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ORIGINAL ARTICLE

Male Infertility

Establishing the lower limits of total serum testosterone among Chinese proven fertile men who received treatment of assisted reproductive technology

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Testosterone (T) plays a crucial role in spermatogenesis because extremely low levels of intratesticular T lead to correspondingly low serum levels of total T (tT), severe disorders of spermatogenesis, and male sterility. However, there is little consensus on the lower limits of serum tT in proven fertile men undergoing assisted reproductive technology treatments in Chinese or other Asian populations. We aimed to establish the reference range of serum tT based on a population of 868 fertile Chinese men undergoing *in vitro* fertilization or intracytoplasmic sperm injection and embryo transfer (IVF/ICSI-ET) treatments. We defined a fertile man as having had a live baby with his partner as recorded in our IVF registration system. The lower limits of serum tT were established using a Siemens IMMULITE 2000 chemiluminescent system. The 1st, 2.5th, and 5th percentiles and their 95% confidence intervals (CIs) were 3.6 (95% CI: 2.7–4.1) nmol l^{-1}, 4.3 (95% CI: 4.1–5.0) nmol l^{-1}, and 5.6 (95% CI: 4.8–5.8) nmol l^{-1}, respectively. Using the linear correlation of serum tT between the Siemens platform and a liquid chromatography–tandem mass spectrometry platform, the calculated lower limits of serum tT were also established for fertile Chinese men undergoing IVF/ICSI-ET treatments, which will benefit the clinical diagnosis and treatment of male infertility during such procedures.

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INTRODUCTION

Testosterone (T) plays a crucial role in spermatogenesis, although the underlying mechanism is not fully understood. The high concentration of T in seminiferous tubules of the testes is necessary to maintain spermatogenesis and suppress germ cell apoptosis.[1] Inhibition of the release of gonadotropins results in declining T levels and reduced sperm production.[2] Extremely low levels of intratesticular T result in severe disorders of spermatogenesis or even male sterility.[3,4]

There are challenges in the interpretation of serum total T (tT) levels. First, this measure varies between ethnic groups. There is also significant variability between laboratories and various assay techniques.[5] To date, there is no acknowledged consensus on the lower limit of serum tT or two-sided reference intervals either worldwide[6] or in China. A lower limit of 8 nmol l^{-1} (230 ng dl^{-1}) is suggested for young men who might benefit from treatment endorsed by the International Society of Andrology, the European Academy of Andrology, and the American Society of Andrology.[7] Similarly, a lower serum tT of 9.7–10.4 nmol l^{-1} was set in the guidelines from the Endocrine Society for therapeutic consideration.[8] Meanwhile, a lower limit (2.5th percentile) of 12.1 nmol l^{-1} was established in 456 healthy men tested on liquid chromatography–tandem mass spectrometry (LC-MS/MS).[9] The discrepancies in defining the lower limits of serum tT increase the difficulties in the diagnosis and treatment of hypogonadism in cases of male infertility.

With the advent of intracytoplasmic sperm injection (ICSI), *in vitro* fertilization (IVF), and embryo transfer (ET) technologies, infertile men still have the opportunity to have their own children if spermatogenesis is present in at least small regions of the testis,

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even though functions of the Sertoli cells and/or interstitial Leydig cells might be impaired. Thus, although their serum tT might be low, infertile men still have a chance to have their own children through such assisted reproductive technology treatments. However, if a man's serum tT is too low to maintain spermatogenesis, there is no need for the couple to be subjected to invasive treatments such as IVF/ICSI-ET.

Here, we aimed to define the lower limit of serum tT in fertile Chinese men who underwent IVF/ICSI-ET protocols and who successfully achieved a live birth with their partners. The research idea was derived from the World Health Organization reference values for human semen characteristics, which set the lower reference limit of semen parameters from fertile men.[10] The fertile men enrolled in that study had a successful pregnancy with their partner, with a known time-to-pregnancy (TTP) of ≥12 months.

PARTICIPANTS AND METHODS

Participants

We aimed to establish reference values based on a population of fertile Chinese men undergoing IVF/ICSI-ET treatments. Cooper *et al*.[10] defined a fertile man as having a currently or formerly pregnant partner with a known TTP of ≥12 months after stopping contraceptive use to achieving a pregnancy. Based on this definition, we defined a fertile man as having had a live baby with his partner using our assisted reproductive technology program as recorded in our IVF/ICSI-ET registration system, with a TTP of ≥30 months, thus guaranteeing that the baby was his own child.

We used the records of 6612 IVF/ICSI-ET treatment cycles from December 2015 to June 2016 collected from our IVF/ICSI-ET registration system in our reproductive center at Peking University Third Hospital, Beijing, China. According to our clinical practice, characteristics of all fresh ET cycles for the female partner were recorded daily into a computerized database by our clinical supporting staff. These included female age; body mass index; basic sex hormone levels; numbers of antral follicles; the causes of infertility; endometrial thickness on human chorionic gonadotropin (hCG) ovulation trigger day; details of the controlled ovarian stimulation protocol; numbers of previous IVF/ICSI-ET attempts; numbers of oocyte retrieved; insemination method; date of insemination; date of ET; numbers of embryos transferred; the serum concentrations of hCG on day 14 and 21 post-ET (hCG_{14} and hCG_{21}, respectively); specific fertilization results; pregnancy types, including ectopic pregnancy; biochemical pregnancy; the first-trimester abortions; ongoing pregnancies; second-trimester abortions; last-trimester abortions; live birth (or not); numbers of gestational sacs; numbers of live births; and any cause of infant mortality. Of these 6612 fresh IVF/ICSI-ET cycles, transfers which met the inclusion and exclusion criteria were selected. Inclusion criteria were: all men whose serum tT level was measured in our reproductive center, no matter whether spermatozoa were retrieved by masturbation or from surgery. Exclusion criteria were: (1) sperm donor cycles or (2) the cycles with male partners whose serum tT test results were not recorded. A total of 2621 own-sperm IVF/ICSI-ET cycles with male endocrine assessments were collected, including 878 live-birth cycles and 1743 nonlive-birth cycles. A flowchart of the data selection strategy is shown in **Figure 1**.

Among these 878 live-birth cycles, 833 men produced semen by masturbation and 45 required surgical sperm retrieval. Of these 45 men, 39 of them underwent testicular sperm extraction (TESE), 4 of them underwent microscopic-TESE, and 2 of them underwent percutaneous epididymal sperm aspiration. Of the men who underwent surgical sperm retrieval, 10 had nonobstructive azoospermia (NOA), 29 had obstructive azoospermia (OA), 3 were unable to produce semen through masturbation on the day of treatment, and 3 had high rates of morphological abnormalities in spermatozoa (teratozoospermia) (**Figure 1**). Furthermore, according to our clinical practice, each man needed to undergo karyotyping under the following circumstances: (1) the sperm concentration was $\leq 10\times10^6$ ml^{-1}; (2) his partner had suffered from two or more spontaneous abortions; (3) a previous failed pregnancy or childbirth; (4) repeated failure of IVF/ICSI-ET protocols; and (5) any abnormalities were discovered in his intelligence or phenotype. The database used in this study contains de-identified data; thus, the need for informed consent by the patients was waived and the institutional review board approval was exempted, which is in line with the Helsinki declaration.

Chemiluminescence immunoassays for measuring sex hormones

In our clinical practice, sex hormones are measured routinely in men who are suspected of having male factor or unexplained infertility. Serum follicle-stimulating hormone (FSH), luteinizing-stimulating hormone (LH), estradiol (E_2), tT, and prolactin (PRL) are measured in the morning. If the tT concentration is <8 nmol l^{-1}, it is retested next visit. Furthermore, considering the daily secretion rhythm of tT, according to our clinical routine, we take blood samples from men between 08:00 and 10:00 a.m.

Circulating tT, FSH, LH, E_2, and PRL levels were measured using a Siemens IMMULITE 2000 immunoassay system (Siemens

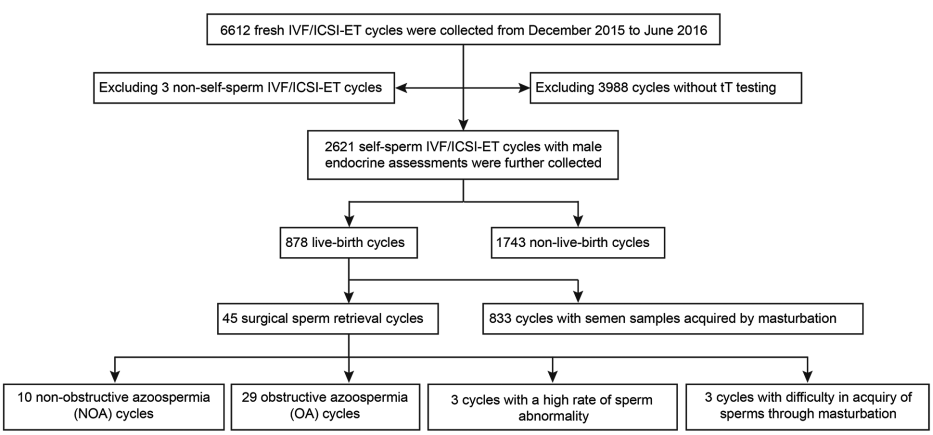

Figure 1: Strategy for patient selection. IVF/ICSI-ET: *in vitro* fertilization or intracytoplasmic sperm injection and embryo transfer; tT: total testosterone.

Healthcare Diagnostics, Shanghai, China). Quality controls used were the Lyphochek trilevel Immunoassay Plus Controls (catalogue 370; lot number 40320; Bio-Rad Laboratories, Hercules, CA, USA). Trilevel controls of the interassay coefficients of variation for tT were ≤10%; those for FSH and PRL were ≤4%; those for LH were ≤6%; and those for E2 were ≤8%.

LC-MS/MS for measuring tT

Twenty samples were tested on both the IMMULITE and LC-MS/MS systems, API4000 triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA), to compare the results for tT concentrations between the two platforms. The raw data of these results are shown in **Supplementary Table 1**. The LC-MS/MS assay for tT measurements was performed by the Clinical Mass Spectrometry Center of Guangzhou Kingmed Center for Clinical Laboratory Co., Ltd. (Guangzhou, China). The procedure included sample preparation and testing, qualitative judgments, and quantitative calculations. Details of the procedure are described in the **Supplementary Participants and Methods**.

Statistical analyses

Statistical analyses were done using JMP PRO version 14.0 software from SAS Institute Inc. (Cary, NC, USA). Normally distributed variables were presented as the mean ± standard deviation (s.d.). Nonnormally distributed variables were presented as medians and quartiles. Categorical variables were described as frequencies and percentages. A two-sided $P < 0.05$ was considered statistically significant. Boxplots were used to display the distributions of different groups showing the median, 25th percentile (Q1), 75th percentile (Q3), Q1–1.5 × interquartile range (IQR), and Q3 + 1.5 × IQR values. If Q1–1.5 × IQR and Q3 + 1.5 × IQR values were not available in the data, then minimal or maximal values are indicated in the boxplot. The serum tT levels were compared between the LC-MS/MS and Siemens IMMULITE platforms using linear regression analysis. According to the recommendation of the Clinical and Laboratory Standards Institute, a minimum reference subject number is 120 for each subgroup,[11] so the 878 cases in this study were sufficient. The Harris and Boyd partitioning method was used to determine whether age subgroup partitioning was needed.[11] The z statistic was calculated for comparing two age groups as: $z = (\overline{x}_1 - \overline{x}_2)/(s_1^2/n_1 + s_2^2/n_2)^{1/2}$. The critical value of the z statistic (z^*) was calculated as: $z^* = 3 \times (n/120)^{1/2}$, where n is the mean number of reference values in the two subgroups. If the calculated z exceeds z^*, then partitioning by age group is recommended.

The percentile method was used to establish one-sided reference intervals. The 1st, 2.5th, and 5th percentiles were established, and a bootstrap method with 1000 times repeated samplings was used to calculate the 95% confidence intervals (CIs) of these percentiles.

RESULTS

Basic characteristics

During the study, 6609 IVF/ICSI-ET cycles were collected and 2621 such cycles with male endocrine assessments tested in our own laboratory were collected. The demographic and baseline characteristics of the male partners in relation to live-birth pregnancy outcomes are shown in **Table 1.** Male partner age was the only factor linked significantly to the live-birth outcome of the couple ($P < 0.001$).

Distribution of serum tT levels in live-birth and nonlive-birth groups

Circulating tT and FSH levels are recommended to be examined during the initial endocrine evaluation of male infertility.[12,13] We hypothesized that a low level of tT would be associated with a low level of live births because the tT level in men suffering from anorchia is extremely low, often undetectable, and these patients are always infertile. However, in our study, the tT level was not significantly associated with the live-birth rate per treatment cycle. This might have been because we aimed to identify factors related to this variable per treatment cycle, and thus many fertile men were probably classified into the nonlive-birth group.

We constructed a boxplot to visualize the distribution of tT levels in each group (**Figure 2**). The minimal values were 1.0 nmol l^{-1} and 2.5 nmol l^{-1} in the nonlive-birth and live-birth groups, respectively. The lower limit of tT was significantly higher in the live-birth cycles than that in nonlive-birth cycles, indicating that too low a level of tT could not maintain spermatogenesis even in small regions of the testes.

Lower limits of serum tT in fertile Chinese men undergoing IVF/ICSI-ET cycles

Low levels of circulating tT are reported to be associated with male infertility;[7,12,13] thus, we concentrated on the lower limit of serum tT among these fertile Chinese men. It is known that tT and male fertility decrease with age;[14,15] therefore, we sought to discover whether it was necessary to group our cohort by age. We compared three subgroups aged <30, 30–39, and ≥40 years. The means, standard deviations, numbers of cases in the three groups, and the z and z^* statistics for each group are listed in **Supplementary Table 2**. However, each z statistic was less than the z^* statistic, indicating that there was no need to partition age groups. The distribution of tT levels of the three age groups is indicated in **Figure 3**. For instance, the column of 7.5–10.0 nmol l^{-1} in serum tT level accounted for the highest percentage in each age group.

Table 1: Male partners' clinical and biological data in relation to live-birth outcome among couples undergoing *in vitro* fertilization or intracytoplasmic sperm injection and embryo transfer protocols

Variable	*Live-birth*		*P*
	No (n=1743)	*Yes (n=878)*	
Age (year), mean±s.d.	34.7±6.0	32.7±4.9	<0.001
BMI (kg m^{-2}), mean±s.d.	24.2±6.8	24.6±5.9	0.200
tT (nmol l^{-1}), mean±s.d.	11.1±4.2	11.4±4.9	0.074
E_2 (pmol l^{-1}), mean±s.d.	153.0±53.7	152.2±57.8	0.610
PRL (ng ml^{-1}), median (quartiles)	7.7 (5.7–11.3)	7.8 (5.6–10.9)	0.410
FSH (IU l^{-1}), median (quartiles)	4.8 (3.4–7.1)	4.8 (3.4–6.8)	0.728
LH (IU l^{-1}), median (quartiles)	3.4 (2.3–4.9)	3.4 (2.4–5.0)	0.491

BMI: body mass index; tT: total serum testosterone; E_2: estradiol; PRL: prolactin; FSH: follicle-stimulating hormone; LH: luteinizing hormone; s.d.: standard deviation

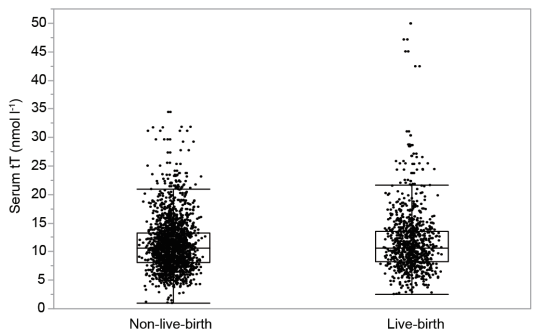

Figure 2: Boxplot displaying the distribution of serum tT levels in nmol l^{-1} in the live-birth and nonlive-birth groups of men among couples undergoing IVF/ICSI-ET treatment. IVF/ICSI-ET: *in vitro* fertilization or intracytoplasmic sperm injection and embryo transfer; tT: total testosterone.

The fertility of men undergoing surgical sperm retrieval is believed to be decreased; thus, we tried to explore whether the serum tT levels were significantly lower in fertile men whose sperm were retrieved surgically than in men who produced semen by masturbation. The medians (quartiles) of serum tT levels among men whose semen samples were retrieved by masturbation were 10.6 (8.2–13.6) nmol l^{-1}, and 12.0 (9.5–14.2) nmol l^{-1} in the men without NOA requiring surgical sperm retrieval, and 5.9 (3.5–11.0) nmol l^{-1} in the men with NOA requiring surgical sperm retrieval. The serum tT level in men with NOA suffered from surgical sperm retrieval was significantly lower than that in the other group, with *P*-value indicated in **Figure 4**. Therefore, the men with NOA were excluded for further analysis because of the heterogeneity in tT level.

We further investigated the percentiles of serum tT levels in these men with live-birth IVF/ICSI-ET cycles whose serum tT was examined using the Siemens IMMULITE 2000 chemiluminescence platform. The 1st, 2.5th, and 5th percentiles and their 95% CIs were 3.6 (95% CI: 2.7–4.1) nmol l^{-1}, 4.3 (95% CI: 4.1–5.0) nmol l^{-1}, and 5.6 (95%CI: 4.8–5.8) nmol l^{-1}, respectively (**Table 2**). As the LC-MS/MS method is acknowledged to be the gold standard for measurement of serum tT,[6,16] we discovered a linear correlation in tT values between the results of the Siemens IMMULITE 2000 chemiluminescence and the LC-MS/MS platforms, with a correlation coefficient of 0.988. If Y and X represent the tT levels produced by the LC-MS/MS and the Siemens IMMULITE 2000 chemiluminescence platforms, respectively, the linear relationship between the two was given by Y = −0.1225 + 1.298X. The calculated lower percentiles in the LC-MS/MS results are shown in **Table 2.**

DISCUSSION

To investigate male factor infertility, the ideal reference cohort is men of proven fertility.[17] Although some men are not strictly fertile via natural intercourse, they can achieve a pregnancy when they undergo IVF/ICSI-ET treatments. Therefore, the reference intervals for serum tT levels we established here could aid in the diagnosis and treatment of male infertility in Chinese or other Asian populations.

Table 2: Lower percentiles of serum total serum testosterone (in nmol l^{-1}) from the two platforms

Percentile	*Siemens IMMULITE 2000*	*LC-MS/MS*
1st (95% CI)	3.6 (2.7–4.1)	4.6 (3.4–5.2)
2.5th (95% CI)	4.3 (4.1–5.0)	5.5 (5.2–6.4)
5th (95% CI)	5.6 (4.8–5.8)	7.1 (6.1–7.4)

tT: total serum testosterone; LC-MS: liquid chromatography–tandem mass spectrometry

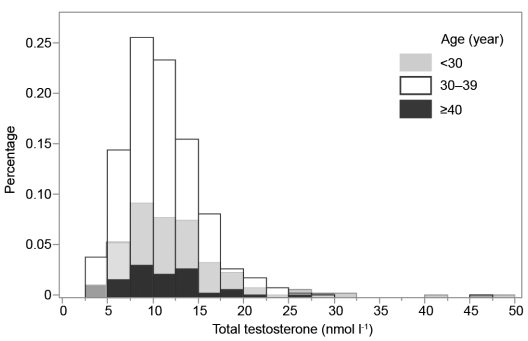

Figure 3: Distribution of serum tT levels in nmol l^{-1} in three age groups among fertile Chinese men from couples undergoing IVF/ICSI-ET treatments. IVF/ICSI-ET: *in vitro* fertilization or intracytoplasmic sperm injection and embryo transfer; tT: total testosterone.

Serum tT assessment is usually performed to discover one reason why a couple cannot conceive naturally. The guidelines suggest that circulating tT levels should be examined when male infertility is first investigated.[13,18] Reference values for tT levels combined with semen analysis and other blood values should be helpful in both clinical and research settings dealing with male infertility. Here, we collected all the serum tT values of proven fertile Chinese men in our reproductive center from December 2015 to June 2016. To our knowledge, this is the first tT reference range for such a cohort.

One-sided distributions of predictive data are preferred when the other side of the distribution is clinically irrelevant.[19] Here, a high concentration of serum tT appeared to be clinically irrelevant to male infertility, so we calculated the 1st, 2.5th, and 5th percentile lower reference limits. All the serum tT values were tested on the same specific chemiluminescence platform, thus minimizing analytical error. The corresponding tT levels from the LC-MS/MS platform calculated using a linear correlation formula given above were also evaluated. As we run one of the largest reproductive centers in China, the large variation in serum tT range we observed in fertile men undergoing IVF/ICSI-ET treatment likely reflects the biological variation of such men, or at least in northern China. Furthermore, the 5th percentiles of serum tT in the fertile men we established here, which were 5.6 nmol l^{-1} from the IMMULITE 2000 platform and 7.1 nmol l^{-1} calculated from the LC-MS/MS platform, were significantly lower than those endorsed by many guideline statements in Europe and the USA (8.0 nmol l^{-1}, or 9.7–10.4 nmol l^{-1}, or 12.1 nmol l^{-1}).[7–9] Differences between ethnic groups, enrolled populations, and examination platforms might help explain these differences.

These reference ranges of serum tT should not be overinterpreted to distinguish fertile men from infertile men accurately. Because even when the function of Leydig cells is normal, they still need the function of Sertoli cells to be normal to generate enough androgen-binding protein to transport T into the seminiferous tubules to enable spermatogenesis. Furthermore, the limits provided here might differ from those of normal healthy men whose partners have conceived spontaneously. Moreover, serum tT values are highly variable within and between men and should be combined with other clinical or laboratory information to help determine the cause of male infertility. Furthermore, it should be noted that serum tT levels within the 95% reference intervals do not guarantee fertility, and any values less than

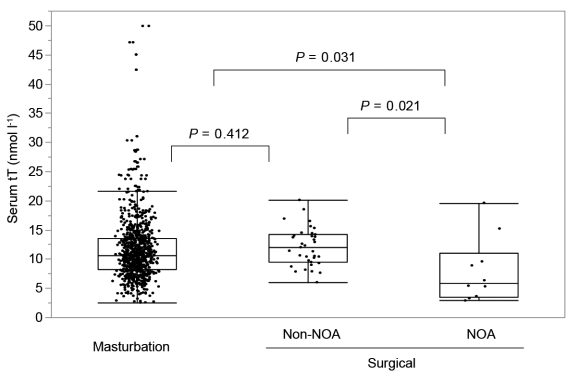

Figure 4: The serum tT levels (nmol l^{-1}) in the groups of men requiring surgical sperm retrieval and those producing semen samples by masturbation, among Chinese fertile men from couples undergoing IVF/ICSI-ET treatment. Masturbation: subjects who produced semen samples by masturbation; surgical: subjects who had their semen samples retrieved by surgery; tT: total testosterone; NOA: nonobstructive azoospermia; IVF/ICSI-ET: *in vitro* fertilization or intracytoplasmic sperm injection and embryo transfer.

the lower limit of the 95% reference intervals do not necessarily suggest male infertility. Indeed, in our data, men with a serum tT level as low as 3.0 nmol l^{-1} could still achieve pregnancy through IVF/ICSI-ET.

Age-related decline in serum tT levels has been studied extensively. There is an average decrease of 0.124 nmol l^{-1} per year in healthy men[20] and 0.8% per year in middle-aged men.[14] However, it was also discovered that serum tT did not fall significantly with age in very healthy men.[21–23] In our study, the group of fertile men undergoing IVF/ICSI-ET cycles contained both normally fertile men and infertile men in terms of the results of natural sexual intercourse. There was high inter-individual variability of tT levels in our cohort, which might be why we found no need for age group partitioning.

The major limitation of this study was that it is a single-center study. However, we run one of the largest reproductive centers in China and there is no strict limit on patient selection. Therefore, we minimized any possible selection bias of different inclusion criteria for men undergoing IVF/ICSI-ET. Thus, our lower limits of serum tT may be closer to the real lower limits of the whole male cohort undergoing such treatments and might be of better clinical significance for the clinical diagnosis and treatment of male infertility in Asian populations.

AUTHOR CONTRIBUTIONS

HYX participated in the design, data collection, and most of the manuscript writing. HJ contributed to the data collection and manuscript writing. GSF was in charge of the statistical analysis and contributed to manuscript writing. YF, YH, WHT, and Hong-Xian Zhang contributed to manuscript writing. FHC, Hong-Xia Zhang, and DFL contributed to data collection. JQ contributed to clinical consultation and design of this study. RL conceived and designed this study, edited it, and finally approved the submission. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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SUPPLEMENTARY

SUPPLEMENTARY PARTICIPANTS AND METHODS

LC-MS/MS METHOD FOR MEASURING SERUM tT LEVELS

The LC-MS/MS assay was performed by the Clinical Mass Spectrometry Center of the Guangzhou Kingmed Center for Clinical Laboratory Co., Ltd. (Guangzhou, P. R. China). Details are as follows.

SAMPLE PREPARATION

A 50 μL aliquot of the internal standard solution (30:70 v/v ratio of acetonitrile to water) containing a $^{13}C_3$-testosterone (T) internal standard (30 μg l^{-1}) was added to a tube containing 200 μL of human serum; then, 1000 μl methyl t-butyl ether was added to precipitate the protein and extract the T. Centrifugation (9391 *g*) was applied for 5 min and then the supernatants were frozen at −70°C for 1 h. These were then vaporized in 50°C nitrogen, and then 100 μL reconstitution solvent (mixture of 35 v/v% methanol in water) was added to obtain the test sample.

SAMPLE TESTING

The test samples were analyzed using a high-throughput LC-MS/MS system (API4000, Applied Biosystems, Foster City, CA, USA). The chromatographic conditions were a C18 column at a temperature of 50°C, and the mobile phase of methanol and water was applied with a flow rate of 0.5 ml min^{-1}. Gradient elution was used; the initial mobile phase was methanol:water with a volume ratio of 35:65. When the analyte peak appeared, the mobile phase volume ratio of methanol and water was changed to 60:40, and then this was returned to the initial mobile phase ratio for 1–2 min; the entire time of gradient elution was 3–8 min.

A positive ion mode was used. Ion source parameters included an ionization source for electrospray ionization; gas curtain pressure 15 psi; heating gas pressure 50 psi; auxiliary heating gas pressure 50 psi; and heating gas temperature 400°C. The gas used was high-purity nitrogen at a pressure of 8 psi and the electrospray needle voltage was 4000 V. A multireaction monitoring (MRM) ion scan of the target quantitative ion pair was used. The target quantitative ion pair cluster voltage was 110 V; the inlet voltage was 10 V; the collision voltage was 32 V; and the outlet voltage was 20 V. The quantitative ion pair cluster voltage for the internal standard T was 100 V; the inlet voltage was 10 V; the collision voltage was 34 V; and the outlet voltage was 12 V.

The parameters for MRM ion scanning of the target quantitative ion pair were as follows: the mass/nuclear ratio of the parent ion was 289.2–290.2, and the mass/nuclear ratio of the corresponding daughter ion was 97.1–98.1. For the internal standard quantitative ion pair MRM ion scan conditions, the mass/nuclear ratio of the internal standard parent ion was 290.2–296.2 of that of the parent ion and the corresponding daughter ion's mass/nuclear ratio was 110.1–116.1. Two sets of liquid chromatography analyses were performed with the same LC conditions, and the liquid collection durations were 2–3 min.

QUALITATIVE JUDGMENT AND QUANTITATIVE CALCULATION

The amount of T was determined from the retention time of the target T and the internal standard $^{13}C_3$-T combined with the abundance of the quantitative ion pair. The tT content in human serum samples was calculated from the peak area ratio of the target T area (Y) and the corresponding T area of the internal standard (X). The equation used was $Y = 0.00187X + 0.000471$; $R = 1.0000$.

Supplementary Table 1: The raw data used for linear correlation analysis between Chemilunimescence platform and liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Sample order	*Gender*	*Chemiluminescence platform (nmol/L)*	*LC-MS/MS (nmol/L)*
1	Male	<0.69	0.18
2	Male	1.48	1.40
3	Male	1.28	1.59
4	Male	2.98	4.10
5	Male	3.88	4.48
6	Male	4.06	5.90
7	Male	6.07	6.07
8	Male	6.34	8.59
9	Male	6.45	9.44
10	Male	7.04	9.05
11	Male	8.15	9.37
12	Male	10.30	12.76
13	Male	10.80	16.35
14	Male	9.64	15.05
15	Male	23.50	28.96
16	Male	0.93	1.63
17	Male	0.69	0.74
18	Male	2.89	2.16
19	Male	3.38	4.23
20	Male	3.74	3.91

Supplementary Table 2: The comparison of z and z* statistics in three age subgroups

	Age <30	*Age 30-39*	*Age >=40*
Means	12	11	11
SD	6.3	4.3	3.9
n	233	570	65
z statistic	3.1©	0.6˚	1.7û
*z** statistic	5.5©	4.9˚	3.3û

SD, standard deviation; N, numbers of cases. ©, difference between age <30 and age 30-39. ˚, difference between age 30-39 and age >=40. û, difference between age <30 and age >=40