subsequently also shown profound suppression, but our short term study allowed a clear distinction between those showing a rapid response (here termed 'suppressors') and those maintaining spermatogenesis at that stage of treatment ('non-suppressors'). These non-suppressors should not be regarded as necessarily having the same characteristics as those who continue to show low rates of spermatogenesis despite more prolonged gonadotrophin withdrawal, but provide a useful group for the investigation of variation in the initial stages of spermatogenic suppression.

Steroidogenic Genes

The most striking changes in gene expression during gonadotrophin withdrawal were in the steroidogenic enzymes CYP17A1 and HSD3B2, with CYP17A1 reduced to <5% of expression in control men. There was no evidence for an additional effect of desogestrel, and no relationship to degree of suppression of spermatogenesis. In rodents Cyp17a1 and Hsd3b2 expression appears unrelated to LH during fetal development [341]. In adult animals however, primary control of both enzymes is via

the LH receptor [48, 342]. These data suggest a similar mechanism of regulation occurs in humans. Progestogens have also been reported to have direct effects on LH receptor expression and to inhibit steroidogenesis in murine Leydig cells [318], but the present results do not provide evidence that such regulation occurs in humans with desogestrel at the dose used here. It is however possible that the very marked degree of suppression of CYP17A demonstrated here has prevented detection of an additional progestogenic effect.

The enzyme 5α-reductase converts testosterone to the more potent androgen DHT. This amplification of androgen action has been suggested to be of importance in the testis in states of testosterone depletion, eg following gonadotrophin suppression, and to be a mechanism whereby some men may maintain low rates of spermatogenesis during contraceptive studies [34, 343]. This has been supported by experimental data in rodents but not by clinical studies involving inhibition of 5α-reductase [36, 314, 344, 345]. In those clinical studies however the type II isoenzyme was preferentially inhibited by the drug finasteride. 5α-reduced steroids within the testis are relatively resistant to gonadotrophin withdrawal [31, 346]. Progesterone and synthetic progestogens have been reported to inhibit 5α-reductase activity in skin [347-349], which predominantly contains the type 1 isoenzyme. The present data demonstrate that SRD5A1 1 gene expression was reduced by administration of desogestrel, but not by gonadotrophin suppression alone. SRD5A1expression was also reduced in relation to the degree of suppression of spermatogenesis. This was clearly the case in men in the CT group, but no difference by spermatogenic suppression was observed in the CTD group. This may reflect the already-present

inhibitory effect of desogestrel, but analysis is also limited by the small group sizes. These data therefore add to the evidence implicating 5α-reduction as a key pathway in the testicular response to hormonal contraceptive regimens, particularly those based on co-administration of a progestogen.

Sertoli Cell Genes

The FSH receptor is only expressed in the Sertoli cells in the testis where FSH acts with testosterone to support spermatogenesis. Withdrawal of FSH and suppression of intratesticular testosterone concentrations induced by the treatment regimens used here might be expected to have marked effects on Sertoli cell function, manifesting as changes in gene expression. However the only Sertoli cell gene demonstrated to change was INHA (encoding inhibin α), which was reduced in both treatment groups. This fall was relatively modest, and was statistically significant only in the CTD group. Additionally, analysis by degree of sperm suppression showed lower INHA expression only in the responders group. This may indicate both a direct effect of progestogen on Sertoli cell function in keeping with the expression of progesterone receptors by human Sertoli cells [317] and a relationship with spermatogenesis. Inhibin B is the biologically active form of inhibin in men consisting of a dimer of the α and β B subunits, and its concentration in blood quantitatively reflects spermatogenesis, comprising the feedback loop linking the functional activity of the seminiferous epithelium with FSH secretion [145, 160, 350]. FSH administration increases inhibin B secretion in hypogonadal but not normal men [149, 351, 352] and there is clear evidence in rodents that inhibin α gene expression is FSH dependent [353, 354]. Similarly FSH administration

increases α subunit concentrations in blood, as does hCG, which may indicate a contribution from Leydig cells [149, 355] which also express INHA [148, 159, 336]. Gonadotrophin withdrawal in men results in a fall in serum inhibin B but only to approximately 50% of normal, even with prolonged treatment [153]. This appears to match the magnitude of the change in gene expression observed in the present study, consistent with the remaining 50% of inhibin expression and production being gonadotrophin-independent in normal men, once spermatogenesis has been established during normal puberty.

Despite the degree of gonadotrophin withdrawal and fall in sperm production induced by these treatment regimens, there were no changes in expression of the other Sertoli cell genes investigated. In particular, we investigated the PEPP1 and PEPP2 genes, as these are the human homologues of the murine *Pem* gene. *Pem* expression is highly androgen-dependent [313, 320], thus we hypothesised that expression of its human homologues would be reduced under the experimental conditions here. These data confirm that PEPP1 and PEPP2 are expressed in the human testis, and that the expression of PEPP2 but not PEPP1 was reduced in treated men compared to controls. Expression of PEPP2 was also reduced in the spermatogenic responder group, but not in non-responders. These data are consistent with PEPP1 expression being androgen-dependent as with *Pem* in the mouse, although the degree of androgen-dependency appears much less in the human. Expression of rat *Pem* in the testis is also less androgen-dependent than in the mouse [319] emphasising the importance of species specificity in the regulation of testicular function.

Expression of other Sertoli cell genes investigated, i.e. FSHR, AR and AMH was similar in controls and treated groups, indicating that expression of these genes is not gonadotrophin dependent, nor does expression appear closely linked to normal spermatogenesis. FSHR expression is FSH-regulated in the mouse [356], but these data suggest that this is not the case in the human. AMH production is low post puberty and suppression is predominantly maintained by high intratesticular testosterone concentrations [182, 188] with additional regulation by germ cells at some stages of development [357]. AMH gene expression was confirmed to be very low in these biopsies of normal men. An increase in AMH might have been observed following gonadotrophin withdrawal, but was not seen in the relatively short period of study.

Germ Cell Genes

MAGE-A4 is expressed by spermatogonia and primary spermatocytes but not by spermatids or Sertoli cells [321] and is therefore a marker for early spermatogenesis. We also investigated expression of ACRBP as a further spermatocyte-specific marker [323, 324]. MAGEA4 expression was similar in the control to the two treatment groups, but was reduced in the responder group. ACRBP showed greater changes, being reduced in the CTD but not CT groups compared to controls, indicating a direct effect of the progestogen. As with MAGEA4, there was markedly lower ACRBP expression in those men in the responder group, but not in non-responders. The greater changes in ACRBP than MAGEA4 may reflect greater changes in spermatocytes than spermatogonia. This is consistent with data from

stereologic analysis of testis biopsies [31] following similar treatment regimens to that used here. Those data indicated that after only 2 weeks of testosterone/progestogen treatment there were fewer type B spermatogonia and early spermatocytes present in the seminiferous epithelium, with inhibition of spermatogonial maturation. This effect was less marked in men treated with testosterone alone, who showed slower gonadotrophin suppression, and with longer treatment durations marked changes were seen in both treatment groups in all germ cell types. Changes in gene expression in spermatogonia and spermatocytes can therefore occur rapidly following gonadotrophin withdrawal and are associated with more rapid suppression of spermatogenesis.

In contrast to these changes in markers of early and mid stages of spermatogenesis, there were no differences in expression of PRM1 either by treatment group or by rate of spermatogenic suppression. PRM1 is expressed in post-meiotic haploid spermatids [326], and is necessary for fertility [358]. Expression (relative to the related gene PRM2) is reduced in infertile men [359]. The maintenance of PRM1 expression here is striking considering the dramatic reduction in sperm output in most men in the treatment groups. These data therefore strongly support the observation that one of the major early defects in the human spermatogenic epithelium following gonadotrophin withdrawal is an inhibition of spermiation [31, 346]. This will result in retention of the cell types expressing PRM1 while at the same time drastically reducing sperm output. This is similar to that observed in the macaque monkey and rodents, in which gonadotrophin withdrawal also results in retention of elongate spermatids [3, 360]. With longer gonadotrophin withdrawal all spermatogenic cell

types are reduced [31], and would be expected to result in a decrease in PRM1 expression, but the short duration of treatment investigated here has allowed demonstration of further evidence of the importance of spermiation failure as a component of the mechanism by which gonadotrophin suppression results in very rapid falls in sperm output in men. It also is in keeping with the rapid return of fertility in some men following withdrawal or omission of hormonal contraception [361].

3.5: Conclusion

These data demonstrate the value of a novel approach to the investigation of the regulation of testicular function in men. Direct evidence for progestogenic effects on all three major compartments of the testis, ie steroidogenesis (5α-reductase), Sertoli cell function (inhibin α) and spermatogenesis (ACRBP), independent of gonadotrophin inhibition is presented. Furthermore, relationships between expression of these and other genes (PEPP2 and MAGEA4) with the rate of initial suppression of sperm output were identified. Notably, these data provide further support for the importance of 5α-reduction and disruption of spermiation as important components of the testicular response to gonadotrophin withdrawal, of direct relevance to understanding of testicular function and the development of novel methods of male contraception.

Chapter 4: Study M017: A single centre study comparing the 24 hour testosterone concentration in two groups of men: men receiving a hormonal contraceptive regimen and a control group.

4.1: Introduction

Diurnal variation of serum testosterone concentration in adult men with a peak in the morning and a nadir in the early evening has been described in many studies [55, 362-365], although its physiological significance, if any, is not understood. Increased understanding of the pattern of testosterone production and thus the correct dose required for physiological replacement will benefit those on long-term testosterone replacement and may aid in the development of male hormonal contraception. Both supraphysiological and subphysiological doses may have adverse effects reflecting the very widespread distribution of androgen receptors. Supraphysiological doses of testosterone are associated with raised haematocrit, decreased HDL levels and an increase in mean blood pressure [366] whereas inadequate replacement results in anaemia, osteoporosis and loss of libido [75, 79, 110].

In this study we re-examined the diurnal variation of testosterone in normal men, and included analysis of both gonadotrophins as main drivers of testicular function, and of inhibin B as the non-steroidal feedback loop from Sertoli cell to the pituitary gland. We have also examined diurnal variation in a further group of normal men after administration of a combination of testosterone and progestogen to suppress gonadotrophin secretion using a dose of testosterone calculated to approximately match physiological production. In this situation the testosterone is delivered constantly throughout the 24 hours so that any variation in its concentration would represent a

change in its metabolic clearance. As circulating testosterone is exogenously derived under those circumstances, we have measured excretion of the specific testicular steroid epitestosterone as a marker of ongoing steroidogenesis in the testis. These data suggest that even without LH stimulation there is a diurnal variation to testicular steroidogenesis, and that under both normal and suppressed states there is a diurnal variation to Sertoli cell function.

4.2: Methods

Subjects

Ten subjects were recruited from the general population to act as controls. Inclusion criteria included age 18-50, BMI 18-32 kg/m^2, normal physical including andrological examination, normal haematological and biochemical parameters and reproductive hormones. Ten subjects (the treated group) were also recruited from a male contraceptive trial (as described in Chapter 2). They received a regime of two etonogestrel implants (each 68mg, Implanon®, Organon NV, Oss, The Netherlands) administered sc into the upper non-dominant arm, in combination with 600mg testosterone pellets (3 x 200mg, Organon NV) administered sc into the lower abdomen. The testosterone implants were repeated every 12 weeks. The investigation was performed midway between the insertion of the testosterone implants i.e. 18 weeks (+/- 7 days) after the administration of etonogestrel. All subjects gave written informed consent, the study received approval from the local ethics committee and was performed according to GCP guidelines.

Study Design

Each of the 20 subjects was admitted for 24 hours from 8.00am to the Welcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh. On admission an indwelling catheter was inserted into a vein in the forearm. Samples of blood (9ml) were collected every hour for the next 24 hours, each sample was centrifuged, separated and serum stored at –20C until hormone assay. Subjects emptied their bladders at 9 am and urine samples were collected at 1pm, 5pm, 9pm, 1am, and 9am, i.e 4 hourly through the day and 8 hours overnight. A record of the total urine volume was kept for each

time-point and aliquots of urine were stored at –20°C. Subjects were allowed to move around freely and retired to bed by 1am.

Assays

All hourly blood samples were analysed for testosterone, LH, FSH, cortisol and prolactin, and for SHBG 3-hourly. Testosterone and cortisol were analysed by radioimmunoassay [335], and LH FSH and SHBG by time-resolved immunofluorometric assay. Assay limits of quantification were 0.3 nmol/l for testosterone, 0.5 nmol/l for SHBG, 0.03IU/l for FSH and 0.15 IU/l for LH. The intra-assay coefficients of variation (CVs) were <10% for testosterone (6.4% between 5 and 100nmol/L), FSH and LH, and 4% for SHBG. The inter-assay CVs were 12.4% for testosterone, <10% for FSH and LH and 8.8% for SHBG. Inhibin B was assayed as previously described [159, 296] with an assay sensitivity of 7.8pg/ml. Testosterone and epitestosterone were measured in urine samples by gas chromatography-mass spectrometry as described and validated previously [367]. For urinary epitestosterone (aglucone plus free fraction) the interassay CV was 13.4% at 1.5 μg/L, the limit of quantification, falling to <10% for concentrations between 10 and 38 μg/L.

Statistical Analysis

Hourly blood data for each subject were analysed by cosinor methods [368] fitting a two-parameter sine curve with 24-hour periodicity. This gave an estimate of the mesor (mean level), acrophase (time of peak level) and amplitude (difference between peak and mean level) as well as a test of significant periodicity for the data from each subject. Throughout the results and discussion mesor is referred to as mean and acrophase as the

mean peak. Rayleigh tests [369] were then used in each of the two treatment groups separately to test for significant directionality in the acrophases, and the estimates of mean and amplitude were compared between the treated and control groups using two-sample t-tests.

Analysis was carried out on the logarithms of the urine measurements since they were positively skewed. Two-factor analysis of variance was used to test for significant daily variation in mass per hour between the five time intervals, and measurements over each time interval were compared between treatment groups by two-sample t-tests. Repeated measures analysis of variance was used to test whether the diurnal variation differed between the two groups.

4.3: Results

Subjects

All 20 subjects were fit and healthy, mean age of controls 29 years (range 24-43) and mean age of the treated group 35 yrs (range 25-50). There were no abnormalities found on biochemical and haematological screening including reproductive hormones and andrological examination and no pre-treatment differences between the two groups (see table 1).

	Control Group	Treatment Group
Age (years)	29.2 ± 2.07	34.9 ± 2.23
Serum LH (IU/l)	4.62 ± 0.57	4.14 ± 0.41
Serum FSH (IU/l)	3.41 ± 0.43	2.43 ± 0.64
Serum T (nmol/l)	23.3 ± 1.99	23.78 ± 3.52

Table 1. Screening values for all subjects data presented as mean ± SEM

Serum Testosterone

Serum testosterone results at the time of 24 hour sampling were similar for the two groups with no significant differences seen in mean concentration (control mean= 16.2 ± 1.5 nmol/L, treated mean=13.9 ±1.7 nmol/L, figure1). When grouped, mean testosterone concentrations (figure 1a) showed a rise in concentration of testosterone in the early morning in the control group but no such overt variation in the treated group. However analysis of individuals demonstrated evidence of periodicity for a substantial proportion of the individual subjects in both groups. There was significant directionality in the mean peaks in the control group ($P<0.01$) with a mean peak at 7am. In contrast although there were individual variations in the concentration of testosterone in the treated group with mean amplitude similar to that in the control

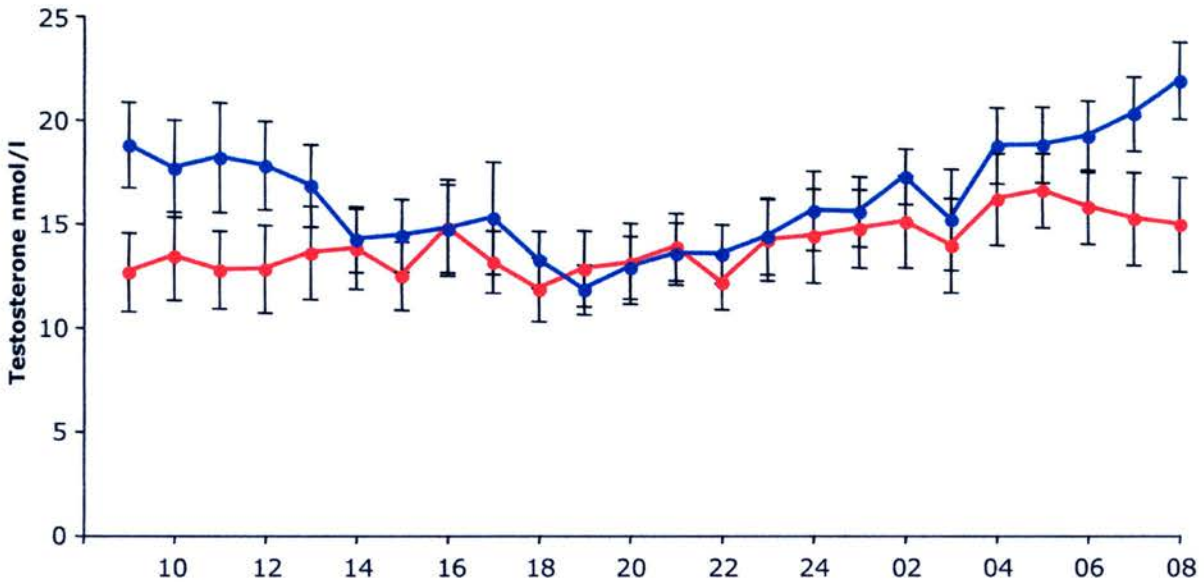


Figure 1. Serum testosterone concentrations over 24 hours in the control (blue) and treated groups (red) presented as mean ± SEM.

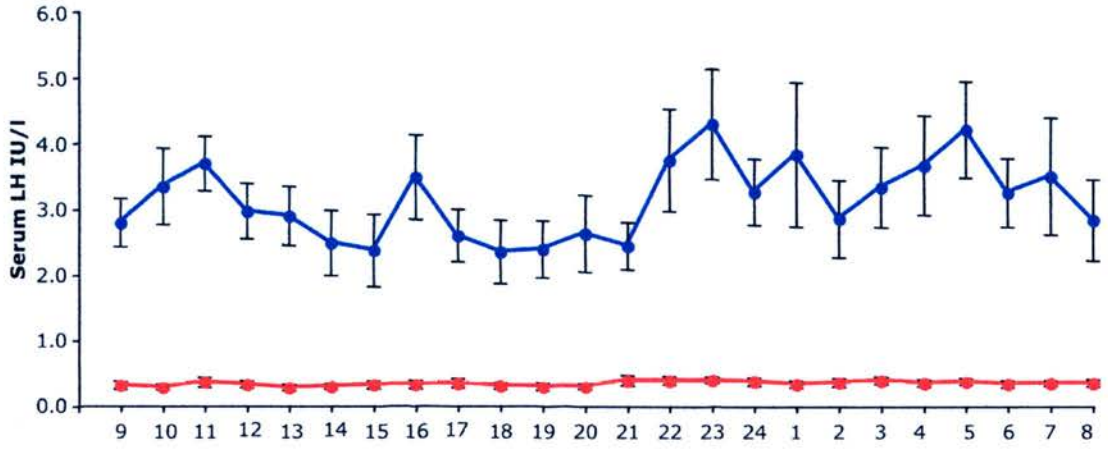
group (control 3.6 ± 0.54 nmol/L, treated 2.5 ± 0.4 nmol/L, NS) there was no clustering and therefore no significant diurnal variation.

Gonadotrophins

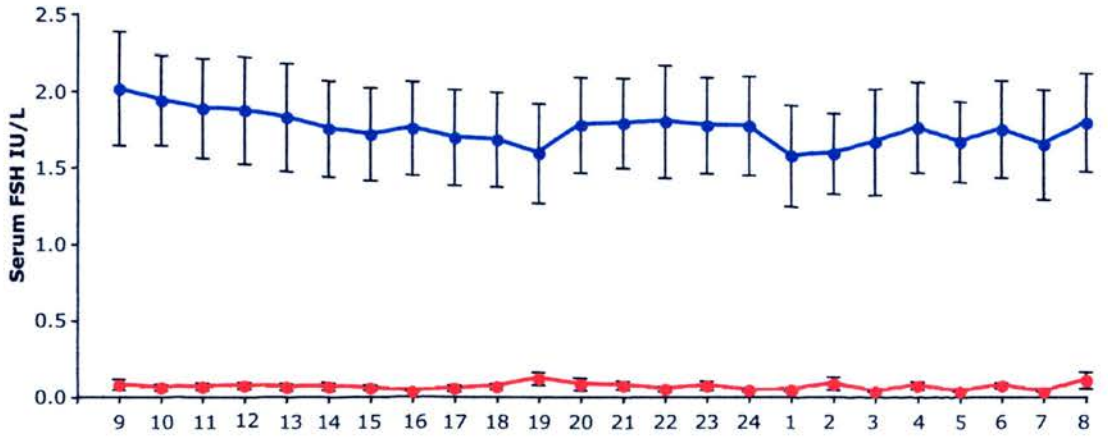
The control group LH results demonstrated a mean LH of 3.1 ± 0.6 IU/l (figure 2a). Although only 3 of the men in this group showed significant periodicity, there was a significant directionality in the peak times ($P<0.01$) with an amplitude of 0.58 IU/l and a mean peak at 5am. FSH results for the control group were within the normal range throughout the 24-hour period (figure 2b) with a mean FSH of 1.7 ± 0.3 IU/l. 5 men showed significant periodicity with an amplitude of 0.21 ± 0.03 IU/L but with no significant directionality. Subjects in the treated group demonstrated highly suppressed LH and FSH as expected with this testosterone/progestogen regime with a mean LH of 0.33 ± 0.06 IU/l ($p<0.001$ vs control group) and mean FSH of 0.06 ± 0.02 IU/l ($p<0.001$) and no significant variability throughout the 24-hour period for either hormone.

Inhibin B

Significant periodicity in inhibin B was detected in 5 of the men in the control group, but there was no evidence of directionality. Overall mean inhibin B concentrations were lower in the treated group (control mean=140.1 ± 3.9, treated mean=94.2 ± 2.4, $P<0.01$, figure 3) but 9/10 men in this group showed significant periodicity albeit with lower amplitude than in the controls (treated amplitude=13.1, control amplitude=30.7, $P<0.01$). Additionally, the treated group demonstrated synchronised



(a)



(b)

Figure 2. Serum concentrations of (a) LH and (b) FSH over 24 hours in the control (blue) and treated groups (red). Mean ± SEM. The control group showed significant diurnal variation in LH but not FSH ($P<0.01$).

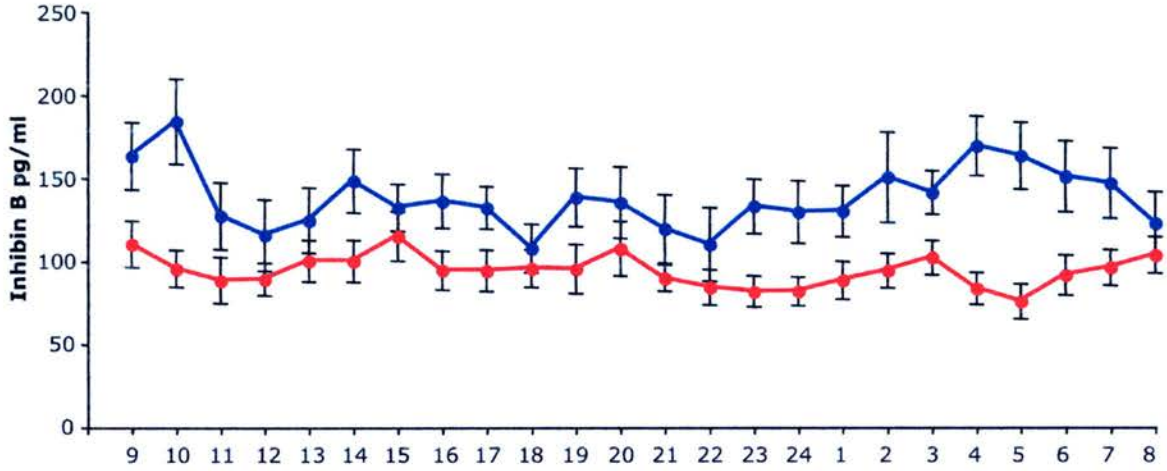


Figure 3. Serum concentrations of inhibin B over 24 hours in the control (blue) and treated groups (red). Mean ± SEM. The treated group showed significant diurnal variation ($P<0.05$) which was not seen in the control group.

diurnal variation with significant directionality of the peak times ($P<0.05$) with a mean peak at 1.13pm.

Urinary Testosterone and EpiTestosterone

Despite the similarity in serum testosterone concentrations between the two groups, urinary excretion was higher in the treated group overall ($P<0.001$) and at all time points (figure 4a and b). There was no significant variation between the samples at different time points in either group, thus urinary testosterone concentrations did not demonstrate significant diurnal variation in either group.

The urinary excretion of epitestosterone (expressed as mass/hr) was 10 fold higher in controls than in the treated group (figure 4c and d). There were therefore highly significant differences ($P<0.001$) between the two groups for each of the five time periods. There was however also significant variation between time points within both groups (both $P<0.05$) with no significant difference between the groups in the pattern of variation over the five time intervals. Thus both groups showed diurnal variation in the excretion of epitestosterone with highest excretion in the morning sample.

Sex hormone binding globulin

SHBG concentrations were higher in the controls than in the treated group (mean 24 hour concentrations 23.6 ± 2.5 vs 17.6 ± 3.3 nmol/L, $P<0.001$) but showed no diurnal variation in either group. Calculated free testosterone [370] however was similar in the two groups (0.40 ± 005 vs. 0.38 ± 0.04 nmol/L).

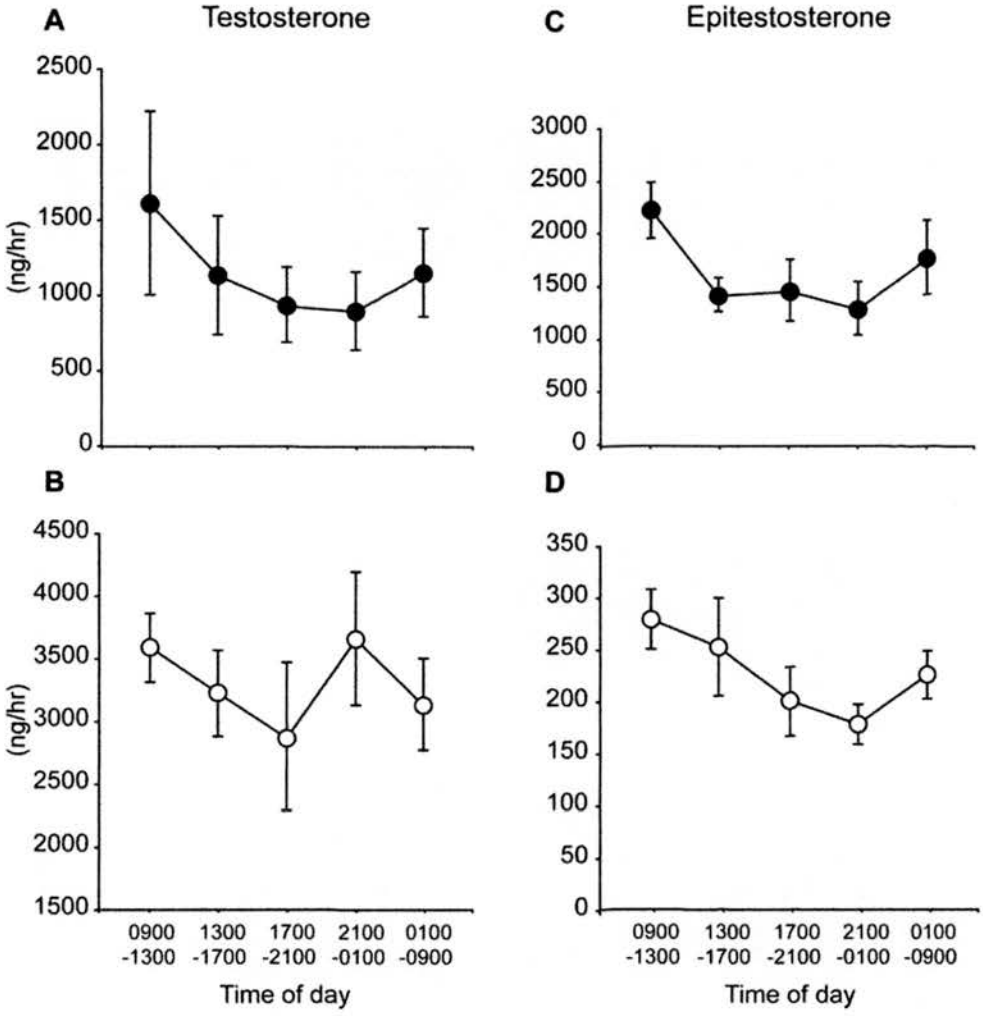


Figure 4: Urinary excretion of testosterone (a and b) and epitestosterone (c and d) over 24 hours in the control (filled symbols) and treated groups (open symbols), both steroids in ng/hr. Time intervals as indicated on abscissa. Mean ± SEM. Testosterone excretion did not show significant diurnal variation in either group, but there was significant diurnal variation in epitestosterone excretion in both control and treated groups ($P<0.05$ in both cases)

Cortisol

Serum cortisol concentrations in both groups demonstrated highly significant diurnal variation with the mean peak mid morning at10am ($P<0.001$ controls, $P<0.01$ treated, figure 5). There was no significant difference in amplitude between the two groups (control amplitude=28.2 ± 1.96, treated amplitude=22.5 ± 3.98) There was a slight difference in average cortisol concentrations between the group (control 47.2 ± 2.0 ng/ml, treated 31.3 ± 1.9 ng/ml $P<0.05$).

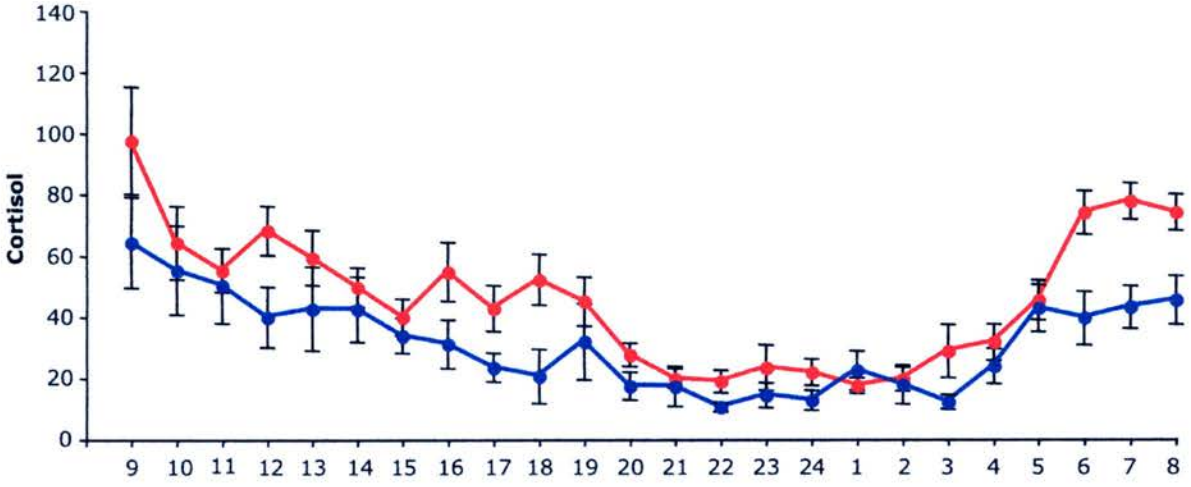


Figure 5. Serum concentrations of cortisol over 24 hours in the control (blue) and treated groups (red). Mean ± SEM. Both groups showed significant diurnal variation ($P<0.001$ control, $P<0.01$ treated groups).

4.4: Discussion

Previous studies have described the diurnal variation of testosterone in healthy men, but none have closely examined the 24-hour hormone profile in men receiving exogenous testosterone as a provisional hormonal contraceptive regimen.

We confirmed that normal men show a diurnal variation in testosterone concentrations, with a peak in the morning (7am). Diurnal variation in serum LH was also detected in the control group, with a peak at 5am, compatible with the influence of LH on testosterone production in normal men. These findings are similar to those reported previously, for both testosterone [56, 365, 371]; and LH [372, 373]. Cortisol was used in this study as an internal control and demonstrated expected diurnal variation in both groups across the 24-hour period with a peak mid-morning. The slightly lower concentrations in the treated group may reflect the mild glucocorticoid activity of etonogestrel [374].

The administration of exogenous testosterone and etonogestrel resulted in similar mean testosterone concentrations to that in the control group. Establishing the correct dosage of testosterone is a fundamental priority for male hormonal contraceptive development. The mean serum T concentration observed in controls was 16 nmol/l (i.e. approx 6ng/ml) and the metabolic clearance rate is approximately1000L/day [375-377]. Therefore, production rate (metabolic clearance rate x serum testosterone concentration) is approximately 6mg/day, as previously determined using isotope infusion [62, 199, 376]. The testosterone pellets used in this study were administered as 600mg every

12weeks. Therefore 900mg was given in 18 weeks: i.e 50mg/week or 7mg/day, which is thus at steady state an appropriate dose for physiological replacement.

The subjects in the treated group displayed serum testosterone concentrations within the normal range throughout the 24-hour period and with a mean value similar to the control group, as anticipated due to the constant release of testosterone from the pellets. Although the concentrations varied through the study period in six out of ten of the treated group the significant synchronised diurnal variation observed in the controls was not seen. Thus the diurnal variation in testosterone concentrations observed in the control group is likely to be due to a change in the secretion of testosterone by the testis rather than an alteration in the metabolic clearance rate; if that was the case a similar pattern should be observed in the treated group.

SHBG concentrations were lower in the treated group, as previously reported in men treated with testosterone plus progestogens [273, 291] likely to account at least in part for the higher urinary testosterone concentrations in those men. Serum free testosterone concentrations were however similar in the two groups.

Epitestosterone (17α-hydroxyandrost-4-en-3-one) is an epimer of testosterone secreted predominantly by the testis [378] which provides a measure of endogenous testicular secretion. Urinary epitestosterone showed a comparable diurnal variation to that in serum testosterone in the control group, with highest excretion in the morning, providing further evidence that this reflects variation in testicular production rather than changing rates of metabolism.

Urinary excretion of epitestosterone was markedly reduced in the treated group, as previously reported in men administered similar testosterone/progestogen regimens [239, 273]. This level of excretion is significantly higher than that in hypogonadal men [378] indicating detectable ongoing Leydig cell steroidogenesis despite markedly suppressed LH concentrations. This is consistent with measurements of testosterone in testicular biopsies from men treated with similar regimens [31, 346] and may contribute to the residual low rates of spermatogenesis in some men [291, 379]. There was clear evidence of a diurnal variation in epitestosterone excretion in the treated group with peak excretion in the morning, as in the control group. Whereas in the controls peak testicular steroidogenesis as detected both in serum and urine may reflect the preceding rise in serum LH, no such rise was seen in the treated group. This suggests that there is an endogenous rhythm within the testis independent of LH although we cannot exclude the presence minor unrecognized fluctuations or bioactivity in the markedly suppressed levels of LH.

The excretion of testosterone in urine did not show significant diurnal variation in either group although in the controls there was a tendency for the values to decline throughout the day to reach their lowest value in the late evening. In contrast the concentration of testosterone in serum peaked in the early morning and reached a nadir in the early evening at 1900. These findings are compatible with the expected delay of a few hours in the excretion of testosterone by the kidney. It demonstrates the relative insensitivity of measurement urinary testosterone as an index of the hour to hour changes in the secretion of testosterone from the testis.

Inhibin B concentrations also showed significant periodicity and synchronised diurnal variation with a mean peak in the early afternoon in the treated but not control group. Inhibin B is partly but not completely under gonadotrophin control, thus prolonged gonadotrophin suppression results in a gradual fall in the concentration of inhibin B (as confirmed here) eventually to approximately 30% of control [147, 152, 153, 273]. A previous study of testicular function in normal men detected a diurnal variation in inhibin B [380]. That study used a more frequent blood sampling regimen than used here, which may have increased their ability to detect any variation. The existence of a diurnal variation in inhibin B in normal men during gonadotrophin suppression has not been previously reported, and as with the epitestosterone data indicates an endogenous diurnal rhythm in the secretion by the testis. Inhibin B production by the testis is independent in the short term of the concentration of gonadotrophins and reflects spermatogenic activity [381]. Thus it appears that the removal of exogenous drivers and regression of spermatogenesis has allowed the detection of an endogenous variation which was masked in the control group with the sampling regimen used here. One possible contributory mechanism is the interaction between Leydig and Sertoli cells, which may also contribute to the regulation of inhibin production [382]. It is noteworthy that the observed peak in inhibin B in the treated group occurs in the early afternoon whereas the peaks in serum testosterone and urinary epitestosterone are in the morning.

4.5: Conclusions

These results raise further questions regarding the control of male reproductive hormones levels over the 24-hour period and in particular indicate the existence of significant intratesticular factors other than the gonadotrophins contributing to Leydig and Sertoli cell function. It may well be that the testis has two intrinsic rhythms, that driven by LH which is dominant and one which is non-gonadotrophin dependent which can be alluded to by the inhibin B and urinary epitestosterone results in the treated group. The nature of these factors and their mechanisms of action remain to be determined.

Chapter 5: Final Summary and Conclusions

The results of the three studies presented here contribute our understanding of the basic underlying mechanisms of action of male hormonal contraceptives and provide further evidence of their efficacy and safety in man.

The data from study M016 provides direct evidence that progestogens have specific intratesticular effects independent of gonadotrophin suppression, which may account for the enhanced suppression of spermatogenesis in male hormonal contraception trial regimens incorporating these hormones [201, 249, 273].

The effective use of a GnRH antagonist in this study adds further support for the effectiveness of these agents in conjunction with testosterone as male hormonal contraceptives [265, 340]. However, their frequent administration regime and high cost may prove prohibitive to their commercial development.

The most evident changes during this study were the marked suppression of the steroidogenic genes CYP17A1 and HSD3B2 during gonadotrophin withdrawal. No additional suppression of these two genes was demonstrated by inclusion of the progestogen desogestrel. However, investigation of the SRD5A1 gene demonstrated that expression was reduced by the addition of desogestrel but not by gonadotrophin withdrawal alone. This provides novel evidence for a progestogenic effect on the expression of 5α-reductase in the testis, which may reduce the amplifying effect of conversion of testosterone to dihydrotestosterone and therefore contribute to greater suppression of spermatogenesis. This data implicates the 5α-reduction pathway as a

key mechanism in the testicular response to testosterone/progestogen combination MHC regimes.

The changes in the gene expression of the germ cell genes also provided for some novel observations. The changes in expression of ACRBP and MAGEA4 may reflect greater changes in spermatocytes than spermatogonia, which are in keeping with previous observations of stereologic analysis [31]. The maintenance of PRM1 expression was interesting considering the significant spermatogenic suppression seen in most men even in this short treatment period. Therefore this data supports previous studies that have demonstrated an inhibition of spermiation as a significant early change in spermatogenic production following gonadotrophin withdrawal [31, 346]. This leads to maintenance of the cell types expressing PRM1 in association with a substantial reduction in sperm production. This maybe one of the mechanisms by which rapid falls in sperm output can be demonstrated after only a short time-period of gonadotrophin suppression. After a longer period of gonadotrophin withdrawal it would be expected that all spermatogenic cell types would demonstrate suppression in keeping with previous observations [31].

M017 investigated closely the diurnal variation in testosterone and its metabolites in normal men and men receiving a contraceptive regime. The diurnal rhythms observed in the control group were in keeping with previous observations [56, 365, 372, 373]. In the treated group it was reassuring to demonstrate that the serum testosterone concentrations remained in the normal range throughout the 24-hour period. Approximation of exogenous doses of testosterone to that observed naturally

is a vital prerequisite of male hormonal contraceptive regimens. The demonstration of diurnal variation in both urinary epitestosterone and serum inhibin B suggests that there is an endogenous rhythm within the testis that is independent of LH control. It is interesting to consider the mechanisms underlying this and how this relates to the interaction between Leydig and Sertoli cells and its implications for the ongoing development of male hormonal contraceptives.

Study M015 investigated further the efficacy and safety of the synthetic androgen MENT in conjunction with etonogestrel in comparison to a regime utilising testosterone pellets. MENT and testosterone alone regimes have both been shown to demonstrate effective spermatogenic suppression but the doses required to achieve this can result in unwarranted non-reproductive effects [204, 232, 234]. The release rates of the MENT implants investigated in this study did not appear to be adequate to maintain normal libido in the men in the study group. Initially however, the degree of spermatogenic suppression achieved was comparable to that of the testosterone group, but it did not continue to be sustained once inconsistent suppression of gonadotrophins was seen. Reassuringly, we did not demonstrate any adverse effects of MENT on prostate activity or bone mass. Increases in haemoglobin and reduction in HDL-C were in keeping with previous observations [234]. The observation of a small but significant increase in systolic blood pressure in the MENT group may have significant long-term health implications but further evidence is necessary. In summary, MENT still has strong potential as a male hormonal contraceptive if formulations to ensure a sustained release rate can be derived. Its non-reproductive effects still require further clarification.

The observations noted in the testosterone group build on data collected in previous studies undertaken by this research group [239, 241, 273, 361]. Consistent and profound spermatogenic suppression was demonstrated with azoospermia in all men. However, no significant advantage was demonstrated over previous regimens investigated. The modest increase in testosterone dosage administered resulted in an increase in haemoglobin and prostate size and decrease in HDL-C that our group had not observed in previous regimens with a lower testosterone dose and comparable or increased dose of progestogen. This highlights that it may be of greater benefit to alter the progestogen dose than that of testosterone to keep to a minimum the undesired non-reproductive effects.

Hormonal male contraceptives remain unavailable at present and it is uncertain whether current ongoing studies will change that within the next 5 years. We should continue to see significant advances in our understanding of androgen action in different tissues possibly aided by the further development of synthetic selective androgens. This will lead to greater sophistication of male hormonal contraceptives, which can minimise safety concerns and may add to health benefits.

The recent 10^{th} Summit meeting on hormonal male contraception has further consolidated guidelines for the development and regulatory approval of these agents. It is widely agreed that large-scale clinical trials are required to bring a product to the market, and a number of specific criteria were agreed [383] :

- It is still considered acceptable to use spermatogenic suppression as the main end point in phase II trials and the goal should be < 1 million/ml.
- All participants should be followed after cessation of treatment until reversibility of sperm production, to levels comparable with normal fertility are demonstrated.
- Only men with sperm concentrations >20 million/ml should participate in trials.
- Open-label non-comparative studies are acceptable if the endpoint is not susceptible to bias.
- To demonstrate contraceptive efficacy, two independent phase III trials for 1 year involving 200 men/couples should be undertaken.
- For safety assurance, trials should involve at least 300-600 men for 6 months, 100 men for 1 year and at least 1500 men in total.
- Long-term safety will be addressed by post-marketing surveillance.

The main areas of focus for safety considerations remain the cardiovascular system, prostate, bone, body composition and behavioural aspects. It is now widely accepted that long-term safety will only be established by post marketing surveillance. There are currently a number of phase III trials being undertaken in Europe/Asia and their outcomes and conclusions will have a great bearing on the future of this method.

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