ENDOGENOUS SERUM TESTOSTERONE IN MAN: AGEING, THE METABOLIC SYNDROME, FUNCTIONAL DECLINE AND THE ROLE OF SUPPLEMENTATION

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CHAPTER 6

6.1 THE EFFECT OF 12-MONTH ORAL TESTOSTERONE UNDECANOATE (ANDRIOL®) ON BODY COMPOSITION AND MUSCLE STRENGTH IN MEN AGED 60 YEARS AND OLDER WITH LOW-NORMAL GONADAL STATUS.

SUMMARY

Loss of muscle mass and strength (sarcopenia) leads to frailty in older men. The decline in testosterone over the lifespan may contribute to this muscle loss. We studied the ability of oral testosterone to prevent muscle and strength loss in older men over a twelve-month period. A standard dose (80mg twice daily) of testosterone undecanoate (TU) or placebo was administered for one year to 76 healthy men 60 years or older. All men had a free androgen index (FAI) of 0.3 – 0.5 which represents a value below the normal lower limit for young men (19-30 years), but within the overall normal male range. Measurements of body composition, muscle strength, hormones, and safety parameters were obtained at 0, 6, and 12 months. Lean body mass increased ($P = 0.0001$) and fat mass decreased ($P = 0.02$) in the testosterone as compared to the placebo treated group. There was no significant overall effect of treatment on muscle strength or serum IGF-1, however change in lean mass was associated with change in quadriceps strength in subjects receiving testosterone. There was a significant increase in haematocrit (0.02%) in the testosterone-treated group ($P = 0.03$). Plasma triglycerides, total cholesterol and LDL cholesterol levels were similar in both groups, but there was a decrease in HDL cholesterol (-0.1mmol/L) at 12 months in the

testosterone as compared to the placebo group (P = 0.026). There were no differences in prostate specific antigen, systolic or diastolic blood pressure between the groups. Oral testosterone administration to older relatively hypogonadal men results in an increase in lean body mass and a decrease in body fat with possible sub-clinical changes in muscle strength in men with the greatest testosterone mediated increases in lean mass.

INTRODUCTION

A decrease in muscle mass (sarcopenia) is a cause of age related frailty in ageing men [264, 325, 326]. In older men there is a relationship between muscle mass and various measures of androgen status including the FAI [94]. Moreover BT, the fraction of testosterone that is not bound to sex hormone binding globulin (SHBG), is associated with both muscle strength and functional status in older males [225]. In men, plasma total testosterone levels decline progressively over the lifespan [35, 47, 54, 59, 67]. Because there is a concomitant increase in plasma SHBG concentration with increasing age, plasma free and bioavailable testosterone decline even more [14, 327]. In young eugonadal men, pharmacological testosterone replacement has been demonstrated to increase both muscle mass and strength [328]. In elderly men with low bioavailable testosterone levels [3, 111, 135] and low-normal total testosterone levels [116], testosterone treatment has been shown to increase muscle mass [111, 135], muscle strength [3, 115, 116] and a measure of functional independence in those undergoing rehabilitation [133]. Other positive effects of testosterone treatment include decreased overall and visceral adipose tissue. Controversy exists, however, concerning the effect of testosterone treatment on body composition and muscle strength in men who have a decrease in plasma testosterone concentration over the lifespan but whose serum levels remain within the normal range. An association between the FAI and muscle strength has been reported [329], but there is no such relationship with total testosterone.

The aim of this study was to determine the effect of oral TU on body composition and muscle strength in healthy men over the age of 60, with a total testosterone greater than 8 nmol/L and an FAI above the

defined lower limit of normal (0.3), and below 0.5, therefore low-normal gonadal status relative to young

men.

METHODS

PARTICIPANT SELECTION

Seventy-six healthy men aged over 60 years (68.5 $\pm$ 6 mean $\pm$ SE, range 60-86) were recruited by community advertisement. Men were included if they had at least two symptoms on the St Louis University Androgen Deficiency in the Aging Male (ADAM) questionnaire [76], an FAI of between 0.3 and 0.5 (based on a single value obtained while fasting between 08.00 and 10.00hrs) and a total T greater than 8 nmol/L. These cut-offs were established in a series of preliminary studies showing that in healthy male Australian Red Cross blood donors (aged 19 – 29 years) the FAI was usually above 0.5, but up to 50% of elderly men had an FAI less than 0.5. In elderly men, when the FAI was above 0.3, the total T was usually greater than 8 nmol/L; the conventional cut-off for the diagnosis and treatment of male hypogonadism in Australia. A second value was obtained at baseline and the mean of these two values is reported. Exclusion criteria included: a history or presence of prostate cancer or a PSA > 5 ng/ml (the upper limit of normal for men aged 60); a score of greater than 20 on the IPSS, suggesting significant urinary obstruction (13); or an abnormal prostate on digital rectal examination. In addition subjects were excluded if they had a history of testicular, liver or renal disease, diabetes mellitus, cardiac failure, severe dysphoria (score of greater than 15 on the Geriatric Depression Scale [330]), joint pain that limited their ability to perform muscle strength testing, prior use of androgen, bisphosphonate, or glucocorticoid treatment (within the preceding six months), or an haematocrit greater than 50%.

The Research Ethics Committee of the Royal Adelaide Hospital approved the study. Informed consent was obtained from all subjects. The study was performed according to the International Conference on Harmonisation (ICH)/Good Clinical Practice.

STUDY DESIGN:

Participants were treated for 12 months with either TU (Andriol[R], Organon, Oss, The Netherlands) 80 mg orally, twice daily, or identical placebo, in a randomized, double blind manner. The testosterone or placebo was taken prior to the onset of breakfast and dinner. Assessments were performed at baseline (0), 1, 3, 6 and 12 months. The dose of testosterone was halved if the haematocrit increased above 50%.

Randomization was achieved with a block design of 4 using the Almedica Drug Labeling System (ADLS), version 5. No person involved in the execution or monitoring of the study had access to the randomization list, other than through the access protected Emergency Drug Identification Record (EDIR). The EDIR identified the treatment code for each individual subject.

MEASUREMENTS:

Body composition

Body fat and lean body mass was measured by whole body DEXA at the Endocrine Bone and Menopause Centre (Norwood, South Australia) as described in Chapter 2.0.

Muscle strength

Bilateral grip peak force was measured as described in Chapter 2.0. Subjects were instructed to complete 15 repetitions on each hand. Lower limb strength testing was performed at the School of Physiotherapy,

University of South Australia. Quadriceps and calf peak torque were measured during concentric and eccentric maximal voluntary contraction (MVC) using a KinCom isokinetic dynamometer (Chattanooga, Tennessee) at 60 degrees/second and 30 degrees/second respectively. Subjects were instructed to move only the dominant limb through a comfortable range of motion and complete 15 repetitions. Prior to the actual test, subjects completed a minimum of three repetitions at 50% of maximum followed by three at 75% and one at maximum. The subjects were then rested for two minutes before starting the test. During the test, subjects were verbally motivated by the tester in order to maintain maximal contraction throughout the 15 repetitions. Complete strength data was not obtained in 9 subjects in the testosterone group and 7 subjects in the placebo group. The inter-class correlation coefficient (ICC) for between-day comparisons of force using the KinCom dynamometer is above 0.99 [331]. During analysis of the KinCom data, torque data was imported into Microsoft Excel and filtered to remove non-isokinetic data ($\pm$ 2.5%).

Androgen deficiency symptoms

Androgen deficiency symptoms were assessed using the St Louis University ADAM questionnaire [Morley, 2000 #230] (See Chapter 1.0, Figure 1.2).

Nutrition

Nutritional status was assessed using the Mini Nutritional Assessment (MNA). This questionnaire identifies both people who are malnourished and those at risk of becoming malnourished and is used as a screening tool in studies in the field of geriatrics [332]. A low MNA score has been found to be predictive of a greater incidence of adverse clinical events during hospitalisation and of higher mortality [333].

Laboratory Assays

Blood was drawn from a forearm vein at 0, 1, 3, 6 and 12 months. At month 1, blood samples for the measurement of plasma testosterone were drawn four hours after the morning dose of testosterone undecanoate. At 3, 6, and 12 months blood samples were obtained fasting, between 08.00 and 0.900 and prior to the morning dose. Total T, SHBG, luteinizing hormone (LH), follicle stimulating hormone (FSH), total prostate specific antigen (PSA), serum lipids, insulin-like growth factor 1 (IGF-1), haematocrit (Hct) and haemoglobin (Hb) were determined as specified in Chapter 2.0. Calculated bioavailable testosterone (cBT) was determined as described in Chapter 3.0.

Safety Monitoring

The International Prostate Symptom Score (IPSS) was used to assess the presence of and change in lower urinary tract symptoms (LUTS) as described in Chaper 2.0. Digital rectal examination to determine irregularities of the prostate was performed by a supervising physician. PSA and Hct were assessed as described in the previous paragraph.

Adverse events (AEs, including serious AEs (SAEs)) were coded as dictionary terms according to the "International monitoring of adverse reactions to drugs: adverse reactions terminology" by the World Health Organisation (WHO). In the event of an (S)AE, the ethics committee was notified within 48 hours of study staff being notified by the participant (or a representative thereof).

STATISTICAL ANALYSES:

Data are reported as mean ± SE except where otherwise specified. Analyses for the primary outcome measures (body composition and muscle strength) were performed using an intent-to-treat approach. Data on all patients randomized were analyzed. Where patients had discontinued, their last observations were carried forward in analyses of subsequent time-points to prevent bias due to differential drop out (last-observation-carried-forward-approach). All other analyses were performed for all subjects treated. The mean change over time between the treatment and placebo groups for continuous variables was compared using a two-tailed independent sample t-test. Categorical data were analyzed by Fisher's exact test. $P < 0.05$ was considered significant.

RESULTS

Of the 76 men enrolled in the study, 39 were treated with testosterone and 37 with placebo. The characteristics of the two groups at baseline are shown in Table 6.1.1. There were no significant differences between the groups. There were 18 early withdrawals, 12 in the placebo group and 6 in the testosterone group (P = 0.11). The reasons for withdrawal are shown in Table 6.1.2.

TREATMENT COMPLIANCE

Of the patients completing the study, 69.7% in the testosterone group and 80% in the placebo group took 90% or more of their tablets, and 27.3% took between 51 and 89% of the dose in the testosterone group and 20% did the same in the placebo group.

	Testosterone (n=39)	Placebo (n=37)	P
Age (years)	69 ± 6 (60-86)	68 ± 5 (60-77)	NS
BMI (kg/m^2)	27 ± 4	29 ± 5	NS
Systolic BP (mmHg)	131 ± 19	134 ± 21	NS
Diastolic BP (mmHg)	79 ± 8	78 ± 8	NS
Smokers (number)	2	1	NS
Mini Nutritional Assessment score	27.9 ± 1.2 (24.5 – 30)	27.3 ± 1.5 (24 – 30)	NS
Currently Exercise (% who responded YES)	78.9	84.2	NS
Relevant Concomitant Meds (number of cases)	7	8	NS
Total testosterone (nmol/L)	17.0 ± 4.4	15.6 ± 4.5	NS
SHBG (nmol/L)	42.5 ± 13	38.9 ± 12	NS
FAI (T/SHBG)	0.41 ± 0.07	0.41± 0.07	NS
cBT (nmol/L)	3.17 ± 0.87	2.82 ± 0.66	NS
LH (IU/L)	5 ± 3	5 ± 3	NS
FSH (IU/L)	7 ± 4	8 ± 5	NS

	Testosterone (n=39)	Placebo (n=37)	P
IGF-1 (nmol/L)	20 ± 8	22 ± 9	NS
Total body mass (kg)	81.0 ± 14.2	84.7 ± 14.1	NS
Lean body mass (kg)	54.8 ± 7.2	56.9 ± 7.9	NS
Fat mass (kg)	23.5 ± 7.9	24.8 ± 8.6	NS
ADAM score (0-10)	5.6 ± 2	5.7 ± 2.1	NS
GDS (0-30)	6.4 ± 3.8	5.6 ± 4.4	NS

Table 6.1.1 Baseline characteristics

of subjects recruited by community advertisement and randomized to receive either oral testosterone or identical placebo for 12 months. Data presented are means ± S.E. except where otherwise specified. Age, BMI, nutritional score (Mini Nutritional Assessment), smoking status and exercise habit at baseline were analysed as potential confounders in an intent-to-treat analysis. Relevant concomitant medications include St Johns Wort, Viagra, Xenical, Caverject and dose change in lipid lowering medications. There were no statistically significant differences between the two groups on any variables. NS = not significant.

	Testosterone	Placebo	P
Number of Withdrawals			
1 month	1	1	NA
3 months	1	4	NA
6 months	0	2	NA
12 months	4	5	NA
Reason for early			
Adverse event	2	4	NA
Intercurrent Illness	0	1	NA
Protocol violations	1	1	NA
Unwilling to continue	3	6	NA
Adverse events			
At least 1 AE	34	31	NS
At least 1 SAE	7	4	NS
Discontinued for an AE	2	4	NS
Drug-related AE's	11	8	NS

Table 6.1.2 Details of early withdrawl

of subjects recruited by community advertisement and randomized to receive either oral testosterone or identical placebo for 12 months. Data presented are counts. There were no statistically significant differences between the two groups on any variables. AE = adverse event, SAE = serious adverse event, NA = statistical differences not analysed, NS = not significant.

PLASMA ANDROGEN LEVELS

To evaluate efficacy of drug absorption, at one-month serum samples were obtained 4 hours after the ingestion of the morning dose taken together with food. Data were analysed for all subjects treated. The FAI was 0.67 ± 0.05 in the testosterone group compared to 0.38 ± 0.02 in the placebo group. This represented an increase of 26.9% from pre-treatment in the testosterone group and a decline of 2.3% in the placebo group (P<0.0001). Calculated BT increased from 3.17 ± 0.15 nmol/L at baseline to 6.52 ± 0.82 nmol/L at one month in the testosterone group, and decreased from 2.82 ± 0.1 nmol/L at baseline to 2.57 ± 0.15 nmol/L at one month in the placebo group (P = 0.016).

At 12 months, total T measured fasting, prior to the morning dose of TU, had declined by 1.7 ± 1.2 nmol/L and 0.7 ± 0.11 nmol/L in the testosterone and placebo groups respectively (P = 0.56). In the testosterone group, SHBG levels declined by 18.6 ± 2.6 % and increased by 4.76 ± 2.7 % in the placebo group (P<0.0001). Accordingly the FAI (P = 0.021) and cBT (P = 0.025) were higher in the testosterone as compared to the placebo groups (Figure 6.1.1).

At 3, 6 and 12 months, LH (P = 0.0000, P = 0.0000 and P = 0. 0002) and FSH (P = 0.0001, P = 0.0000, P = 0.0015) were lower in the testosterone group, as compared to the placebo group (Figure 6.1.2).
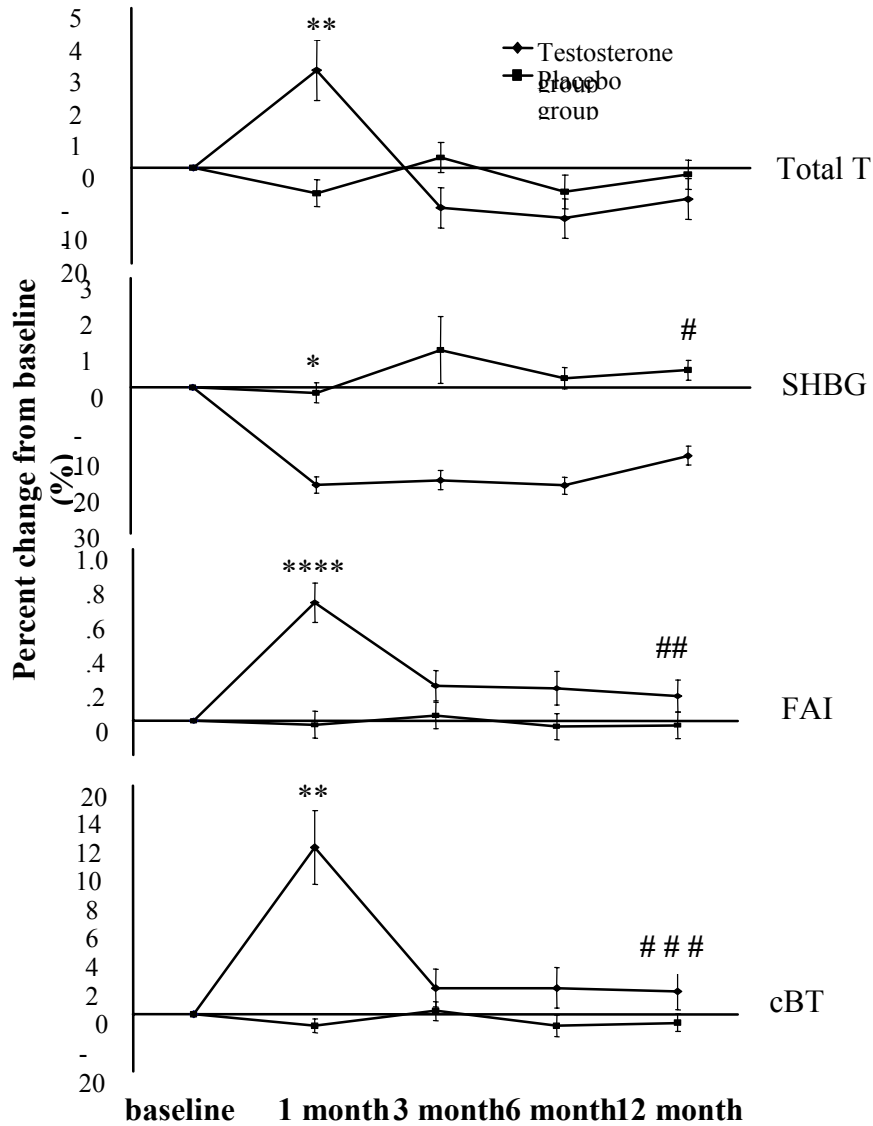
Figure 6.1.1 Plasma concentrations of total T, SHBG, FAI and cBT at baseline, 1 month, 3 months, 6 months and 12 months in Testosterone and Placebo groups.

Month 1 data are plasma levels 4-hours post treatment dose, month 3, 6 and 12 data are fasting morning blood samples prior to the morning treatment dose: *P = 0.032, **P = 0.016, ***P = 0.0005, **** P<0.0001, #P = 0.057 (NS), ##P = 0.025, ###P = 0.021.
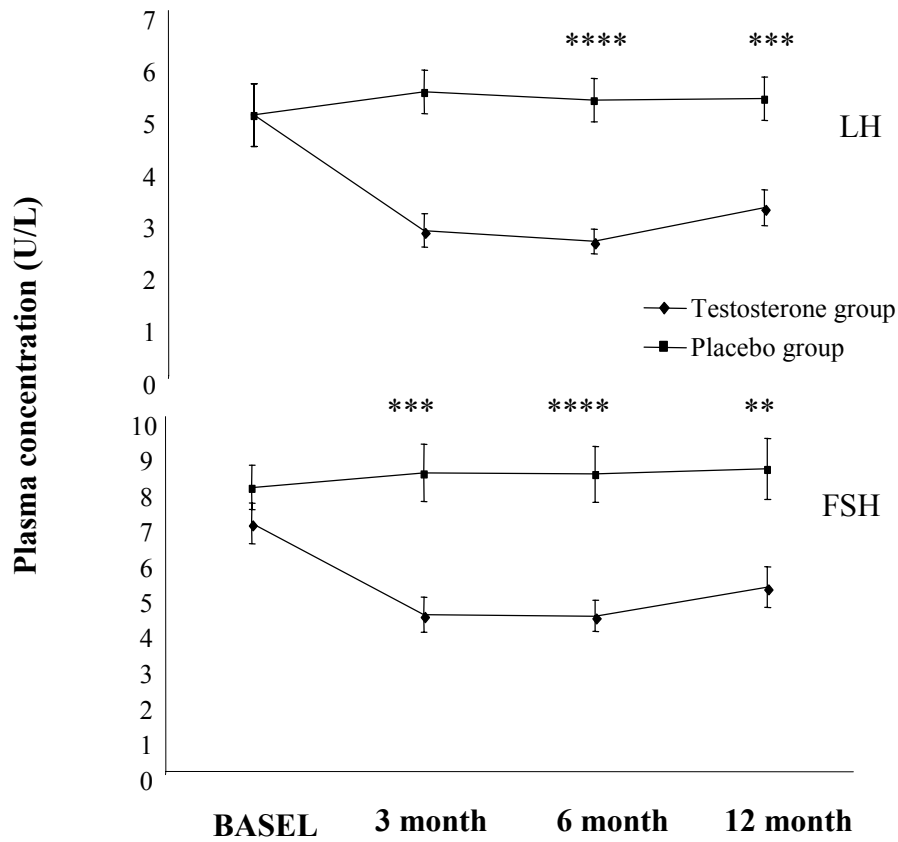
Figure 6.1.2 Plasma concentrations of LH and FSH at baseline, 3 months, 6 months and 12 months in Testosterone and Placebo groups.

Data presented are mean ± SEM. LH (top panel) and FSH (bottom panel) both decreased by month 3 in the Testosterone group and remained significantly lower than the Placebo group at month 12. **P<0.01, ***P<0.001, ****P<0.0001.

BODY COMPOSITION

There was no change in body weight during the course of the study. After 6 months, lean body mass decreased by $1.47 \pm 0.52\%$ (0.91 ± 0.03 kg) and increased by $2.16 \pm 0.51\%$ (1.04 ± 0.07 kg), from baseline, in the placebo and testosterone groups respectively ($P < 0.00001$). By month 12, lean body mass had decreased by $1.65 \pm 0.49\%$ (0.98 ± 0.08 kg) in the placebo group and increased by $1.54 \pm 0.58\%$ (0.67 ± 0.05 kg) in the testosterone group from baseline ($P < 0.0001$, Figure 6.1.4). There was no significant change in LBM between 6 and 12 months in either the placebo or testosterone groups.

After 6 months, fat mass decreased by $4.31 \pm 1.63\%$ (0.2 ± 0.1 kg) and increased by $4.21 \pm 1.13\%$ (0.85 ± 0.19 kg), from baseline, in the testosterone and placebo groups respectively ($P < 0.0001$). By 12 months, fat mass declined by $1.07 \pm 1.33\%$ (0.05 ± 0.07 kg) in the testosterone group and increased by $4.61 \pm 1.96\%$ (0.7 ± 0.13 kg) in the placebo group, from baseline ($P < 0.01$, Figure 6.1.3). These changes were significant and independent of age, BMI, nutritional status, smoking, physical activity and concomitant medications.
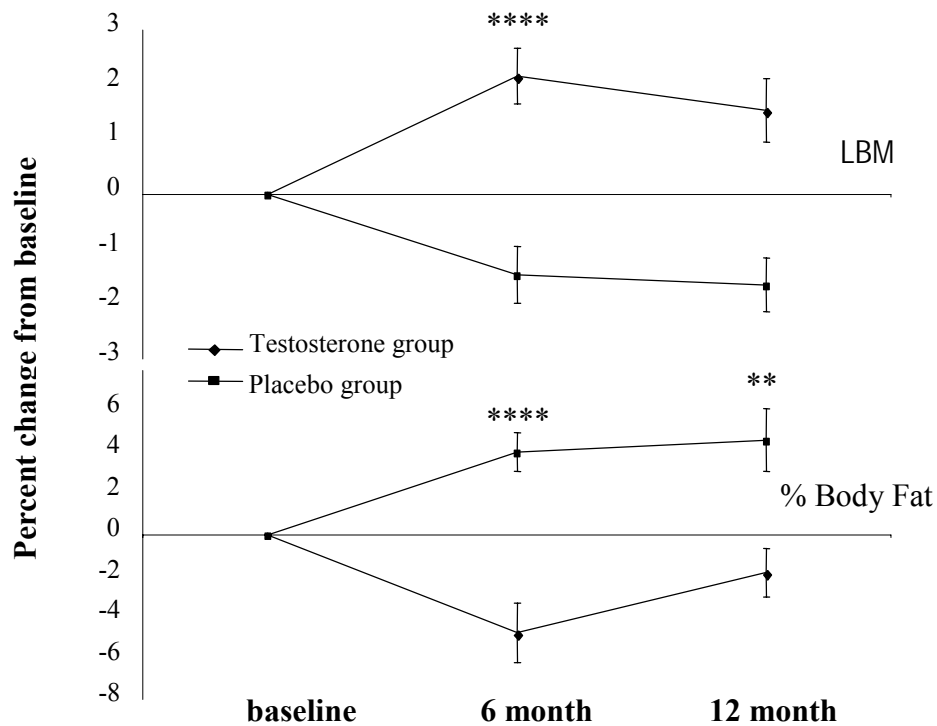
Figure 6.1.3 Percent change from baseline in lean body mass (LBM) and body fat after 6 months and 12 months of treatment with Testosterone or Placebo.

LBM (top panel) increased in the Testosterone group and decreased in the Placebo group by month 6 and remained significantly different at month 12. Percent body fat (bottom panel) decreased in the Testosterone group and increased in the Placebo group by month 6 and remained significantly different at month 12. **P<0.01, ***P<0.001, ****P<0.0001.

MUSCLE STRENGTH

There were no significant differences in grip, quadriceps or calf strength, between the treatment and placebo groups at either 6 or 12 months (Table 6.1.3). In both the testosterone and placebo groups, concentric quadriceps strength improved from baseline to month 6 (P = 0.002 and P = 0.02 respectively) and month 12 (both P's < 0.0001). There was also a decrease in eccentric quadriceps strength at month 12 in the testosterone (P = 0.037) but not in the placebo (P = 0.39) group and a decrease in concentric and eccentric calf strength in the placebo (P = 0.039 and P = 0.043 respectively) but not in the testosterone (P = 0.63 and P = 0.51 respectively) group.

In a *post-hoc* analysis to determine if changes in LBM were associated with changes is muscle strength there was a significant positive correlation between increase in lean body mass and increase in quadriceps strength in the testosterone, but not in the placebo group (Testosterone group R=0.46, P=0.02; Placebo group R=0.336, P=0.16).

	Right grip (Kg)		Left grip (Kg)		Quadriceps (Nm)		Calf (Nm)	
	T	Placebo	T	Placebo	T	Placebo	T	Placebo
Baseline	37.5 (1.3)	38.4 (1.6)	36.1 (1.3)	36.1 (1.4)	124.7 (9.78)	125.9 (10.24)	217.1 (12.88)	248.1 (12.93)
6 month	39.9 (1.5)	38.6 (2.1)	37.9 (1.5)	38.9 (1.7)	168.1 (11.35)**	159.8 (9.6)*	224.1 (11.84)	251.6 (12.61)
12 month	40.9 (1.4)	40.7 (2.0)	38.0 (1.3)	39.6 (1.7)	184.4 (10.52)†	181.8 (9.36)†	212.36 (12.60)	223.7 (10.63)*

Table 6.1.3 Maximal isometric right and left grip strength and maximal concentric, isokinetic quadriceps and calf strength (dominant limb only) at baseline, month 6 and month 12 in Testosterone (T) and Placebo treated subjects.

Data shown are mean (± S.E.). The mean differences between baseline-month 6, month 6-month 12 and baseline-month 12 were compared between the Testosterone and Placebo groups using a two-tailed independent samples t-test. There were no significant differences between the Testosterone and Placebo groups at any time point. Significant within group improvements were observed in quadriceps strength for both T and Placebo groups from baseline to months 6 and 12. A significant decline in calf strength from baseline to month 12 was observed in the Placebo group. *$P < 0.05$, **$P = 0.002$, †$P < 0.0001$

INSULIN GROWTH FACTOR 1

At baseline, plasma IGF-1 levels were 20.4 ± 7.7 nmol/L and 21.7 ± 8.8 nmol/L in the testosterone and placebo treated groups respectively. At month 3, 6 and 12, IGF-1 levels were 22.3 ± 7.1 nmol/L, 22.0 ± 6.7 nmol/L and 21.7 ± 7.5 nmol/L in the testosterone group, and 22.6 ± 4.8 nmol/L, 22.4 ± 7.7 nmol/L, and 21.7 ± 8.1 nmol/L in the placebo group. There were no significant differences either between or within these groups at any time.

SAFETY MEASUREMENTS

The results of safety parameter monitoring are shown in Table 6.1.4. There was a small but non-significant improvement in total IPSS score from baseline to 12 months in the testosterone as compared to the placebo treated group (-0.3 ± 4.0 vs 0.9 ± 5.1, (mean change in score ± SD), P = 0.25). In absolute terms, at 3 months, 2 subjects (6.3%) and at 12 months, 1 subject (4.0%) in the placebo group had significant LUTS, whereas no patient had significant LUTS in the testosterone group at any time.

In the testosterone treated group there was a small, but non-significant increase in PSA of 0.34 ± ng/L, 0.42 ± ng/L, and 0.38 ± ng/L at months 1, 3 and 6 respectively. The change in plasma PSA levels from baseline to 12 months was similar (0.1 ± 0.8 vs 0.4 ± 1.2 ng/L), in the testosterone and placebo groups respectively (P = 0.47). A similar number of subjects in each group (6 in the testosterone treated group and 5 in the placebo group) developed an elevated PSA (>5 ng/L) at some time during the study.

At 12 months, there were no differences in either plasma LDL cholesterol or triglyceride levels between the groups (P = 0.21 & P = 0.26, respectively). Plasma HDL levels decreased by 0.128 ± 0.03 mmo/L and

increased by 0.148 ± 0.0 mmo/L in the testosterone and placebo groups respectively (P = 0.03). Haematocrit increased by 2% over 12 months in the testosterone group and did not change in the placebo group (P = 0.026). After 6 and 12 months, 5 (14.7%) and 2 (6.3%) subjects, respectively in the testosterone treated group had a Hct greater than 50%, whereas none of the subjects in the placebo group did (P = 0.012). There were no changes in either systolic or diastolic blood pressure at any time during the course of the study in either group.

	Testosterone		Placebo		P
	Mean	SD	Mean	SD	
Haematocrit (%)	0.02	0.03	0.00	0.02	0.026
PSA (ng/L)	0.09	0.83	0.36	1.19	0.47
Urine flow (I-PSS, 0-30)	-0.30	4.00	0.90	5.10	0.25
Systolic BP (mmHg)	8.00	23.00	3.00	16.00	0.40
Diastolic BP (mmHg)	2.00	10.00	1.00	11.00	0.65
HDL (mmol/L)	-0.10	0.20	0.00	0.10	0.03
LDL (mmol/L)	-0.10	0.60	-0.30	0.50	0.21
Cholesterol total (mmol/L)	-0.20	0.60	-0.20	0.60	0.81
Triglycerides (mmol/L)	0.00	0.50	-0.10	0.70	0.26

Table 6.1.4 Change from baseline to month 12 for safety measures in the Testosterone and Placebo-treated groups.

Data presented are mean changes from baseline ± S.D. Haematocrit increased and HDL cholesterol decreased significantly in the Testosterone group when compared to the Placebo group (P = 0.026 & P = 0.03, respectively).

DISCUSSION

This study has demonstrated that a standard dose of oral TU, administered for 12 months to older men without overt hypogonadism, increases lean body mass and decreases fat mass. The decrease in body fat may be of significance as it has been shown that the combination of adipose excess and muscle loss in older persons (the fat frail) results in markedly increased morbidity [334]. The aim of this study was to investigate men with borderline low plasma testosterone concentrations resulting from normal ageing and to exclude those who were frankly hypogonadal. Due to the increase in SHBG with age, the FAI was used to define our population group. Although the FAI has limitations and its use is controversial in men [16, 335], it does correlate significantly with a marker of testosterone action, namely muscle mass [94] and no better method was available at the time of recruitment. The cut-off range (0.3 - 0.5) was based on a study of 214 healthy men aged 19 - 83 years. When the FAI was above 0.3, the total T was always above 8nmol/L, the cut-off used to diagnose and treat hypogonadism in Australia. Of those aged 19 - 29 years (N = 56), all had an FAI above 0.5 and a cBT above 3.09 nmol/L (the lower reference value for cBT, calculated as two standard deviations below the mean for male blood donors aged 19-29 years, as shown in Chapter 3.0). The cBT values of the ageing men included in our study were low relative to healthy young males, and also correlated well with the calculated FAI.

The dose of oral TU (Andriol^R) chosen has been widely used throughout the world to treat hypogonadal men [336]. In accordance with known pharmacokinetics of oral Andriol® [337-339],plasma total T, FAI and cBT increased four hours after the morning dose. Trough levels of total testosterone were decreased due to a significant and sustained reduction in SHBG. The suppression of LH and FSH reflected the efficacy of the dose used.

The effect of oral testosterone treatment to decrease body fat is consistent with observations on the effects of supplemental testosterone in either hypogonadal, or a mixed population of eugonadal and hypogonadal men [3, 111, 131, 135].

Testosterone replacement in young hypogonadal men [113, 119] and supraphysiological treatment in eugonadal young men have been shown to increase muscle mass and strength [328]. In older men with low bioavailable testosterone [3] or low-normal total testosterone levels [115, 116], replacement therapy increases muscle mass and strength. In longitudinal studies, men have been shown to have a decline in testosterone with increasing age at the rate of approximately 0.3 nM/year [47]; a cluster of men become relatively hypogonadal compared to when they were young and exhibit such symptoms as a decline in muscle mass and strength, low libido, fatigue and depressed mood. The finding of an increase in lean body mass in this study in response to supplemental testosterone is therefore not surprising. Moreover it is in keeping with epidemiological studies suggesting that the FAI is a predictor of both muscle mass and strength [94]. The present study, however, did not show an increase in muscle strength, a finding that is in accordance with other studies where a large proportion of men with normal total testosterone levels were included [111, 135]. However, it was shown that change in lean mass did correlate with change in quadriceps strength from baseline to month 12 in the testosterone but not in the placebo group. Thus sub-clinical improvements in muscle strength may have occurred at least in the quadriceps. A subjective improvement in physical functioning in response to testosterone as compared to the placebo has been reported [111]. Bhasin et al. (2001) have recently shown, in young men, that the muscle response to testosterone is related to dose [340]. Therefore if we had used a higher dose of oral testosterone it is possible that we would have seen a greater effect on muscle strength, as well as muscle mass. However, comparison of muscle strength between the two groups may not be clear because there was an apparent increase in muscle strength in both the placebo and treatment groups, raising the possibility that a neural

learning effect may confound the data. In addition, the relevance of muscle function testing in the elderly is confounded by wide variability in most measures. Motivation, tolerance to pain and potential learning effects may be some of the major factors limiting the ability of these tests to identify differences between the treatment groups in this study. Accordingly large study groups may be required to determine small treatment benefits [93]. Furthermore, it should be noted that isokinetic movement rarely occurs in actual everyday tasks, limiting the interpretation of the testing used in this study. Nevertheless, quadriceps strength measured on the Kin-Com isokinetic dynamometer has been show to relate to gait time on a standardized walk-turn-walk test at maximal gait speed [341]

Urban et al. (1999) reported previously that testosterone increased muscle IGF-1 mRNA [342] and Snyder et al. (1999) reported an increase in serum IGF-1 in men receiving testosterone [111]. There was no effect of testosterone therapy on IGF-1 levels in this study, possibly due to the dose of testosterone used. Much recent research has focused on the testosterone and IGF-1 stimulation of myogenic satellite cell activity in aged skeletal muscle. Satellite cells are responsible for muscle regeneration and repair after injury or atrophy. Satellite cells are decrease in number and proliferative activity in older muscle. Skeletal muscle is responsible for the production of 25% of circulating IGF-1. There are two muscle isoforms; one similar to liver IGF-1 and the other (IGF-IEc: mechano-growth factor) has local actions on muscle [268]. Exercise (stretch) leads to upregulation of the mRNA for both muscle isoforms and reduced muscle IGF-1 signaling leads to muscle atrophy. Hormones (growth hormone, testosterone, insulin and vitamin D) and exercise regulate muscle IGF-1 [343]. In rats, IGF-1 enhances muscle growth partially by increasing satellite cell production [344] and electroporation of IGF-1 stimulates muscle fiber hypertrophy [345]. IGF-IEc also stimulates protein synthesis in muscle [346]. A localized IGF-IEc transgene prevented age-related muscle atrophy and allowed older animals to develop a similar proliferative response to muscle injury as that seen in younger animals [347].

There were minimal adverse effects of testosterone supplementation. Testosterone increased haematocrit, as has been demonstrated in most [112, 113, 115, 348, 349] but not all studies [119, 135]. Levels of prostate specific antigen increased transiently but not significantly and there was trend towards a reduction in symptom scores on the IPSS with testosterone treatment. Previously testosterone replacement has been shown to have either no effect, or to decrease the occurrence of benign prostatic hyperplasia [112].

There were no significant changes in plasma total, LDL cholesterol or triglyceride levels in this study. However, a small, but significant, decrease in HDL cholesterol occurred. Small decreases in plasma total and LDL cholesterol have been found in some [131, 132, 144] but not all [3, 126, 341] studies of testosterone treatment in older men, and the majority have not found a significant decrease in HDL cholesterol [3, 90, 126, 132, 144](32,16,35,36,37). There is very little other published data on the effect of oral TU on plasma lipids. Uyanik et al (36) reported that oral testosterone undecanoate decreased plasma LDL cholesterol, but had no effect on HDL cholesterol in healthy older men, but the dose used (120mg/day) was lower than in our study and the duration (90 days) was shorter [144]. The most important factor in determining the effect on plasma lipids appears to be mode of delivery, although the dose may also be a significant factor. Intramuscular testosterone administration has consistently been reported to decrease total, LDL and HDL cholesterol in both eugonadal and hypogonadal men [350]. In contrast, transdermal testosterone has been found not to effect plasma HDL cholesterol either in eugonadal, mildly hypogonadal, or profoundly hypogonadal men irrespective of age [90, 113, 126]. However, a recently reported randomised controlled trial did show a decrease in HDL cholesterol after 12 months of transdermal testosterone treatment [127]. There are no long-term studies on the cardiovascular risk of testosterone replacement in older men. However Andriol® has been shown to decrease the symptoms of angina in

elderly men [351] and a transdermal delivery system of testosterone decreases ST segment during stress testing [145].

Testosterone therapy in this study was associated with half the number of dropouts compared to placebo, suggesting that patients had some perceived benefit from the therapy. Furthermore, because there were significantly more drop-outs in the placebo than in the testosterone treated group and because an intent-to-treat analysis was used, it is possible that significant changes may have either been over-estimated or obscured.

In conclusion this study has demonstrated that oral testosterone therapy increased muscle mass and decreased fat mass in older persons with minimal side effects. If this benefit is sustained, the development of age related sarcopenia and frailty, may be delayed or prevented.

6.2 THE EFFECT OF 12-MONTH ORAL TESTOSTERONE UNDECANOATE (ANDRIOL®) ON SYMPTOMS OF ANDROGEN DEFICIENCY IN MEN AGED 60 YEARS AND OLDER WITH LOW-NORMAL GONADAL STATUS.

SUMMARY

Relative androgen deficiency in ageing males is assumed to have adverse health effects. This study assessed the effect of 12 months standard dose, oral testosterone, on symptoms attributed to testosterone deficiency in older men with plasma testosterone levels in the low-normal range for young men. Testosterone undecanoate (TU, 80 mg bid) or placebo was administered orally for one year to 76 healthy men, 60 years or older with a free androgen index (FAI) of 0.3 – 0.5 and significant symptoms on a questionnaire designed to evaluate androgen deficiency in the ageing male (ADAM). The ADAM was completed at baseline, 6 and 12-months. Hormone and safety data were collected at baseline, 1, 3, 6 and 12-months. After 12-months, plasma total testosterone was unchanged in both groups and sex hormone binding globulin decreased in the testosterone group (P = 0.01). FAI and calculated bioavailable testosterone (cBT) were greater in the testosterone as compared to the placebo group (P = 0.021 and P = 0.025 respectively). There was no significant difference in total symptom score between testosterone and placebo groups after 12 months of oral TU. However, there were trends toward improvements in sadness/grumpy (P = 0.063), reduced erection strength (P = 0.059) and decreased work performance symptoms (P = 0.077), in the testosterone group as compared to the placebo group, particularly in men with baseline cBT levels below 3.1 nmol/L. This study concludes that 80 mg bid of oral TU does not improve overall ADAM questionnaire scores in older men with low-normal gonadal

status. Oral TU may preserve mood and erectile function, as assessed by this questionnaire, particularly in men with the lowest testosterone levels.

INTRODUCTION

Plasma total testosterone (total T) levels decline progressively, but variably, over the male lifespan [35, 47, 54, 59, 67]. A concomitant increase in plasma sex hormone binding globulin (SHBG) concentration results in an even greater decline in the plasma free and bioavailable fractions of testosterone [14, 327]. Relative androgen deficiency in ageing males is often assumed to have adverse health effects. Efforts to identify the symptom complex associated with low testosterone in older men, if one exits, have been made but are complicated by the slow rate of decline in testosterone levels, concomitant disease, changes in dietary and exercise habits and the multitude of other physiological changes that occur along a similar time course [167, 352].

In order to identify clinical signs and symptoms of relative androgen deficiency, 3 different screening questionnaires have been developed and used to identify men most likely to have low testosterone levels. Morley and colleagues developed a questionnaire based on 10 symptoms that, in their clinical experience, were commonly observed in older males with low bioavailable testosterone (BT) levels. This questionnaire, called the St Louis University ADAM questionnaire for Androgen Deficiency in the Ageing Male, was reported to have 88% sensitivity and 60% specificity in identifying Canadian physicians with BT < 70 ng/dL (2.43 nmo/L) [76]. Furthermore, treatment with testosterone cypionate (200mg i.m.) improved ADAM questionnaire scores, in men with borderline low BT levels (< 85 ng/dL, (2.95 nmol/L)) [76]. Using a cut-off of 5 on a questionnaire developed from Massachusetts Male Aging study data, McKinlay and colleagues reported a sensitivity of 71%, a specificity of 53% and a positive predictive value of 27.9% in identifying men with total T < 12.1 nmol/L [110]. A screening questionnaire that consisted of 17 questions covering physical, vasomotor, psychological and sexual symptoms had a 90% sensitivity, 86% specificity and a positive predictive value of 75% for identifying relative androgen

deficiency (plasma testosterone concentration not specified) when a questionnaire score of 18 was used as the cut-off in 32 Lebanese men [353]. These authors further reported that questionnaire scores improved from baseline after 8 months of oral testosterone undecanoate (TU) treatment (120 mg/day) in 76 out of 87 men aged 50 - 70 years [353]. This study was, however, open, and non-comparative in design, thereby limiting interpretation of the data. The Ageing Male Symptoms Scale (AMS), has also been used to evaluate the symptoms of relative hypogonadism in ageing men with comparable performance to the ADAM questionnaire [354, 355].

The aim of the present study was to assess the effect of 12 months of treatment with oral TU on symptoms of testosterone deficiency, in elderly men with 2 or more symptoms on the St Louis University ADAM questionnaire, and a testosterone level in the low-normal range relative to young men.

MATERIALS & METHODS

PARTICIPANT SELECTION

Seventy-six healthy men aged 60 years or older (68.5 ± 6 years, range 60-86) were recruited by community advertisement. Men were included if they had at least two symptoms on the St Louis University ADAM questionnaire [76], an FAI between 0.3 and 0.5 (based on a single value obtained while fasting between 08.00 and 10.00hrs) and total T greater than 8 nmol/L. These cut-offs were established in a series of preliminary studies as described previously in Chapter 6.1. A second value was obtained at baseline and we have reported the mean of these two values. Exclusion criteria were described in Chapter 6.1. and briefly included: a history or presence of prostate cancer or a PSA > 5 ng/ml a score of greater than 20 on the International Prostate Symptom Score (IPSS), an abnormal prostate on digital rectal examination, history of testicular, liver or renal disease, diabetes mellitus, cardiac failure, a score of greater than 15 on the Geriatric Depression Scale, significant joint pain, prior use of androgen, bisphosphonates, oral, intravenous or intraarticular glucocorticoid within the preceding six months or an haematocrit greater than 50%. The baseline characteristics of these subjects were described in Chapter 6.1 (Table 6.1.1).

The Research Ethics Committee of the Royal Adelaide Hospital approved the study. Informed consent was obtained from all subjects. The study was performed according to International Conference on Harmonisation (ICH)/Good Clinical Research Practice (GCRP).

STUDY DESIGN:

Subjects were treated for 12 months with either TU (Andriol®, Organon, Oss, The Netherlands) 80 mg orally, twice daily, or identical placebo, in a randomized, double blind manner. The testosterone or placebo was taken prior to the onset of breakfast and dinner. Assessments were performed at baseline, 1, 3, 6 and 12 months. The dose of testosterone was halved if the haematocrit increased above 50%.

Randomization was done using a block design of 4 in the Almedica Drug Labeling System (Version 5). The drug was well tolerated; treatment compliance and adverse event details were reported in Chapter 6.1

MEASUREMENTS

St Louis University ADAM Questionnaire

Androgen deficiency symptoms were assessed using the St Louis University ADAM questionnaire [76] at screening, 6 and 12-months.

Laboratory Assays

Assay protocols for this study are described in Chapter 2.0 and further in Chapter 6.1.

Calculated bioavailable testosterone (cBT)

Bioavailable testosterone was calculated from total T and SHBG concentrations as described in Chapter 3.0. The correlation between cBT values and BT values obtained by the ammonium sulphate precipitation method was 0.96 in 143 healthy male blood donors aged 19 – 65 years and 0.79 in 131 clinic patients aged 60 - 88 years, self-selected for symptoms of androgen deficiency (Chapter 3.0).

STATISTICAL ANALYSES

Data are reported as mean ± SE except where otherwise specified. Analyses for the primary outcome measures (St Louis University ADAM symptoms) were performed using an intent-to-treat approach. Where patients had discontinued, their last observations were carried forward in analyses of subsequent time-points to prevent bias due to differential drop out. All other analyses were performed for all subjects treated. The mean change over time between the treatment and placebo groups for total St Louis University ADAM score was compared using a two-tailed independent sample t -test. The effect of treatment on individual symptoms was analysed using Chi-squared tests. Within the Andriol treated group, the effect of low (<3.1 nmol/L) or normal (≥ 3.1 nmol/L) baseline cBT levels on each individual symptom was also analysed using Chi-squared tests. P < 0.05 was considered significant.

RESULTS

BASELINE

There were no differences in total T or cBT between those answering positively and those answering negatively to particular symptoms on the ADAM questionnaire with the exception, those who were sad and/or grumpy had slightly higher cBT (P = 0.052) and those falling asleep after dinner had lower total T (P = 0.006) (Table 6.2.1). Total ADAM scores were slightly, but not significantly higher in men with cBT < 3.1 nmol/L at baseline (Table 6.2.2).

The frequency of positive ADAM symptoms was similar between the testosterone and placebo groups, with the exception that decreased libido was greater in the placebo group (P = 0.009) and lack of energy tended to be more common in the testosterone group (Table 6.2.1).

MONTH-12

There were similar frequencies of positively reported symptoms between the two groups, but decreased libido was more frequently reported in the placebo group (P = 0.034) (Table 6.2.1). When chi-squared analysis was employed on data transformed to show change from baseline to month-12 (ie: symptom improved, worsened or remained unchanged) there were no significant differences in the change from baseline in any of the ADAM symptoms between the testosterone and placebo groups. However, there were trends towards fewer reports of sadness/grumpiness (Chi-2 = 5.54, P = 0.063) and fewer complaints of decreased strength of penile erection (Chi-2 = 5.67, P = 0.059) in the testosterone compared to the placebo group. However, changes over time occurred in both groups. In the placebo treated group there were fewer reports of decreased libido (97% at baseline down to 83% at month-12,

P = 0.047), lack of energy (65% down to 37%, P = 0.021) and decreased strength/endurance (86% down to 53%, P = 0.0023) at the end of the 12 months compared to baseline (Table 6.2.1).

In a *post-hoc* analysis of subjects with a cBT < 3.1 nmol/L at baseline, there was a trend toward better performance at work (P = 0.077) and improved penile erection strength (P = 0.092) with testosterone treatment (Table 6.2.1).

St Louis University ADAM Question	Yes		No		Testosterone		Placebo		Testosterone		Placebo	
	Baseline				Percentage (%) of subjects responding "yes"							
	Total T	cBT	Total T	cBT	BL	M12	BL	M12	BL	M12	BL	M12
1. Do you have a decrease in libido (sex drive)?	16.3	2.98	15.7	3.08	77‡	59§	97	83	79	50	95	81
2. Do you have a lack of energy?	15.9	3	17.2	3	82	54	65	37	84	67	64	31
3. Do you have a decrease in strength and/or endurance?	16.2	2.97	16.8	3.2	90	51	86	53	95	61	82	50
4. Have you lost height?	17.1	3.09	15.8	2.96	11	27	35	17	33	39	32	13
5. Have you noticed a decreased "enjoyment of life"?	15.6	3	16.6	3.02	28	22	35	20	37	22	45	25
6. Are you sad and/or grumpy?	15.1	3.28	16.6	2.92*	26	11	19	23	28	11	18	25
7. Are your erections less strong?	16.3	2.99	14.6	3.19	95	86	95	93	100	89	95	94
8. Have you noted a recent deterioration in your ability to play sports?	15.6	2.93	16.9	3.08	49	48	49	40	50	35	59	38
9. Are you falling asleep after dinner?	14.8	3.1	17.4†	2.91	38	46	49	37	37	44	45	25
10. Has there been a recent deterioration in your work performance?	15.8	2.86	16.5	3.08	44	24	35	20	53	17	36	19

Table 6.2.1 Mean total T and cBT levels for "yes" and "no" responses to symptoms at baseline and the percentages of positively reported ADAM symptoms at baseline (BL) and month 12 (M12), in the testosterone and placebo groups for all subjects and for those with baseline cBT < 3.1 nmol/L.

Data presented are mean hormone levels and percentages of those who responded "yes" to the ADAM question. At baseline, decreased libido was less in the testosterone (30/39) than in the placebo (36/37) group (chi2 = 6.9, P = 0.009). There was a trend towards a difference between the testosterone (32/39) and placebo (24/37) groups for lack of energy (chi2 = 2.9, P = 0.089). All other individual symptoms of the ADAM were reported with similar proportions. At month-12, decreased libido was less in the testosterone (22/37) than in the placebo (25/30) group (chi2 = 4.51, P = 0.034), but the proportion of subjects experiencing ADAM symptoms 2 to 10, were similar. In subjects with cBT < 3.1 nmol/L at baseline, there were trends towards reduction in the number reporting decreased performance at work (P = 0.077) and decreased penile erection strength (P = 0.092) with testosterone treatment at month 12. *P = 0.052, §P = 0.034, ‡P = 0.009, †P = 0.006.

	Mean total ADAM score	
Baseline cBT	< 3.1 nmol/L	≥ 3.1 nmol/L
All subjects	5.8	5.43
Testosterone group	5.89	5.35
Placebo group	5.73	5.53

Table 6.2.2 Comparison of mean total ADAM scores in all subjects and in testosterone and placebo groups, by baseline cBT.

There were no differences in total ADAM score within or between the two groups at baseline.

DISCUSSION

This study shows that the overall symptom complex as defined by the St Louis University ADAM questionnaire was not greatly improved by 12-months of oral TU treatment in older men with low-normal testosterone levels. Some changes in individual symptoms occurred in both placebo and testosterone treated subjects.

The present study enrolled subjects who had at least 2 positive symptoms on the ADAM questionnaire at screening rather than the criteria set by Morley et al. (2000). Morley at al. (2000) observed that 18 out of 21 patients experienced improvement in ADAM symptoms after 3 to 4 months of testosterone treatment (TC, 200mg i.m.) [76]. While these men had similar testosterone levels and similar total ADAM scores (5.8 ± 0.5) to the men in the present study, they were younger (57.5 ± 1.6 years), a much higher dose of testosterone was used and this was an uncontrolled, single-group, observational study. In a group of men with much lower total T levels (mean 7.07 nmol/L) Ghanem et al. (2001) showed improvement in similar symptoms in 71 out of 86 men aged 50 - 70 years [353] but once again this was an uncontrolled observational study. Improvements in scores obtained on the Ageing Male Symptoms Scale (AMS), which has been used to evaluate the symptoms of relative hypogonadism in ageing men have also been noted in uncontrolled studies [354, 355]. Similarly, the study of Park et al. (2003) that reported improved St Louis University ADAM scores after 3 months of oral TU treatment (160 mg/day) in 10 men with primary hypogonadism and 29 older men with relative androgen deficiency [136] was single-blind as opposed to the present study's double-blind, placebo controlled design.

The differences at month-12 in sadness/grumpiness and strength of penile erections between the testosterone and placebo treated groups in the present study, was primarily due to the increased incidence of the symptoms in the placebo group after 12 months, rather than a decreased prevalence due to improvement in the testosterone group. There was, however, a trend to an absolute improvement in erectile strength and work performance, as determined by a change in response to the questions relating to erectile function and work performance on the ADAM questionnaire, in testosterone treated men with a baseline cBT < 3.1 nmol/L. There was, however, no improvement in any other symptom on the questionnaire with testosterone as opposed to placebo treatment regardless of the baseline testosterone level.

Aversa et al. (2000) showed that in men with erectile dysfunction, low free testosterone levels, independent of age, correlated with impaired relaxation of cavernous endothelial and corporeal smooth muscle cells [283]. Moreover, a follow-up study in men with arteriogenic erectile dysfunction, testosterone levels in the lower quartile of the normal range, and who were non-responsive to sildenafil treatment after six attempts, demonstrated that one month of transdermal testosterone supplementation improved erectile response to sildenafil [139]. Tariq, et al. (2003) also reported reversal of sildenafil failure following testosterone therapy [356]. A number of other studies have suggested enhanced strength and/or maintenance of erections following testosterone or dihydrotestosterone replacement [147, 161, 357]. It is therefore plausible that exposure to testosterone supplementation over the 12-month period may have protected against both decreased cavernosal blood flow, and impaired relaxation of cavernosal endothelial and corporeal smooth muscle, resulting in maintenance of erectile strength. Furthermore a meta-analysis of the usefulness of androgen replacement for erectile dysfunction, showed that testosterone treated patients improve significantly more than placebo treated patients and that patients with primary testicular failure respond better to treatment than those with secondary testicular failure. Moreover, transdermal therapy is more effective than oral or intramuscular therapy [358].

Novak et al. (2002) reported that both patients and physicians considered that decreased energy levels and impaired sexual performance had the greatest adverse effect on well-being and quality of life. Moreover, patients generally felt that testosterone replacement led to improvement in energy level in addition to improvements in libido and erectile function, albeit to a lesser degree [359]. Unlike other studies [2, 130, 147, 161, 360], the present study did not observe enhanced libido with testosterone replacement in older males. Whether the improvement in feelings of sadness and grumpiness in the testosterone treated subjects is directly related to improvements in erectile function or an independent effect of testosterone cannot be determined from these data. Testosterone supplementation has been reported to decrease anger, irritability, sadness, tiredness and nervousness and to increase energy level, friendliness and sense of well-being in younger hypogonadal men after six months of various modes and doses of testosterone supplementation [121]. The present study did not show any benefit on energy levels, strength and endurance, enjoyment of life or ability to play sports, although a small but non-significant improvement in performance at work was observed.

It is important to note that over the treatment period there was also a trend for some of the responses on the questionnaire to improve on placebo alone, highlighting the importance of appropriate placebo controlled trials in this field. It also suggests that many purported relative androgen deficiency symptoms may be driven by psychosocial factors rather than by low plasma testosterone. There is potential for depression to lead to positive answers on the ADAM questionnaire; particularly for lack of energy, decreased enjoyment of life and sad and/or grumpy items. Tan et al. (2004) report the confounding nature of depressive symptoms and concurrent medical conditions in determining whether low androgen levels are responsible for symptoms such as loss of libido, erectile dysfunction and fatigue, in a primary care setting [361]. Mood disorders may be a major reason for the low specificity of the ADAM questionnaire. The confounding effect

of depression in the present study is likely to be minimal as depression, as defined by a GDS > 15 was a criteria for exclusion.

The methods used to measure plasma testosterone and the cut-offs that are used to identify low testosterone levels differ widely and potentially influence the outcomes of replacement studies. More sophisticated methods such as the measurement of free testosterone by equilibrium dialysis are not in routine clinical use in most laboratories.

In general, oral testosterone had no significant effect on symptoms of hypogonadism as evaluated by the St Louis University ADAM questionnaire in this group of men with borderline low testosterone levels. The data do indicate associations of erectile strength and some aspects of mood as assessed by the ADAM questionnaire, with serum testosterone levels and response to testosterone supplementation in men with the cBT < 3.1 nmol/L. Nevertheless, taken together, these data do not support the use of the ADAM questionnaire in the clinical evaluation of any particular testosterone level in ageing men and moreover, do not support a positive ADAM questionnaire as an indication for testosterone replacement therapy in older Australian men with low-normal testosterone levels.

6.3 THE EFFECT OF 12-MONTH ORAL TESTOSTERONE UNDECANOATE (ANDRIOL®) ON VISUOSPATIAL COGNITION, MOOD, WELLBEING QUALITY OF LIFE IN MEN AGED 60 YEARS AND OLDER WITH LOW-NORMAL GONADAL STATUS.

SUMMARY

The effect of supplemental testosterone on visuospatial cognition, mood and wellbeing in ageing men is unclear. This study aimed to assess the effect of 12-months of oral testosterone supplementation on visuospatial ability, mood and quality of life in elderly men with low-normal gonadal status, not specifically selected for cognitive or mood defects. A standard oral dose (80mg twice daily) of testosterone undecanoate (TU) or placebo was administered for one year to 76 healthy men 60 years or older. All men had a free androgen index (FAI) of 0.3 – 0.5, which represents a value below the normal lower limit for young men (19-29 years), but remains within the overall normal male range. A neuropsychological assessment including the trail making test - part B (TMT-B), visuospatial (VSP) block design test, mini mental state exam (MMSE), Geriatric Depression Scale (GDS), a 5-point Likert and a 10-point visual analogue quality of life (QoL) scale, along with serum hormone measurements were obtained at baseline, 6, and 12 months. Testosterone treatment compared to placebo, resulted in improved scores on the GDS and Likert wellbeing scale after 12-months of treatment. There were no effects of treatment on trail making or VSP block tests, MMSE, or VAS QoL scale scores. There was no relationship between baseline cBT or FAI and treatment effect for any of the outcome measures. In conclusion, men with relatively low testosterone levels, symptoms of androgen deficiency and free of clinically defined depression, show measurable improvements in mood and well-being but not in visuospatial cognition or quality of life after 12 months of standard dose TU treatment.

INTRODUCTION

As previously state in Chapters 1.0-6.2, plasma total testosterone (total T) levels decline progressively over the lifespan [35, 47, 54, 67, 362] and this occurs to a greater extent in some men than others. A concomitant increase in plasma sex hormone binding globulin (SHBG) concentration with increasing age results in an even greater decline in the plasma free and bioavailable fractions of testosterone [14, 327]. The effect of this decline on cognitive function, mood, and quality of life, in ageing men is presently unclear. This issue is of importance since testosterone is considered by many to be of benefit in improving mood and health-related quality of life[136, 359, 363].

The domain of cognitive function that seems to be most closely related to circulating testosterone levels is visuospatial function. In addition to effects on processing, androgens have a permanent organising effect on some cognitive abilities. They may be important in a developmental sense, but have little role in maintenance as men get older. This is evidenced through a study by Moffat & Hampson (1996) where men with the idiopathic form of hypogonadism showed markedly impaired spatial ability that did not improve with testosterone therapy, whereas visuospatial ability in men with acquired hypogonadism is similar to controls [86]. The relationship between both total and bioavailable testosterone (BT) and visuospatial ability seems to be quadratic (U-shaped); men with markedly high or low serum levels of testosterone have been shown to have impairment in visuospatial ability [84, 85].

In response to testosterone supplementation, visuospatial ability has been shown to improve in older men [85, 141]. Kenny et al. (2002) showed that there was no difference in the change in scores on a number of tests of cognitive function (digit symbol, digit span or trail making tests (TMT's)) after 12 months, in 67 men aged 65 – 87 years, with BT levels "below the lower limit of the normal adult range" (128 ng/dL ≈ 4.44 nmol/L), randomised to receive testosterone or placebo transdermal patches [302]. In older hypogonadal men (BT < 60 ng/dL ≈ 2.08 nmol/L) Sih et al. (1997) found no change in memory, recall, or verbal fluency tests in 15 men randomised to placebo and 17 men to 200 mg testosterone

cypionate bi-weekly for 12 months [3]. In contrast, Janowsky et al. (1994) reported improved performance on the visuospatial block design test in 56 healthy men aged 60-75 years of age treated with testosterone, sufficient to increase levels by 150% in comparison to placebo treated men [85] and Cherrier et al. (2001) showed improvement in both spatial (block design) and verbal memory in 25 healthy community-dewelling men randomised to placebo or 100 mg of testosterone enanthate for six weeks [141].

Hypogonadal men have been shown to be more depressed, angered, fatigued and confused than infertile, treated eugonadal or normal men [82] and positive relationships between androgen levels and mood and wellbeing have been reported [50, 83]. It has been suggested that the age-related decline in testosterone levels in men may be involved in decrease mood and feelings of wellbeing as well as muscle strength and sexual function, and consequently overall quality of life. In a cross-sectional study of 856 community-dwelling men aged 50-89 years, Barrett-Conner et al. (1999) reported an inverse relationship between BT level and score on the Beck Depression Inventory that was independent of age, weight change and physical activity; BT was 17% lower in categorically defined depressives than in normal healthy men [50]. Rabijewski et al. (1998) found increased positive and decreased negative mood variables after testosterone administration [161]. Seidman et al. (2001) found no effect of testosterone in patients with major depression [129]. The cause and effect relationships and the response to testosterone supplementation of mood and wellbeing remain unclear.

The aim of this study was to determine the effect of oral TU on visuospatial test scores, mood and quality of life scores in healthy men over the age of 60, with total T greater than 8 nmol/L and FAI above the defined lower limit of normal (0.3), and below 0.5, therefore low relative to young men.

MATERIALS AND METHODS

PARTICIPANT SELECTION

As described previously in Chapters 6.1 & 6.2, seventy-six healthy men aged over 60 years (68.5 ± 6 years, range 60-86) were recruited by community advertisement. Men were included if they had at least two symptoms on the St Louis University Androgen Deficiency in the Aging Male (ADAM) questionnaire [76], an FAI between 0.3 and 0.5 (based a single value obtained while fasting between 08.00 and 10.00hrs) and total T greater than 8 nmol/L. A second value was obtained at baseline and the mean of these two values is reported. Exclusion criteria were described in Chapters 6.1 & 6.2. Briefly, subjects were excluded if they had a history or presence of prostate cancer or a prostate specific antigen (PSA) > 5 ng/ml, International Prostate Symptom Score (IPSS) > 20,[364], abnormal prostate on digital rectal examination, history of testicular, liver or renal disease, diabetes mellitus, cardiac failure, severe dysphoria (Geriatric Depression Scale (GDS) > 15 [83]), joint pain that limited their ability to perform muscle strength testing, prior androgen, bisphosphonate, or glucocorticoid treatment (within the preceding six months), or an haematocrit greater than 50%.

The Research Ethics Committee of the Royal Adelaide Hospital approved the study. Informed consent was obtained from all subjects. The study was performed according to International Conference on Harmonisation (ICH)/Good Clinical Practice.

STUDY DESIGN:

Subjects were treated for 12 months with either testosterone undecanoate (TU) (Andriol[R], Organon, Oss, The Netherlands) 80 mg orally, twice daily, or identical placebo, in a randomized, double-blind manner. The testosterone or placebo capsules were taken with breakfast and dinner. The dose of

testosterone was halved if the haematocrit increased above 50%. Androgens, liver function tests, PSA, haemoglobin and haematocrit and the IPSS were collected at 0 (baseline), 1, 3, 6 and 12 months. Visuospatial tests, the Mini Mental State Examination (MMSE [365]), the GDS and quality of life scales were measured at 0, 6 and 12 months. As stated in Chapter 6.1 androgen levels at month-1 were taken 4-hours post-dose of TU, taken together with breakfast (non-fasting) for pharmicokinetic analysis.

Randomization was done using a block design of 4 and the randomization was performed in the Almedica Drug Labeling System (version 5). No person involved in the execution or monitoring of the study had access to the randomization list, other than in emergencies through the Emergency Drug Identification Record that identified the treatment code for each individual subject.

MEASUREMENTS

Trail making test part B (TMT- B)

The TMT-B, a test of visuomotor tracking and attention [366, 367], was conducted as described in Chapter 2.0. Lower scores on the TMT-B indicate better performance and better visuomotor tracking and attention ability. Results on this test can be confounded by the examiners reaction time in noticing mistakes and the time taken by the participant to comprehend and then correct their mistake. In order to minimise these biases the same examiner was used throughout the trial.

Visuospatial (VSP) block design test

The VSP block test is a test of visuospatial ability from the Wechsler Adult Intelligence Scale [368, 369]. It involves the construction of patterns using blocks with two red sides, two white sides and two red/white sides. The test consists of nine trials. In trials 1 and 2, subjects were presented with four blocks; two red sides, one white side and one red/white side were facing up. Subjects were asked to copy a 4-block design created by the investigator. In trials 3-5, subjects were presented with four blocks

and the same up-facing sides as in the previous two trials and instructed to copy block designs drawn on cards. Trials 6-9 began with 9 blocks: three red, three white and three red/white sides facing up and subjects were asked to reconstruct 9-block designs from cards. Timing commenced following the instruction to begin. If the subject constructed an incorrect design or failed to complete the design within the cut-off time (60 seconds for trials 1-5 and 120 seconds for trials 6-9), the trial was recorded as a failure. Each subject was given two attempts to successfully complete a particular design; if they failed on the second attempt the test was terminated. Time taken for each trial was recorded and summed at the end to give a total VSP block score in seconds. Lower VSP block scores indicate better performance and better visuospatial ability.

Mini Mental State Exam (MMSE)

The MMSE is a tool used to detect cognitive deficits seen in syndromes of dementia and delirium and for measuring these cognitive changes over time [370]. A score of 23 or below is 87% sensitive and 82% specific in detecting dementia and delirium when a psychiatrist's standardized clinical diagnosis is used as the criterion [365].

Geriatric Depression Scale (GDS)

Mood was assessed using the 30-item, Yesavage Geriatric Depression Scale (GDS) that detects the presence of negative mood or dysphoria [83].

Quality of Life (QoL) and well-being

A five-point Likert scale was used to categorise the well-being of subjects. They were asked the following question: "If you had to characterise your life at this time, would you say you were?" They were required to respond as either content, anxious, happy, depressed or worried. A ten-point visual analogue scale (VAS) was also used to rate quality of life (QoL). Subjects were asked "on a scale of 1 to 10, with 10 being the best, how would you rate your overall quality of life?" They were then required

to mark the point along the line corresponding to level of quality of life they believed they experienced, where 0 = poorest and 10 = highest quality of life.

Laboratory Assays

The laboratory assays were previously described in Chapters 6.1 & 6.2.

Calculated bioavailable testosterone (cBT)

Bioavailable testosterone was calculated from total T and SHBG concentrations as described in Chapter 3.0.

STATISTICAL ANALYSES:

Data are reported as mean $\pm$ SE unless otherwise specified. Analyses for the primary outcome measures (TMT-B, VSP blocks, MMSE, GDS and QoL scales) were performed using an intent-to-treat approach. Data on all patients randomized were analyzed. Where patients had discontinued, their last observations were carried forward in analyses of subsequent time-points to prevent bias due to differential drop out (last-observation-carried-forward-approach). All other analyses were performed for all subjects treated. The change over time and the effect of treatment were assessed using a mixed plot analysis of variance (ANOVA). $P < 0.05$ was considered significant. A *post-hoc* comparison of means between subjects with cBT above and below 3.1 nmo/L at baseline was performed for all variables at month-12 using student's t-test. Linear regression analysis was also performed on month-12 data and on the change in androgen levels from baseline to month-12 and the change in outcome variables over the same period.

RESULTS

Of the 76 men enrolled in the study, 39 were treated with testosterone and 37 with placebo. Baseline characteristics of the two groups were described in Chapter 6.1 (Table 6.1.1). Further, baseline visuospatial cognition tests, mood and quality of life scores are described, by treatment group, in Table 6.3.1. At baseline, there were no differences between the treatment and placebo groups for hormone levels, TMT-B, VSP block test, MMSE, GDS or QoL scales.

TREATMENT COMPLIANCE AND ADVERSE EVENTS

Treatment compliance, adverse events, safety data and early study withdrawal details were reported in Chapter 6.1. Briefly, there was no statistically significant difference in the change in the IPSS, PSA, LDL cholesterol, triglycerides, systolic or diastolic blood pressure between testosterone and placebo groups from baseline to months 6 or 12. At month-12, plasma HDL levels were lower and haematocrit was higher in the testosterone as compared to the placebo group ($P = 0.03$ and $P = 0.026$ respectively).

	Testosterone (n=39)	Placebo (n=37)	P
GDS (0-30)	6.28 ± 3.8	5.7 ± 4.4	NS
TMT-B (sec)	164.28 ± 78.42	152.56 ± 62.98	NS
VSP Block test (sec)	431.1 ± 107.83	416.89 ± 104.67	NS
MMSE (0-30)	29.15 ± 0.99	29.16 ± 1.21	NS
Likert well-being (1-5)	1.46 ± 0.94	1.94 ± 1.22	NS
QoL (VAS) (0-10)	7.79 ± 1.34	7.42 ± 1.18	NS

Table 6.3.1 Baseline visuospatial cognition, mood and quality of life data of all subjects randomised to the Testosterone and Placebo treatment groups.

Data are expressed as mean ± SD. No differences were observed between the two groups at baseline. GDS = Geriatric Depression Scale, TMT-B = Trail-Making Test part B, VSP = Visuospatial (block test), MMSE = Mini Mental State Examination, QoL = Quality of Life, VAS = Visual Analogue Scale and NS = Non-Significant.

SERUM ANDROGEN LEVELS

The effect of 12-month oral TU treatment on serum testosterone, SHBG, LH and FSH levels were reported on Chapter 6.1.

VISUOSPATIAL TESTS, MOOD, WELL-BEING AND QOL SCALES

The changes from baseline to month-6 and month-12 on TMT-B, VSP blocks, MMSE, GDS, Likert well-being scale and QoL VAS are shown in Table 6.3.2. There was a significant effect of time on VSP block scores ($F_{(2, 204)} = 3.19$, $P = 0.04$) and significant effects of treatment on depression scores and Likert well-being ratings ($F_{(1, 206)} = 4.54$, $P = 0.03$ & $F_{(1, 208)} = 6.04$, $P = 0.01$ respectively). In addition, at month-6, one subject in the testosterone group and none in the placebo group experienced clinically relevant depression, defined as a score of >15 on the GDS. At month-12, no clinically relevant depression was observed.

POST-HOC ANALYSES

In a post hoc analysis, subjects were divided into two groups according to cBT levels at baseline (low: <3.1 nmo/L and normal: ≥ 3.1 nmol/L). There were no significant differences between men with low baseline cBT and those with normal baseline cBT for any of the cognitive, mood or QoL measures within the testosterone or placebo treated groups at month-6 or month-12 (Table 6.3.3). There was also no relationship between baseline cBT or FAI and response to treatment for any of the measures (cognitive test scores, mood or QoL ratings) at either month-6 or month-12.

From regression analyses on month-12 data, there was a trend toward an inverse linear relationship between TMT-B scores and serum total T ($R^2 = 0.06$, $P = 0.051$) and a significant positive relationship with SHBG ($R^2 = 0.1$, $P = 0.009$) but not cBT ($R^2 = 0.001$, $P = 0.8$) or FAI ($R^2 = 0.0002$, $P = 0.91$). VSP

block scores were not related to total T ($R^2 = 0.0003$, P = 0.89), cBT ($R^2 = 0.003$, P = 0.68) or FAI ($R^2 = 0.005$, P = 0.6), but a trend towards an inverse relationship with SHBG ($R^2 = 0.056$, P = 0.058) was evident. Scores on the MMSE, Likert scale, VAS and GDS were not related to the level of any of the hormones measured.

The magnitude of change in total T levels from baseline to month 12 was positively associated with change in Likert QoL rating (R2 = 0.07, P = 0.03). No other hormonal changes were associated with change in any of the outcomes over the 12-month treatment period.

| | Testosterone | Placebo | Absolute change from baseline | | | | P | P |
| | | | Testosterone | | Placebo | | | |
	Baseline		Month-6	Month-12	Month-6	Month-12	Treatment	Time
TMT-B (time, sec)	164.28	152.56	-28.2	-18.4	-13.9	-27.1	0.20	0.16
VSP blocks (time, sec)	431.10	416.89	54.3	16.8	40.3	7.1	0.14	0.04*
MMSE (0-30)	29.15	29.16	-0.06	-0.4	-0.13	-0.27	0.39	0.36
GDS (0-30)	6.28	5.70	-0.54	-0.95	-1.69	-1.27	0.01*	0.1
Likert well-being (1-5)	1.46	1.94	-0.19	0.19	0.15	-0.07	0.03*	0.59
VAS QoL (0-10)	7.79	7.42	-0.19	-0.16	0.29	0.14	0.78	0.81

Table 6.3.2 Mean change from baseline to month-6 and month-12 for visuospatial tests, mood, well-being and quality of life scales in Testosterone and Placebo groups.

TMT-B = Trail-Making Test part B, VSP = Visuospatial (block test), MMSE = Mini Mental State Examination, GDS = Geriatric Depression Scale, QoL = Quality of Life, VAS = Visual Analogue Scale. *P < 0.05.

	Baseline				Month 6				Month 12			
	Testosterone		Placebo		Testosterone		Placebo		Testosterone		Placebo	
Baseline cBT level	< 3.1	> 3.1	< 3.1	> 3.1	< 3.1	> 3.1	< 3.1	> 3.1	< 3.1	> 3.1	< 3.1	> 3.1
TMT-B	157.9 (88.3)	170.4 (69.5)	155.5 (75.9)	148.0 (36.7)	135.6 (14.7)	144.2 (21.2)	149.0 (20.6)	120.8 (6.1)	157.3 (28.3)	141.9 (18.9)	137.4 (16.7)	107.8 (8.2)
VSP block test	417.5 (95.3)	444.0 (119.6)	401.0 (117.0)	441.8 (79.3)	474.7 (32.1)	493.9 (23.7)	454.1 (30.4)	452.5 (22.5)	432.1 (25.0)	461.3 (24.2)	425.9 (37.4)	419.7 (21.3)
MMSE	29.3 (0.67)	29.0 (1.21)	29.1 (1.32)	29.2 (1.08)	28.9 (0.29)	29.3 (0.32)	29.1 (0.23)	29.4 (0.20)	28.8 (0.25)	28.7 (0.31)	29.2 (0.21)	29 (0.43)
GDS	5.74 (3.68)	6.80 (3.85)	6.77 (4.79)	4.13 (3.40)	6.33 (0.9)	5.37 (0.76)	3.7 (0.82)	3.85 (0.82)	4.67 (0.78)	6.16 (0.96)	4.38 (1.11)	3.79 (0.87)
Likert QoL	1.42 (1.07)	1.50 (0.83)	2.18 (1.37)	1.57 (0.85)	1.28 (0.16)	1.32 (0.17)	1.5 (0.22)	2.17 (0.27)	1.56 (0.20)	1.74 (0.26)	1.56 (0.26)	1.50 (0.23)
VAS QoL	7.49 (1.21)	8.07 (1.42)	7.22 (1.15)	7.72 (1.20)	7.52 (0.31)	7.80 (0.33)	7.55 (0.37)	8.24 (0.28)	7.47 (0.32)	7.86 (0.28)	7.35 (0.38)	8.21 (0.24)

Table 6.3.3 Visuospatial cognition, mood and quality of life scores, by baseline cBT, in Testosterone and Placebo groups at baseline, month-6 and month-12.

Values presented are means (± S.E.). There were no significant differences within treatment groups (Testosterone / Placebo) between low (<3.1 nmol/L) or normal (≥ 3.1 nmol/L) baseline cBT levels for any of the outcome measures. cBT = calculated Bioavailable Testosterone, TMT-B = Trail-Making Test part B, VSP = Visuospatial (block test), MMSE = Mini Mental State Examination, GDS = Geriatric Depression Scale, QoL = Quality of Life, VAS = Visual Analogue Scale.

DISCUSSION

A number of population-based cohort and cross-sectional studies have reported relationships between endogenous serum testosterone levels and scores on cognitive tests of visuospatial ability and memory, as well as mood inventories and quality of life scales. In a cross-sectional analysis of 547 community-dwelling men aged 59-89 years, Barrett-Conner et al. (1999) reported better long-term memory in men with higher levels of BT [84]. In a similar study design, Yaffe et al. (2002) reported better scores on the MMSE, TMT-B and digit symbol test in men with BT levels in the highest tertile [301]. In a longitudinal analysis of 407 men aged 50-91 years at baseline and followed for an average of 10 years, higher FAI was associated with better scores on visual and verbal memory tests, visuospatial functioning and visuomotor scanning and a reduced rate of longitudinal decline in visual memory. No associations, however, were reported between either total T or FAI and measures of verbal knowledge, mental status or depressive symptoms [300].

In the present study, there was no improvement in visuospatial ability in older men with low-normal testosterone levels after a conventional dose of oral TU for 12 months. Kenny et al. (2002) showed that men receiving either transdermal testosterone or a placebo patch had improved scores on the TMT-B test. Although the scores after 12-months of treatment were correlated with 12-month testosterone levels (P = 0.016), there was no significant difference in scores on the TMT-B test between the two groups at any time point during the study [302]. Similarly, in the present study there was a significant correlation between the TMT-B test scores and total T levels at month-12, but no difference in the change in TMT-B test scores between testosterone and placebo groups. The men studied by Kenny et al (2002), like the men in the present study, were selected purely on the basis of their androgen status and not on the basis of visuospatial ability nor on the basis of their mood or general well being. Men with

severe dysphoria or hypogonadism were excluded from the present study. Therefore it remains possible that men selected for either relative or absolute hypogonadism with one or more cognitive abnormalities or a mood disorder may derive benefit from androgen supplementation. In contrast to our findings, Janowsky et al. (1994) showed that in men of a similar age to those in the present study and without pre-existing cognitive abnormalities, 3 months of testosterone supplementation using a scrotal patch that delivered 15 mg/day of testosterone improved spatial cognition (Block Design test). This effect was however not related to a change in testosterone, but rather to a change in oestradiol levels. Furthermore, cognitive flexibility (TMT-B test), motor speed (Grooved Pegboard test) and mood, remained unchanged after 3-months of treatment [85]. In the present study, performances on the VSP block and TMT-B tests were not related to oestradiol levels at month-12 (data no shown). It is possible that a greater level of biotransformation of testosterone to oestradiol occurred in Janowsky's study than in our study, but it does not explain the discrepancy with the study of Kenny et al (2002), where a similar biotransformation to oestradiol would have been anticipated. Moreover, in the negative study by Sih et al. (1997) the high intramuscular dose of testosterone would be expected to result in a significantly higher degree of aromatization to oestradiol [3].

Supplemental testosterone has been shown to have positive effects on mood in hypogonadal men when hormone levels are well below the normal male range, but has no effect when levels are within or above this range [371]. In the present study, there was a significant effect of treatment on sub-clinical depression scores and well-being ratings. Wang et al. (1996) demonstrated positive correlations between testosterone levels (area-under-the-curve) and friendliness and sense of well-being and inverse correlations with nervousness, irritability and tiredness at baseline [113]. Additionally, Wang and co-workers showed that testosterone replacement (TE 200 mg i.m. once every 20 days, or sublingual T (SLT) 2.5 mg tid, or SLT 5 mg tid) led to a decrease in anger, tiredness, sadness, irritability and nervousness and a significant improvement in energy level, friendliness and sense of well-being, in hypogonadal older men [113]. Similarly, high dose (240 mg/day) methyltestosterone administration to

healthy normal males with no psychiatric history increased scores on positive mood ratings of euphoria, energy and sexual arousal, however it also increased negative parameters of irritability, mood swings, violent feelings and hostility and increased cognitive impairment in the domains of distractibility, forgetfulness and confusion [372]. A low dose regimen (40 mg/day) of the same drug did not produce the same dramatic mood and cognitive changes, but still resulted in a significant increase in sexual arousal and depression score on the Hamilton Depression Rating Scale [372].

The present study did not demonstrate significant change in a crude measure of mental health (MMSE) or in quality of life scores. Although all subjects had symptoms suggestive of androgen deficiency based on the ADAM [76], no men had clinically defined dementia, delirium or depression and MMSE cores were very high; thus there was little potential for positive change. Unlike the GDS, the MMSE may not have been sensitive enough to detect change in mental state in these highly screened men. Thus this trial, as a function of its design may be limited in its ability to detect improvement on this mental state examination.

The men in the present study had relative rather than absolute hypogonadism and a relatively low dose of TU was used that did not significantly increase the serum levels of total T. Nevertheless the treatment dose was associated with significant improvements in mood (even in the sub-clinical dysphoric range), wellbeing, biochemical and physical effects (decreased fat mass and increased lean body mass) in the same group, as described in Chapter 6.1. Park et al. (2003) reported an improvement in health related QoL after 3 months of oral TU administration (160 mg/day), the same dose as in the current study, in 39 men (10 with primary hypogonadism and 29 with androgen levels low relative to young men and selected for symptoms that may be attributable to androgen deficiency). This was, however, a single blind study, with fewer men, at least a third of who were unequivocally hypogonadal [136].

It is important to note that early withdrawals in the present study represented 25% of the men enrolled and that half of those who withdrew before completing the 12-month protocol were receiving placebo and half of these again, withdrew due to a lack of perceived benefit.

Taken together, it would appear that men with relatively low testosterone levels, symptoms of androgen deficiency and free of clinically defined depression, show measurable improvements in mood and well-being but not in visuospatial cognition or quality of life after 12 months of standard dose TU treatment.

CHAPTER 7.0 THE DEVELOPMENT OF AN EMPIRICAL MEASURE OF TESTOSTERONE BIOACTION

SUMMARY

There is no well-validated, objective measure of testosterone action by which to gauge the effects of gradually declining serum testosterone levels with age and there are limitations to the current methods for measuring total and tissue-available testosterone levels in human plasma. The aim of this study was to develop an in-vitro transactivation assay of testosterone in human plasma. This chapter reports preliminary, feasability studies of androgen receptor (AR) mediated transcription of a luciferase reporter gene in various cell-lines. Initially, four different cell lines were studied. From initial experiments in the different cell lines, it was concluded that the degree of testosterone/DHT stimulated luciferase induction was not only dependent on the amount of co-transfected human AR, but also on the type of cell used. This work has determined the optimal number of CHO-K1 cells to seed and the optimal amounts of hAR and ARR3-tk-luc to transfect in order to induce the greatest luciferase gene transcription. Some suppression of induction has also been shown with the anti-androgen flutamide. A very similar and novel assay for the determination of androgen bioactivity in human serum was published during the course of the present studies [373]. The authors reported enhanced sensitivity of the assay by using a co-activating factor, AR-interacting protein 3 (ARIP3). The bioassay of Raivio and colleagues has a level of sensitivity below 0.1 nmo/L. The intra-assay CV was 8.3% at 4.9 nmol/L and the inter-assay CV was 21% [373]. The bioassay system of the present study may have a potential advantage over that of Raivio and colleagues in the co-transfection of full-length human AR as opposed to co-transfection of fragments of rat AR. Taken together, the current data suggest that it is feasible and possible to establish

a cell-culture based bioassay, utilising a transfected androgen responsive promoter, luciferase reporter gene.

INTRODUCTION

In contrast to the acute reduction in oestrogen production in women at the menopause, the age-associated decline in testosterone in men is slow and progressive from around the age of thirty [63, 64] through to old age. Furthermore, in contrast to the menopause where clinically identifiable signs such as the cessation of periods and hot flashes accompany reduced oestrogen levels, the progressive reduction of plasma testosterone in men does not appear to coincide with such obvious and specific clinical manifestations. However, it is widely accepted that apart from their role in the masculinization of male genitalia *in utero* and the development of secondary sex characteristics in boys at puberty, androgens are also important for the maintenance of male sexual function, muscle and bone mass and muscle and cognitive function in adult life.

Hormone replacement therapy for women has been extensively studied and apart from recent concerns about long-term safety, has shown to be of benefit. However, supplemental testosterone for older men is a relatively new area of interest and the benefits and risks remain unclear. The interpretation of currently available placebo controlled trials is confounded by the absence of a globally accepted definition of relative androgen deficiency in the ageing male and the greatest response to treatment seems to occur in men with the lowest baseline testosterone levels as discussed in Chapters 6.1 – 6.3. The absence of an empirical marker of testosterone action limits the ability to define inappropriate testosterone levels more accurately, thus at present statistically derived cut-off's, such as those shown in Chapter 3.0, are commonly used with no physiological basis.

On entering target cells, androgens bind to the androgen receptor (AR), a ligand dependent transcription factor. After binding of the hormone, AR enters the nucleus and binds to the regulatory region of the target gene as a homodimer. AR belongs to the superfamily of nuclear receptors that also

includes receptors for vitamin D_3, thyroid hormones, retinoids and steroid hormones [374]. These receptors have conserved DNA-binding (DBD) and ligand binding domains (LBD) and variable hinge and N-terminal regions. The N-terminal region of AR encompasses the primary transcriptional activation domain. Androgen binding has been shown to cause the LBD and N-terminal regions of AR to interact, facilitating the dimerisation of AR [375, 376], modulating transcriptional activity [377] and stablilising the receptor at low ligand concentrations [378]. SHBG receptors have also been identified on the plasma membranes of some cells (e.g. prostate) and initiate non-genomic signalling pathways subsequent to initial SHBG and secondary testosterone binding [379].

There is no well-validated, objective measure of testosterone action by which to gauge the effects of gradually declining testosterone levels with age and there are limitations to the current methods for measuring total and tissue-available testosterone levels in human plasma. This is of great clinical importance as androgen supplementation in Australia is only available for men over the age of forty with a plasma total T level of 8 nmol/L or lower, or 8-15 nmol/L in the presence of an LH level > 1.5 times the upper limit of the reference range for young men (termed "hypogonadism"). The basis for these criteria appear to be arbitrary and as shown in Chapter 3.0 total T of 8 nmol/L represents the 5th percentile of values obtained in blood donors aged 19-65 years.

The aim of this study was to develop an in-vitro transactivation assay of testosterone in human plasma. It is expected that this assay will provide a sensitive and reliable measure of androgen receptor (AR) mediated transcriptional activity in cells transfected with standard concentrations of human AR (hAR) and androgen response element (ARE). The application of this method will be limited to cases in which normal AR expression and functioning can be assumed.

MATERIALS AND METHODS

CELL LINES AND ANDROGEN GENE REGULATION

A bioassay system such as the one proposed requires a cell line that is easy to culture, has good transfection efficiency and has the transcription factors involved in androgen receptor mediated gene regulation. It was therefore important to establish which types of cells would be best for the purpose of the proposed assay system. Due to immediate availability Human colon adenocarcinoma (CaCo2), Human epithelial cervix carcinoma (HeLa), human prostate adenocarcinoma metastatic in bone (PC3) and (Chinese hamster ovary) CHO-K1 cell lines were studied.

Maintenance of cells

All cell lines were routinely grown in a 5% CO_2 humidified atmosphere at 37°C and maintained in either Dulbecco's modified Eagle's medium (DMEM)/F12 with 20mM HEPES, supplemented with 10% foetal calf serum (FCS), penicillin, and gentamicin (CaCo2, HeLa, PC3) or Ham's F12 with the same additions (CHO-K1). Both culture media were free of phenol red. For passaging, cells were grown in a 175cm^2 flask to 80% confluency, removed by trypsinisation and seeded into 175cm^2 flasks at a density of 2-2.5 $\times$ 10^5 cells/flask. For induction studies, cell cultures were used between passage 4 and 12.

Reporter vectors

ARE-luc

Initial experiments in CaCo2, HeLa and PC3 cells (reported in this Chapter as Experiment 1 determination of optimal cell line) were performed using an ARE with a luciferse reporter gene that was generously supplied by Prof. Wayne Tilley (Flinders University of South Australia). This response element was of unknown origin and electrophoretic gel studies were unable to determine, unequivocally,

whether it was of rat prostate (similar to ARR3-tk-luc described below) or a human PSA origin. For this reason the highly responsive ARE detailed below (ARR3-tk-luc) was obtained.

ARR3-tk-luc (p(-244/-96)₃T81Luc)

The ARR3-tk-luc reporter gene was generously supplied by Dr RJ Matusik (Vanderbuilt University) under a standard materials transfer agreement. The construct contains a triple repeat of the Probasin (PB) derived androgen response element (-244/-96) from the rat ventral prostate. The triple repeat is 474 bp in size and is linked by HindIII restriction enzymes within the ampicillin resistant pT81Luc host vector [380]. In PC3 cells with AR expression vectors, 200-300 fold induction occurs with DHT or R1881 treatment (Personal communication, RJ Matusik, July 2001).

pRL-TK-Luc (Renilla luciferase)

The pRL-TK vector contains the herpes simplex virus thymidine kinase (HSV-YK) promoter region upstream of *Renilla luciferase.* The HSV-TK promoter provides low-level, constitutive expression in mature mammalian tissues [381].

Transfection protocol

Cells were grown to 80% confluency, trypsinised and counted in a hemocytometer in triplicate before seeding, initially at 1.5×10^5 cells/well into 24 well plates (17mm diameter/well, Falcon). Cells were transiently transfected with varying amounts of the experimental plasmid and 50 ng of reference plasmid, pRL-TK-Luc (to assess transfection efficiency) and co-transfected with varying amounts of wild type human androgen receptor (hAR) using a cationic liposome mediated transfection reagent (DOTAP, Roche), according to manufacturers protocol. After initial experiments all cells were co-transfected with 400 ng of hAR. Transfections were conducted at 80% confluency, which was approximately 24 hours after plating. During experiments (from transfection of plasmids to lysis of cells, 48 hours) cells were

maintained in phenol and serum free media. Cells were treated with ethanol vehicle, testosterone (T), dihydrotestosterone (DHT) or, androgen in combination with flutamide (FLUT) at the indicated concentrations and incubated for 24 hours before harvesting. All androgens and anti-androgens were dissolved in 100% ethanol.

Luciferase assay

Cells were washed once with phosphate-buffered saline (PBS) and treated with Cell Passive Lysis Buffer (Promega) for 15 minutes on a rocking platform at room temperature. Cell lysates were collected and luciferase activity determined using the dual-luciferase assay kit (Promega) and a TD 20/20 Luminometer (Quantum Scientific).

STUDY PLAN AND ORDER OF EXPERIMENTS

The principle of the trans-activation assay is that androgens from the media environment enter cells and induce the interaction between the LBD and the N-terminal of the AR. The complexes bind to Gal4-binding sites located in the pT81-Luc reporter construct and the transcriptional activation domains are linked to the luciferase gene promoter, leading to activation of luciferase gene transcription.

Experiments 1 to 5 involved the transient transfection of ARE-luc (unknown origin) or ARR3-tk-Luc, PRL-tk-luc and hAR into cultured cells, treatment with androgens and the measurement of transcriptional activity using the Dual-Luciferase® Reporter assay system (Promega, Madison, Wisconsin, USA). Experiment 6 involved the transfection of optimal amounts of reporter genes and hAR, treatment with human serum and measurement of transcriptional activity.

Experiment 1. Determination of optimal cell line

Caco-2, HeLa, PC3 and CHO-K1 cells were plated at 150,000 cells/well, into 24-well cell culture plates. Standard cell culture environment was maintained as described above. CaCo2, HeLa and PC3 cells were plated in DMEM/F12 media while CHO-K1 cells were plated in Ham's F12. All cells lines were exposed to similar experimental conditions of 200 ng of ARE-luc or 400 ng of ARR3-tk-luc, titrated amounts of co-transfected hAR and treatment with testosterone or DHT at a concentration of 10^{-7} M. HeLa cells co-transfected with 100 ng of hAR were also exposed to male and female human serum.

Experiment 2. Determination of optimal plating density for CHO-K1 cells

CHO-K1 cells were plated at 50, 100, 200 and 400 thousand cells/well in Ham's F12 media. All cells were transfected with 400 ng of ARR3-tk-luc, co-transfected with 1ng hAR and treated with DHT at a concentration of 10^{-7} M.

Experiment 3. Determination of the level of co-transfected hAR required for optimal induction of ARR3-tk-luc

CHO-K1 cells were plated at 100,000 cells/well in Hams F12 media. The amount of hAR required for optimal stimulation of ARR3-luc was determined. The amount of hAR was titrated from 0 ng to 400 ng. The amount of ARR3-tk-luc (400 ng), and concentration of DHT treatment (10^{-7} M) remained constant.

Experiment 4. Determination of the level of ARR3-tk-luc required for optimal luciferase activity

CHO-K1 cells were plated at 100,000 cells/well in Hams F12 media. The amount of ARR3-tk-luc required to achieve maximal induction with androgen treatment was investigated. The amount of ARR3-

tk-luc was titrated from 50 ng to 500 ng. Cells were co-transfected with 1ng hAR and treated with DHT at a concentration of 10^{-7} M.

Experiment 5. Determination of dose response of ARR3-tk-luc to testosterone and DHT

CHO-K1 cells were plated at 100,000 cells/well in Hams F12 media. Cells were transfected with 400 ng of ARR3-tk-luc and co-transfected with 1ng of hAR. The dose response pattern of ARR3-tk-luc induction to testosterone and DHT was investigated. The treatment doses of testosterone and DHT were titrated as follows:

Testosterone: 10^{-7} to 10^{-15}

DHT: 10^{-9} to 10^{-17}

The anti-androgen flutamide (FLUT) was also added at concentrations of 10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} M to determine if blocking of transactivation could be achieved.

Experiment 6. Application to measurement of testosterone in human serum

CHO-K1 cells were plated at 100,000 cells/well in Hams F12 media. Cells were transfected with 400 ng of ARR3-tk-luc and co-transfected with 1ng of hAR. The ability of the assay to detect variations in the level of testosterone in human plasma was tested. Cells were treated with male and female plasma of varying testosterone concentrations and 1 row of cells was treated with DHT at a concentration of 10^{-7} M as a positive control.

STATISTICAL ANALYSES

Calculation of relative induction of the luciferase gene linked to the ARE and TK was performed using a formula driven MS Excel spreadsheet. Effect of androgen treatment is reported as a fold-induction above ethanol treated controls.

RESULTS

EXPERIMENT 1. DETERMINATION OF OPTIMAL CELL LINE

Caco-2 cells

In testosterone compared to ethanol treated Caco-2 cells, co-transfection of 100 ng and 10 ng of hAR resulted in 4.5-fold and 1.5-fold induction in ARE-luc respectively, with no significant induction at 5, 1 or 0 ng of hAR (Figure 7.0.1 panel A).

HeLa cells

In testosterone compared to ethanol treated HeLa cells, the greatest degree of ARE-luc induction (3.75-fold) was observed in cells free of co-transfected hAR. Co-transfection of 10, 100, 200 and 400 ng of hAR resulted in 3, 2.75, 1.25 and 2-fold inductions of ARE-luc respectively (Figure 7.0.1 2nd panel). Treatment of cells with 100 ng of hAR with human male and female serum did not result in significant induction of ARE-luc above that of ethanol treatment (Figure 7.0.1 panel B).

PC3 cells

In testosterone treated PC3 cells, significant induction of ARE-luc compared to ethanol treated controls was observed only in cells co-transfected with 5 ng of hAR (1.45-fold). Co-transfection of 0, 10, 100, 200 or 400 ng of hAR yielded no significant induction (Figure 7.0.1 panel C).

CHO-K1 cells

In DHT compared to ethanol treated CHO-K1 cells, 7.2-fold induction of the ARE-luc was observed in cells co-transfected with 5 ng of hAR. Co-transfection with increasing amounts of hAR (10, 20, 50 and

100 ng) yielded a dose response pattern of reduced induction (4.1, 2.6, 1.8 and 1.6-fold respectively) (Figure 7.0.1 panel D).
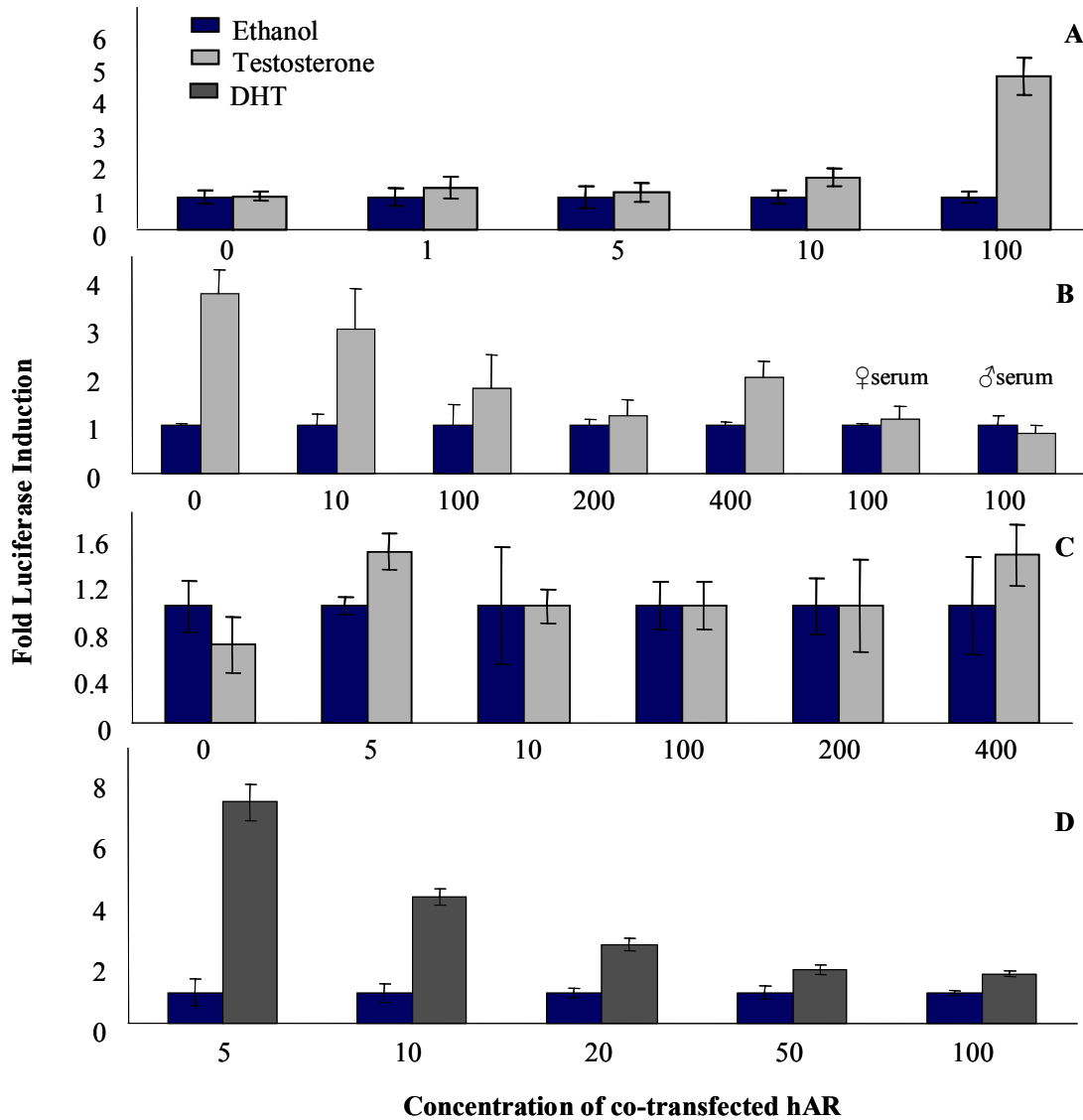
Figure 7.0.1 The effect of Testosterone or DHT on luciferase induction of transfected ARE-luc in CaCo2 (panel A), HeLa (panel B), PC3 (panel C) and CHO-K1 (panel D) cell lines, co-transfected with varying concentrations of human androgen recepto (hAR).

CaCo2 (A), HeLa (B) and PC3 (C) cells were transfected with 200 ng of an ARE-luc of unknown origin. CHO-K1 (D) cells were transfected with 400 ng of ARR3-tk-luc derived from the probasin gene of rat ventral prostate. HeLa cells (panel B) co-transfected with 100 ng of hAR were also exposed to male (♂) and female (♀) human serum.

EXPERIMENT 2. DETERMINATION OF OPTIMAL PLATING DENSITYFOR CHO-K1 CELLS

Cells plated at 50,000 cells per well, were 90% - 100% confluent at the time of DHT treatment, 24 hours prior to lysis. Cells plated at 100,000 cells per well were 100% confluent at this time and cells plated at 200,000 and 400,000 cells per well were over confluent.

The greatest induction was observed in cells plated at 200,000 cells per well, however there was much variation between wells (23.6 ± 25.1-fold induction). Plating cells at 50,000 and 100,000 cells per well resulted in 22 ± 6.8-fold and 19 ± 2.4-fold inductions respectively with significantly less variation (Figure 7.0.2). Plating cells at 100,000 cells per well is highly practical and this seeding concentration was used in subsequent experiments.
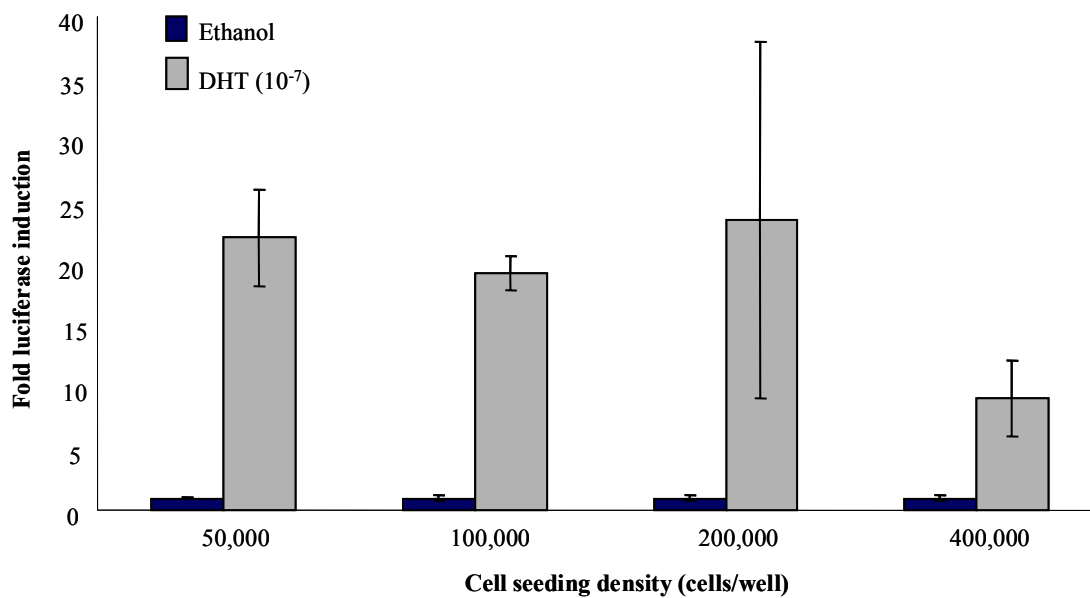
Figure 7.0.2 The effect of cell seeding density on ARR3-tk-luc induction in cells transfected with 400 ng of ARR3-tk-luc, co-transfected with 1ng hAR and treated with DHT (10^{-7} M) or ethanol vehicle.

EXPERIMENT 3. DETERMINATION OF THE LEVEL OF CO-TRANSFECT hAR REQUIRED FOR OPTIMAL INDUCTION OF ARR3-TK-LUC

In experiment 1 (Figure 7.01, panel D), the highest induction of ARR3-tk-luc occurred in cells co-transfected with 5 ng of hAR (7.2 ± 0.6). A follow-up experiment using lower levels of co-transfected hAR showed a 5.3 ± 1.3-fold induction in cells co-transfected with 2.5 ng of hAR (Figure 7.0.3 panel A). Co-transfection with 1 ng or 5 ng hAR yielded 3.5-fold and 3.2-fold inductions respectively and 10 ng hAR resulted in a modest 1.9-fold induction. No ARR3-tk-luc induction was observed in cells free of co-transfection with hAR. A subsequent experiment confirmed that maximum ARR3-tk-luc induction occurred when cells were co-transfected with either 0.5 ng or 1 ng hAR (21.0 ± 5.0-fold and 21.3 ± 3.9-fold) using serum free media during the treatment incubation (Figure 7.0.3 panel C).

EXPERIMENT 4. DETERMINATION OF THE LEVEL OF ARR3-TK-LUC REQUIRED FOR OPTIMAL LUCIFERASE ACTIVITY

Transfection of 400 ng of ARR3-tk-luc resulted in the highest luciferase induction 14.7 ± 2.2-fold. Lower levels of ARR3-tk-luc transfection (50 ng, 100 ng and 200 ng) resulted dose-dependent inductions of 1-fold, 2.2-fold and 5.2-fold respectively (Figure 7.0.4). Transfection with 500 ng, 600 ng and 700 ng resulted in similar dose-dependent inductions similar to those observed from transfection of 50 ng, 100 ng and 200 ng of ARR3-tk-luc.
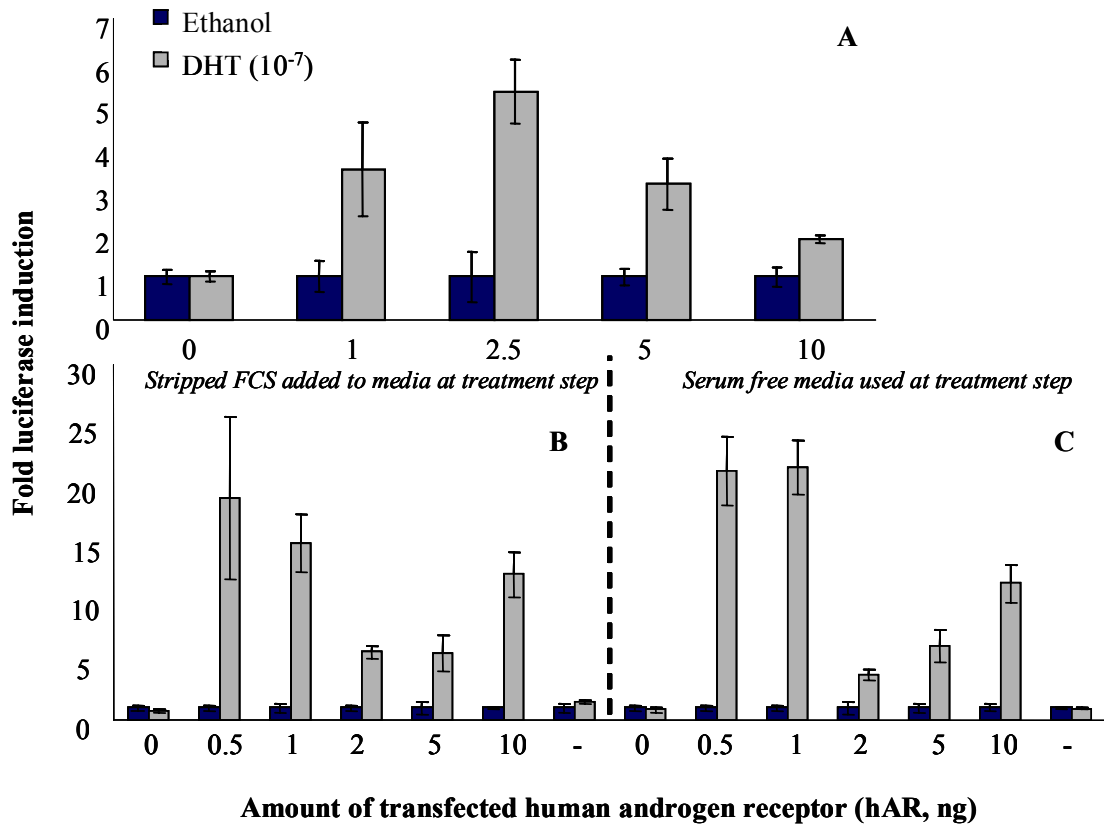
Figure 7.0.3 The effect of co-transfection concentration of human androgen receptor (hAR) on ARR3-tk-luc induction in cells transfected with 400 ng of ARR3-tk-luc and treated with DHT (10^{-7} M) or ethanol vehicle.

In panel B, cells were exposed to media containing fetal calf serum (FCS) stripped of steroids and in panel C, to serum free media the at the treatment step. In panels B & C (-) indicates than no ARR3-tk-luc or hAR was transfected into cells, representing background luciferase activity.
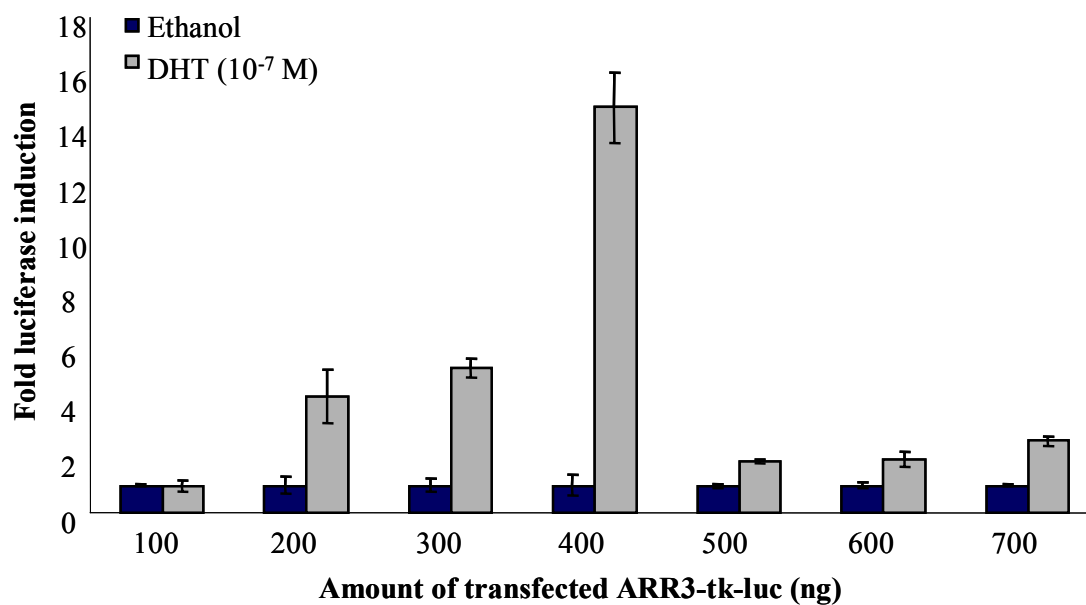
Figure 7.0.4 The effect of transfection concentration of ARR3-tk-luc on induction in cells co-transfected with 1 ng of hAR and treated with DHT (10^{-7} M) or ethanol vehicle.

Experiment 5. Determination of dose response of ARR3-tk-luc to testosterone and DHT

The dose response of the experimental bioassay system to DHT and T is shown in Figure 7.0.5. Treatment with DHT at concentrations of 10^{-11}, 10^{-9} and 10^{-7} M resulted in relative luciferase induction of 1.1-fold, 12.0-fold and 32.2-fold respectively, above that of ethanol. The addition of the anti-androgen Flutamide at 10^{-7} M slightly reduced induction to a level of 27.3-fold in cells exposed to 10^{-7} M DHT. The dose response pattern with Testosterone treatment was similar (10^{-11} M, 0.4-fold; 10^{-9} M, 9.1-fold; 10^{-7} M, 8.7-fold) but of lower magnitude, however the addition of Flutamide at 10^{-7} M increased induction to 19.1 fold, twice that of the induction achieved by Testosterone at 10^{-7} M without Flutamide.

Experiment 6. Application to measurement of testosterone in human serum

Treatment with human male and female serum resulted in 9.1-fold and 12.5-fold luciferase inductions respectively (Figure 7.0.6). As a quality control measure a 29-fold induction of ARR3-tk-luc was observed with DHT treatment at a concentration of 10^{-7} M.
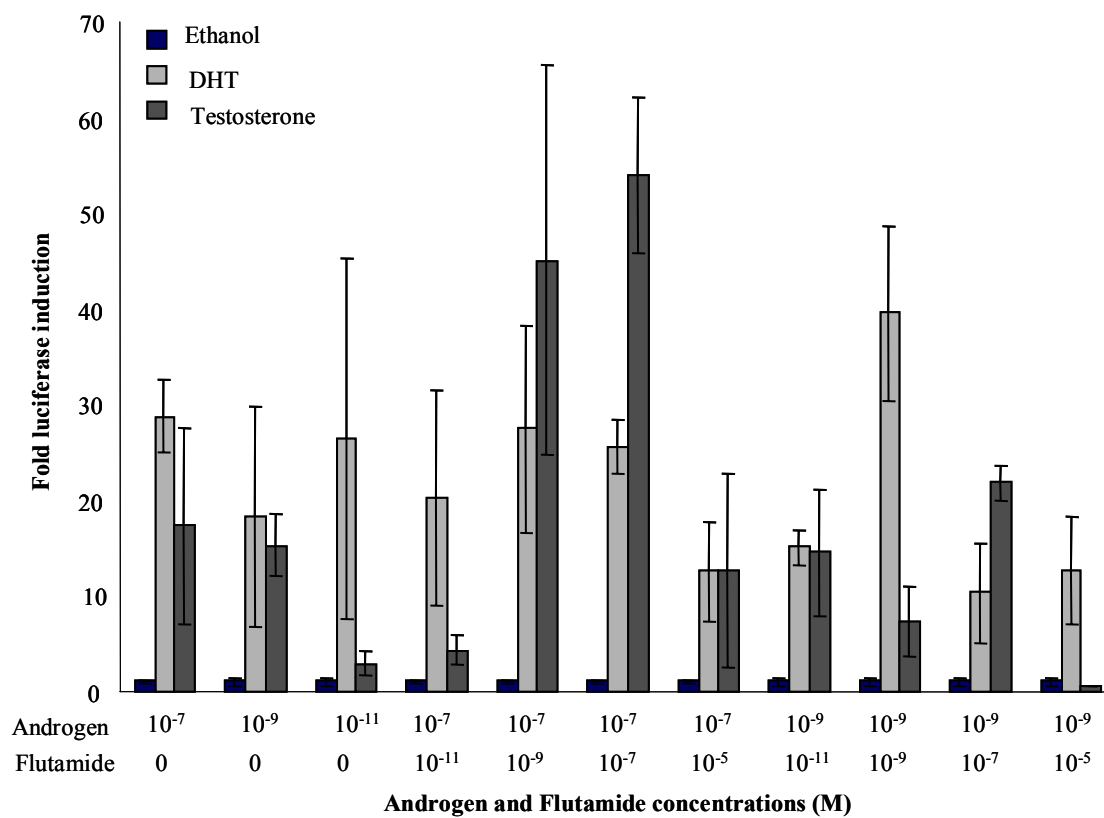
Figure 7.0.5 The dose-response effect of Testosterone and DHT and suppression with Flutamide, on 400 ng ARR3-tk-luc induction in CHO-K1 cells co-transfected with 1 ng of hAR.
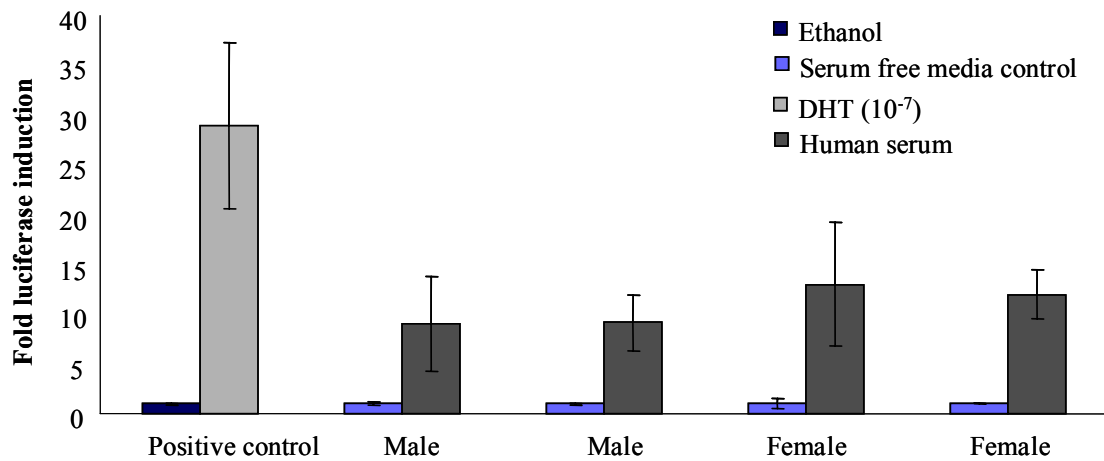
Figure 7.0.6 The effect of human male and female serum on 400 ng ARR3-tk-luc induction in CHO-K1 cells co-transfected with 1 ng of hAR.

Serum free media was used as a control for human serum treated cells from which the fold luciferase induction was calculated. As a positive control in the assay, a row of cells was exposed to DHT (10^{-7} M) treatment with a standard ethanol control.

DISCUSSION

The aim of this work was to develop a novel approach to the measurement of testosterone bioactivity in human serum. Historical assays for the measurement of androgenic bioactivity were based on androgen-dependent responses of living organisms such as the growth of the rooster's comb [382, 383], the deer's antlers [384-387] or the rat's prostate [388]. The assay system used in this work utilises modern cell culture techniques and a commercially available dual luciferase reporter assay.

Initially, four different cell lines were studied due to their immediate availability and current use in the laboratory. From initial experiments in the different cell lines, it was concluded that the degree of testosterone/DHT stimulated luciferase induction was not only dependent on the amount of co-transfected hAR, but also on the type of cell used. The expected consistent dose-response relationship between hAR and induction of luciferase occurred in CaCo-2 and HeLA cells treated with testosterone and in CHO-K1 cells treated with DHT; in HeLa and CHO-K1 cell lines the dose-response relationship was negative rather than positive. CaCo-2 cells display variability in their morphology and great care is needed in standardizing culture and transfection conditions to ensure experimental reproducibility [389]. Significant induction was observed in HeLa cells with no co-transfected hAR. Being of cervical epithelial origin, it is likely that these cells contain their own androgen transcription factors. PC3 cells were extremely non-responsive to testosterone treatment at any level of co-transfected hAR. Of the four cell lines studied, the CHO-K1 cell line seemed to perform most satisfactorily. CHO-K1 cells, being of ovarian origin, may have co-regulators of AR dependent transcription that regulate the stimulation and inhibition of transcription when androgen binds

to the ligand-binding domain (LBD) of the ARE [390], this could also include regulation via the estrogen receptor (ER) and ER transcription co-factors [391, 392].

A very similar and novel assay for the determination of androgen bioactivity in human serum [373] was published during the course of the present studies. The authors reported enhanced sensitivity of the assay by using a co-activating factor, AR-interacting protein 3 (ARIP3), a 572 amino acid nuclear protein that enhances the androgen-dependent interaction between the LBD and N-terminal of the AR [393]. Co-transfection of ARIP3 allowed Raivio and colleagues to demonstrate a maximal fold luciferase induction of 745 after treatment with 100 nmol/L of testosterone compared to charcoal-stripped foetal calf serum without added testosterone [373]. In our experiments, where no co-activating proteins were added, the maximum fold luciferase induction we were able to demonstrate was 29, with 10^{-7} M DHT; a much more highly potent androgen than testosterone. The aim of an assay such as this requires sufficient sensitivity to determine differences in testosterone levels at nanomolar concentrations in human serum. The bioassay of Raivio and colleagues has a level of sensitivity below 0.1 nmo/L. The intra-assay CV was 8.3% at 4.9 nmol/L and the inter-assay CV was 21% [373].

Ravio et al. (2001) developed their bioassay in the mammalian COS-1 cell line; derived from the kidney of an African green monkey. The reason for using this particular type of cell was unclear, but American Type Culture Collection Global Bioresource Center specify's its application as a transfection host (http://www.atcc.org/SearchCatalogs/CellBiology.cfm ATCC #: CRL-1650). CHO-K1 cells have been the line of choice for the development of stably transfected cell lines for the assessment of biological andro- and antiandrogenic, estro- and antiestrogenic effects of various environmental, food and medicinal compounds [394, 395].

The purpose of the preliminary studies reported in this chapter was to develop and understanding of the characteristics of the cell lines and the hAR and ARE's plasmids. This work has determined the optimal number of CHO-K1 cells to seed and the optimal amounts of hAR and ARR3-tk-luc to transfect in order to induce the greatest luciferase gene transcription. Some suppression of induction has also been shown with the anti-androgen flutamide. It is suspected that administration of the active alpha-hydroxylated metabolite, hydroxyflutamide, may have resulted in more reliable and possibly complete suppression of AR-induced luciferase gene transcription. Flutamide may not be hydroxylated within the CHO-K1 cell. It has also been demonstrated that this system can detect androgen present in male and female human serum. It is interesting, and confusing that CHO-K1 bioassay system has a trend (although not statistically significant) towards greater induction of luciferase transcription with exposure to female as compared to male human serum. Being of ovarian origin, this cell-line may have specific transcription co-factors that produce a male-like transactivation of the luciferase gene from the female hormonal milieu. ERα and functional Erβ mRNA isoforms and transcription co-factors have been identified in CHO-K1 cells [391, 392]. These transcription factors may enhance intracellular signalling pathways acting synergystically with testosterone induction of ARE's. The bioassay system of the present study may have a potential advantage over that of Raivio and colleagues in the co-transfection of full-length human AR as opposed to co-transfection of fragments of rat AR.

Nevertheless, the aim of this work was to provide a bio-indicator of the biologically important component of testosterone in human plasma. Raivio et al. report that the serum androgen bioactivity measured by the luciferase bioassay correlated strongly with serum total testosterone concentration in 13 boys with constitutional delay of puberty and 9 pre-pubertal boys with cryptorchidism (R = 0.93, P < 0.0001), but surprisingly, not with 5α-DHT, dehydroepiandrosterone (DHEA) or androstenedione levels [373]. Moreover,

the addition of testosterone to SHBG-containing serum resulted in a 75% reduction in androgen bioactivity compared to diethyl ether extracted testosterone reconstitution in charcoal-stripped FCS [373].

The COS-1 cell bioassay of Raivio et al. confirms the current belief that the SHBG bound component of testosterone in plasma is not biologically available. This system has not yet been evaluated with respect to correlation with non-SHBG bound or free testosterone. However, a 7-fold increase in serum testosterone bioactivity using the bioassay system, was demonstrated after 6-months of daily topical DHT gel treatment in 14 men (aged 51 – 63 years) with symptoms of relative androgen deficiency and baseline serum total T < 15 nmol/L and/or SHBG > 30 nmol/L [396]. Unfortunately, the authors did not report this change in association with any symptomatic or function effects of treatment.

Taken together, the current data suggest that it is feasible and possible to establish a cell-culture based bioassay, utilising a transfected androgen responsive promoter, luciferase reporter gene.

CHAPTER 8.0 GENERAL DISCUSSION

This thesis has described the problems associated with the laboratory and clinical diagnosis of androgen deficiency in the ageing male. In Chapter 3.0, the ammonium sulphate precipitation method for the measurement of bioavailable testosterone (BT) was described and laboratory specific, age-related references ranges were reported. Increasingly, in North America, particularly in Canada, laboratories are implementing this method and increasing numbers of physicians and family practitioners are ordering this test for older male patients with symptoms of androgen deficiency. An inherent problem is the variation in measurement between laboratories and it is essential that each laboratory establish its own reference ranges based on serum from healthy volunteers and not patient samples. Calibration against a national standard would also be beneficial. At present, in Australia, BT is not routinely available to physicians and general practitioners. Recently, at the North American Congress on the Aging Male (Vancouver, Canada, February 2-5, 2005) much debate focussed on which method of testosterone measurement should be used in determining which men should be offered testosterone replacement therapy. In addition to the ammonium sulphate measurement of BT, calculated BT and calculated free testosterone are also offered by laboratories. The calculations are problematic as they rely on binding constants of SHBG and albumin for testosterone. During ageing there are many potential factors that may alter SHBG and albumin affinities for testosterone. Unpublished data from Morley and colleagues shows tighter SHBG binding in serum from older men and similarly, as shown in Chapter 3.0 (Figure 3.1) the dissociation constant of SHBG for testosterone was 5.88 nM in young men and 10.34 nM in men over 65 years with andropausal symptoms. Thus, the current recommendation is to measure BT (where available) in patients with androgen deficiency symptoms and to make treatment decisions based on laboratory specific references ranges (as opposed to

cut-off values reported in the literature) and clinical symptomology. New methods for measuring biologically active levels of testosterone in serum are needed. The luciferase-reporter bioassay techniques described in Chapter 7.0 are exciting and correlations with current assays and calculations should be assessed. Unfortunately, like the equilibrium dialysis measurement of free testosterone, the luciferase reporter bioassay is labour intensive, expensive and not practical as a routine assay. New kit assays are also being developed for the measurement of salivary testosterone that reportedly are highly sensitive and relate to biological effects of testosterone. This remains an important and central area of research in ageing male medicine.

Data from the Florey Adelaide Ageing Male Study (FAMAS) showed that the measurement of BT was more strongly associated with ageing per se` than was either total or calculated free testosterone (Chapter 5.1). This is due to the strong effect of age on increasing SHBG levels and possibly due to tighter binding to SHBG with increasing age. Low total testosterone was strongly associated with a metabolic cluster of abdominal obesity, high triglycerides and high HbA1c. These data suggest that measurement of total T may be useful in detecting chronic disease associated testosterone decline, but that a more sensitive measure such as BT may be required to detect age-related alterations in plasma binding when chronic disease is less of a factor, but androgen deficiency symptoms are still present. Thus for many men with androgen deficiency symptoms and clinically relevant chronic disease (metabolic ie: diabetes, obesity, hyperlipidaemia) a total T measurement may be sufficient, whereas in men without apparent disease but complaining of androgen deficiency symptoms, measurement of BT may be required. Supporting this idea is the predictive value of low total testosterone and SHBG, but not bioavailable or free T levels with abdominal obesity, measured by DEXA (Chapter 5.2). Moreover, lower total T and SHBG independently predict a lower percentage of total lean body mass. In addition, metabolic disease was independently predictive of lower lean body mass, reduced muscle strength and poorer physical function and it is likely that reduced

testosterone levels and other growth factors, such as IGF-1 and growth hormone, play a key role in the mechanisms of this association. As shown in Chapter 6.1 testosterone supplementation in older men with low-normal gonadal status increased lean mass and decreased fat mass. However, no overall changes in serum IGF-1 levels or muscle strength were observed as a result of the 160 mg/day dose of oral testosterone undecanoate formulation. In the group receiving testosterone however, changes in quadriceps muscle strength were correlated with changes in lean body mass, so some variable functional improvement may have occurred. There may be potential for testosterone therapy, along with that of other growth factors, such as IGF-1 and vitamin D, to delay or prevent sarcopenia and frailty through effects on muscle protein synthesis and increased activity of muscle satellite cells. The effect of these therapies in conjunction with resistance exercise in the elderly warrants further intense scientific scrutiny. Moreover, the identification of altered gene expression between young and old, mobile and immobilised and testosterone/IGF-1 exposed and non-exposed skeletal muscle, using microarray and proteomic technology will help to improve knowledge on the mechanisms of sarcopenia and may lead to potential gene therapies.

Findings from the FAMAS also included an independent association between all measures of serum testosterone and sexual desire but not with erectile function (Chapter 5.3). There were slight differences in the associations between the testosterone measurements and sexual desire when classified as either solitary of dyadic (partner related desire). BT was not associated with partner related desire but all measures of T were associated with solitary desire. The mechanisms regulating these different domains of desire are not understood and are likely to involve complex psychosocial, sexual and physiological factors. Few studies have attempted to define the two elements of desire. Many older men, complaining of loss of sexual desire may have lost there partners and it may be extremely important to establish level of solitary desire for a full investigation of sexual dysfunction or monitoring of treatment effects. As shown in Chapter 6.2, 160 mg/day of oral testosterone undecanoate administration to older men with low-normal gonadal

status did not improve sexual desire but showed a trend towards improved erectile strength as measured by the St. Louis University Androgen Deficiency in the Aging Male (ADAM) questionnaire. The sexual desire tool used to measure effect in this study was probably inadequate as sexual desire was not a primary outcome of this trial. Had a well validated measure of sexual desire been used (such as the SDI-2 used in the FAMAS study), improvement due to testosterone replacement may have been detected. There is no doubt that adequate levels of androgens are required for penile erection to occur and be sustained for successful sexual intercourse. It is not clear, however, what this adequate level is. It has been shown that testosterone in conjunction with phosphodiesterase-5 (PDE-5) inhibitors improves penile erection in men with erectile dysfunction and low testosterone levels [139]. Moreover, healthy neural signalling and vascular stability is needed for successful erection and erectile dysfunction therapeutic development is now concentrated on nitric oxide- and neural independent cyclic guanosine monophosphate stimulation in penile endothelial and smooth muscle cells. This technology may restore erectile function to men with severe penile vascular insufficiency and post radical prostatectomy ED.

Lower urinary tract symptoms (LUTS) and reduced maximal urinary flow rates (Qmax) were not associated with any measure of serum testosterone or SHBG levels in the FAMAS cohort (Chapter 5.4). Having an enlarged prostate and higher free PSA levels were the major factors, other than age, in LUTS and reduced Qmax respectively. As shown in Chapter 6.1, LUTS were not adversely affected by testosterone supplementation and if anything, testosterone supplementation was associated with better lower urinary tract function. Testosterone administration was not associated with a significant increase in serum total PSA. The literature is now strongly in favour of a neutral effect of physiological dose testosterone supplementation on the prostate. Evidence is even growing towards a beneficial effect of treating men with androgen deficiency symptoms and asymptomatic benign prostatic hyperplasia with testosterone. Hypogonadism has been implicated in the development of more aggressive, high Gleeson grade adenomas

and as such, testosterone supplementation may protect against prostate cancer. Moreover, in men with organ confined prostate cancer and hypogonadism, post-radical prostatectomy testosterone replacement therapy is currently being debated. However, at present insufficient data exists to recommend this line of treatment.

The various measures of serum testosterone and SHBG were not associated with neuropsychological function independent of covariates, cross-sectionally, in the FAMAS cohort (Chapter 5.5). There was however a non-significant trend towards an association between low serum BT and higher depression scores. This association, as well as those of lower BT and cBT with better memory performance on the ineffective reminder component of the Fuld object memory evaluation, were associated prior to controlling for confounders. No measure of visuospatial cognition was improved with 160 mg/day of oral testosterone undecanoate in older men with low-normal gonadal status (Chapter 6.3). These men, however, did not have clinically significant impairments in visuospatial cognition at baseline and thus any improvement that may have occurred would have been difficult to detect. Mood and well-being however were improved by testosterone supplementation in this study even though men with clinically relevant depression were not included. Thus reduced levels of biologically available testosterone are associated with reduced mood and this can be ameliorated by returning serum bioavailable testosterone to normal levels with standard dose oral testosterone undecanoate. This appears to be a safe and efficacious therapy in older men.

In conclusion, reduced serum testosterone was directly associated with clustering of the metabolic disorders: obesity, diabetes and poor glycaemic control and hypertrigyceridaemia (Figure 8.0.1). Through this association and also through increasing age, serum testosterone plays a role in sarcopenia and ultimately the onset of frailty (Figure 8.0.1 panel A). Testosterone supplementation (orally with testosterone undecanoate) results in moderate elevations in serum bioavailable testosterone, reduced SHBG levels,

reductions in fat mass and increased lean body mass with variable improvements in muscle strength that are associated with increased lean body mass. This may lead to prevention of sarcopenia and frailty (Figure 8.0.1 panel B). Measurable improvements were also seen in mood and well-being which are most likely physiological responses as a trend towards an association between lower serum BT and higher Beck Depression Inventory scores was observed in a model also including components of the metabolic syndrome (diabetes and serum triglyceride levels). Testosterone supplementation in older men with low-normal testosterone levels also marginally improved erectile function but no improvements were observed in sexual desire or visouspatial cognition.
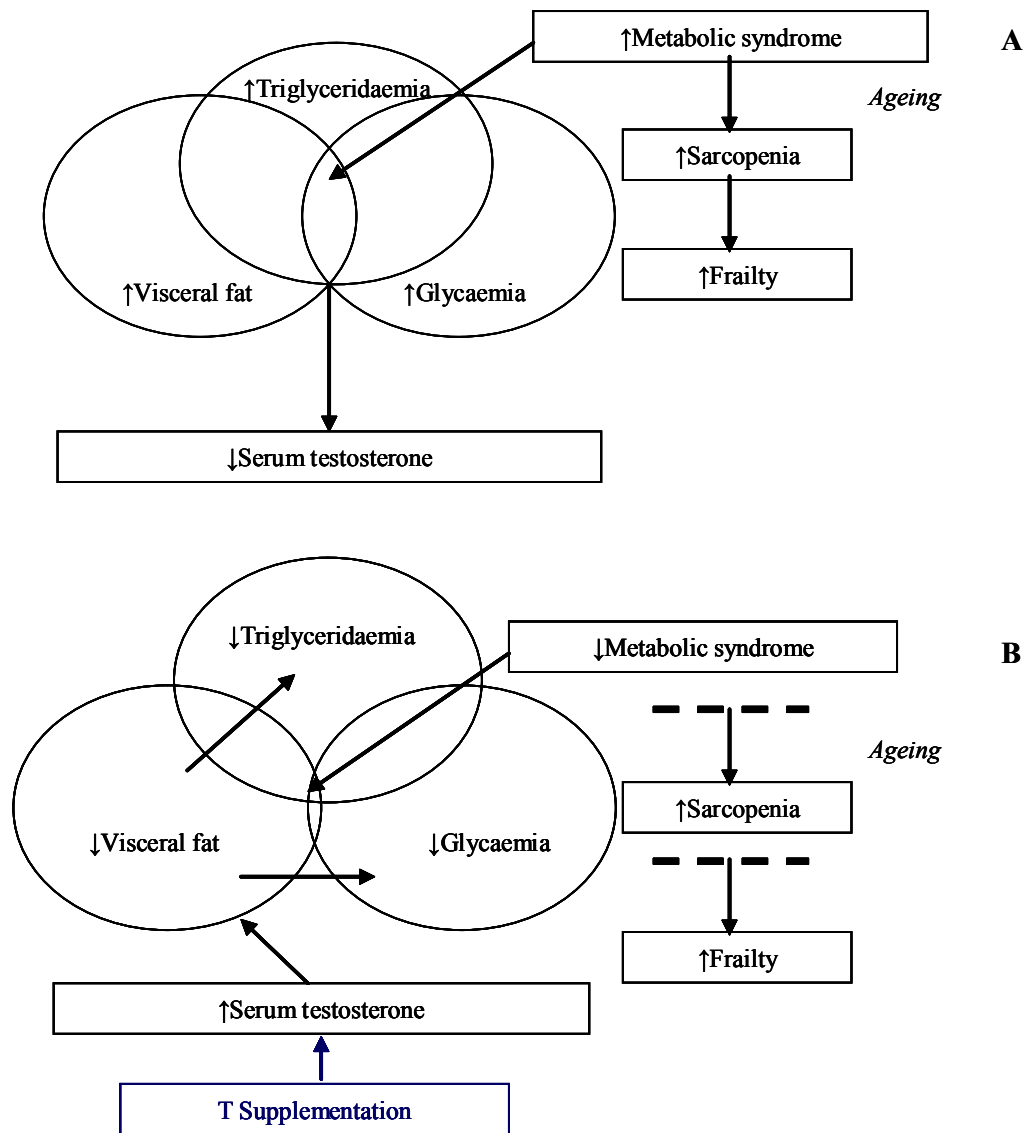
Figure 8.0.1 The associations between serum testosterone, the metabolic syndrome and age-associated sarcopenia and frailty.

Increased visceral obesity associated with reduced testosterone levels and the metabolic syndrome leads to sarcopenia and frailty with advancing age (panel A). Testosterone supplementation increases serum testosterone levels, decreases visceral fat, improves components of the metabolic syndrome potentially preventing sarcopenia and frailty in advancing age (panel B).indicates potential prevention of disease progression.