Age-related Changes in Luteinizing Hormone and Testosterone Levels in Korean Men

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한국 남성의 혈중 Luteinizing Hormone과 Testosterone 수준의 연령-관련 변화

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ABSTRACT : Changes in luteinizing hormone (LH), serum testosterone (T), and salivary T levels with age were examined in Korean men. Serum was obtained from 167 Korean men of different ages (20~69 y), and the serum LH and T levels were measured. Saliva samples were also obtained, and the salivary T level was determined. The LH levels did not change considerably until 40 y of age (20s, 2.5±1.0; 30s, 2.7±1.5; and 40s, 2.5±1.8 mIU/mL) but increased significantly around 50 y (50s, 3.7±1.8 and 60s, 3.1±1.7 mIU/mL). Further, the serum T levels also did not change until 40 y of age (20s, 5.3±2.6, 30s, 4.4±1.4, 40s, 4.1±1.5 ng/mL) but decreased significantly at 50 y (50s, 3.4±1.5; 60s, 2.6±0.8 ng/mL). The salivary T levels also showed small changes until the age of 40 y (20s~40s, 0.11±0.015 ng/mL) but decreased significantly at 50 y (0.08±0.03 ng/mL). Thus, the relative ratio of salivary T to serum T levels did not change significantly in all the ages examined (2.4±0.9%). Linear regression analysis predicted that the LH levels increased 1.5%/y while the serum and salivary T levels decreased 1%/y and 0.8%/y, respectively. The serum T/LH ratio did not change considerably until the age of 40 y (20s~40s; 2.27±0.14) but decreased significantly (1.2±1.0) at 50 y. Age-related changes in the salivary T/LH ratio were very similar to those in the serum T/LH ratio. These results demonstrated that LH and T levels in serum or saliva did not change considerably until 40 y of age; instead, in Korean men, from 50 y of age, the LH level increased, while the T level decreased. This suggests that primary testicular failure that occurred due to aging (approximately 50 y) and caused this phenomenon. The present study also shows that the salivary T level can be an indicator of the free T level in serum although the salivary T level correlates weakly with the total T level in serum (r=0.53). Thus, information regarding salivary T levels may be useful for studying the age-related changes occurring in male testicular physiology.

    Key words : Serum and salivary testosterone, Luteinizing hormone, Age-related changes.

요 약 : 한국 남성의 연령 증가에 따른 혈중 luteinizing hormone (LH), testosterone (T), 그리고 타액 T 수준 변화를 조사하였다. 혈중LH 수준은 40대까지 유의한 변화를 보이지 않았으나(20s, 2.5±1.0; 30s, 2.7±1.5; and 40s, 2.5±1.8 mIU/mL), 50대 이상에서 유의하게 증가하였다(50s, 3.7±1.8 and 60s, 3.1±1.7 mIU/mL). 또한, 혈중 T 수준도 40대까지는 변화하지 않았으나(20s, 5.3±2.6, 30s, 4.4±1.4, 40s, 4.1±1.5 ng/mL), 50대 이후 유의하게 감소하였다(50s, 3.4±1.5;

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60s, 2.6± 0.8 ng/mL). 타액 T 수준 또한 40대까지 약간의 변화가 나타났으나(20s~40s, 0.11±0.015 ng/mL), 50대에 유의하게 감소하였다(0.08±0.03 ng/mL). 타액 T 대 혈중 T의 상대적인 비율은 모든 연령대에서 유의한 변화가 없었다(2.4±0.9%). 직선회귀 분석(Linear regression analysis)에서 혈중 LH 수준은 매년 1.5%씩 증가하고 혈중 T와 타액 T 수준은 각각 매년 1%와 0.8%씩 감소하는 것으로 예측되었다. 혈중 T/LH 비율은 40대까지 유의한 변화가 없었으나(20s~40s; 2.27±0.14) 50대에 유의하게 감소하였다(1.2±1.0). 연령과 관련된 타액 T/LH 비율은 혈중 T/LH 비율과 대단히 유사하였다. 본 연구 결과에서 혈중 LH와 T, 그리고 타액 T 수준이 한국인 남성에서 40대까지는 유의하게 변화하지 않았고, 50대부터는 LH 수준이 증가하고 T 수준이 감소하였다. 이 결과는 50대경 노화과정에 의해 일어나는 원발성 정소부전(primary testicular failure)이 원인인 것으로 보인다. 또한, 본 연구결과는 비록 타액 T 수준이 혈중 총 T 수준과 약한 상관관계(r=0.53)를 갖지만, 혈중 free T 수준을 나타내는 것으로 보인다. 그러므로, 타액 T 수준에 대한 정보는 연령과 관련해서 나타나는 남성 정소생리를 연구하는데 유용할 것으로 사료된다.

INTRODUCTION

Since long, numerous investigators have focused on the changes occurring in male reproductive physiology with aging. It is well-known that the total, bioavailable, and free testosterone (T) levels decrease gradually with advancing age (Vermeulen, 1991; Lamberts et al., 1997). Some studies showed that the decrease in the total T levels commenced at 30 y of age with a decline rate of 0.4~1.6% per year (Feldman et al., 2002; Gray et al., 1991). Andropause is a clinical syndrome associated with aging and is characterized by a deficiency in serum T levels. Signs or symptoms such as a decrease in libido and sexual function are typically exhibited during andropause (Morley et al., 2006). The cause for T deficiency in men with advance in age is believed to be due to a disorder of the hypothalamic-pituitary axis or testis, or a combination of both. Structural and physiological changes in the testis are believed to be the primary cause for the decrease in testicular T production with advancing age because the Leydig cell numbers are negatively correlated with aging (Neaves et al., 1985; 1987) and the responsiveness of these cells to gonadotropins diminishes with aging (Harman & Tsitouras, 1980; Veldhuis et al., 2005). Studies in a particular population showed that LH and follicle-stimulating hormone (FSH) levels increased with aging, and this increase correlated negatively with T levels (Feldman et al., 2002; Gray et al., 1991; Pincus et al., 1997; Nahoul & Roger, 1990). Serum T levels in healthy young men show-

ed a typical circadian rhythm, i.e., highest in the morning and lowest in the late afternoon (Barberia et al., 1973); however, the early morning rise in T levels was not observed in old age (Bremner et al., 1983; Marrama et al., 1982). In order to determine whether the decrease in T levels in old age is due to testicular failure or a hypothalamic-pituitary disorder (Carnegie, 2004; Matsumoto, 2002), some investigators suggested that serum T levels should be measured in the early morning and compared to LH or FSH levels (Carnegie, 2004; Matsumoto, 2002). It is difficult to diagnose andropause because its signs and symptoms are nonspecific and overlap with those of other common syndromes. Furthermore, there are three types of T (bioavailable, free, and protein-bound T), and the type of T that is important for the diagnosis of andropause remains to be determined. The levels of bioavailable T and free T, rather than that of total T in serum, are considered as biomarkers for the diagnosis of T deficiency in elderly men because the total T level is not indicative of the levels of bioactive hormones, such as the sex hormone-binding globulin (SHBG), that increase with age (Feldman et al., 2002; Gray et al., 1991; Morley et al., 2006). However, measuring the bioavailable T and free T levels is restricted due to the difficulty in separating bioavailable T and free T from protein-bound T.

An assay for the salivary T level has several advantages over an assay for the serum T level. It is believed that hormone concentrations in saliva are representative of the concentrations of non-protein-bound forms of hormones in

serum (Ohzeki et al., 1991; Sannikka et al., 1983). Repeated collection of saliva samples is much easier than that of blood samples since there is no restriction with regard to collection time and collecting saliva does not require skilled personnel. The levels of most steroid hormones can be measured in saliva and used for assessing endocrine function (Kaufman & Lamster, 2000). We have shown that salivary adrenal steroid(cortisol and dehydroepiandrosterone (DHEA)) levels are indicative of the levels of these steroids in serum, and both, the salivary and serum, levels of steroids declined with advancing age in Korean men (Ahn et al., 2007). In this study, we measured the serum T and LH levels in Korean men of different age groups and found that the serum T levels decreased, while the LH levels increased significantly at 50 y of age; this phenomenon was due to testicular failure in old men. Free T levels in saliva were also measured to estimate the true levels of bioavailable T. Measuring salivary T levels will provide valuable information for the diagnosis of T deficiency in men and for distinguishing between primary testicular failure and hypothalamic-pituitary disorders. Salivary T levels were also measured to evaluate the feasibility of using of salivary T levels for the diagnosis of T deficiency in men. Therefore, correlation coefficient between salivary and serum total T levels was determined and compared to the physiological changes in total T in serum.

MATERIALS AND METHODS

1. Subjects

We recruited 167 city-dwelling volunteers from July to September in 2004 from among the applicants for a medical examination in the CHA health-promoting center. Their saliva and blood samples were collected between 10 AM and 11 AM. None of the participants selected presented a diagnosis for diabetes or hypo- or hypertension. Further, those undergoing hormone replacement therapy or taking sleeping pills were excluded from our study. Volunteers gave informed consent and were provided with information on their hormonal values. General information regarding the health of the volunteers is summarized in a previous study (Ahn et al., 2007).

2. Saliva and Blood Collection

We have described methods for the collection of saliva and blood samples in a previous study (Ahn et al., 2007). In brief, saliva and blood samples were collected between 10 AM and 11 AM. The subjects were requested to rinse their mouth with water before collecting the saliva, and a minimum volume of 1 mL saliva was directly obtained when the subjects expectorated into a collecting tube. Because salivary steroid levels are independent of flow rate and sugarless gum does not interfere with the salivary assay (Kirschbaum & Hellhammer, 1994), the subjects were permitted to chew sugarless gum if needed to stimulate saliva flow. During the simultaneous collection of saliva and blood from a subject, the saliva was always collected first. Saliva samples contaminated with blood were excluded from this study. The collected saliva samples were subjected to two freezing-thawing cycles in order to precipitate mucins; the samples were then centrifuged (10,000× g 15 min, 4℃) (Harman & Tsitouras, 1980). The supernatants were collected and stored at −70℃ until the day of the assay. Blood samples were collected between 10 AM and 11 AM and allowed to clot at room temperature. The samples were then centrifuged to obtain serum that was stored at −70℃ until the day of the assay.

3. Measurement of serum T and LH Levels

To determine the total T levels in serum, serum samples were extracted three times with three volumes of diethyl ether. The bioavailable T or free T in serum was not measured in this study. The ether extracts were dried using a speed vacuum evaporator and reconstituted with gelatin-containing (0.1%, w/v) phosphate-buffered saline (GPBS, pH=7.2). Approximately 10,000 cpm of tritium-labeled T (2,4,6,7-^{3}H-testosterone, PerkinElmer Life and

Analytical Sciences, Boston, MA, USA) was added to the pooled serum, and the same extraction procedure as described above was followed to determine the recovery rate of T. The recovery rate of T in serum was relatively constant (95±2%, n=10). The total T and LH levels in serum were measured using commercially available radio-immunoassay (RIA) and immunoradiometric assay (IRMA) kits, respectively (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The intra-assay coefficient of variation for total T in serum was 3.5% (n=10) and that for serum LH was 3.7% (n=10). The inter-assay coefficient of variation for total T in serum was 4.5% (n=4) and that for serum LH was 4.3% (n=4).

4. Measurement of Salivary T levels

The commercially available kits for T RIA were not sensitive enough for measuring salivary T levels and more sophisticated assay techniques were required the analysis of salivary T. Therefore, we developed a hormone assay system based on the liquid phase-double antibody method. Since the T standards in the commercially available kits were not adequate for assaying salivary T levels, the kit standards were further diluted with GPBS to final concentrations of 0, 1, 10, 50, 100, 250, and 500 pg/0.1 mL. I^{125}-labeled T (testosterone-3-(O-caboxymethyl oximino- (2-^{125}I iodohistamine) was obtained from Amersham Biosciences (Buckinghamshire, UK). T antiserum was purchased from Biogenesis (Oxford, UK). It cross-reacts 16% with 5α-dihydrotestosterone, 5.8% with 5α-androstane-3α,17β-diol, 3.7% with 5α-androstane-3β,17β-diol, 2.1% with androstenedione, 0.04% with DHEA, and less than 0.01% with cortisol. General assay procedures were adapted from those described in our previous reports (Ahn et al., 2007). The intra-assay coefficients of variation for salivary T were 7.7% (n=20) at 50 pg/0.1 mL and inter-assay coefficients of variation for salivary T were 5.8% (n=8) at 50 pg/0.1mL and 13.4% (n=8) at 5 pg/0.1 mL. The analytical sensitivity for T was 0.5 pg/tube.

5. Preparation of Steroid-Free Saliva and Determination of RIA Accuracy

Steroid-free saliva was prepared as follows. Approximately 50,000 cpm of tritium-labeled T (2,4,6,7-^{3}H-testosterone, Perkin-Elmer Life and Analytical Sciences) was added to 20 mL of each saliva sample. To each tube, 20% (w/v) activated charcoal powder (Sigma-Aldrich Chemical Co.) was added and stirred at 4℃ overnight. The tubes were centrifuged (15,000×g, 30 min, 4℃), and the supernatants were collected. The residual radioactivity in the supernatants was counted using a liquid scintillation analyzer (Tricarb 2900, PerkinElmer Life and Analytical Sciences). The charcoal-stripping procedures were repeated until there were not more than 100 cpm of tritium-labeled T remaining in the 1 mL samples. Each charcoal-stripped sample was filtered through a 0.45-μm filter and stored at −70℃ until use. To determine the accuracy of the RIA, unlabeled T was added to the charcoal- stripped saliva, and the steroid level was determined using the RIA procedure described above. The T levels in the charcoal-stripped saliva were non-detectable. Further, exogenously added T (100 pg/0.1 mL) ranging from 84 to 116 pg/0.1 mL (96.5±8.5 pg/0.1 mL, n=15) was detected.

6. Statistical Analyses

Conventional methods (mean and SD and analysis of variance (ANOVA)-Tukey's HSD test for unequal sample number) were used to analyze the differences in hormone levels between age groups; the level of significance was set at $p<0.01$, and STATISTICA version 5.1 for Windows (Tulsa, OK, USA) was used for the analysis. Linear regression analysis was carried out to determine the relationship between hormone levels and age by using STATISTICA. Pearson's correlation (r, P) was calculated by using GraphPad Prism version 4 for Windows (San Diego, CA, USA) to correlate serum and salivary hormone levels.

RESULTS

1. Changes in the Serum and Salivary T Levels among Groups of Different Ages

In order to examine whether T levels change with age or not, the total T levels in serum and in saliva were determined in groups of different ages. Fig. 1 shows the
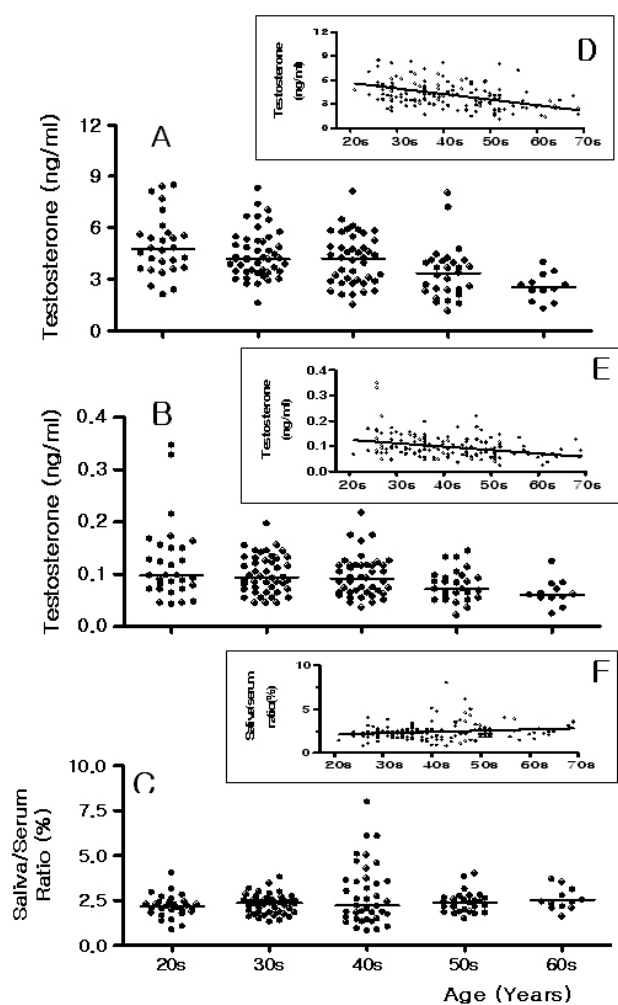

Fig. 1. Changes in the levels of serum and salivary T in Korean men of different age groups. Samples were collected between 10 AM and 11AM, and T levels in serum (n=158) and saliva (n=157) samples were determined by an RIA. The serum T level the serum of each subject is depicted in A, and the salivary T level is depicted in B. The relative ratio of salivary T to serum T, i.e., the salivary/serum T ratio in paired samples (n=152) is depicted in (C). The line in Fig. D, E, and F represents the best fit linear regression with age. Each point on the figure represents the individual value for each participant.

total T levels in serum (A), salivary T levels (B), and the relative ratio of the salivary T to serum T (C) in the groups of different ages comprising men aged from 20 to 69 y (sample collection, 10 AM~11 AM). The serum T levels of the men in their 20s were relatively higher (5.3±2.6 ng/mL) than those of men of other ages (30s, 4.4±1.4; 40s, 4.1±1.4; 50s, 3.4±1.5; and 60s, 2.6±0.8 ng/mL) (Fig. 1A). The total T levels in serum were significantly lower at 50 y of age than at 20 y ($p<0.01$ by Tukey's HSD test). Linear regression analysis showed an overall decrease in serum T levels with advancing age (slope=−0.07) (Fig. 1D). Similarly, salivary T levels of men in their 20s and 40s (0.12~0.095 ng/mL) were similar but those of men in their 50s were significantly lower (0.078 ng/mL, $p<0.01$, by Tukey's HSD test) (Fig. 1B). Lineage regression analysis also showed that the salivary T levels decreased with advancing age (slope=−0.0014, Fig. 1E). Although the ratio of serum T to salivary T appeared to increase with age (slope=0.014) (Fig. 1F), the change in the ratio was small for the different ages (2.1~2.7%, $p=0.08$) (Fig. 1C).

2. Changes in LH Levels and in the Ratio of Serum and Salivary T/LH in Groups of Different Ages

LH levels were determined by using an IRMA to examine whether the LH level changed with age (Fig. 2A), and the serum T/LH ratio was calculated for paired samples (Fig. 2B). The salivary T/LH ratio was also calculated to evaluate the availability of salivary T level measurement (Fig. 2C). The LH levels did not change in men until 40 y of age (2.5~2.7 mIU/mL, $p=0.101$), but it increased significantly in men aged 50 y (3.7±1.8 mIU/mL, $p<0.01$, by Tukey's HSD test) (Fig. 2A). Lineage regression analysis showed an overall increase in LH levels with age (slope=0.026) (Fig. 2D). The serum T/LH ratio did not change significantly in men until 40 y of age ($p=0.72$), but decreased significantly at 50 y ($p<0.01$) (Fig. 2B). Lineage regression analysis also showed a decrease of the serum T/LH ratio with age (slope=−0.037, Fig. 2E). The salivary
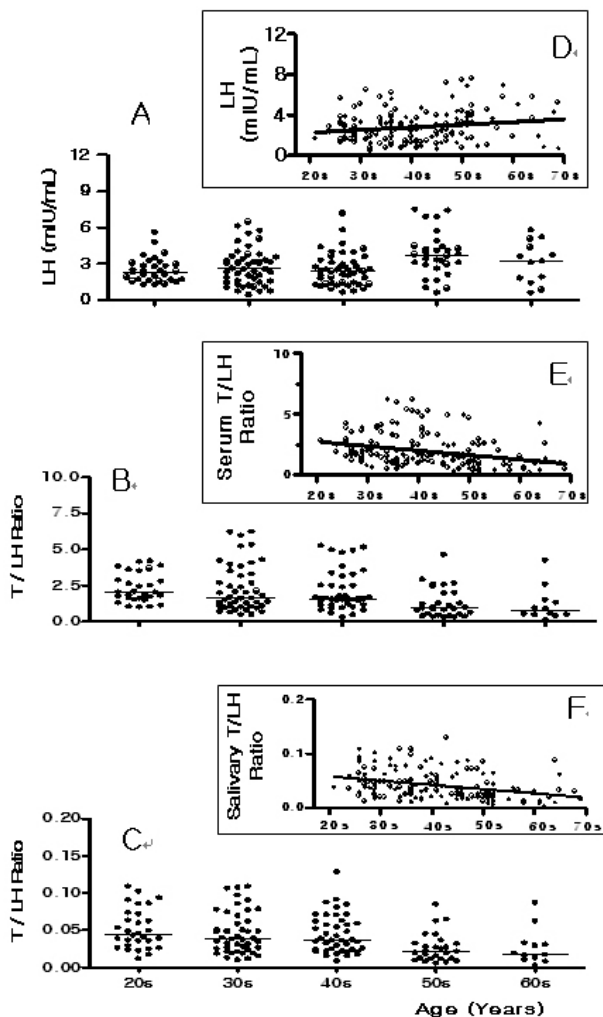
Fig. 2. Changes in LH levels, total T/LH ratio, and salivary T/LH ratio in Korean men of different age groups. Samples were collected between 10 AM and 11 AM, and the LH levels in the serum samples (n=166) were determined by an IRMA. The LH levels are depicted in A, the serum T/LH ratio in B, and the salivary T/LH ratio in C. The line depicted in D, E, and F represents the best fit linear regression with age. Each point on the figure represents the individual value for each participant.

T/LH ratio was a good indicator of the serum T/LH ratio, which did not change significantly until men were 40 y old (0.044∼0.051, p=0.53). However, it decreased markedly when men were in their 50s (0.026, p<0.01) (Fig. 2C). Lineage regression analysis also showed a decrease in the salivary T/LH ratio with advancing age (slope=−0.0008,
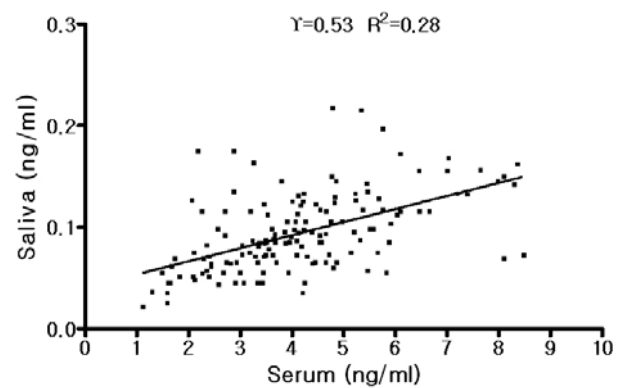

Fig. 3. Correlation of salivary and serum T levels. Data was collected from 151 paired (saliva and serum) samples, and correlation coefficients (Pearson's correlation coefficient r and P) were evaluated. Regression statistics; slope= 0.013, Y intercept=0.041 ng/mL, R^2=0.29, Sy·x=0.032, Pearson's correlation coefficient r=0.53, 95% CI=0.41∼ 0.64, p<0.0001.

Fig. 2c).

3. Correlation between Serum and Salivary T Levels

Fig. 3 shows the correlation between the serum and salivary T levels. The data presented here were obtained only from paired samples of saliva and serum from each person. Thus, the results show a direct comparison of T levels in saliva with those in simultaneously obtained serum samples. Pearson's correlation was calculated to compare the serum and salivary T levels. The salivary T levels correlated weakly with the serum T levels (Fig. 3; r=0.53, R^2=0.28, p<0.0001).

DISCUSSION

The present study shows that the serum and salivary T levels decreased with advancing age, but the LH levels increased and correlated negatively with T levels as aging progressed. The serum or salivary T levels did not change until 40 y of age but decreased significantly at approximately 50 y. Many studies have already shown that there are age-related declines in T levels among old men in western (Feldman et al., 2002; Gray et al., 1991; Harman

& Tsitouras, 1980; Harman et al., 2001) and oriental countries (Okamura et al., 2005; Kang et al., 2003). However, the decreasing rates of total T with advancing age differed among studies such as 1%/y (Gray et al., 1991), 1.6%/y (Feldman et al., 2002), 0.7% (Zumoff et al., 1982; Gray et al., 1983), and 0.5% (Simon et al., 1992; Ferrini et al., 1998). In contrast, some studies have demonstrated that the total T levels do not decrease significantly with advancing age in healthy men (Harman et al., 1980; Li et al., 2005; Sparrow et al., 1980). Linear regression analysis shows that the total T levels decreased by 0.98%/y (95% confidence intervals (CI); 0.6% ~1.3%/y) between 20 to 60 years of age (Fig. 1D) and by 1.1%/y (95% CI, 0.26%~2.95%/y) in the group of men aged between 40 to 50 y. Thus, the T levels decrease more rapidly in old men (40~50 y). Fig. 1A also showed that the total T levels decreased significantly around 50 y of age.

The present study also showed that the salivary T levels decreased significantly at approximately 50 y of age (Fig. 1B) and declined by 0.86%/y between the ages of 20~69 y (Fig. 1E). The predicted decline rate of this study was similar to those of previous reports, i.e., 0.68%~1%/y (Morley et al., 2006; Lukas et al., 2004). It is generally accepted that the total serum T or salivary T levels and age -related decline rates are not uniform across all populations even though the samples were collected using identical protocols and were assayed in the same laboratory (Ellison et al., 2002). Declines of T levels in men with age are thought to be caused by decline of testicular function. It is well-known that the number of Leydig cells (Neaves et al., 1985, 1987), the responsiveness to LH (Mulligan et al., 2001; Veldhuis et al., 2005), and the binding site for LH (Chen et al., 2002) and steroidogenic enzyme proteins (Luo et al., 2001) in Leydig cells decrease significantly in old men.

Some investigators recommended routine measurement of T in a sample obtained at early morning (Carnegie et al., 2004) because serum T levels show circadian variation; highest levels in the morning and lowest levels in the late afternoon (Albertsson-Wikland et al., 1997). Collecting saliva is advantageous because repeated collections that are necessary for the analysis of diurnal rhythm are possible. T-deficiency patients have shown blunt diurnal rhythms because they have a less evident T peak at post-awakening, whereas healthy men have the highest levels of T at post-awakening (Goncharov et al., 2006). When the T level values are low, repeated measurement of serum FSH and LH levels is also recommended to distinguish between primary testicular failure and hypothalamic-pituitary disorders (Christ-Crain et al., 2005). Low T level is accompanied by an increase in the levels of FSH and LH in primary testicular failure, whereas low T level is accompanied by low to normal FSH and LH levels in hypothalamic-pituitary disorders (secondary hypogonadism). This study showed that the serum LH levels increased with age (Fig. 2A). In contrast, the serum (Fig. 2B) and salivary T levels decreased with advancing age (Fig. 2C). Thus, LH levels are negatively correlated with serum and salivary T levels. The increase in the LH levels with ageing that was observed in Korean men (1.6%/y) was similar to that observed in other large-scale longitudinal population studies (Feldman et al., 2002; Gray et al., 1991). The T/LH ratio decreases in cases of primary testicular failure. Fig. 2B and C indicated that in Korean men, primary testicular failure is responsible for the decrease in T levels with advancing age.

It is believed that the hormone levels in saliva are indicative of the concentration of the free-form of these hormones in blood (Quissell, 1993). Some investigators have shown that the salivary T level is weakly correlated with the total serum T level but strongly correlated with the level of free T or bioavailable T in serum (Morley et al., 2006; Jinrui et al., 1994; Vittek et al., 1985). Correlation coefficient between salivary T and total serum T in this study was not high (r=0.53, Fig. 4); hence, the distribution of the serum T/LH and salivary T/LH ratios differed slightly among individuals in same-aged groups (Fig. 2B and C). However, the age-related decline patterns

of serum and salivary T/LH ratios are very similar.

In summary, the present study demonstrated that in Korean men, the salivary and serum T levels decrease, whereas the LH levels increase significantly at around 50 y of age. This suggests that physiological changes occur due to primary testicular failure rather than secondary hypogonadism in old men. In addition, we found that measuring salivary T levels is indicative of the levels of free T in serum and is thus a useful to tool for understanding the hormonal changes occurring with the progression of age.

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