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Pharmacokinetics of Testosterone Undecanoate Injected Alone or In Combination with Norethisterone Enanthate in Healthy Men*

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Abstract

Long acting injectable testosterone undecanoate (TU) is a promising androgen for male hormonal contraception. As a prerequisite for a planned multicenter male contraceptive efficacy study, we studied the pharmacokinetics of two doses of TU alone or in combination with Norethisterone enanthate (NET-E) in a prospective two center study, randomized for TU dose in each center. 20 healthy male volunteers in each center were administered intramuscular injections of 750 or 1000 mg TU alone or in combination with 200 mg of NET-E IM every 8 weeks for three injections. There were no significant differences in maximum concentration and area under the curve (AUC) for serum total and free testosterone (T) between TU 750 and 1000 mg groups irrespective of whether TU was administered with 200 mg of NET-E. TU 1000 mg IM alone or with NET-E at 8 weekly intervals resulted in linear increases in average concentration and AUC of serum total and free T with each injection. Accumulation ratios of serum total and free T levels (calculated as 8 weeks post- to pre-injection levels) for each period showed significant increases in the TU+ NET-E groups. Serum gonadotropins levels and sperm concentration were more consistently suppressed in the TU 1000mg +NET-E group. We conclude that despite some accumulation of T, TU 1000 mg + NET-E 200 mg administered every 8 weeks may be preferable for the future contraceptive efficacy study because of more complete suppression of gonadotropins and spermatogenesis.

Introduction

Reliable, reversible, safe and preferably long-acting methods of hormonal male contraception might allow men to participate in family planning with higher compliance. At the present, all potential male hormonal contraceptives require an androgen for suppression of gonadotropins and spermatogenesis while maintaining androgenicity of healthy adult men. Testosterone enanthate (TE) administered as an intramuscular (im) injection once every two to three weeks is the most common injectable androgen used for the treatment of hypogonadal men (Snyder and Lawrence, 1980; Sokol et al, 1982). Testosterone Undecanoate (TU), formulated as a longer lasting injectable preparation was first studied in Chinese hypogonadal men. In these studies, 500 or 1000 mg intramuscular (im) TU injections in tea seed oil resulted in serum T levels within the adult male range for about four to six weeks (Zhang et al, 1998). Subsequent studies in Europe (using a preparation in castor oil that was different from the formulation developed in China) with single and repeated 1000 mg im injections of TU maintained normal adult male physiological serum T levels in hypogonadal men for 12 weeks (Behre et al, 1999; Nieschlag et al, 1999; Schubert et al, 2004; von Eckardstein and Nieschlag, 2002; Von Eckardstein and Nieschlag, 2002). Recent studies showed that TU injections improved sexual function and muscle and bone mass in hypogonadal men (Jockenhovel, 2004; Qoubaitary, Swerdloff, and Wang, 2005; Schubert et al, 2004). These studies provided evidence that TU could maintain serum T within or above the adult range with much less frequent injections than was required for TE; the need for less frequent injections suggested a more patient convenient regimen that could improve adherence to treatment for hypogonadism and male contraception. TU was first

utilized in male contraception clinical trials in Chinese men when administration of TU 500 mg and 1000 mg im injections monthly led to azoospermia in 11/12 volunteers in the 500 mg and all volunteers in the1000 mg group respectively (Zhang et al, 1999). A large multicenter efficacy study involving 308 men showed that azoospermia was achieved in 97% of Chinese men when TU was administered with an initial loading dose of 1000 mg followed by 500 mg at monthly intervals (Gu et al, 2003).

The efficacy of TU in suppressing spermatogenesis was also demonstrated in 14 white men who were administered TU 1000 mg every 6 weeks where 86% of the men became severely oligozoospermic (Kamischke et al, 2000). It is generally recognized from prior studies that Asian men respond to exogenous T injections with more efficacious suppression of spermatogenesis than non-Asian men (World Health Organization Task Force on methods for the regulation of male fertility, 1990; World Health Organization Task Force on methods for the regulation of male fertility, 1996). The relative less sperm suppression of androgens alone in non-Asian men led to the concept of combined preparations whereby a second gonadotropin suppressor (i.e. progestin or GnRH analogue) is added to the androgen to optimize sperm suppression (Amory and Bremner, 2003; Anderson and Baird, 2002; Meriggiola and Bremner, 1997; Nieschlag, Zitzmann, and Kamischke, 2003; Wang and Swerdloff, 2004).

Norethisterone Enanthate (NET-E) is a progestin that has weak androgenic and estrogenic activity and has been used as a two monthly injectable female contraceptive in many countries (Fotherby et al, 1984; Kesseru-Koos et al, 1973; Sang et al, 1981). When NET-E 200mg was combined with TU 1000 mg injections every six Weeks, suppression of spermatogenesis was enhanced compared to TU alone (Kamischke et

al, 2001; Kamischke et al, 2002). In a more recent study, this high efficacy of spermatogenic suppression was maintained even when the frequency of injections was extended to once every eight weeks (Meriggiola et al, 2005). Based on these promising data on relatively few men, a proposed large international multicenter study to examine the contraceptive efficacy in many couples utilizing a combination of 8-weekly intervals of TU and NET-E injections has been planned. The dose of TU had not been determined, 1000 mg was proposed but data from a lower dose of TU such as 750 mg had not been tested. To ensure that TU administered im every 8 weeks will provide adequate T levels without any significant accumulation of the steroid while suppression of spermatogenesis is optimized, a detailed pharmacokinetics study of TU in healthy men was warranted. The purpose of this study was to characterize pharmacokinetics of TU administered at 750 or 1000 mg im every 8 weeks that would be optimal for male contraceptive clinical studies either alone or in combination with NET-E administered at the same intervals in healthy male volunteers.

Subjects and Methods

Subjects

40 (20 in each center) healthy men aged between 18 and 50 years were enrolled in the study (**Table 1**). In Los Angeles, 7 of the volunteers were white, 6 Hispanic, 4 African American, 1 Asian and 2 Pacific islanders whereas in Bologna all subjects were white, All men were in good general health as confirmed by medical history and physical examination. They had normal baseline hematology, blood chemistry, urinalysis, fasting lipid profile, a prostate-specific antigen level of less than 4 ng/ mL, and a urine flow rate

of over 15 mL/s. All volunteers had normal reproductive hormones and two normal semen analyses. As the end points of the study included serum hormone concentrations and semen quality, the study did not require the participants to have proven fertility. Men with history of chronic diseases, positive hepatitis serology or drug screen were excluded. Digital rectal examination was performed at the beginning and end of study and any abnormality was noted. Testis volume was assessed by the Prader orchidometer (Test-Size Orchidometer from Accurate Surgical & Scientific Instruments Corp, Westbury, New York, USA) (Prader, 1966) at Los Angeles site by two moderately experienced physicians and at Bologna by one experienced physician. The physicians did not have an opportunity to compare their assessment on the same subject between the centers.

Study design

This was a two center prospective study consisting of a 2-week baseline period, 24-week treatment period and 8-week recovery period which was extended until each subject had sperm counts above 20 million / ml . We have recently shown that if sperm concentrations returned to 20 million/ml, it is most likely that the sperm concentration will return to the baseline concentration (Liu et al, 2006). The two centers were the Division of Endocrinology, Department of Medicine, Harbor-UCLA Medical Center, Los Angeles, United States and the Department of Obstetrics and Gynecology, University of Bologna, S. Orsola Hospital, Italy. Subjects studied in Los Angeles were randomized to receive three injections of 750 mg or 1000 mg TU at 8 weekly intervals (TU alone group). Because of drug regulatory limitations, it was not possible to use NET-E in the United States and the study of TU plus Net-E was performed in Italy.

Subsequently, in Bologna the same protocol was utilized to study the pharmacokinetics of TU at doses of 750 and 1000 mg together with 200 mg NET-E im every 8 weeks for three injections (TU + NET-E group). Subjects in Bologna were also randomized to receive either TU 750 or 1000 mg injections. Blood samples were drawn between 7 and 10 AM for serum total and free T, 5-alpha dihydrotestosterone (DHT) and estradiol (E_2) were drawn at day 0 and then at weekly intervals. Serum FSH, LH and sex hormone binding globulin (SHBG) were measured at monthly intervals. Serum hormones were also drawn at week 32 during recovery. Semen analyses were obtained every four weeks during the treatment period and every 8 weeks during recovery. Physical examination and safety laboratory tests were done before, at week 12 and 24 of treatment and at week 32 during recovery.

Medications

Testosterone undecanoate (TU) was supplied by Schering AG (Berlin, Germany) and through the Contraceptive Research and Development program (CONRAD) program (Arlington, VA). Each ampoule contained 1000 mg of TU dissolved in 4 mL of castor oil. This preparation used I the present study is the same as that reported in the European studies (Behre et al, 1999; Kamischke et al, 2001; Kamischke et al, 2000; Nieschlag et al, 1999) and different form the formulation used in China. The preparation was shaken vigorously before injection. For the 1000 mg dose, 4 mL was administered and for 750 mg dose, 3 mL was given. The injections were given as deep IM injections into the gluteal regions slowly to avoid pain associated with the injection. The same batch of TU was used throughout the study. TU is absorbed into the circulation and rapidly metabolized into the active unesterified T and the undecanoate side-chain. The

undecanoate moiety undergoes ß-oxidation with two carbon pieces entering the citric acid cycle. The residual 3 carbon piece (Propionyl- CoA) is converted to Propionyl-carnitine and excreted in the urine. The undecanoate moiety has no biological action (Information from Schering AG). Norethisterone Enanthate (NET-E) was supplied by Schering AG (Berlin, Germany) to Dr Meriggiola. For the 200 mg dose, 1 mL was administered. The injections were given as deep IM injections into the gluteal regions separate from the TU injections. Experienced nurses at both centers gave all the injections under the supervision of the investigators.

Methods

Serum samples form Bologna was stored at -20 ° C and shipped frozen in batches to Los Angeles. All samples from a subject were measured in the same assay to reduce inter-assay variation. All hormone and SHBG assays used validated methods established at Harbor-UCLA Endocrine Research laboratory. The methods used to measure these hormones as well as SHBG had been previously described (Swerdloff et al, 2000; Wang et al, 2004) except serum total and free T which had been modified and briefly described below. Serum T levels were measured by a specific RIA using kit from Diagnostic Product Corporation (Los Angeles, CA). The lower limit of quantitation (LLOQ) of serum T measured for this assay was 0.43 nmol/ liter. All results below this value were reported as 0.43 nmol/ liter. The mean accuracy (recovery) of the T assay, determined by spiking steroid free serum with varying amounts of T (0.9 nmol/ liter to 56 nmol/liter), was 104% (range 95 to 114%). The intra-and inter-assay coefficients of variation for the T assay were 4.0 and 5.8 %, respectively at the normal adult male range (established by obtaining sera from over 120 healthy men of mixed ethnicity who

had normal physical examination, semen analyses and normal serum gonadotropin levels) which in our laboratory were 9.4 to 30.9 nmol/ liter (271 to 892 ng/dl). Serum free T was measured by the equilibrium dialysis method using purified radioactive labeled T and dialyzed overnight in dialysis cells at 37^{o}C. The labeled T that was in the dialysate expressed as a percent of the total amount of labeled added to the serum was used to calculate the percent free T. The free T concentration was then derived by serum total T concentration x percent free. The intra- and inter-assay precisions (CV) of percent free T were 6.3% and 10.6%. The adult male reference range for free T values in our laboratory was 0.127 to 0.576 nmol/liter (3.66 to 16.62 ng/dl).

Semen Analyses were performed using methods described by the WHO Manual for the Examination of Human semen and sperm Cervical Mucus Interaction (World Health Organization, 1999). The Harbor-UCLA Andrology participated in the external proficiency testing provided by the College of American Pathologists and the Bologna center participated in "VEQ - Gruppo Controllo Qualita' Analitico Azienda Ospedaliero-Universitaria di Bologna, Policlinico Sant'Orsola-Malpighi". All safety laboratories including serum PSA were measured at each center's clinical biochemistry laboratories. At Harbor-UCLA Medical Center, the PSA was quantitated using two-site chemiluminescent Beckman Access Hybritech total PSA assay (Beckman Coulter, Fullerton, CA; inter-assay CV 5.2 and 4.2 % at low and high PSA levels) and in Bologna, immunofluorescent assays (KRYPTOR; CIS-Bio International, Oris Group, Gif-sur-Yvette, France; inter-assay CV 2.1 % for both high and low range).

Statistical analyses

For each of the three 8-week injection periods, and for each of the four subject groups,

derived pharmacokinetics measures for testosterone (T) and free T were calculated. These measures include C_{avg}=mean concentration, C_{max}=maximum concentration, AUC=area under the curve using the trapezoidal method, accumulation ratio= ratio of the 8-week post-injection concentration to pre-injection concentration, and the response ratio=ratio of the 1-week post-injection concentration to pre-injection concentration. Testosterone, free T, SHBG, DHT, E2, sperm concentration, and baseline FSH and LH were log-transformed prior to analysis and are summarized as geometric means. All other measures are summarized as arithmetic means, except post-treatment LH and FSH, for which medians were used for summarization since many values were at the lower limit of quantification of the assay. (Note that in the figures, for simplicity mean and SEM were shown except for serum LH and FSH levels when medians and box plots were used). Baseline subject characteristics were compared between Los Angeles and Bologna subjects with t-tests. Correlation between testis volume and other parameters were by Pearson correlation analyses. Comparison of pharmacokinetic measures over the three 8-week injection periods and between subject groups were performed with repeated measures analysis of variance (ANOVA) using period as a repeated measure and group as a classification factor, and using linear contrasts to assess trends over subsequent injection periods. Baseline BMI was added to these models to adjust group differences in pharmacokinetic measures for BMI, which tended to be greater in the Los Angeles subjects. Post-treatment FSH and LH were compared between subject groups with non-parametric Wilcoxon tests. Percentages of subjects attaining azoospermia or oligozoospermia were compared between groups with Fisher's exact tests. Trends over time in body weight, cholesterol (total, LDL and HDL),

serum chemistry, liver functions tests, hematocrit, hemoglobin, PSA, and testes volume were assessed with repeated measures ANOVA for separate subject groups.

Results

Subjects

All subjects completed the study. Summary baseline demographic, clinical, and hormonal characteristics of the subjects at the time of randomization are shown in **Table 1**. All parameters were within the normal range. Mean body weight and BMI were significantly higher in the subjects in Los Angeles. Mean serum free T and LH levels, were significantly higher in the subjects in Bologna, and mean sperm concentration and testes volume were significantly higher in the subjects in Los Angeles; all other baseline hormone levels and semen parameters were similar between the two groups. It is well recognized that measurement of testis volume using orchidometers has large inter- and intrar-observer variances and may be dependent on the experience of the observers (Behre, Nashan, and Nieschlag, 1989) The difference in the mean testes volume, though significant, might be due to a systematic measuring difference between the two centers. On further analyses the participants in this study showed pre-treatment positive associations of sperm concentration (and also total sperm count per ejaculate) with testes volume (Pearson correlation>0.48; $p<0.002$) and with abstinence time (Pearson correlation>0.29; $p<0.06$), and that larger men had larger testes (BMI-testes volume Pearson correlation=0.39; $p=0.01$). However, these subject characteristics were not at all associated with treatment effect. For example, mean testes volume, BMI, and abstinence time were almost identical for subjects who were severely oligozoospermic

compared to those who were not at 24 weeks (45.7 vs. 46.5 ml, 26.6 vs. 26.7 kg/m^2, and 2.5 vs. 2.5 days, respectively; t-tests p>0.65).

Serum Testosterone

Fig. 1. (**top panel)** shows serum T concentrations after injections of TU 750 mg or 1000 mg IM alone or with NET-E 200 mg im every 8 weeks. The maximum (C_{max}) serum T concentrations and area under the serum T curve (AUC) were similar between the 750 and 1000 mg dose irrespective of whether TU was administered alone or with NET-E (**Table 2**). The average concentrations of serum T (C_{avg}) were higher in the TU 1000 mg compared to 750 mg group when administered alone after the second (p=0.03) and third injections (p=0.01) (**Table 2**). Mean C_{avg} and AUC of serum total T increased steadily over the three periods for the 1000 mg dose TU groups irrespective of whether NET-E was co-administered (p≤0.02). These parameters did not significantly increase over injection periods for the 750 mg dose groups, although a similar trend was present. Note from figure 1 that the mean serum T levels 8 weeks after the first TU injection were lower than pre- first injection (baseline levels); the mean pre- and 8 weeks post-second injection serum T levels were approximately equal; and the mean serum T concentrations 8 weeks after the third injection was greater than pre-third injection levels for both 750 mg dose groups and in the 1000 mg TU + NET-E group. Thus, mean accumulation ratios (defined as the ratio of serum T level at 8 weeks post injection to pre-injection level) significantly increased with subsequent injections for all groups except the TU 1000 mg alone group.

Serum Free T

The serum free T levels mimicked the serum total T levels (**Fig. 1, lower panel**). There were no significant differences in mean C_{avg}, C_{max}, and AUC for free T between the two dose groups with only TU, or between the two TU +NET-E groups. The mean immediate response ratios significantly increased with each injection in the TU +NET-E groups ($p \leq 0.03$), with similar, but non-significant, trends for the TU only groups. The C_{avg} ($p<0.01$) , AUC ($P<0.02$) and accumulation ratio ($p<0.01$) increased significantly with repeated injections for both TU +NET-E groups, with similar, but non-significant, trends for the TU only groups. TU alone and TU + NET-E groups did not differ significantly, with the following exceptions. The mean C_{avg} for free T was significantly greater for TU + NET-E compared with TU alone after each 1000mg TU injection ($p \leq 0.03$) and after second ($p<0.04$) and third $p<0.01$) 750mg TU injections, and mean AUC for TU + NET-E was significantly greater than TU only groups after the second and third injections ($p<0.02$). These differences (except Cavg after the second injection) in serum free T parameters between the TU alone vs. TU + NET-E were markedly attenuated to become non-significant after adjustment for BMI, which tended to be greater in the TU groups in Los Angeles.

Serum DHT and E_2

Serum DHT (**Fig. 2, upper panel**) and E_2 (**Fig. 2, lower panel**) levels paralleled those shown by serum total T concentrations. There were no significant differences in mean serum DHT (C_{avg}) and DHT AUC between the two doses of TU when TU was administered with NET-E ($p>0.37$), but were greater in the 1000 mg TU group compared to the 750 mg TU group without NET-E after the second ($p<0.05$) and third injection ($p<0.005$). There were no significant differences in mean serum E_2 C_{avg} and E_2 AUC

between the two doses of TU when TU was administered without NET-E ($p>0.28$), but were greater with the 1000 mg TU group compared to the 750 mg TU group with concurrent NET-E administration after the third injection ($p<0.05$), but not with the first two injections ($p>0.15$).

Serum SHBG

Fig. 3 shows that there were no significant differences in the time course of serum SHBG levels between the two doses of TU whether or not NET-E was given concurrently ($p>0.24$). Serum SHBG was not significantly suppressed with TU alone ($p=0.20$). AS anticipated from our knowledge of androgenic progestin effects on SHBG levels, serum SHBG levels were significantly ($p<0.0001$) suppressed to an average of 58% and 61% of baseline at 4 weeks after each injection of 1000 and 750 mg of TU + NET-E respectively.

Serum Gonadotropins

The changes in serum gonadotropins (median with 25 and 75 percentiles shown in the box plots) are shown in **Fig. 4 a and b.** At 4 weeks after the first injection median serum LH concentrations were suppressed below 0.6 IU/liter and reached 0.1 IU/liter after the second and the third injections of either 750 and 1000mg of TU. Median serum LH levels rebounded after the first and second injections in both TU 750 mg and 1000 mg alone groups though the rebound became less with each injection. Addition of NET-E induced suppression of LH to median levels of 0.1 IU/liter 4 weeks after each injection in both TU dose groups. Median serum LH remained suppressed to this very low level after the second injection in the TU 1000 mg +NET-E group but not in the TU 750mg +NET-E group. There were no significant differences in serum LH levels between TU

1000 and 750mg dose groups used alone or with NET-E, except at week 16 for the NET-E groups, when the 1000 mg dose had a significantly (p=0.02) lower median LH than the 750 mg dose. Serum FSH followed a similar pattern as serum LH (**Fig. 4b**.). Median serum FSH was suppressed at 4 weeks and rebounded at 6 to 8 weeks after each injection. Only in the TU 1000mg +NET-E group were median serum FSH levels persistently suppressed to 0.1 IU/liter from week 20 onwards. Median serum FSH levels were lower ($p \leq 0.06$) in the TU 1000 mg +NET-E when compared to TU 750 mg +NET-E group at all time points. Serum FSH were similar ($p \geq 0.12$) at all times for TU 1000 mg and TU 750 mg groups.

Sperm Concentration

Sperm concentrations fell significantly in all subjects. All subjects recovered to over 20 million /ml **(Fig. 5)**. Median 24 week sperm concentrations were zero in both 1000mg TU groups (though three subjects in the TU 1000 mg only group had sperm concentration over 20 million/ml), and 1.41 and 0.10 million/ml for the 750 mg TU and 750mg TU + NETE-E groups , respectively (p=0.46). The median time of recovery to 20 million/ml was week 40 (24 weeks post-third dose) in the TU alone groups and also week 40 in the TU +NET-E groups. **Fig. 6** shows the percentages of subjects with sperm concentration suppressed to 0 and < 1 million/ml. At some time during treatment, 3/10 and 5/10 subjects in the TU 750 mg group and 6/10 and 8/10 in the TU 1000 mg group achieved azoospermia or < 1 million/ml respectively. Whereas, 5/10 and 7/10 in the TU 750 mg + NET-E and 7/10 and 10/10 subjects in the TU 1000 mg +NET-E group achieved azoospermia and < 1 million/ml respectively. Because the study is not

powered to examine differences in suppression of spermatogenesis, the differences between the groups were not statistically significant.

Safety parameters and adverse events

There were no significant changes serum chemistry and liver functions tests in all four groups of subjects. Serum total and LDL cholesterol did not change in all treatment groups whereas in both NET-E groups, but in neither TU only groups. Mean serum HDL cholesterol decreased during treatment and partially rebounded during recovery for the TU 1000 mg + NET-E group (p=0.0002) and for the TU 750 mg + NET-E group (p=0.01) (**Table 3**). In the TU 750 mg NET-E group, serum calcium decreased during treatment: pre-treatment, 12 week, and 24 week respectively (p=0.004). The changes in calcium levels were very small not clinically significant. TU 750 mg administered every 8 weeks alone or with NET-E did not result in significant increases in hematocrit or hemoglobin. In contrast, significant increases in hematocrit and hemoglobin were observed in both the TU 1000 mg alone group (p=0.01) and TU 1000 mg + NET-E group (p=0.006) (**Table 3**). Hemoglobin followed the same trend with mean increases of 0.7 (p=0.005) and 1.0 g/dl (p=0.01) in the TU 1000 mg alone or with NET-E groups respectively. There was one serious adverse event of Penicillin hypersensitivity, which was considered to be unrelated to drug exposure. Three subjects complained of transient pain and swelling at the injection sites. The pain was mild in severity and resolved spontaneously with no treatment. Other side effects of androgen treatment included oily skin that were mild and required no treatment. Overall, approximately twice as many subjects gained weight as lost weight (26 gained, 2 stable, 12 lost), with a significant mean increase, although subjects were very heterogeneous in their weight changes

(mean ± SD = 1.7±3.7 Kg; $p<0.05$). There were no significant differences according to dose or center/use of progestin or their interaction (ANOVA $p>0.15$). Specifically, mean (range) weight changes were 1.7 (-7.7 to 6.9), 1.4 (-4.0 to 7.0), 0.49 (-2.8 to 5.1), and 3.4 (-4.0 to 11.0) Kg for 750 mg TU , 750mg TU +NET-E, 1000 mg TU, and 1000 mg TU +NETE-E groups respectively. None of volunteers developed gynecomastia, prostate enlargement estimated by digital rectal examination, significant changes in urine flow or increases in serum PSA levels. Changes in sexual function or mood were not reported. Mean testes volume decreased from baseline to 12 weeks to 24 weeks in both the TU without NET-E group (52.3 ± 1.7, 48.5 ± 2.2, and 47.6 ± 2.4 ml, respectively; $p=0.01$) and in the TU + NET-E group (39.9 ± 0.41, 38.3 ± 0.50, and 37.4 ± 0.70 ml, respectively; $p=0.0005$).

Discussion

In this study, we determined pharmacokinetics of TU injections administered at 750 and 1000 mg im either alone or in combination with NET-E every 8 weeks for three injections in healthy male volunteers. The study was initiated in Los Angeles and because NET-E is not available in the United States, the Bologna center joined the study for the groups being administered the combination of TU and NET-E using an identical protocol to that in Los Angeles. This study was done to determine the optimal dose of TU to be used in combination with 8 weekly injections of NET-E in a planned late phase 2 contraceptive efficacy trial involving a relatively large number of couples. The goal was to achieve optimal suppression of gonadotropins and spermatogenesis with the lowest possible amount of T to be delivered to the body to maintain eugonadal

state while enhancing the effect of NET-E on gonadotropin and spermatogenic suppression. The duration of 8 weeks was chosen because pharmacokinetics of NET-E in prior studies in women (Fotherby et al, 1984; Sang et al, 1981) suggested that a longer interval might result in inadequate level of NET for suppression of gonadotropins. Moreover, a previous preliminary report showed that NET-E 200 mg administered with TU 1000 mg at eight weekly intervals induced a profound sperm suppression that was not maintained when the injection interval was extended to 12 weeks (Meriggiola et al, 2005). Though serum T levels had been studied in eugonadal subjects (Kamischke et al, 2000; Zhang et al, 1999) after administration of TU 1000 mg and 500 mg injections every 4 to 6 weeks, the dose of 750mg has never been administered before to normal or hypogonadal men and detailed pharmacokinetics were not available for TU 1000 mg administered im every 8 weeks in healthy men.

We showed that there were no significant differences in C_{max} and AUC between the two doses of TU injections irrespective of whether the TU was given concurrently with NET-E. The C_{avg} for serum T and DHT was significantly higher in the TU 1000mg group when administered alone after the second and third injections but this was not observed when NET-E was added. . For both doses there was an accumulation of serum T after each injection which was more pronounced when NET-E was given in addition to the TU injections. Linear increases in $C_{avg,,}$ AUC and immediate response ratios suggested there was accumulation of T with both doses but more with the 1000mg dose. The accumulation of serum T was relatively small as the serum T level at week 24 (8 weeks after the third injection) was not significantly different from baseline levels in all

treatment groups. Subtle differences in the pharmacokinetic measures might not have been detected in this study because of the small group size of ten men. In our experimental paradigm, no loading dose of TU was administered. This resulted in lower serum T levels at 8 weeks after the first injection compared to pre-injection baseline. The pre-dose serum T levels rose after each injection to reach baseline levels by the third injection. Because of this characteristic of TU, a loading dose may prevent the serum T levels falling to below baseline before the next scheduled injection. The recommended dose of TU for androgen replacement in hypogonadal men by the manufacturer of TU (package insert for Nebido® injections) is to give a second 1000 mg of TU 6 weeks after the initial TU 1000 mg injection and then followed by maintenance injections at 12 weekly intervals (Jockenhovel, 2004; Qoubaitary, Swerdloff, and Wang, 2005). Furthermore, the reported contraceptive efficacy trial in China also employed a loading dose of 1000 mg followed by 500 mg TU every 4 weeks (Gu et al, 2003). Our study did not include a loading dose of TU with the intention to keep the proposed hormonal contraception regimen as simple as possible for the proposed large multicenter study. Serum free T followed the same pattern as serum T. Apparent higher serum free T levels were detected in the group where TU was administered with NET-N. One reason for this difference could be due to the suppressed SHBG levels occurring after NET-E administration resulting in more free T in the groups administered the progestin in addition to the androgen. In this study serum total T, however, was not different between the groups receiving TU alone or TU+ NET-E where the more suppressed SHBG should result in a lower serum total T level in the TU +NET-E group. Subjects were not randomized to whether NET-E was administered, and thus TU +

NET-E vs. TU only group differences may be attributable to subject differences as well as to the effect of NET-E, and this confounding can be only partially examined with statistical adjustment. When we examined the subjects in Los Angeles (TU alone) and Bologna (TU +NET-E), we noted that while their mean height was not different but the body weight and BMI were significantly greater in the men in Los Angeles and their baseline free T levels were lower. The baseline free T levels were significantly higher in the Italian men. The Italian subjects had lower body weight and BMI but they were healthy and not undernourished, whereas the subjects in Los Angeles were generally heavier. It is well known that higher body weight and BMI are inversely related to total serum T and free T (Gapstur et al, 2002; Glass et al, 1977; Jensen et al, 2004; Vermeulen, Kaufman, and Giagulli, 1996). Such differences in serum total T levels have recently been reported in a prior study utilizing testosteterone and levonorgestral implants between leaner men in Nanjing, China and heavier non-Asian men in Los Angeles (Wang et al, 2006). When statistical adjustment for subject differences in BMI was made, the significance of the differences were attenuated and BMI largely explained the differences in free T Cavg levels between the Los Angeles and Bologna subjects after all three injections of the 750 mg TU dose ($p>0.79$), but not for the 1000 mg dose ($0.03\leq p\leq 0.08$). The remaining differences could be related to the more significant suppression of SHBG in those receiving TU+NET-E, an androgenic progestin.

At baseline serum LH was higher in the subjects in Bologna despite a higher serum free T level. The reason for this difference between the subjects is not clear and is probably not clinically significant. The subjects in Bologna had lower mean testes

volume and mean sperm concentration than the subjects in Los Angeles. The difference in testes volume may be due to variances in measurement by different observers. However analyses showed significant positive correlations between sperm count, total sperm count, BMI and testis volume indicating that the observed differences are influenced by body size and spermatogenic rate. Such associations had been previously reported in many ethics groups (Aribarg et al, 1986; Handelsman et al, 1984; Ku et al, 2002)It has also been reported both in Europe and in the United States that geographical differences in sperm concentration do occur (Jorgensen et al, 2001; Jorgensen et al, 2002; Swan et al, 2003). Despite no apparent differences in pharmacokinetics were found between the two doses of TU, the suppression of both gonadotropins to very low levels was significantly better achieved by the TU 1000 mg both with and without NET-E. Only in the group receiving TU 1000mg + NET-E were the gonadotropins persistently suppressed after the second injection to levels that were close to the limit of detection. As a corollary to the more persistent gonadotropin suppression, TU 1000 mg + NET-E 200 mg administered every 8 weeks led to the consistent suppression of sperm concentration to $< 1 \times 10^6$ / ml in all subjects at 24 weeks of treatment, This dose, however, as discussed above caused some accumulation of serum total and free T levels though serum T levels at the end of treatment were similar to those at baseline. The higher dose of TU 1000 mg every 8 weeks also resulted in a linear trend for increases in hematocrit and hemoglobin by a small amount which remained within the physiological range of adult healthy men. There was mild weight gain which was not significantly different among the treatment groups. The lower dose of TU 750 mg + NET-E 200 mg maintained T levels within the

physiologic range, however, serum FSH and LH levels rebounded 6 to 8 weeks after each injection. Fewer subjects achieved suppression of sperm concentration < 1 million/ml at 24 weeks of treatment. The differences in sperm suppression may become less apparent with more prolonged use of TU + NET-E, however, during the 6 months of treatment in this study the suppression of spermatogenesis with the lower dose would generally be considered inadequate for male contraception. One may also suggest that increasing the dose of NET-E may blunt this gonadotropin rebound. Previous studies testing the dose of NET-E 400 mg every 8 weeks did not offer an advantage in spermatogenic suppression over NET-E 200 mg (Kamischke et al, 2002). We noted that the TU and NET-E injections were well tolerated by the subjects during the study period. TU alone did not cause any changes in serum cholesterol levels but addition of NET-E resulted in statistically significant suppression of HDL-Cholesterol as reported for other androgenic progestins such as levonorgestrel (Anawalt et al, 1999; Kamischke et al, 2001; Wu et al, 1999). Only three subjects expressed some mild and transient pain and swelling at the injection site after a 4 ml injection. There were no clinical significant adverse effects related to the testosterone during the study.

We conclude that the detailed pharmacokinetics analyses of TU injections, given at 750 mg and 1000 mg every 8 weeks for three injections showed no detectable dose response difference in normal volunteers. The higher dose of TU 1000 mg may result in more accumulation of T though the serum level was not different from baseline after three injections. We only examined a course of three injections, so accumulation may become more pronounced with a more long term regimen of injections every 8 weeks resulting in serum T concentrations towards the upper half of the adult male range. The

higher dose also resulted in elevated hematocrit which remained in the physiological range. However in view of the more consistent suppression of gonadotropins without rebound and consequently more inhibition of spermatogenesis, we recommend that the phase 2 studies should consider using TU 1000 mg with NET-E every 8 weeks to attain optimal efficacy. During the treatment duration, pre-injection serum T levels and red cell parameters should be monitored to assess whether changes in these parameters are persistent. The alternative of administering 750 mg TU every 6 weeks or using a loading dose of TU 1000 mg followed by maintenance with 750 mg was not tested in the study or TU 1000 mg every 10 weeks was not considered because of the known pharmacokinetics of the accompanying NET-E necessitating injections every 8 weeks.

Acknowledgments

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Legend for the Figures

Fig. 1. Serum total (top panel) and Free T (bottom panel) levels in the subjects. Subjects in Los Angeles were administered TU 1000 or 750 mg every 8 weeks for three injections and subjects in Bologna received NET-E 200 mg at the same time as the TU injections.

Fig. 2. Serum DHT (top panel) and E_2 (bottom panel) levels in the subjects administered TU alone or TU with NET-E. .

Fig. 3. Serum SHBG levels in the subjects administered TU alone or TU with NET-E..

Fig. 4. Serum LH (Fig. 4a.) and FSH (Fig. 4b.) levels in the subjects administered TU alone or TU with NET-E. The line within the box represents the median, the box the 25^{th} and 50^{th} percentiles and the whiskers 10^{th} and 90^{th} percentiles.

Fig. 5. Sperm concentrations in subjects administered TU alone or TU with NET-E. .

Fig. 6. Percentage subjects achieving azoospermia or severe oligozoospermia (< 1million/ml) after administration of TU alone or TU with NET-E.

508

Table 1. Baseline clinical and biochemical parameters of subjects

	Los Angeles		Bologna		
	Mean	**Range**	**Mean**	**Range**	**p-value**
Age (years)	31.7	24 – 47	33.5	20 – 46	0.42
Weight (kg)	91.5	55 – 125	76.4	60 – 96	0.004
Height (cm)	177	167 – 184	178	166 – 192	0.66
BMI (kg/m^2)	29.1	19.8 – 39.5	24.1	20.2 – 31.1	0.001
Serum T (nmol/liter)	16.1	5.4 – 31.4	19.0	12.3 – 30.3	0.13
Free T (nmol/liter)	0.26	0.10 – 0.43	0.34	0.18 – 0.66	0.02
SHBG (nmol/liter)	27.9	13.5 – 67.9	30.3	12.5 – 62.6	0.54
DHT (nmol/liter)	3.62	1.26 – 7.62	4.29	1.27 – 14.2	0.33
E_2 (pmol/liter)	159	128 – 257	156	104 – 262	0.75
FSH (IU/liter)	2.68	1.21 – 6.86	2.91	1.12 – 4.88	0.56
LH (IU/liter)	2.86	1.31 – 5.63	4.07	1.52 – 7.89	0.01
Testes Volume (ml)	52.3	40 – 70	39.9	36 – 44	<0.0001
Sperm Concentration (million/ml)	80.7	16 – 182	40.4	21 – 102	0.0002

Table 2. Mean pharmacokinetic parameters for serum T after TU injection with or without NET-E injections co-administered at weeks 0, 8, and 16.

	TU 1000 mg	TU 750 mg	TU 1000 mg + NET-E 200 mg	TU 750 mg + NET-E 200 mg
C_{avg} (nmol/liter)				
0-8 weeks	17.9	14.8	16.7	15.9
8-16 weeks	19.6*	14.5*	18.0	17.0
16-24 weeks	21.1*	15.8*	20.0	18.4
p-value for trend	0.01	0.20	0.02	0.06
C_{max} (nmol/liter)				
0-8 weeks	32.4	29.8	27.0	25.0
8-16 weeks	34.5	32.2	38.1	29.5
16-24 weeks	37.2	31.5	28.6	28.7
p-value for trend	0.21	0.66	0.52	0.26
AUC (nmol•wk /l)				
0-8 weeks	154.4	140.4	136.7	138.4
8-16 weeks	175.5	149.2	154.3	152.6
16-24 weeks	185.2	155.7	163.7	160.3
p-value for trend	0.02	0.16	0.01	0.08
Accumulation Ratio				
Wk 8 / Wk 0	0.92	0.82	0.72	0.72
Wk 16 / Wk 8	0.92	0.90	1.00	1.06
Wk 24 / Wk 16	1.04	1.11	1.27	1.23
p-value for trend	0.33	0.03	<0.0001	0.003
Response Ratio				
Wk 1/Wk 0	1.85	1.88	1.26	1.25
Wk 9/Wk 8	2.26	2.33	2.11	2.08
Wk 17/Wk 16	2.65	2.57	1.82	1.66
p-value for trend	0.03	0.14	0.06	0.02

* $p<0.05$ for TU 1000 mg vs. TU 750 mg.

Table 3. Safety parameters after TU and NET-E injections

Measurement (units)	Visits	Los Angeles (TU Only)		Bologna (TU+NET-E)	
	Week	750 mg	1000 mg	750 mg	1000 mg
Serum Calcium (mmol/liter)					
	Screen	2.35±0.02	2.38±0.02	2.34±0.03	2.33±0.04
	12	2.35±0.03	2.34±0.03	2.26±0.02	2.30±0.03
	24	2.33±0.02	2.37±0.02	2.35±0.03	2.32±0.04
	32	2.33±0.04	2.33±0.03	2.34±0.03	2.32±0.02
Serum Total Cholesterol (mmol/liter)					
	Screen	5.20±0.33	4.83±0.19	4.50±0.38	4.65±0.32
	12	5.36±0.40	5.21±0.26	4.49±0.34	4.13±0.32
	24	5.23±0.32	5.04±0.25	4.62±0.34	4.42±0.29
	32	5.42±0.34	5.15±0.19	4.59±0.38	4.55±0.28
Serum HDL Cholesterol (mmol/liter)					
	Screen	1.13±0.06	0.98±0.08	1.45±0.11	1.35±0.08
	12	1.10±0.06	1.03±0.11	1.24±0.07	1.15±0.06
	24	1.12±0.09	1.02±0.12	1.38±0.11	1.24±0.06
	32	1.13±0.07	1.04±0.12	1.41±0.11	1.40±0.07
Serum LDL Cholesterol (mmol/liter)					
	Screen	3.55±0.29	3.23±0.18	2.59±0.30	2.75±0.31
	12	3.77±0.37	3.64±0.29	2.89±0.27	2.49±0.29
	24	3.58±0.26	3.40±0.25	2.71±0.23	2.61±0.25
	32	3.71±0.30	3.39±0.22	2.70±0.33	2.63±0.29
Hematocrit (liter/liter)					
	Screen	0.44±0.006	0.45±0.007	0.44±0.009	0.43±0.009
	12	0.46±0.010	0.46±0.004	0.43±0.011	0.45±0.017
	24	0.45±0.009	0.47±0.005	0.45±0.014	0.46±0.015
	32	0.44±0.010	0.45±0.006	0.45±0.012	0.44±0.014
Hemoglobin (g/liter)					
	Screen	150.8±2.3	152.4±2.7	151.7±2.9	143.4±4.6
	12	155.0±3.7	155.4±2.1	147.5±4.2	149.4±6.6
	24	152.6±3.7	159.7±2.2	154.6±4.8	153.0±5.9
	32	149.7±3.8	153.0±2.4	154.2±4.1	149.2±6.1
Serum PSA (ug/liter)					
	Screen	0.56±0.06	0.56±0.10	0.69±0.12	0.68±0.10
	12	0.93±0.19	0.64±0.11	0.84±0.13	0.92±0.17
	24	0.61±0.08	0.58±0.09	0.74±0.13	1.05±0.32
	32	0.57±0.06	0.77±0.24	0.72±0.13	0.78±0.14

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Fig. 1

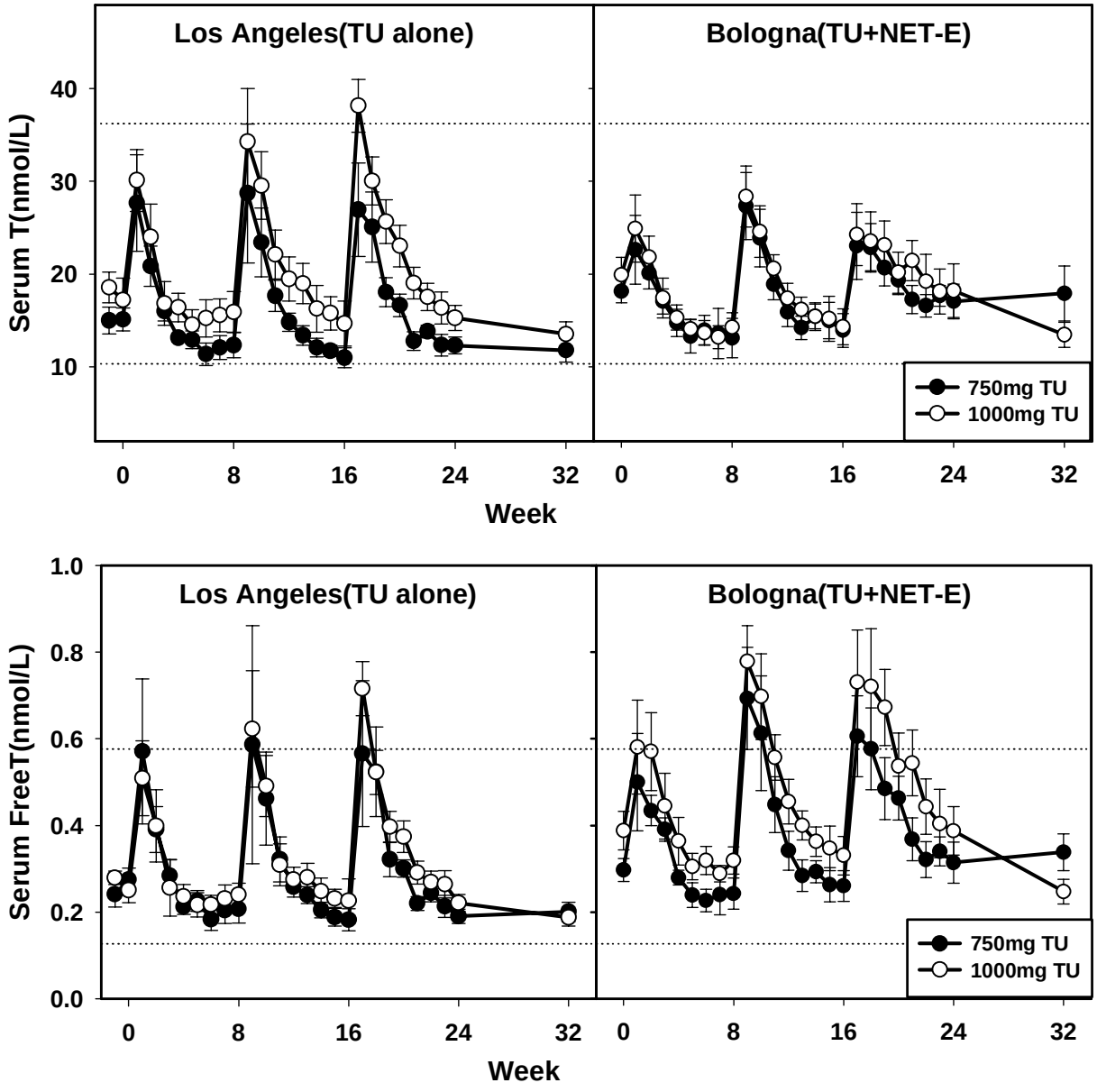

Los Angeles(TU alone)
Bologna(TU+NET-E)
Serum T(nmol/L)
40
30
20
10
0
8
16
24
32
Week
750mg TU
1000mg TU
Los Angeles(TU alone)
Bologna(TU+NET-E)
Serum FreeT(nmol/L)
1.0
0.8
0.6
0.4
0.2
0.0
0
8
16
24
32
Week
750mg TU
1000mg TU

Fig. 2

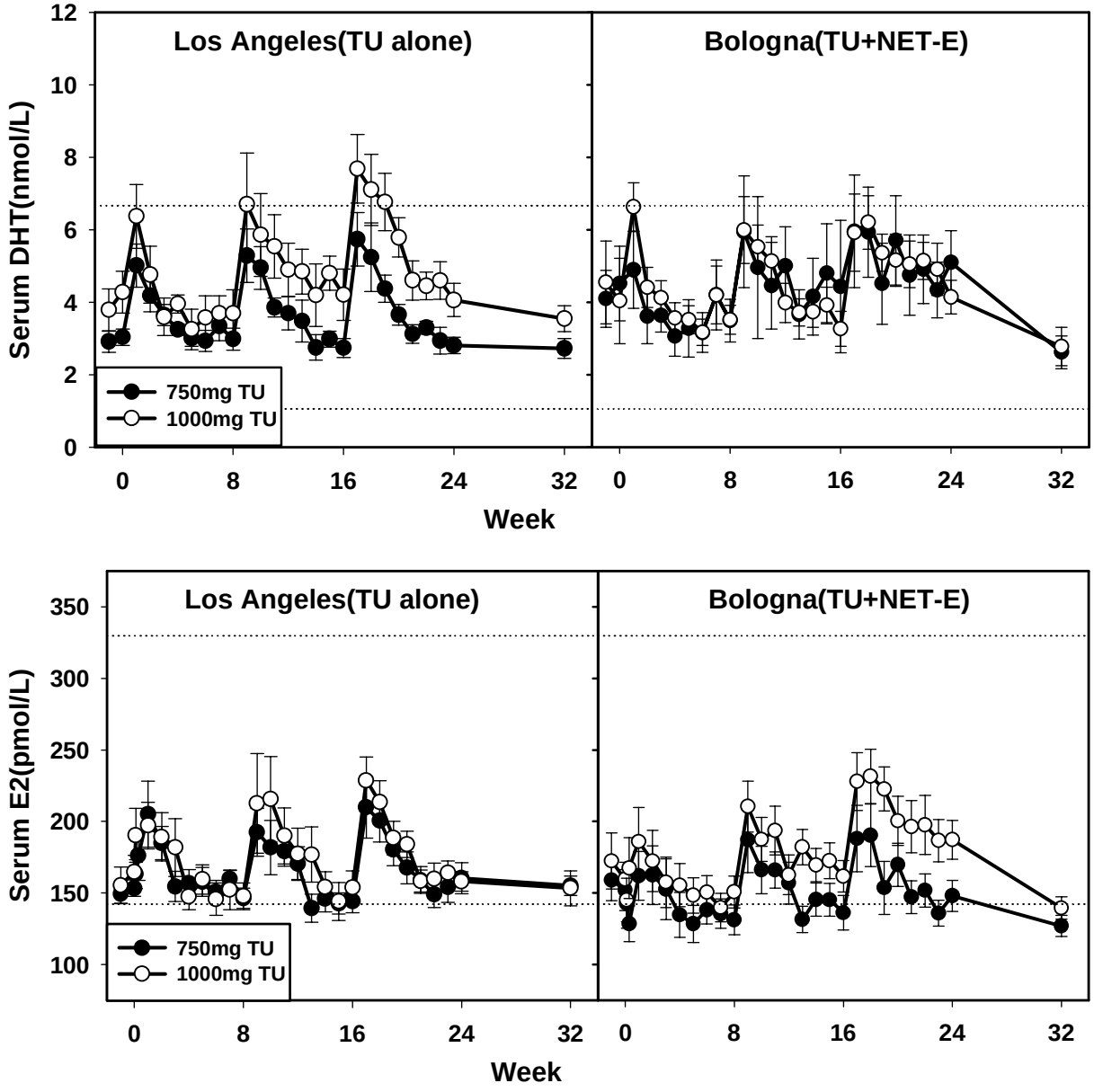

Los Angeles(TU alone)
Bologna(TU+NET-E)
Serum DHT(nmol/L)
750mg TU
1000mg TU
Week
Los Angeles(TU alone)
Bologna(TU+NET-E)
Serum E2(pmol/L)
750mg TU
1000mg TU
Week

Fig. 3

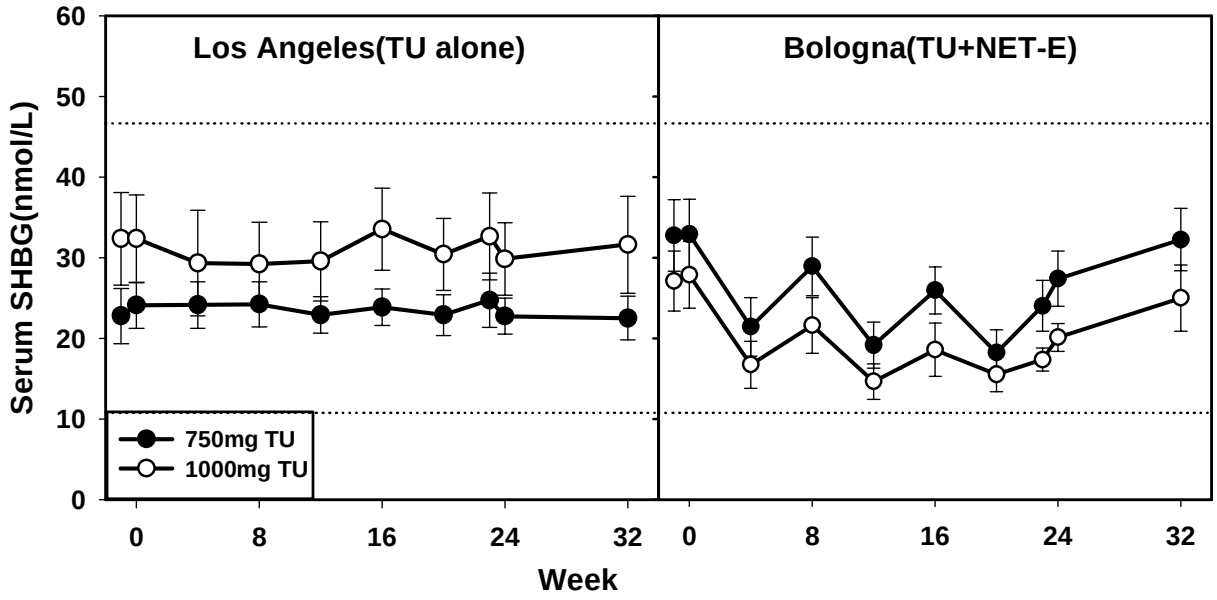

Los Angeles(TU alone)
Bologna(TU+NET-E)
Serum SHBG(nmol/L)
60
50
40
30
20
10
0
750mg TU
1000mg TU
0
8
16
24
32
Week

Fig. 4a.

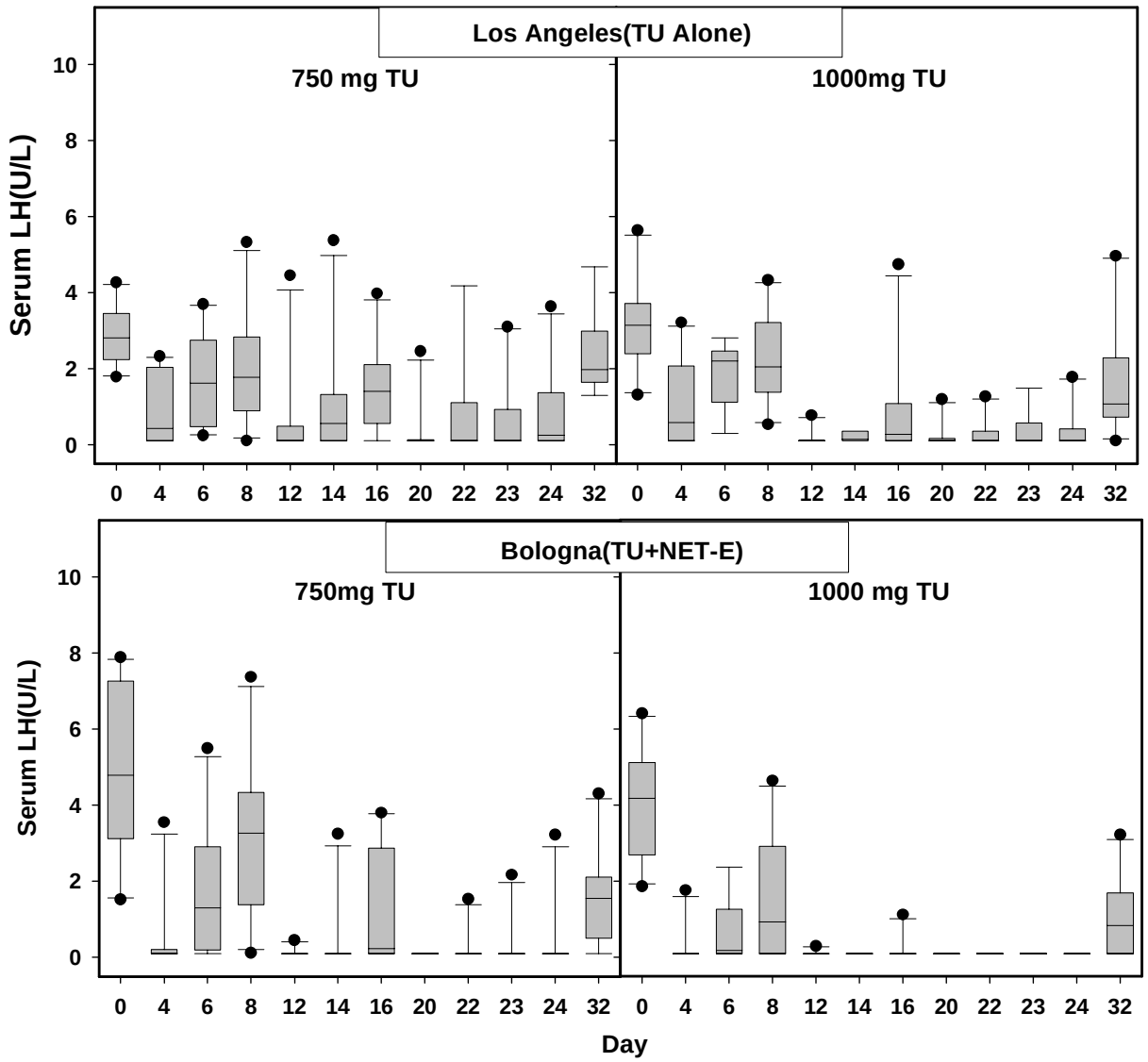

Los Angeles(TU Alone)
750 mg TU
1000mg TU
Serum LH(U/L)
Bologna(TU+NET-E)
750mg TU
1000 mg TU
Day

Fig. 4b.

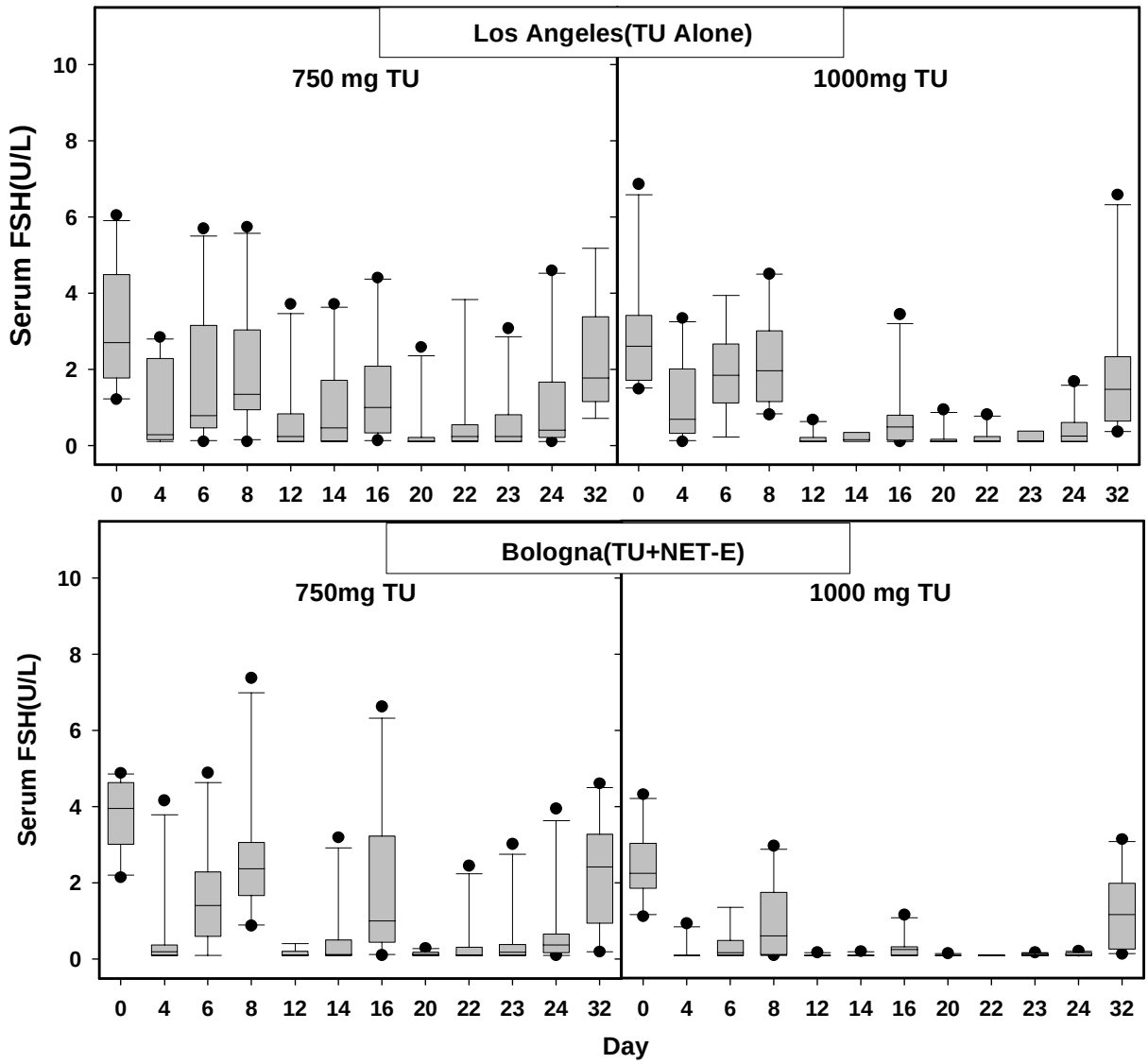

Los Angeles(TU Alone)
750 mg TU
1000mg TU
Serum FSH(U/L)
0
2
4
6
8
10
0 4 6 8 12 14 16 20 22 23 24 32 0 4 6 8 12 14 16 20 22 23 24 32
Bologna(TU+NET-E)
750mg TU
1000 mg TU
Serum FSH(U/L)
0
2
4
6
8
10
0 4 6 8 12 14 16 20 22 23 24 32 0 4 6 8 12 14 16 20 22 23 24 32
Day

Fig. 5

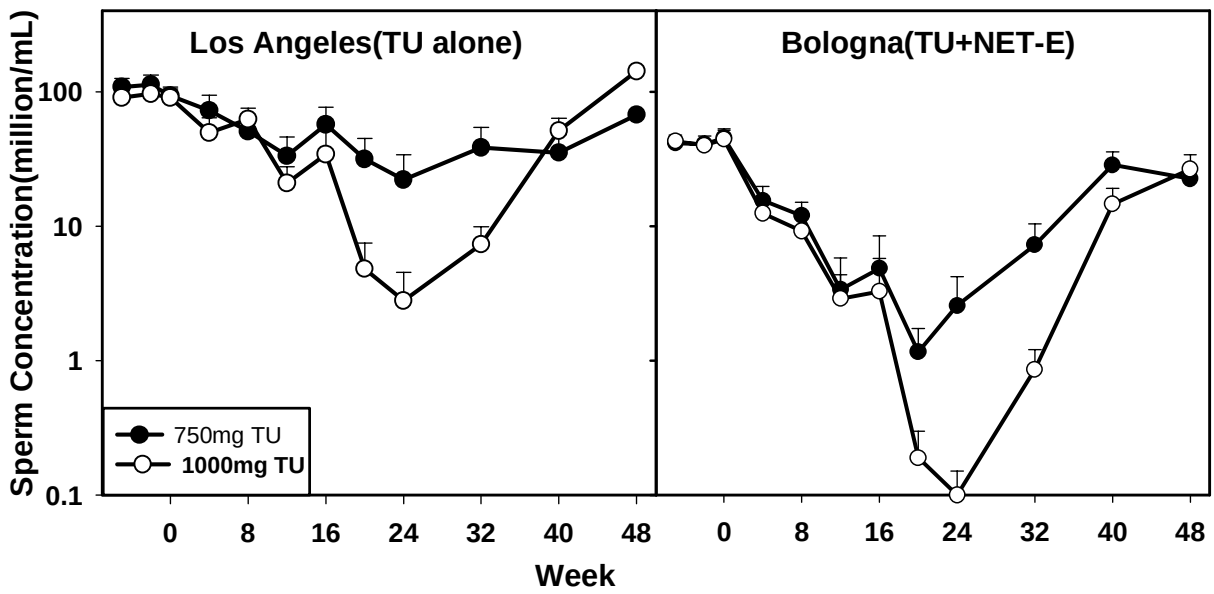
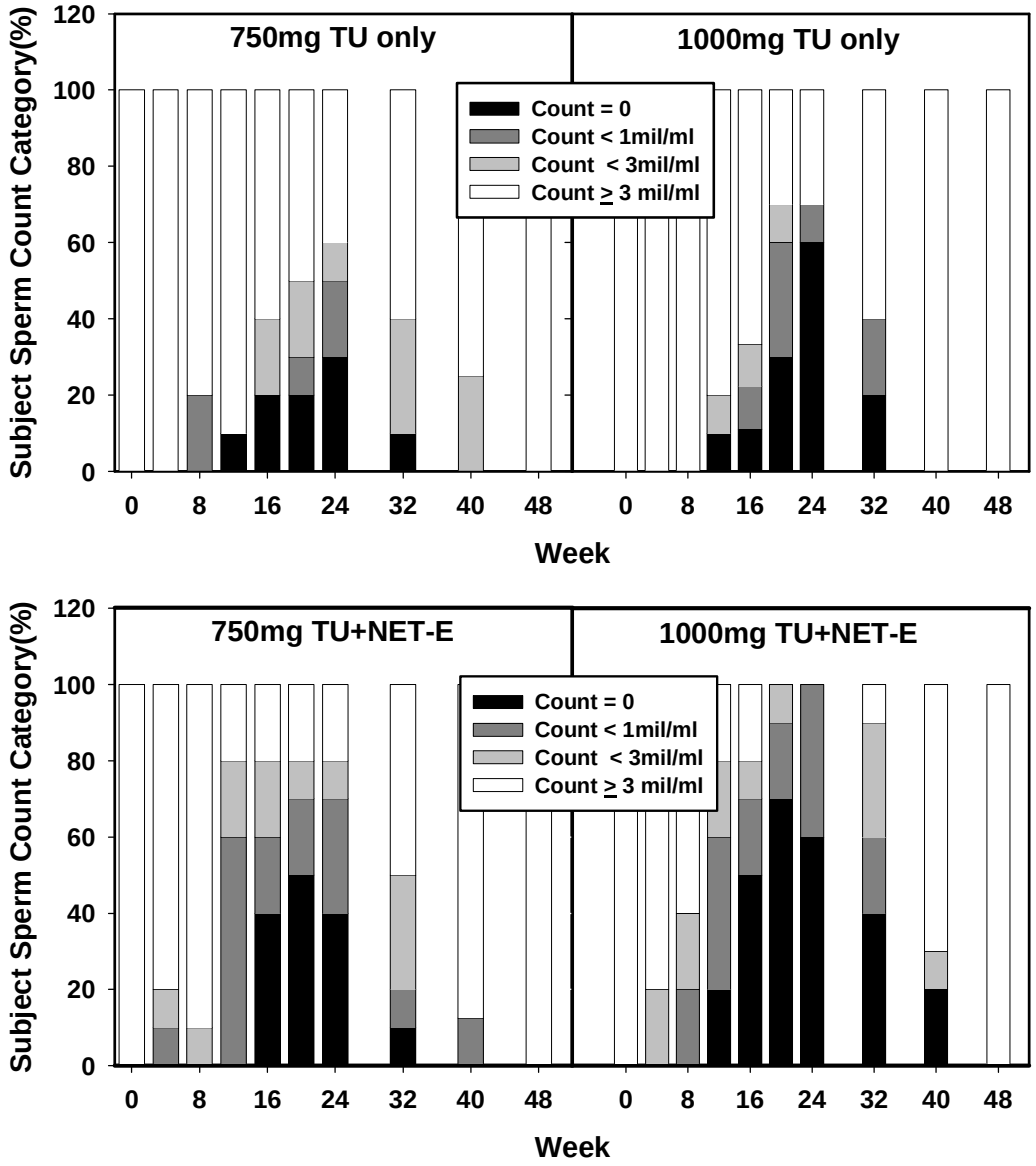

Los Angeles(TU alone)
Bologna(TU+NET-E)
Sperm Concentration(million/mL)
100
10
1
0.1
750mg TU
1000mg TU
0
8
16
24
32
40
48
Week

Fig. 6


750mg TU only
1000mg TU only
Count = 0
Count < 1mil/ml
Count < 3mil/ml
Count ≥ 3 mil/ml
Subject Sperm Count Category(%)
120
100
80
60
40
20
0
0
8
16
24
32
40
48
Week
750mg TU+NET-E
1000mg TU+NET-E
Count = 0
Count < 1mil/ml
Count < 3mil/ml
Count ≥ 3 mil/ml
Subject Sperm Count Category(%)
Week