

# **Studies in male hormonal contraception**

Dr W. Morton Hair BSc., MB. ChB.

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## Declaration

This thesis has been composed by me and is my own work. It has not been submitted for any other degree or postgraduate qualification.

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Dr W. Morton Hair BSc, MB ChB.

## Abstract

Early studies aimed at producing a reversible contraceptive for men involved treatments with injectable testosterone esters which induce azoospermia in only a proportion of subjects. Androgens remain a central part of any hormonal contraceptive for men although understanding the heterogeneity of the suppression response within populations may be pivotal and require consideration of other hormonal regulatory systems. Development of minimally invasive, long acting androgen formulations is also necessary to provide an acceptable, convenient and reliable form of androgen delivery which men themselves can administer. The first study describes a clinical trial employing a wholly subject administered, non-invasive hormonal contraceptive regime which combined transdermal T patches with the oral progestin desogestrel (DSG). The study shows that oral DSG combined with transdermal T produces suppression of gonadotrophins and spermatogenesis but is less effective than regimens incorporating injectable T and serves to further emphasise the critical role of T delivery. Studies in animals have established prolactin as a progonadal hormone in the testis and accessory glands. To explore the role of prolactin in men we first investigated the localization of prolactin receptor expression in the human testis and accessory tissues by immunohistochemistry and found it to be localized to the Leydig cells and differentiating cells of the testis, the epithelium of vas deferens, epididymis, prostate and seminal vesicles. Expression of prolactin receptor mRNA was identified in human testis and vas deferens by RT-PCR. Functional activation of prolactin receptor was demonstrated in human vas deferens by examination of the Janus Kinase/Signal Transducer and Activator of Transcription and Mitogen Activated Protein kinase and Extracellular signal-Regulated Kinase signalling pathways. The demonstration of function and localization of the prolactin receptor presented here suggests multiple roles for prolactin in the human male reproductive tract. The final study investigated whether concomitant suppression of PRL with the non-ergot, dopamine receptor agonist quinagolide (Q) in healthy male volunteers, would enhance spermatogenic suppression by testosterone (T). The results suggest that inhibition of PRL does not confer additional efficacy in spermatogenic suppression by T although difficulty in consistently suppressing PRL in eugonadal men did not allow unequivocal testing of the hypothesis. It is hoped that these studies address some of the challenges in this area and contribute toward the ambition of a safe, reversible contraceptive for men.

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## List of abbreviations used in this thesis

CPA	cyproterone acetate
DHT	dihydrotestosterone
DSG	desogestrel
DMPA	depot medroxyprogesterone acetate
ENG	etonogestrel
ERK	extracellular signal-related kinase
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
HDL-C	high density lipoprotein-cholesterol
JAK	janus kinase
LDL	low density lipoprotein
LH	luteinizing hormone
LNG	levonorgestrel
MENT	7 $\alpha$ -methyl-19-nortestosterone
19-NT	19-nortestosterone
PRL	prolactin
Q	quinagolide
STAT	signal transducer and activator of transcription
T	testosterone
TE	testosterone enanthate
TU	testosterone undecanoate

*“Men take a pill? They can’t even remember to put the toilet seat down”*

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## Introduction

The female combined oral contraceptive pill is widely recognized as one of the pivotal scientific and cultural developments of the modern era.<sup>1</sup> Developed in the middle of last century by Gregory Pincus and Carl Djerrassi it allowed many women to reliably control their fertility for the first time and arguably to gain fuller control over their lives.

Developments in the sphere of human male reproductive biology have been less marked: the most widely used male-directed methods today (condoms and vasectomy) were developed in the 16<sup>th</sup> and 19<sup>th</sup> century respectively. However, recent estimates indicate that some 45 million men have had a vasectomy with similar numbers using condoms, which together with the substantial number still relying on withdrawal, constitute at least one-third of total contraceptive usage in the world today.<sup>2</sup> It is clear that, despite contrary perceptions in some quarters, men are active and willing participants in the practice of contraception, whose needs and demands are not met by current methods.

Discussions on the subject of male hormonal contraception inevitably turn to the twin issues of whether men would use it and whether women would trust them to. The definitive answer will have to wait until such products are a commercial reality but recently published data suggests support for the concept and that a market for such a product exists.<sup>3,4</sup> Questionnaire based surveys have been carried out to ascertain the attitudes of both men and women to male contraception in four culturally and economically diverse cities: Edinburgh, Cape Town, Hong Kong and Shanghai. The principal finding of these studies was that the majority of men would look favourably

on new hormonal methods of contraception with 44-83% adding they would definitely or probably use a male pill. The women surveyed were similarly positive with over 84% in the four centres agreeing that availability of a hormonal preparation for men would facilitate sharing of responsibility for contraception.

Spermatogenic suppression following testosterone administration was first reported in the first half of last century and administration of exogenous androgen remains a universal feature of effective prototype regimes examined to date. Despite progress in the half century which has passed since the first studies, a commercially available, safe, effective, reversible hormonal contraceptive for men remains elusive.

In the review that follows, the published data in the field of male hormonal contraception will be examined in some detail. Three recurring themes emerge:

- Finding a means of effective, sustained, non-invasive androgen delivery
- Need for combination therapies to lower the dosage of exogenous testosterone without compromising efficacy
- Need to improve efficacy such that treatment is consistently, reliably and absolutely effective – with the female combined oral contraceptive pill as the yardstick.

The following studies attempt to address one or all of these difficulties. The consistent, reproducible finding that a cohort of men fail to suppress spermatogenesis to azoospermia in the absence of gonadotrophins challenges current concepts of

testicular regulation in the human. Prolactin is a recognised progonadal hormone in many animals and receptor localisation and activation is becoming more clearly understood. In seasonal animals following experimental withdrawal of gonadotrophins, persistent, prolactin driven testicular cycles are found suggesting a facilitatory role for prolactin in conjunction with the established gonadotrophins FSH and LH. Clinical data supports a similar progonadal role for prolactin in humans. It is postulated that experimental manipulation of prolactin in conjunction with the classical gonadotrophins will result in more effective suppression of spermatogenesis. The following studies describe the localization and distribution of the prolactin receptor in human male reproductive tract using histochemical and molecular biology laboratory methods. These studies have established the presence and distribution of the prolactin receptor in key structures in the human male reproductive tract and provide a sound foundation for the subsequent clinical trial. The clinical trial that followed was conducted over 18 months to explore the effects on spermatogenesis of combining a prolactin inhibitor, quinagolide with testosterone implants: a well established, effective method of androgen replacement in contraceptive research and clinically proven in androgen replacement in hypogonadal men. The final study describes a clinical trial which employs a wholly subject administered combination contraceptive drug regimen. Transdermal androgen replacement offers the hope of simple, non-invasive androgen replacement in combination with desogestrel – a highly effective progestagen whose efficacy had already been established in earlier studies where, in combination with injectable testosterone, complete azoospermia was achieved in all subjects. If such regimes are to become a commercial and scientific reality, they must be able to withstand the rigours of testing “in the field” with the attendant problems of subject compliance and reliability. An effective regime will

have tolerances in-built to allow a margin of safety i.e. that efficacy remains uncompromised when subjects are outwith the laboratory setting and compliance may on occasions be less than optimal. This study uses a downward dose finding regime of desogestrel to find the minimum effective dose of progestagen in combination with transdermal testosterone.

The following studies hope to offer novel approaches to established biological problems in the area of male hormonal contraceptive research.

### References

1. Achievements of the Millennium The Economist December 2000
2. Men: new focus for family planning programs. *Population Reports*. 1986; 14:J889.
3. Martin CW, Anderson RA, Cheng L, Ho PC, van der Spuy Z, Smith KB et al. 2000. Potential impact of hormonal male contraception: cross cultural implications for development of novel preparations. *Human Reprod*. 15:637-45.
4. Glasier A, Anakwe R, Everington D, Martin CW, van der Spuy Z, Cheng L et al. 2000. Would women trust their partners to use a male pill? *Human Reprod* 15:646-9.

# **Chapter 1**

## **Review of male contraception**

The world's population currently stands at around 6 billion and, unless global fertility rates are curtailed, will more than triple to 19 billion by 2100 (Population Studies, 1990). Such growth is unsustainable in natural resource terms as well as its projected impact on issues of global poverty and pollution. This realization is a key factor underpinning research efforts directed towards the regulation of human fertility, which began in the second half of the twentieth century. In the last two decades this field has enjoyed a broader perspective, with agreement through the Cairo Programme of Action that a shift in emphasis was warranted (Cairo Programme of Action, 1994). Thus, research efforts initially directed at the control of processes in the female were expanded to include regulation of male fertility in general and male hormonal contraception in particular with drug industry interest showing a parallel increase. In order to appreciate the challenges inherent in producing an effective male hormonal contraceptive, it is appropriate to review our understanding of the physiological regulation of the male reproductive axis.

### **Physiological basis for a male hormonal contraceptive**

#### **Spermatogenesis and steroidogenesis**

Spermatogenesis in the testis is dependent on intratesticular testosterone concentrations 100-fold in excess of those in peripheral blood. Testosterone production is activated by the action of LH from the anterior pituitary on the Leydig

cells of the testis and acts in conjunction with pituitary FSH on the Sertoli cell to promote normal spermatogenesis within the seminiferous tubules. FSH and LH release is regulated by hypothalamic GnRH which is in turn regulated by negative feedback from testosterone which also feeds back on FSH and LH at the level of the anterior pituitary. Inhibition of both FSH and LH is mandatory to achieve maximal suppression of spermatogenesis but the parallel fall in testosterone production results in diminished libido, mood changes and fatigue, with decreased bone and muscle mass and disruption to haemopoiesis as longer term effects. Androgen replacement is thus a central component to all contraceptive regimes of this type. Exogenous testosterone at sufficient doses provides effective suppression of FSH and LH via classical negative feedback pathways but when such supraphysiological doses are employed there may be unwanted extragonadal effects e.g acne, changes in libido and excess sebum production. The challenge for any contraceptive drug regimen is thus to achieve a testosterone dose which will suppress gonadotrophins whilst avoid symptoms of hypogonadism. Combination therapies may allow the simultaneous achievement of these apparently conflicting objectives.

Testosterone mediates a number of peripheral, extra gonadal actions on bone, liver, muscle and prostate either directly or following  $5\alpha$ -reduction to dihydrotestosterone (DHT) or following aromatisation to oestradiol.

#### *5 $\alpha$ -reductase*

The enzyme  $5\alpha$ -reductase converts testosterone to DHT, a steroid with a greater potency of action than testosterone. It is highly expressed in some peripheral tissues such as the prostate and has been demonstrated in human testis (Payne et al., 1973, Rivarola et al., 1973). Under normal circumstances, DHT is not thought to make a

significant contribution to maintenance of spermatogenesis, but following experimental suppression of endogenous testosterone by a hormonal contraceptive regimen, conversion to DHT may become a more important factor in supporting spermatogenesis. The enzyme has two isoforms and in the rat testis, the type 1 isoform predominates. In humans, characterization of the isoforms, and their regulation are less clearly understood: activity and mRNA levels are said to be low but it is thought that humans may bear the type 2 isoform (Russel et al., 1994). Variation in suppression of spermatogenesis between and within population groups has been ascribed to differences in  $5\alpha$ -reductase activity and clinical studies based on this postulate are described later. Peripheral tissue conversion to DHT may underlie some of the unwanted effects of exogenous testosterone therefore synthetic androgens which do not undergo  $5\alpha$ -reduction may offer the advantage of fewer extra-gonadal effects e.g. prostatic hypertrophy.

#### *Aromatase and oestrogens*

A role for oestrogen in support of spermatogenesis has begun to emerge in the last decade. Testosterone is converted to oestradiol by the enzyme aromatase. Estrogen receptor  $\alpha$  (ER $\alpha$ ) knockout mice are found to have dysfunctional seminiferous tubules and epididymis and impaired fertility as a consequence (Lubhan et al., 1998, Hess et al., 1997, Korach et al., 1996). Individual humans with rare mutations in the gene coding for aromatase and for the oestrogen receptor showed similar impaired fertility (Smith et al., 1994, Morishima et al., 1995, Carani et al., 1997). Combined oestradiol and testosterone implants have been shown in the rat (Ewing et al., 1977) and the monkey (Lobl et al., 1988) to very effectively suppress spermatogenesis without affecting mating behaviour suggesting oestrogen blockade as a potential target for contraception. Conversely, aromatisation of testosterone to oestrogen has

also been found to be highly important in the mediation of the feedback effect of testosterone on FSH secretion (Hayes et al., 2000). Improved FSH negative feedback, and consequently, spermatogenic suppression through addition of low dose oestradiol has been demonstrated in animal studies. Similar contraceptive studies in men are described below.

Although incomplete, our knowledge of male reproductive physiology has developed significantly in recent years. In addition to the classical gonadotrophins, regulation by other hormone systems may play an important role in the development of an effective hormonal contraceptive for men.

### ***Extragonadal effects of androgen administration***

Intramuscular testosterone has been shown to produce markedly fluctuating levels of testosterone with repeated high peaks which lead to weight gain, effects on lipid metabolism (lowering of high-density lipoprotein-cholesterol (HDL-C)), skin (increased sebum production leading, in some cases, to acne), liver (raised transaminases) (Wu et al., 1996) and haemopoiesis (raised hemoglobin, red cell count, and haematocrit) (Anderson et al., 1995). All changes proved to be transient and had returned to normal within 3 months of discontinuing treatment. Such changes have been reported in the great majority of studies of this kind in the last decade regardless of the agents employed. (Wu et al., 1999)

HDL-C has an important role in removal of cholesterol to the liver and is controlled by physiological levels of sex steroids via their effect on hepatic triglycerides lipase activity. Low concentrations of HDL-C have been epidemiologically linked to coronary artery disease although there is no established link between testosterone concentrations and cardiovascular disease. (Barrett-Connor, 1999)

SHBG concentrations also change following administration of exogenous sex steroids showing a decline following administration of either testosterone or progestins and may be useful as a marker of unwanted hepatic steroid effects on the liver.

(Handelsman 1992)

It is also well known that androgens are stimulators of erythropoiesis manifest by increases in haematocrit and haemoglobin concentrations following TE administration. (Wu et al., 1996) Antiandrogens such as CPA produce the opposite effect. (Meriggiola et al., 1998) Clearly, the availability of improved methods for the delivery of testosterone at doses that more closely resemble physiological values and awareness of the extra-gonadal effects of other agents in the combination regimes described below is crucial for the continued development of safe hormonal contraceptives that are acceptable to healthy young men.

## **Male contraception**

Three broad approaches to male fertility regulation are currently being pursued: (i) interruption of sperm transport (ii) disruption of sperm maturation and/or function and, (iii) suppression of sperm production.

### *Vasectomy and Condoms*

Interruption of sperm transport by physical barriers such as vasectomy and condoms or by coitus interruptus have been the mainstay of male contraceptive methods thus far and are the contraceptive method of choice for a third of users worldwide. These are the only two reliable methods currently available to men (condoms and vasectomy) are based on historical practices developed from the 16th and 19th centuries respectively. Since the 1960s, vasectomy has played an increasingly

important role as the principal method for male contraception in men who have completed their family. Some 45 million men are estimated to have undergone vasectomy. The single most important advantage of the method is the very high efficacy (1-2 failures per 1000 procedures). Complications include haematoma or infection in 5%, recanalisation in up to 3% and sperm granulomata varying from 3 - 75%, with no reported mortality. Vasectomy has at various times been linked to increased disease risks (atherosclerosis, diabetes, immunological disorders) but has generally been shown to be a reassuringly safe (Nienhuis et al., 1992). Retrospective surveys have suggested a modest increased predisposition to prostatic (Giovannuci et al., 1993) and testicular tumours in vasectomised men in the USA. However, the reported relationships were weak and have not been universally observed. On the basis of current data, no changes in vasectomy practice are warranted although ongoing prospective studies should further clarify this situation (Farley et al., 1993). Results of vasectomy reversal are highly variable depending on the time interval since the original procedure, the skill and experience of the operator and the type of procedure. In general, much more favourable results are obtained with microsurgical techniques within 2 years after vasectomy. Potential patients should expect to achieve patency rates of 80% and pregnancy rates of 60% if reversal is within 5 years after vasectomy. Two main factors limit the wider acceptability of surgical vasectomy: the need for skin incisions and the uncertainty of reversibility. New techniques such as no-scalpel vasectomy and percutaneous transluminal vas occlusion have been or are being developed to make the procedure simpler, less invasive as well as more predictably reversible (Xiaozhang and Sunqiang, 1993).

Barrier contraception has been employed in ancient Greece, Egypt and China in form of animal bladder, intestines and silk paper sheaths during sexual intercourse. But in

more recent times, it is disease prevention that promoted the use of sheath-type barriers. The condom is the only male contraceptive method effective in protecting against sexually transmitted diseases (STDs) and should have a pre-eminent role in men who have multiple, short-term or irregular sexual partners. However, despite the danger of HIV infections, poor user compliance and low consumer acceptability have continued to produce high failure rates and undermine the potential usefulness of condoms. Currently available condoms made from vulcanised latex rubber are further hampered by high slippage/breakage rates, incompatibility with oil-based lubricants, latex allergy and limited shelf life. These factors have prompted efforts to develop non-latex polyurethane condoms that have higher tensile strength and provide greater transmission of heat and tactile sensations. They are less susceptible to degradation and are unaffected by oil-based lubricants. Several polyurethane products are currently undergoing clinical testing and some are commercially available.

#### *Epididymal agents*

The epididymis would appear to be a promising target for a contraceptive agent, by preventing the acquisition of motility and fertilizing potential through disruption of epididymal function. Loss of sperm motility through interference with energy metabolism, or disruption of sperm surface protein chemistry causing impaired sperm-ovum binding, acrosome reaction and fertilizing capacity are all possible modes of action for prototype contraceptive drugs.

A few compounds which inhibit the oxidative metabolism of spermatozoa, and hence inhibit motility, have been evaluated.  $\alpha$ -Chlorohydrin and 6-chloro-6-deoxyglucose are metabolised to 3-chlorolactaldehyde, a compound with the same stereochemistry as R-glyceraldehyde - a key substrate in the glycolytic pathway. Administration of these compounds results in competitive inhibition of the key enzyme, glyceraldehyde-

3-phosphate dehydrogenase, and glycolysis (and therefore sperm motility) is inhibited.

Evaluation of these and other novel compounds as potential post-testicular contraceptive agents in rats and mice remains an active area of enquiry. (Bone et al., 2002, Jones et al., 2002, Cooper 2001). However, given the effects on a core biochemical process (i.e. glycolysis), many of these compounds have serious systemic side effects, particularly on the nervous system and bone marrow which may limit further exploration of their contraceptive potential in humans (Ford and Waites 1986).

The antifungal agent ketoconazole is known to accumulate in seminal plasma and inhibit sperm motility post-ejaculation. This property has been ascribed to the imidazole ring structure in ketoconazole and consequently, a variety of substituted imidazole compounds were evaluated as orally-administered, rather than topical, inhibitors of sperm motility (Vickery et al., 1986). There have been no studies using these compounds reported in the last decade.

Nitroimidazole derivatives, often used as antibiotics and anti-protozoan treatments suppress fertility by inhibiting sperm maturation in the epididymis though once again, its broad side-effect profile makes long-term use in contraception impractical (Nieschlag et al., 1997).

## **Hormonal Methods**

Hormonal methods of suppressing spermatogenesis have shown the most promise as adjuncts to existing barrier based methods and merit review in detail.

Injectable androgen-only regimes and alternative androgen delivery methods

From studies in the 1970's it was found that supraphysiological doses of testosterone alone can consistently and reliably suppress spermatogenesis to azoospermia in 40 to 70% of Caucasian males (Scheerer et al., 1978). The most effective regimen was intramuscular testosterone enanthate (TE), 200 mg weekly. This was chosen as the prototype candidate for subsequent male hormonal contraceptive trials because of its well-documented safety over many years in the treatment of hypogonadal men, despite its suboptimal pharmacokinetics.

### Testosterone enanthate

Between 1986 and 1990, the first ever study to examine the contraceptive efficacy of testosterone-induced azoospermia was conducted by the World Health Organization (WHO) in 271 healthy volunteers in 7 countries using TE, at a dose of 200 mg weekly (WHO, 1990). There were 157 men who achieved azoospermia, and these men entered the 12-month efficacy phase, in which the weekly testosterone injections were the only form of contraception used. Only 1 pregnancy occurred during the 1486 months of exposure, giving a Pearl rate of 0.8 (95% CI 0.02 to 4.5). This established that hormonal suppression to azoospermia yielded an efficacy rate comparable to female injectables, slightly better than female oral contraception and substantially better than the condom.

While azoospermia has been confirmed to be the logical target for inducing infertility, a substantial minority of men remained oligospermic despite maximal sex steroid suppression. It is interesting to note that even when men are severely oligospermic, the remaining sperm are functionally normal (Wang et al., 1997).

A second WHO trial was conducted to investigate the contraceptive efficacy of TE-induced oligospermia (WHO, 1995). In this study, 349 out of 358 (98%) healthy men

in 9 countries were suppressed to azoospermia or oligospermia ( $< 5 \times 10^6/\text{ml}$ ) using TE at 200 mg i.m, weekly. They accumulated 283.5 years of exposure during which there were 9 pregnancies. It became clear that pregnancy was related to sperm concentrations below  $5 \times 10^6/\text{ml}$ , which enabled a threshold sperm density of below  $3 \times 10^6/\text{ml}$  to be identified, above which the failure rate was deemed unacceptably high compared to existing reversible contraceptive methods. Thus a total of 4 pregnancies during 49.5 years of exposure contributed by men with sperm densities between 0.1 and  $3 \times 10^6/\text{ml}$  yielded a Pearl rate of 8.1 (2.2 to 20.7 per 100 person years). This study yielded a Pearl rate for the method as a whole of 1.4 per 100 person years for those men with sperm densities below  $3 \times 10^6/\text{ml}$  (i.e., azoospermia to  $3 \times 10^6/\text{ml}$ ) which is comparable with the failure rate of current female reversible contraceptives (combined pill, injectables and medicated intrauterine devices) and is superior to the condom (12 per 100 person years).

These trials became the benchmark against which future contraceptive studies may be compared.

### Testosterone implants

Testosterone implants are small cylindrical pellets of crystalline testosterone which are implanted surgically, usually under local anesthesia, into the subcutaneous fat of the anterior abdominal wall. This form of T delivery was the earliest means of providing effective, long lasting steroid replacement in hypogonadal men and have been in use since the 1950's. Kinetics approximate to first-order release, yielding 0.65 mg/day for 100 mg pellets and 1.5 mg/day for 200 mg pellets, with near complete bioavailability (Handelsman et al., 1990). A 600 mg dose of implants will give physiological replacement over a period of 4 to 6 months (Cantrill et al., 1984,

Conway et al., 1988). However, a supraphysiological dose of 1200 mg is required to produce azoospermia in 65% of men although fewer androgenic side-effects than weekly injections of TE were reported (Handelsman et al., 1992). Implants are relatively inexpensive to produce and despite the fact that a minor surgical procedure is required, this is a popular method of androgen replacement although the method is complicated by an extrusion rate of around 7% which in recent studies remains unchanged by presoaking with antibiotics or varying insertion trajectory (Kelleher et al., 2001, Kelleher et al., 2002).

### Testosterone buciclate

Testosterone-17 $\beta$ -trans-4-n-butyl-cyclohexylcarboxylate (testosterone buciclate) is a long-acting ester of testosterone developed jointly by the WHO and the National Institute for Child Health and Human Development, USA, for specific use as a male contraceptive. The ester is formulated as finely milled crystals (10 to 15  $\mu$ m particle size) and is administered as an aqueous suspension by i.m injection. Single-dose injections of 600 mg and 1000 mg raised plasma testosterone in hypogonadal men into the low/normal range and maintained a stable concentration for 16 and 20 weeks respectively (Behre et al., 1992). A single dose of 1200 mg testosterone buciclate produced azoospermia in 3 out of 8 volunteers whose plasma testosterone levels remained within the normal range (Behre et al., 1995). At the time of writing it is unavailable due to persistent formulation difficulties.

### Testosterone undecanoate (TU)

TU has a long aliphatic side chain, which increases its lipid solubility. It was initially formulated as a capsule in arachis oil which facilitates oral absorption (*vide infra*). In a castor oil or tea seed oil base, it may be given intramuscularly, and has been shown

to have a significantly longer half-life ( $t_{1/2}$ ) than TE (Partsch et al., 1995). These initial studies in orchidectomized monkeys have more recently been extended to androgen replacement in hypogonadal men (Zhang et al 1998). TU kinetics show both a long duration of effectiveness (up to 8 weeks from a single i.m injection of 1000 mg), plus the absence of the initial supraphysiological peak seen with TE, thereby reducing the likelihood of androgenic side-effects.

In a clinical study in China, 11 out of 12 volunteers became azoospermic after receiving TU injections at a dose of 500mg every 4 weeks with all 12 subjects achieving azoospermia at the higher dose of 1000mg/4weekly (Zhang et al., 1999). Increasing the dosage interval to 6 weeks in a recent study in Caucasians receiving 1000mg/6weekly resulted in poorer rates of azoospermia (Kamischek et al., 2001). However the same authors, employing TU in combination with long acting injectable progestin NET enanthate, found 13 out of 14 subjects became azoospermic with the remaining subject suppressing to a sperm concentration of less than 1 million/ml (Kamischke et al., 2002).

### Methyl-nortestosterone

7 $\alpha$ -methyl-19-nortestosterone (MENT) is a highly potent synthetic androgen which, like testosterone, is a free steroid rather than an ester and consequently is usually formulated as a subdermal implant. MENT is not 5 $\alpha$ -reduced to dihydrotestosterone (DHT), and in the rat has been found to be 12-fold more potent than testosterone in suppression of gonadotrophins, whilst it is 4-fold more potent in the maintenance of prostate and seminal vesicle weight and 10-fold more potent in maintaining muscle weights (Kumar et al., 1992). It is resistant to 5 $\alpha$ -reduction but aromatises to oestrogen (Kumar et al., 1992, Lamorte et al., 1994). As a result of its increased

potency and tissue selectivity, this compound has, theoretically, the potential advantage of being able to suppress gonadotrophin production and maintain adult sexual function and muscle mass at relatively low doses that would not stimulate the prostate (Sundaram et al.,1993).

Recent studies in non-human primates have been encouraging in this respect: MENT was found to be 10-fold more potent than testosterone in the suppression of gonadotrophins but only twice as potent in stimulating prostate growth (Cummings et al., 1998). However, it was also found to be 10-fold more potent than testosterone in its effects on lipids, causing decreases in both HDL-C and total cholesterol. Phase I pharmacokinetic studies in healthy men using micronized MENT (2 to 8 mg), given as a single i.m injection have recently been completed showing effective suppression of gonadotrophins without reported side-effects (Suvisaari et al., 1997). MENT acetate implants in healthy men have resulted in dose dependent suppression of gonadotrophins and testosterone (Noe et al.,1999). Dose-finding contraceptive studies with MENT in men have recently been reported using implants left *in situ* for 180 days. Rates of azoospermia using 4 x 135mg MENT acetate implants alone are comparable with those obtained for weekly TE injections or 6-weekly TU injections (von Eckardstein et al., 2001). These implants may prove useful as part of a future combination regimen.

### 19-Nortestosterone (19-NT)

19-NT (See Fig 1) or nandrolone, is one of a group of non-aromatisable anabolic steroids which have been in clinical use for over 30 years. Given that 19-NT possesses a higher affinity for the androgen receptor and is 10-fold more progestagenic than testosterone, it is a potentially attractive antifertility drug, either as

a single agent or in combination with another progestagen. 19-NT alone (Knuth et al., 1985) or in combination with depot of the progestagen medroxy-progesterone acetate (Knuth et al., 1989) induced suppression of gonadotrophins and testosterone for 12 weeks and achieved azoospermia in 6 and 8 out of 12 volunteers, respectively. 19-NT may, therefore, have a role as a single-agent contraceptive, providing it is able to maintain essential androgen-dependent functions without producing undesirable metabolic, prostatic and behavioral effects which have not been studied to date. Reports of its action in combination with GnRH antagonists are described later. Table 1 below summarises and evaluates the androgens described thus far.

**Table 1: Evaluation of commonly used androgens in male contraception**

Androgen	Advantages	Disadvantages
Testosterone enanthate	Effective suppression of gonadotrophins. Moderately high rates of azoospermia when given alone.	Short acting. High peak levels and extragonadal effects e.g. on lipids
Testosterone buciclate	Long acting. Effective androgen conc. in hypogonadal men. Produced azoospermia in single dose studies	Persistent formulation difficulties. Uncertain future.
Testosterone undecanoate	Longer half-life than TE. Lower post injection peaks than TE (fewer androgenic side effects)	Ester given by injection. Poor oral bioavailability.
19-NT	Established use for 30 years in hypogonadal men. Higher affinity for T receptor than T itself. Progestational properties may be useful in contraception.	Optimal suppression lasts less than 12 weeks.
MENT	Free steroid not subject to $5\alpha$ -reduction. 12 times more potent than T in gonadotrophin suppression. Better tissue selectivity than T. Aromatises to oestrogen – may be beneficial in contraceptive regimens.	Potent adverse effects on lipids.
Testosterone implants	Good pharmacokinetics. Established use. No need for daily compliance following insertion.	Minor surgical procedure required. Occasional extrusions. Dosage interval still too short (12 weeks).

## Other androgen delivery Systems

### Transdermal testosterone

Delivery of testosterone via the transdermal route is a more recent development. Compared to transdermal estrogen delivery in female hormone replacement therapy which requires 50 to 100  $\mu\text{g}$  of steroid daily, much higher amounts - around 3 to 10 mg of testosterone - are required to maintain androgen-dependent physical functions. The rich vascular network of the scrotal skin is particularly suited to rapid drug absorption and is able to facilitate the increased delivery of steroid required (Sitruke-Ware et al., 1989).

### Scrotal Systems

This method was initially employed in the treatment of hypogonadal men using Testoderm - testosterone transdermal system (TTS, ALZA Corporation, Palo Alto, California, USA) (Place et al., 1990). This system contains testosterone incorporated in a layer of self-adhering ethylene vinyl acetate co-polymer with a soft flexible bonded polyester backing which is applied to shaved scrotal skin and proximal penile shaft and worn for 22 h before it is replaced. Two sizes are available delivering 2.4 mg and 3.6 mg testosterone per day, which allows testosterone levels to be maintained in the adult physiological range over a 24 h period without fluctuations outside this normal range, representing an improvement over injectable testosterone. DHT levels in these patients are elevated - up to three times the normal range, yielding a DHT/testosterone ratio of 0.5 (normal range 0.1 to 0.2) which is a reflection of the rich  $5\alpha$ -reductase activity within scrotal skin. The consequences regarding unwanted effects on DHT-dependent tissues are not yet known.

## Non-Scrotal Systems

More recently, non-scrotal transdermal delivery systems have been developed for use in androgen replacement therapy in hypogonadal men (Miekle et al., 1998).

Androderm Testosterone Transdermal Systems (SmithKline Beecham) is one such system containing testosterone in a permeation-enhancing vehicle composed of water, ethyl alcohol, glycerine, glycerol mono-oleate, methyl laurate and pharmaceutical gelling agents (Miekle et al., 1992). Two types of system are available, delivering 2.5 mg or 5 mg of testosterone. For adult hypogonadal men, 2 of the lower strength patches or 1 of the higher strength provides adequate androgen replacement without alteration of the DHT/testosterone ratio. However, the alcoholic excipients employed have caused considerable problems. A recent study reported adverse effects in 84% of hypogonadal men receiving androgen replacement by this method, most commonly dermatological problems and of these, 72% elected to return to injected depot preparations (Parker et al., 1999). This system, marketed as Andropatch in Europe, has been recently evaluated as part of a contraceptive study (Hair et al., 2001) and will be described in detail in this thesis.

A similar study in Europe using LNG has also been reported with broadly similar findings (Buchter et al., 1999). Other systems producing a more physiological, pulsatile release of testosterone are under development (Misra et al., 1997).

A hydroalcoholic gel containing a 2.5% solution of  $5\alpha$ -DHT (Andractim, Besins Iscovesco, France) is currently in use in Europe for androgen replacement. If applied over a sufficiently large surface area, e.g., chest and abdomen, adequate androgen substitution is achieved (Sitruk-Ware et al., 1989). Phase 1 trials of a 2.5% testosterone gel have very recently been reported with satisfactory replacement after

daily application of only 5g of gel and may find later use in contraceptive studies (Rolf et al., 2002).

### Testosterone microspheres

Testosterone encapsulated into biodegradable polylactide-glycolide co-polymer microspheres can be administered as a depot injection, yielding a biphasic pattern of testosterone release over 11 weeks (Burriss et al., 1998). By varying the particle size, a second-generation microsphere system containing 315 mg testosterone has recently been shown to provide approximately zero-order testosterone release at physiological levels for 70 days (Bahsin et al., 1992). Recent animal studies have confirmed that the microspheres are completely degraded in this particular study after a period of 10 weeks (Kobayashi et al., 1998). There have been no recent developments employing this approach in human subjects.

### Orally-active testosterone

Orally administered free testosterone is efficiently absorbed from the intestine but undergoes virtually complete hepatic first-pass metabolism. Avoiding first-pass metabolism, orally administered TU (see above) is absorbed into lymph and enters the thoracic duct and thence to the systemic circulation via the subclavian vein. TU (40 mg) dissolved in arachis oil (Restandol, Organon NV) is available in capsule form. However, its variable reabsorption coupled with a short  $t_{1/2}$  and duration of action requires that patients take two to four capsules per day to achieve adequate androgen substitution (Schaison et al., 1998). Testosterone complexed with an oligosaccharide ring can, when administered sublingually, give rise to short-lived (2 h) peaks in testosterone. However, once again, multiple (3-times/daily) administrations are

required for adequate androgen replacement and this will limit their practical use (Salehian et al., 1994).

### Inhaled preparations

Using a new type of aerosol characterized by particles of small mass and large size, it has been shown (Edwards et al., 1997) that inhalation of such particles complexed with testosterone yielded high systemic bioavailability of the hormone and avoids first-pass metabolism. This system offers a potential non-invasive means of androgen replacement as part of a hormonal contraceptive regime though, as yet, no such studies have been reported.

### **Combined hormonal regimes**

Experience with androgen-only regimes indicates that supraphysiological levels of testosterone are required for maximal suppression of gonadotrophins. Due to the direct stimulatory action of testosterone on spermatogenesis, especially at high doses, the ability of these regimes to suppress spermatogenesis remains sub-optimal. Dose-dependent, androgen-related side effects already described, provide further impetus to lower the testosterone content of future male contraceptives. In addition, until unequivocal long-term safety data is available with regard to the effect of testosterone on conditions such as benign and malignant disease of the prostate, cardiovascular disease and behavioral disturbance, it seems prudent to pursue alternative regimens which mimic physiological levels as closely as possible. Combination regimes have been examined with the dual objectives of improving efficacy and lowering the dose of testosterone or androgen required to minimize extra-gonadal steroid effects.

## Progestogen and androgen combinations

Progestogens are potent inhibitors of gonadotrophin secretion in men (Kjeld et al., 1979) and may also directly suppress spermatogenesis (Goldzieher et al 1984). By acting synergistically or additively with androgens they permit lower doses of each steroid to be used. Synthetic steroids such as levonorgestrel (LNG), desogestrel, gestodene and norgestimate and medroxyprogesterone acetate (DMPA) are highly potent progestational compounds that are effective in microgram quantities with only moderate androgenic properties. Structural modification of the parent molecules from which they are derived namely, 19-nortestosterone and 17-hydroxyprogesterone, has allowed the development of these orally active hormones. The comparatively low mass of drug required has also allowed the successful development of long-acting subdermal implants such as Norplant (levonorgestrel, Wyeth) and Implanon (3-ketodesogestrel, Organon NV).

### *DMPA and testosterone*

A single depot injection of the progestin DMPA, in combination with testosterone (800 mg) implants (Handelsman et al., 1996) showed that the addition of the DMPA markedly increased the rate of azoospermia compared to testosterone alone although did not reduce the time course over which this was achieved. Indonesian men receiving DMPA combined with testosterone undecanoate injections showed rates of azoospermia of 80% compared with 0% in the testosterone alone group (Moeloek et al., 2001).

### *LNG and testosterone*

Initial studies using LNG (Foegh et al., 1980) in combination with subreplacement doses of testosterone produced results that did not improve on those expected for

androgen alone. Similar results were obtained using DMPA (Schearer et al., 1978), danazol (Skoglund et al., 1973) and Norethisterone (Soufir et al., 1983).

Later studies are more encouraging; Bebb reports a study (Bebb et al., 1996) in which volunteers received 500 µg LNG in combination with higher doses of TE (100 mg/week). With this regime they found significantly greater and faster suppression of spermatogenesis than with the same dose of testosterone alone. However, HDL-C levels were significantly reduced in those patients receiving LNG plus testosterone though not in the control group receiving androgen alone.

Combining LNG implants (Norplant II) with transdermal T patches (10mg/day) yielded poor results with only 35% of subjects achieving azoospermia compared to 93% when the implants were combined with 100mg weekly TE injections (Gonzalo et al., 2002). Poor results have also been reported in a dose study finding using up to four LNG implants with transdermal DHT gel with none of the subjects achieving azoospermia (Pollanen et al., 2001). These studies confirm our earlier findings that, as yet, transdermal T delivery is not sufficiently effective for contraception regardless of the progestin with which it is combined.

#### *Desogestrel and testosterone*

Studies have recently been completed showing oral desogestrel (DSG) (300 µg/day) in combination with low-dose testosterone (50 mg/weekly i.m injection) provides effective suppression of gonadotrophins and rates of spermatogenesis superior to androgen only regimes and earlier studies using progestin/androgen combinations (Wu et al., 1999). The rate of azoospermia for this regime (100% in the above treatment group) also compares very favourably with more recent studies using progestin/androgen combinations, described above: LNG (67% azoospermia) (Bebb

et al., 1996); and, DMPA (90%) (Handelsman et al., 1996). As with LNG, there was a significant reduction in HDL-C levels in those patients receiving desogestrel. It remains to be seen whether employing lower doses of desogestrel or newer progestagens can minimize these lipid effects without compromising contraceptive efficacy.

A novel feature of this study was the phased treatment regimen in which testosterone treatment was initiated after 3 weeks of pre-treatment using desogestrel alone. By virtue of the phased introduction of androgen the authors were able to demonstrate the relative contributions made by each drug to suppression of spermatogenesis and gonadotrophins.

Interestingly, it was found that desogestrel alone, even at maximal dose, suppressed the hypothalamo-pituitary-testicular axis only partially and that the addition of testosterone was crucial in achieving maximal suppression. This demonstrates that testosterone is not only essential for maintenance of androgen-dependent physiological functions but also to achieve optimal suppression of gonadotrophins. Two recent studies in Edinburgh have further evaluated the DSG/T combination. In the first, subjects received implants containing etonogestrel (the active metabolite of DSG) in combination with 400mg T implants (Anderson et al., 2002). Thirteen of the fourteen subjects displayed suppressed sperm concentrations to less than 0.1million/ml with 75% of the group achieving azoospermia. The second study evaluated oral DSG at a dose of 150µg or 300µg in combination with 400mg T implants administered at 12 week intervals for 24 weeks (Kinniburgh et al., 2002). Azoospermia was achieved by all subjects in the higher dose DSG group. A large (but not complete) cohort of men in 150µg DSG group also attained azoospermia and were recruited to a extended treatment arm of the study lasting a further 24 weeks. All

subjects who completed the treatment remained azoospermic until they discontinued the study medication. These results are encouraging insofar as they demonstrate effective long term suppression of spermatogenesis but both studies are still confounded by marked heterogeneity of response within the subject groups. The combination studies reviewed thus far have employed a number of permutations with respect to progestagen, androgen and modes of delivery. Table 2 overleaf summarises the principal studies and suggests desogestrel as optimal progestagen.

**Table 2: Summary of androgen/progestin combinations employed in recently published male contraceptive studies and reported rates of azoospermia.**

<b>Authors and date of study</b>	<b>Androgen progestin combination</b>	<b>Maximal reported rates of azoospermia (dose regimen)*</b>
Wu et al.,1999.	Daily oral DSG and weekly injected TE	100% (300µg DSG +TE 50mg)
Kinniburgh et al., 2002	Daily oral DSG and 12-weekly T implants	100% (300µg DSG+400mg T)
Hair et al., 2001	Daily oral DSG and daily transdermal T patches.	57% (300µg DSG+5mg T)
Anderson et al., 2002	Once only ENG implants and T implants	75% (136mg ENG +400mg T)

\* These rates are for individual treatment arms of each study and not the overall rates of azoospermia for the study as a whole

Abbreviations: T testosterone, TE testosterone enanthate, TU Testosterone undecanoate, DHT dihydrotestosterone, DSG desogestrel, ENG etonogestrel, DMPA depot medroxyprogesterone acetate, LNG levonorgestrel, NET norethisterone.

**Table 2: Continued.**

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Moeloek et al., 2001	12-weekly DMPA injections and TU injections 6-weekly	80% (TU500mg+DMPA300mg)
Bebb et al., 1996.	Oral LNG daily and weekly TE injections	67% (TE 1000mg+LNG 500µg)
Buchter et al., 1999	Oral LNG daily and daily transdermal T patches	18% (T 5mg+LNG 500mg)
Pollanen et al., 2002.	Once only LNG implants and daily transdermal DHT gel	0%
Gonzalo et al., 2002	Once only LNG implants and weekly TE injections	93% (Norplant II+TE 100mg)
Kamischke et al., 2002	NET injections and injected TU	93% (1000mg TU+200mg NETE)

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Abbreviations: T testosterone, TE testosterone enanthate, TU Testosterone undecanoate, DHT dihydrotestosterone, DSG desogestrel, ENG etonogestrel, DMPA depot medroxyprogesterone acetate, LNG levonorgestrel, NET norethisterone

## **Other combined contraceptive regimens**

As well as androgen/progestin combinations, a number of other combined steroid and non-steroid regimens have been evaluated.

### **Anti-androgen/androgen combination**

Cyproterone acetate (CPA) is a synthetic steroid which blocks testosterone effects on the testis, through competitive binding at the androgen receptor, as well as suppressing gonadotrophin production by the pituitary. It has been used in Europe for the treatment of hirsutism in females (Dianette, Schering) and prostatic malignancy in males (Cyprostat, Schering). When CPA alone is orally administered to men in doses from 5 mg to 20 mg daily, disruption of sperm motility and morphology occurs, but with associated symptoms of androgen deficiency (Wang et al., 1980, Foegh et al., 1979, Roy et al., 1976, Molz et al., 1980). More recent studies have combined low-dose CPA with androgen supplementation to good effect (Merrigiola et al., 1996). Initial studies employed a regime of CPA (50 or 100 mg/day) and weekly injections of TE (100 mg/week), which caused complete azoospermia in the small number of volunteers treated with either dose of CPA. In a more recent study by the same group, an oral regime was employed for the first time (Merrigiola et al., 1997). Using a lower dose of oral CPA (25 mg/day) in combination with oral capsules of TU (160 mg/day), however, produced much lower rates of azoospermia (only 12.5% of subjects). Also of note is the significant decrease in haemoglobin and haematocrit seen in subjects treated with CPA in all studies so far reported, which in the long-term may produce anaemia. It remains to be seen whether lower doses of CPA may elicit the desired effect on spermatogenesis without manifesting these important effects on haemopoiesis.

## Oestrogen/androgen combinations

In man, the counterbalancing effects of small amounts of estrogens on lipid and bone metabolism as well as on improved gonadotrophin suppression may, theoretically, produce an ideal formulation for the suppression of spermatogenesis. Such studies have recently been reported evaluating a combination of implants of oestradiol and testosterone in men (Handelsman et al., 2000). Whilst oestradiol was indeed found to augment the suppressive qualities of testosterone, the narrow therapeutic index and dose limiting side effects of oestrogen as well as safety concerns regarding thromboembolism make it highly unlikely that such regimes will have a practical future.

## 5 $\alpha$ -Reductase inhibitor/steroid regimes

The enzyme 5 $\alpha$ -reductase converts testosterone to DHT, a steroid, which, in some tissues exhibits a greater potency than testosterone, e.g., prostate, seminal vesicles, and hair follicles. Within-group variation in 5 $\alpha$ -reductase (described below) activity was thought to account for the incomplete suppression of spermatogenesis in some individuals via residual DHT activity. Studies to evaluate the effects of the (type 2) 5 $\alpha$ -reductase inhibitor finasteride (Merck & Co) in conjunction with androgen/progestins however showed no enhancement of the rate of spermatogenesis in the finasteride group (Kinniburgh et al., 2001). Similarly disappointing results have also been reported in men receiving testosterone implants and finasteride raising doubts over the extent to which 5 $\alpha$ -reductase supports spermatogenesis in man (Mclachlan et al., 2000). A fuller understanding of the 5 $\alpha$ -reductase isoforms and their regulation in men may yet lead to improved suppression, perhaps through the use of specific type1 inhibitors.

## Gonadotrophin-releasing hormone (GnRH) agonists

Abolition of gonadotrophin secretion and therefore of spermatogenesis can be produced by GnRH agonists. They achieve this by down regulation of pituitary GnRH receptors and have the advantage that this is a highly specific action, thereby eliminating unwanted actions on metabolism, haemostasis, and the cardiovascular system.

A number of studies have explored the potential of GnRH agonists in suppressing sperm production (Cummings et al., 1994). In total, only 23% of subjects achieved azoospermia. These results are clearly disappointing, although it may be argued that the dose of agonist employed was not adequate or that there may have been persistence (or escape) of FSH activity during GnRH agonist suppression (Pavlou et al., 1988, Santen et al., 1984). The GnRH agonist leuprolide has recently been found to produce effective reversible azoospermia in dogs (Inaba et al., 1996) though as yet, no human studies have been reported. Further studies are required to more fully evaluate the true potential of GnRH agonists.

## GnRH antagonists

These are capable of reversibly suppressing gonadotrophins completely and rapidly (Bagatell et al., 1989). They have higher affinity and slower dissociation rates than either GnRH itself or any of the GnRH agonists (Heber et al., 1982, Pavlou et al., 1989). The principal drawback of GnRH antagonists is low potency, necessitating milligram amounts to be administered systemically via complex delivery systems and low oral bioavailability (Pavlou et al., 1991). Given that most are produced by substitution of the natural decapeptide with unnatural, synthetic amino acids (Karten et al., 1986), they are also extremely expensive to produce, have low solubility and

are difficult to formulate, which currently limits their potential for large-scale clinical trials. Despite these constraints, the effects of daily, subcutaneous injections of Nal-Glu GnRH antagonist given in combination with weekly injections of low-dose TE (50 mg i.m/weekly) were examined in men and showed greater suppression of gonadotrophins than with T alone (Bagatell et al., 1995, Tom et al., 1992).

More recent studies using the same antagonist showed that azoospermia was achieved more rapidly and at higher incidence than TE alone (Swerdloff et al., 1998). In addition, having suppressed spermatogenesis during the combination induction phase, it remained suppressed when maintenance treatment of TE alone (100 mg/week) was continued. This reduction of lag time between treatment and response addresses criticisms of earlier regimes in which maximal suppression of spermatogenesis took up to 3 months to achieve.

Combining the novel GnRH antagonist Cetrorelix with the androgenic steroid 19-Nortestosterone produced azoospermia in all subjects over the 3 month period of combination therapy but was not sustained by 19 NT alone following cessation of the Cetrorelix (Behre et al., 2001). Future developments in pharmaceutical technology may allow more economical production of these compounds as well as orally active non-peptide GnRH antagonists, making them more attractive prospects for use in male fertility regulation.

### Selective FSH suppression

Selective action against gonadotrophins has been explored but with disappointing results.

Monkey studies in which inhibition of FSH alone (achieved by raising antibodies against FSH) causes reduction in sperm concentration but not azoospermia (Nieschlag 1985). Further evidence may be found in studies of men with mutations of FSH receptors. The authors report that although sperm production was quantitatively impaired, absence of FSH activity in these men did not abolish spermatogenesis (Tapanainen et al., 1997). Accordingly, peptides such as inhibin and follistatin, which selectively suppress FSH production, are not considered promising candidates as male contraceptives.

### **Heterogeneity of response to hormonal suppression of spermatogenesis**

A striking finding from the studies described thus far is the marked difference both between and within population groups in the degree of steroid-induced suppression of spermatogenesis.

#### **Variation between population groups**

One of the surprising findings from the WHO trials was that Asian (Chinese, Indonesian and Thai) men showed a consistently higher rate of spermatogenic suppression (89.9%) when compared with European or American (74.3%) men ( $p < 0.001$ ) [30]. Given the much greater efficacy of testosterone-alone regimes in these countries, the national authorities of China, Indonesia and India have initiated large-scale phase III trials evaluating testosterone as a single-agent contraceptive. The source of this heterogeneity has been extensively investigated, though the underlying reasons remain unclear. Characteristics such as body size, mass, serum biochemistry, as well as baseline endocrine and semen parameters do not explain the observed differences (Handelsman et al., 1995).

A recent study examining the comparative rates of androgen production and metabolism in Caucasian and Chinese subjects showed increased levels of  $5\alpha$ -reduced metabolites in Caucasians (Santner et al., 1998). However, this was not found to be due to a difference in  $5\alpha$ -reductase activity or testosterone to dihydrotestosterone conversion ratios in the two groups. The study did show, however, raised levels of androgenic steroid precursors, which serve as substrates for these metabolites in the Caucasian group, and interestingly, in Chinese men resident in the USA. The authors postulate that this is indicative of an environmental or dietary origin to these population differences. Differences in the gonadotrophin negative feedback response to exogenous testosterone in Asian and Caucasian men have also been reported (Wang et al., 1998).

Differences in testicular structure between Asian men and Caucasians have also been observed (Johnson et al., 1998). Histological examination of testes taken from post-mortem examination of previously healthy Asian males dying from sudden traumatic injury has demonstrated that Asian males have fewer spermatogonia, smaller diameter seminiferous tubules and fewer Sertoli cells per man than do Caucasians. This variation in testicular structure results in differences in spermatogenic potential between the two groups, and is tendered by the authors as a potential explanation as to why Asian men exhibit more complete suppression of testicular function in response to exogenous testosterone administration.

Recent studies in African men have also yielded interesting results with one treatment group (n=18) who received oral DSG plus T implants for a whole year achieving azoospermia in all subjects suggesting perhaps enhanced efficacy in African males (Anderson et al., 2002).

## Variation within population groups

It is known that supraphysiological doses of testosterone induce rates of azoospermia ranging from 40% to 70% in Caucasian men, with the remainder becoming oligospermic (WHO 1990). The reasons for this variation in spermatogenic suppression are not entirely understood although it has been shown recently that it is not accounted for in differences or daily fluctuations in gonadotrophins or circulating testosterone concentrations (Amory et al., 2001). It is thought by many that a fuller understanding of this variation is a key step towards perfecting male hormonal contraceptive regimens.

Earlier studies have shown that men who become oligospermic during testosterone treatment exhibit a higher  $5\alpha$ -reductase activity than those who develop azoospermia which may cause altered intratesticular androgen levels (Anderson et al., 1996). This higher residual androgenic action on the seminiferous tubules may cause resistance to complete suppression of spermatogenesis although the apparent failure of the combined steroid/ $5\alpha$ -reductase inhibitor studies (*vide supra*) suggests a more complex relationship.

Other studies report the histological evaluation of the effects of testosterone treatment on spermatogenesis using open testicular biopsy material from healthy males awaiting vasectomy pre-treated with weekly doses of intramuscular testosterone enanthate TE (Zhengwei et al., 1998). This study demonstrates that the most consistent lesion induced by TE treatment was in the reduction in formation of type B spermatogonia - the germ cells that enter meiosis. Studies in the rat support the view that the development of A to B spermatogonia is androgen dependent and can be impaired by

a  $5\alpha$ -reductase inhibitor and that the extent of  $5\alpha$  reduction may be an important determinant of the extent of spermatogenic suppression induced by exogenous testosterone based contraceptives (O'Donnell et al., 1999). Studies from the same group comprehensively assessed a group of men awaiting vasectomy who received an experimental hormonal contraceptive regimen (testosterone  $\pm$  DMPA). These subjects provide a very useful experimental model offering the opportunity of detailed histological evaluation of cellular responses from testicular biopsy material taken prior to vasectomy, analysis of intratesticular hormone levels from testis homogenates as well as established parameters such as sperm concentration in the ejaculate and peripheral blood hormone measurements.

Recently published data demonstrated more rapid onset of gonadotrophin secretion in subjects treated with T+DMPA than those with T alone with a more marked impairment of spermatogonial development although germ cell numbers and sperm count did not differ. (McLachlan et al., 2002). Testicular DHT and androstendione levels were maintained despite markedly reduced levels of T, FSH and LH. The authors postulate up-regulation of  $5\alpha$ -reductase activity and the mechanism and inter-subject differences in this ability as a possible determinant of the differential response to contraceptive regimens.

Despite disappointing results from clinical studies using  $5\alpha$ -reductase inhibitors, these recent data suggest that  $5\alpha$ -reductase and variations in its activity play an important role in within (and perhaps between) group variation. A fuller understanding of the distribution of the isoforms of this enzyme and careful choice of appropriate inhibitors may yet resolve this issue although very recently published animal data from independent groups challenges the concept. Both studies, performed in cynomolgus

monkeys contend that variation in T induced inhibition of spermatogenesis is more closely related to FSH suppression than to intratesticular androgen and, by implication, intratesticular 5 $\alpha$ -reductase activity (Weinbauer et al., 2001, Narula et al., 2002). A re-evaluation of FSH suppression in human contraceptive studies may help to resolve this issue.

Genetic polymorphism of the androgen receptor has also been recently postulated as a source of heterogeneity in population response although once again, there does not appear to be a consensus view. Initial studies reported that CAG repeat length in the androgen receptor (RNA) correlates inversely with sperm concentrations and that this polymorphism may contribute to the efficiency of spermatogenesis and by implication, the ease of its suppression in normal men (Von Eckardstein et al., 2001, Von Eckardstein et al., 2002 ). More recent studies challenge this view citing data from infertile men and fertile controls in which no relationship between CAG repeat length and fertility was shown. These authors further cite retrospective analysis from published European studies in over 600 men in support of their hypothesis (Rajpert-de Meyts et al., 2002). As yet, there are no published data linking such polymorphism to responder/non-responder status in contraceptive studies.

## **Conclusions**

These studies show that experimental suppression of human spermatogenesis to achieve effective contraception is a reality though several problems persist.

Exogenous androgen administration remains central to all effective regimes examined thus far. Clearly, development of minimally invasive, long acting formulations is necessary to provide an acceptable, convenient and reliable form of androgen delivery which, ideally, should be self-administered. Understanding the heterogeneity of the

suppression response between and within populations is pivotal to the development of an effective contraceptive and may require changes to the orthodox views of testicular regulation in man. Consideration of other hormonal regulatory systems is part of the challenge to that orthodoxy.

## References

**Amory JK, Anawalt BD, Bremner WJ, Matsumoto AM. (2001).** Daily testosterone and gonadotrophin levels are similar in azoospermic and nonazoospermic normal men administered weekly testosterone: implications for male contraceptive development. *22(6):1053-60.*

**Anderson RA, Ludlum CA, Wu FCW (1995).** Haemostatic effects of supraphysiological levels of testosterone in normal men. *Thromb Haemost. 74:693-697.*

**Anderson RA, Wallace AM, Wu FCW. (1996)** Comparison between testosterone enanthate induced azoospermia and oligospermia in a male contraceptive study. 3. Higher 5 alpha reductase activity in oligospermic men administered supraphysiological doses of testosterone *J Clin Endocrinol Metab. 81: 902-908.*

**Anderson RA, Kinniburgh D, Baird DT. (2002)** Suppression of spermatogenesis by etonogestrel implants with depot testosterone: potential for long-acting male contraception. *J Clin Endocrinol Metab. 87: 3640-9.*

**Anderson RA, Van Der Spuy ZM, Dada OA, Tregoning SK, Tregoning Sk, Zinn PM, Adeniji OA, Fakoya TA, Smith KB, Baird DT. (2002)** Investigation of hormonal male contraception in African men: suppression of spermatogenesis by oral desogestrel with depot testosterone. *Hum. Reprod. 17: 2869-2877.*

**Bagatell CJ, McLachlan RI, de Kretser DM, et al. (1989)** A comparison of the suppressive effects of testosterone and a potent new GnRH antagonist on gonadotropin and inhibin levels in normal men. *J Clin Endocrinol Metab* **69**:43-48.

**Bagatell CJ, Rivier JE, Bremner WJ. (1995)** Dose Effects of the gonadotrophin-releasing-hormone antagonist Nal-Glu, combined with testosterone enanthate on gonadotrophin levels in normal men. *Fertil Steril* **64**: 139-145.

**Barrett-Connor E (1996)** Testosterone, HDL-cholesterol and cardiovascular disease in men. In: Bahsin S, Gabelnick HL, Speiler JM, Swerdloff RS, Wangg C, Kelly C. (Eds) *Pharmacology, Biology and Clinical Applications of Androgens: Current Status and future Prospects*. New York: Wiley-Liss: 215-23.

**Bebb RA, Bradley D, Anawalt R, Christensen RB, Paulsen CA, Bremner WJ, Matsumoto A. (1996)** Combined administration of levonorgestrel and testosterone induces more rapid and effective suppression spermatogenesis than testosterone alone: a promising male contraceptive approach. *J Clin Endocrinol Metabolism*. **81**: 757-762.

**Behre HM, Nieschlag E. (1992)** Testosterone buciclate in hypogonadal men: Pharmacokinetics and pharmacodynamics of the new long-acting androgen ester. *J Clin Endocrinol Metab* **75**:1204-1210.

**Behre HM, Baus S, Kliesch S et al., (1995)** Potential of testosterone buciclate for male contraception: endocrine differences between responders and non-responders. *J Clin Endocrinol Metab* **80**: 2394-2403.

**Behre HM, Kliesch S, Lemcke b, von Eckardstein S, Neischlag E. (2001).**  
Suppression of spermatogenesis to azoospermia by combined administration of GnRH antagonist and 19-nortestosterone cannot be maintained by this non-aromatisable androgen alone. *Hum Reprod.* **16**:2570-7

**Bhasin S, Swerdloff RS, Steiner B, et al., (1992)** A biodegradable testosterone microcapsule formulation provides uniform eugonadal levels of testosterone for 10-11 weeks in hypogonadal men. *J Clin Endocrinol Metab.* **74**:75-83.

**Bone W, Jones AR, Cooper TG. (2002)** The effect of (R,S)-ornidazole on the fertility of male mice and the excretion and metabolism of <sup>36</sup>Cl-(R,S)-ornidazole and <sup>36</sup>Cl-(R,S)-alpha-chlorohydrin in male mice and rats. *Int. J. Androl.* **25(2)**: 94-99.

**Burris AS, Ewing LL, Sherins RJ. (1998)** Initial trial of slow-release testosterone microsphere in hypogonadal men. *Fertil Steril.* **50**:493-497.

**Buchter D, Von Eckardstein S, Von Eckardstein A, (1999)** Clinical trial of transdermal testosterone and oral levonorgestrel for male contraception. *J Clin Endocrinol.* **84**:1244-1249.

**Cairo Programme of Action.** International Conference on population and Development. Cairo, Egypt: 1994

**Carani C, Qin K, Simoni M et al. (1997).** Effect of testosterone and estradiol in a man with aromatase deficiency. *N. Engl. J. Med.* **337**:91-95.

**Cantrill JA, Dewis P, Large DM, et al. (1984):** Which testosterone replacement therapy? *Clin Endocrinol.* **21**:97-107.

**Castelli WP, Garrison RJ, Wilson PWF, Abbott RD, Kalousdian S, Kannel WB. (1986)** Incidence of coronary heart disease and lipoprotein cholesterol levels – the Framingham study: *JAMA* **256**: 2835-2838.

**Conway AJ, Boyland LM, Howe C, et al. (1988)** Randomized clinical trial of testosterone replacement therapy in hypogonadal men. *Int J Androl.* **11**:247-264

**Cooper TG, Yeung CH. (2001)** Approaches to post-testicular contraception. *Asian J Androl.* **1**: 29-36.

**Cummings DE, Kumar N, Bardin CW, Sundaram K, Bremner WJ. (1998)** Prostate-sparing effects of the potent androgen 7 $\alpha$ -methyl-19-nortestosterone: a potential alternative to testosterone for androgen replacement and male contraception. *J Clin Endocrinol Metab.* **83**: 4212-4219.

**Cummings DE, Bremner WJ. (1994)** Prospects for new hormonal male contraceptives. In *Clinical Andrology*, ed. WJ Bremner, *Endocrinol Metab Clin North Am.* **22**: 893-922.

**Edwards DA, Hanes J, Caponetti G, Hrkach J, Ben-Jebria A, Eskew ML, Mintzes J, Deaver D, Lotan N, Langer R. (1997)** Large porous particles for pulmonary drug delivery. *Science* **276**: 1868-1871.

**Ewing LL, Desjardin C, Irby DC, Robaire B (1997)** Synergistic interaction of testosterone and estradiol inhibits spermatogenesis in rats. *Nature* **269**:409-411.

**Farley TMM, Merik O, Mehta S, Waites GMH (1993).** The safety of vasectomy: recent concerns. *Bulletin of the world Health Organization* **71**: 413-419.

**Foegh M, Corker CS, Hunter WM, et al. (1979):** The effects of low doses of cyproterone acetate on some functions of the reproductive system in normal men. *Acta Endocrinol (Copenh)* **91**:545-552.

**Foegh M, Nichol M, Letterson IB, Schou G. (1980)** Clinical evaluation of long-term treatment with levo-norgestrel and testosterone enanthate in normal men. *Contraception* **21**:631-640.

**Ford WCL, Waites GMH. (1986)** Sperm maturation and the potential for contraceptive interference. In: Zatuchni GI, Goldsmith A, Speiler JM, et al., editors.

*Male contraception: advances and future prospects*. Philadelphia: Harper and Row: 89-106.

**Giovannuci E. et al. (1993)** A retrospective cohort study of vasectomy and prostate cancer in US men. *JAMA*. **269**: 878-882.

**Goldzieher JW, Castracane VD (1984)** Antifertility effects of progestational steroids. In Benagiano H, Diczfalusy E (eds): *Endocrine Mechanisms in Fertility regulation*. New York, Raven Press, 49.

**Gonzalo ITG, Swerdloff RS, Nelson AL, Clevenger B, Garcia R, Berman N, Wang C. (2002)** Levonorgestrel implants (Norplant II) for male contraception clinical trials: combination with transdermal and injectable testosterone. *J Clin Endocrinol Metab*. **87**: 3562-3572.

**Hair WM, Kitteridge K, O'Connor D, Wu FCW. (2001)** A novel male contraceptive pill/patch combination: oral desogestrel and transdermal testosterone in suppression of spermatogenesis in normal men. *J Clin Endocrinol Metab*. **86**:5201-5209.

**Handelsman DJ, Farley TMM, Peregoudov A, Waites GMH and World Health Organisation Task Force on Methods for the Regulation of Male Fertility(1995):** Factors in non-uniform induction of azoospermia by testosterone enanthate in normal men. *Fertil Steril*. **63**:125-133.

**Handelsman DJ, Conway AJ, Howe CJ, Turner L, Mackey MA (1996)**

Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J Clin Endocrinol Metab.* **81**: 4113-4121.

**Handelsman DJ, Wishart S, Conway AJ. (2000).** Oestradiol enhances testosterone-induced suppression of human spermatogenesis. *Hum. Reprod.* **15**:672-679

**Handelsman DJ, Conway AJ, Boylan LM. (1990)** Pharmacokinetics and pharmacodynamics of testosterone pellets in man. *J Clin Endocrinol Metab* **70**:216-222.

**Handelsman DJ, Conway AJ, Boylan LM. (1992)** Suppression of human spermatogenesis by testosterone implants. *J Clin Endocrinol Metab.* **75**:1326-1332.

**Hayes FJ, Seminara SB, Decruz S, Beopple PA, Crowley WF. (2000).** Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback. *J. Clin. Endocrinol. Metab.* **85**:3027-3035.

**Heber D, Dodson R, Swerdloff RS. (1982)** Pituitary receptor site blockade by gonadotropin-releasing hormone antagonist in vivo: Mechanism of action. *Science* **216**:420-421.

**Hess RA, Bunick D, Lee KH et al. (1997).** A role for estrogens in the male reproductive system. *Nature.* **390**:509-512.

**Inaba T, Umehara T, Mori J, Torii R, Tamada H, Sawada T. (1996)** Reversible suppression of pituitary-testicular function by a sustained release formulation of a GnRH agonist (leuprolide acetate) in dogs. *Theriogenology* **46**: 671-677.

**Johnson L, Barnard J, Rodriguez L, Smith E, Swerdloff R, Wang X, Wang C. (1998)** Ethnic differences in testicular structure and spermatogenic potential may predispose testes of asian men to a high sensitivity to steroidal contraceptives. *J Androl.* **19**:348-357.

**Jones AR, Morin C, Bone W, Cooper TG. (2002)** Lack of antifertility properties of novel halogenated glycolytic inhibitors and the urinary excretion and metabolism of 1,6-dichloro-1,6 dideoxy-D-fructofuranose in the male rat. *Int. J. Androl.* **25**: 168-174.

**Karten MJ, Rivier JE. (1986)** Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: Rationale and perspective. *Endocr Rev* (1986) **7**:44-66.

**Kamischke A, Venherm S, Ploger D, von Eckardstein S, Nieschlag E. (2001)** Intramuscular testosterone undecanoate and norethisterone enanthate in a clinical trial for male contraception. *J Clin Endocrinol Metab.* **86**(1): 303-9.

**Kamischke A, Huerman T, Kruger K, von Eckardstein S, Schnellschmidt I, Rubig A, Nieschlag E. (2002)** An effective hormonal male contraceptive using

testosterone undecanoate with oral or injectable norethisterone preparations. *J Clin Endocrinol Metab.* **87**: 530-9.

**Kelleher S, Conway AJ, Handelsman DJ. (2002).** A randomized controlled clinical trial of antibiotic impregnation of testosterone pellet implants to reduce extrusion rate. *Eur J Endocrinol* **146**: 513-8.

**Kelleher S, Conway AJ, Handelsman DJ. (2001).** Influence of implantation site and track geometry on the extrusion rate and pharmacology of testosterone implants. *Clin Endocrinol.* **55**: 531-6

**Kinniburgh D, Zhu H, Cheng L, Kicman AT, Baird DT, Anderson RA. (2002)** Oral desogestrel with testosterone pellets induces consistent suppression of spermatogenesis to azoospermia in both Caucasian and Chinese men. *Hum. Reprod.* **17**: 1490-501.

**Kinniburgh D, Anderson RA, Baird DT. (2001)** Suppression of spermatogenesis with desogestrel and testosterone pellets is not enhanced by addition of finasteride. *J. Androl.* **22**:88-95.

**Kjeld JM, Puaah CM, Kaufman B, et al. (1979)** Effects of norgestrel and ethinyloestradiol ingestion on serum levels of sex hormones and gonadotropins in men. *Clin Endocrinol.* **11**:497-504.

**Knuth UA, Behre H, Belkien L, et al. (1985)** Clinical trial of 19-nortestosterone-hexoxyphenyl-propionate (Anadur) for male fertility regulation. *Fertil Steril.* **44**:814-821.

**Knuth UA, Yeung C, Nieschlag E. (1989)** Combination of 19-nortestosterone-hexoxyphenyl-propionate (Anadur) and depot medroxyprogesterone-acetate (Clinovir) for male contraception. *Fertil Steril.* **51**:1011-1018.

**Kobayashi D, Tsubuku S, Yamanaka H, Asano M, Miyajima M, Yoshida M. (1998)** In vivo characteristics of injectable poly(DL-lactic acid) microspheres for long acting drug delivery. *Drug Dev Ind Pharm.* **24**:819-825.

**Kumar N, Didolkar AK, Monder C, et al. (1992)** The biological activity of 7-methyl-19-nortestosterone is not amplified in male reproductive tract as is that of testosterone. *Endocrinology* **130**: 3677-3683.

**Korach KS, Couse JF, Curtis SW et al. (1996)** Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog. Horm. Res.* **51**:159-186.

**Lamorte A, Kumar N, Bardin CW, Sundaram K. (1994).** Aromatization of 7 $\alpha$ -methyl-19-nortestosterone by human placental microsomes *in vitro*. *J Steroid Biochem. Mol Biol.* **48**:297-304.

**Lobl TJ, Kirton KT, Forbes AD et al. (1988)** Contraceptive efficacy of testosterone-estradiol implants in male Rhesus monkeys. *Contraception* **27**:383-389.

**Lubhan DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. (1998)** Cloning of human androgen receptor complementary DNA and localization of the X-chromosome. *Science* **240**:327-330

**McLachlan RI, McDonald J, Rushford D, Robertson DM, Garret C, Baker HWG. (2000)** Efficacy and acceptability of testosterone implants, alone or in combination with a 5 $\alpha$ -reductase inhibitor for male contraception. *Contraception*. **62**:73-78.

**McLachlan RI, O'Donnell L, Stanton PG, Balourdous G, Frydenberg M, de Kretser DM, Robertson DM. (2002)** Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones and germ cell populations in normal young men. *J Clin Endocrinol Metab* **87**:546-56.

**Meikle WA (1998)** A permeation-enhanced non-scrotal testosterone transdermal system for the treatment of male hypogonadism. In Nieschlag and Behre Eds. *Testosterone: Action, Deficiency, Substitution* 2<sup>nd</sup> Edition (1998) Springer

**Meikle WA, Mazer NA, Moeller JF, et al. (1992)** Enhanced transdermal delivery of testosterone across nonscrotal skin produces physiological concentrations of testosterone and its metabolites in hypogonadal men. *J Clin Endocrinol Metab* **74**:623-628.

**Merrigiola CM, Bremner WJ, Paulsen CA, Valdisserri A, Incorvaia L, Motta R, Pavani A, Capelli M, Flamigni C. (1996)** A combined regimen of cyproterone acetate and testosterone enanthate as a potentially highly effective male contraceptive. *J Clin Endocrinol Metab.* **81**:3018-3023.

**Merrigiola CM, Bremner WJ, Costantino A, Pavani A, Capelli M, Flamigni C (1997)** An oral regimen of cyproterone acetate and testosterone undecanoate for spermatogenic suppression in men. *Fertil Steril.* **68**: 844-850.

**Merigiola MC, Bremner WJ, Constantino A, Di Cintio G, Flamigni C. (1998)** Low dose of cyproterone acetate and testosterone enanthate for contraception in men. *Hum. Reprod.* **13**:1225-9.

**Misra A, Pal R, Majumdar SS, Talwar GP, Singh O (1997)** Biphasic testosterone delivery profile observed with two different transdermal formulations. *Pharm Res* (1997) **14**:1264-1268.

**Moelock N, Pujianto DA, Augustin R. (2001)** Achieving azoospermia by injections of testosterone undecanoate only or combined with depot medroxyprogesterone acetate in Indonesian men (Jakarta Centre Study). *J Androl. (Proceedings supplement)* May/June 2001. **P1/2-133**.

**Moltz L, Rommler A, Post K, et al. (1980)** Medium-dose cyproterone acetate (CPA): Effects on hormone secretion and on spermatogenesis in men. *Contraception* **21**:393-413.

**Morishima A, Grimbach MM, Simpson ER, Fisher C, Qin K. (1995).** Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J. Clin. Endocrinol. Metab.* **80**:3689-3698.

**Narula A, Gu YQ, O'Donnel L, Stanton PG, Robertson DM, McLachlan RI, Bremner WJ. (2002)** Variability in sperm suppression during testosterone administration to adult monkeys is related to follicle stimulating hormone suppression and not to intratesticular androgens. *J Clin Endocrinol Metab.* **87**: 3399-406

**Nienhuis H. et al. (1992)** Incidence of disease after vasectomy: a record linkage retrospective cohort study. *British Medical Journal* **304**:743-746.

**Nieschlag E, Behre HM, Engelmann U, Hertle L. (1997)** Male contribution to Contraception in Nieschlag E, Behre HM. Eds *Andrology: Male reproductive health and reproduction.* (1997) Springer.

**Nieschlag E. (1985)** Reasons for abandoning immunization against FSH as an approach to fertility regulation. In Zatuchini GI, Goldsmith A, Spieler JM, et al (eds): *Male Contraception: Advances and Future Prospects.* Philadelphia, Harper & Row (1985): 395.

**Noe G, Suvisaari J, Martin C et al. (1999).** Gonadotrophin and testosterone suppression by 7 $\alpha$ -methyl-19-nortestosterone acetate administered by subdermal implant to healthy men. *Human Reprod.* **14**:2200-2206.

**O'Donnel L, Kyriakos P, Stanton PG, Robertson DM, McLachlan RI. (1999)**

Testosterone dependent restoration of spermatogenesis in adult rats is impaired by a 5 $\alpha$ -reductase inhibitor. *J Androl.* **20**: 109-117.

**Parker S, Armitage M (1999)** Experience with transdermal testosterone replacement therapy for hypogonadal men. *Clin Endocrinol.* **50**:57-62.

**Partsch CJ, Weinbauer GF, Fang RY, Nieschlag E (1995)** Injectable testosterone undecanoate has more favourable pharmacokinetics and pharmacodynamics than testosterone enanthate. *Eur J Endocrinol.* **132**: 514-519.

**Pavlou SN, Dahl KD, Wakefield G, et al. (1988)**: Maintenance of the ratio of bioactive to immunoreactive follicle-stimulating hormone in normal men during chronic luteinizing hormone releasing hormone agonist administration. *J Clin Endocrinol Metab.* **66**:1005.

**Pavlou S, Wakefield G, Schlechter NL, et al. (1989)** Mode of suppression of pituitary and gonadal function after acute or prolonged administration of a luteinizing hormone-releasing hormone antagonist in normal men. *J Clin Endocrinol Metab* **68**:446-454.

**Pavlou SN, Brewer K, Farley MG, et al. (1991)** Combined administration of a gonadotropin-releasing hormone antagonist and testosterone in men induces

reversible azoospermia without loss of libido. *J Clin Endocrinol Metab.* **73**:1360-1369.

**Payne AH, Kawano A, Jaffe RB (1973)** Formation of dihydrotestosterone and other  $5\alpha$ -reduced metabolites by isolated seminiferous tubules and suspension of interstitial cells in a human testis. *J. Clin. Endocrinol. Metab.* **37**:448-453

**Place VA, Atkinson L, Prather DA et al. (1990).** Transdermal testosterone replacement through genital skin. In Nieschlag E & Behre HM (eds), *Testosterone action.deficiency substitution* :165-182 Berlin: Springer-Verlag.

**Pollanen P, Nikkanen V, Huhtaniemi I. (2001)** Combination of subcutaneous levonorgestrel implants and transdermal dihydrotestosterone gel for male hormonal contraception. *Int. J Androl.* **24**:369-380.

**Population Studies:** *World Population Prospects 1990.* New York, United Nations, (1991):5.

**Rajpert-De Meyts E, Leffers H, Petersen JH, Andersen AG, Carlsen E, Jorgensen N, Skakkebaek NE. (2002)** CAG repeat length in androgen-receptor gene and reproductive variables in fertile and infertile men. *Lancet* **359**: 44-46.

**Rivarola MA, Podesta EJ, Chemes HE, Aguilard D. (1973)** *In vitro* metabolism of testosterone by whole human testis, isolated seminiferous tubules and interstitial tissue. *J. Clin. Endocrinol. Metab.* **37**:448-453.

**Rolf C, Kemper S, Lemnitz G, Eickenberg U, Nieschlag E. (2002)**

Pharmacokinetics of a new transdermal gel in gonadotrophin-suppressed normal men.

*Eur J Endocrinol.* **146(5)**: 673-9.

**Roy S, Chatterjee S, Prasad MRN, et al. (1976)** Effects of cyproterone acetate on reproductive functions in normal human males. *Contraception* **14**:403-420.

**Russel DW, Wilson JD. (1994)**: Steroid 5 $\alpha$ -reductase: two genes/two enzymes.

*Annu. Rev. Biochem.* **63**:25-61.

**Salehian B, Wang C, Davidson T, et al. (1994)** Comparison of pharmacokinetics of testosterone after sublingual testosterone or testosterone enanthate injections. *Clin Res* **42**:74.

**Santen RJ, Demers LM, Max DT (1984)**: Long term effects of administration of a gonadotrophin releasing hormone superagonist analogue in men with prostatic carcinoma. *J Clin Endocrinol Metab.* **58**:397-400.

**Santner SJ, Albertson B, Zhang GY, Zhang GH, Santulli M, Wang C, Demers LM, Shackleton C, Santen RJ (1998)** Comparative rates of androgen production and metabolism in Caucasian and Chinese subjects. *J Clin Endocrinol Metab* **83**:2104-2109.

**Schaison G, Couzinet B. (1998)** Percutaneous dihydrotestosterone treatment. In Nieschlag and Behre Eds. *Testosterone: Action, Deficiency, Substitution* 2<sup>nd</sup> Edition Springer 423-436.

**Schearer SB, Alvarez-Sanchez F, Anselmo J, et al. (1978)** Hormonal contraception for men. *Int J Androl* 2(Suppl):680

**Sitruk-Ware R (1989)** Transdermal delivery of steroids. *Contraception* 39: 1-20.

**Skoglund RD, Paulsen CA (1973)** Danazol-testosterone combination: A potentially effective means of reversible male contraception. A preliminary report. *Contraception* 7:357.

**Smith EP, Boyd J, Frank GR et al. (1994)** Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N. Engl. J. Med.* 331:1056-1061

**Soufir J-C, Jounnet P, Marson J, Soumah A. (1983)** Reversible inhibition of sperm production and gonadotrophin secretion in men following combined oral medroxyprogesterone acetate and percutaneous testosterone treatment. *Acta Endocrinol (Copenh)* 102:625-632.

**Sundaram K, Kumar N, Bardin CW. (1993)** 7 $\alpha$ -methyl-19-nortestosterone (MENT); the optimal androgen for male contraception. *Ann Med* 25:199-205.

**Suvisaari J, Sundaram K, Noe G, Kumar N, Aguilleaume C, Tsong YY, Lahteenmaki P, Bardin CW (1997)** Pharmacokinetics and pharmacodynamics of 7 $\alpha$ -methyl-19-nortestosterone after intramuscular administration in healthy men. *Human Reproduction* **12**: 967-973.

**Swerdloff RS, Bagatell CJ, Wang C, Anawalt BD, Berman N, Steiner B, Bremner WJ (1998)** Suppression of spermatogenesis in man induced by Nal-Glu-gonadotrophin releasing hormone antagonist and testosterone enanthate (TE) is maintained by TE alone. *J Clin Endocrinol Metabol.* **83**: 3527-3533.

**Tapanainen JS, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi I (1997)** Men homozygous for an inactivating mutation of the follicle stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. *Nature Genet.* **15**:205-206.

**Tom L, Bhasin S, Salameh E, et al. (1992)** Induction of azoospermia in normal men with combined Nal-Glu gonadotropin-releasing hormone antagonist and testosterone enanthate. *J Clin Endocrinol Metab.* **75**:476-483.

**Vickery BH, Grigg MB, Goodpasture JC, et al. (1986)** Towards a same-day, orally administered male contraceptive. In: Zatuchni GI, Goldsmith A, Speiler JM, et al., editors. *Male contraception: advances and future prospects*. Philadelphia: Harper and Row. 271-92.

**Von Eckardstein S, Syska A, Gromoli J, Kamischke A, Simoni M, Nieschlag E. (2001).** Inverse correlation between sperm concentration and number of androgen receptor CAG repeats in normal men. *J. Clin. Endocrinol. Metab.* **86(6):**2585-90.

**Von Eckardstein S, Nieschlag E, Croxatto H, Noe G, Brache V, Alvarez F, Moo-Young A, Sundaram K. (2001)** 7 $\alpha$ -Methyl-19-Nortestosterone (MENT™) implants for male contraception: a dose finding study. *J Androl. Proceeding Supplement*, VIIth International Congress Andrology. May/June 2001. **P1/2-140.**

**Von Eckardstein S, Schmidt A, Kamischke A, Simoni M, Gromoli J, Nieschlag E. (2002)** CAG repeat length in the androgen receptor gene and gonadotrophin suppression influence the effectiveness of hormonal male contraception. *Clin Endocrinol* **57(5):** 647-55

**Wang LZ:** The therapeutic effect of domestically produced testosterone undecanoate in Klinefelter syndrome. *New Drugs Market* **8:** 28-32

**Wang C, Yeung RTT. (1980)** Use of low-dosage oral cyproterone acetate as a male contraceptive. *Contraception* **21:**245-269.

**Wang C, Berman N, Veldhuis JD, Der T, McDonald V, Steiner B, Swerdloff R (1998):** Graded testosterone infusions distinguish gonadotrophin negative feedback responsiveness in asian and white men- a clinical research center study. *J Clin Endocrinol Metab* (1998) **83:**870-876.

**Wang C, Leung A, Superlano L, Steiner B, Swerdloff RS (1997)** Oligozoospermia induced by exogenous testosterone is associated with normal functioning residual spermatozoa. *Fertil Steril* **68**:149-153.

**Weinbauer GF, Schlatt S, Walter V, Neischlag E. (2001)** Testosterone-induced inhibition of spermatogenesis is more closely related to suppression of FSH than to testicular androgen levels in the cynomolgus monkey model (*Macaca fascicularis*). *J Endocrinol.* **168**: 25-38.

**World Health Organisation Task Force on Methods for the Regulation of Male Fertility (1990)** Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet* **335**:955.

**World Health Organisation Task Force on Methods for the Regulation of Male Fertility (1995)** Rates of testosterone-induced suppression to severe oligozoospermia or azoospermia in two multicentre clinical studies. *Int J Androl* **18**:157-165.

**Wu FCW, Farley TMM, Peregoudov A, Waites GMH, Zhang GY, Li GZ, Gottlieb C, Handlesman DJ, Huhtaniemi I (1996):** Effects of testosterone enanthate in normal men: Experience from a multicentre contraceptive efficacy study. *Fertil Steril* **65**:626-636.

**Wu FCW, Balasubramanian R, Mulders T, Coelinh-Bennink H. (1999).** Oral progestogen combine with testosterone as a potential male contraceptive: additive effects between desogestrel and testosterone enanthate in suppression of

spermatogenesis, pituitary testicular axis and lipid metabolism. *J Clin Endocrinol Metab* **84**:112-122.

**Xiaozhang L, Sunqiang L. (1993)** Vasal sterilization in China: *Contraception* **48**: 255-265.

**Zhang GY, Gu YQ, Wang XH, Cui YG, Bremner WJ. (1998)** A pharmacokinetic study of injectable testosterone undecanoate in hypogonadal men. *J. Androl.* **19**:761-8.

**Zhang GY, Gu YQ, Wang XH, Cui YG, Bremner WJ. (1999)** A clinical trial of injectable testosterone undecanoate as a potential male contraceptive in normal Chinese men. *J. Clin. Endocrinol. Metab.* **84(10)**: 3642-7.

**Zhengwei Y, Wreford NG, Royce P, de Krester DM, McLachlan R (1998):** Stereological evaluation of human spermatogenesis by testosterone treatment: heterogeneous pattern of spermatogenic impairment. *J Clin Endocrinol Metab.* **83**:1284-1291.

## Chapter 2

### **Prolactin receptor expression in human testis and accessory tissues: localization and function.**

#### **Abstract**

Experimental studies in animals have established prolactin as a progonadal hormone that promotes the function of the testis and reproductive accessory glands. The present study investigated the localization of prolactin receptor expression in the human testis and accessory tissues. Expression of prolactin receptor was identified in human testis and vas deferens by RT-PCR. Expression of prolactin receptor was further localized by immunohistochemistry to the Leydig cells and differentiating cells of the testis germ cell (developmental stages extending from pachytene spermatocytes to elongating spermatids). Positive staining for prolactin receptor was also clearly evident in the epithelium of vas deferens, epididymis, prostate and seminal vesicles. Functional activation of prolactin receptor was demonstrated in fresh samples of vas deferens collected at vasectomy by examination of the JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) and MAP (Mitogen Activated Protein) kinase ERK (Extracellular signal-Regulated Kinase) signalling pathways. Within the vas deferens, prolactin induced rapid tyrosine phosphorylation of JAK 2 and STAT 5 (after 10 and 20 min respectively), and tyrosine and threonine phosphorylation of ERK 1 and 2 (after 5 min). The demonstration of function and localization of the prolactin receptor presented here suggests multiple roles for prolactin in the human male reproductive tract.

## **Introduction**

Experimental studies using animal models have established that prolactin acts in concert with the classical gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), to stimulate full testicular function in the adult male rat, mouse and hamster (Bartke, 1971, Zipf et al., 1978, Bartke et al., 1975, Dombrowicz et al., 1992). In the ram, a functional role for prolactin in the testis is indicated by the observation that hypothalamo-pituitary disconnected rams (HPD rams), that permanently lack gonadotrophin secretion due to blockade of gonadotrophin releasing hormone (GnRH) secretion, continue to express cycles in testicular size in response to photoperiod-induced changes in prolactin secretion. These gonadal changes are minor and occur with a long latency, however, indicating that prolactin is a weak gonadotrophin in the absence of LH and FSH (Lincoln et al., 1996).

Prolactin receptor gene expression in the testis has been demonstrated in different species including rat, ram and red deer (Ouhit et al., 1993, Hondo et al., 1995, Jabbour et al., 1998a, Jabbour and Lincoln, 1999). These studies show prolactin receptor localized to the Leydig cells in the interstitium and the germ cells within the seminiferous tubules (Jabbour et al., 1999). The addition of prolactin to testicular explants of ruminant species induces phosphorylation of JAK (Janus Kinase) and STAT (Signal Transducer and Activator of Transcription) signalling proteins, consistent with a functional prolactin receptor in the testis (Jabbour et al. 1999). Prolactin is believed to stimulate testicular steroidogenesis by regulating LH receptors (Bex and Bartke, 1977, Takase et al., 1990), or androgen/oestrogen biosynthesis through the control of rate-limiting enzymes in the Leydig cells (Takeyama et al., 1986; Chandrashekar and Bartke, 1988). However, the mechanism of action of

prolactin on spermatogenesis remains to be clarified.

Expression of prolactin receptor has also been demonstrated in the rat dorsal and lateral prostate, and seminal vesicles (Ouhtit et al., 1993; Nevalainen et al., 1996). In cultured prostatic cells, androgens and oestrogens stimulate the expression of prolactin receptor (Nevalainen et al., 1996), and both gonadal steroids and prolactin, induce the secretion of prostate-specific proteins (Costello and Franklin, 1994). In transgenic mice engineered to over-express the prolactin gene, the prostate gland becomes grossly enlarged illustrating the importance of prolactin in the control of accessory gland physiology (Wennbo et al., 1997). Chronic suppression of blood concentrations of prolactin secretion in the ram produces a decrease in size and fructose content of the seminal vesicles, with no change in testosterone secretion (Ravault et al., 1977), and manipulations of prolactin and androgens in the macaque monkey affect seminal vesicular enzymes (Arunakran et al., 1988), thus prolactin may promote the function of various androgen-dependant male accessory structures.

Clinical observations also support a role for prolactin in the regulation of the testis and accessory glands in man. For example, the restoration of normal prolactin levels in a cohort of subfertile, hypoprolactinemic men caused an increase in sperm density and quality, and restored fertility (Ufearo et al., 1995). In another study, suppression of gonadotrophins and prolactin secretion in eugonadal men treated for prostatic carcinoma, caused a more marked reduction in testicular weight and spermatogenesis, than suppression of gonadotrophin treatment alone (Huhtaniemi, 1991). Both observations are consistent with a progonadal role of prolactin in the testis. Early studies using I<sup>125</sup> labelled prolactin demonstrated the presence of a prolactin binding

protein/receptor in human prostate (Leake et al., 1983). In addition, other studies suggest that prolactin may play a role in the aetiology of benign prostatic hyperplasia and cancer (Kandar et al., 1988; Nevalainen et al., 1997) and the synchronous reduction in both prolactin and androgen improves the efficacy of the treatment of prostatic carcinoma (Rana et al., 1995).

The purpose of the present study was to provide direct evidence for a role of prolactin in the regulation of the testis and reproductive tract in man. To this end, the expression of the prolactin receptor gene was investigated by RT-PCR, using RNA extracted from human testis and vas deferens. The localization of expression of the prolactin receptor protein was further studied using immunohistochemistry in sections prepared from the human testis, epididymis, vas deferens, prostate and seminal vesicles. Lastly, functional prolactin receptor was identified in the vas deferens by investigating activation of the JAK/STAT and MAP (Mitogen Activated Protein) kinase ERK (Extracellular signal-Regulated Kinase) specific intracellular signalling pathways. Activation of JAK/STAT and ERK proteins, following binding of prolactin to its receptor, mediates both proliferative and differentiating effects in target cells (Finidiori and Kelly, 1995; Lewis et al., 1998).

## **Material and Methods**

### *Subjects and Tissues*

Testicular tissue (n=6), exhibiting normal morphology, was obtained by biopsy from men exhibiting unexplained azoospermia. Vas deferens tissue (n=10) was obtained from normal men undergoing vasectomy. Left and right vas deferens were used for comparison between treatment and control. Ethical approval was obtained from

Lothian Paediatric and Reproductive Medicine Research Ethics Subcommittee, and written informed consent was obtained from each subject. Prostate and bladder tissues were obtained from archival surgical resection specimens stored by the Department of Pathology, Western General Hospital, Edinburgh. Epididymis and seminal vesicles were obtained from a commercially available human cadaver tissue library.

#### *Tissue culture*

Following collection, vas deferens tissue was washed in PBS twice and subsequently minced thoroughly with fine scissors. Four aliquots of each tissue (approximately 0.17 g each) were then incubated overnight in 2 ml serum free RPMI 1640 medium (Sigma Chemical Co., Dorset, UK) containing 100 U/ml penicillin and 100 µg/ml streptomycin in a 37°C incubator with 5% CO<sub>2</sub>. The following day, samples were treated with 100 ng/ml human prolactin (hPRL-SIAFP-B2, donated by NIDDK, NIH) for 0, 5, 10 and 20 min. The tissue was stored at -70°C prior to analysis by immunoprecipitation and/or western blotting.

#### *RNA extraction and RT-PCR*

Total RNA was extracted from testis and vas deferens using the guanidinium thiocyanate method as previously described (Chomczynski and Sacchi, 1987). Polyadenylated RNA (poly A<sup>+</sup>) was purified on oligo(deoxythymidine)-cellulose affinity columns (Pharmacia Biotech, St Albans, Herts, England); the yield and purity of RNA was estimated by spectrophotometry. Single strand cDNA was generated from 5 µg poly A<sup>+</sup> RNA by reverse transcription using 1.6 ng oligo (deoxythymidine)<sub>12-18</sub> primer and Superscript reverse transcriptase, according to the manufacturer's instructions (Gibco BRL, Paisley, Scotland). cDNA (10 ng) was then diluted 25 times in ddH<sub>2</sub>O and amplified by PCR using primers corresponding to

nucleotide positions 182-201 (5'-CA-CCT-CCT-GAA-AAA-CCC-AAG-3'; forward primer) and 724-743 (5'-CC-ATG-GTC-TGG-CTT-GCA-GCG-3'; reverse primer) of the prolactin receptor open reading frame. The reaction was carried out in PCR buffer (50 mM KCl, 2 mM MgCl<sub>2</sub> and 20 mM Tris-HCl, pH 8.3), 200 μM deoxy-NTPs, 25 pmol forward and reverse primers and 1U Taq polymerase (Perkin-Elmer, Warrington, Cheshire, England) in a total volume of 50 μl. Samples were subjected to 35 cycles of: 94 °C for 40 sec, 52 °C for 75 sec and 72°C for 2 min 30 sec. After a 10 min final extension at 72°C, the products were visualised on a 1% agarose gel using ethidium bromide staining.

#### *Histology and immunohistochemistry*

Testis, vas deferens and other tissues were placed immediately in Bouins fixative for 6 h before transfer to 70% ethanol, and subsequent dehydration and embedding in wax blocks. Sections were cut and mounted on slides coated with 2% TESPA in acetone. Slides were then dried overnight at 50°C before dewaxing in histoclear (National Diagnostics, Hull, UK). Tissues were rehydrated in graded ethanol and washed in water followed by TBS (0.05 M Tris.HCl pH 7.4, 0.85% NaCl). Sections were treated with 10% hydrogen peroxide in methanol for 30 min and blocked for 30 min with normal swine serum (NSS) diluted 1:5 in TBS with 5% BSA. The primary antibody for the prolactin receptor (kindly donated by Dr PM Ingleton, School of Medicine, University of Sheffield) was raised against a 16 amino acid synthetic peptide corresponding to residues 53-68 of the external domain of the rat prolactin receptor (Nevalainen et al 1996). The polyclonal antibody was diluted in NSS/TBS/5% BSA (as above) and incubated overnight at 4°C. Control sections were incubated with non-immune rabbit serum. All sections were washed twice in TBS (5 min each), incubated

for 30 min with biotinylated swine anti-rabbit immunoglobulin (Dako, Bucks, UK), diluted 1:500 in NSS/TBS. Sections were washed again twice in TBS (5 min each) and incubated with peroxidase conjugated to avidin-biotin complex (Dako, Bucks, UK) for 30 min at room temperature. Colour reaction was developed by incubation in a mixture of 0.05% 3, 3'-Diaminobenzidine (DAB; Sigma) in 10 ml 0.05 M Tris.HCl buffer (pH 7.4) and 0.033% hydrogen peroxide. Sections were subsequently counterstained using haematoxylin.

#### *Immunoprecipitation and western blotting*

Tissue was homogenised and lysed in 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 10% glycerol, 0.6% NP40, 10 µg/ml aprotinin, 1 mM PMSF and 1 mM Sodium orthovanadate. Cytoplasmic extracts were prepared by centrifugation for 2 min at 14000 rpm. For analysis of phosphorylation of JAK 2 and STAT 5, 50 µg of protein was incubated with 10 µl monoclonal anti-phosphotyrosine antibody (5 µg/ml; Affiniti, Exeter, UK) for 1 h at 4°C. Samples were then incubated overnight at 4 °C with M-450 Dynabeads conjugated to rat anti-mouse IgG2b (Dynal, Wirral, UK). The complexes were washed three times in PBS using a Dynal MPC magnet (Dynal, Wirral, UK), and boiled for 5 min in sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 2.5% dithiothreitol, 20% glycerol, 0.05% bromophenol blue). For western blotting, samples were subjected to SDS-PAGE and then transferred to PVDF membrane (Millipore, Bedford, MA). For analysis of ERK phosphorylation, 50 µg of protein from each sample was analysed. Membranes were incubated with antibodies against ERK 1/2, JAK 2, STAT 5 (Santa Cruz Biotechnology, Santa Cruz CA, USA) or phosphorylated ERK (T202/Y204, Cell Signaling, New England Biolabs, Beverly, MA), each diluted 1000 fold in 2% dried skimmed milk/TBST (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.1% tween 20). Membranes were washed briefly in TBST and

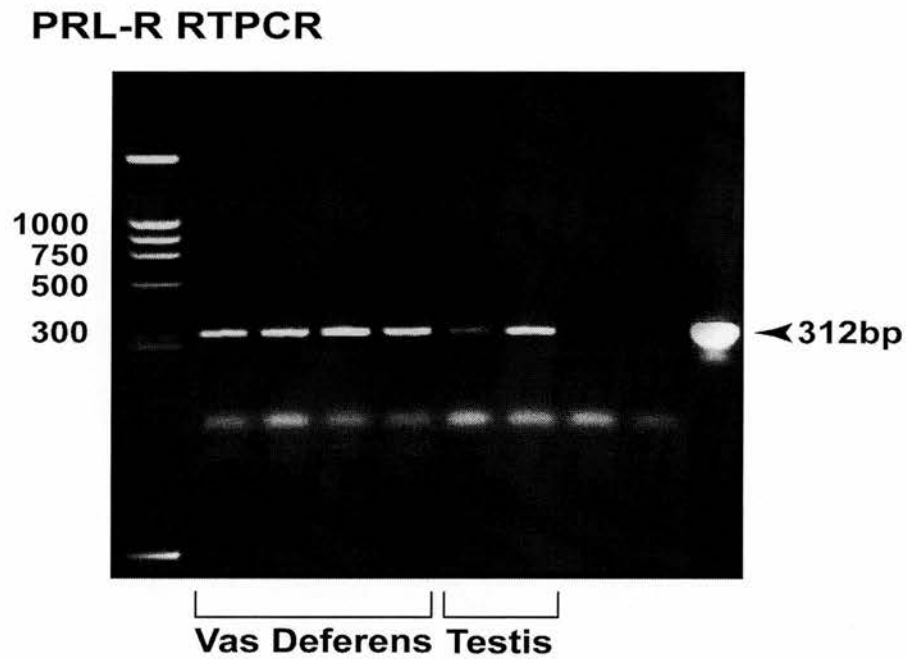
incubated with secondary antibodies conjugated to HRP (Amersham plc, Buckinghamshire, UK), in 2% milk/TBST. Membranes were again washed in TBST and proteins detected using the ECL+ detection kit (Amersham plc, Buckinghamshire, UK).

## **Results**

### **RT-PCR of prolactin receptor mRNA**

The expression of the prolactin receptor was measured initially by RT-PCR, using RNA extracted from testis and vas deferens. A single 312 bp transcript, corresponding to a region in the extracellular domain of the human prolactin receptor, was amplified in all samples of both testis and vas deferens tissue, indicative of the presence of PRL receptor mRNA in these tissues (Fig.1).

*Figure 1. RT-PCR of prolactin receptor mRNA*

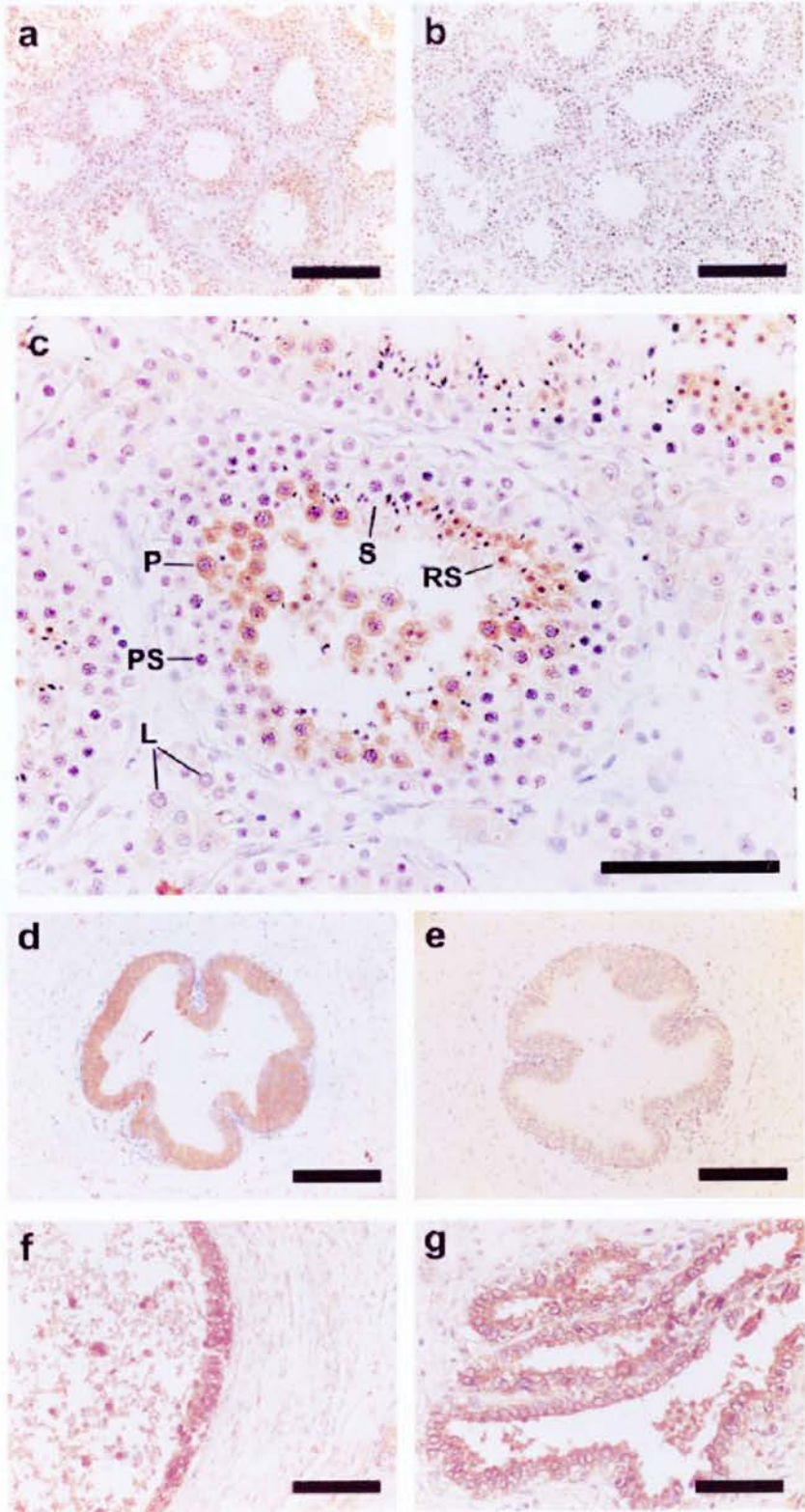


**Figure 1.** RT-PCR of prolactin receptor from RNA purified from human vas deferens and testis. Lane 1: DNA size marker, lanes 2-5: RNA from vas deferans, lanes 6 and 7: RNA from testis, lanes 8 and 9: no reverse transcriptase added, lane 10: positive control using PRLR cDNA as template. The predicted size of the PCR product corresponding to the extracellular domain of the prolactin receptor mRNA is marked as 312 bp.

## Immunohistochemistry of prolactin receptor

To determine the localisation of prolactin receptor, immunohistochemistry was performed using sections prepared from human testis, vas deferens, epididymis, prostate, seminal vesicle and bladder. In the testis biopsies of all 6 subjects, immunostaining was localised to the Leydig cells in the interstitial tissue and to the germ cells at different stages of spermatogenesis in the seminiferous tubules. Representative micrographs are shown in Fig. 2 (panels a,c). The most marked staining was evident in the cytoplasm of the germ cells in the developmental stages extending from pachytene spermatocytes to elongating spermatids. The spermatogonia, early dividing spermatocytes and final differentiating spermatozoa were devoid of staining, as were the Sertoli cells (Fig 2c). Control sections of testis were totally devoid of staining (Fig 2b). In the vas deferens, epididymis and seminal vesicle samples, immunostaining for the prolactin receptor was consistently evident in the epithelium, but not in the underlying stroma and muscle tissue (Fig 2d,f,g); control sections of vas deferens were again unstained (Fig 2e). Immunostaining for the prolactin receptor was also present in the epithelium of the prostate (data not shown as demonstrated previously, Nevalainen et al., 1997).

Figure 2: Immunohistochemistry of PRL-R

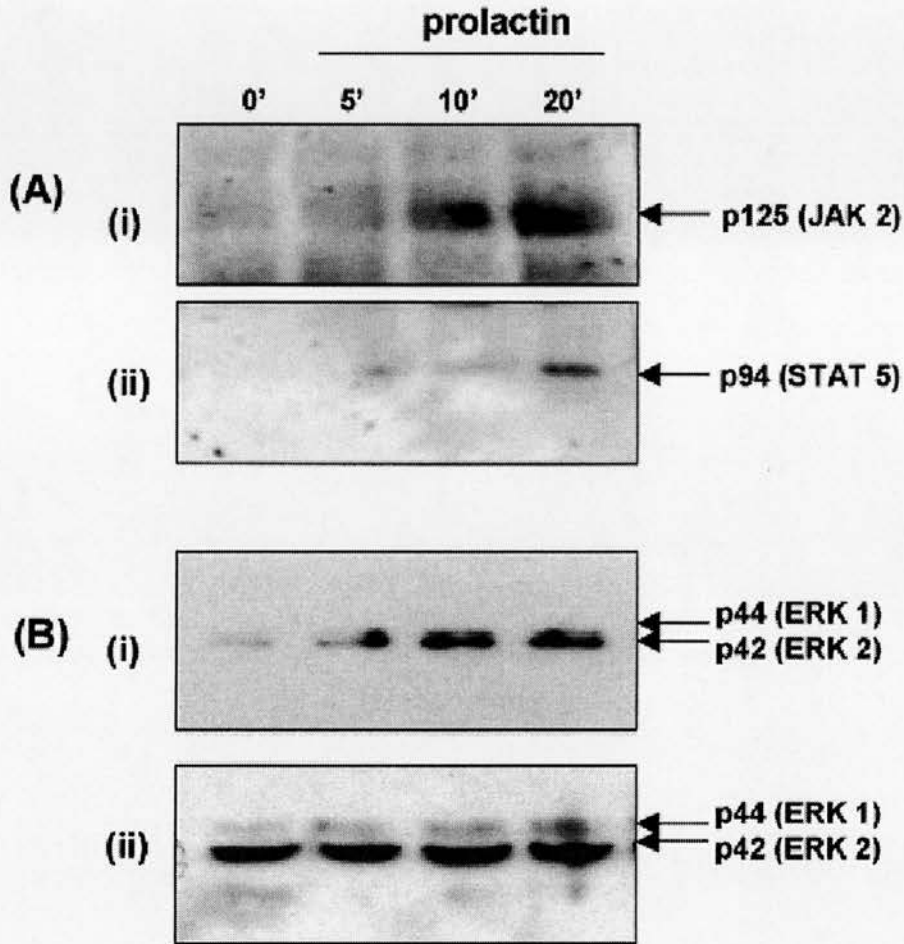


**Figure 2.** Immunohistochemistry of the prolactin receptor (PRL-R) in human male reproductive tissues. Human testis was immunostained with **(a)** and without **(b)** PRL-R antibody and examined at X20 magnification. Panel **(c)** corresponds to testis immunostained with PRL-R and examined at X40 magnification. Expression of PRL-R is evident in Leydig cells (L) in the interstitial tissue, pachytene spermatocytes (P), and round spermatids (RS) in the seminiferous tubules. There was no apparent staining in the spermatocytes at early stages of spermatogenesis (PS) and differentiated sperm (S). Vas deferens was immunostained with **(d)** and without **(e)** PRL-R antibody and examined at X40 magnification. Panel **(f)** human epididymis and **(g)** human seminal vesicle stained for PRL-R. Size bar=100mm.

## Intracellular signalling in the vas deferens

Intracellular signalling pathways were examined by investigating tyrosine phosphorylation of JAK 2 and STAT 5 and tyrosine and threonine phosphorylation of ERK 1 and 2, following stimulation of fresh vas deferens tissue with human prolactin (Fig. 3). Tyrosine phosphorylation of JAK 2 and STAT 5 was measured by an immunoprecipitation procedure using an antibody against phosphorylated tyrosine, followed by western blotting using antibodies against JAK 2 and STAT 5. Phosphorylation of ERK 1 and 2 were measured by western blotting using an antibody raised against ERK, phosphorylated on specific tyrosine and threonine residues. Rapid phosphorylation of ERK 1 and 2 (evident from 5 min), JAK 2 (10 min), and STAT 5 (20 min) was observed following treatment with prolactin (Fig. 3). No increase in phosphorylated proteins was observed in the absence of prolactin.

**Figure 3: Intracellular signalling in the human vas deferens**



**Figure 3.** Phosphorylation of JAK 2, STAT 5 and ERK1/2 in the human vas deferens, in response to prolactin. Tissue was serum starved and incubated with 100ng/ml of human prolactin for 0,5,10 or 20 mins. (A) Proteins immunoprecipitated with anti-phospholipid antibody and analysed by Western blotting using antibodies against JAK 2(i) and STAT5 (ii). (B) Proteins analysed by Western blotting using antibodies against phosphorylated (T202)/Y204) ERK (i) and non-phosphorylated ERK (ii).

## **Discussion**

The data presented here demonstrate that prolactin receptors are expressed in the human testis and in various male accessory glands. This is based on RT-PCR amplification of the receptor mRNA extracted from testis and vas deferens, and the localization of the receptor protein by immunocytochemistry in a wide range of reproductive tissues. Furthermore, the incubation studies with fresh tissues of human vas deferens show that the addition of prolactin activates phosphorylation of several proteins known to mediate the intracellular effects of prolactin in target cells. These data provide clear evidence for functional activation of the prolactin receptor in a clinically accessible human male reproductive tissue.

Various isoforms of the prolactin receptor, differing in the intracellular domain, have been characterised in man, and these are differentially expressed in the many prolactin target organs (Kline et al., 1999, Hu et al., 2001). Long forms are expressed in the testis, and long and intermediate forms in the prostate. The methodology used in the current study was directed at the extracellular domain of the prolactin receptor (cDNA primers for RT-PCR, and antibody for immunohistochemistry), thus it was not possible to confirm the expression of the different isoforms of the prolactin receptor in the human testis and other tissues. In rodent models, different isoforms of the prolactin receptor have been identified, and both are expressed in the testis and accessory glands (Ouhtit et al., 1993). Hence, it is plausible that similar diversity of prolactin receptor expression exists in human testis and accessory glands.

The current immunohistochemical studies localized for the first time the prolactin

receptor protein to the Leydig cells and germ cells of the human testis, and confirmed the receptor expression in the secretary, adsorptive or lining epithelium in the various accessory male structures. The pattern of immunostaining in the testis is similar to that described in the red deer and ram (Jabbour et al 1996; Jabbour et al 1999). Expression of the receptor in Leydig cells is consistent with a role of prolactin in the control of testicular steroidogenesis. Evidence in rodent species suggests that prolactin influences testosterone secretion through the stimulation and maintenance of expression of LH receptors in Leydig cells (Klemcke et al., 1984; Takase et al., 1990) or by regulating specific enzymatic steps in androgen biosynthesis (Chandrashekar et al., 1988). The effect of prolactin on steroidogenesis in the adrenal gland is also well documented in animals and man (Glasow et al., 1996). The presence of prolactin receptors in the differentiating germ cells in the testis is consistent with the view that prolactin acts directly within the seminiferous tubules to affect spermatogenesis, as well as acting indirectly through gonadal steroid secretion. In the immature hypophysectomised rat, treatment with prolactin stimulated an increase in the number of primary spermatocytes (Dombrowicz et al., 1992), while in hypoprolactinemic subfertile men, exogenous prolactin, or treatment with metoclopramide to promote prolactin secretion, increased sperm density, reduced sperm abnormalities and acted to restore fertility (Ufearo et al., 1995). Other studies in the ram indicate that the effects of prolactin in the testis are largely dependent on the concurrent secretion of LH and FSH (Lincoln et al., 2001). Taken together, the data indicate that prolactin acts within the testis to facilitate and augment the actions of LH and FSH promoting full testicular activity.

The universal distribution of the prolactin receptor in the epithelium of the

epididymis, vas deferens, seminal vesicles and prostate confirms that prolactin also plays a role in the regulation of accessory gland function in man. Animal studies clearly demonstrate that prolactin acts in association with androgens to stimulate the activity of the accessory glands. For example, the production of secreted proteins from cultured rat prostate cells is markedly enhanced by addition of prolactin (Nevalainen et al., 1996). In transgenic rodent models, over-expression of prolactin, or its receptor, is associated with enlargement of the accessory glands, while under-expression is associated with reduced functional activity (Wennbo et al., 1997). The clinical observation that prolactin enhances the effectiveness of steroid withdrawal for the treatment of malignant disease of the prostate (Rana et al., 1995) also supports an action for prolactin in the human accessory glands. The presence of prolactin receptor in the epithelial cells in the tissues of the male reproductive tract suggests that prolactin is potentially involved in the regulation of secretion, absorption, and/or the control of transport of fluids across the cell membrane. This is consistent with a conserved function of prolactin in epithelial tissues across species (Nicoll, 1974).

As well as demonstrating functional prolactin receptors within the vas deferens, the current results support the view that prolactin signals through more than one intracellular pathway to regulate the tissue responses. In the vas deferens, prolactin activated rapid tyrosine phosphorylation of JAK 2 and STAT 5 protein. The temporal sequence is similar to that observed in other prolactin target-tissues including rat mammary gland (Jahn et al., 1997) and ovary (Ruff et al., 1996), ram testis (Jabbour et al., 1999) and human endometrium (Jabbour et al., 1998b). Upon activation, STAT proteins dimerise, translocate to the nucleus and bind to STAT-regulatory elements in the promoters of target genes to influence transcription (Schindler and Darnell, 1995).

In mammary gland, activation of STAT 1 and STAT 5 proteins up-regulate transcription of genes for  $\beta$ -casein (Gouilleux et al.,1994),  $\beta$ -lactoglobulin (Burdon et al.,1994) and whey acidic protein (Li and Rosen, 1995) to mediate the effects of prolactin on milk synthesis and secretion. The target genes for prolactin in the vas deferens epithelium are still unknown, but are likely to include genes encoding secretory proteins.

The demonstration of rapid phosphorylation of ERK proteins in response to prolactin in human vas deferens indicates that prolactin also acts via the ERK pathway to affect the function of the secretory epithelium. The involvement of both the JAK/STAT and ERK pathways acting together to influence differentiation has been documented in other cell types (Lewis et al., 1998). In the human endometrium prolactin induces the JAK/STAT and ERK pathways in the epithelial cells during the secretory phase of the menstrual cycle (Jabbour et al., 1998b; Gubbay et al., 2001). The activation of ERK by prolactin in terminally differentiated secretory epithelium, as in the human vas deferens, indicates that prolactin signals through ERK, as well as through the JAK/STAT pathway, to provide divergent control of more than one cellular response. In the current study, only fresh tissue from human vas deferens was available for tissue culture, thus it was not possible to establish the pattern of phosphorylation of the second messenger proteins in the different male reproductive tissues.

In conclusion, this study provides clear support for the view that prolactin is a regulator of reproductive function in the human male. Prolactin appears to act in Leydig cells of the testis to promote steroidogenesis, and in the germ cells of the seminiferous tubules of the testis to promote the efficiency of spermatogenesis,

actions that augment the more dominant progonadal effects of LH and FSH. Prolactin also acts in the epithelia of the efferent ducts and the male accessory sex glands, in conjunction with the gonadal steroid hormones, to regulate the secretory/adsorptive functions of these male tissues. Our results are consistent with the pleiotrophic character of prolactin, as a hormone with multiple target tissues signalling through multiple pathways. A role for prolactin within the male reproductive tract, suggests that abnormalities in circulating levels of prolactin within the body, or abnormalities in prolactin receptor function, may have a significant impact on male fertility.

## References

- Arunakran J., Balasubramanian K., Srinivasan N et al. (1988).** Effects of androgens, prolactin and bromocryptine on seminal vesicular enzymes of the pyruvate malate cycle involved in lipogenesis in castrated mature monkeys, *Macaca radiata*. *Int J Andrology* **11**: 133-139.
- Bartke, A. (1971).** Effect of prolactin on spermatogenesis in hypophysectomised mice. *J Endocrinol* **49**: 311-316.
- Bartke, A., Croft, B.T., Dalterio, S. (1975).** Prolactin restores plasma testosterone levels and stimulates testicular growth in hamsters exposed to short day-length. *Endocrinology* **97**: 1601-1604.
- Bex, F.K., Bartke, A. (1977).** Testicular LH binding in the hamster: modification by photoperiod and prolactin. *Endocrinology* **100**, 1223-1226.
- Burdon, T.G., Demmer, J., Clarke J.A., Watson, C.J. (1994).** The mammary factor MPBF is a prolactin-induced transcription regulator which binds to STAT factor recognition sites. *Fed Eur Biochem Soc* **350**, 177-182
- Chandrashekar, V., Bartke, A. (1988).** Influence of endogenous prolactin on luteinising hormone stimulation of testicular steroidogenesis and the role of prolactin in the adult male rat. *Steroids* **5**: 559-576.

**Chomczynski, P., Sacchi, N. (1987).** Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. *Anal Biochem* **162**: 156-159.

**Costello, L.C., Franklin, R.B. (1994).** Effect of prolactin on the prostate. *Prostate* **24**: 162-166.

**Dombrowicz, D., Sente, B., Closset, J. et al. (1992).** Dose-dependent effects of human prolactin on the immature hypophysectomised rat testis. *Endocrinology* **130**: 695-700.

**Findiori, J., Kelly, P.A. (1995).** Cytokine receptor signalling through two novel families of transducer molecules: Janus kinase and signal transducers and activation of transcription. *J Endocrinol* **147**: 11-23.

**Glasow, A., Breidert, M., Haidan, A. et al. (1996).** Functional aspects of the effect of prolactin (PRL) on adrenal steroidogenesis and distribution of PRL receptor in the human adrenal gland. *J Clin Endocrinol Metab* **81**: 3103-3111.

**Gouilleux, F., Wakao, H., Mundt, M. Groner, B. (1994).** Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J*, **13**, 4361-4369.

**O Gubbay, HOD Critchley, JM Bowen, A King, and HN Jabbour.(2001)**

Prolactin induces ERK phosphorylation in epithelial and CD56+ natural killer cells of

the human endometrium. *J Clin Endocrinol Metab*. Submitted.

**Hondo, E., Kurohmaru, M., Sakai, S. et al. (1995).** Prolactin receptor expression in rat spermatogenic cells. *Biol Reprod* **52**: 1284-1290.

**Hu, Z.Z., Meng, J., Dufau, M.L. (2001).** Isolation and characterization of two novel forms of the human prolactin receptor generated by alternative splicing of a newly identified exon 11. *J Biol Chem* **276**: 41068-41094.

**Huhtaniemi, I., Kurohmaru, M., Venho, P. et al. (1991).** Combination of a GnRH agonist with an antiandrogen or bromocriptine in the treatment of prostatic cancer; slight potential for antigonadal effects. *Int J Androl* **14**: 374-386.

**Jabbour, H.N., Clarke, L.A., McNeilly, A.S. et al. (1998a).** Is prolactin a gonadotrophic hormone in red deer (*Cervus elaphus*)? Pattern of expression of the prolactin receptor in the testis and epididymis. *J Mol Endocrinol* **20**: 175-182.

**Jabbour, H.N., Critchley, H.O.D., Boddy, S.C., (1998b).** Expression of functional prolactin receptors in non-pregnant human endometrium: Janus kinase-2, signal transducer and activator of transcription-1 (STAT1), and STAT5 proteins are phosphorylated after stimulation with prolactin. *J Clin Endocrinol Metab* **83**: 2545-2553.

**Jabbour, H.N., Lincoln, G.A. (1999).** Prolactin receptor expression in the testis of the ram: localisation, functional activation and the influence of gonadotrophins. *Mol*

*Cell Endocrinol* **148**: 151-161.

**Jahn, G.A., Daniel, N., Jolivet, G. et al. (1997).** In vivo study of prolactin (PRL) intracellular signalling during lactogenesis in the rat: Jak/Stat pathway is activated by PRL in the mammary gland but not in the liver. *Biol Reprod* **57**: 894-900.

**Kandar, T., Ben-David, M., Pontes, J.E. et al. (1988).** Prolactin and luteinizing hormone-releasing hormone receptors in human benign prostatic hyperplasia and prostatic cancer. *Prostate* **12**: 299-307.

**Klemcke, H.G., Bartke, A., Borer, K.Y. (1984).** Regulation of testicular prolactin and luteinising hormone receptors in the golden hamster. *Endocrinology* **114**: 594-603.

**Kline, J.B., Rochrs, H., Clevenger, C.V. (1999).** Functional characterization of the intermediate isoform of the human prolactin receptor. *J Biol Chem* **274**: 35461-35468.

**Leake, A., Chrisholm, G.D., Habib, F.K. (1983).** Characterization of the prolactin receptor in human prostate. *J Endocrinol* **99**: 321-328.

**Lewis, T.S., Shapiro, P.S., Ahn, N.G. (1998).** Signal transduction through MAP kinase cascades. *Adv Cancer Res.* **74**:49-139.

**Li, S., Rosen, J.M. (1995).** CTF/NF1 and mammary gland factor (STAT5) play a critical role in regulating rat whey acidic protein gene expression in transgenic mice.

*Mol Cell Biol* **15**, 2063-2070.

**Lincoln, G.A., Clarke, I.J., Sweeney, T. (1996).** Hamster-like cycles in testicular size in the absence of gonadotrophin secretion in HPD rams exposed to long-term changes in photoperiod and treatment with melatonin. *J Neuroendocrinol* **8**, 855-866.

**Lincoln, G.A., Townsend, J., Jabbour, H.N. (2001).** Prolactin actions in the sheep testis: a test of the priming hypothesis. *Biol Reprod* **65**: 936-943.

**Nevalainen, M.T., Valve, E.M., Ingleton, P.M. et al. (1996).** Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. *Endocrinology* **137**: 3078-3088.

**Nevalainen, M.T., Valve EM., Ingleton, P.M. et al. (1997).** Prolactin and prolactin receptors are expressed and functioning in the human prostate. *J Clin Invest* **99**: 618-627.

**Nicoll, C. (1974).** Physiological actions of prolactin. In Handbook of Physiology, Eds E. Knobil & W.H. Sawyer. American Physiological Society, Washington, DC.

**Ouhtit, A., Morel, G., Kelly, P.A. (1993).** Visualisation of gene expression of short and long forms of prolactin receptors in rat reproductive tissues. *Biol Reprod* **49**: 528-536.

**Rana, A., Habib, F.K., Halliday, P. et al. (1995).** A case for synchronous reduction

of testicular androgen, adrenal androgen and prolactin for the treatment of advanced carcinoma of the prostate. *Eur J Cancer* **31A**:859-860

**Ravault, J.P., Courot, M., Garnier, D. et al. (1977).** Effect of 2-bromo- $\alpha$ -ergocryptine (CB154) on plasma prolactin, LH and testosterone levels, accessory reproductive glands and spermatogenesis in lambs during puberty. *Biol Reprod* **17**: 192-197.

**Ruff, S.J., Leers-Sucheta, S., Melner, M.H., et al. (1996).** Induction and activation of Stat5 in the ovaries of pseudopregnant rats. *Endocrinology* **137**: 4095-4099.

**Schindler, C., Darnell, Jr J.E. (1995).** Transcriptional responses to polypeptide ligands: the Jak-Stat pathway. *Ann Rev Biochem* **64**: 621-651.

**Takase, M., Tsutsui, K., Kawashima, S. (1990).** Effects of prolactin and bromocriptine on the regulation of testicular luteinising hormone receptors in mice. *J Exp Zool* **6**: 219-229.

**Takeyama, M., Nagareda, T., Takatsuka, D. et al. (1986).** Stimulatory effect of prolactin on luteinising hormone-induced testicular 5- $\alpha$ -reductase activity in hypophysectomised adult rats. *Endocrinology* **118**: 2268-2275.

**Ufearo, C.S., Orisakwe, O.E. (1995).** Restoration of normal sperm characteristics in hypoprolactineamic infertile men treated with metaclopramide and exogenous prolactin. *Clin Pharmacol Therapeut* **58**: 354-359.

**Wennbo, H., Kindblom, J., Isaksson, O.G.P. et al. (1997).** Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland. *Endocrinology* **138**: 4410-4415.

**Zipf, W.B., Payne, A.H., Kelch, R.P. (1978).** Prolactin, growth hormone and luteinising hormone in the maintenance of testicular luteinising hormone receptor. *Endocrinology* **103**: 595-600.

## Chapter 3

### **An investigation of the effectiveness of testosterone implants in combination with the prolactin inhibitor quinagolide in the suppression of spermatogenesis in men**

#### **Abstract**

BACKGROUND Administration of testosterone (T) inhibits gonadotrophin secretion and spermatogenesis in men but the degree of response is highly variable. This treatment also stimulates prolactin, itself a progonadal hormone in animals. This study investigated whether concomitant suppression of prolactin (PRL) with the non-ergot, dopamine receptor agonist quinagolide (Q), would enhance the efficacy of T in its inhibition of spermatogenesis in healthy eugonadal men. METHODS Forty-six men were randomised to three treatment groups: Group 1, T1200: 1200mg T implant plus daily oral placebo; Group 2, T1200+Q: 1200mg T plus oral Q 75 $\mu$ g/day; Group 3, T800+Q: T 800mg plus oral Q 75  $\mu$ g/day. Treatments were for 24 weeks followed by an 8-week recovery period. RESULTS Total number of subjects that achieved severe oligospermia ( $\leq$ 1million/ml including azoospermia) from weeks 8-16: 11/13 [85%], 11/12 [92%], 8/13 [61.5%] in the 3 groups respectively. CONCLUSIONS The results show that inhibition of PRL does not to confer additional efficacy in spermatogenic suppression in men. However, Q did not totally block PRL secretion in the subjects, possibly because T replacement itself stimulated PRL by a direct action on the lactotroph, thus the effectiveness of dual inhibition of gonadotrophin and PRL could not be fully investigated.

## Introduction

Androgen administration aimed at producing a reversible contraceptive for men acts by inhibition of FSH and LH secretion to suppress spermatogenesis. It does not however suppress PRL secretion, which at least in animal models, is a weak gonadotrophin (Bartke et al., 1975, Ouhtit et al., 1993, Lincoln et al., 1996, Jabbour and Lincoln, 1999). Initial studies showed that long-term treatments with testosterone (T) esters, even at high doses, induce azoospermia in only a proportion (ca. 65%) of subjects with notable differences between ethnic groups (World Health Organization Task Force, 1990; Handelsman et al., 1992; Sundaram et al., 1993, Behre et al., 1995). More recently, steroid treatments involving T combined with progestins, have been shown to be more effective in the suppression of spermatogenesis (Merrigiolla et al., 1996, Handelsman et al., 1996, Bebb et al., 1996, Wu et al., 1999, Martin et al., 2000). Treatments with anti-androgenic progestins (e.g. cyproterone acetate), however, produce undesirable changes in the haemopoietic system (Merrigiola et al., 1996), and progestins may produce effects on mood as is well demonstrated in women (Pearlstein, 1995). In addition these treatments have not been shown to produce universal azoospermia (Merrigiola et al., 1996, Handelsman et al., 1996, Bebb et al., 1996, Wu et al., 1999, Martin et al., 2000). A number of suggestions have been proposed to explain the heterogeneity in the contraceptive response to steroid treatments. These include differing SHBG levels and responsiveness to gonadotropin suppression (Behre et al., 1995, Wang et al., 1998), differing 5-alpha reductase activity in the testis and its impact on intratesticular androgen levels (Anderson et al., 1996), structural differences in testicular morphology between ethnic groups (Zhengwei et al., 1998), and possible differences in sex steroid metabolism and/or diet (Santner et al., 1998).

An additional explanation for the failure to induce complete azoospermia is that it may be necessary to inhibit PRL, in addition to the classical gonadotrophins, to fully block spermatogenesis. This is because PRL potentially acts in the testis to stimulate both androgenic and spermatogenic functions based on studies in rodents (Hondo et al., 1995). In man the progonadal role of PRL is less clear. Early studies using I<sup>125</sup>-PRL, failed to demonstrate PRL binding in the human testis, in contrast to the situation in the rat (Wahlstrom et al., 1983). More recently, mRNA for the PRL receptor has been characterised in the human testis (Kline et al., 1999), and immunocytochemistry has revealed that PRL receptors are weakly expressed in the Leydig cells in the interstitial tissue and more strongly expressed in germ cells undergoing spermatogenesis in the seminiferous tubules (Hair et al., 2002). Functional activation of these receptors and their second messenger systems Jak/STAT and ERK by PRL has also been demonstrated in human testis and vas deferens. Furthermore, there is clinical data indicating that PRL may promote spermatogenesis. In one study, treatment with exogenous PRL, or a dopamine antagonist, to increase circulating PRL concentrations was shown to restore testicular function and fertility in hypoprolactinaemic infertile men (Ufearo and Orisakwe, 1995), and in another study, combined suppression of gonadotrophins and PRL in eugonadal men treated for prostatic carcinoma produced a more marked reduction in testicular weight than gonadotrophin suppression alone (Huhtaniemi et al., 1991). Based on these observations, and the demonstration that the administration of T and oral progestin stimulates PRL secretion (Bellis and Wu, 1998), we infer that suppression of PRL may enhance the effectiveness of sex steroid in inducing spermatogenic suppression in man.

The purpose of the present study was to test this hypothesis. Healthy male volunteers were treated orally with the non-ergot dopamine receptor agonist quinagolide, to chronically suppress PRL secretion. This drug has been shown to inhibit PRL production with minimal effects on the gastrointestinal tract and on nausea, mood and sleep behaviours (Brownell et al., 1996). At the start of the treatment, the volunteers also received subcutaneous implants of T to suppress gonadotrophin secretion. Implants were used rather than intermittent injections to provide a smooth androgen profile with less inconvenience to the subjects. A high and an intermediate dose of T was selected to establish whether PRL inhibition would act in synergy with the degree of gonadotrophin suppression and perhaps allow use of a lower dose of androgen to induce azoospermia in men.

A preliminary report on this study has been published as an abstract (Hair et al., 2000).

## **Materials and Methods**

### *Subjects*

The study medication and design, information for volunteers, method of recruitment, and the reimbursement of expenses were all approved by the Central Manchester Ethical Committee for Medical Research and by the Medical Research Council. Prospective volunteers were recruited from the community through local media advertising. Written and verbal information as to the nature of the study was provided to 480 men. Prospective candidates were then interviewed during which a full medical history was obtained and thereafter underwent physical examination. Routine haematology, biochemistry and semen analysis were then performed as screening tests on two occasions 2 weeks apart, to determine if subjects met the inclusion

criteria. The subjects were required to be within the age range 19-50, free of chronic disease, on no long-term medication, with normal haematology and biochemical screening tests and a normal semen profile based on World Health Organization guidelines (WHO Manual, 1999). The subjects were also required to be willing to continue with their existing contraceptive method. Following recruitment, 46 volunteers entered the study.

### *Medications*

Quinagolide (Q) was used to chronically inhibit PRL secretion in the volunteers. The drug tablets were supplied by Novartis (Norprolac, Novartis Pharmaceuticals UK Ltd, Frimley Business Park, Frimley, Camberly, Surrey GU16 5SG). Subjects initially received a Starter Pack containing two tablets each of 25, 50 and 75  $\mu\text{g}$  of Q to allow graded introduction of the medication. Following the introduction over 6 days, the subjects took one 75- $\mu\text{g}$  tablet per day. This dose was selected based on manufacturers recommendations in the treatment of hyperprolactinaemia to effectively suppress PRL secretion and minimise side effects. A small pilot study in 5 normal men was also conducted. This demonstrated that daily administration of 75  $\mu\text{g}$  Q suppressed blood plasma PRL concentration by >80% for the 9-day treatment period with a carryover effect for 3 days. For the main clinical trial, tablets were taken after food and before bed. Placebo tablets supplied by our hospital pharmacy were taken by one group of subjects as a control.

200 mg crystalline T implants were generously donated by Organon (NV Organon, Oss, Netherlands; Product licence No. 0065/5084R). Implants were inserted using a trocar with sterile technique in the subcutaneous fat of the anterior abdominal wall with local anaesthesia using 1% Lignocaine. In the high dose treatment the subjects

received 6 implants (1200 mg), and in the lower dose they received 4 implants (800 mg). These doses were selected on the basis that 1200 mg T produces severe oligospermia in normal men, while 800 mg T less completely suppresses sperm numbers.

### ***Study design***

Subjects were randomised to one of three treatment groups in a single blind parallel group design: Group 1 – T 1200 mg plus daily oral placebo (T1200), Group 2 – T 1200 mg plus oral quinagolide 75 µg/ day (T1200+Q) and Group 3 – T 800 mg plus oral quinagolide 75 µg/day (T800+Q). A more balanced design would have utilised a T800+placebo group but this was not included due to constraints on the number of volunteers and the power calculation requirement of no fewer than 12 subjects per group. The protocol involved a pre-treatment phase of 4 weeks, a treatment phase of 24 weeks and a recovery phase of 8-12 weeks (see Figure 1). The treatment phase was initiated by the insertion, on one occasion, of either 4 or 6 T implants according to treatment-group. The subjects also commenced daily oral Q, or placebo tablets, which were dispensed monthly at the clinical monitoring visit. Treatment of the subjects did not occur simultaneously but was staggered according to time of recruitment. The recovery phase was determined by the time to re-attainment of the pre-treatment sperm concentration in the ejaculate, or to 2 consecutive sperm counts >20million/ml.

### ***Clinical monitoring***

Every 4 weeks throughout the study each subject was weighed, and pulse and blood pressure measured. They were then interviewed to record evidence of compliance, unused tablets were returned and any notable effects of the treatment were recorded.

A blood sample was obtained for hormone measurements, and a semen sample was produced for analysis. The blood samples were heparinised, centrifuged within 30 minutes of collection and stored at  $-20^{\circ}\text{C}$  until analysis on completion of the full study. Every 8 weeks, additional blood samples were obtained for monitoring of haematological parameters, renal and liver function tests, and serum lipid concentrations. Testis size was measured using an orchidometer. At the beginning and end of the study a standard rectal examination of the prostate was carried out.

#### *Semen Analysis*

Semen samples were obtained by masturbation after 48hours abstinence and analysis of semen volume, pH, sperm concentration, motility and morphology was carried out within 60 minutes of collection according to the World Health Organization manual (WHO Manual, 1999). Azoospermia was verified by centrifugation of the whole semen sample and microscopic examination of the re-suspended pellet residue.

#### *Blood analysis*

Full blood counts, glucose, HbA1c, urea, electrolytes, liver enzymes and lipid profiles were measured by a hospital autoanalyser. LDL-cholesterol was derived using the Friedwald formula. On completion of the clinical study the blood plasma concentrations of PRL, FSH and LH were assayed in all subjects by highly sensitive immunofluorometric assays (Delfia, Pharmacia-Wallac, Turku, Finland) with an assay sensitivity of 9.0 mU/L, 0.125 IU/L, 0.125 IU/L, respectively. Total T was determined by previously described radioimmunoassay (Corker and Davidson, 1978) with detection limit of 0.3 nmol/L.

### *Statistical analysis*

Results are expressed as group mean  $\pm$  SEM. Data were analysed by two-way ANOVA with repeated measures using Statistica software (version 4.0) to identify significant time by group interactions with Tukey's post-hoc comparisons. In addition, ANOVA with repeated measures was performed within each group to detect significant effects of treatment. Baseline levels for each variable were defined as the arithmetic mean of the two pre-treatment samples. Recovery levels were defined as those obtained at 4 -12 weeks following completion of treatment according to time at which the recovery criteria were met. Severe oligospermia was defined as a specimen with sperm present in the ejaculate but at a concentration of <1million/ ml. Testis volume was defined as the sum of volume measurements for the left and right testis. The 8-20 week period was selected as the period of maximum sperm suppression.

## **RESULTS**

### **Subjects**

Of the 46 men who entered, 38 completed the study. Five men failed to attend regular clinical appointments and were lost to follow-up after 4-20 weeks without explanation. One man was withdrawn from the study due to extrusion of 2 T implants and due to his lack of compliance with oral Q. Two subjects did not tolerate oral Q: in one case, the treatment caused symptoms of nausea and vomiting during the first few days of treatment and in the second case the treatment caused perceived effects on mood and libido from week 4 and the subject subsequently withdrew at week 16. After these losses the group sizes were 13,12,13 for T1200, T1200+Q and T800+Q treatments respectively.

In the 38 men who completed the study, the oral Q and T implants were generally well tolerated and there were no serious adverse effects reported by the participants. Transient morning nausea in the first week was reported by 2 subjects taking oral Q and by 1 subject taking placebo. Mild acne was reported in 4 subjects and increased libido at the start of the study was reported in 16 subjects. There was no apparent difference in these symptoms in the three treatment groups. The men did not differ significantly between groups in age ( $30.77\pm 1.99$ ,  $33.36\pm 1.5$ ,  $34.75\pm 1.23$  yrs, Group T1200+placebo, Group T1200+Q, Group T800+Q, respectively), body weight ( $83.54\pm 4.54$ ,  $81.33\pm 4.82$ ,  $76.36\pm 3.37$  kg) and testis volume ( $45.83\pm 2.6$ ,  $49.64\pm 2.43$ ,  $44.55\pm 2.73$  ml). All subjects had a pre-treatment sperm concentration  $>20$ million/ml in the ejaculate.

#### **PRL/T manipulations**

Q treatment. Blood plasma concentrations of PRL are shown in Figure 1 (upper left panel). The treatment with Q suppressed PRL concentrations but with notable inter-subject variability. The statistical analysis of the PRL profiles revealed a significant ( $P<0.05$ ) time by treatment interaction. PRL concentrations were significantly ( $P<0.05$ ) decreased at weeks 4-24 (throughout the treatment period) in the T1200+Q and T800+Q groups, compared with the T1200+placebo group. The mean PRL concentrations for the treatment period were as follows:  $43.5\pm 18.6$ ,  $52.6\pm 21.4$  and  $162.3\pm 14.9$  mU/L for the T1200+Q, T800+Q and T1200+placebo groups, respectively. There was no significant difference in the PRL concentrations between the two groups that received Q. PRL was suppressed by at least 80% (compared to pre-treatment values) in 9/12 (75%) subjects in the T1200+Q group, and in 9/13 (69%) subjects in the T800+Q group. The remaining subjects suppressed to a mean

47.73±8.26% (range 11.2-72.2%). There was no disclosed non-compliance to explain this variability in PRL suppression.

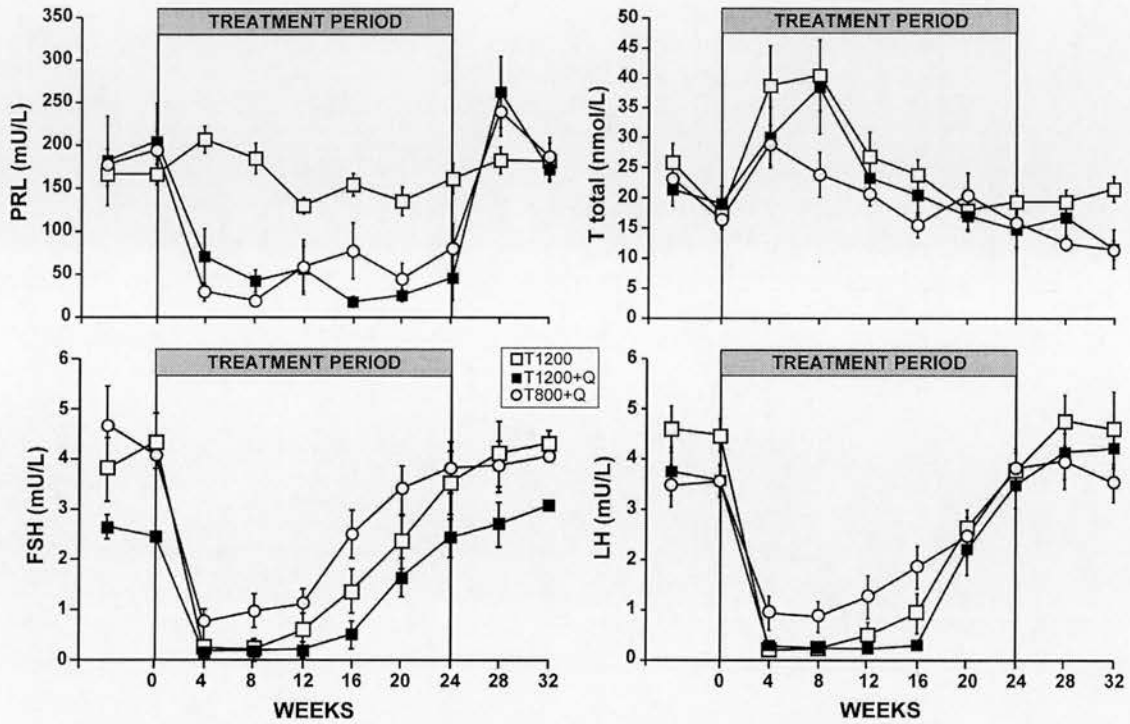
There was also evidence that T alone increased PRL secretion. In the T 1200+placebo group, plasma PRL concentrations increased significantly ( $p<0.05$ ) relative to pre-treatment values at week 4 following insertion of the T implants. In the T 1200+Q and T800+Q groups, PRL concentrations also increased significantly ( $p<0.05$ ) above the placebo group at week 28, as a rebound response to cessation of Q.

T Implantation. After insertion of the T implants, total T concentration increased above baseline for 4-8 weeks in all groups (Figure 1, upper right panel). The analysis of the total T concentration in blood plasma showed a significant ( $p<0.001$ ) effect of time but no statistical differences between groups, although plasma concentrations of T were lower in the T800 group.

### **Gonadotrophin suppression**

Plasma LH and FSH concentrations were markedly suppressed by the T treatment in all groups (Figure 1, lower panels). Concentrations were lowest at week 4 and remained suppressed until week 12 before increasing to pre-treatment values by week 24. The analysis revealed a significant ( $p<0.001$ ) time by treatment interaction; the plasma LH and FSH concentrations were significantly ( $p<0.05$ ) lower in both T1200 groups compared with the T800+Q group from weeks 4-16. There was no significant difference in gonadotrophin concentrations between the T1200+ placebo and the T 1200+Q groups during the phases of suppression and recovery.

**Figure 1: Plasma Hormone concentrations (group means)**



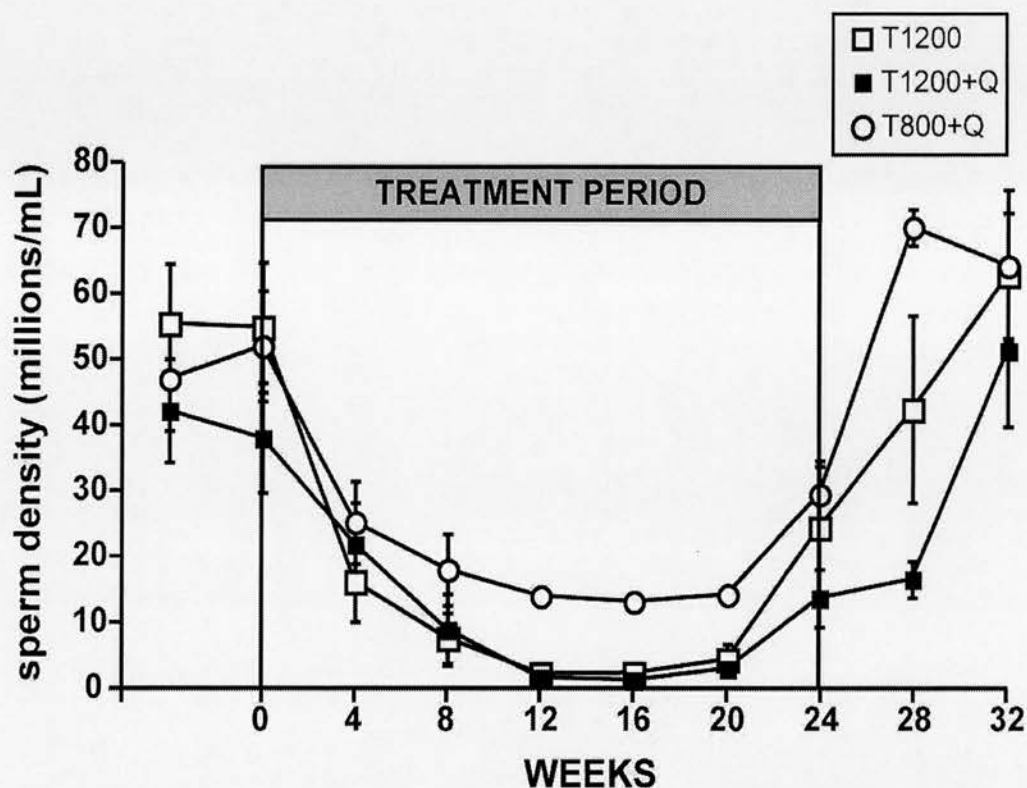
**Figure 1.** Long term changes in the blood plasma concentrations of prolactin (PRL), testosterone (T), FSH and LH in groups of male volunteers treated with T implants with either quinagolide (Q) or placebo as follows: i) T1200mg+daily oral placebo (open square symbol), ii) T1200mg + 75 $\mu$ g daily oral Q (closed square symbol), iii) T800mg+75 $\mu$ g daily oral Q (open round symbol). The T implants were inserted once only at week 0 and the Q treatment was continued for 24 weeks (horizontal bar – treatment period). Values are mean  $\pm$  SEM. n=13, 12, 13 for the 3 groups respectively.

### **Sperm suppression and recovery**

Pre-treatment sperm concentrations in the ejaculate were similar in the 3 treatment groups (group means:  $55.19 \pm 8.09$ ,  $40.62 \pm 6.97$ ,  $51.08 \pm 9.36$  millions/ml for T1200+placebo, T1200+Q and T800+Q respectively) (Figure 2). Sperm concentration fell sharply in all groups by week 4 of treatment and continued to decline until weeks 12-16 before increasing by week 24. Sperm concentrations returned to pre-treatment values by weeks 28-32. The analysis demonstrated a significant ( $p < 0.001$ ) time by treatment interaction, with sperm concentrations significantly ( $p < 0.05$ ) reduced in both T1200 groups, compared with the T800+Q group from weeks 12-20. There was no significant difference in sperm concentrations between the T1200+placebo and the T1200+Q groups during the phases of suppression and recovery.

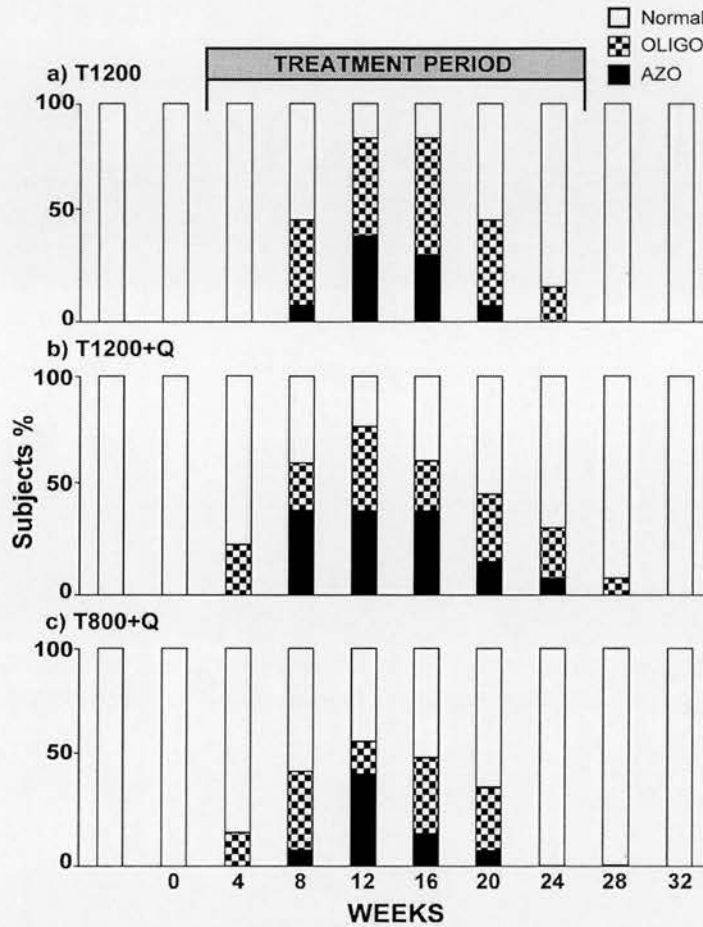
The proportion of subjects in each group who achieved severe oligospermia/azoospermia (sperm concentration  $< 1$  million/ml) for at least 4 weeks was 11/13 (85%), 11/12 (92%), 8/13 (61.5%) in T1200+placebo, T1200+Q and T800+Q groups respectively. The corresponding numbers achieving azoospermia were 5/13 (38%), 6/12 (50%), 5/13 (38%). The group profiles for the incidence of oligospermia and azoospermia are summarised in Figure 3. Inhibition was maximal at weeks 8-16 with a similar pattern in the 3 treatment groups; the incidence of sustained oligo/azoospermia was least in the T800+Q group. In this group, the subjects in which PRL secretion was maximally inhibited (suppression  $> 80\%$  of pre-treatment,  $n=9$ ), the degree of spermatogenic suppression was notably greater than in those subjects in whom PRL was inadequately suppressed ( $n=4$ ). Severe oligospermia/azoospermia was achieved in 78% of the PRL inhibited group but only

Figure 2: sperm density (group means)



**Figure 2.** Long term changes in sperm density in the ejaculate of groups of male volunteers treated with T implants with either quinagolide (Q) or placebo as follows: i) T1200mg+daily oral placebo (open square symbol), ii) T1200mg + 75 $\mu$ g daily oral Q (closed square symbol), iii) T800mg+75 $\mu$ g daily oral Q (open round symbol). The T implants were inserted once only at week 0 and the Q treatment was continued for 24 weeks (horizontal bar – treatment period). Values are mean  $\pm$  SEM. n=13, 12, 13 for the 3 groups, respectively

**Figure 3: Group sperm suppression profiles**



**Figure 3.** Percentage of subjects achieving severe oligospermia (sperm concentration in the ejaculate <1million/ml – hatched histogram) and azoospermia (absence of sperm in ejaculate – filled histogram), in groups of male volunteers treated with T implants with either quinagolide (Q) or placebo as follows: (a) T1200mg+daily oral placebo, (b) T1200mg + 75µg daily oral Q and (c) T800mg+75µg daily oral Q. The T implants were inserted once only at week 0 and the Q treatment was continued for 24 weeks (horizontal bar – treatment period).

25% in the poorly inhibited group; this apparent association between PRL and sperm production was not evident in the T 1200+Q group.

Progressive motility (WHO categories, a+b) was unaffected by treatment in the 3 groups. Ejaculate volume was marginally decreased in the 3 treatment groups from weeks 12-20 (range 8-29%) with no difference between groups. Semen pH was unaffected by treatment.

### **Metabolic, haematological and general effects of treatment**

Blood plasma concentration of HDL-cholesterol was decreased at week 12-20 during treatment with no significant differences between the 3 groups. Concentrations had not returned to pre-treatment values by the end of the recovery period. Total cholesterol, triglycerides and LDL-cholesterol concentrations were not affected by the treatments. Body weight, blood pressure, pulse, liver function tests (albumin, AST, ALT, Alkaline phosphatase and albumin), glucose metabolism (fasted blood glucose, HbA1c) and renal function (electrolytes, urea, creatinine) were also unaffected by the treatments. Haemoglobin concentrations were significantly ( $p < 0.01$ ) increased at week 12 in all groups, in parallel with experimentally induced changes in T, but were otherwise unaffected. Other haematology indices (white blood cell count, platelets) were unaffected. Prostate examination revealed no changes related to any treatment.

## **DISCUSSION**

The aim of this study was to establish whether suppression of PRL enhances the effectiveness of exogenous T in inhibiting sperm production in normal men. This was based on the potential progonadal effects of PRL, and the presumption that the

simultaneous suppression of both PRL, and the classical gonadotrophins LH and FSH, would induce more complete spermatogenic arrest. The overall results do not support the hypothesis. The degree of spermatogenic arrest and the period of induced azoo/oligospermia was similar in the T1200 and T1200+Q groups, and the effects in the T800+Q group were comparable to those previously reported for men receiving this dose of T alone (Handelsman et al., 1992). There was therefore, no significant effect on the spermatogenic profiles associated with the chronic treatment with our selected dose of quinagolide.

The current experiment was designed to compare treatments with and without quinagolide in the presence of a standardised high dose of T (T1200mg). This was based on the expectation that this level of T would produce sub-maximal sperm suppression and allow us to detect an additional effect due to Q. However, the T1200mg treatment alone in this study produced a very high degree of sperm suppression, leaving little scope to observe an additional effect of Q during the treatment phase. We therefore looked for an effect of the PRL manipulation on the pattern of recovery at the time when the T implants were becoming exhausted (weeks 12-24) and daily quinagolide treatment continued. Again, there was no significant difference between the T1200+placebo and T1200+Q groups in this pattern of recovery, although the T1200+Q subjects were the last to return to pre-treatment values, and there was a period at weeks 24-28 where there was no overlap in sperm concentrations between the two groups that is suggestive of a marginal effect. The only evidence that the Q treatment may have had some androgen dose-sparing effect was obtained from the T800+Q group. In this group, there was marked individual variation in the degree of inhibition of PRL and degree of spermatogenic suppression.

In the subjects in which PRL secretion was maximally inhibited (suppression >80% of pre-treatment, n=9), the degree of spermatogenic suppression was greater than in those subjects in whom PRL was inadequately suppressed (n=4). Severe oligospermia was achieved in 78% of the PRL inhibited group, which was comparable with 85% for the T1200+placebo group. This provides minimal support for the view that only in partial hypogonadotrophic states when gonadotrophin concentrations are sub-maximally reduced (as in the T800 group), is it possible to observe any concomitant effect of PRL withdrawal. A subtle interactive effect of PRL and gonadotrophin status has previously been described in animal models where the effects LH and FSH on testicular physiology always predominate (Bartke, 1999; Lincoln et al., 2001).

The unexpected finding in our trial was that the oral quinagolide was not fully effective at blocking PRL and there was marked variability in the degree of PRL suppression between subjects in both the T800+Q and T1200+Q groups. This was despite the findings of the short pilot study clearly showing that oral quinagolide at 75 $\mu$ g/d markedly suppressed blood PRL concentrations towards the minimum assay detection limit. There are several possible explanations to account for this anomaly. The first is that the subjects failed to comply with the experimental protocol of taking daily quinagolide. However, monthly tablet returns and rigorous questioning at the monthly clinical monitoring did not suggest a significant level of non-compliance. Given that the elimination half-life of quinagolide is 17.5 hours (Brownell et al., 1995), the omission of occasional tablets is unlikely to have had a significant long-term effect on PRL control. Moreover, at the end of the treatment period, there was a notable increase in PRL concentrations in all subjects that received Q. This is a rebound response to chronic dopamine receptor agonist withdrawal and further

supports our contention that subject compliance was good. The second explanation is that chronic quinagolide treatment resulted in the development of refractoriness to PRL inhibition. Quinagolide is routinely used clinically in the treatment of hyperprolactinaemia in both men and women without clear evidence of long-term loss of responsiveness (Homburg et al., 1990; Schultz et al., 2000). Such refractoriness, however, may obtain in the suppression of PRL in men with normal physiological concentrations of PRL (Rana et al., 1995).

The third, and perhaps most important factor, is that the T treatments themselves activated PRL secretion, thus rendering the quinagolide less effective. Previous work has shown that plasma PRL concentrations are significantly increased in men treated with T enanthate injections (Bellis et al., 1998). In the current study, blood PRL concentrations were significantly increased in the placebo treated subjects 4 weeks after the administration of the 1200mg T implants. Furthermore, it is well known from pituitary cell culture studies using animal tissues that both testosterone and oestradiol potently stimulate PRL synthesis and release due to a direct genomic effect on the lactotroph (Shull et al., 1985; Lambert and McLeod, 1990). This can occur independently of the inhibitory effect of dopamine receptor activation. Clinical studies also support the view that sex steroid status affects PRL secretion. For example, the efficacy of bromocriptine in the suppression of PRL secretion is reduced in hyperprolactinaemic women during pregnancy when oestradiol concentrations are increased and PRL secretion decreases in the menopause and after pregnancy when sex steroid concentrations decline (Karunakaran et al., 2001). Such steroid effects on PRL secretion in our trial subjects would render quinagolide inhibition of PRL less

effective. These effects of chronic treatment in normal men were not anticipated, but clearly compromised the objective of this study.

A final point is that quinagolide was well tolerated with no serious adverse effects reported. Prolactin acts in multiple target tissues including brain, liver, skin, prostate and accessory glands. Despite this, chronic PRL suppression using quinagolide had no demonstrable effects on semen volume, sexual behaviour and a wide range of haematological and biochemical indices demonstrating desirable specificity of action on the reproductive axis. Treatment with T produced the expected effects on HDL-C (Wallace and Wu, 1990), but this was not blocked or enhanced by the manipulation of PRL. There were a number of casual observations on the effect of chronic PRL suppression. The incidence of acne was lower than expected and two subjects noted an improvement in the condition of their skin and hair.

## **Conclusions**

The current study investigated for the first time whether manipulation of PRL would enhance the efficacy of T in the suppression of spermatogenesis in men. The results did not support this idea. Unexpectedly it proved difficult to achieve consistent long-term inhibition of PRL secretion in the men. Future studies will need to establish a means of totally blocking PRL release, or to utilise a cross-over design in which all subjects receive both PRL inhibitor and placebo, to test whether concomitant T and PRL blockade will induce complete spermatogenic arrest. The idea of employing lower levels of T, and reducing PRL due to its trophic effects on the prostate, make a combined preparation of this kind an attractive prospect in the regulation of male fertility.

## References

- Anderson, R.A., Wallace, E.M. and Wu, F.C.W. (1996)** Comparison between testosterone enanthate induced azoospermia and oligospermia in a male contraceptive study III. Higher 5-alpha reductase activity in oligospermic men administered supraphysiological doses of testosterone. *J. Clin. Endocrinol. Metab.* **81**, 902-908.
- Bartke, A., Croft, B.T. and Dalterio, S. (1975)** Prolactin restores plasma testosterone levels and stimulates testicular growth in hamsters exposed to short day-length. *Endocrinology* **97**, 1601-1604.
- Barke, A. (1999)** Role of growth hormone and prolactin in the control of reproduction: what are we learning from transgenic and knockout animals? *Steroids* **64**, 598-604.
- Bebb, R.A., Anawalt, B.D., Christensen, R.B., Paulsen, C.A., Bremner, W.J. and Matsumoto, A.M. (1996)** Combined administration of levonorgestrel and testosterone induces more rapid and effective suppression of spermatogenesis than testosterone alone: a promising male contraceptive approach. *J. Clin. Endocrinol. Metab.* **81**, 757-762.
- Behre, H.M., Baus, S., Klesch, S., Keck, C., Simoni, M. and Nieschlag, E. (1995)** Potential of testosterone buciclate for male contraception: endocrine differences between responders and non-responders. *J. Clin. Endocrinol. Metab.* **80**, 2394-2403.

**Bellis, A and Wu, F.C.W. (1998)** Prolactin levels increase as a result of administration of a combination of testosterone enanthate and oral desogestrel as a contraceptive regime in normal men. IV Europ. Congr. Endocrinol. Abstract P1-296.

**Bex, F.K and Bartke, A. (1977)** Testicular LH binding in the hamster: modification by photoperiod and prolactin. *Endocrinology*. **100**:1223-1226

**Brownell, J, van der Lely, A.J. and Lamberts, S.W.J. (1996)** The treatment of hyperprolactinaemia. In J Brownell (ed) *Norprolac (quinagolide): an update*. Medical Forum International, Zeist, The Netherlands.

**Corker, C.S. and Davidson, D.W. (1978)** Radioimmunoassay of testosterone in various biological fluids without chromatography. *J. Steroid Biochem.* **9**,373-374.

**Hair, W.M., Gubbay, O., Jabbour,H. and Lincoln, G.A. (2002)** Prolactin receptor expression in human testis and accessory tissues: localization and function. *Mol. Hum. Reprod.* **8**, 606-611.

**Hair, W.M., Wu, F.C.W. and Lincoln, G.A (2000)** Subcutaneous testosterone combined with the prolactin inhibitor quinagolide: a novel hormonal contraceptive regime in men. *Int. J. Androl.* **23**, 30, P051.

**Handelsman, D.J., Conway, A.J. and Boylan, L.M. (1992)** Suppression of human spermatogenesis by testosterone implants. *J. Clin. Endocrinol. Metab.* **75**,1326-1332.

- Handelsman, D.J., Conway, A.J., Howe, C.J., Turner, L. and Mackey, M.A. (1996)** Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J. Clin. Endocrinol. Metab.* **81**, 4113-4121.
- Hondo, E., Kurohmaru, M., Sakai, S., Ogawa, K. and Hatashi, Y. (1995)** Prolactin receptor expression in rat spermatogenic cells. *Biol. Reprod.* **52**, 1284-1290.
- Homburg, R., West, C., Brownell, J. and Jacobs H.S. (1990)** A double-blind study comparing a new non-ergot long acting dopamine agonist CV205-502 with bromocriptine in women with hyperprolactinaemia. *Clin. Endocrinol.* **32**, 565-571.
- Huhtaniemi, I., Parvinen, M Venho, P. and Rannikkos, S.(1991).** Combination of a GnRH agonist with an antiandrogen or bromocriptine in the treatment of prostatic cancer; slight potential for antigonadal effects. *Int. J. Androl.* **14**: 374-386.
- Jabbour,H.N. and Lincoln,G.A. (1999).** Prolactin receptor expression in the testis of the ram: localisation, functional activation and the influence of gonadotrophins. *Mol. Cell Endocrinol.*, **148**, 151-161.
- Karunakaran, S., Page, R.C.L. and Wass, J.A.H. (2001)** The effect of the menopause on prolactin levels in patients with hyperprolactinaemia. *Clin. Endocrinol.*, **54**, 295-300.

**Kline, J.B., Roehrs, H and Clevenger, C.V. (1999)** Functional characterization of the intermediate isoform of the human prolactin receptor. *J. Biol. Chem.*, **274**, 35461-35468.

**Lambert, W.J. and Mcleod, R.,M. (1990)** regulation of prolactin secretion at the level of the lactotroph. *Physiol. Rev.*, **70**, 279-305.

**Lincoln, G.A., Clarke, I.J. and Sweeney, T. (1996)** Hamster-like cycles in testicular size in the absence of gonadotrophin secretion in HPD rams exposed to long-term changes in photoperiod and treatment with melatonin. *J. Neuroendocrinol.*, **8**, 855-866.

**Lincoln, G.A., Townsend, J. and Jabbour,H.N. (2001)** Prolactin actions in the sheep testis: a test of the priming hypothesis. *Biol. Reprod.*, **65**, 936-943.

**Martin, C.W., Riley, S.C., Everington, D., Groome, N.P., Riemersa, R.A., Baird, D.T. and Anderson, R.A. (2000)** Dose-finding study of oral desogestrel with testosterone pellets for suppression of the pituitary testicular axis in normal men. *Hum. Reprod.* **15**, 1515-1524.

**Meriggiola, C.A., Bremner,W.,J., Paulsen, C.A., Valdiserri, A., Incorvaia, L., Motta, R., Pavani, A., Capelli, M. and Flamigni, C. (1996)** A combined regimen of cyproterone acetate and testosterone enanthate as a potentially highly effective male contraceptive. *J. Clin. Endocrinol. Metab.*, **81**, 3018-3023.

**Ouhtit, A., Morel, G. and Kelly, P.A. (1993)** Visualisation of gene expression of short and long forms of prolactin receptors in rat reproductive tissues. *Biol. Reprod.*, **49**, 528-536.

**Pearlstein, T.B. (1995)** Hormones and depression: what are the facts about premenstrual syndrome, menopause and hormone replacement therapy? *Am. J. Obstetrics Gynecol.*, **173**, 646-653.

**Rana, A., Habib, F.K., Halliday, P., Ross, M., Wild, R., Elton, R.A. and Chisholm, G.D. (1995)** A case for synchronous reduction of testicular androgen, adrenal androgen and prolactin for the treatment of advanced carcinoma of the prostate. *Eur. J. Cancer*, **31A**, 871-875.

**Santner, S.J., Albertson, B., Zhang, G.Y., Zhang, G.H., Santulli, M., Wang, C., Demers, L.M., Shackelton, C. and Santen, R.J. (1995)** Comparative rates of androgen production and metabolism in Caucasian and Chinese subjects. *J. Clin. Endocrinol. Metab.*, **83**, 2104-2109.

**Schultz, P.N., Ginsberg, L., McCutcheon, I.E., Samaan, N., Leavens, M and Gagel, R.F. (2000)** Quinagolide in the management of prolactinoma. *Pituitary*, **3**, 239-49.

**Shull J.D. and Gorski J. (1985)** Estrogen regulates the transcription of the rat prolactin gene in vivo through at least two independent mechanisms. *Endocrinology*, **116**, 2456-2462.

**Sundaram, K., Kumar, N. and Bardin, C.W. (1993)** 7-alpha-methyl-nortestosterone (MENT): the optimal androgen for male contraception. *Ann. Med.*, **25**, 199-205.

**Ufearo, C.S. and Orisakwe, O.E. (1995).** Restoration of normal sperm characteristics in hypoprolactineamic infertile men treated with metaclopramide and exogenous prolactin. *Clin. Pharmacol. Therapeut.*, **58**, 354-359.

**Wahlstrom, T., Huhtaniemi, I., Hovatta, O., and Seppalla, M. (1983)** Localization of luteinizing hormone, follicle-stimulating hormone, prolactin, and their receptors in human and rat testis using immunohistochemistry and radioreceptor assay. **57**: 825-830.

**Wallace, E.M. and Wu, F.C.W. (1990)** Effect of medroxyprogesterone acetate and testosterone enanthate on serum lipoproteins in man. *Contraception*, **41**, 63-71.

**Wang, C., Berman, N.G., Veldhuis, J.D., Der, T., McDonald, V., Steiner, B. and Swerdloff, R.S. (1998)** Graded testosterone infusions distinguish gonadotrophin negative feedback responsiveness in Asian and white men: a clinical research center study. *J. Clin. Endocrinol. Metab.*, **83**, 870-876.

*World Health Organization Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 1999* 4<sup>th</sup> ed. Cambridge: Cambridge University Press

**World Health Organization Task Force On Methods For The Regulation of Male Fertility. (1990)** Contraceptive efficacy of testosterone induced azoospermia in normal men. *Lancet*, **336**, 955-959.

**Wu, F.C.W., Balasubramanian, R., Mulders, T.M.T., Coelingh-Bennink, H.J. (1999)** Oral progestagen combined with testosterone as a potential male contraceptive: additive effects between desogestrel and testosterone enanthate in suppression of spermatogenesis, pituitary-testicular axis, and lipid metabolism. *J. Clin. Endocrinol. Metab.*, **84**, 112-122.

**Zhengwei, Y., Wreford, N.G., Royce, P., deKretser and McLachlan, R.I. (1998)** Stereological evaluation of human spermatogenesis by testosterone treatment: heterogeneous pattern of spermatogenic impairment. *J. Clin. Endocrinol. Metab.*, **83**, 1284-1291.

## Chapter 4

### **A novel male contraceptive pill-patch combination: oral desogestrel and transdermal testosterone in the suppression of spermatogenesis in normal men.**

#### **Abstract**

This study investigated the effect of transdermal testosterone and oral desogestrel on the reproductive axis and extra-gonadal metabolism of healthy men. 23 men were randomised to one of three treatment groups and received a daily transdermal testosterone patch plus oral desogestrel at a dose of 75 $\mu$ g, 150 $\mu$ g or 300 $\mu$ g/day for 24 weeks. Baseline blood and semen samples were obtained and then 4 weekly thereafter for 32 weeks. The outcome measures were sperm density, plasma levels of FSH, LH, total and free T and lipids. The results show dose dependent suppression of spermatogenesis and gonadotrophins. Seven of the seventeen subjects became azoospermic. Desogestrel 300 $\mu$ g daily, in combination with 5mg daily transdermal T, was the most effective (57% azoospermic) while 75 $\mu$ g was ineffective (0% azoospermic). Total and free plasma T were reduced by approximately 50%. HDL-cholesterol was significantly reduced. No serious side effects were encountered. We conclude that daily self – administered desogestrel with transdermal T is capable of suppressing the male reproductive axis although the efficacy was less marked and less consistent than injectable regimes. The lower efficacy is likely to be due to failure of the transdermal T system to maintain circulating T levels consistently in the required range.

## **Introduction**

The principle that exogenous sex steroid-induced oligo- and azoospermia can confer effective, reversible contraceptive protection in men was established in two multi-centre trials employing an androgen-only prototype regime of intramuscular (i.m.) injections of testosterone (T) enanthate (WHO Task Force 1990, 1995). The pharmacokinetics of T enanthate are such that a relatively high dose (200mg), administered at weekly intervals, is required to ensure maximum suppression of gonadotrophins and spermatogenesis (Schearer et al., 1978). This regimen produced repeated supraphysiological peaks and markedly fluctuating levels of T (Anderson et al., 1995), which induced significant extra-gonadal androgenic effects on lipid metabolism, skin, muscle, liver and haemopoiesis (Wu et al., 1996). These unwanted effects, coupled with the impracticality of uncomfortable weekly i.m. injections, underline the need to use lower doses of testosterone with more stable delivery in hormonal male contraception.

Spermatogenesis can be effectively suppressed by combining a reduced dose of testosterone with a second anti - gonadotrophic agent such as a progestagen (Handelsman et al., 1996) or GnRH antagonist (Tom et al., 1992). It has been previously reported that i.m. T enanthate at the relatively low doses of only 100mg or 50 mg weekly, if combined with a 19-nortestosterone derived oral synthetic progestin, desogestrel (DSG), can suppress spermatogenesis very effectively in men; the best dose combination, 300µg DSG daily with 50 mg of testosterone enanthate weekly, induced consistent azoospermia in all subjects (Wu et al., 1999). This study also showed that DSG and testosterone both contributed additively and interchangeably to the reproductive as well as the non-reproductive metabolic effects e.g. reduction of HDL-cholesterol and SHBG levels. T enanthate 50 mg i.m. weekly, delivering 5mg of free T daily, is regarded as the minimal effective dose and also the minimal dose required to maintain androgen sufficiency. Although it can be surmised that lower

doses of DSG or alternate formulations of T, which can reproduce stable physiological (rather than sharply - fluctuating) levels, may reduce these unwanted actions, the minimally effective dose combination is currently unknown.

Transdermal T delivery systems have been recently developed for use as a non-invasive method of androgen replacement therapy in hypogonadal men (Miekle 1998, Miekle et al., 1992). Daily self - application of these systems offer the potential of maintaining stable levels of testosterone within the normal range with a small diurnal fluctuation which closely mimics the physiological pattern. Transdermal T systems has not been studied in male contraception.

To date, few male contraceptive studies have employed an entirely subject-administered regime. Since this is likely to be preferred by the majority of potential users, it is important to determine if daily self-administration, independent of provider or research personnel, can still be effective. This will give important clues about the tolerance or margin of safety in hormonal male contraceptive regimes against a backdrop of the varying levels of compliance which will inevitably be encountered with preventative medications across any population.

We have conducted a study which employs a novel, non-invasive, daily, self-administered treatment regimen to effect reversible suppression of spermatogenesis in healthy male volunteers. The specific aims of the study were:

1. To evaluate the effects of oral desogestrel combined with a non-scrotal transdermal testosterone delivery on gonadotrophin secretion and spermatogenesis.

2. To compare the effects of reducing doses of desogestrel combined with a fixed dose of T designed for physiological androgen replacement.
3. To determine the minimally effective contraceptive combination and the tolerance for breakthrough of suppression in this self-administered regimen
4. To assess the non-reproductive effects of these combinations in men.

## **Materials and Methods**

### *Subjects*

Of 101 respondents to our advertisements, 33 were suitable for screening. After the initial interview and screening tests, 10 were excluded because of low sperm counts (n = 5) or high cholesterol (n=5) leaving 23 Caucasian men, mean age  $34.2 \pm 7.0$  year (range 20 - 43) to take part in the study.

### *Study Design*

Subjects who met the admission criteria similar to our previous study were randomised into one of three treatment groups to receive: 1) oral DSG 300 $\mu$ g daily and transdermal T 5mg daily (n=7), 2) oral DSG 150 $\mu$ g daily and transdermal T 5mg daily (n=6), or 3) oral DSG 75 $\mu$ g daily and transdermal T 5mg daily (n=4) for 24 weeks in a single blind parallel group design.

Subjects were studied in three phases: 1) Control phase: medical screening examination, two baseline semen analyses and hormonal and biochemical assessments were carried out over 4 weeks; 2) Treatment phase: each subject was randomly allocated to one of the three treatment groups described above. Both transdermal T and DSG were administered for twenty-four weeks. Medical review including

physical examination, blood sampling and semen analyses were performed every four weeks. 3) Recovery phase: all subjects were monitored every 4 weeks by medical review, semen analysis and blood sampling until recovery criteria were satisfied namely; geometric mean pre-treatment sperm density was reached or two consecutive specimens showed sperm density greater than 20 million/ml.

All subjects provided informed written consent and were advised to continue with their existing forms of contraception during the study. The study was approved by the Central Manchester Healthcare Trust ethical committee for medical research.

### *Medications*

Desogestrel (75µg and 150µg tablets) were supplied by NV Organon, Oss, Netherlands. Each subject received one tablet per day in the case of the 75µg and 150µg or two 150µg tablets in the case of the 300µg/day group. Tablets were taken in the evening before bed. Testosterone was administered by the volunteers as two transdermal delivery systems (Andropatch, SKB, Welwyn Garden City, England) applied to skin of the upper back, legs or flanks, changed daily at bedtime, employing a different site at each application.

### *Clinical Monitoring*

Subjects were interviewed monthly with particular emphasis on eliciting any side effects and monitoring sexual function. Body weight, pulse and blood pressure were measured monthly and testicular size (by orchidometer) measured 3-monthly. A digital prostate examination was carried out pre-treatment and on recovery.

### *Semen analysis*

Semen collection and analysis of semen volume, sperm density and motility were carried out according to the WHO Laboratory Manual for the Examination of Human Semen and sperm-cervical mucus interaction 3<sup>rd</sup> edition 1993. The suppression targets are defined as:- azoospermia - complete absence of spermatozoa in the ejaculate verified by centrifugation of a whole semen sample; severe oligospermia - sperm concentration of less than 1 million/mL; oligospermia - sperm concentration of less than 3 million/mL in one sample.

### *Blood Tests*

Blood samples were obtained twice pre-treatment and at four-weekly intervals thereafter for hormone measurements (testosterone, LH, FSH, sex hormone binding globulin) and haematological (haemoglobin, haematocrit, and white cell count), biochemical (urea, electrolytes, liver enzymes, glucose, and haemoglobin A1c) and lipid profiles (total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglyceride and Apolipoprotein A1).

### *Hormone Assays*

All plasma samples were stored at -20 C until assay. Plasma gonadotrophins were assayed by previously reported highly sensitive immunofluorimetric assays (Delfia, Pharmacia-wallac, Turku, Finland (Miekle et al., 1992) with an assay sensitivity of 0.05 IU/ml for both LH and FSH. Testosterone was determined by previously described RIA with an assay sensitivity of 0.3nmol/L (Wu et al., 1991). SHBG was determined by an immunoradiometric assay (Farnos Diagnostica, Oulun Salo, Finland). Free Testosterone was kindly measured in an external laboratory by Dr

Christina Wang, using an established equilibrium dialysis technique (Wang et al., 1998). All serial samples from one individual were assayed in a single batch to reduce variability.

### *Biochemical analyses*

Full blood count, glucose, haemoglobin A1c, lipids (total cholesterol, HDL-C, and triglyceride) and renal and liver function was measured by routine autoanalyser methods. LDL-C was derived from the other lipid indices using Friedwalds formula.

### *Statistical analyses*

The data were analysed by repeated measures ANOVA, paired *t* tests, one-way ANOVA with Tukeys's *post-hoc* test for continuous variables with statistical significance set at  $p < 0.05$ . Values were expressed as the arithmetical mean  $\pm$  SEM. LH and FSH below the sensitivity of the assay were allocated a value of 0.05 U/L, the lower limit of detection.

## **Results**

### *Spermatogenesis*

Seventeen subjects completed the suppression phase and six subjects were discontinued (See below). The mean sperm densities before, during and after DSG and transdermal T administration in each of the three treatment groups are shown in Fig. 1a. The rates of suppression to 3 target sperm densities i.e. azoospermia (no sperm), severe oligospermia ( $< 1$ million/mL) and oligospermia ( $< 3$ million/mL) are shown in fig. 1b. Sperm density was significantly ( $P < 0.05$ ) reduced in all treatment groups with respect to baseline showing a clear dose – dependent although the

differences between treatment groups failed to achieve significance. Seven (41%) of the seventeen subjects who completed the suppression phase achieved azoospermia. 9 (53%) of the seventeen achieved suppression to less than 1 million/mL with no additional subjects achieving suppression to less than 3 million/mL. The most effective drug regimen was DSG 300 $\mu$ g and 5mg transdermal T daily in which four (57%) of the seven subjects achieved azoospermia, the earliest by week 8 and the remainder by week 16. One further subject suppressed to less than 1 million/mL by week 12 giving a total of 71% for this group. In the DSG 150 $\mu$ g group, 3 (50%) of the 6 subjects achieved azoospermia, the earliest by week 8, the others by week 12 whilst the remainder failed to suppress to oligospermia. In the DSG 75 $\mu$ g group none of the four subjects achieved azoospermia although one became severely oligospermic (<1million/mL) by week 12 while the others (75 %) remained outside the oligospermic range with sperm densities above 10 M/mL throughout treatment.

Pre – treatment sperm densities were not significantly different between the azoospermic or oligozoospermic responders and the non – suppressors.

Examination of the individual sperm density profiles in each group demonstrates marked heterogeneity of response within treatment groups (Fig. 1a). In 4 of the seven subjects who achieved azoospermia there was escape from suppression before the end of the treatment period – the others remaining fully suppressed until treatment ended. This phenomenon was also observed in one of the two subjects who became severely oligospermic. Sperm density values began to recover within four weeks of cessation of therapy. All but one of the subjects achieved the recovery criteria by week 48 (i.e. 24 weeks after end of treatment). One subject, despite normal gonadotrophins did not recover until week 64. His testosterone levels were also subnormal over a similar time course although his gonadotrophins were normal.

**Figure 1: Suppression of spermatogenesis**

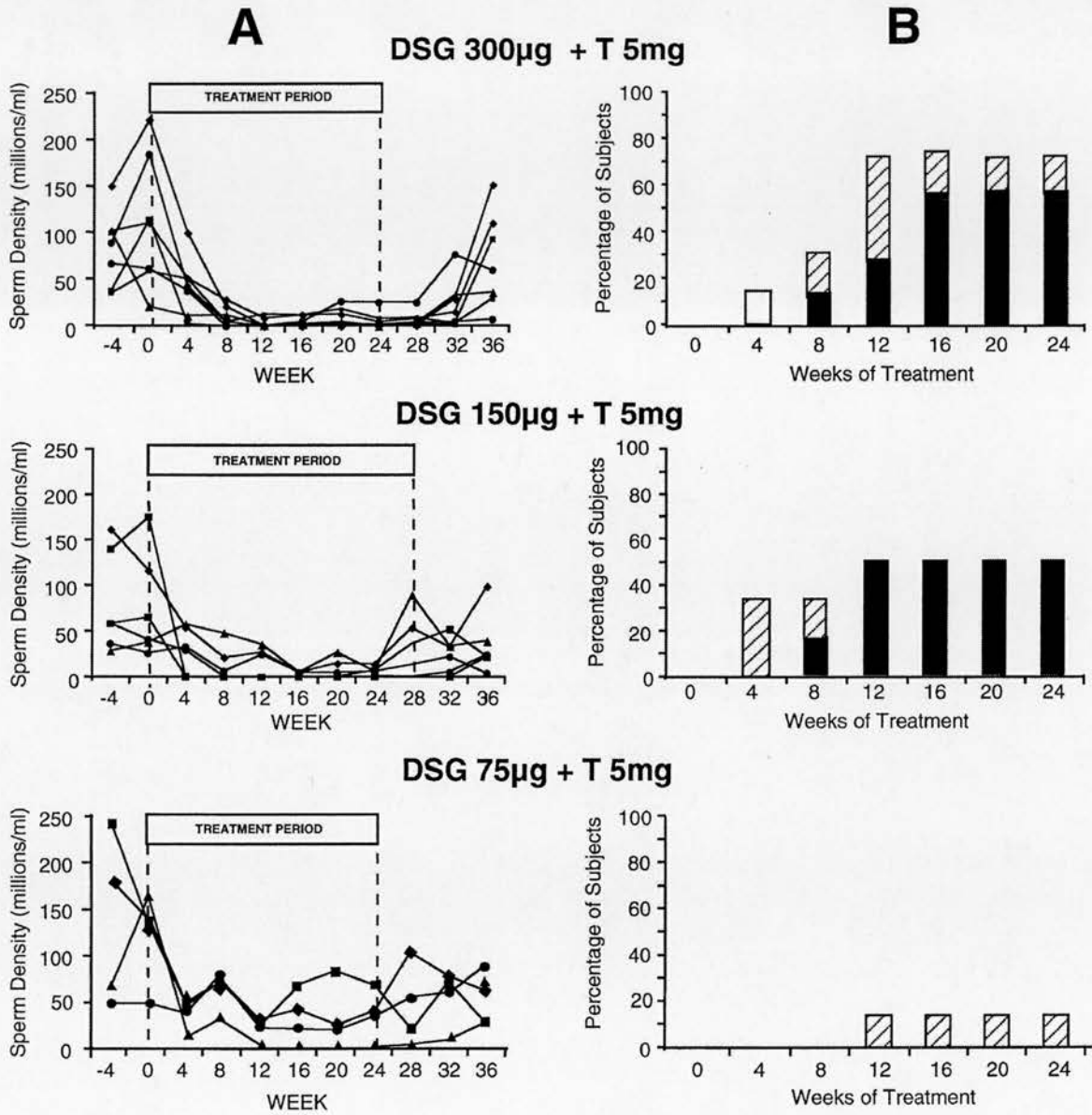


Figure 1. Suppression of spermatogenesis. Column A shows individual sperm density profiles for subjects in each of the three treatment groups at 4 week intervals during 24 weeks of treatment.

Figure 1. continued. Column B shows rates of suppression of spermatogenesis indicated by the percentage of subjects attaining azoospermia, (closed bar) oligospermia <3million/mL (open bar) and <1 million/mL (hatched bar) over the same time course. Subjects received transdermal T 5mg/day plus daily oral DSG at a dose of either 300µg or 150µg or 75µg.

## *LH*

LH levels were significantly ( $P < 0.05$ ) suppressed during the treatment phase in all treatment groups from Week 4 onwards. Suppression was dose - dependent, the most effective being in the DSG 300 $\mu$ g group although the difference between treatment groups did not achieve statistical significance (fig. 2). In the DSG 300 $\mu$ g group, LH was significantly ( $P < 0.05$ ) suppressed from a baseline mean of  $4.5 \pm 0.7$  U/L to nadir of  $0.2 \pm 0.1$  U/L (Week 8), whilst in the DSG 150 $\mu$ g group mean levels fell from  $4.5 \pm 1.0$  U/L to nadir  $0.8 \pm 0.3$  U/L (Week 4). In the DSG 75 $\mu$ g group LH levels were suppressed from baseline mean of  $4.6 \pm 1.5$  U/L to  $0.9 \pm 0.7$  U/L (Week 12). Suppression of LH was not fully maintained during continued treatment. In the 300  $\mu$ g DSG group, LH decreased to below the assay detection limit initially in four and to  $< 0.3$  U/L by week 8 in the other three subjects. Between week 8 and end of treatment, all subjects in this group showed some degree of escape, mostly transient and below 0.8 U/L, except for one which broke through to 1.6 U/L at week 16 having had undetectable LH four weeks earlier. In the 150 $\mu$ g DSG group, LH suppressed to below assay detection in four of the six subjects two of whom escaped while the other two maintained undetectable LH until end of treatment. In the 75  $\mu$ g DSG group, suppression was variable and none reached the detection limit. Pre - treatment LH was significantly higher in subjects who did not respond compared to those that suppressed to azoospermia and oligozoospermia ( $< 3$  M/mL). This was largely attributable to one unresponsive subject with a baseline LH of 9.1U/L. In all subjects LH recovered to baseline within 4 weeks after cessation of treatment. There was no significant difference in the rate of recovery in the three treatment groups

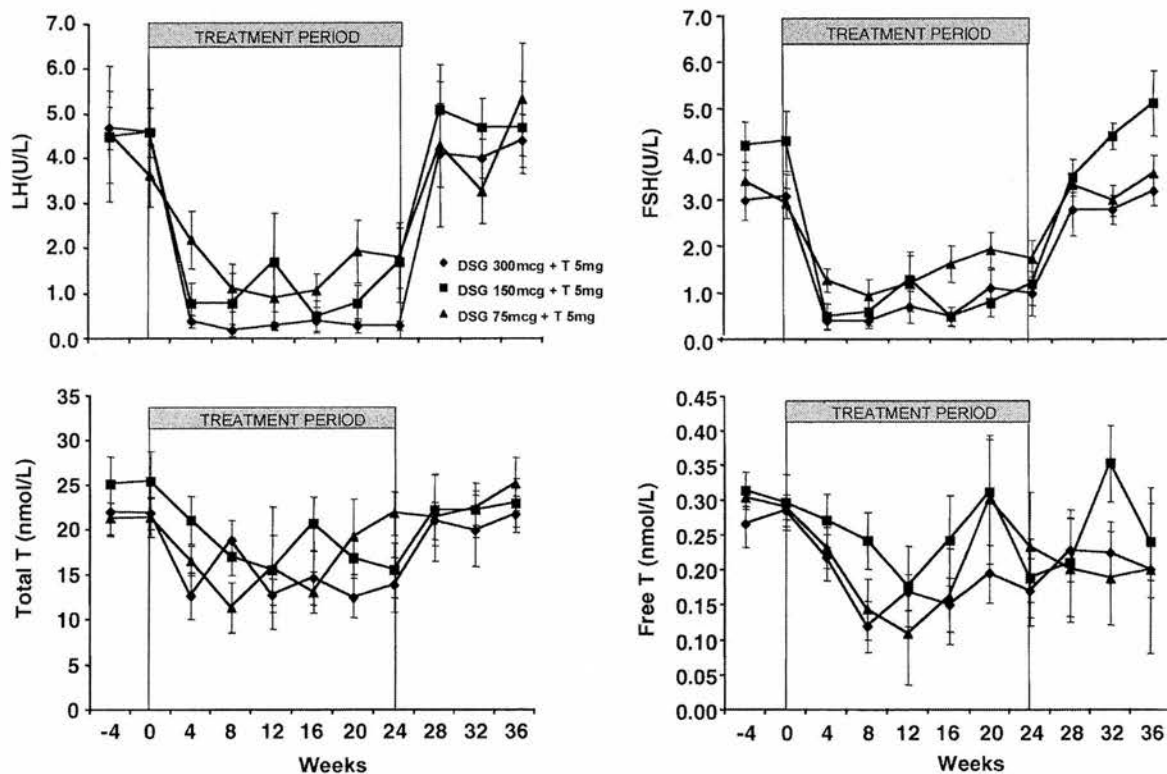
## *FSH*

FSH levels were significantly ( $P < 0.05$ ) suppressed during the treatment phase in all treatment groups. Suppression was dose - dependent, the most effective being in the DSG 300 $\mu$ g group although the difference between treatment groups did not achieve statistical significance (Figure 2). In the 300 $\mu$ g DSG group, FSH was significantly suppressed from a baseline mean of  $2.7 \pm 0.3$  U/L to a nadir of  $0.4 \pm 0.2$  U/L (Week 8) whilst in the DSG 150 $\mu$ g group, mean levels fell from a baseline of  $4.2 \pm 0.5$  U/L to a nadir of  $0.5 \pm 0.3$  U/L (Week 4). In the 75 $\mu$ g group mean FSH levels fell from a baseline of  $3.4 \pm 0.4$  to nadir of  $0.9 \pm 0.4$  (week 8). There was a consistent pattern of gradual escape from suppression after week 8 in all three groups.

In both the DSG 300 $\mu$ g and 150 $\mu$ g groups, FSH suppressed to below assay detection limits in three subjects but only one in each group could maintained the suppression until the end of treatment. In the 75 $\mu$ g DSG group, FSH suppressed to under 1.0 U/L in two subjects but only transiently.

There was no difference in pre - treatment FSH levels in subjects who suppressed to azoospermia and oligozoospermia ( $< 3$  M/mL) compared to those that did not. Mean FSH during treatment was significantly lower in azoospermic and oligozoospermic responders compared to non - responders. FSH recovered rapidly on cessation of treatment reaching normal levels by week 28 i.e. the first post-treatment assessment. There was no significant difference in the rate of recovery in the three treatment groups.

**Figure 2: Hormone data**



**Figure 2.** Hormone data: group means. Figure shows the levels of plasma FSH, LH, total and free T at 4 week intervals during the 24 week treatment period and subsequent recovery. Groups received transdermal T 5mg/day plus daily oral desogestrel at a dose of 300 $\mu$ g or 150 $\mu$ g or 75 $\mu$ g. Values are mean $\pm$ SEM. Subjects received transdermal T 5mg daily plus oral desogestrel at a dose of either 300 $\mu$ g (solid rhomboids), 150 $\mu$ g (solid squares), or 75 $\mu$ g (solid triangles).

### *Testosterone*

Total testosterone levels were significantly ( $p < 0.05$ ) decreased during the treatment phase compared to baseline in all three groups though they remained within the normal physiological range throughout the study. There was no significant difference in total T between the three groups. In the 300 $\mu$ g DSG group, T levels fell from a baseline of  $22.0 \pm 2.7$  nmol/L to treatment mean of  $14.2 \pm 2.5$  (64.5% of basal) and nadir of  $12.5 \pm 2.2$  nmol/L (week 20), whilst in the 150 $\mu$ g DSG group, levels fell from a baseline of  $25.2 \pm 3.1$  nmol/L to a mean of  $17.8 \pm 2.7$  (70.6% of basal) and a nadir of  $15.5 \pm 3.1$  nmol/L (week 24). In the 75 $\mu$ g DSG group T levels decreased from a baseline of  $21.3 \pm 1.8$  nmol/L to a mean of  $16.4 \pm 2.4$  (76.8% of basal) and a nadir of  $11.4 \pm 2.8$  nmol/L (week 8).

Free T levels also decreased significantly ( $p < 0.05$ ) during the treatment phase. In the 300 $\mu$ g DSG group levels fell from baseline mean of  $8.0 \pm 0.8$  ng/dL to treatment period mean of  $4.9 \pm 0.4$  ng/dL (61.6% of basal). In the 150 $\mu$ g DSG group free T levels fell from  $8.8 \pm 0.9$  to  $6.9 \pm 1.2$  ng/dL (78.4%) and in the DSG 75 $\mu$ g group, from  $8.6 \pm 0.4$  to  $5.7 \pm 1.0$  ng/dL (66.3% of basal). There was no difference in pre – treatment total T levels in subjects who suppressed to azoospermia and oligozoospermia ( $< 3$  M/mL) compared to those that did not. Mean T during treatment was not significantly different in azoospermic and oligozoospermic responders compared to non - responders. Total and free T levels returned to pre-treatment levels by the end of the recovery period in all groups.

### *SHBG*

SHBG levels were significantly reduced ( $P < 0.05$ ) in all treatment groups during the treatment phase although there was no significant difference between treatment group

in this regard. In the DSG 300µg group, mean levels fell from a baseline of 31.3±4.8 nmol/L to a nadir of 20.7±3.9 (week 12), whilst in the DSG 150µg group levels fell from baseline mean of 30.2±6.7 nmol/L to a nadir of 22.5±5.5 nmol/L (week 24). In the DSG 75µg group, levels fell from a baseline mean of 35±8.92nmol/L to a nadir of 27.8±6.3 nmol/L (week 12). SHBG levels returned to pre-treatment levels by the end of the recovery period in all groups.

### **Biochemical and haematological parameters**

There were no significant changes in plasma urea, creatinine, sodium, potassium, calcium, alkaline phosphatase, AST, ALT, GGT, bilirubin, glucose, HbA1c, haemoglobin, haematocrit, white cell count and platelets during treatment. (Data not shown).

### *Lipids*

HDL-cholesterol was significantly decreased ( $P<0.05$ ) with respect to baseline during treatment in all groups. In the DSG 300µg group, HDL-C fell from a baseline of 1.34± 0.10 mmol/L to a nadir of 1.11±0.06 mmol/L (week 24), a fall of 17%. In the DSG 150µg group, HDL-C fell from a baseline of 1.29±0.15mmol/L to a nadir of 0.86±0.06 mmol/L (week 16) representing a fall of 33%. In the DSG 75µg group, HDL-C fell from a baseline of 1.18±0.17 mmol/L to a nadir of 0.90±0.02 mmol/L (week 12), a fall of 24%. The differences in HDL-C suppression between treatment groups did not achieve statistical significance. Overall, the mean decrease in HDL-C levels at the end of the treatment period was 12 ±3.2%.

There were no significant changes in total cholesterol, LDL-C, triglycerides or Apoprotein A1. Total cholesterol was found to be significantly increased in the

recovery period compared to baseline – this significance disappeared on removing one outlier from the analysis. All lipid parameters returned to pre-treatment levels by the end of the recovery phase in all treatment groups.

### **Physical changes**

There was a small but significant ( $P<0.001$ ) increase of  $1.94\pm 0.56$  kg (range  $-4$  to  $+7$ kg) in body weight by the end of the treatment phase. This gain persisted, the average weight gain at the end of the recovery period being  $2.53\pm 0.67$ kg (range  $-3$  to  $+9.5$ kg). There was no significant difference between treatment groups in this regard. Testicular volume decreased by an average of  $3.3\pm 1.1$ ml (range  $0-7.25$ ml) during treatment and returned to pre-treatment values by the end of the recovery phase. There were no significant changes in systolic or diastolic blood pressure throughout the study.

### **Discontinuations and side effects.**

There were six discontinuations - two due to marked and persistent skin reaction to the T patches. The remaining subjects were either lost to follow-up before completion of the study ( $n=2$ ) or withdrew for non-treatment related reasons i.e. job relocation or marital discord ( $n=2$ ). During the treatment phase 11 subjects reported side effects, namely increased sex drive ( $n=4$ ), decreased sex drive ( $n=7$ ), emotional lability ( $n=2$ ), irritability ( $n=5$ ) and tiredness ( $n=2$ ) though these were transient in nature. In addition 15 (65%) of the subjects reported skin reactions of varying degree to the T patches. In three subjects the reaction was classified as mild (transient erythema), whilst in a further 8 subjects the reaction was classified as moderate (marked erythema and itch requiring treatment with topical hydrocortisone). The remaining four subjects were

classified as having severe reactions, manifest as marked skin erythema and blistering.

## **Discussion**

Previous studies in hormonal male contraception have overwhelmingly employed i.m. injections of T (enanthate in particular) in doses (200mg weekly) which generated supraphysiological levels in the circulation (Hair et al., 1999). The thrust of these earlier endeavours has been to ensure maximal suppression of spermatogenesis. Consequently, not only have side effects been observed, but also the minimal effective dose for spermatogenesis suppression has remained undefined. In the development of new therapies, especially novel contraceptives, once primary efficacy has been ascertained, it is important to demonstrate the extent of the safety margin for breakthrough and the tolerance to variable/suboptimal compliance. This can only be revealed by systematically investigating the effects of reducing doses and establishing the minimally effective dose level. In a previous study (Wu et al., 1999), it was shown that spermatogenesis suppression could be effectively achieved even when i.m. T enanthate was reduced to the lowest physiological maintenance dose of 50mg weekly (equivalent to 5mg of unesterified T daily) when combined with the synthetic oral progestin, desogestrel. However, despite substantially reducing the total dose, the suboptimal pharmacokinetics of T enanthate inevitably produced sharp fluctuations with supraphysiological post - injection peak T levels (Snyder et al., 1980). This contributed to demonstrable non - reproductive effects and rendered interpretation of minimal effective doses of drug combinations difficult. At the equivalent dose of 5 mg of T daily, transdermal systems can maintain stable physiological levels of T and

offer the opportunity to investigate the minimal dose combination of progestin required to suppress spermatogenesis effectively.

In the present study a downward dose - ranging design was employed to determine the threshold dose of desogestrel, combined with a fixed daily amount of 5mg of transdermally – delivered T, for suppression of spermatogenesis. We have demonstrated a trend of progressively declining efficacy in achieving the three target levels of sperm density with reducing doses of DSG. 300 and 150µg of DSG daily induced azoospermia in 57 and 50% of subjects respectively. This is approaching the range of azoospermic suppression observed with levonorgestrel 500 µg with T enanthate 100mg weekly (67%) (Anawalt et al., 1999), T enanthate 200mg weekly (WHO Task Force 1990) (65%) and T implants 1200mg (56%) (Handelsman et al., 1992) in Caucasian men. Suppression to oligozoospermia (either <1 or <3 M/mL) in the present study (71 and 50% with 300 and 150µg of DSG respectively) however was clearly inferior to the other regimens (Wu et al., 1999, Meriggiola et al., 1998, Pavlou et al., 1991) which can achieve the <3M/mL target in 94-100% of subjects. Below 150µg/day of desogestrel, there was a marked drop in effectiveness with none of the subjects achieving azoospermia. Nevertheless, even at this suboptimal dose, one of the four subjects suppressed to <1 million/mL consistently while the other three failed to reach sperm densities less than 10 M/mL. This divergence between responders and non – responders was also observed in the 150µg but not the 300µg DSG group. Thus, in contrast to previous studies that emphasised the resistance to achieving azoospermia in a minority of men receiving maximal doses (Anderson et al., 1996), we have demonstrated a marked between – subject variation in sensitivity to hormonal suppression by exploring threshold (150µg DSG daily) to sub – threshold (75µg DSG daily) doses of treatment. This heterogeneous response pattern suggests

that a substantial proportion, may be up to 50%, of healthy men are able to respond to much lower doses of exogenous sex steroids than customarily used before in attaining effective contraception. These susceptible individuals however cannot be easily identified by any baseline characteristics such as sperm density, LH, FSH or T levels but they tend to show a more precipitous decline in sperm density in within the first 8 weeks after starting treatment.

Suppression of spermatogenesis with daily oral DSG and transdermal T was also less effective than similar doses of DSG combined with weekly i.m. injections of T Enanthate (Table 1) although the speed of decline in sperm density was not different (Wu et al., 1999, Anawalt et al., 1999). This was particularly true for the oligospermic targets. In the two previous studies using DSG, the least effective combination employing 150µg of DSG daily with 50 mg of T Enanthate weekly, produced suppression rates similar to the best results obtained in the group receiving 300mg DSG with 5mg of transdermal T. Furthermore, the breakthrough of suppression in 3 of the 7 men who reached azoospermia and the partial recovery or 'escape' before the end of the treatment period in most subjects was not observed previously with DSG and T Enanthate or Enanthate alone. It appears that substituting transdermal T for i.m. T enanthate has resulted in a loss of efficacy in spermatogenesis suppression.

Similarly, inhibition of gonadotrophins by DSG and transdermal T was less effective and less consistent than previous regimes employing i.m. T enanthate (Handelsman et al 1995, Anawalt et al., 2000). Suppression of gonadotrophins showed the same dose – related trend as spermatogenesis suppression with LH and FSH reaching assay sensitivity in 57 and 43% of subjects respectively in the 300µg DSG group and none in the 75µg group. In line with sperm density, very few subjects maintained consistent suppression of gonadotrophin, most showing transient escape or partial recovery

**Table 1.** Comparison of spermatogenesis suppression between oral DSG combined with transdermal T daily and oral DSG daily with im T enanthate weekly

Transdermal T	Suppression targets:			Intramuscular T	Suppression targets:		
Treatment	Oligo <3	Oligo <1	Azoospermia	Treatment	Oligo <3	Oligo <1	Azoospermia
DSG (300 µg/d)+ TTS (5 mg/d)	71 (9.6 ± 1.6)	71 (10.4 ± 0.1)	57 (10.6 ± 1.9)	DSG (300 µg/day)+ TE (100 mg/week) <sup>1</sup>	88 (10.9 ± 1.1)	75 (10.7 ± 1.3)	75 (14.0 ± 2.7)
DSG (150 µg/d)+ TTS (5 mg/d)	50 (5.3 ± 1.3)	50 (5.3 ± 1.3)	50 (10.6 ± 1.3)	DSG (150 µg/day)+ TE (100 mg/week) <sup>1</sup>	100 (10.0 ± 0.8)	100 (10.5 ± 0.7)	57 (13.0 ± 1.0)
DSG (75 µg/d)+ TTS (5 mg/d)	25 (12)	25 (16)	0	DSG (300 µg/day)+ TE (50 mg/week) <sup>1</sup>	100 (8.0 ± 0.8)	100 (9.0 ± 0.7)	100 (12.5 ± 1.2)
				DSG (150 µg/day)+ TE (50 mg/week) <sup>2</sup>	78	75 (11.0 ± 1.9)	57 (13.2 ± 2.7)

Values given are percentages; figures in *parentheses* are the mean ± SEM time in weeks taken to achieve the stated sperm density targets. Oligo <3, Oligozoospermia sperm density less than 3 million/ml; Oligo <1, oligozoospermia sperm density less than 1 million/ml.

<sup>1</sup> Data are from Wu *et al.* (8). <sup>2</sup> Data are from Anawalt *et al.* (28).

before the end of treatment. Unsurprisingly, azoospermic and oligospermic responders achieved significantly lower levels of gonadotrophins than non – responders during treatment. It is therefore highly probable that the inadequate suppression of spermatogenesis engendered by reducing doses of DSG and transdermal T is due to incomplete inhibition of gonadotrophins.

The torso transdermal testosterone delivery system has been shown to produce physiological circulating T levels in the mid – normal range for long – term maintenance of sexual and other androgen – dependent functions in hypogonadal men (Miekle 1998, Miekle et al., 1998, Dobs et al., 1999). In eugonadal men rendered hypogonadal by exogenous sex steroid in the present study however, total T decreased from baseline by about 30% during treatment but mean levels at  $16.1 \pm 2.4$  nmol/L (all 3 groups combined) (normal 10 – 35 nmol/L) remained within the physiological range. None of the subjects reported symptoms of androgen deficiency and haemoglobin and haematocrit did not fall during the treatment phase. Equilibrium dialysis - measured free T levels also decreased significantly by similar extents as total T. Lower SHBG concentration, associated with DSG and other oral synthetic progestin treatment, therefore did not correct for the low total T and an absolute decline in circulating bioavailable T levels was extant during treatment. Insufficient T may therefore be one explanation for the lower efficacy in gonadotrophin and spermatogenesis suppression compared with other studies using same doses of oral DSG (Table 1). Nevertheless, this study showed that improved suppression of spermatogenesis can be obtained with DSG and transdermal T compared to LNG combined with a similar dose but a different transdermal T preparation (Buchter et al., 1999) and also compared to CPA and oral T Undecanoate (Merrigiola et al., 1997) (Table 2). This may be related to the varying biopotencies and efficiencies of the

**Table 2.** Comparison of efficacy of spermatogenesis suppression between the present study and other progestin and T combinations as indicated by the maximal percentage of subjects achieving oligozoospermia and azoospermia

Oral progestin:	DSG (300 µg/d)	DSG (300 µg/d) <sup>1</sup>	LNG (250/500 µg) <sup>2</sup>	LNG (250 mg/d) <sup>3</sup>	CPA (12.5 mg daily) <sup>4</sup>	CPA (25 mg/d) <sup>5</sup>
T:	TTS T (5 mg/d)	TE (50 mg/wk)	TTS T (5 mg/d)	TE (100 mg/wk)	Oral TU (80 mg daily)	TE (100 mg/wk)
Oligo <3 M/ml	71	100	45	89	63	100
Oligo <1 M/ml	71	100	45	89	38	100
Azoospermia	57	100	18	78	13	100

Loss of efficacy in regimens employing noninjectable T preparations is apparent regardless of the type of progestin used in the combinations. Oligo, Oligozoospermia; DSG, desogestrel; LNG, levonorgestrel; CPA, cyproterone acetate; TTS T, transdermal therapeutic system T; TU, T undecanoate; TE, im T enanthate; M, million.

<sup>1</sup> Wu *et al.* (n = 8; 24-wk treatment).

<sup>2</sup> Buchter *et al.* (n = 11; 24-wk treatment).

<sup>3</sup> Anawalt *et al.* (n = 18; 24-wk treatment).

<sup>4</sup> Meriggiola *et al.* (n = 8; 16-wk treatment).

<sup>5</sup> Meriggiola *et al.* (n = 5; 16-wk treatment).

different progestins and/or preparations of non-injection T. Our results suggest that self – administration of male hormonal contraceptive steroids is potentially viable particularly in more responsive individuals. However, the transdermal systems are clearly less reliable than injectable T regimens (see later). The loss of efficacy when weekly i.m. injections of T enanthate is substituted by daily self - administered non – injection preparations of T is a consistent finding across all three paired comparisons (Table 2). This highlighted the critical role of T action and some important practical issues in the development of male hormonal contraception.

When the supraphysiological dose of 100mg T enanthate (Snyder et al., 1980) was co-administered with LNG or CPA, the much higher dose and the higher peak levels of T compared with the transdermal dose of 5mg daily and the T Undecanoate dose of 80mg bid may account for the discrepancy in efficacy. In our two studies on DSG and T (Table 2), we deliberately targeted our comparisons with a lower yet effective dose of 50mg weekly of T Enanthate i.m. which delivers 5mg of unesterified T daily, an amount equivalent to the daily delivery rates of transdermal system studied. T enanthate 50mg i.m. weekly produced pre – injection trough levels of T at 11 nmol/L (Wu et al., 1999), peaks of around 22 nmol/L and mean levels of 16 - 17 nmol/L (extrapolated from Snyder et al., 1980). This is not significantly different from mean T level of  $16.1 \pm 2.4$  nmol/L encountered during DSG and transdermal T treatment in the present study. The decline in efficacy in non – injection delivery of T is therefore unlikely to be due to lower mean levels of T and other explanations have to be considered. The fluctuating T levels giving high post – injection peaks with T Enanthate are probably not required for maximal gonadotrophin suppression since relatively stable T levels generated by 800mg of T implants when combined with DMPA was highly effective (Handelsman et al., 1996). Scrutiny of individual profiles

showed that many subjects in our study had transient, decreases in total and free T levels to the hypogonadal range with concomitant or subsequent escape in gonadotrophins and sperm density (Table 3. Subject 1). The relatively low nadir T levels during treatment in all three groups is a reflection of this. In contrast, the few individuals whose T levels were maintained in the normal range throughout the treatment phase were able to sustain suppression of gonadotrophins and spermatogenesis throughout treatment (Table 3. Subject 2). Analogously, the short half - life of oral T Undecanoate and its unpredictable absorption (Behre et al., 1998) make it unlikely that twice-daily administration can maintain stable T levels within the physiological range (Skakkebaek et al., 1981). These observations suggest that the common thread that links the low efficacy in the three studies using non – injectable T preparations is likely to be non – maintenance of adequate T levels in the mid – normal range which may have resulted from delivery problems or subject non-compliance. All these daily regimens of T are liable to the vagaries of inconsistent/unreliable delivery and non-compliance with daily self – administration in healthy volunteers. The consequence of these intermittent plunges in circulating T may not be important or clinically detectable in hypogonadal replacement but is likely to be of critical importance in maintenance of spermatogenesis suppression for contraception. The cumulative effects of repeated troughs in T (probably underestimated by monthly monitoring) provide the most likely explanation not only for the lower initial efficacy but also the subsequent breakthrough and progressive escape seen across all three studies using non - injectable T preparations (Table 2).

This study also shows that as we descend to lower threshold doses of DSG, the ‘safety margin’ is gradually eroded and the tolerance of the combination regimen to fluctuations in T levels becomes increasingly tested. The margin of safety for

**Table 3.** Individual hormone profiles for two subjects

	WK										
	-4	-2	4	8	12	16	20	24	28	32	
<b>Subject 1</b>											
LH <sup>1</sup>	2.9	3.5	0.05	0.05	0.05	0.05	0.3	0.05	5.2	2.7	
FSH	2.8	3.1	0.12	0.09	0.08	0.08	1.6	0.05	3.8	2.8	
Free T <sup>2</sup>	10.1	8.19	7.72	3.99	3.27	0.3	0.29	8.75	6.53	5.7	
Sperm Density <sup>3</sup>	89	184	42	5	0.1	0	2	1.2	4.2	4.4	
<b>Subject 2</b>											
LH	1.5	1.1	0.05	0.05	0.05	0.05	0.05	0.05	3.1	4.4	
FSH	2.5	1.4	0.05	0.05	0.05	0.05	0.05	0.05	6.3	3.8	
Free T	8.78	9.79	9.14	11.17	9.85	8.6	9.12	8.08	9.6	9.6	
Sperm Density	58	65	0.8	0	0	0	0	0	0.1	0.1	

<sup>1</sup> Units for gonadotrophins are international units per ml. <sup>2</sup> Nanomoles per litre. <sup>3</sup> Millions per ml.

suppression of spermatogenesis to maintain contraceptive efficacy is relatively narrow. Doses of T adequate for physiological replacement (5-6 mg daily) represent the minimum requirement but only if drug delivery is strictly reliable. This raises the important issue of compliance in potential contraceptive users who are healthy young men unaccustomed to daily self – medication. Although most of our subjects claimed full compliance with the drug regimen, closer questioning revealed problems with the transdermal patches other than the significant skin reaction. These included patch removal to go swimming, playing sport or lovemaking, patch detachment with excess perspiration or showering and poor patch adhesion to hirsute skin and in hot weather. Daily oral DSG administration is more likely to be complied with and not subject to the same problems as transdermal systems. The full efficacy when oral DSG was combined with i.m. T enanthate 50mg weekly, the well maintained plasma levels of etonogestrel after 6 month's treatment (the active metabolite of DSG) and the persistent lowering of SHBG attest to a high degree of compliance (Wu et al., 1999). We have previously shown that DSG alone decreased levels of HDL-C as well as apolipoprotein A1, an effect augmented by co-administration of T Enanthate. In the present study with transdermal T, HDL-C was reduced significantly in all treatment groups. This confirms that, in absence of high peak T levels, reduction of HDL-C is due to the action of oral DSG on hepatic lipid metabolism.

Side effects were relatively common with skin irritation the most widely reported. Our findings that 65% of subjects experienced skin irritation of varying degrees is in agreement with recent experience with the same preparation in hypogonadal men (Parker et al., 1999). These local problems with currently available transdermal systems for T further undermine the appeal for this mode of delivery in male contraception.

In conclusion, we have shown that oral DSG combined with a non-scrotal transdermal T delivery system produces suppression of gonadotrophins and spermatogenesis but is less effective than regimens incorporating injectable T. The minimally effective dose of desogestrel is 150 $\mu$ g. Escape from suppression is apparent in those subjects in whom T replacement is inconsistent or inadequate at all dose levels of DSG. These findings serve to further emphasise the critical role of T delivery and highlight some important practical issues concerning daily self – administered regimens for hormonal male contraception. In the current state of patch technology, the transdermal route of delivery may not be optimal for male contraception.

## References

**Anawalt BD, Bebb RA, Bremner WJ, Matsumoto AM. (1999)** A lower dosage levonorgestrel and testosterone combination effectively suppress spermatogenesis and circulating gonadotrophin levels with fewer metabolic effects than higher dosage combinations. *J Androl* **20**:407-414.

**Anderson RA, Ludlum CA, Wu FCW. (1995)** Haemostatic effects of supraphysiological levels of testosterone in normal men. *Thrombosis and Haemostasis*. **74**:693-697.

**Anderson RA Wallace EM, Wu FCW. (1996)** Comparison between testosterone enanthate induced azoospermia and oligospermia in a male contraceptive study. 3. Higher 5-alpha reductase activity in oligospermic men administered supraphysiological doses of testosterone. *J Clin Endocrinol Metab* **81**:902-908.

**Behre HM , Nieschlag E. (1998)** Comparative pharmacokinetics of testosterone esters. In Testosterone: action, deficiency and substitution. Behre HM, Nieschlag E Eds. 2<sup>nd</sup> edition 1998 Springer. 293-328.

**Buchter D, von Eckardstein S, von Eckardstein A. et al. (1999)** Clinical trial of transdermal testosterone and oral levonorgestrel for male contraception. *J Clin Endocrinol Metab* **84**:1244-1249.

**Dobs AS, Meikle AW, Arver S, Sanders SW, Caramelli KE, Mazer NA. (1999)** Pharmacokinetics, efficacy and safety of a permeation enhanced testosterone transdermal system in comparison with bi-weekly injections of testosterone enanthate for the treatment of hypogonadal men. *J Clin Endocrinol Metab* **84**:3469-3478

**Hair WM, Kitteridge K, Wu FCW. (1999)** A novel contraceptive pill-patch combination as a potential hormonal contraceptive in men. 81<sup>st</sup> Meeting of Endocrine Society. San Diego.P3-374

**Hair WM, Wu FCW (1999)** The role of drugs in male contraception *Curr Opin Oncol Endo Metab Investig Drugs* **1**: 50-59

**Handelsman DJ, Conway AJ, Howe CJ, Turner L, Mackey MA. (1996)** Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J Clin Endocrinol Metab* **81**: 4113-4121.

**Handelsman DJ, Conway AJ, Howe CJ, Turner L, Mackey M-A. (1996)** Establishing a minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J Clin Endocrinol Metab* **81**:4113-4121.

**Handelsman DJ, Conway AJ, Boylan LM. (1992)** Suppression of human spermatogenesis by testosterone implants. *J Clin Endocrinol Metab* **75**: 1326-1332.

**Merrigiola MC, Bremner WJ, Paulsen CA et al. (1996)** A combined regimen of cyproterone acetate and testosterone enanthate as a potentially highly effective male contraceptive. *J Clin Endocrinol Metab.* **81**: 3018-3023.

**Merrigiola CM, Bremner WJ, Costantino A, Pavani A, Capelli M, Flamigni C. (1997)** An oral regimen of cyproterone acetate and testosterone undecanoate for spermatogenic suppression in men. *Fert Steril* **68**: 844-850.

**Miekle WA. (1998)** A permeation-enhanced non-scrotal testosterone transdermal system for the treatment of male hypogonadism. In Nieschlag and Behre Editors. Testosterone: Action, Deficiency, Substitution. 2<sup>nd</sup> Edition Springer.

**Meikle AW, Mezer NA, Moellemer JF et al. (1992)** Enhanced transdermal delivery of testosterone across non-scrotal skin produces physiological concentrations of testosterone and its metabolites in hypogonadal men. *J Clin Endocrinol Metab* **74**:623.

**Parker S, Armitage M. (1999)** Experience with transdermal testosterone replacement therapy for hypogonadal men. *Clin Endocrinol* **50**: 57-62.

**Pavlou SN, Brewer K, Farley M, et al. (1991)** Combined administration of a gonadotrophin-releasing hormone antagonist and testosterone in men induces reversible azoospermia without loss of libido. *J Clin Endocrinol Metab* **73**: 1360-1368.

**Scheerer SB, Alvarez-Sanchez F, Anselmo J et al. (1978)** Hormonal contraception for men. *Int. J. Androl. (Suppl)*. **2**:680-712

**Skakkebaek NE, Bancroft J, Davidson DW, Warner P. (1981)** Androgen replacement with oral testosterone undecanoate in hypogonadal men: a double blind controlled study. *Clin Endocrinol* **14**: 49-61

**Snyder PJ, Lawrence DA. (1980)** Treatment of male hypogonadism with testosterone enanthate. *J Clin Endocrinol Metab* **51**:1335-1339.

**Tom L, Bhasin S, Salameh E et al. (1992)** Induction of azoospermia in normal men with combined Nal-Glu gonadotropin-releasing hormone antagonist and testosterone enanthate. *J Clin Endocrinol Metab* **75**:476-483

**Wang C, Iranmesh N, Berman N et al. (1998)** Comparative pharmacokinetics of three doses of percutaneous dihydrotestosterone gel in healthy elderly men- a clinical research center study. *J Clin Endocrinol Metab* **83**:2749-2757.

**World Health Organisation Task Force on Methods for the Regulation of Male Fertility (1990):** Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet*, **335**:955.

**World Health Organisation Task Force on Methods for the Regulation of Male Fertility (1995).** Rates of testosterone –induced suppression to severe oligospermia or azoospermia in two multicentre clinical studies. *International Journal of Andrology* **18**:157-165.

**Wu FCW, Balasubramanian R, Mulders T, Coelingh-bennink JT. (1999)** Oral progestagen combined with testosterone as a potential male contraceptive: additive effects between desogestrel and testosterone enanthate in suppression of spermatogenesis, pituitary testicular axis and lipid metabolism. *J Clin Endocrinol Metab.* **84**: 112-122.

**Wu FCW, Butler GE, Kelnar CJH, Stirling HF, Huhtaneimi I. (1991)** Patterns of pulsatile luteinizing hormone and follicle stimulating hormone secretion in prepubertal (mid-childhood) boys and girls with idiopathic hypogonadotropic hypogonadism (Kallmann's syndrome): a study using an ultrasensitive time resolved immunofluorometric assay. *J Clin Endocrinol Metab* **72**:1229-1237.

**Wu FCW, Farley TMM, Peregoudov A et al., (1996).** Effects of testosterone enanthate in normal men: Experience from a multicentre contraceptive efficacy study. *Fert Steril.* **65**:626-636.

## Chapter 5

### Summary and Conclusions

The literature review of male contraceptive studies describes increasing research activity over several decades and it is clear that a number of challenges remain:

- Need for effective, minimally –invasive, long acting androgen delivery
- Between-subject variation in sensitivity to spermatogenic suppression
- A lack of consistent reliable suppression to azoospermia in large numbers of subjects

The requirement for effective, self-administered, long-term androgen delivery forms the basis for the transdermal patch study which for the first time tested the effectiveness of transdermal testosterone in combination with oral desogestrel as a male contraceptive. This study employed a wholly subject administered drug regimen and was set against the backdrop of previously reported studies which employed T implants or i.m injections of T enanthate, alone or in combination with various progestins often administered by researchers themselves. The rates of azoospermia achieved were comparable with some previously reported androgen/progestin regimes. It is clear however, that suppression of spermatogenesis with daily oral DSG and transdermal T was less effective than similar doses of DSG combined with weekly i.m. injections of T enanthate at doses which deliver the same amount of daily unesterified T. It seems likely that the transdermal system failed to produce smooth, sustained delivery of T sufficient to consistently suppress gonadotrophins and leading to intermittent escape and restoration of spermatogenesis. The consequence of these

intermittent falls in circulating T may not be important or clinically detectable in hypogonadal replacement but may be pivotal in maintenance of spermatogenesis suppression for contraception. Nevertheless, the study demonstrated that, on the whole, men, are dependable users of this type of contraception and have sufficient motivation to self medicate in a reliable way.

Understanding the heterogeneity of the suppression response between and within populations remains central to the development of an effective contraceptive and challenges conventional views of testicular regulation in man. Consideration of other hormonal regulatory systems is part of that challenge and forms the basis for the two studies which examine the role of prolactin as a putative progonadal hormone in man. The laboratory studies described have unequivocally shown that prolactin receptors are expressed in the human testis and in male accessory glands based on RT-PCR amplification of the receptor mRNA, the localization of the receptor protein by immunocytochemistry and the functional activation of the receptor demonstrated by the second messenger experiments. The methodology used in the current study (using primers or antibodies directed at the extracellular domain of the prolactin receptor) precluded the demonstration different isoforms of the prolactin receptor in the human testis and other tissues. Various isoforms of the prolactin receptor, differing in the *intracellular* domain, have been characterised in man, and these are differentially expressed in the many prolactin target organs. Hence, it is plausible that similar diversity of prolactin receptor expression exists in human testis and accessory glands.

The current immunohistochemical studies localized for the first time the prolactin receptor protein to the Leydig cells and germ cells of the human testis, and confirmed

receptor expression in the epithelium in various accessory male structures. The pattern of immunostaining in the human testis is similar to that described in other mammals and points to a putative role for prolactin in the control of testicular steroidogenesis. Such a role in humans has not yet been demonstrated but is an attractive prospect for future studies. For example the immunostaining images for Leydig cells might be further strengthened through the use of in-situ hybridisation techniques although the technical challenges may prove considerable.

As well as demonstrating functional prolactin receptors within the vas deferens, the current results suggest that prolactin signals through more than one intracellular pathway to regulate the tissue responses. In the vas deferens, prolactin activated rapid phosphorylation of JAK 2, STAT 5 and ERK proteins. The temporal sequence is similar to that observed in other prolactin target-tissues in both animals and man. The involvement of both the JAK/STAT and ERK pathways acting together to influence differentiation has been documented in other cell types and suggests that PRL acts to provide divergent control of more than one cellular response. The target genes for prolactin in the vas deferens epithelium are still unknown, but on the basis of these and earlier studies are likely to include genes encoding secretory proteins. In the current study, only fresh tissue from human vas deferens was available for tissue culture, thus it was not possible to establish the pattern of phosphorylation of the second messenger proteins in the different male reproductive tissues, but this, subject to adequate sourcing of fresh tissue, may be considered for future work.

A role for prolactin within the human male reproductive tract suggests that manipulation of circulating levels of prolactin within the body may have a significant

impact on male fertility and forms the basis for the subsequent clinical trial. This study set out to establish whether suppression of PRL enhances the effectiveness of exogenous T in inhibiting sperm production in normal men. This was based on the potential progonadal effects of PRL, and the presumption that the simultaneous suppression of both PRL, and the classical gonadotrophins LH and FSH, would more completely effectively suppress spermatogenesis. The overall results did not support the hypothesis: the degree of spermatogenic suppression arrest was similar in the T1200 and T1200+Q groups, and the effects in the T800+Q group were comparable to those previously reported for men receiving this dose of T alone. The experiment was designed to compare treatments with and without quinagolide in the presence of a standardised high dose of T (T1200mg) based on the expectation that this level of T would produce sub-maximal sperm suppression and allow us to detect an additional effect due to Q. However, the T1200mg treatment alone in this study produced an unexpectedly high degree of sperm suppression, leaving little scope to observe an additional effect of Q during the treatment phase.

The only evidence that the Q treatment may have had some androgen dose-sparing effect was obtained from the T800+Q group. At the time of the study design, this group was introduced to ascertain whether addition of Q allowed for dose sparing of androgen in the regimen without compromising efficacy. No control group (i.e. T800 alone) was therefore felt to be necessary and in retrospect it is disappointing that such a group was not included. In the T800+Q group, there was marked individual variation in the degree of inhibition of PRL and degree of spermatogenic suppression. Steroid effects on PRL secretion in the trial subjects appeared to render quinagolide

inhibition of PRL less effective. These effects of chronic treatment in normal men were not anticipated, but clearly compromised the objective of this study.

Interestingly, in the subjects in which PRL secretion was maximally inhibited, the degree of spermatogenic suppression was greater than in those subjects in whom PRL was inadequately suppressed. Although subject numbers were too small to allow robust comparisons, this provides support, albeit rather tentative, for the view that only in partial hypogonadotrophic states when gonadotrophin concentrations are sub-maximally reduced is it possible to observe any concomitant effect of PRL withdrawal. Such a subtle interactive effect of PRL and gonadotrophin status has previously been described in animal models where, like man, the effects LH and FSH on testicular physiology appear always to predominate. Future studies will need to establish a means of totally blocking PRL release and be carefully design to test whether concomitant T and PRL blockade will induce complete spermatogenic arrest. The idea of employing lower levels of T, and reducing PRL due to its trophic effects on the prostate, make a combined preparation of this kind an attractive prospect in the regulation of male fertility.

The studies described here have shown that men are enthusiastic and willing participants in contraception. Despite troublesome skin effects, the subjects in the patch study persisted with treatment over 36 weeks and were highly supportive of the idea of hormonal contraception for men. To address the continuing issue of inter-subject variability and a lack of a comprehensive azoospermic drug regimen, other hormonal regulatory systems have been considered. It was postulated and subsequently demonstrated that functional prolactin receptors are distributed in the

human male reproductive tract. To further test the concept of prolactin as a progonadal hormone in men, the clinical study set out to test the idea that combined suppression of prolactin and gonadotrophins would produce more effective suppression of spermatogenesis. Difficulties with prolactin suppression in normal healthy subjects did not allow this question to be unequivocally answered by the study with the data providing only rather tentative support for the concept of prolactin as a progonadal hormone in men. It is the ambition of researchers in this field to develop an effective safe, reversible hormonal contraceptive which men can administer themselves. It is hoped that these studies contribute to the realisation of that ambition.

## **Appendix: Published papers from these studies**

# The role of drugs in male contraception

<sup>1</sup>WM Hair & <sup>2</sup>FCW Wu

## Address

<sup>1</sup>Department of Reproductive Medicine  
Sub-fertility Laboratory  
Second floor-Old Building  
St Mary's Hospital  
Whitworth Park  
Manchester  
M13 0JH  
UK  
Email: w.morton.hair@man.ac.uk

<sup>2</sup>Department of Endocrinology

Manchester Royal Infirmary  
Oxford Road  
Manchester  
M13 9WL  
UK  
Email: frederick.wu@man.ac.uk

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*Initial studies undertaken by the World Health Organisation demonstrated the efficacy of testosterone enanthate administered weekly by intramuscular injection as a potential hormonal male contraceptive. In the decade following these multicenter trials, alternative methods of testosterone delivery have been developed with improved pharmacokinetics and patient acceptability. Combination therapies are also under evaluation with the promise of improved contraceptive efficacy whilst curtailing unwanted extratesticular effects. This review systematically discusses and evaluates these drug regimens with respect to contraceptive efficacy, unwanted effects and future developments.*

## Introduction

Overcrowding is a key global health issue contributing to many major worldwide problems such as poverty, resource depletion and pollution. The world's population currently stands at around 6 billion and, unless global fertility rates are curtailed, will more than triple to 19 billion by 2100 [1]. This realization is a key factor underpinning research efforts directed towards the regulation of human fertility, which began in the second half of the twentieth century. In the last two decades, this field has enjoyed a broader perspective, moving from research principally directed at the control of processes in the female to include regulation of male fertility in general, and male hormonal contraception in particular. Three broad approaches are currently being pursued: (i) suppression of sperm production; (ii) disruption of sperm maturation and/or function; and, (iii) interruption of sperm transport.

## Physiological principles underlying the approach to male contraception

Normal spermatogenesis is absolutely dependent on sufficient concentrations of testosterone within the testis, normally 50- to 100-fold higher than in peripheral blood, in concert with the action of follicle-stimulating hormone (FSH). Testosterone production is maintained by luteinizing hormone (LH) stimulation of Leydig cells within the testis. Hormonal suppression of spermatogenesis therefore requires that intratesticular testosterone be profoundly inhibited. This is achieved indirectly through suppression of both LH and FSH, which then results in inhibition of testicular steroidogenesis and spermatogenesis. The

implication of this is that exogenous testosterone supplementation will be an invariant feature of any effective male hormonal contraceptive regime in order to maintain extratesticular functions such as libido, bone and muscle metabolism and hemopoiesis. Gonadotrophin withdrawal and the resultant depletion of intratesticular testosterone arrests spermatogenesis without affecting the stem cell population. This explains why hormonal suppression of spermatogenesis is invariably reversible.

The epididymis would appear to be an ideal target for a contraceptive agent, by preventing the acquisition of motility and fertilizing potential through disruption of epididymal function. Loss of sperm motility through interference with energy metabolism, or disruption of sperm surface protein chemistry causing impaired sperm-ovum binding, acrosome reaction and fertilizing capacity are all possible modes of action for such drugs.

Interruption of sperm transport by physical barriers, such as vasectomy and condoms or by coitus interruptus have been the mainstay of male contraceptive methods thus far. Improvement of these techniques may be brought about through developments such as non-latex condoms with virucidal spermicides and immunization against specific sperm surface antigens to interfere with the sperm-ovum interactions required for fertilization.

## Hormonal methods

Pituitary gonadotrophin secretion can be inhibited by a number of agents, some of which have been investigated as potential male hormonal contraceptives.

### Androgen-only regimes

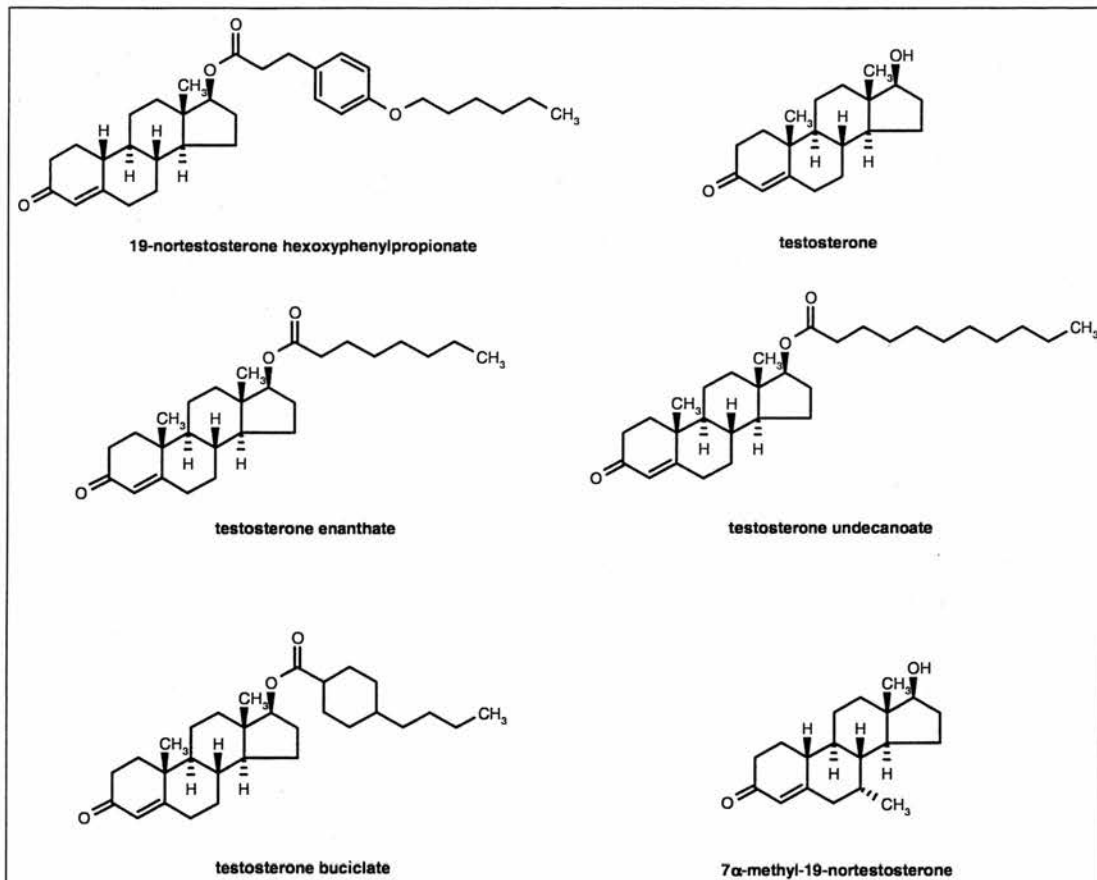
#### Testosterone enanthate

From studies in the 1970s it was found that supraphysiological doses of testosterone alone can consistently and reliably suppress spermatogenesis to azoospermia in 40 to 70% of Caucasian males [2]. The most effective regimen was intramuscular testosterone enanthate (TE), 200 mg weekly (Figure 1). This was chosen as the prototype candidate for subsequent male hormonal contraceptive trials because of its well-documented safety over many years in the treatment of hypogonadal men, despite its suboptimal pharmacokinetics.

Between 1986 and 1990, the first ever study to examine the contraceptive efficacy of testosterone-induced azoospermia was conducted by the World Health Organization (WHO) in 271 healthy volunteers in seven countries using TE, at a dose of 200 mg weekly [3••].

A total of 157 men achieved azoospermia, and these men entered the 12-month efficacy phase, in which the weekly testosterone injections were the only form of contraception used. Only one pregnancy occurred during the 1486 months of exposure, giving a Pearl rate of 0.8 (95% CI 0.02 to 4.5) ( $\text{Pearl rate} = \frac{(\text{number of pregnancies})}{(\text{number of woman months of pregnancy risk})} \times 1200$ ). This established that hormonal suppression to azoospermia yielded an efficacy rate comparable to female injectables, slightly better than female oral contraception, and substantially better than the condom.

Figure 1. Molecular structures of testosterone and other androgen preparations used in male contraception.



While azoospermia has been confirmed as the logical target for inducing infertility, a substantial minority of men remain oligospermic despite maximal sex steroid suppression. It is interesting to note that even when men are severely oligospermic, the remaining sperm are functionally normal [4].

A second WHO trial was conducted to investigate the contraceptive efficacy of TE-induced oligospermia [5••]. In this study, 349 of 358 (98%) healthy men in nine countries were suppressed to azoospermia or oligospermia ( $< 5 \times 10^6/\text{ml}$ ) using TE at 200 mg im weekly. They accumulated 283.5 years of exposure during which there were nine pregnancies. It became clear that pregnancy was related to sperm concentrations below  $5 \times 10^6/\text{ml}$ , which enabled a threshold sperm density of below  $3 \times 10^6/\text{ml}$  to be identified, above which the failure rate was deemed unacceptably high compared to existing reversible contraceptive methods. Thus a total of 4 pregnancies during 49.5 years of exposure contributed by men with sperm densities between 0.1 and  $3 \times 10^6/\text{ml}$  yielded a Pearl rate of 8.1 (2.2 to 20.7 per 100 person-years). This study yielded a Pearl rate for the method as a whole of 1.4 per 100 person-years for those men with sperm densities below  $3 \times 10^6/\text{ml}$  (ie, azoospermia to  $3 \times 10^6/\text{ml}$ ), which is comparable with the failure rate of current female reversible contraceptives (combined pill, injectables and medicated intrauterine devices) and is superior to the condom (12 per 100 person-years). These trials have become the benchmark against which future contraceptive studies may be compared.

Intramuscular TE produced markedly fluctuating levels of testosterone with repeated high peaks which predisposed subjects to androgen-related effects on lipid metabolism (lowering of high-density lipoprotein-cholesterol (HDL-C)), skin (increased sebum production leading, in some cases, to acne), liver (raised transaminases) [6•] and hemopoiesis (raised hemoglobin, red cell count, and hematocrit) [7]. Clearly, the availability of improved methods for the delivery of testosterone is crucial for the continued development of safe hormonal contraceptives that are acceptable to healthy young men.

#### Testosterone implants

Testosterone implants are small cylindrical pellets of crystalline testosterone which are implanted surgically, usually under local anesthesia, into the subcutaneous fat of the anterior abdominal wall. Kinetics approximate to first-order release, yielding 0.65 mg/day for 100 mg pellets and 1.5 mg/day for 200 mg pellets, with near complete bioavailability [8]. A 600 mg dose of implants will give physiological replacement over a period of four to six months [9,10]. However, a supraphysiological dose of 1200 mg is required to produce azoospermia in 65% of men. Fewer androgenic side-effects than weekly injections of TE were reported [11]. Implants are relatively inexpensive to produce and despite the fact that a minor surgical procedure is required, this is a popular method of androgen replacement in Europe and Australia, although this is not the case for the rest of the world [9].

### **Testosterone buciclate**

Testosterone-17 $\beta$ -trans-4-n-butyl-cyclohexylcarboxylate (testosterone buciclate, Figure 1) is a long-acting ester of testosterone developed jointly by the WHO and the National Institute for Child Health and Human Development, USA, for specific use as a male contraceptive. The ester is formulated as finely-milled crystals (10 to 15  $\mu$ m particle size) and is administered as an aqueous suspension by im injection. Single-dose injections of 600 mg and 1000 mg raised plasma testosterone in hypogonadal men into the low/normal range and maintained a stable concentration for 16 and 20 weeks, respectively [12]. A single dose of 1200 mg testosterone buciclate produced azoospermia in 3 of 8 volunteers whose plasma testosterone levels remained within the normal range [13]. At the time of writing it is unavailable due to persistent formulation difficulties. Further studies of this promising long-acting ester are planned.

### **Testosterone undecanoate**

Testosterone undecanoate (TU, Figure 1) has a long aliphatic side chain, which increases its lipid solubility. It was initially formulated as a capsule in arachis oil which facilitates oral absorption (*vide infra*). In a castor oil or tea oil base, it may be administered im, and has a significantly longer half-life ( $t_{1/2}$ ) than TE [14]. These initial studies in orchidectomized monkeys have more recently been extended to androgen replacement in hypogonadal men [15]. TU kinetics show both a long duration of effectiveness (up to 8 weeks from a single im injection of 1000 mg), plus the absence of the initial suprphysiological peak seen with TE, thereby reducing the likelihood of androgenic side-effects. A multicenter contraceptive study in China is ongoing.

### **Methyl-nortestosterone**

7 $\alpha$ -Methyl-19-nortestosterone (MENT) is a highly potent synthetic androgen which, like testosterone, is a free steroid rather than an ester, and consequently is usually formulated as a subdermal implant. MENT is not 5 $\alpha$ -reduced to dihydrotestosterone (DHT), and in the rat has been found to be 12-fold more potent than testosterone in suppression of gonadotrophins, whilst it is 4-fold more potent in the maintenance of prostate and seminal vesicle weight and 10-fold more potent in maintaining muscle weights [16]. As a result of its increased potency and tissue selectivity, this compound has, theoretically, the potential advantage of being able to suppress gonadotrophin production and maintain adult sexual function and muscle mass at relatively low doses that would not stimulate the prostate [17].

Recent studies in non-human primates have been encouraging in this respect; MENT was 10-fold more potent than testosterone in the suppression of gonadotrophins but only twice as potent in stimulating prostate growth [18]. However, it was also found to be 10-fold more potent than testosterone in its effects on lipids, causing decreases in both HDL-C and total cholesterol.

Phase I pharmacokinetic studies in healthy men using micronized MENT (2 to 8 mg), given as a single im injection have recently been completed showing effective suppression of gonadotrophins without reported side-effects [19]. Further clinical studies in hypogonadal men are planned.

### **19-Nortestosterone**

19-Nortestosterone (19-NT, nandrolone; Figure 1) is one of a group of anabolic steroids which have been in clinical use for over 30 years. Given that 19-NT possesses a higher affinity for the androgen receptor and is 10-fold more progestagenic than testosterone, it is a potentially attractive antifertility drug, either as a single agent or in combination with another progestagen.

19-NT alone [20] or in combination with a depot of the progestagen, medroxy-progesterone acetate [21] induced suppression of gonadotrophins and testosterone for 12 weeks and achieved azoospermia in 6 and 8 out of 12 volunteers, respectively. 19-NT may, therefore, have a role as a single-agent contraceptive, providing it is able to maintain essential androgen-dependent functions without producing undesirable metabolic, prostatic and behavioral effects which have not been studied to date.

### **Transdermal testosterone**

Delivery of testosterone via the transdermal route is a more recent development. Compared to transdermal estrogen delivery in female hormone replacement therapy which requires 50 to 100  $\mu$ g of steroid daily, much higher amounts - around 3 to 10 mg of testosterone - are required to maintain androgen-dependent physical functions. The rich vascular network of the scrotal skin is particularly suited to rapid drug absorption and is able to facilitate the increased delivery of steroid required [22].

### **Scrotal systems**

This method was initially employed in the treatment of hypogonadal men using Testoderm - testosterone transdermal system (TTS, ALZA Corporation) [23]. This system contains testosterone incorporated in a layer of self-adhering ethylene vinyl acetate co-polymer with a soft flexible bonded polyester backing which is applied to shaved scrotal skin and proximal penile shaft and worn for 22 h before it is replaced. Two sizes are available delivering 2.4 mg and 3.6 mg testosterone per day, which allows testosterone levels to be maintained in the adult physiological range over a 24-h period without fluctuations outside this normal range, representing an improvement over injectable testosterone. DHT levels in these patients are elevated - up to three times the normal range - yielding a DHT/testosterone ratio of 0.5 (normal range 0.1 to 0.2) which is a reflection of the rich 5 $\alpha$ -reductase activity within scrotal skin. The consequences regarding unwanted effects on DHT-dependent tissues are not yet known.

### **Non-scrotal systems**

More recently, non-scrotal transdermal delivery systems have been developed for use in androgen replacement therapy in hypogonadal men [24]. Androderm Testosterone Transdermal Systems (TheraTech) is one such system containing testosterone in a permeation-enhancing vehicle composed of water, ethyl alcohol, glycerine, glycerol mono-oleate, methyl laurate and pharmaceutical gelling agents [25]. Two types of system are available, delivering 2.5 mg or 5 mg of testosterone. For adult hypogonadal men, two of the lower strength patches or one of the higher strength provides adequate androgen replacement without alteration of the DHT/testosterone ratio. This system, marketed as Andropatch in Europe, is currently under evaluation in a male contraceptive study.

However, the alcoholic excipients employed have caused considerable problems. A recent study reported adverse effects in 84% of hypogonadal men receiving androgen replacement by this method (most commonly dermatological problems) and of these, 72% elected to return to injected depot preparations [26•]. Other systems producing a more physiological, pulsatile release of testosterone are under development [27].

A hydroalcoholic gel containing a 2.5% solution of 5 $\alpha$ -DHT (Andractim, Unimed Pharmaceuticals Inc) is currently in use in Europe. If applied over a sufficiently large surface area, eg, chest and abdomen, adequate androgen substitution is achieved [22]. More recent studies describe an improved formulation employing a 0.7% DHT hydroalcoholic gel (Besco Icovesco, France) delivering 16 mg DHT as a metered dose [28]. The study reports no skin irritation or adverse hematological or biochemical events. Studies are currently in progress to determine its efficacy as a method of androgen replacement.

### **Testosterone microspheres**

Testosterone encapsulated into biodegradable polylactide-glycolide co-polymer microspheres can be administered as a depot injection, yielding a biphasic pattern of testosterone release over 11 weeks [29]. By varying the particle size, a second-generation microsphere system containing 315 mg testosterone has recently been shown to provide approximately zero-order testosterone release at physiological levels for 70 days [30]. Recent animal studies have confirmed that the microspheres are completely degraded in this particular study after a period of 10 weeks [31]. There have been no recent developments employing this approach in human subjects.

### **Orally-active testosterone**

Orally-administered free testosterone is efficiently absorbed from the intestine but undergoes virtually complete hepatic first-pass metabolism. Avoiding first-pass metabolism, orally-administered TU (see above) is absorbed into lymph and enters the thoracic duct and thence to the systemic circulation via the subclavian vein. TU (40 mg) dissolved in arachis oil (Restandol, Organon NV) is available in capsule form. However, its variable reabsorption coupled with a short  $t_{1/2}$  and duration of action requires that patients take two to four capsules per day to achieve adequate androgen substitution [32•].

Testosterone complexed with hydroxy- $\beta$ -cyclodextrins, an oligosaccharide ring digested by bacteria, can, when administered sublingually, give rise to short-lived (2 h) peaks in testosterone. However, once again, multiple (three times daily) administrations are required for adequate androgen replacement [33].

### **Inhaled preparations**

Using a new type of aerosol characterized by particles of small mass and large size, it has been shown [34] that inhalation of such particles complexed with testosterone yielded high systemic bioavailability of the hormone and avoids first-pass metabolism. This system offers a potential non-invasive means of androgen replacement as part of a hormonal contraceptive regime though, as yet, no such studies have been reported.

## **Combined hormonal regimes**

Experience with androgen-only regimes indicates that supraphysiological levels of testosterone are required for maximal suppression of gonadotrophins. Due to the direct stimulatory action of testosterone on spermatogenesis, especially at high doses, the ability of these regimes to suppress spermatogenesis remains suboptimal. Dose-dependent, androgen-related side-effects already described, provide further impetus to lower the testosterone content of future male contraceptives. In addition, until unequivocal long-term safety data are available with regard to the effect of testosterone on conditions such as benign and malignant disease of the prostate, cardiovascular disease and behavioral disturbance, it seems prudent to pursue alternative regimens which minimize the amount of exogenous testosterone that is employed.

### **Progestogen and androgen combinations**

Progestogens are potent inhibitors of gonadotrophin secretion in men [35] and may also directly suppress spermatogenesis [36]. By acting synergistically or additively with androgens, they permit lower doses of each steroid to be used. Synthetic steroids such as levonorgestrel (LNG), desogestrel, gestodene and norgestimate and medroxyprogesterone acetate (DMPA) are highly potent progestational compounds that are effective in microgram quantities with only moderate androgenic properties. Structural modification of the parent molecules from which they are derived, namely 19-nortestosterone and 17-hydroxyprogesterone, has allowed the development of these orally-active hormones. The comparatively low mass of drug required has also allowed the successful development of long-acting subdermal implants such as Norplant (levonorgestrel, Wyeth Ayerst) and Implanon (3-ketodesogestrel, Organon NV).

### **DMPA and testosterone**

A single depot injection of the progestin DMPA, in combination with testosterone (800 mg) implants [37] showed that the addition of DMPA markedly increased the rate of azoospermia compared to testosterone alone, although did not reduce the time course over which this was achieved.

### **LNG and testosterone**

Initial studies using LNG [38] in combination with subreplacement doses of testosterone produced results that did not improve on those expected for androgen alone. Similar results were obtained using DMPA [2], danazol [39] and Norethisterone [40].

Recent studies have been more encouraging; Bebb reports a study [41] in which volunteers received 500  $\mu$ g LNG in combination with higher doses of TE (100 mg/week). With this regime, significantly greater and faster suppression of spermatogenesis than with the same dose of testosterone alone was observed. However, HDL-C levels were significantly reduced in those patients receiving LNG plus testosterone though not in the control group receiving androgen alone. Reduced HDL-C levels have been associated with increased risk of atherosclerotic heart disease [42].

### **Desogestrel and testosterone**

Studies have recently been completed showing oral desogestrel (300  $\mu$ g/day) in combination with low-dose testosterone (50 mg/weekly im injection) provides effective

suppression of gonadotrophins and rates of spermatogenesis superior to androgen only regimes and earlier studies using progestin/androgen combinations [43•]. The rate of azoospermia for this regime (100% in the above treatment group) also compares very favorably with more recent studies using progestin/androgen combinations, described above: LNG (67% azoospermia) [41]; and, DMPA (90%) [37]. As with LNG, there was a significant reduction in HDL-C levels in those patients receiving desogestrel. It remains to be seen whether employing lower doses of desogestrel or newer progestagens can minimize these lipid effects without compromising contraceptive efficacy.

A novel feature of this study was the phased treatment regimen in which testosterone treatment was initiated after 3 weeks of pre-treatment using desogestrel alone. By virtue of the phased introduction of androgen the authors were able to demonstrate the relative contributions made by each drug to suppression of spermatogenesis and gonadotrophins. Interestingly, it was found that desogestrel alone, even at maximal dose, suppressed the hypothalamo-pituitary-testicular axis only partially and that the addition of testosterone was crucial in achieving maximal suppression. This demonstrates that testosterone is not only essential for maintenance of androgen-dependent physiological functions but also to achieve optimal suppression of gonadotrophins.

#### **Anti-androgen/androgen combination**

Cyproterone acetate (CPA) is a synthetic steroid which blocks testosterone effects on the testis, through competitive binding at the androgen receptor, as well as suppressing gonadotrophin production by the pituitary. It has been used in Europe for the treatment of hirsutism in females (Dianette; Schering) and prostatic malignancy in males (Cyprostat; Schering).

When CPA alone is administered orally to men at doses of 5 mg to 20 mg daily, disruption of sperm motility and morphology occurs, but with associated symptoms of androgen deficiency [44-47]. More recent studies have combined low-dose CPA with androgen supplementation to good effect [48]. Initial studies employed a regime of CPA (50 or 100 mg/day) and weekly injections of TE (100 mg/week), which caused complete azoospermia in the small number of volunteers treated with either dose of CPA. In a more recent study by the same group, an oral regime was employed for the first time [49•]. Using a lower dose of oral CPA (25 mg/day) in combination with oral capsules of TU (160 mg/day), however, produced much lower rates of azoospermia (only 12.5% of subjects).

Also of note is the significant decrease in hemoglobin and hematocrit seen in subjects treated with CPA in all studies so far reported, which in the long-term may produce anemia. It remains to be seen whether lower doses of CPA may elicit the desired effect on spermatogenesis without manifesting these important effects on hemopoiesis.

#### **Estrogen/androgen combination**

Combined estradiol and testosterone implants have been shown in the rat [50] and the monkey [51] to effectively suppress spermatogenesis without affecting mating behavior.

In man, the counterbalancing effects of small amounts of estrogens on lipid and bone metabolism complemented by the anti-estrogenic properties of testosterone may, theoretically, produce an ideal formulation for the suppression of spermatogenesis.

Studies have recently begun evaluating the combination of implants of estradiol and testosterone.

#### **5 $\alpha$ -Reductase inhibitor/androgen combination**

The enzyme 5 $\alpha$ -reductase converts testosterone to DHT, a steroid, which, in some tissues exhibits a greater potency than testosterone, eg, prostate, seminal vesicles and hair follicles.

Studies to evaluate the effects of the 5 $\alpha$ -reductase inhibitor finasteride (Merck & Co) in conjunction with testosterone are currently in progress. It is hoped that the attenuation of DHT production will diminish unwanted extra-testicular androgen effects without impacting on suppression of gonadotrophins and spermatogenesis.

#### **Non-steroidal hormonal agents**

##### **Gonadotrophin-releasing hormone agonists**

Abolition of gonadotrophin secretion and therefore of spermatogenesis can be produced by gonadotrophin-releasing hormone (GnRH) agonists, which downregulate pituitary GnRH receptors and have the advantage that this is a highly specific action, thereby eliminating unwanted actions on metabolism, hemostasis, and the cardiovascular system.

A number of studies have explored the potential of GnRH agonists in suppressing sperm production [52]. In total, only 23% of subjects achieved azoospermia. These results are clearly disappointing, although it may be argued that the dose of agonist employed was not adequate or that there may have been persistence (or escape) of FSH activity during GnRH agonist suppression [53,54].

The GnRH agonist leuprolide (Takeda Chemical Industries) has recently been found to produce effective reversible azoospermia in dogs [55] though as yet, no human studies have been reported. Further studies are required to more fully evaluate the true potential of GnRH agonists.

##### **GnRH antagonists**

These are capable of the complete and rapid reversible suppression of gonadotrophins [56]. They have higher affinity and slower dissociation rates than either GnRH itself or any of the GnRH agonists [57,58]. The principal drawback of GnRH antagonists is low potency, necessitating milligram amounts to be administered systemically via complex delivery systems and low oral bioavailability [59]. Given that most are produced by substitution of the natural decapeptide with unnatural, synthetic amino acids [60], they are also extremely expensive to produce, have low solubility and are difficult to formulate, which currently limits their potential for large-scale clinical trials.

Despite these constraints, the effects of daily, subcutaneous injections of Nal-Glu GnRH antagonist given in combination with weekly injections of low-dose TE (50 mg im/week) were examined in men and showed greater suppression of gonadotrophins than with testosterone alone [61,62].

More recent studies using the same antagonist showed that azoospermia was achieved more rapidly and at higher incidence than TE alone [63•]. In addition, having suppressed spermatogenesis during the combination induction phase, it remained suppressed when maintenance treatment of TE alone (100 mg/week) was continued. This reduction of lag time between treatment and response addresses criticisms of earlier regimes in which maximal suppression of spermatogenesis took up to three months to achieve. Future developments in pharmaceutical technology may allow more economical production of these compounds as well as orally-active, non-peptide GnRH antagonists, making them more attractive prospects for use in male fertility regulation.

### **Selective FSH suppression**

FSH is currently not considered to be essential for maintenance of spermatogenesis in the presence of LH. This is suggested by monkey studies in which inhibition of FSH alone (achieved by raising antibodies against it) causes reduction in sperm concentration but not azoospermia [64].

Further evidence may be found in studies of men with mutations of FSH receptors. Tapanainen *et al* report that although sperm production was quantitatively impaired, absence of FSH activity in these men did not abolish spermatogenesis [65•]. Accordingly, peptides such as inhibin and follistatin, which selectively suppress FSH production, are not considered promising candidates as male contraceptives.

## **Heterogeneity of response to hormonal suppression of spermatogenesis**

### **Variation between population groups**

A striking finding from the studies described thus far is the marked difference both between and within population groups in the degree of steroid-induced suppression of spermatogenesis. It is known that supraphysiological doses of testosterone induce rates of azoospermia ranging from 40% to 70% in Caucasian men, with the remainder becoming oligospermic [3••]. The reasons for this variation in spermatogenic suppression are not entirely understood although there are a number of studies which address this question.

Men who become oligospermic during testosterone treatment exhibit a higher  $5\alpha$ -reductase activity than those who develop azoospermia which may cause altered intratesticular androgen levels [66•]. This higher residual androgenic action on the seminiferous tubules may cause resistance to complete suppression of spermatogenesis. More recent studies report the histological evaluation of the effects of testosterone treatment on spermatogenesis using open testicular biopsy material from healthy males awaiting vasectomy pre-treated with weekly doses of im TE [67]. This study demonstrates that the most consistent lesion induced by TE treatment was in the reduction in formation of type B spermatogonia - the germ cells that enter meiosis. Studies in the rat support the view that the development of A to B spermatogonia is androgen-dependent and can be impaired by a  $5\alpha$ -reductase inhibitor [68•].

One of the surprising findings from the WHO trials was that Asian (Chinese, Indonesian and Thai) men showed a consistently higher rate of spermatogenic suppression (89.9%) when compared with European or American (74.3%) men ( $p < 0.001$ ) [30]. Given the much greater efficacy of testosterone-alone regimes in these countries, the national authorities of China, Indonesia and India have initiated large-scale phase III trials evaluating testosterone as a single-agent contraceptive. The source of this heterogeneity has been extensively investigated, though the underlying reasons remain unclear. Characteristics such as body size, mass, serum biochemistry, as well as baseline endocrine and semen parameters do not explain the observed differences [69].

A recent study examining the comparative rates of androgen production and metabolism in Caucasian and Chinese subjects showed increased levels of  $5\alpha$ -reduced metabolites in Caucasians [70]. However, this was not found to be due to a difference in  $5\alpha$ -reductase activity or testosterone to dihydrotestosterone conversion ratios in the two groups. The study did show, however, raised levels of androgenic steroid precursors, which serve as substrates for these metabolites in the Caucasian group, and interestingly, in Chinese men resident in the USA. The authors postulate that this is indicative of an environmental or dietary origin to these population differences rather than differences in  $5\alpha$ -reductase activity. Differences in the gonadotrophin negative feedback response to exogenous testosterone in Asian and Caucasian men have also been reported [71].

More recently, differences in testicular structure between Asian men and Caucasians have been observed [72]. Histological examination of testes taken from post-mortem examination of previously healthy Asian males dying from sudden traumatic injury has demonstrated that Asian males have fewer spermatogonia, smaller diameter seminiferous tubules and fewer Sertoli cells per man than do Caucasians. This variation in testicular structure results in differences in spermatogenic potential between the two groups, and is tendered by the authors as a potential explanation as to why Asian men exhibit more complete suppression of testicular function in response to exogenous testosterone administration.

Further studies are currently directed towards a more complete understanding of these population differences.

## **Non-hormonal drugs**

Serendipity has played its role in uncovering the fertility-suppressing side-effects of compounds developed for other uses. The majority of these have not showed promise, and indeed, many have unacceptable levels of toxicity [73-75]. The most important of these compounds will briefly be reviewed.

## **Anti-spermatogenic agents**

### **Plant-based compounds**

In the 1970s, more than 8000 Chinese men were given gossypol, a polyphenolic plant pigment derived from cottonseed oil, with a view to determining its contraceptive activity. Side-effects, such as hypokalemia coupled with its high incidence of irreversibility, saw gossypol abandoned as a potential male contraceptive in 1986.

Triptolide and triptolide are oxygenated diterpine compounds derived from the plant *Trypterium wilfordii* whose prime use is as a traditional Chinese medicine in the treatment of inflammatory and dermatological diseases. They are among several compounds which have now been extracted in sufficient amounts to allow more detailed evaluation of their chemical structure, actions and side-effects [76]. Initial animal studies have shown effective suppression of spermatogenesis but a narrow therapeutic index: immunosuppression was seen at doses of 5- to 12-fold greater than those required to produce azoospermia [76].

Recent studies in the rat have demonstrated that triptolide was moderately effective in the suppression of spermatogenesis and also completely inhibited motility in the epididymis without effects on gonadotrophins, giving a so-called post-testicular contraceptive [77]. Careful toxicology studies are required before these compounds are more fully evaluated in men, although the low therapeutic index coupled with a recent report of infantile meningoencephalocele in a pregnant woman taking triptolide for rheumatoid arthritis [78] is less than encouraging.

#### **Antifolate drugs**

Dihydrofolate (DHF) reductase inhibitors such as the early sulpha-containing antibiotics are known to have modest antifertility effects. Similarly, the antimalarial drug pyrimethamine (also a DHF reductase inhibitor), reversibly abolishes fertility in male rats [79], resulting in a search for analogous, but more potent, compounds through, eg, substitution of the basic diaminopyrimidine structure which will, it is hoped, improve the selectivity of these compounds [72].

#### **Epididymal agents**

A few compounds which inhibit the oxidative metabolism of spermatozoa, and hence inhibit motility, have been evaluated.  $\alpha$ -Chlorohydrin and 6-chloro-6-deoxyglucose are metabolized to 3-chlorolactaldehyde, a compound with the same stereochemistry as R-glyceraldehyde - a key substrate in the glycolytic pathway. Administration of these compounds results in competitive inhibition of the key enzyme, glyceraldehyde-3-phosphate dehydrogenase, and glycolysis, thereby inhibiting sperm motility. Perhaps not surprisingly, given the effects on a core biochemical process, these compounds have serious systemic side-effects, particularly on the nervous system and bone marrow precluding further exploration of their contraceptive potential [80].

The antifungal agent ketoconazole is known to accumulate in seminal plasma and inhibit sperm motility post-ejaculation. This property has been ascribed to the imidazole ring structure in ketoconazole and consequently, a variety of substituted imidazole compounds were evaluated as orally-administered, rather than topical, inhibitors of sperm motility [81]. There have been no studies using these compounds reported in the last decade.

Nitroimidazole derivatives, often used as antibiotics and anti-protozoan treatments suppress fertility by inhibiting sperm maturation in the epididymis though once again, the broad side-effect profile makes long-term use in contraception impractical [82].

## **Conclusions**

In the decade since the publication of the initial WHO study reporting the contraceptive efficacy of testosterone-induced azoospermia, substantial progress has been made towards a fuller understanding of the control of male fertility. Improved androgen delivery systems and combination therapies promise greater efficacy, whilst efforts are ongoing to reduce the impact of these therapies on lipids and other extra-testicular metabolic processes. Drug industry interest, although slow to develop, is now becoming established. The development of diverse and improved methods of male fertility control increases choice for both men and women and provides potentially rich benefits for all.

## **References**

- of outstanding interest
  - of special interest
1. **Population Studies: World Population Prospects 1990.** New York, United Nations, (1991): 5.
  2. Schearer SB, Alvarez-Sanchez F, Anselmo J, Brenner P, Coutinho E, Latham-Faundes A, Frick J, Heinild B, Johansson ED: **Hormonal contraception for men.** *Int J Androl* (1978) 2(Suppl):680-712.
  3. World Health Organisation Task Force on Methods for the Regulation of Male Fertility: **Contraceptive efficacy of testosterone-induced azoospermia in normal men.** *Lancet* (1990) 335:955.
  - *The first study to examine the efficacy of testosterone as a contraceptive, and with [5] remains the benchmark against which subsequent studies are compared.*
  4. Wang C, Leung A, Superlano L, Steiner B, Swerdloff RS: **Oligozoospermia induced by exogenous testosterone is associated with normal functioning residual spermatozoa.** *Fertil Steril* (1997) 68:149-153.
  5. World Health Organisation Task Force on Methods for the Regulation of Male Fertility: **Rates of testosterone-induced suppression to severe oligozoospermia or azoospermia in two multicentre clinical studies.** *Int J Androl* (1995) 18:157-165.
  - *The first study to examine the efficacy of testosterone as a contraceptive, and with [3] remains the benchmark against which subsequent studies are compared.*
  6. Wu FCW, Farley TMM, Peregoudov A, Waites GMH, Zhang GY, Li GZ, Gottlieb C, Handlesman DJ, Hutaniemi I: **Effects of testosterone enanthate in normal men: Experience from a multicentre contraceptive efficacy study.** *Fertil Steril* (1996) 65:626-636.
  - *A comprehensive look at findings of the WHO studies and a discussion of the non-contraceptive effects of testosterone.*
  7. Anderson RA, Ludlum CA, Wu FCW: **Haemostatic effects of supraphysiological levels of testosterone in normal men.** *Thromb Haemost* (1995) 74:693-697.
  8. Handelsman DJ, Conway AJ, Boylan LM: **Pharmacokinetics and pharmacodynamics of testosterone pellets in man.** *J Clin Endocrinol Metab* (1990) 70:216-222.
  9. Cantrill JA, Dewis P, Large DM, Newman M, Anderson DC: **Which testosterone replacement therapy?** *Clin Endocrinol* (1984) 21:97-107.
  10. Conway AJ, Boyland LM, Howe C, Ross G, Handelsman DJ: **Randomized clinical trial of testosterone replacement therapy in hypogonadal men.** *Int J Androl* (1988) 11:247-264

11. Handelsman DJ, Conway AJ, Boylan LM: **Suppression of human spermatogenesis by testosterone implants.** *J Clin Endocrinol Metab* (1992) **75**:1326-1332.
12. Behre HM, Nieschlag E: **Testosterone buciclate in hypogonadal men: Pharmacokinetics and pharmacodynamics of the new long-acting androgen ester.** *J Clin Endocrinol Metab* (1992) **75**:1204-1210.
13. Behre HM, Baus S, Kliesch S, Keck C, Simoni M, Nieschlag E: **Potential of testosterone buciclate for male contraception: endocrine differences between responders and non-responders.** *J Clin Endocrinol Metab* (1995) **80**:2394-2403.
14. Partsch CJ, Weinbauer GF, Fang RY, Nieschlag E: **Injectable testosterone undecanoate has more favourable pharmacokinetics and pharmacodynamics than testosterone enanthate.** *Eur J Endocrinol* (1995) **132**:514-519.
15. Wang LZ: **The therapeutic effect of domestically produced testosterone undecanoate in Klinefelter syndrome.** *New Drugs Market* **8**:28-32
16. Kumar N, Didolkar AK, Monder C, Bardin CW, Sundaram K: **The biological activity of 7-methyl-19-nortestosterone is not amplified in male reproductive tract as is that of testosterone.** *Endocrinology* (1992) **130**:3677-3683.
17. Sundaram K, Kumar N, Bardin CW: **7 $\alpha$ -methyl-19-nortestosterone (MENT); the optimal androgen for male contraception.** *Ann Med* (1993) **25**:199-205.
18. Cummings DE, Kumar N, Bardin CW, Sundaram K, Bremner WJ: **Prostate-sparing effects of the potent androgen 7 $\alpha$ -methyl-19-nortestosterone: a potential alternative to testosterone for androgen replacement and male contraception.** *J Clin Endocrinol Metab* (1998) **83**:4212-4219.
19. Suvisaari J, Sundaram K, Noe G, Kumar N, Aguilhaume C, Tsong YY, Lahteenmaki P, Bardin CW: **Pharmacokinetics and pharmacodynamics of 7 $\alpha$ -methyl-19-nortestosterone after intramuscular administration in healthy men.** *Human Reproduction* (1997) **12**:967-973.
20. Knuth UA, Behre H, Belkien L, Bents H, Nieschlag E: **Clinical trial of 19-nortestosterone-hexoxyphenyl-propionate (Anadur) for male fertility regulation.** *Fertil Steril* (1985) **44**:814-821.
21. Knuth UA, Yeung C, Nieschlag E: **Combination of 19-nortestosterone-hexoxyphenyl-propionate (Anadur) and depot medroxyprogesterone-acetate (Clinovir) for male contraception.** *Fertil Steril* (1989) **51**:1011-1018.
22. Sitruk-Ware R: **Transdermal delivery of steroids.** *Contraception* (1989) **39**:1-20.
23. Place VA, Atkinson L, Prather DA, Trunnel N, Yates FE: **Transdermal testosterone replacement through genital skin.** In: *Testosterone: Action, deficiency, substitution.* Nieschlag E, Behre HM (Eds), Springer-Verlag, Berlin (1990):165-182.
24. Miekle WA: **A permeation-enhanced non-scrotal testosterone transdermal.** In: *Testosterone: Action, deficiency, substitution.* Nieschlag E, Behre HM (Eds), Springer-Verlag, Berlin (1990):389-422.
25. Meikle AW, Mazer NA, Moellemer JF, Stringham JD, Tolman KG, Sanders SW, Odell WD: **Enhanced transdermal delivery of testosterone across nonscrotal skin produces physiological concentrations of testosterone and its metabolites in hypogonadal men.** *J Clin Endocrinol Metab* (1992) **74**:623-628.
26. Parker S, Armitage M: **Experience with transdermal testosterone replacement therapy for hypogonadal men.** *Clin Endocrinol* (1999) **50**:57-62.  
• *Study reporting substantial problems with andropatch use in hypogonadal men.*
27. Misra A, Pal R, Majumdar SS, Talwar GP, Singh O: **Biphasic testosterone delivery profile observed with two different transdermal formulations.** *Pharm Res* (1997) **14**:1264-1268.
28. Wang C, Iranmanesh N, Berman N, McDonald V, Steiner B, Ziel F, Faulkner SM, Dudley RE, Veldhuis JD, Swerdloff RS: **Comparative pharmacokinetics of three doses of percutaneous dihydrotestosterone gel in healthy elderly men- a clinical research center study.** *J Clin Endocrinol Metab* (1998) **83**:2749-2757.
29. Burris AS, Ewing LL, Sherins RJ: **Initial trial of slow-release testosterone microsphere in hypogonadal men.** *Fertil Steril* (1998) **50**:493-497.
30. Bhasin S, Swerdloff RS, Steiner B, Peterson MA, Meridores T, Galmirini M, Pandian MR, Goldberg R, Berman N: **A biodegradable testosterone microcapsule formulation provides uniform eugonadal levels of testosterone for 10-11 weeks in hypogonadal men.** *J Clin Endocrinol Metab* (1992) **74**:75-83.
31. Kobayashi D, Tsubuku S, Yamanaka H, Asano M, Miyajima M, Yoshida M: **In vivo characteristics of injectable poly(DL-lactic acid) microspheres for long acting drug delivery.** *Drug Dev Ind Pharm* (1998) **24**:819-825.
32. Schaison G, Couzinet B: **Percutaneous dihydrotestosterone treatment.** In: *Testosterone: Action, deficiency, substitution. 2<sup>nd</sup> Edition.* Nieschlag E, Behre HM (Eds), Springer-Verlag, Berlin (1998):423-436.  
• *A comprehensive discussion of scrotal transdermal systems.*
33. Salehian B, Wang C, Davidson T, Umana V, Steiner B, Peterson M, Sinhahikim I, Swerdloff RS: **Comparison of pharmacokinetics of testosterone after sublingual testosterone or testosterone enanthate injections.** *Clin Res* (1994) **42**:A74.
34. Edwards DA, Hanes J, Caponetti G, Hrkach J, Ben-Jebria A, Eskew ML, Mintzes J, Deaver D, Lotan N, Langer R: **Large porous particles for pulmonary drug delivery.** *Science* (1997) **276**:1868-1871.
35. Kjeld JM, Puah CM, Kaufman B, Loizou S, Vlotides J, Gwee HM, Kahn F, Sood R, Joplin GF: **Effects of norgestrel and ethinyloestradiol ingestion on serum levels of sex hormones and gonadotropins in men.** *Clin Endocrinol* (1979) **11**:497-504.
36. Goldzieher JW, Castracane VD: **Antifertility effects of progestational steroids.** In: *Endocrine Mechanisms in Fertility Regulation.* Benagiano H, Diczfalusy E (Eds), Raven Press, New York (1984): 49.
37. Handelsman DJ, Conway AJ, Howe CJ, Turner L, Mackey MA: **Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot.** *J Clin Endocrinol Metab* (1996) **81**:4113-4121.
38. Føgh M, Nichol M, Lettersen IB, Schou G: **Clinical evaluation of long-term treatment with levo-norgestrel and testosterone enanthate in normal men.** *Contraception* (1980) **21**:631-640.
39. Skoglund RD, Paulsen CA: **Danazol-testosterone combination: A potentially effective means of reversible male contraception. A preliminary report.** *Contraception* (1973) **7**:357.

40. Soufir J-C, Jounnet P, Marson J, Soumah A: **Reversible inhibition of sperm production and gonadotrophin secretion in men following combined oral medroxyprogesterone acetate and percutaneous testosterone treatment.** *Acta Endocrinol (Copenh)* (1983) 102:625-632.
41. Bebb RA, Bradley D, Anawalt R, Christensen RB, Paulsen CA, Bremner WJ, Matsumoto A: **Combined administration of levonorgestrel and testosterone induces more rapid and effective suppression spermatogenesis than testosterone alone: a promising male contraceptive approach.** *J Clin Endocrinol Metab* (1996) 81:757-762.
42. Castelli WP, Garrison RJ, Wilson PWF, Abbott RD, Kalousdian S, Kannel WB: **Incidence of coronary heart disease and lipoprotein cholesterol levels – the Framingham study:** *JAMA* (1986) 256:2835-2838.
43. Wu FCW, Balasubramanian R, Mulders T, Coelinh-Bennink H: **Oral progestogen combined with testosterone as a potential male contraceptive: additive effects between desogestrel and testosterone enanthate in suppression of spermatogenesis, pituitary testicular axis and lipid metabolism.** *J Clin Endocrinol Metab* (1999) 84(1):112-122.
- *Use of a novel phased treatment regime showing for the first time, the relative contributions made by testosterone and progestogen in suppression of spermatogenesis.*
44. Wang C, Yeung RTT: **Use of low-dosage oral cyproterone acetate as a male contraceptive.** *Contraception* (1980) 21:245-269.
45. Føgh M, Corker CS, Hunter WM, McLean H, Philip J, Schou G, Skakkebaek NE: **The effects of low doses of cyproterone acetate on some functions of the reproductive system in normal men.** *Acta Endocrinol (Copenh)* (1979) 91:545-552.
46. Roy S, Chatterjee S, Prasad MRN, Poddar AK, Pandey DC: **Effects of cyproterone acetate on reproductive functions in normal human males.** *Contraception* (1976) 14:403-420.
47. Moltz L, Rommler A, Post K, Schwartz U, Hammerstein J: **Medium-dose cyproterone acetate (CPA): Effects on hormone secretion and on spermatogenesis in men.** *Contraception* (1980) 21:393-413.
48. Merrigiola CM, Bremner WJ, Paulsen CA, Valdisserri A, Incorvaia L, Motta R, Pavani A, Capelli M, Flamigni C: **A combined regimen of cyproterone acetate and testosterone enanthate as a potentially highly effective male contraceptive.** *J Clin Endocrinol Metab* (1996) 81:3018-3023.
49. Merrigiola CM, Bremner WJ, Costantino A, Pavani A, Capelli M, Flamigni C: **An oral regimen of cyproterone acetate and testosterone undecanoate for spermatogenic suppression in men.** *Fertil Steril* (1997) 68:844-850.
- *First study employing an entirely oral treatment regimen.*
50. Ewing LL, Desjardin C, Irby DC, Robaire B: **Synergistic interaction of testosterone and estradiol inhibits spermatogenesis in rats.** *Nature* (1977) 269:409-411.
51. Lobl TJ, Kirton KT, Forbes ADEwing LL, Kemp PL, Desjardins C: **Contraceptive efficacy of testosterone-estradiol implants in male Rhesus monkeys.** *Contraception* (1983) 27:383-389.
52. Cummings DE, Bremner WJ: **Prospects for new hormonal male contraceptives.** *Endocrinol Metab Clin North Am* (1994) 22:893-922.
53. Pavlou SN, Dahl KD, Wakefield G, Rivier J, Vale W, Hsueh AJ, Lindner J: **Maintenance of the ratio of bioactive to immunoreactive follicle-stimulating hormone in normal men during chronic luteinizing hormone releasing hormone agonist administration.** *J Clin Endocrinol Metab* (1988) 66:1005.
54. Santen RJ, Demers LM, Max DT: **Long term effects of administration of a gonadotrophin releasing hormone superagonist analogue in men with prostatic carcinoma.** *J Clin Endocrinol Metab* (1984) 58:397-400.
55. Inaba T, Umehara T, Mori J, Torii R, Tamada H, Sawada T: **Reversible suppression of pituitary-testicular function by a sustained release formulation of a GnRH agonist (leuprolide acetate) in dogs.** *Theriogenology* (1996) 46:671-677.
56. Bagatell CJ, McLachlan RI, de Kretser DM, Burger HG, Vale WW, Rivier JE, Bremner WJ: **A comparison of the suppressive effects of testosterone and a potent new GnRH antagonist on gonadotropin and inhibin levels in normal men.** *J Clin Endocrinol Metab* (1989) 69:43-48.
57. Heber D, Dodson R, Swerdloff RS: **Pituitary receptor site blockade by gonadotropin-releasing hormone antagonist in vivo: Mechanism of action.** *Science* (1982) 216:420-421.
58. Pavlou SN, Wakefield G, Schlechter NL, Lindner J, Souza KH, Kamilaris TC, Konidaris S, Rivier JE, Vale WW, Togliola M: **Mode of suppression of pituitary and gonadal function after acute or prolonged administration of a luteinizing hormone-releasing hormone antagonist in normal men.** *J Clin Endocrinol Metab* (1989) 68:446-454.
59. Pavlou SN, Brewer K, Farley MG, Lindner J, Bastias MC, Rogers BJ, Swift LL, Rivier JE, Vale WW, Conn PM: **Combined administration of a gonadotropin-releasing hormone antagonist and testosterone in men induces reversible azoospermia without loss of libido.** *J Clin Endocrinol Metab* (1991) 73:1360-1369.
60. Karten MJ, Rivier JE: **Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: Rationale and perspective.** *Endocr Rev* (1986) 7:44-66.
61. Bagatell CJ, Rivier JE, Bremner WJ: **Dose Effects of the gonadotrophin-releasing-hormone antagonist Nal-Glu, combined with testosterone enanthate on gonadotrophin levels in normal men.** *Fertil Steril* (1995) 64:139-145.
62. Tom L, Bhasin S, Salameh E, Steiner B, Peterson M, Sokol RZ, Rivier J, Vale W, Swerdloff RS: **Induction of azoospermia in normal men with combined Nal-Glu gonadotropin-releasing hormone antagonist and testosterone enanthate.** *J Clin Endocrinol Metab* (1992) 75:476-483.
63. Swerdloff RS, Bagatell CJ, Wang C, Anawalt BD, Berman N, Steiner B, Bremner WJ: **Suppression of spermatogenesis in man induced by Nal-Glu-gonadotrophin releasing hormone antagonist and testosterone enanthate (TE) is maintained by TE alone.** *J Clin Endocrinol Metabol* (1998) 83:3527-3533.
- *Interesting study employing a phased treatment regime showing brisk suppression using combined therapy, which is then maintained by TE alone.*
64. Nieschlag E: **Reasons for abandoning immunization against FSH as an approach to fertility regulation.** In: *Male Contraception: Advances and Future Prospects.* Zatuchini GI, Goldsmith A, Spieler JM et al (Eds), Harper & Row, Philadelphia (1985):395.
65. Tapanainen JS, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi I: **Men homozygous for an inactivating mutation of the follicle stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility.** *Nature Genet* (1997) 15:205-206.
- *Demonstrates that FSH is not required for spermatogenesis.*
66. Anderson RA, Wallace AM, Wu FCW: **Comparison between testosterone enanthate induced azoospermia and oligospermia in a male contraceptive study. 3. Higher 5 alpha reductase activity in oligospermic men administered supraphysiological doses of testosterone** *J Clin Endocrinol Metab* (1996) 81:902-908.
- *Study which begins to answer the question of heterogeneity of response to testosterone in contraceptive regimens.*

67. Zhengwei Y, Wreford NG, Royce P, de Krester DM, McLachlan R: **Stereological evaluation of human spermatogenesis by testosterone treatment: heterogeneous pattern of spermatogenic impairment.** *J Clin Endocrinol Metab* (1998) **83**:1284-1291.
68. O'Donnel L, Kyriakos P, Stanton PG, Robertson DM, McLachlan RI: **Testosterone dependent restoration of spermatogenesis in adult rats is impaired by a 5 $\alpha$ -reductase inhibitor.** *J Androl* (1999) **20**:109-117.
- *Animal study which, like [66], suggests that intratesticular DHT levels contribute to variation in spermatogenic response to testosterone.*
69. Handelsman DJ, Farley TMM, Peregoudov A, Waites GMH and World Health Organisation Task Force on Methods for the Regulation of Male Fertility: **Factors in non-uniform induction of azoospermia by testosterone enanthate in normal men.** *Fertil Steril* (1995) **63**:125-133.
70. Santner SJ, Albertson B, Zhang GY, Zhang GH, Santulli M, Wang C, Demers LM, Shackleton C, Santen RJ: **Comparative rates of androgen production and metabolism in Caucasian and Chinese subjects.** *J Clin Endocrinol Metab* (1998) **83**:2104-2109.
71. Wang C, Berman N, Veldhuis JD, Der T, McDonald V, Steiner B, Swerdloff R: **Graded testosterone infusions distinguish gonadotrophin negative feedback responsiveness in asian and white men- a clinical research center study.** *J Clin Endocrinol Metab* (1998) **83**:870-876.
72. Johnson L, Barnard J, Rodriguez L, Smith E, Swerdloff R, Wang X, Wang C: **Ethnic differences in testicular structure and spermatogenic potential may predispose testes of Asian men to a high sensitivity to steroidal contraceptives.** *J Androl* (1998) **19**:348-357.
73. Wu FCW: **Male contraception: current status and future prospects.** *Clin Endocrinol* (1998) **29**:443-465.
74. Swerdloff RS, Wang C, Bhasin S: **Male contraception: 1988 and beyond.** In: *The Testis*. Burger H, de Krester D (Eds), Raven Press, New York (1989):547.
75. Matlin SA: **Prospects for pharmacological male contraception.** *Drugs* (1994) **48**:851-863.
76. Zhen QS, Ye X, Wei ZJ: **Recent progress in research on Tripterygium - a male antifertility plant.** *Contraception* (1995) **51**:121-129
77. Lue YH, Hikim APS, Wang C, Leung A, Baravarian S, Reutrakul V, Sangawan R, Chaichana S, Swerdloff RS: **Triptolide: a potential male contraceptive.** *J Androl* (1998) **19**:479-486.
78. Takei A, Nagahima G, Suzuki R, Hokaku H, Takahashi M, Miyo T, Asai J, Sanada Y, Fujimoto T: **Meningoencephalocele associated with Tripterygium wilfordii treatment.** *Paediatric Neurosurgery* (1997) **27**:45-48.
79. Cosentino MJ, Pakyz RE, Fried J: **Pyrimethamine: an approach to the development of a male contraceptive.** *Proc Nat Acad Sci USA* (1990) **87**:1431-1435.
80. Ford WCL, Waites GMH: **Sperm maturation and the potential for contraceptive interference.** In: *Male contraception: advances and future prospects*. Zatuchni GI, Goldsmith A, Speiler JM *et al* (Eds), Harper & Row, Philadelphia (1986):89-106.
81. Vickery BH, Grigg MB, Goodpasture JC, Bergstrom KK, Walker K: **Towards a same-day, orally administered male contraceptive.** In: *Male contraception: advances and future prospects*. Zatuchni GI, Goldsmith A, Speiler JM *et al* (Eds), Harper & Row, Philadelphia (1986):271-92.
82. Nieschlag E, Behre HM, Engelmann U, Hertle L: **Male contribution to contraception.** In: *Andrology: Male reproductive health and reproduction*. Nieschlag E, Behre HM (Eds), Springer-Verlag, Berlin (1997): 377-392.

# Prolactin receptor expression in human testis and accessory issues: localization and function

N.M.Hair, O.Gubbay, H.N.Jabbour and G.A.Lincoln<sup>1</sup>

MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, UK

To whom correspondence should be addressed. E-mail: g.lincoln@hrsu.mrc.ac.uk

Experimental studies in animals have established prolactin (PRL) as a progonadal hormone that promotes the function of the testis and reproductive accessory glands. The present study investigated the localization of PRL receptor (PRL-R) expression in the human testis and accessory tissues. Expression of PRL-R was identified in human testis and vas deferens by RT-PCR, and further localized by immunohistochemistry to the Leydig cells and differentiating germ cells of the testis (developmental stages extending from pachytene spermatocytes to elongating spermatids). Positive staining for PRL-R was also clearly evident in the epithelium of vas deferens, epididymis, prostate and seminal vesicles. Functional activation of PRL-R was demonstrated in fresh samples of vas deferens collected at vasectomy by examination of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAP (mitogen-activated protein) kinase ERK (extracellular signal-regulated kinase) signalling pathways. Within the vas deferens, PRL induced rapid tyrosine phosphorylation of JAK 2 and STAT 5 (after 10 and 20 min respectively), and tyrosine and threonine phosphorylation of ERK 1 and 2 (after 5 min). The demonstration of function and localization of PRL-R presented here suggests multiple roles for PRL in the human male reproductive tract.

**Key words:** male reproductive tract/prolactin/prolactin receptor/testis

## Introduction

Experimental studies in animals have established that prolactin acts in concert with the classical gonadotrophins, LH and FSH, to stimulate full testicular function in the adult male rat, mouse and hamster (Bartke, 1971; Bartke *et al.*, 1975; Zipf *et al.*, 1978; Dombrowicz *et al.*, 1992). In the ram, a functional role for prolactin (PRL) in the testis is indicated by the observation that hypothalamo-pituitary disconnected rams, that permanently lack gonadotrophin due to a blockade of GnRH secretion, continue to express cycles in testicular size in response to photoperiod-induced changes in PRL secretion. These gonadal changes are minor and occur with a long latency, but they indicate that PRL is a weak gonadotrophin in the absence of LH and FSH (Lincoln *et al.*, 1996).

Prolactin receptor (PRL-R) gene expression in the testis has been demonstrated in different species including rat, ram and red deer (Ouhtit *et al.*, 1993; Hondo *et al.*, 1995; Jabbour *et al.*, 1998a; Jabbour and Lincoln, 1999). These studies show PRL-R to be localized to the Leydig cells in the interstitium and germ cells within the seminiferous tubules (Jabbour and Lincoln, 1999). The addition of PRL to testicular explants of ruminant species induces phosphorylation of JAK (Janus kinase) and STAT (signal transducer and activator of transcription) signalling proteins, consistent with a functional PRL-R in the testis (Jabbour *et al.*, 1998b). PRL is believed to stimulate testicular steroidogenesis by regulating LH receptors (Bex and Bartke, 1977; Takase *et al.*, 1990), or androgen/estrogen biosynthesis through the control of rate-limiting enzymes in the Leydig cells (Takeyama *et al.*, 1986; Chandrashekar and Bartke, 1988). However, the mechanism of action of PRL on spermatogenesis remains to be clarified.

Expression of PRL-R has also been demonstrated in the rat dorsal and lateral prostate, and seminal vesicles (Ouhtit *et al.*, 1993; Nevalainen *et al.*, 1996). In cultured prostatic cells, androgens and estrogens stimulate the expression of PRL-R (Nevalainen *et al.*, 1996), and both gonadal steroids and PRL, induce the secretion of prostate-specific proteins (Costello and Franklin, 1994). In transgenic mice engineered to over-express the PRL gene, the prostate gland becomes grossly enlarged illustrating the importance of PRL in the control of accessory gland function (Wennbo *et al.*, 1997). Chronic suppression of blood concentrations of PRL secretion in the ram produces a decrease in size and fructose content of the seminal vesicles, with no change in testosterone secretion (Ravault *et al.*, 1977), and manipulations of PRL and androgens in the macaque monkey affect seminal vesicular enzymes (Arunakaran *et al.*, 1988); thus, PRL may promote the function of various androgen-dependant male accessory structures.

Clinical observations also support a role for PRL in the regulation of the testis and accessory glands in man. For example, the restoration of normal PRL levels in a cohort of subfertile, hypoprolactinaemic men caused an increase in sperm density and quality, and restored fertility (Ufearo *et al.*, 1995). In another study, suppression of gonadotrophins and PRL secretion in eugonadal men treated for prostatic carcinoma caused a more marked reduction in testicular weight and spermatogenesis than suppression of gonadotrophin secretion alone (Huhtaniemi *et al.*, 1991). Both observations are consistent with a progonadal role of PRL in the testis, although early studies using I<sup>125</sup>-iodo PRL failed to demonstrate the presence of PRL binding in the human testis, in contrast to the situation in the rat (Wahlstrom *et al.*, 1983). PRL binding has been demonstrated in the human prostate (Leake *et al.*, 1983), and other studies suggest that

PRL may play a role in the aetiology of benign prostatic hyperplasia and cancer (Kadar *et al.*, 1988; Nevalainen *et al.*, 1997). The synchronous reduction in both PRL and androgen improves the efficacy of the treatment of prostatic carcinoma (Rana *et al.*, 1995).

The purpose of the present study was to provide direct evidence for a role of PRL in the regulation of the testis and reproductive tract in man. To this end, the expression of the PRL-R gene was investigated by RT-PCR using RNA extracted from human testis and vas deferens. The localization of expression of the PRL-R protein was further studied using immunohistochemistry in sections prepared from the human testis, epididymis, vas deferens, prostate and seminal vesicles. Lastly, a functional PRL-R was identified in the vas deferens by investigating activation of the JAK/STAT and MAP (mitogen activated protein) kinase ERK (extracellular signal-regulated kinase) specific intracellular signalling pathways. Activation of JAK/STAT and ERK proteins, following binding of PRL to its receptor, mediates both proliferative and differentiating effects in target cells (Findiori and Kelly, 1995; Lewis *et al.*, 1998).

## Materials and methods

### Subjects and tissues

Testicular tissue ( $n = 6$ ), exhibiting normal morphology, was obtained by biopsy from men exhibiting unexplained infertility. Vas deferens tissue ( $n = 10$ ) was obtained from normal men undergoing vasectomy. Left and right vas deferens were used for comparison between treatment and control. Ethical approval was obtained from Lothian Paediatric and Reproductive Medicine Research Ethics Subcommittee, and written informed consent was obtained from each subject. Prostate and bladder tissues were obtained from archival surgical re-section specimens stored by the Department of Pathology, Western General Hospital, Edinburgh. Epididymis and seminal vesicles were obtained from a commercially available human cadaver tissue library.

### Tissue culture

Following collection, vas deferens tissue was washed in phosphate-buffered saline (PBS) twice and subsequently minced thoroughly with fine scissors. Four aliquots of each tissue (~0.17 g each) were then incubated overnight in 2 ml serum-free RPMI 1640 medium (Sigma Chemical Co., Dorset, UK) containing 100 IU/ml penicillin and 100 mg/ml streptomycin in a 37°C incubator with 5% CO<sub>2</sub>:95% air. The following day, samples were treated with 100 ng/ml human PRL (hPRL-SIAFP-B2; donated by NIDDK, NIH) for 0, 5, 10 and 20 min. The tissue was stored at -70°C prior to analysis by immunoprecipitation and/or Western blotting.

### RNA extraction and RT-PCR

Total RNA was extracted from testis and vas deferens using the guanidinium thiocyanate method as previously described (Chomczynski and Sacchi, 1987). Polyadenylated RNA (poly A<sup>+</sup>) was purified on oligo(deoxythymidine)-cellulose affinity columns (Pharmacia Biotech, St Albans, Herts, UK); the yield and purity of RNA was estimated by spectrophotometry. Single strand cDNA was generated from 5 µg poly A<sup>+</sup> RNA by reverse transcription using 1.6 ng oligo (deoxythymidine)<sub>12-18</sub> primer and Superscript reverse transcriptase, according to the manufacturer's instructions (Gibco BRL, Paisley, UK). cDNA (10 ng) was then diluted 25 times in double-distilled H<sub>2</sub>O and amplified by PCR using primers corresponding to nucleotide positions 182-201 (5'-CACCTCCTGAAAAACCCAAG-3'; forward primer) and 724-743 (5'-CCATGGTCTGGCTGCAGCG-3'; reverse primer) of the PRL-R open reading frame. The reaction was carried out in PCR buffer (50 mmol/l KCl, 2 mmol/l MgCl<sub>2</sub> and 20 mmol/l Tris-HCl, pH 8.3), 200 µmol/l deoxy-NTPs, 25 pmol forward and reverse primers and 1 IU Taq polymerase (Perkin-Elmer, Warrington, Cheshire, UK) in a total volume of 50 µl. Samples were subjected to 35 cycles of 94°C for 40 s, 52°C for 75 s and 72°C for 150 s. After a 10 min final extension at 72°C, the products were visualized on a 1% agarose gel using ethidium bromide staining.

### Histology and immunohistochemistry

Testis, vas deferens and other tissues were placed immediately in Bouin's fixative for 6 h before transfer to 70% ethanol, and subsequent dehydration and embedding in wax blocks. Sections were cut and mounted on slides coated with 2% 3-aminopropyltriethoxysilane (TESPA) in acetone. Slides were then dried overnight at 50°C before dewaxing in histoclear (National Diagnostics, Hull, UK). Tissues were rehydrated in graded ethanol and washed in water followed by Tris-buffered saline (TBS; 0.05 mol/l Tris-HCl, pH 7.4, 0.85% NaCl). Sections were treated with 10% hydrogen peroxide in methanol for 30 min and blocked for 30 min with normal swine serum (NSS) diluted 1:5 in TBS with 5% bovine serum albumin (BSA).

The primary antibody for PRL-R (kindly donated by Dr P.M. Ingleton, School of Medicine, University of Sheffield) was raised against a 16 amino acid synthetic peptide corresponding to residues 53-68 of the external domain of the rat PRL-R (Nevalainen *et al.*, 1996). The polyclonal antibody was validated for use in human tissue based on the manner that pre-absorption of the antibody with the corresponding synthetic peptide totally blocked the immunostaining (Nevalainen *et al.*, 1999). The polyclonal antibody was diluted in NSS/TBS/5% BSA (as above) and incubated overnight at 4°C. Control sections were incubated with non-immune rabbit serum. All sections were washed twice in TBS (5 min each), incubated for 30 min with biotinylated swine anti-rabbit Ig (Dako, Bucks, UK), diluted 1:500 in NSS/TBS. Sections were washed again twice in TBS (5 min each) and incubated with peroxidase conjugated to avidin-biotin complex (Dako) for 30 min at room temperature. Colour reaction was developed by incubation in a mixture of 0.05% 3,3'-diaminobenzidine (DAB; Sigma) in 10 ml 0.05 mol/l Tris-HCl buffer (pH 7.4) and 0.033% hydrogen peroxide. Sections were subsequently counterstained using haematoxylin.

### Immunoprecipitation and Western blotting

Tissue was homogenized and lysed in 150 mmol/l NaCl, 10 mmol/l Tris (pH 7.4), 1 mmol/l EDTA, 10% glycerol, 0.6% NP40, 10 µg/ml aprotinin, 1 mmol/l phenylmethylsulphonyl fluoride and 1 mmol/l sodium orthovanadate. Cytoplasmic extracts were prepared by centrifugation for 2 min at 10 000 g. For analysis of phosphorylation of JAK 2 and STAT 5, 50 µg of protein was incubated with 10 µl monoclonal anti-phosphotyrosine antibody (5 mg/ml; Affiniti, Exeter, UK) for 1 h at 4°C. Samples were then incubated overnight at 4°C with M-450 Dynabeads conjugated to rat anti-mouse IgG2b (Dyna, Wirral, UK). The complexes were washed three times in PBS using a Dynal MPC magnet (Dyna), and boiled for 5 min in sample buffer [125 mmol/l Tris-HCl, pH 6.8, 4% sodium dodecyl sulphate (SDS), 2.5% dithiothreitol, 20% glycerol, 0.05% Bromophenol Blue].

For Western blotting, samples were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). For analysis of ERK phosphorylation, 50 µg of protein from each sample was analysed. Membranes were incubated with antibodies against ERK 1/2, JAK 2, STAT 5 (Santa Cruz Biotechnology, Santa Cruz CA, USA) or phosphorylated ERK (T202/Y204, Cell Signaling, New England Biolabs, Beverly, MA, USA), each diluted 1000-fold in 2% dried skimmed milk/TBST (20 mmol/l Tris-HCl pH 7.4, 500 mmol/l NaCl, 0.1% Tween 20). Membranes were washed briefly in TBST and incubated with secondary antibodies conjugated to horse radish peroxidase (Amersham, Buckinghamshire, UK), in 2% milk/TBST. Membranes were again washed in TBST and proteins detected using the ECL+ detection kit (Amersham).

### Statistical analysis

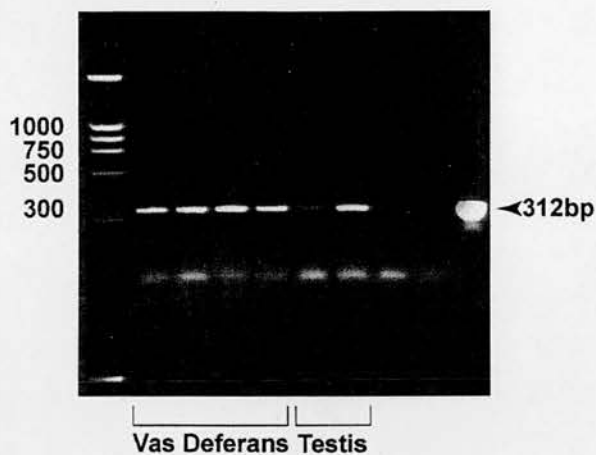
The degree of phosphorylation of proteins in response to PRL was measured at four time points by densitometry, and the significance of the changes was analysed by analysis of variance (ANOVA) with repeated measures.

## Results

### RT-PCR of PRL-R mRNA

The expression of PRL-R was analysed initially by RT-PCR, using RNA extracted from testis and vas deferens. A single 312 bp transcript, corresponding to a region in the extracellular domain of the human PRL-R mRNA, was amplified in all samples of both testis and vas

## PRL-R RTPCR



**Figure 1.** RT-PCR analysis of prolactin receptor (PRL-R) from RNA purified from human vas deferens and testis. Lane 1: DNA size marker, lanes 2–5: RNA from vas deferens, lanes 6 and 7: RNA from testis, lanes 8 and 9: no reverse transcriptase added, lane 10: positive control using PRL-R cDNA as template. The predicted size of the PCR product corresponding to the extracellular domain of the PRL-R mRNA is marked as 312 bp.

deferens tissue, indicative of the presence of PRL-R mRNA in these tissues (Figure 1).

### Immunohistochemistry of PRL-R

To determine the localization of PRL-R, immunohistochemistry was performed using sections prepared from human testis, vas deferens, epididymis, prostate, seminal vesicle and bladder. In the testis biopsies of all six subjects, weak but consistent immunostaining was localized to the Leydig cells in the interstitial tissue and stronger staining localized to the germ cells at different stages of spermatogenesis in the seminiferous tubules. Representative micrographs are shown in Figure 2a,c. The most marked staining was evident in the cytoplasm of the germ cells in the developmental stages extending from pachytene spermatocytes to elongating spermatids. The spermatogonia, early dividing spermatocytes and final differentiating sperm were devoid of staining, as were the Sertoli cells (Figure 2c). Control sections of testis were totally devoid of staining (Figure 2b). In vas deferens collected from 10 different subjects, and in epididymis and seminal vesicle samples collected from three other subjects, immunostaining for PRL-R was consistently evident in the epithelium, with minimal staining in the underlying stroma and muscle tissue (Figure 2d,f,g); control sections of vas deferens were again unstained (Figure 2e). Immunostaining for PRL-R was also consistently present in the epithelium of the prostate in three subjects (data not shown; demonstrated previously) (Nevalainen *et al.*, 1997).

### Intracellular signalling in the vas deferens

Intracellular signalling pathways were examined by investigating tyrosine phosphorylation of JAK 2 and STAT 5 and tyrosine and threonine phosphorylation of ERK 1 and 2, following stimulation of fresh vas deferens tissue with human PRL (Figure 3). This was repeated using fresh tissue from three different subjects attending for vasectomy. Tyrosine phosphorylation of JAK 2 and STAT 5 was measured by an immunoprecipitation procedure using an antibody against phosphorylated tyrosine, followed by Western blotting using antibodies against JAK 2 and STAT 5. Phosphorylation of ERK 1 and 2 were measured by Western blotting using an antibody raised against ERK, phosphorylated on specific tyrosine and threonine residues, and the density of the signal was measured by densitometry.

Rapid phosphorylation, with a significant increase in signal intensity ( $P < 0.05$ , ANOVA), was observed following treatment with PRL for ERK 1 and 2 (evident from 5 min), JAK 2 (10 min) and STAT 5 (20 min) (Figure 3). No increase in phosphorylated proteins was observed in the absence of PRL.

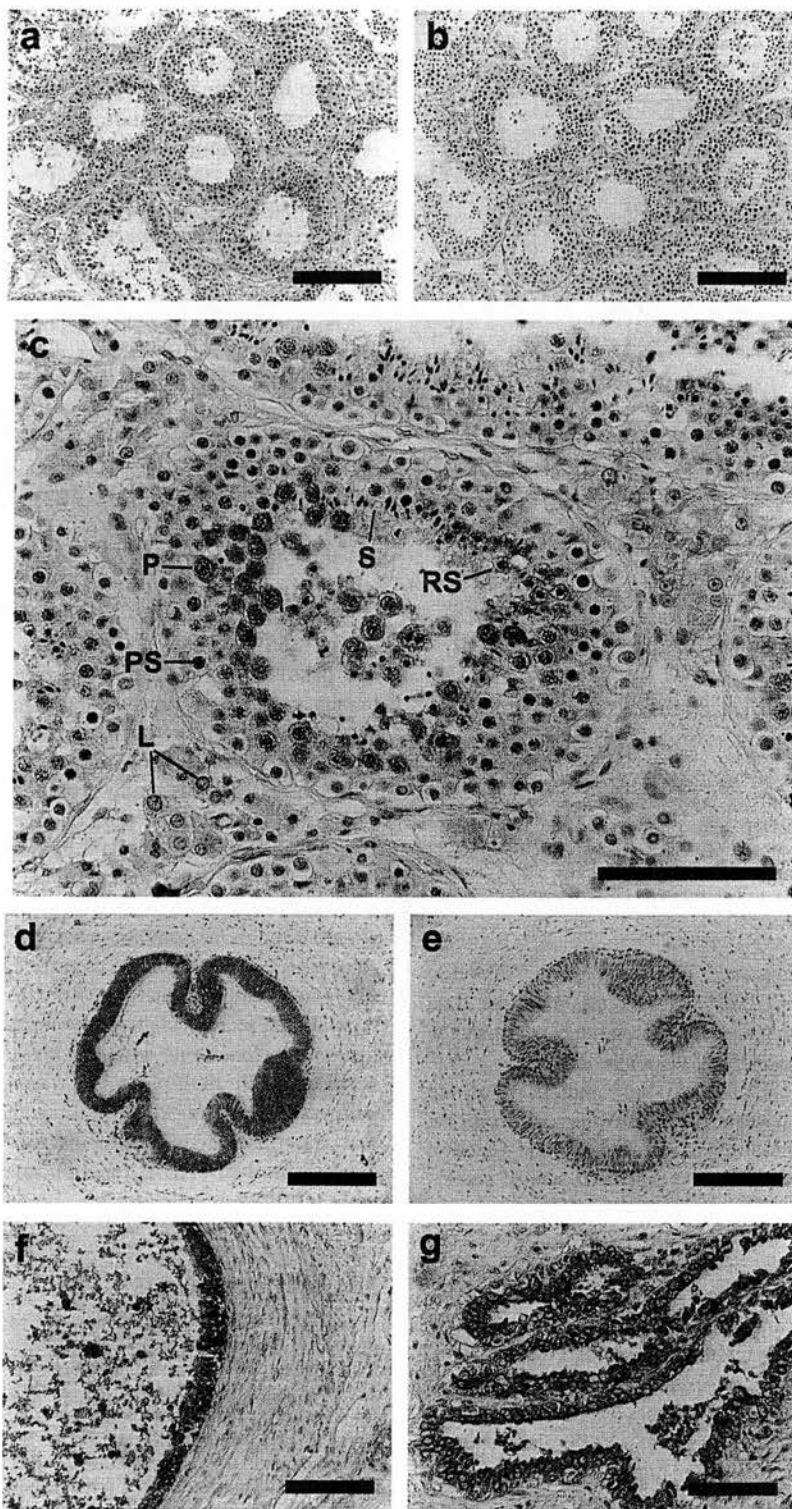
### Discussion

The data presented here demonstrate that PRL-R is expressed in the human testis and in various male accessory glands. This is based on RT-PCR amplification of the receptor mRNA extracted from testis and vas deferens, and the localization of the receptor protein by immunocytochemistry in a range of reproductive tissues. Furthermore, incubation studies with fresh tissues of human vas deferens show that the addition of PRL activates phosphorylation of several proteins known to mediate the intracellular effects of PRL in target cells. These data provide clear evidence for functional activation of PRL-R in a clinically accessible human male reproductive tissue.

Various isoforms of PRL-R, differing in the intracellular domain, have been characterized in man, and these are differentially expressed in the many PRL target organs (Kline *et al.*, 1999; Hu *et al.*, 2001). Long forms are expressed in the testis, and long and intermediate forms in the prostate. In the human testis, the abundance of PRL-R mRNA is low compared with many tissues (Kline *et al.*, 1999), and there is evidence that a consistent portion of PRL-R present in the testis is in a truncated form which may block, or alter, the function of the long form of the receptor (Hu *et al.*, 2001). The methodology used in the current study was directed at the extracellular domain of PRL-R (cDNA primers for RT-PCR, and antibody for immunocytochemistry). Thus, it was not possible to confirm the expression of the different isoforms of PRL-R in the human testis and other tissues. In the rat, different isoforms of PRL-R have been identified, and both are expressed in the testis and accessory glands (Ouhtit *et al.*, 1993). Hence, it is likely that a similar diversity of PRL-R expression exists in human testis and accessory glands; this may permit tissue-specific responses to PRL within the male reproductive tract.

The current immunohistochemical studies localized for the first time the PRL-R protein to the Leydig cells and germ cells of the human testis. This is contrary to the results of an earlier study which failed to detect PRL-R in human testicular tissue using a radioreceptor method, although the receptor was readily demonstrated in the rat (Wahlstrom *et al.*, 1983). This was despite the use of fresh human testicular tissue, and a comprehensive study of the binding characteristics of human PRL in testis homogenates. The most likely explanation for the discordance is that PRL-R is expressed at low abundance in the human testis, and it requires a sensitive immunohistochemical or molecular amplification method to resolve the localization or gene expression in the tissue. The current study also confirmed PRL-R expression in the secretory, adsorptive or lining epithelium in the various accessory male structures. The pattern of immunostaining in the testis is similar to that described in the red deer and ram (Jabbour *et al.*, 1998a; Jabbour and Lincoln, 1999). Expression of the receptor in Leydig cells is consistent with a role of PRL in the control of testicular steroidogenesis. Evidence in rodent species suggests that PRL influences testosterone secretion through the stimulation and maintenance of expression of LH receptors in Leydig cells (Klemcke *et al.*, 1984; Takase *et al.*, 1990) or by regulating specific enzymatic steps in androgen biosynthesis (Chandrashekar and Bartke, 1988). The effect of PRL on steroidogenesis in the adrenal gland is also well documented in animals and man (Glasow *et al.*, 1996).

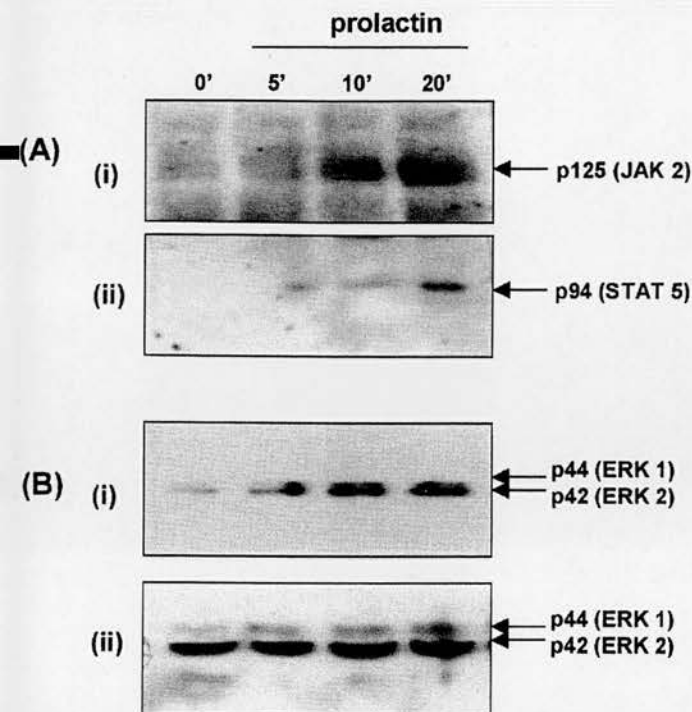
The presence of PRL-R in the differentiating germ cells in the testis is consistent with the view that PRL acts directly within the



**Figure 2.** Immunohistochemistry of the prolactin receptor (PRL-R) in human male reproductive tissues. Human testis was immunostained with (a) and without (b) PRL-R antibody and examined at  $\times 20$  magnification. Panel (c) corresponds to testis immunostained with PRL-R and examined at  $\times 40$  magnification. Expression of PRL-R is evident in Leydig cells (L) in the interstitial tissue, pachytene spermatocytes (P), and around spermatids (RS) in the seminiferous tubules. There was no apparent staining in the spermatocytes at early stages of spermatogenesis (PS) and differentiated sperm (S). Vas deferens was immunostained with (d) and without (e) PRL-R antibody and examined at  $\times 40$  magnification. Panel (f) human epididymis and panel (g) human seminal vesicle stained for PRL-R. Size bar = 100  $\mu\text{m}$ .

seminiferous tubules to affect spermatogenesis, as well as acting indirectly through gonadal steroid secretion. In the immature hypophysectomized rat, treatment with PRL stimulated an increase in the number of primary spermatocytes (Dombrowicz *et al.*, 1992), while

in hypoprolactinaemic subfertile men, exogenous PRL, or treatment with metoclopramide to promote PRL secretion, increased sperm density, reduced sperm abnormalities and acted to restore fertility (Uferio *et al.*, 1995). Other studies in the ram indicate that the effects



**Figure 3.** Phosphorylation of JAK 2, STAT 5 and ERK1/2 in the human vas deferens, in response to prolactin. Tissue was serum starved and incubated with 100 ng/ml of human prolactin for 0, 5, 10 or 20 min. (A) Proteins immunoprecipitated with anti-phosphotyrosine antibody and analysed by Western blotting using antibodies against JAK 2 (i) and STAT5 (ii). (B) Proteins analysed by Western blotting using antibodies against phosphorylated (T202/Y204) ERK (i) and (non-phosphorylated) ERK (ii).

of PRL in the testis are largely dependent on the concurrent secretion of LH and FSH (Lincoln *et al.*, 2001). Taken together, the data indicate that PRL acts within the testis to facilitate and augment the actions of LH and FSH in promoting full testicular activity.

The universal distribution of PRL-R in the epithelium of the epididymis, vas deferens, seminal vesicles and prostate confirms that PRL also plays a role in the regulation of accessory gland function in man. Animal studies clearly demonstrate that PRL acts in association with androgens to stimulate the activity of the accessory glands. For example, the production of secreted proteins from cultured rat prostate cells is markedly enhanced by addition of PRL (Nevalainen *et al.*, 1996). In transgenic rodent models, over-expression of PRL, or its receptor, is associated with enlargement of the accessory glands, while under-expression is associated with reduced functional activity (Wennbo *et al.*, 1997). The clinical observation that suppression of PRL enhances the effectiveness of steroid withdrawal for the treatment of malignant disease of the prostate (Rana *et al.*, 1995) also supports an action for PRL in the human accessory glands. The presence of PRL-R in the epithelial cells in the tissues of the male reproductive tract suggests that PRL is potentially involved in the regulation of secretion, absorption, and/or the control of transport of fluids across the cell membrane. This is consistent with a conserved function of PRL in epithelial tissues across species (Nicoll, 1974).

As well as demonstrating functional PRL-R within the vas deferens, the current results support the view that PRL signals through more than one intracellular pathway to regulate the tissue responses. In the vas deferens, PRL activated rapid tyrosine phosphorylation of JAK 2 and STAT 5 protein. The temporal sequence is similar to that observed in other PRL target-tissues including rat mammary gland (Jahn *et al.*, 1997) and ovary (Ruff *et al.*, 1996), ram testis (Jabbour and Lincoln, 1999) and human endometrium (Jabbour *et al.*, 1998b).

Upon activation, STAT proteins dimerize, translocate to the nucleus and bind to STAT-regulatory elements in the promoters of target genes to influence transcription (Schindler and Darnell, 1995). In the mammary gland, activation of STAT 1 and STAT 5 proteins up-regulate transcription of genes for  $\beta$ -casein (Gouilleux *et al.*, 1994),  $\beta$ -lactoglobulin (Burdon *et al.*, 1994) and whey acidic protein (Li and Rosen, 1995) to mediate the effects of PRL on milk synthesis and secretion. The target genes for PRL in the vas deferens epithelium are still unknown, but are likely to include genes encoding secretory proteins.

The demonstration of rapid phosphorylation of ERK proteins in response to PRL in human vas deferens indicates that PRL also acts via the ERK pathway to affect the function of the secretory epithelium. The involvement of both the JAK/STAT and ERK pathways acting together to influence differentiation has been documented in other cell types (Lewis *et al.*, 1998). In the human endometrium, PRL induces the JAK/STAT and ERK pathways in the epithelial cells during the secretory phase of the menstrual cycle (Jabbour *et al.*, 1998b; Gubbay *et al.*, 2001). The activation of ERK by PRL in terminally differentiated secretory epithelium, as in the human vas deferens, indicates that PRL signals through ERK, as well as through the JAK/STAT pathway, to provide divergent control of more than one cellular response. In the current study, only fresh tissue from human vas deferens was available for tissue culture, thus it was not possible to establish the pattern of phosphorylation of the second messenger proteins in the different male reproductive tissues.

In conclusion, this study provides clear support for the view that PRL is a regulator of reproductive function in the human male. PRL appears to act in the Leydig cells of the testis to promote steroidogenesis, and in the germ cells of the seminiferous tubules of the testis to promote the efficiency of spermatogenesis, actions that augment the more dominant progonadal effects of LH and FSH. PRL also acts in the epithelia of the efferent ducts and the male accessory sex glands, in conjunction with the gonadal steroid hormones, to regulate the secretory/adsorptive functions of these male tissues. Our results are consistent with the pleiotrophic character of PRL, as a hormone with multiple target tissues signalling through multiple pathways. A role for PRL within the male reproductive tract, suggests that abnormalities in circulating levels of PRL within the body, or abnormalities in PRL-R function, may have a significant impact on male fertility.

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## References

- Arunakaran J., Balasubramanian K., Srinivasan, N., Aruldas, M.M. and Govindarajulu, P. (1988) Effects of androgens, prolactin and bromocriptine on seminal vesicular enzymes of the pyruvate malate cycle involved in lipogenesis in castrated mature monkeys, *Macaca radiata*. *Int. J. Androl.*, **11**, 133–139.
- Bartke, A. (1971) Effect of prolactin on spermatogenesis in hypophysectomised mice. *J. Endocrinol.*, **49**, 311–316.
- Bartke, A., Croft, B.T. and Dalterio, S. (1975) Prolactin restores plasma testosterone levels and stimulates testicular growth in hamsters exposed to short day-length. *Endocrinology*, **97**, 1601–1604.

- Bex, F.K. and Bartke, A. (1977) Testicular LH binding in the hamster: modification by photoperiod and prolactin. *Endocrinology*, **100**, 1223–1226.
- Burdon, T.G., Demmer, J., Clarke J.A. and Watson, C.J. (1994) The mammary factor MPBF is a prolactin-induced transcription regulator which binds to STAT factor recognition sites. *Fed. Eur. Biochem. Soc.*, **350**, 177–182.
- Chandrashekar, V. and Bartke, A. (1988) Influence of endogenous prolactin on luteinising hormone stimulation of testicular steroidogenesis and the role of prolactin in the adult male rat. *Steroids*, **5**, 559–576.
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
- Costello, L.C. and Franklin, R.B. (1994) Effect of prolactin on the prostate. *Prostate*, **24**, 162–166.
- Dombrowicz, D., Sente, B., Closset, J. and Hennen, G. (1992) Dose-dependent effects of human prolactin on the immature hypophysectomised rat testis. *Endocrinology*, **130**, 695–700.
- Findiori, J. and Kelly, P.A. (1995) Cytokine receptor signalling through two novel families of transducer molecules: Janus kinase and signal transducers and activation of transcription. *J. Endocrinol.*, **147**, 11–23.
- Glasow, A., Breidert, M., Haidan, A., Andereg, U., Kelly, P.A. and Bornstein, S.R. (1996) Functional aspects of the effect of prolactin (PRL) on adrenal steroidogenesis and distribution of PRL receptor in the human adrenal gland. *J. Clin. Endocrinol. Metab.*, **81**, 3103–3111.
- Gouilleux, F., Wakao, H., Mundt, M. and Groner, B. (1994) Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J.*, **13**, 4361–4369.
- Gubbay, O., Critchley, H.O.D., Bowen, J.M., King, A. and Jabbour, H.N. (2001) Prolactin induces ERK phosphorylation in epithelial and CD56+ natural killer cells of the human endometrium. *J. Clin. Endocrinol. Metab.*, in press.
- Hondo, E., Kurohmaru, M., Sakai, S., Ogawa, K. and Hatashi, Y. (1995) Prolactin receptor expression in rat spermatogenic cells. *Biol. Reprod.*, **52**, 1284–1290.
- Hu, Z.Z., Meng, J. and Dufau, M.L. (2001) Isolation and characterization of two novel forms of the human prolactin receptor generated by alternative splicing of a newly identified exon 11. *J. Biol. Chem.*, **276**, 41068–41094.
- Huhtaniemi, I., Parvinen, M., Venho, P. and Rannikkos, S. (1991) Combination of a GnRH agonist with an antiandrogen or bromocriptine in the treatment of prostatic cancer; slight potentiation of antigonadal effects. *Int. J. Androl.*, **14**, 374–386.
- Jabbour, H.N. and Lincoln, G.A. (1999) Prolactin receptor expression in the testis of the ram: localization, functional activation and the influence of gonadotrophins. *Mol. Cell. Endocrinol.*, **148**, 151–161.
- Jabbour, H.N., Clarke, L.A., McNeilly, A.S., Edery, M. and Kelly, P.A. (1998a) Is prolactin a gonadotrophic hormone in red deer (*Cervus elaphus*)? Pattern of expression of the prolactin receptor in the testis and epididymis. *J. Mol. Endocrinol.*, **20**, 175–182.
- Jabbour, H.N., Critchley, H.O.D. and Boddy, S.C. (1998b) Expression of functional prolactin receptors in non-pregnant human endometrium: Janus kinase-2, signal transducer and activator of transcription-1 (STAT1), and STAT5 proteins are phosphorylated after stimulation with prolactin. *J. Clin. Endocrinol. Metab.*, **83**, 2545–2553.
- Jahn, G.A., Daniel, N., Jolivet, G., Belair, L., Bole-Feysot, C., Kelly, P.A. and Djiane, J. (1997) *In vivo* study of prolactin (PRL) intracellular signalling during lactogenesis in the rat: Jak/Stat pathway is activated by PRL in the mammary gland but not in the liver. *Biol. Reprod.*, **57**, 894–900.
- Kadar, T., Ben-David, M., Pontes, J.E., Fekete, M. and Schally, A.V. (1988) Prolactin and luteinizing hormone-releasing hormone receptors in human benign prostatic hyperplasia and prostatic cancer. *Prostate*, **12**, 299–307.
- Klemcke, H.G., Bartke, A. and Borer, K.Y. (1984) Regulation of testicular prolactin and luteinising hormone receptors in the golden hamster. *Endocrinology*, **114**, 594–603.
- Kline, J.B., Roehrs, H. and Clevenger, C.V. (1999) Functional characterization of the intermediate isoform of the human prolactin receptor. *J. Biol. Chem.*, **274**, 35461–35468.
- Leake, A., Chrisholm, G.D. and Habib, F.K. (1983) Characterization of the prolactin receptor in human prostate. *J. Endocrinol.*, **99**, 321–328.
- Lewis, T.S., Shapiro, P.S. and Ahn, N.G. (1998) Signal transduction through MAP kinase cascades. *Adv. Cancer Res.*, **74**, 49–139.
- Li, S. and Rosen, J.M. (1995) CTF/NF1 and mammary gland factor (STAT5) play a critical role in regulating rat whey acidic protein gene expression in transgenic mice. *Mol. Cell. Biol.*, **15**, 2063–2070.
- Lincoln, G.A., Clarke, I.J. and Sweeney, T. (1996) Hamster-like cycles in testicular size in the absence of gonadotrophin secretion in HPD rams exposed to long-term changes in photoperiod and treatment with melatonin. *J. Neuroendocrinol.*, **8**, 855–866.
- Lincoln, G.A., Townsend, J. and Jabbour, H.N. (2001) Prolactin actions in the sheep testis: a test of the priming hypothesis. *Biol. Reprod.*, **65**, 936–943.
- Nevalainen, M.T., Valve, E.M., Ingleton, P.M. and Harkonen, P.L. (1996) Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. *Endocrinology*, **137**, 3078–3088.
- Nevalainen, M.T., Valve, E.M., Ingleton, P.M., Nurmi, M., Martikainen, P.M. and Harkonen, P.L. (1997) Prolactin and prolactin receptors are expressed and functioning in the human prostate. *J. Clin. Invest.*, **99**, 618–627.
- Nicoll, C. (1980) Ontogeny and evolution of prolactins' function. *Fed. Proc.*, **39**, 2563–2566.
- Ouhtit, A., Morel, G. and Kelly, P.A. (1993) Visualisation of gene expression of short and long forms of prolactin receptors in rat reproductive tissues. *Biol. Reprod.*, **49**, 528–536.
- Rana, A., Habib, F.K., Halliday, P., Ross, M., Wild, R., Elton, R.A. and Chisholm, G.D. (1995) A case for synchronous reduction of testicular androgen, adrenal androgen and prolactin for the treatment of advanced carcinoma of the prostate. *Eur. J. Cancer*, **31A**, 859–860.
- Ravault, J.P., Courot, M., Garnier, D., Pelletier, J. and Terqui, M. (1977) Effect of 2-bromo- $\alpha$ -ergocryptine (CB154) on plasma prolactin, LH and testosterone levels, accessory reproductive glands and spermatogenesis in lambs during puberty. *Biol. Reprod.*, **17**, 192–197.
- Ruff, S.J., Leers-Sucheta, S., Melner, M.H. and Cohen, S. (1996) Induction and activation of Stat5 in the ovaries of pseudopregnant rats. *Endocrinology*, **137**, 4095–4099.
- Schindler, C. and Darnell, Jr J.E. (1995) Transcriptional responses to polypeptide ligands: the Jak-Stat pathway. *Ann. Rev. Biochem.*, **64**, 621–651.
- Takase, M., Tsutsui, K. and Kawashima, S. (1990) Effects of prolactin and bromocriptine on the regulation of testicular luteinising hormone receptors in mice. *J. Exp. Zool.*, **6**, 219–229.
- Takeyama, M., Nagareda, T., Takatsuka, D., Namiki, M., Koizumi, K., Aono, T. and Matsumoto, K. (1986) Stimulatory effect of prolactin on luteinising hormone-induced testicular 5- $\alpha$ -reductase activity in hypophysectomised adult rats. *Endocrinology*, **118**, 2268–2275.
- Ufearo, C.S. and Orisakwe, O.E. (1995) Restoration of normal sperm characteristics in hypoprolactinaemic infertile men treated with metaclopramide and exogenous prolactin. *Clin. Pharmacol. Therapeut.*, **58**, 354–359.
- Wahlstrom, T., Huhtaniemi, I., Hovatta, O. and Seppala, M. (1983) Localization of luteinizing hormone, follicle-stimulating hormone, prolactin, and their receptors in human and rat testis using immunohistochemistry and radioreceptor assay. *J. Clin. Endocrinol. Metab.*, **57**, 825–830.
- Wennbo, H., Kindblom, J., Isaksson, O.G.P. and Tornell, J. (1997) Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland. *Endocrinology*, **138**, 4410–4415.
- Zipf, W.B., Payne, A.H. and Kelch, R.P. (1978) Prolactin, growth hormone and luteinising hormone in the maintenance of testicular luteinising hormone receptor. *Endocrinology*, **103**, 595–600.

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# An investigation of the effectiveness of testosterone implants in combination with the prolactin inhibitor quinagolide in the suppression of spermatogenesis in men

W.Morton Hair<sup>1</sup>, Frederick C.W.Wu<sup>2</sup> and Gerald A.Lincoln<sup>1,3</sup>

<sup>1</sup>MRC Human Reproductive Sciences Unit, The University of Edinburgh, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB and <sup>2</sup>University Department of Endocrinology, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL, UK

<sup>3</sup>To whom correspondence should be addressed. E-mail: g.lincoln@hrsu.mrc.ac.uk

**BACKGROUND:** Administration of testosterone inhibits gonadotrophin secretion and spermatogenesis in men but the degree of response is highly variable. This treatment also stimulates prolactin, itself a progonadal hormone in animals. This study investigated whether concomitant suppression of prolactin (PRL) with the non-ergot, dopamine receptor agonist quinagolide (Q), would enhance the efficacy of testosterone in its inhibition of spermatogenesis in healthy eugonadal men. **METHODS:** A total of 46 men were randomized to three treatment groups: Group 1, T1200: 1200 mg testosterone implant plus daily oral placebo; Group 2, T1200 + Q: 1200 mg testosterone plus oral Q 75 µg/day; Group 3, T800 + Q: testosterone 800 mg plus oral Q 75 µg/day. After an initial pre-treatment period of 4 weeks, subjects were treated for 24 weeks followed by an 8-week recovery period. **RESULTS:** The total numbers of subjects that achieved severe oligospermia ( $\leq 10^6$ /ml including azoospermia) from weeks 8–16 were 11/13 (85%), 11/12 (92%), 8/13 (61.5%) in the three groups respectively. **CONCLUSIONS:** The results show that inhibition of PRL does not confer additional efficacy in spermatogenic suppression in men. However, Q did not totally block PRL secretion in the subjects, possibly because testosterone replacement itself stimulated PRL by a direct action on the lactotroph, thus the effectiveness of dual inhibition of gonadotrophin and PRL could not be fully investigated.

*Key words:* prolactin inhibition/quinagolide/spermatogenesis/testosterone

## Introduction

Androgen administration aimed at producing a reversible contraceptive for men acts by inhibition of FSH and LH secretion to suppress spermatogenesis. It does not however suppress PRL secretion, which, at least in animal models, is a weak gonadotrophin (Bartke *et al.*, 1975; Ouhtit *et al.*, 1993; Lincoln *et al.*, 1996; Jabbour and Lincoln, 1999). Initial studies showed that long-term treatments with testosterone esters, even at high doses, induce azoospermia in only a proportion (~65%) of subjects with notable differences between ethnic groups (World Health Organization Task Force, 1990; Handelsman *et al.*, 1992; Sundaram *et al.*, 1993; Behre *et al.*, 1995). More recently, steroid treatments involving testosterone combined with progestins, have been shown to be more effective in the suppression of spermatogenesis (Bebb *et al.*, 1996; Handelsman *et al.*, 1996; Meriggiola *et al.*, 1996; Wu *et al.*, 1999; Martin *et al.*, 2000). Treatments with anti-androgenic progestins (e.g. cyproterone acetate) however, produce undesirable changes in the haemopoietic system (Meriggiola *et al.*, 1996), and progestins may produce effects on mood, as is well demonstrated in women (Pearlstein, 1995). In addition these treatments have not been shown to produce universal

azoospermia (Bebb *et al.*, 1996; Handelsman *et al.*, 1996; Meriggiola *et al.*, 1996; Wu *et al.*, 1999; Martin *et al.*, 2000). A number of suggestions have been proposed to explain the heterogeneity in the contraceptive response to steroid treatments. These include differing sex hormone-binding globulin (SHBG) levels and responsiveness to gonadotrophin suppression (Behre *et al.*, 1995; Wang *et al.*, 1998), differing 5- $\alpha$  reductase activity in the testis and its impact on intra-testicular androgen levels (Anderson *et al.*, 1996), structural differences in testicular morphology between ethnic groups (Zhengwei *et al.*, 1998) and possible differences in sex steroid metabolism and/or diet (Santner *et al.*, 1998).

An additional explanation for the failure to induce complete azoospermia is that it may be necessary to inhibit PRL, in addition to the classical gonadotrophins, to fully block spermatogenesis. This is because PRL potentially acts in the testis to stimulate both androgenic and spermatogenic functions, based on studies in rodents (Hondo *et al.*, 1995). In man the progonadal role of PRL is less clear. Early studies using I<sup>125</sup>-PRL failed to demonstrate PRL binding in the human testis, in contrast to the situation in the rat (Wahlstrom *et al.*, 1983). More recently, mRNA for the PRL receptor has been

characterized in the human testis (Kline *et al.*, 1999), and immunocytochemistry has revealed that PRL receptors are weakly expressed in the Leydig cells in the interstitial tissue and more strongly expressed in germ cells undergoing spermatogenesis in the seminiferous tubules (Hair *et al.*, 2002). Functional activation of these receptors and their secondary messenger systems JAK-STAT and the extracellular signal-regulated kinase (ERK) by PRL has also been demonstrated in human testis and vas deferens. Furthermore, there is clinical data indicating that PRL may promote spermatogenesis. In one study, treatment with exogenous PRL, or a dopamine antagonist to increase circulating PRL concentrations was shown to restore testicular function and fertility in hypoprolactinaemic infertile men (Ufearo and Orisakwe, 1995), and in another study, combined suppression of gonadotrophins and PRL in eugonadal men treated for prostatic carcinoma produced a more marked reduction in testicular weight than gonadotrophin suppression alone (Huhtaniemi *et al.*, 1991). Based on these observations, and the demonstration that the administration of testosterone and oral progestin stimulates PRL secretion (Bellis and Wu, 1998), we infer that suppression of PRL may enhance the effectiveness of sex steroid in inducing spermatogenic suppression in man.

The purpose of the present study was to test this hypothesis. Healthy male volunteers were treated orally with the non-ergot dopamine receptor agonist quinagolide (Q), to chronically suppress PRL secretion. This drug has been shown to inhibit PRL production with minimal effects on the gastrointestinal tract and on nausea, mood and sleep behaviours (Brownell *et al.*, 1996). At the start of the treatment, the volunteers also received s.c. implants of testosterone to suppress gonadotrophin secretion. Implants were used rather than intermittent injections to provide a smooth androgen profile with less inconvenience to the subjects. A high and an intermediate dose of testosterone was selected to establish whether PRL inhibition would act in synergy with the degree of gonadotrophin suppression and perhaps allow use of a lower dose of androgen to induce azoospermia in men.

A preliminary report on this study has been published as an abstract (Hair *et al.*, 2000).

## Materials and methods

### Subjects

The study medication and design, information for volunteers, method of recruitment and the reimbursement of expenses were all approved by the Central Manchester Ethical Committee for Medical Research and by the Medical Research Council. Prospective volunteers were recruited from the community through local media advertising. Written and verbal information as to the nature of the study was provided to 480 men. Prospective candidates were then interviewed during which a full medical history was obtained and thereafter underwent physical examination. Routine haematology, biochemistry and semen analysis were then performed as screening tests on two occasions 2 weeks apart, to determine if subjects met the inclusion criteria. The subjects were required to be within the age range 19–50 years, free of chronic disease, on no long-term medication, with normal haematology and biochemical screening tests and a normal semen profile based on World Health Organization (WHO) guidelines (World Health

Organization, 1999). The subjects were also required to be willing to continue with their existing contraceptive method. Following recruitment, 46 volunteers entered the study.

### Medications

Quinagolide (Q) was used to chronically inhibit PRL secretion in the volunteers. The drug tablets were supplied by Novartis (Norprolac; Novartis Pharmaceuticals UK Ltd, Frimley, Surrey, UK). Subjects initially received a Starter Pack containing two tablets each of 25, 50 and 75 µg of Q to allow graded introduction of the medication. Following the introduction over 6 days, the subjects took one 75 µg tablet per day. This dose was selected based on manufacturer's recommendations in the treatment of hyperprolactinaemia to effectively suppress PRL secretion and minimize side effects. A small pilot study in five normal men was also conducted. This demonstrated that daily administration of 75 µg Q suppressed blood plasma PRL concentration by >80% for the 9 day treatment period with a carryover effect for 3 days. For the main clinical trial, tablets were taken after food and before bed. Placebo tablets supplied by our hospital pharmacy were taken by one group of subjects as a control.

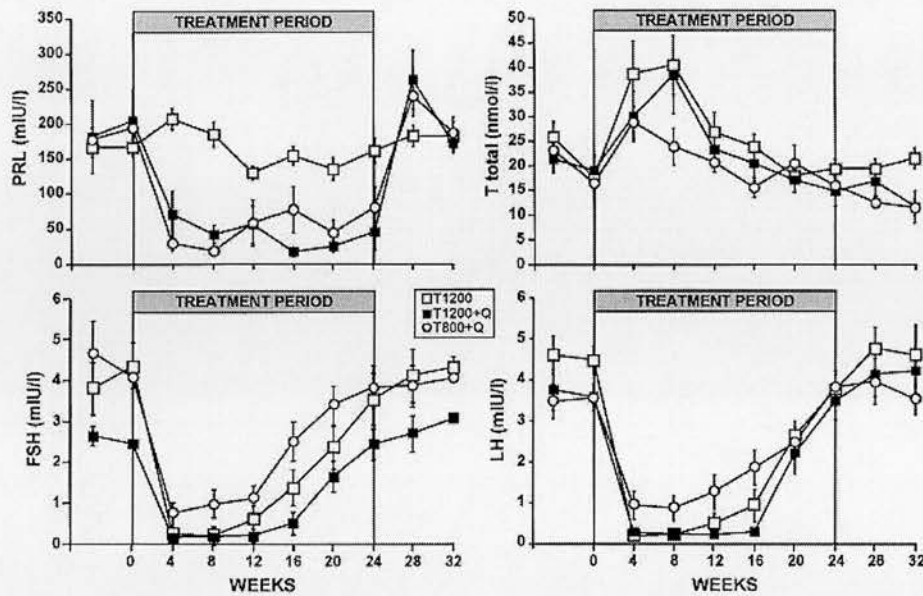
Crystalline testosterone implants (200 mg) were generously donated by Organon (Product licence No. 0065/5084R; NV Organon, Oss, Netherlands). Implants were inserted using a trocar with sterile technique in the s.c. fat of the anterior abdominal wall with local anaesthesia using 1% lignocaine. In the high dose treatment the subjects received six implants (1200 mg), and in the lower dose they received four implants (800 mg). These doses were selected on the basis that 1200 mg testosterone produces severe oligospermia in normal men, while 800 mg testosterone less completely suppresses sperm numbers.

### Study design

Subjects were randomized to one of three treatment groups in a single blind parallel group design: Group 1; testosterone 1200 mg plus daily oral placebo (T1200); Group 2; testosterone 1200 mg plus oral Q 75 µg/day (T1200 + Q); and Group 3; testosterone 800 mg plus oral Q 75 µg/day (T800 + Q). A more balanced design would have utilized a T800 + placebo group but this was not included due to constraints on the number of volunteers and the power calculation requirement of no fewer than 12 subjects per group. The protocol involved a pre-treatment phase of 4 weeks, a treatment phase of 24 weeks and a recovery phase of 8–12 weeks (see Figure 1). The treatment phase was initiated by the insertion, on one occasion, of either four or six testosterone implants according to treatment-group. The subjects also commenced daily oral Q, or placebo tablets, which were dispensed monthly at the clinical monitoring visit. Treatment of the subjects did not occur simultaneously but was staggered according to time of recruitment. The recovery phase was determined by the time to re-attainment of the pre-treatment sperm concentration in the ejaculate, or to two consecutive sperm counts >20 × 10<sup>6</sup>/ml.

### Clinical monitoring

Every 4 weeks throughout the study each subject was weighed and pulse and blood pressure measured. They were then interviewed to record evidence of compliance, unused tablets were returned and any notable effects of the treatment were recorded. A blood sample was obtained for hormone measurements, and a semen sample was produced for analysis. The blood samples were heparinized, centrifuged within 30 min of collection and stored at –20°C until analysis on completion of the full study. Every 8 weeks, additional blood samples were obtained for monitoring of haematological parameters, renal and liver function tests and serum lipid concentrations. Testis size was measured using an orchidometer. At the



**Figure 1.** Long term changes in the blood plasma concentrations of prolactin (PRL), testosterone (T), FSH and LH in groups of male volunteers treated with testosterone implants with either quinagolide (Q) or placebo as follows: (i) T1200 mg + daily oral placebo (open square symbol), (ii) T1200 mg + 75 µg daily oral Q (closed square symbol), (iii) T800 mg + 75 µg daily oral Q (open round symbol). The testosterone implants were inserted once only at week 0 and the Q treatment was continued for 24 weeks (horizontal bar = treatment period). Values are mean  $\pm$  SEM.  $n = 13, 12$  and  $13$  for the three groups respectively.

beginning and end of the study a standard rectal examination of the prostate was carried out.

#### Semen analysis

Semen samples were obtained by masturbation after 48 h abstinence and analysis of semen volume, pH, sperm concentration, motility and morphology was carried out within 60 min of collection according to WHO guidelines (World Health Organization, 1999). Azoospermia was verified by centrifugation of the whole semen sample and microscopic examination of the re-suspended pellet residue.

#### Blood analysis

Full blood counts, glucose, HbA1c, urea, electrolytes, liver enzymes and lipid profiles were measured by a hospital auto-analyser. Low-density lipoprotein (LDL) cholesterol was derived using the Friedwald formula. On completion of the clinical study the blood plasma concentrations of prolactin (PRL), FSH and LH were assayed in all subjects by highly sensitive immunofluorometric assays (Delfia, Pharmacia-Wallac, Turku, Finland) with an assay sensitivity of 9.0, 0.125 and 0.125 IU/l, respectively. Total testosterone was determined by previously described radioimmunoassay (Corker and Davidson, 1978) with a detection limit of 0.3 nmol/l.

#### Statistical analysis

Results are expressed as group mean  $\pm$  SEM. Data were analysed by two-way ANOVA with repeated measures using Statistica software (version 4.0) to identify significant time-by-group interactions with Tukey's post-hoc comparisons. In addition, ANOVA with repeated measures was performed within each group to detect significant effects of treatment. Baseline levels for each variable were defined as the arithmetic mean of the two pre-treatment samples. Recovery levels were defined as those obtained at 4–12 weeks following completion of treatment according to time at which the recovery criteria were met. Severe oligospermia was defined as a specimen with sperm present in

the ejaculate but at a concentration of  $<1 \times 10^6$ /ml. Testis volume was defined as the sum of volume measurements for the left and right testis. The 8–20 week period was selected as the period of maximum sperm suppression.

## Results

### Subjects

Of the 46 men who entered, 38 completed the study. Five men failed to attend regular clinical appointments and were lost to follow-up after 4–20 weeks without explanation. One man was withdrawn from the study due to extrusion of two testosterone implants and due to his lack of compliance with oral Q. Two subjects did not tolerate oral Q: in one case, the treatment caused symptoms of nausea and vomiting during the first few days of treatment and in the second case the treatment caused perceived effects on mood and libido from week 4 and the subject subsequently withdrew at week 16. After these losses the group sizes were 13, 12 and 13 for T1200, T1200 + Q and T800 + Q treatments respectively.

In the 38 men who completed the study, the oral Q and testosterone implants were generally well tolerated and there were no serious adverse effects reported by the participants. Transient morning nausea in the first week was reported by two subjects taking oral Q and by one subject taking the placebo. Mild acne was reported in four subjects and increased libido at the start of the study was reported in 16 subjects. There was no apparent difference in these symptoms in the three treatment groups. The men did not differ significantly between groups in age (Group T1200 + placebo,  $30.77 \pm 1.99$  years; Group T1200 + Q,  $33.36 \pm 1.5$  years and Group T800 + Q,  $34.75 \pm$

1.23 years), body weight ( $83.54 \pm 4.54$ ,  $81.33 \pm 4.82$ ,  $76.36 \pm 3.37$  kg respectively) and testis volume ( $45.83 \pm 2.6$ ,  $49.64 \pm 2.43$ ,  $44.55 \pm 2.73$  ml). All subjects had a pre-treatment sperm concentration  $>20 \times 10^6$ /ml in the ejaculate.

### PRL/testosterone manipulations

#### Q treatment

Blood plasma concentrations of PRL are shown in Figure 1 (upper left panel). The treatment with Q suppressed PRL concentrations but with notable inter-subject variability. The statistical analysis of the PRL profiles revealed a significant ( $P < 0.05$ ) time-by-treatment interaction. PRL concentrations were significantly ( $P < 0.05$ ) decreased at weeks 4–24 (throughout the treatment period) in the T1200 + Q and T800 + Q groups, compared with the T1200 + placebo group. The mean PRL concentrations for the treatment period were as follows:  $43.5 \pm 18.6$ ,  $52.6 \pm 21.4$  and  $162.3 \pm 14.9$  mIU/l for the T1200 + Q, T800 + Q and T1200 + placebo groups respectively. There was no significant difference in the PRL concentrations between the two groups that received Q. PRL was suppressed by at least 80% (compared to pre-treatment values) in 9/12 (75%) subjects in the T1200 + Q group, and in 9/13 (69%) subjects in the T800 + Q group. The remaining subjects suppressed to a mean  $47.73 \pm 8.26\%$  (range 11.2–72.2%). There was no disclosed non-compliance to explain this variability in PRL suppression.

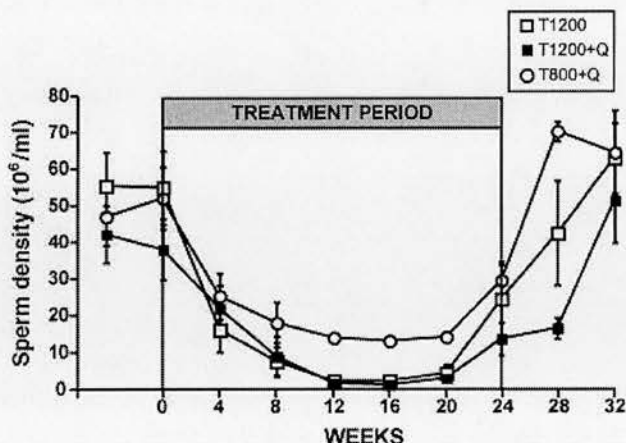
There was also evidence that testosterone alone increased PRL secretion. In the testosterone 1200 + placebo group, plasma PRL concentrations increased significantly ( $P < 0.05$ ) relative to pre-treatment values at week 4 following insertion of the testosterone implants. In the testosterone 1200 + Q and T800 + Q groups, PRL concentrations also increased significantly ( $P < 0.05$ ) above the placebo group at week 28, as a rebound response to cessation of Q.

#### Testosterone implantation

After insertion of the testosterone implants, total testosterone concentration increased above baseline for 4–8 weeks in all groups (Figure 1, upper right panel). The analysis of the total testosterone concentration in blood plasma showed a significant ( $P < 0.001$ ) effect of time but no statistical differences between groups, although plasma concentrations of testosterone were lower in the T800 + Q group.

#### Gonadotrophin suppression

Plasma LH and FSH concentrations were markedly suppressed by the testosterone treatment in all groups (Figure 1, lower panels). Concentrations were lowest at week 4 and remained suppressed until week 12 before increasing to pre-treatment values by week 24. The analysis revealed a significant ( $P < 0.001$ ) time-by-treatment interaction; the plasma LH and FSH concentrations were significantly ( $P < 0.05$ ) lower in both T1200 groups compared with the T800 + Q group from weeks 4–16. There was no significant difference in gonadotrophin concentrations between the T1200 + placebo and the testosterone 1200 + Q groups during the phases of suppression and recovery.

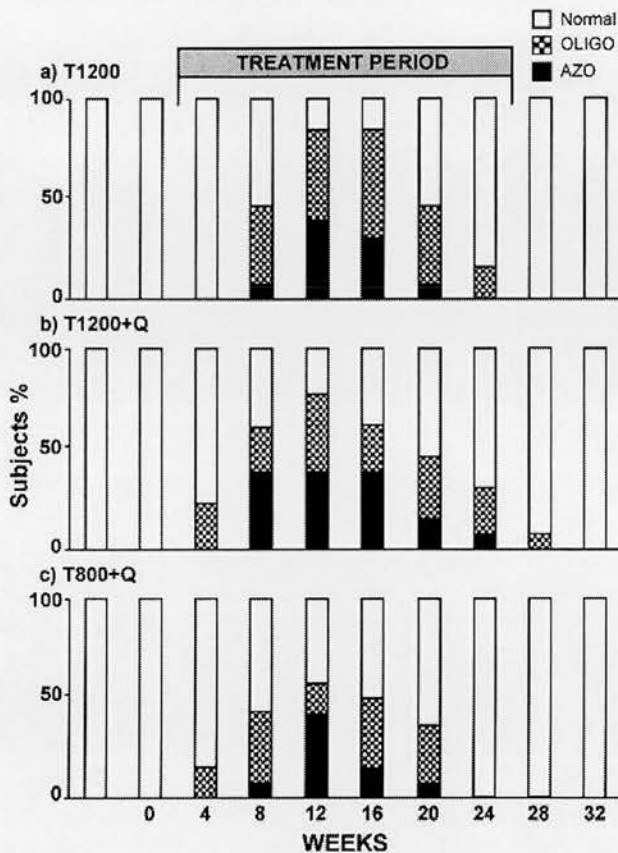


**Figure 2.** Long term changes in sperm density in the ejaculate of groups of male volunteers treated with testosterone (T) implants with either quinagolide (Q) or placebo as follows: (i) T1200 mg + daily oral placebo (open square symbol), (ii) T1200 mg + 75 µg daily oral Q (closed square symbol), (iii) T800 mg + 75 µg daily oral Q (open round symbol). The testosterone implants were inserted once only at week 0 and the Q treatment was continued for 24 weeks (horizontal bar = treatment period). Values are mean  $\pm$  SEM.  $n = 13$ , 12 and 13 for the three groups respectively.

#### Sperm suppression and recovery

Pre-treatment sperm concentrations in the ejaculate were similar in the three treatment groups (group means:  $55.19 \pm 8.09$ ,  $40.62 \pm 6.97$ ,  $51.08 \pm 9.36$  millions/ml for T1200 + placebo, T1200 + Q and T800 + Q respectively) (Figure 2). Sperm concentration fell sharply in all groups by week 4 of treatment and continued to decline until weeks 12–16 before increasing by week 24. Sperm concentrations returned to pre-treatment values by weeks 28–32. The analysis demonstrated a significant ( $P < 0.001$ ) time-by-treatment interaction, with sperm concentrations significantly ( $P < 0.05$ ) reduced in both T1200 groups, compared with the T800 + Q group from weeks 12–20. There was no significant difference in sperm concentrations between the T1200 + placebo and the testosterone 1200 + Q groups during the phases of suppression and recovery.

The proportion of subjects in each group who achieved severe oligospermia/azoospermia (sperm concentration  $<1 \times 10^6$ /ml) for at least 4 weeks was 11/13 (85%), 11/12 (92%), 8/13 (61.5%) in T1200 + placebo, T1200 + Q and T800 + Q groups respectively. The corresponding numbers achieving azoospermia were 5/13 (38%), 6/12 (50%) and 5/13 (38%). The group profiles for the incidence of oligospermia and azoospermia are summarized in Figure 3. Inhibition was maximal at weeks 8–16 with a similar pattern in the three treatment groups; the incidence of sustained oligo/azoospermia was least in the testosterone 800 + Q group. In this group, the subjects in which PRL secretion was maximally inhibited (suppression  $>80\%$  of pre-treatment,  $n = 9$ ), the degree of spermatogenic suppression was notably greater than in those subjects in whom PRL was inadequately suppressed ( $n = 4$ ). Severe oligospermia/azoospermia was achieved in 78% of the PRL inhibited group but only 25% in the poorly inhibited



**Figure 3.** Percentage of subjects achieving severe oligospermia (OLIGO) (sperm concentration in the ejaculate  $<1 \times 10^6/\text{ml}$ , hatched histogram) and azoospermia (AZO) (absence of sperm in ejaculate, filled histogram), in groups of male volunteers treated with testosterone implants with either quinagolide (Q) or placebo as follows: (i) T1200 mg + daily oral placebo, (ii) T1200 mg + 75  $\mu\text{g}$  daily oral Q and (iii) T800 mg + 75  $\mu\text{g}$  daily oral Q. The testosterone implants were inserted once only at week 0 and the Q treatment was continued for 24 weeks (horizontal bar = treatment period).

group; this apparent association between PRL and sperm production was not evident in the testosterone 1200 + Q group.

Progressive motility (WHO categories, a + b) was unaffected by treatment in the three groups. Ejaculate volume was marginally decreased in the three treatment groups from weeks 12–20 (range 8–29%) with no difference between groups. Semen pH was unaffected by treatment.

#### Metabolic, haematological and general effects of treatment

Blood plasma concentration of high-density lipoprotein (HDL) cholesterol was decreased at week 12–20 during treatment with no significant differences between the three groups. Concentrations had not returned to pre-treatment values by the end of the recovery period. Total cholesterol, triglycerides and LDL cholesterol concentrations were not affected by the treatments. Body weight, blood pressure, pulse, liver function tests (albumin, aspartate amino transferase, alanine amino transferase, alkaline phosphatase and albumin), glucose

metabolism (fasted blood glucose, HbA1c) and renal function (electrolytes, urea, creatinine) were also unaffected by the treatments. Haemoglobin concentrations were significantly ( $P < 0.01$ ) increased at week 12 in all groups, in parallel with experimentally induced changes in testosterone, but were otherwise unaffected. Other haematology indices (white blood cell count and platelets) were unaffected. Prostate examination revealed no changes related to any treatment.

#### Discussion

The aim of this study was to establish whether suppression of PRL enhances the effectiveness of exogenous testosterone in inhibiting sperm production in normal men. This was based on the potential progonadal effects of PRL, and the presumption that the simultaneous suppression of both PRL, and the classical gonadotrophins LH and FSH, would induce more complete spermatogenic arrest. The overall results do not support the hypothesis. The degree of spermatogenic arrest and the period of induced azoo/oligospermia was similar in the T1200 and T1200 + Q groups, and the effects in the T800 + Q group were comparable to those previously reported for men receiving this dose of testosterone alone (Handelsman *et al.*, 1992). There was, therefore, no significant effect on the spermatogenic profiles associated with the chronic treatment with our selected dose of Q.

The current experiment was designed to compare treatments with and without Q in the presence of a standardized high dose of testosterone (T1200 mg). This was based on the expectation that this level of testosterone would produce sub-maximal sperm suppression and allow us to detect an additional effect due to Q. However, the T1200 mg treatments in this study produced a very high degree of sperm suppression, leaving little scope to observe an additional effect of Q during the treatment phase. We therefore looked for an effect of the PRL manipulation on the pattern of recovery at the time when the testosterone implants were becoming exhausted (weeks 12–24) and daily Q treatment continued. Again, there was no significant difference between the T1200 + placebo and T1200 + Q groups in this pattern of recovery, although the T1200 + Q subjects were the last to return to pre-treatment values, and there was a period at weeks 24–28 where there was no overlap in sperm concentrations between the two groups, that is suggestive of a marginal effect. The only evidence that the Q treatment may have had some androgen dose-sparing effect was obtained from the T800 + Q group. In this group, there was marked individual variation in the degree of inhibition of PRL and degree of spermatogenic suppression. In the subjects in which PRL secretion was maximally inhibited (suppression  $>80\%$  of pre-treatment,  $n = 9$ ), the degree of spermatogenic suppression was greater than in those subjects in whom PRL was inadequately suppressed ( $n = 4$ ). Severe oligospermia was achieved in 78% of the PRL inhibited group, which was comparable with 85% for the T1200 + placebo group. This provides minimal support for the view that only in partial hypogonadotrophic states when gonadotrophin concentrations are sub-maximally reduced (as in the T800 group), is it possible to observe any concomitant effect of PRL

withdrawal. A subtle interactive effect of PRL and gonadotrophin status has previously been described in animal models where the effects of LH and FSH on testicular physiology always predominate (Bartke, 1999; Lincoln *et al.*, 2001).

The unexpected finding in our trial was that the oral Q was not fully effective at blocking PRL and there was marked variability in the degree of PRL suppression between subjects in both the T800 + Q and T1200 + Q groups. This was despite the findings of the short pilot study clearly showing that oral Q at 75 µg/day markedly suppressed blood PRL concentrations towards the minimum assay detection limit. There are several possible explanations to account for this anomaly. The first is that the subjects failed to comply with the experimental protocol of taking daily Q. However, monthly tablet returns and rigorous questioning at the monthly clinical monitoring did not suggest a significant level of non-compliance. Given that the elimination half-life of quinagolide is 17.5 h (Brownell *et al.*, 1996), the omission of occasional tablets is unlikely to have had a significant long-term effect on PRL control. Moreover, at the end of the treatment period, there was a notable increase in PRL concentrations in all subjects that received Q. This is a rebound response to chronic dopamine receptor agonist withdrawal and further supports our contention that subject compliance was good. The second explanation is that chronic Q treatment resulted in the development of refractoriness to PRL inhibition. Q is routinely used clinically in the treatment of hyperprolactinaemia in both men and women without clear evidence of long-term loss of responsiveness (Homburg *et al.*, 1990; Schultz *et al.*, 2000). Such refractoriness, however, may obtain in the suppression of PRL in men with normal physiological concentrations of PRL (Rana *et al.*, 1995).

The third, and perhaps most important factor, is that the testosterone treatments themselves activated PRL secretion, thus rendering the Q less effective. Previous work has shown that plasma PRL concentrations are significantly increased in men treated with testosterone enanthate injections (Bellis *et al.*, 1998). In the current study, blood PRL concentrations were significantly increased in the placebo treated subjects 4 weeks after the administration of the 1200 mg testosterone implants. Furthermore, it is well known from pituitary cell culture studies using animal tissues that both testosterone and estradiol potently stimulate PRL synthesis and release due to a direct genomic effect on the lactotroph (Shull *et al.*, 1985; Lambert and McLeod, 1990). This can occur independently of the inhibitory effect of dopamine receptor activation. Clinical studies also support the view that sex steroid status affects PRL secretion. For example, the efficacy of bromocriptine in the suppression of PRL secretion is reduced in hyperprolactinaemic women during pregnancy when estradiol concentrations are increased and PRL secretion decreases in the menopause and after pregnancy when sex steroid concentrations decline (Karunakaran *et al.*, 2001). Such steroid effects on PRL secretion in our trial subjects would render Q inhibition of PRL less effective. These effects of chronic treatment in normal men were not anticipated, but clearly compromised the objective of this study.

A final point is that Q was well tolerated with no serious adverse effects reported. Prolactin acts in multiple target tissues including brain, liver, skin, prostate and accessory glands. Despite this, chronic PRL suppression using Q had no demonstrable effects on semen volume, sexual behaviour and a wide range of haematological and biochemical indices demonstrating desirable specificity of action on the reproductive axis. Treatment with testosterone produced the expected effects on HDL cholesterol (Wallace and Wu, 1990), but this was not blocked or enhanced by the manipulation of PRL. There were a number of casual observations on the effect of chronic PRL suppression. The incidence of acne was lower than expected and two subjects noted an improvement in the condition of their skin and hair.

### Conclusions

The current study investigated for the first time whether manipulation of PRL would enhance the efficacy of testosterone in the suppression of spermatogenesis in men. The results did not support this idea. Unexpectedly it proved difficult to achieve consistent long-term inhibition of PRL secretion in the men. Future studies will need to establish a means of totally blocking PRL release, or to utilise a cross-over design in which all subjects receive both PRL inhibitor and placebo, to test whether concomitant testosterone and PRL blockade will induce complete spermatogenic arrest. The idea of employing lower levels of testosterone, and reducing PRL due to its trophic effects on the prostate, make a combined preparation of this kind an attractive prospect in the regulation of male fertility.

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### References

- Anderson, R.A., Wallace, E.M. and Wu, F.C.W. (1996) Comparison between testosterone enanthate induced azoospermia and oligospermia in a male contraceptive study III. Higher 5- $\alpha$  reductase activity in oligospermic men administered supraphysiological doses of testosterone. *J. Clin. Endocrinol. Metab.*, **81**, 902-908.
- Bartke, A. (1999) Role of growth hormone and prolactin in the control of reproduction: what are we learning from transgenic and knockout animals? *Steroids*, **64**, 598-604.
- Bartke, A., Croft, B.T. and Dalterio, S. (1975) Prolactin restores plasma testosterone levels and stimulates testicular growth in hamsters exposed to short day-length. *Endocrinology*, **97**, 1601-1604.
- Bebb, R.A., Anawalt, B.D., Christensen, R.B., Paulsen, C.A., Bremner, W.J. and Matsumoto, A.M. (1996) Combined administration of levonorgestrel and testosterone induces more rapid and effective suppression of

- spermatogenesis than testosterone alone: a promising male contraceptive approach. *J. Clin. Endocrinol. Metab.*, **81**, 757–762.
- Behre, H.M., Baus, S., Klesch, S., Keck, C., Simoni, M. and Nieschlag, E. (1995) Potential of testosterone buciclate for male contraception: endocrine differences between responders and non-responders. *J. Clin. Endocrinol. Metab.*, **80**, 2394–2403.
- Bellis, A. and Wu, F.C.W. (1998) Prolactin levels increase as a result of administration of a combination of testosterone enanthate and oral desogestrel as a contraceptive regime in normal men. *IV Europ. Congr. Endocrinol.*, Abstract P1-296.
- Brownell, J., van der Lely, A.J. and Lamberts, S.W.J. (1996) The treatment of hyperprolactinaemia. In Brownell, J. (ed) *Norprolac (quinagolide): an update*. Medical Forum International, Zeist, The Netherlands.
- Corker, C.S. and Davidson, D.W. (1978) Radioimmunoassay of testosterone in various biological fluids without chromatography. *J. Steroid Biochem.*, **9**, 373–374.
- Hair, W.M., Gubbay, O., Jabbour, H. and Lincoln, G.A. (2002) Prolactin receptor expression in human testis and accessory tissues: localization and function. *Mol. Hum. Reprod.*, **8**, 606–611.
- Hair, W.M., Wu, F.C.W. and Lincoln, G.A. (2000) Subcutaneous testosterone combined with the prolactin inhibitor quinagolide: a novel hormonal contraceptive regime in men. *Int. J. Androl.*, **23**, 30, P051.
- Handelsman, D.J., Conway, A.J. and Boylan, L.M. (1992) Suppression of human spermatogenesis by testosterone implants. *J. Clin. Endocrinol. Metab.*, **75**, 1326–1332.
- Handelsman, D.J., Conway, A.J., Howe, C.J., Turner, L. and Mackey, M.A. (1996) Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J. Clin. Endocrinol. Metab.*, **81**, 4113–4121.
- Hondo, E., Kurohmaru, M., Sakai, S., Ogawa, K. and Hatashi, Y. (1995) Prolactin receptor expression in rat spermatogenic cells. *Biol. Reprod.*, **52**, 1284–1290.
- Homburg, R., West, C., Brownell, J. and Jacobs, H.S. (1990) A double-blind study comparing a new non-ergot long acting dopamine agonist CV205–502 with bromocriptine in women with hyperprolactinaemia. *Clin. Endocrinol.*, **32**, 565–571.
- Huhtaniemi, I., Parvinen, M., Venho, P. and Rannikko, S. (1991) Combination of a GnRH agonist with an antiandrogen or bromocriptine in the treatment of prostatic cancer; slight potential for antigonadal effects. *Int. J. Androl.*, **14**, 374–386.
- Jabbour, H.N. and Lincoln, G.A. (1999). Prolactin receptor expression in the testis of the ram: localisation, functional activation and the influence of gonadotrophins. *Mol. Cell Endocrinol.*, **148**, 151–161.
- Karanakaran, S., Page, R.C.L. and Wass, J.A.H. (2001) The effect of the menopause on prolactin levels in patients with hyperprolactinaemia. *Clin. Endocrinol.*, **54**, 295–300.
- Kline, J.B., Roehrs, H. and Clevenger, C.V. (1999) Functional characterization of the intermediate isoform of the human prolactin receptor. *J. Biol. Chem.*, **274**, 35461–35468.
- Lambert, W.J. and McLeod, R.M. (1990) regulation of prolactin secretion at the level of the lactotroph. *Physiol. Rev.*, **70**, 279–305.
- Lincoln, G.A., Clarke, I.J. and Sweeney, T. (1996) Hamster-like cycles in testicular size in the absence of gonadotrophin secretion in HPD rams exposed to long-term changes in photoperiod and treatment with melatonin. *J. Neuroendocrinol.*, **8**, 855–866.
- Lincoln, G.A., Townsend, J. and Jabbour, H.N. (2001) Prolactin actions in the sheep testis: a test of the priming hypothesis. *Biol. Reprod.*, **65**, 936–943.
- Martin, C.W., Riley, S.C., Everington, D., Groome, N.P., Riemersma, R.A., Baird, D.T. and Anderson, R.A. (2000) Dose-finding study of oral desogestrel with testosterone pellets for suppression of the pituitary testicular axis in normal men. *Hum. Reprod.*, **15**, 1515–1524.
- Meriggola, C.A., Bremner, W.J., Paulsen, C.A., Valdiserri, A., Incorvaia, L., Motta, R., Pavani, A., Capelli, M. and Flamigni, C. (1996) A combined regimen of cyproterone acetate and testosterone enanthate as a potentially highly effective male contraceptive. *J. Clin. Endocrinol. Metab.*, **81**, 3018–3023.
- Ouhtit, A., Morel, G. and Kelly, P.A. (1993) Visualisation of gene expression of short and long forms of prolactin receptors in rat reproductive tissues. *Biol. Reprod.*, **49**, 528–536.
- Pearlstein, T.B. (1995) Hormones and depression: what are the facts about premenstrual syndrome, menopause and hormone replacement therapy? *Am. J. Obstetrics Gynecol.*, **173**, 646–653.
- Rana, A., Habib, F.K., Halliday, P., Ross, M., Wild, R., Elton, R.A. and Chisholm, G.D. (1995) A case for synchronous reduction of testicular androgen, adrenal androgen and prolactin for the treatment of advanced carcinoma of the prostate. *Eur. J. Cancer*, **31A**, 871–875.
- Santner, S.J., Albertson, B., Zhang, G.Y., Zhang, G.H., Santulli, M., Wang, C., Demers, L.M., Shackelton, C. and Santen, R.J. (1995) Comparative rates of androgen production and metabolism in Caucasian and Chinese subjects. *J. Clin. Endocrinol. Metab.*, **83**, 2104–2109.
- Schultz, P.N., Ginsberg, L., McCutcheon, I.E., Samaan, N., Leavens, M. and Gagel, R.F. (2000) Quinagolide in the management of prolactinoma. *Pituitary*, **3**, 239–249.
- Shull, J.D. and Gorski, J. (1985) Estrogen regulates the transcription of the rat prolactin gene in vivo through at least two independent mechanisms. *Endocrinology*, **116**, 2456–2462.
- Sundaram, K., Kumar, N. and Bardin, C.W. (1993) 7- $\alpha$ -methyl-nortestosterone (MENT): the optimal androgen for male contraception. *Ann. Med.*, **25**, 199–205.
- Ufearo, C.S. and Orsakwe, O.E. (1995). Restoration of normal sperm characteristics in hypoprolactinaemic infertile men treated with metaclopramide and exogenous prolactin. *Clin. Pharmacol. Therapeut.*, **58**, 354–359.
- Wahlstrom, T., Huhtaniemi, I., Hovatta, O., and Seppala, M. (1983) Localization of luteinizing hormone, follicle-stimulating hormone, prolactin, and their receptors in human and rat testis using immunohistochemistry and radioreceptor assay. *J. Clin. Endocrinol. Metab.*, **57**, 825–830.
- Wallace, E.M. and Wu, F.C.W. (1990) Effect of medroxyprogesterone acetate and testosterone enanthate on serum lipoproteins in man. *Contraception*, **41**, 63–71.
- Wang, C., Berman, N.G., Veldhuis, J.D., Der, T., McDonald, V., Steiner, B. and Swerdloff, R.S. (1998) Graded testosterone infusions distinguish gonadotrophin negative feedback responsiveness in Asian and white men: a clinical research center study. *J. Clin. Endocrinol. Metab.*, **83**, 870–876.
- World Health Organization (1999) World Health Organization Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 4th edn. Cambridge University Press, Cambridge, UK.
- World Health Organization (1990) World Health Organization Task Force On Methods For The Regulation of Male Fertility. Contraceptive efficacy of testosterone induced azoospermia in normal men. *Lancet*, **336**, 955–959.
- Wu, F.C.W., Balasubramanian, R., Mulders, T.M.T. and Coelingh-Bennink, H.J. (1999) Oral progestagen combined with testosterone as a potential male contraceptive: additive effects between desogestrel and testosterone enanthate in suppression of spermatogenesis, pituitary-testicular axis, and lipid metabolism. *J. Clin. Endocrinol. Metab.*, **84**, 112–122.
- Zhengwei, Y., Wreford, N.G., Royce, P., deKretser and McLachlan, R.I. (1998) Stereological evaluation of human spermatogenesis by testosterone treatment: heterogeneous pattern of spermatogenic impairment. *J. Clin. Endocrinol. Metab.*, **83**, 1284–1291.

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## A Novel Male Contraceptive Pill-Patch Combination: Oral Desogestrel and Transdermal Testosterone in the Suppression of Spermatogenesis in Normal Men

W. MORTON HAIR, KAY KITTERIDGE, DARYL B. O'CONNOR, AND FREDERICK C. W. WU

Department of Endocrinology, Manchester Royal Infirmary, University of Manchester, Manchester, United Kingdom M13 9WL

This study investigated the effect of transdermal T and oral desogestrel on the reproductive axis of healthy men. Twenty-three men were randomized to 1 of 3 treatment groups and received a daily transdermal T patch plus oral desogestrel at a dose of 75, 150, or 300  $\mu\text{g}/\text{d}$  for 24 wk. Baseline blood and semen samples were obtained and then every 4 wk thereafter for 32 wk. The outcome measures were sperm density and plasma levels of FSH, LH, total and free T. The results show a dose-dependent suppression of spermatogenesis and gonadotropins. Seven of the 17 subjects became azoospermic. Desogestrel (300  $\mu\text{g}$  daily) in combination with 5 mg daily transdermal T was the most effective (57% azoospermic), whereas

a dose of 75  $\mu\text{g}$  was ineffective (0% azoospermic). Total and free plasma T were reduced by approximately 30%. High density lipoprotein cholesterol was significantly reduced. No serious side-effects were encountered. We conclude that daily self-administered desogestrel with transdermal T is capable of suppressing the male reproductive axis, although the efficacy was less marked and less consistent than injectable regimens. The lower efficacy is likely to be due to failure of the transdermal T system to maintain circulating T levels consistently in the required range. (*J Clin Endocrinol Metab* 86: 5201-5209, 2001)

THE PRINCIPLE THAT exogenous sex steroid-induced oligo- and azoospermia can confer effective, reversible contraceptive protection in men was established in two multicenter trials employing an androgen-only prototype regimen of im injections of T enanthate (1, 2). The pharmacokinetics of T enanthate are such that a relatively high dose (200 mg), administered at weekly intervals, is required to ensure maximum suppression of gonadotropins and spermatogenesis (3). This regimen produced repeated supraphysiological peaks and markedly fluctuating levels of T (4) which induced significant extra-gonadal androgenic effects on lipid metabolism, skin, muscle, liver and hemopoiesis (5). These unwanted effects coupled with the impracticality of uncomfortable weekly im injections underline the need to use lower doses of T with more stable delivery in hormonal male contraception.

Spermatogenesis can be effectively suppressed by combining a reduced dose of T with a second antigonadotropic agent such as a progestagen (6) or GnRH antagonist (7). We previously reported that im T enanthate at the relatively low doses of only 100 or 50 mg weekly, if combined with a 19-nortestosterone-derived oral synthetic progestin, desogestrel (DSG), can suppress spermatogenesis very effectively in men; the best dose combination, 300  $\mu\text{g}$  DSG daily with 50 mg T enanthate weekly, induced consistent azoospermia in all subjects (8). This study also showed that DSG and T both contributed additively and interchangeably to the reproductive as well as the nonreproductive metabolic effects, e.g. reduction of high density lipoprotein cholesterol (HDL-C) and SHBG levels. T enanthate (50 mg, im, weekly), delivering

5 mg free T daily, is regarded as the minimal effective dose and also the minimal dose required to maintain androgen sufficiency. Although it can be surmised that lower doses of DSG or alternate formulations of T, which can reproduce stable physiological (rather than sharply fluctuating) levels, may reduce these unwanted actions, the minimally effective dose combination is currently unknown.

Transdermal T delivery systems have been recently developed for use as a noninvasive method of androgen replacement therapy in hypogonadal men (9, 10). Daily self-application of these systems offers the potential of maintaining stable levels of T within the normal range with a small diurnal fluctuation that closely mimics the physiological pattern.

To date, few male contraceptive studies have employed an entirely subject-administered regimen. As this is likely to be preferred by the majority of potential users, it is important to determine whether daily self-administration, independent of provider or research personnel, can still be effective. This will give important clues about the tolerance or margin of safety in hormonal male contraceptive regimens against a backdrop of the varying levels of compliance that will inevitably be encountered with preventative medications across any population.

We have conducted a study that employs a novel, non-invasive, daily, self-administered treatment regimen to effect reversible suppression of spermatogenesis in healthy male volunteers. The specific aims of the study were 1) to evaluate the effects of oral DSG combined with a nonscrotal transdermal T delivery on gonadotropin secretion and spermatogenesis; 2) to compare the effects of reducing doses of DSG combined with a fixed dose of T designed for physiological androgen replacement; 3) to determine the minimally effective

Abbreviations: DSG, Desogestrel; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

tive contraceptive combination and the tolerance for breakthrough of suppression in this self-administered regimen; and 4) to assess the nonreproductive effects of these combinations in men.

## Subjects and Methods

### Subjects

Of 101 respondents to our advertisements, 33 were suitable for screening. After the initial interview and screening tests, 10 were excluded because of low sperm counts ( $n = 5$ ) or high cholesterol ( $n = 5$ ), leaving 23 Caucasian men (mean age,  $34.2 \pm 7.0$  yr; range, 20–43 yr) to take part in the study.

### Study design

Subjects who met the admission criteria similar to our previous study (8) were randomized into one of three treatment groups to receive 1) oral DSG (300  $\mu\text{g}$  daily) and transdermal T (5 mg daily;  $n = 7$ ), 2) oral DSG (150  $\mu\text{g}$  daily) and transdermal T (5 mg daily;  $n = 6$ ), or 3) oral DSG (75  $\mu\text{g}$  daily) and transdermal T (5 mg daily;  $n = 4$ ) for 24 wk in a single blind, parallel group design.

Subjects were studied in three phases. 1) In the control phase medical screening examination, two baseline semen analyses, and hormonal and biochemical assessments were carried out over 4 wk. 2) In the treatment phase each subject was randomly allocated to one of the three treatment groups described above. Both transdermal T and DSG were administered for 24 wk. Medical review, including physical examination, blood sampling, and semen analyses, were performed every 4 wk. 3) In the recovery phase all subjects were monitored every 4 wk by medical review, semen analysis, and blood sampling until recovery criteria were satisfied, namely, geometric mean pretreatment sperm density was reached or two consecutive specimens showed sperm density greater than 20 million/ml.

All subjects provided informed written consent and were advised to continue with their existing forms of contraception during the study. The study was approved by the Central Manchester Healthcare Trust ethical committee for medical research.

### Medications

Desogestrel (75- and 150- $\mu\text{g}$  tablets) were supplied by NV Organon (Oss, The Netherlands). Each subject received one tablet per d in the case of the 75- and 150- $\mu\text{g}$  tablets or two 150- $\mu\text{g}$  tablets in the case of the 300  $\mu\text{g}/\text{d}$  group. Tablets were taken in the evening before bed. T was administered by the volunteers as two transdermal delivery systems (Andropatch, SKB, Welwyn Garden City, UK) applied to skin of the upper back, legs, or flanks and changed daily at bedtime, employing a different site at each application.

### Clinical monitoring

Subjects were interviewed monthly, with particular emphasis on eliciting any side-effects, monitoring sexual function, and checking medication compliance. The latter consisted of direct questioning and counting tablets and patches remaining in returned containers. Each subject was also asked to record the occasions they missed their medications in writing. Body weight, pulse, and blood pressure were measured monthly, and testicular size (by orchidometer) was measured every 3 months. A digital prostate examination was carried out before treatment and on recovery.

### Semen analysis

Semen collection and analysis of semen volume, sperm density, and motility were carried out according to the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. The suppression targets are defined as: azoospermia, complete absence of spermatozoa in the ejaculate verified by centrifugation of a whole semen sample; severe oligospermia, sperm concentration of less than 1 million/ml; and oligospermia, sperm concentration of less than 3 million/ml in one sample.

### Blood tests

Blood samples were obtained twice before treatment and at 4-wk intervals thereafter for hormone measurements (T, LH, FSH, and SHBG) and hematological (hemoglobin, hematocrit, and white cell count), biochemical (urea, electrolytes, liver enzymes, glucose, and hemoglobin  $A_{1c}$ ), and lipid profiles [total cholesterol, low density lipoprotein cholesterol (LDL-C), HDL-C, triglycerides, and apolipoprotein A1].

### Hormone assays

All plasma samples were stored at  $-20^\circ\text{C}$  until assay. Plasma gonadotropins were assayed by previously reported highly sensitive immunofluorometric assays (Delfia, Pharmacia-Wallac, Inc., Turku, Finland) (10) with an assay sensitivity of 0.05 IU/ml for both LH and FSH. T was determined by previously described RIA with an assay sensitivity of 0.3 nmol/liter (11). SHBG was determined by an immunoradiometric assay (Farnos Diagnostica, Oulun Salo, Finland). Free T was determined using an established equilibrium dialysis technique (12). All serial samples from one individual were assayed in a single batch to reduce variability.

### Biochemical analyses

Full blood count, glucose, hemoglobin  $A_{1c}$ , lipids (total cholesterol, HDL-C, and triglyceride), and renal and liver function was measured by routine autoanalyzer methods. LDL-C was derived from the other lipid indices using Friedwald's formula.

### Statistical analyses

The data were analyzed by repeated measures ANOVA, paired *t* tests, and one-way ANOVA with Tukey's *post-hoc* test for continuous variables with statistical significance set at  $P < 0.05$ . Values were expressed as the arithmetical mean  $\pm$  SEM. LH and FSH levels below the sensitivity of the assay were allocated a value of 0.05 U/liter, the lower limit of detection.

## Results

### Spermatogenesis

Seventeen subjects completed the suppression phase, and 6 subjects were discontinued (see below). The mean sperm densities before, during, and after DSG and transdermal T administration in each of the 3 treatment groups are shown in Fig. 1A. The rates of suppression to three target sperm densities, *i.e.* azoospermia (no sperm), severe oligospermia ( $<1$  million/ml), and oligospermia ( $<3$  million/ml), are shown in Fig. 1B. Sperm density was significantly ( $P < 0.05$ ) reduced in all treatment groups with respect to baseline showing a clear dose dependence, although the differences between treatment groups failed to achieve significance. Seven (41%) of the 17 subjects who completed the suppression phase achieved azoospermia. Nine (53%) of the 17 achieved suppression to less than 1 million/ml, with no additional subjects achieving suppression to less than 3 million/ml. The most effective drug regimen was 300  $\mu\text{g}$  DSG and 5 mg transdermal T daily in which 4 (57%) of the 7 subjects achieved azoospermia, the earliest by wk 8 and the remainder by wk 16. One additional subject suppressed to less than 1 million/ml by wk 12, giving a total of 71% for this group. In the 150  $\mu\text{g}$  DSG group, 3 (50%) of the 6 subjects achieved azoospermia, the earliest by wk 8, and the others by wk 12, while the remainder failed to suppress to oligospermia. In the 75  $\mu\text{g}$  DSG group none of the 4 subjects achieved azoospermia, although 1 became severely oligospermic ( $<1$  million/ml) by wk 12, whereas the others (75%) remained

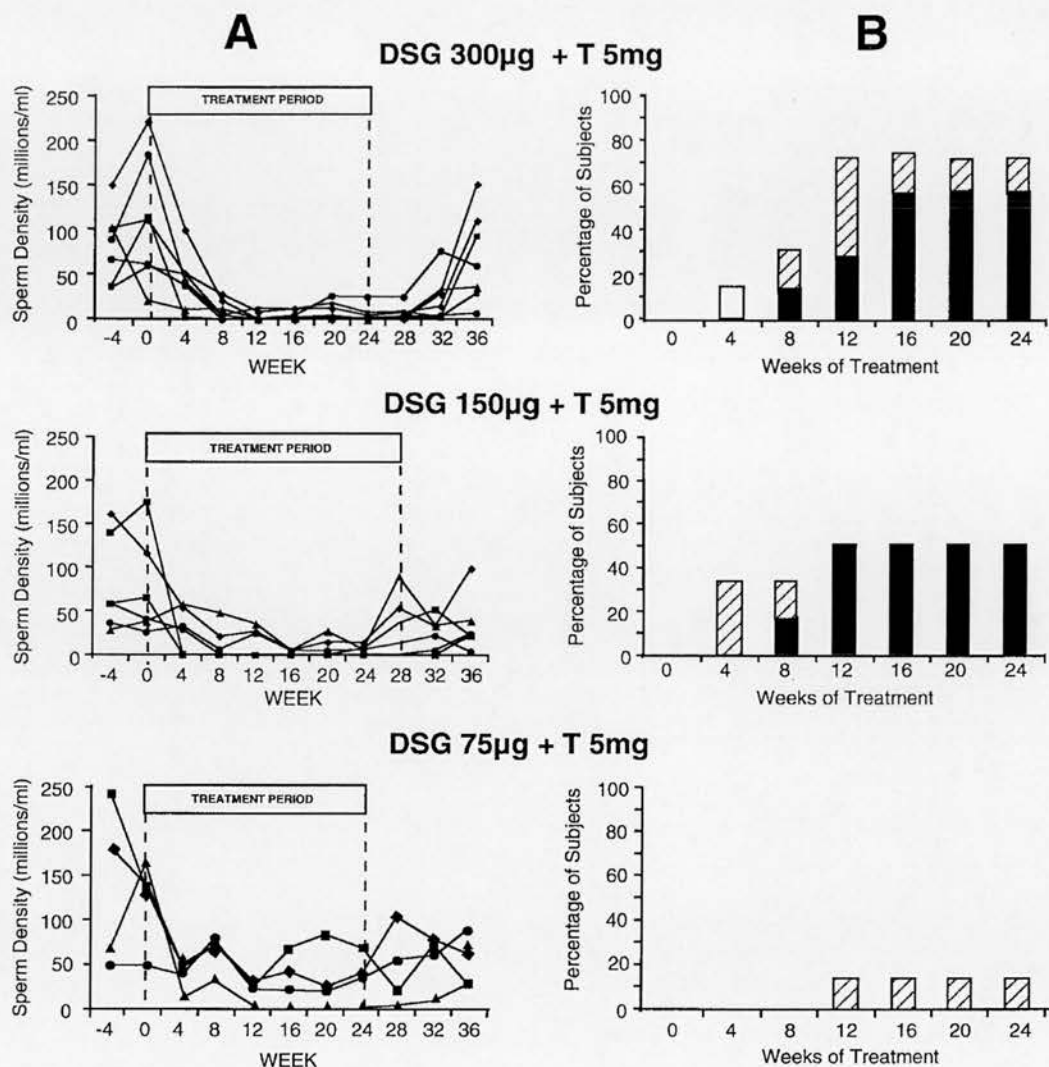


FIG. 1. Suppression of spermatogenesis. A, Individual sperm density profiles for subjects in each of the three treatment groups at 4-wk intervals during 24 wk of treatment. B, Rates of suppression of spermatogenesis indicated by the percentage of subjects attaining azoospermia (■) or oligozoospermia below 1 million/ml (▨) and below 3 million/ml (□) over the same time course. Subjects received transdermal T (5 mg/d) plus daily oral DSG at a dose of 300, 150, or 75 µg.

outside the oligospermic range, with sperm densities above 10 M/ml throughout treatment. Pretreatment sperm densities were not significantly different between the azoospermic or oligozoospermic responders and the nonsuppressors.

Examination of the individual sperm density profiles in each group demonstrates marked heterogeneity of response within treatment groups (Fig. 1A). In 4 of the 7 subjects who achieved azoospermia there was escape from suppression before the end of the treatment period; the others remained fully suppressed until treatment ended. This phenomenon was also observed in 1 of the 2 subjects who became severely oligospermic. Sperm density values began to recover within 4 wk of cessation of therapy. All but 1 of the subjects achieved the recovery criteria by wk 48 (*i.e.* 24 wk after the end of treatment). One subject, despite normal gonadotropin levels,

did not recover until wk 64. His T levels were also subnormal over a similar time course, although his gonadotropin levels were normal.

#### LH

LH levels were significantly ( $P < 0.05$ ) suppressed during the treatment phase in all treatment groups from wk 4 onward. Suppression was dose dependent, the most effective being in the 300 µg DSG group, although the difference between treatment groups did not achieve statistical significance (Fig. 2). In the 300 µg DSG group, LH was significantly ( $P < 0.05$ ) suppressed from a baseline mean of  $4.5 \pm 0.7$  U/liter to a nadir of  $0.2 \pm 0.1$  U/liter (wk 8), whereas in the 150 µg DSG group mean levels fell from  $4.5 \pm 1.0$  U/liter to

a nadir of  $0.8 \pm 0.3$  U/liter (wk 4). In the 75  $\mu$ g DSG group LH levels were suppressed from a baseline mean of  $4.6 \pm 1.5$  to  $0.9 \pm 0.7$  U/liter (wk 12). Suppression of LH was not fully maintained during continued treatment. In the 300  $\mu$ g DSG group, LH decreased to below the assay detection limit initially in four subjects and to less than 0.3 U/liter by wk 8 in the other three subjects. Between wk 8 and the end of treatment, all subjects in this group showed some degree of escape, mostly transient and below 0.8 U/liter, except for one which broke through to 1.6 U/liter at wk 16 having had undetectable LH 4 wk earlier. In the 150  $\mu$ g DSG group, LH suppressed to below assay detection in four of the six subjects, two of whom escaped while the other two maintained undetectable LH until the end of treatment. In the 75  $\mu$ g DSG group, suppression was variable, and no subject had detectable levels.

The pretreatment LH level was significantly higher in subjects who did not respond compared with those that suppressed to azoospermia and oligozoospermia ( $<3$  M/ml). This was largely attributable to one unresponsive subject with a baseline LH of 9.1 U/liter. The mean LH level during treatment was significantly lower in azoospermic and oligozoospermic responders compared with nonresponders. In all subjects LH recovered to baseline within 4 wk after cessation of treatment. There was no significant difference in the rate of recovery in the three treatment groups.

### FSH

FSH levels were significantly ( $P < 0.05$ ) suppressed during the treatment phase in all treatment groups. Suppression was dose dependent, the most effective being in the 300  $\mu$ g DSG group, although the difference between treatment groups did not achieve statistical significance (Fig. 2). In the 300  $\mu$ g DSG group, FSH was significantly suppressed from a baseline mean of  $2.7 \pm 0.3$  U/liter to a nadir of  $0.4 \pm 0.2$  U/liter (wk 8), whereas in the 150  $\mu$ g DSG group, mean levels fell from a baseline of  $4.2 \pm 0.5$  U/liter to a nadir of  $0.5 \pm 0.3$  U/liter (wk 4). In the 75  $\mu$ g group mean FSH levels fell from a baseline of  $3.4 \pm 0.4$  to a nadir of  $0.9 \pm 0.4$  (wk 8). There was a consistent pattern of gradual escape from suppression after wk 8 in all three groups.

In both the 300 and 150  $\mu$ g DSG groups, FSH suppressed to below assay detection limits in three subjects, but only one in each group maintained the suppression until the end of treatment. In the 75  $\mu$ g DSG group, FSH suppressed to less than 1.0 U/liter in two subjects, but only transiently.

There was no difference in pretreatment FSH levels in subjects who suppressed to azoospermia and oligozoospermia ( $<3$  M/ml) compared with those who did not. The mean FSH level during treatment was significantly lower in azoospermic and oligozoospermic responders compared with nonresponders. FSH recovered rapidly on cessation of treatment, reaching normal levels by wk 28, *i.e.* the first post-

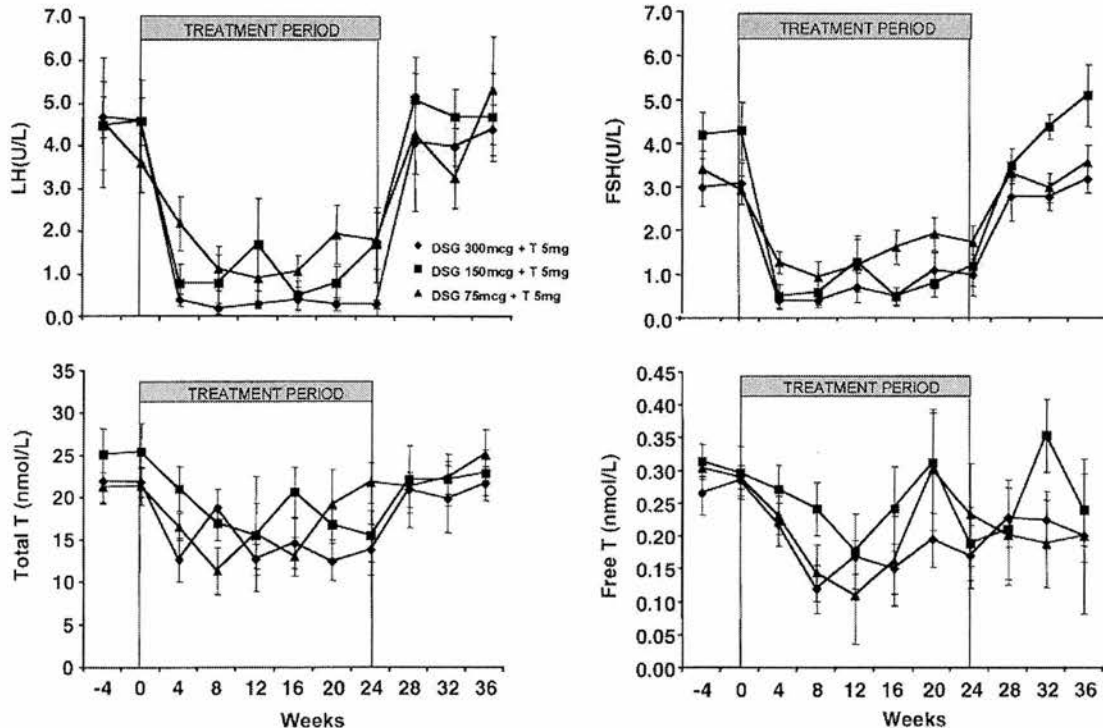


FIG. 2. Hormone data: group means. The levels of plasma FSH, LH, and total and free T at 4-wk intervals during the 24-wk treatment period and the subsequent recovery period are shown. Groups received transdermal T (5 mg/d) plus daily oral DSG at a dose of 300, 150, or 75  $\mu$ g. Values are the mean  $\pm$  SEM. Subjects received transdermal T (5 mg daily) plus oral DSG at a dose of 300  $\mu$ g (solid rhomboids), 150  $\mu$ g (solid squares), or 75  $\mu$ g (solid triangles).

treatment assessment. There was no significant difference in the rate of recovery in the three treatment groups.

### T

Mean total T levels were significantly ( $P < 0.05$ ) decreased during the treatment phase compared with baseline in all three groups, although they remained within the normal physiological range throughout the study. There was no significant difference in total T between the three groups. In the 300  $\mu\text{g}$  DSG group, T levels fell from a baseline of  $22.0 \pm 2.7$  nmol/liter to treatment mean of  $14.2 \pm 2.5$  (64.5% of basal) and a nadir of  $12.5 \pm 2.2$  nmol/liter (wk 20), whereas in the 150  $\mu\text{g}$  DSG group, levels fell from a baseline of  $25.2 \pm 3.1$  nmol/liter to a mean of  $17.8 \pm 2.7$  (70.6% of basal) and a nadir of  $15.5 \pm 3.1$  nmol/liter (wk 24). In the 75  $\mu\text{g}$  DSG group T levels decreased from a baseline of  $21.3 \pm 1.8$  nmol/liter to a mean of  $16.4 \pm 2.4$  (76.8% of basal) and a nadir of  $11.4 \pm 2.8$  nmol/liter (wk 8).

Free T levels also decreased significantly ( $P < 0.05$ ) during the treatment phase. In the 300  $\mu\text{g}$  DSG group levels fell from baseline mean of  $0.28 \pm 0.03$  nmol/liter to a treatment period mean of  $0.17 \pm 0.01$  nmol/liter (61.6% of basal). In the 150  $\mu\text{g}$  DSG group free T levels fell from  $0.31 \pm 0.03$  to  $0.24 \pm 0.04$  nmol/liter (78.4%) and in the DSG 75  $\mu\text{g}$  group T levels fell from  $0.30 \pm 0.01$  to  $0.20 \pm 0.03$  nmol/liter (66.3% of basal).

There was no difference in pretreatment total T levels in subjects who suppressed to azoospermia and oligozoospermia ( $<3$  m/ml) compared with those who did not. The mean T during treatment was not significantly different in azoospermic and oligozoospermic responders compared with nonresponders. Total and free T levels returned to pretreatment levels by the end of the recovery period in all groups.

### SHBG

SHBG levels were significantly reduced ( $P < 0.05$ ) in all treatment groups during the treatment phase, although there was no significant difference between treatment groups in this regard. In the 300  $\mu\text{g}$  DSG group, mean levels fell from a baseline of  $31.3 \pm 4.8$  nmol/liter to a nadir of  $20.7 \pm 3.9$  (wk 12), whereas in the 150  $\mu\text{g}$  DSG group levels fell from baseline mean of  $30.2 \pm 6.7$  nmol/liter to a nadir of  $22.5 \pm 5.5$  nmol/liter (wk 24). In the 75  $\mu\text{g}$  DSG group, levels fell from a baseline mean of  $35 \pm 8.92$  nmol/liter to a nadir of  $27.8 \pm 6.3$  nmol/liter (wk 12). SHBG levels returned to pretreatment levels by the end of the recovery period in all groups.

### Biochemical and hematological parameters

There were no significant changes in plasma urea, creatinine, sodium, potassium, calcium, alkaline phosphatase, aspartate amino transferase, alanine amino transferase,  $\gamma$ -glutamyl transpeptidase, bilirubin, glucose, HbA<sub>1c</sub>, hemoglobin, hematocrit, white cell count, or platelets during treatment (data not shown).

### Lipids

HDL-C was significantly decreased ( $P < 0.05$ ) with respect to baseline during treatment in all groups. In the 300  $\mu\text{g}$  DSG group, HDL-C fell from a baseline of  $1.34 \pm 0.10$  mmol/liter

to a nadir of  $1.11 \pm 0.06$  mmol/liter (wk 24), a fall of 17%. In the 150  $\mu\text{g}$  DSG group, HDL-C fell from a baseline of  $1.29 \pm 0.15$  mmol/liter to a nadir of  $0.86 \pm 0.06$  mmol/liter (wk 16), representing a fall of 33%. In the 75  $\mu\text{g}$  DSG group, HDL-C fell from a baseline of  $1.18 \pm 0.17$  mmol/liter to a nadir of  $0.90 \pm 0.02$  mmol/liter (wk 12), a fall of 24%. The differences in HDL-C suppression between treatment groups did not achieve statistical significance. Overall, the mean decrease in HDL-C levels at the end of the treatment period was  $12 \pm 3.2\%$ .

There were no significant changes in total cholesterol, LDL-C, triglycerides, or apolipoprotein A1. Total cholesterol was significantly increased in the recovery period compared with baseline; this significance disappeared on removing one outlier from the analysis. All other lipid parameters returned to pretreatment levels by the end of the recovery phase in all treatment groups.

### Physical changes

There was a small, but significant ( $P < 0.001$ ), increase of  $1.94 \pm 0.56$  kg (range,  $-4$  to  $+7$ kg) in body weight by the end of the treatment phase. This gain persisted; the average weight gain at the end of the recovery period was  $2.53 \pm 0.67$  kg (range,  $-3$  to  $+9.5$ kg). There was no significant difference between treatment groups in this regard. Testicular volume decreased by an average of  $3.3 \pm 1.1$  ml (range,  $0$ – $7.25$  ml) during treatment and returned to pretreatment values by the end of the recovery phase. There were no significant changes in systolic or diastolic blood pressure throughout the study.

### Discontinuations, side-effects, and compliance

There were 6 discontinuations; 2 were due to marked and persistent skin reaction to the T patches, and the remaining 4 subjects were either lost to follow-up before completion of the study ( $n = 2$ ) or withdrew for nontreatment-related reasons, *i.e.* job relocation or marital discord ( $n = 2$ ). During the treatment phase 11 subjects reported side-effects, namely increased sex drive ( $n = 4$ ), decreased sex drive ( $n = 7$ ), emotional lability ( $n = 2$ ), irritability ( $n = 5$ ), and tiredness ( $n = 2$ ), although these were transient in nature and unrelated to treatment groupings. In addition, 15 (65%) of the subjects reported skin reactions of varying degree to the T patches. In 3 subjects the reaction was classified as mild (transient erythema), whereas in an additional 8 subjects the reaction was classified as moderate (marked erythema and itch requiring treatment with topical hydrocortisone). The remaining 4 subjects were classified as having severe reactions, manifest as marked skin erythema and blistering.

Most of our subjects reported full compliance with the drug regimen. Closer questioning, however, revealed problems with the transdermal patches other than the significant skin reaction. These included patch removal to swim, play sports, or participate in lovemaking; patch detachment with excess perspiration or showering; and poor patch adhesion to hirsute skin and in hot weather. It was therefore difficult to accurately assess the degree of noncompliance with the T patch. As daily oral DSG administration was not subject to the same problems as transdermal application and skin reaction, there was no reason to doubt the high degree of

compliance reported, and this was confirmed by the tablet counts. This showed that compliance with oral medication was 90–95%.

### Discussion

Previous studies in hormonal male contraception have overwhelmingly employed im injections of T (enanthate in particular) in doses (200 mg weekly) that generated supra-physiological levels in the circulation (13). Consequently, not only have side-effects been observed, but the minimal effective dose for spermatogenesis suppression has remained undefined. In the development of new therapies, it is important to demonstrate the extent of the safety margin for breakthrough and the tolerance to variable/suboptimal compliance. This can only be revealed by systematically investigating the effects of reducing doses and establishing the minimally effective dose level. In our previous study (8) we showed that spermatogenesis suppression could be effectively achieved even when im T enanthate was reduced to the lowest physiological maintenance dose of 50 mg weekly (equivalent to 5 mg unesterified T daily) when combined with the synthetic oral progestin, DSG. However, despite substantially reducing the total dose, the suboptimal pharmacokinetics of T enanthate inevitably produced sharp fluctuations with supraphysiological postinjection peak T levels (14). This contributed to demonstrable nonreproductive effects and rendered interpretation of minimal effective doses of drug combinations difficult. At the equivalent dose of 5 mg T daily, transdermal systems can maintain stable physiological levels of T and offer the opportunity to investigate the minimal dose combination of progestin required to suppress spermatogenesis effectively.

In the present study a downward dose-ranging design was employed to determine the threshold dose of DSG combined with a fixed daily amount of 5 mg transdermally delivered T for suppression of spermatogenesis. We have demonstrated a trend of progressively declining efficacy in achieving the three target levels of sperm density with reducing doses of DSG. Three hundred and 150  $\mu$ g DSG daily induced azoospermia in 57% and 50% of subjects, respectively. This is approaching the range of azoospermic suppression observed with 500  $\mu$ g levonorgestrel with 100 mg T enanthate weekly (67%) (15), 200 mg T enanthate weekly (1) (65%), and 1200 mg T implants (16) (56%) in Caucasian men. Suppression to oligozoospermia (either  $<1$  or  $<3$  M/ml) in the present study (71% and 50% with 300 and 150  $\mu$ g DSG, respectively), however, was clearly inferior to the other regimens (8, 17, 18), which can achieve the less than 3 M/ml target in 94–100% of subjects. Below 150  $\mu$ g/d DSG, there was a marked drop in effectiveness, with none of the subjects achieving azoospermia. Nevertheless, even at this suboptimal dose, one of the four subjects suppressed to less than 1 million/ml consistently, whereas the other three failed to reach sperm densities less than 10 M/ml. This divergence in responsiveness was also observed in the 150  $\mu$ g, but not the 300  $\mu$ g, DSG group. Thus, in contrast to previous studies that emphasized the resistance to achieving azoospermia in a minority of men receiving maximal doses (19), we have demonstrated a marked between-subject variation in sensitivity

to hormonal suppression by exploring threshold (150  $\mu$ g DSG daily) to subthreshold (75  $\mu$ g DSG daily) doses of treatment. This heterogeneity in suppression suggests that a substantial proportion, perhaps up to 50%, of healthy men are able to respond to much lower doses of exogenous sex steroids than customarily used in attaining effective contraception in previous studies. In common with others (20) we have not been able to identify these susceptible individuals by any baseline characteristics, such as sperm density, gonadotropins, T levels, or body mass index, but they tend to show a more precipitous decline in sperm density within the first 8 wk after starting treatment.

Suppression of spermatogenesis with daily oral DSG and transdermal T was also less effective than with similar doses of DSG combined with weekly im injections of T enanthate (Table 1), although the speed of decline in sperm density was not different (8, 21). This was particularly true for the oligospermic targets. In the previous studies using DSG (8, 21) the least effective combination employing 150  $\mu$ g DSG daily with 50 mg T enanthate weekly produced suppression rates similar to the best results obtained in the group receiving 300 mg DSG with 5 mg transdermal T. Furthermore, the breakthrough of suppression in three of the seven men who reached azoospermia and the partial recovery or escape before the end of the treatment period in most subjects was not observed previously with DSG and T enanthate or T enanthate alone. It appears that substituting transdermal T for im T enanthate has resulted in a loss of efficacy in spermatogenesis suppression.

Similarly, inhibition of gonadotropins by DSG and transdermal T was less effective and less consistent than previous regimens employing im T enanthate. Suppression of gonadotropins showed the same dose-related trend as spermatogenesis suppression, with LH and FSH reaching assay sensitivity in 57% and 43% of subjects, respectively, in the 300  $\mu$ g DSG group and none in the 75  $\mu$ g group. In line with sperm density, very few subjects maintained consistent suppression of gonadotropin, most showing transient escape or partial recovery before the end of treatment. Unsurprisingly, azoospermic and oligozoospermic responders achieved significantly lower levels of gonadotropins than nonresponders during treatment. It is therefore highly probable that the inadequate suppression of spermatogenesis engendered by reducing doses of DSG and transdermal T is due to incomplete inhibition of gonadotropins.

The torso transdermal T delivery system has been shown to produce physiological circulating T levels in hypogonadal men (9, 10, 22). In eugonadal men rendered hypogonadal by exogenous sex steroid in the present study, however, total T decreased from baseline by about 30% during treatment, but mean levels at  $16.1 \pm 2.4$  nmol/liter (all three groups combined; normal, 10–35 nmol/liter) remained within the physiological range. Equilibrium dialysis-measured free T levels also decreased significantly by similar extents as total T. A lower SHBG concentration, associated with DSG and other oral synthetic progestin treatment, therefore did not correct for the low total T, and an absolute decline in circulating bioavailable T levels was extant during treatment. Insufficient T may therefore be one explanation for the lower efficacy in gonadotropin and spermatogenesis suppression

**TABLE 1.** Comparison of spermatogenesis suppression between oral DSG combined with transdermal T daily and oral DSG daily with im T enanthate weekly

Treatment	Transdermal T			Intramuscular T		
	Suppression targets:			Suppression targets:		
	Oligo <3	Oligo <1	Azoospermia	Oligo <3	Oligo <1	Azoospermia
DSG (300 µg/d) + TTS (5 mg/d)	71 (9.6 ± 1.6)	71 (10.4 ± 0.1)	57 (10.6 ± 1.9)	DSG (300 µg/day) + TE (100 mg/week) <sup>a</sup>	88 (10.9 ± 1.1)	75 (14.0 ± 2.7)
DSG (150 µg/d) + TTS (5 mg/d)	50 (5.3 ± 1.3)	50 (5.3 ± 1.3)	50 (10.6 ± 1.3)	DSG (150 µg/day) + TE (100 mg/week) <sup>a</sup>	100 (10.0 ± 0.8)	57 (13.0 ± 1.0)
DSG (75 µg/d) + TTS (5 mg/d)	25 (12)	25 (16)	0	DSG (300 µg/day) + TE (50 mg/week) <sup>a</sup>	100 (8.0 ± 0.8)	100 (12.5 ± 1.2)
				DSG (150 µg/day) + TE (50 mg/week) <sup>b</sup>	78	57 (13.2 ± 2.7)

Values given are percentages; figures in parentheses are the mean ± SEM time in weeks taken to achieve the stated sperm density targets. Oligo <3, Oligozoospermia sperm density less than 3 million/ml; Oligo <1, oligozoospermia sperm density less than 1 million/ml.

<sup>a</sup> Data are from Wu et al. (8).

<sup>b</sup> Data are from Anawalt et al. (28).

compared with other studies using the same doses of oral DSG (Tables 1 and 2). Nevertheless, this study showed that improved suppression of spermatogenesis can be obtained with DSG and transdermal T compared with levonorgestrel combined with a similar dose but a different transdermal T preparation (23) and also compared with cyproterone acetate and oral T undecanoate (24) (Table 2). This may be related to the varying biopotencies and efficiencies of the different progestins and/or preparations of noninjection T. Our results suggest that self-administration of male hormonal contraceptive steroids is potentially viable, particularly in more responsive individuals. However, the transdermal systems are clearly less reliable than injectable T regimens (see below). The loss of efficacy when weekly im injections of T enanthate is substituted by daily self-administered noninjection preparations of T is a consistent finding across all three paired comparisons (Table 2). This highlights the critical role of T in male hormonal contraception.

The lower efficacy associated with noninjection delivery of T merits further consideration. When the supraphysiological dose of 100 mg T enanthate (14) was coadministered with LNG or CPA, the higher dose and the higher peak levels of T compared with the transdermal dose of 5 mg daily and the T undecanoate dose of 80 mg twice daily may account for the superior efficacy. However, in our two studies with DSG and T (Table 2), we deliberately targeted comparisons with a lower, yet effective, dose of 50 mg T enanthate weekly, im, which delivers 5 mg unesterified T daily, an amount equivalent to the daily delivery rates of the transdermal system studied. The mean T levels, therefore, should not be very different. The fluctuating T levels giving high postinjection peaks with T enanthate are probably not required for maximal gonadotropin suppression, as relatively stable T levels generated by 800-mg T implants combined with depo medroxyprogesterone acetate was highly effective (25). Scrutiny of individual profiles showed that many subjects in our study had transient decreases in total and free T levels to the hypogonadal range with concomitant or subsequent escape in gonadotropins and sperm density (Table 3, subject 1). In contrast, the few individuals whose T levels were maintained in the normal range throughout the treatment phase were able to sustain suppression of gonadotropins and spermatogenesis throughout treatment (Table 3, subject 2). Analogously, the short half-life of oral T undecanoate and its unpredictable absorption (26) make it unlikely that twice daily administration can maintain stable T levels within the physiological range (27). These observations suggest that the common thread that links the low efficacy in the three studies using noninjectable T preparations is likely to be nonmaintenance of adequate T levels, which may have resulted from pharmacokinetic and delivery problems or noncompliance. The consequence of these intermittent troughs in circulating T may not be important or clinically evident in hypogonadal replacement, but is likely to be of critical importance in the maintenance of spermatogenesis suppression for contraception. The cumulative effects of repeated falls in T (probably underestimated by monthly monitoring) provide the most likely explanation not only for the lower initial efficacy, but also the subsequent breakthrough and progressive escape

**TABLE 2.** Comparison of efficacy of spermatogenesis suppression between the present study and other progestin and T combinations as indicated by the maximal percentage of subjects achieving oligozoospermia and azoospermia

Oral progestin: T:	DSG (300 µg/d) TTS T (5 mg/d)	DSG (300 µg/d) <sup>a</sup> TE (50 mg/wk)	LNG (250/500 µg) <sup>b</sup> TTS T (5 mg/d)	LNG (250 mg/d) <sup>c</sup> TE (100 mg/wk)	CPA (12.5 mg daily) <sup>d</sup> Oral TU (80 mg daily)	CPA (25 mg/d) <sup>e</sup> TE (100 mg/wk)
Oligo <3 M/ml	71	100	45	89	63	100
Oligo <1 M/ml	71	100	45	89	38	100
Azoospermia	57	100	18	78	13	100

Loss of efficacy in regimens employing noninjectable T preparations is apparent regardless of the type of progestin used in the combinations. Oligo, Oligozoospermia; DSG, desogestrel; LNG, levonorgestrel; CPA, cyproterone acetate; TTS T, transdermal therapeutic system T; TU, T undecanoate; TE, im T enanthate; M, million.

<sup>a</sup> Wu et al. (n = 8; 24-wk treatment).

<sup>b</sup> Buchter et al. (n = 11; 24-wk treatment).

<sup>c</sup> Anawalt et al. (n = 18; 24-wk treatment).

<sup>d</sup> Meriggiola et al. (n = 8; 16-wk treatment).

<sup>e</sup> Meriggiola et al. (n = 5; 16-wk treatment).

**TABLE 3.** Individual hormone profiles for two subjects

	Wk									
	-4	-2	4	8	12	16	20	24	28	32
Subject 1										
LH <sup>a</sup>	2.9	3.5	0.05	0.05	0.05	0.05	0.3	0.05	5.2	2.7
FSH	2.8	3.1	0.12	0.09	0.08	0.08	1.6	0.05	3.8	2.8
Free T <sup>b</sup>	10.1	8.19	7.72	3.99	3.27	0.3	0.29	8.75	6.53	5.7
Sperm Density <sup>c</sup>	89	184	42	5	0.1	0	2	1.2	4.2	4.4
Subject 2										
LH	1.5	1.1	0.05	0.05	0.05	0.05	0.05	0.05	3.1	4.4
FSH	2.5	1.4	0.05	0.05	0.05	0.05	0.05	0.05	6.3	3.8
Free T	8.78	9.79	9.14	11.17	9.85	8.6	9.12	8.08	9.6	9.6
Sperm Density	58	65	0.8	0	0	0	0	0	0.1	0.1

<sup>a</sup> Units for gonadotropins are international units per ml.

<sup>b</sup> Nanomoles per liter.

<sup>c</sup> Millions per ml.

seen across all three studies using noninjectable T preparations.

This study also shows that as we descend to lower threshold doses of DSG, the safety margin is gradually eroded, and the tolerance of the combination regimen to fluctuations in T levels becomes increasingly tested. The margin of safety for suppression of spermatogenesis to maintain contraceptive efficacy is relatively narrow. Doses of T adequate for physiological replacement (5–6 mg daily) represent the minimum requirement, but only if drug delivery is strictly reliable. This raises the important issue of compliance in potential contraceptive users who are healthy young men unaccustomed to daily self-medication. There was serious doubt concerning the efficacy and acceptability of transdermal T delivery due to the difficulties experienced with patch application and the high incidence (65% of subjects) of skin reactions in agreement with recent experience with the same preparation in hypogonadal men (28). These factors will conspire to discourage high compliance, and it is our view that the transdermal route will not be the most satisfactory for drug delivery in male contraception unless the current therapeutic systems are improved considerably. In our previous study with daily oral DSG combined with weekly im T enanthate, high compliance with the oral medication was confirmed by the well maintained plasma levels of etonogestrel (the active metabolite of DSG) after 6-month treatment and the persistent lowering of SHBG levels (8). In the current study with a similar cohort of men, compliance with daily oral DSG

administration was good. Although etonogestrel levels were not measured, the consistent suppression of SHBG indirectly attests to satisfactory compliance. Daily oral drug administration does not appear to be problematical for healthy men seeking contraception.

We have previously shown that DSG alone decreased levels of HDL-C as well as apolipoprotein A1, an effect augmented by coadministration of T enanthate. In the present study with transdermal T, HDL-C was reduced significantly in all treatment groups. This confirms that in the absence of high peak T levels, reduction of HDL-C is due to the action of oral DSG on hepatic lipid metabolism.

Side-effects during this study were relatively uncommon apart from skin irritation. Symptoms that may be attributable to androgen deficiency were encountered in seven subjects. However, these were transient in nature, and temporal correlation with changes in T levels was inconsistent.

In conclusion, we have shown that oral DSG combined with a nonsrotal transdermal T delivery system produces suppression of gonadotropins and spermatogenesis, but is less effective than regimens incorporating injectable T. The minimally effective dose of DSG is 150 µg. Escape from suppression is seen at all dose levels of DSG, particularly in those subjects in whom T replacement is not well maintained. These findings serve to further emphasize the critical role of T delivery and highlight some important practical issues concerning daily self-administered regimens for hormonal male contraception. In the current state of patch technology,

the transdermal route of delivery may not be optimal for male contraception.

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Address all correspondence and requests for reprints to: Dr. Frederick C. W. Wu, Department of Endocrinology, Manchester Royal Infirmary, Oxford Road, Manchester, United Kingdom M13 9WL.

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### References

- WHO Task Force on Methods for the Regulation of Male Fertility 1990 Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet* 335:955
- WHO Task Force on Methods for the Regulation of Male Fertility 1995 Rates of testosterone-induced suppression to severe oligospermia or azoospermia in two multicentre clinical studies. *Int J Androl* 18:157-165
- Scheerer SB, Alvarez-Sanchez F, Anselmo J, Brenner P, Coutinho E, Lathoen-Faundes A, Frick J, Heinild B, Johansson EDB 1978 Hormonal contraception for men. *Int J Androl* 2(Suppl):680-712
- Anderson RA, Ludlum CA, Wu FCW 1995 Haemostatic effects of supraphysiological levels of testosterone in normal men. *Thromb Haemost* 74:693-697
- Wu FCW, Farley TMM, Peregoudov A, Waites GM 1996 Effects of testosterone enanthate in normal men: experience from a multicentre contraceptive efficacy study. *Fertil Steril* 65:626-636
- Handelsman DJ, Conway AJ, Howe CJ, Turner L, Mackey MA 1996 Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J Clin Endocrinol Metab* 81:4113-4121
- Tom L, Bhasin S, Salameh W, Steiner B, Peterson M, Sokol RZ, Rivier J, Vale W, Swerdloff RS 1992 Induction of azoospermia in normal men with combined Nal-Glu gonadotropin-releasing hormone antagonist and testosterone enanthate. *J Clin Endocrinol Metab* 75:476-483
- Wu FCW, Balasubramanian R, Mulders T, Coelingh-Bennink JT 1999 Oral progestagen combined with testosterone as a potential male contraceptive: additive effects between desogestrel and testosterone enanthate in suppression of spermatogenesis, pituitary testicular axis and lipid metabolism. *J Clin Endocrinol Metab* 84:112-122
- Miekle WA 1998 A permeation-enhanced non-scrotal testosterone transdermal system for the treatment of male hypogonadism. In: Nieschlag E, Behre H, eds. *Testosterone: action, deficiency, substitution*, 2nd Ed. New York: Springer, 390-419
- Meikle AW, Mazer NA, Moellemer JF, Stringham JD, Tolman KG, Sanders SW, Odell WD 1992 Enhanced transdermal delivery of testosterone across non-scrotal skin produces physiological concentrations of testosterone and its metabolites in hypogonadal men. *J Clin Endocrinol Metab* 74:623
- Wu FCW, Butler GE, Kelnar CJH, Stirling HF, Huhtaneimi I 1991 Patterns of pulsatile luteinizing hormone and follicle stimulating hormone secretion in prepubertal (mid-childhood) boys and girls with idiopathic hypogonadotropic hypogonadism (Kallmann's syndrome): a study using an ultrasensitive time resolved immunofluorometric assay. *J Clin Endocrinol Metab* 72: 1229-1237
- Wang C, Iranmesh N, Berman N, McDonald V, Steiner B, Ziel E, Faulkner SM, Dudley RE, Veldhuis JD, Swerdloff RS 1998 Comparative pharmacokinetics of three doses of percutaneous dihydrotestosterone gel in healthy elderly men—a clinical research center study. *J Clin Endocrinol Metab* 83:2749-2757
- Hair WM, Wu FCW 1999 The role of drugs in male contraception *Curr Opin Oncol Endocrinol Metab Invest Drugs* 1:50-59
- Snyder PJ, Lawrence DA 1980 Treatment of male hypogonadism with testosterone enanthate. *J Clin Endocrinol Metab* 51:1335-1339
- Anawalt BD, Bebb RA, Bremner WJ, Matsumoto AM 1999 A lower dosage levonorgestrel and testosterone combination effectively suppress spermatogenesis and circulating gonadotrophin levels with fewer metabolic effects than higher dosage combinations. *J Androl* 20:407-414
- Handelsman DJ, Conway AJ, Boylan LM 1992 Suppression of human spermatogenesis by testosterone implants. *J Clin Endocrinol Metab* 75:1326-1332
- Merigiola MC, Bremner WJ, Costantino A, Di Cintio G, Flamigni C 1998 Low dose of cyproterone acetate and testosterone enanthate for contraception in men. *Hum Reprod* 13:1225-1229
- Pavlou SN, Brewer K, Farley MG, Lindner J, Bastias MC, Rogers BJ, Swift LL, Rivier JE, Vale WW 1991 Combined administration of a gonadotrophin-releasing hormone antagonist and testosterone in men induces reversible azoospermia without loss of libido. *J Clin Endocrinol Metab* 73:1360-1368
- Anderson RA, Wallace EM, Wu FCW 1996 Comparison between testosterone enanthate induced azoospermia and oligospermia in a male contraceptive study. III. Higher 5- $\alpha$  reductase activity in oligospermic men administered supraphysiological doses of testosterone. *J Clin Endocrinol Metab* 81:902-908
- Handelsman DJ, Farley TMM, Peregoudov A, Waites GMH, WHO Task Force on Methods for the Regulation of Male Fertility 1995 Factors in non-uniform induction of azoospermia by testosterone enanthate in normal men. *Fertil Steril* 63:125-133
- Anawalt BD, Herbst KL, Matsumoto AM, Mulders TM, Coelingh-Bennink HJ, Bremner WJ 2000 Desogestrel plus testosterone effectively suppresses spermatogenesis but also causes modest weight gain and high-density lipoprotein suppression. *Fertil Steril* 74:707-714
- Dobs AS, Meikle AW, Arver S, Sanders SW, Caramelli KE, Mazer NA 1999 Pharmacokinetics, efficacy and safety of a permeation enhanced testosterone transdermal system in comparison with bi-weekly injections of testosterone enanthate for the treatment of hypogonadal men. *J Clin Endocrinol Metab* 84:3469-3478
- Buchter D, von Eckardstein S, von Eckardstein A, et al. 1999 Clinical trial of transdermal testosterone and oral levonorgestrel for male contraception. *J Clin Endocrinol Metab* 84:1244-1249
- Merrigiola CM, Bremner WJ, Costantino A, Pavani A, Capelli M, Flamigni C 1997 An oral regimen of cyproterone acetate and testosterone undecanoate for spermatogenic suppression in men. *Fertil Steril* 68:844-850
- Handelsman DJ, Conway AJ, Howe CJ, Turner L, Mackay M-A 1996 Establishing a minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J Clin Endocrinol Metab* 81:4113-4121
- Behre HM, Nieschlag E 1998 Comparative pharmacokinetics of testosterone esters. In: Behre HM, Nieschlag E, eds. *Testosterone: action, deficiency and substitution*, 2nd Ed. New York: Springer, 293-328
- Skakkebaek NE, Bancroft J, Davidson DW, Warner P 1981 Androgen replacement with oral testosterone undecanoate in hypogonadal men: a double blind controlled study. *Clin Endocrinol (Oxf)* 14:49-61
- Parker S, Armitage M 1999 Experience with transdermal testosterone replacement therapy for hypogonadal men. *Clin Endocrinol (Oxf)* 50:57-62