The optimal time-lag for testosterone challenge research based on salivary profiles following different doses of transdermal testosterone administrations

Yan Wu [a, b, c, d], Yin Wu [e], Liuxi Chu [a, b, c], Haoran Yang [a, b, c], Wei Wang [a, b, c], Huihua Deng [a, b, c]*

[a] *Department of Brain and Learning Science, School of Biological Science & Medical Engineering, Southeast University, Nanjing 210096, China.*

[b] *Key Laboratory of Child Development and Learning Science (Southeast University), Ministry of Education, Nanjing 210096, China.*

[c] *Institute of Child Development and Education, Southeast University, Nanjing 210096, China.*

[d] *Nanjing Jiangbei New Area Biophamaceutical Public Service Platform Co., Ltd, Nanjing 210000, China.*

[e] *Department of Applied Social Sciences, Hong Kong Polytechnic University, Hong Kong 999077, China.*

*Corresponding author

Tel: 86-25-83795664;

Fax: 86-25-83793779;

Email: dengrcls@seu.edu.cn.

Abstract

In recent decades, testosterone challenge research examining the effects of testosterone on human neuropsychological behaviors has rapidly grown with the development of a single-dose transdermal testosterone administration paradigm. However, the optimal time-lag between testosterone administration and behavioral measurement is not unified, partly hindering causal understanding of the "testosterone effect". The present study aimed to investigate the optimal time-lag through LC-MS/MS-based salivary profiles of ten biomarkers among healthy males following administration of different doses of transdermal testosterone (i.e., 450- and 150-mg [Androgel®]). Results revealed that testosterone administration significantly increased salivary testosterone levels, reaching maximum levels 2 hours after 450-mg testosterone administration and 1 hour after 150-mg testosterone administration, respectively. Salivary androstenedione and DHEA increased synchronously with testosterone following administration. Moreover, the ratios of testosterone to androstenedione, DHEA, estradiol, and of androstenedione to estrone significantly elevated 1 hour after testosterone administration. In contrast, salivary cortisol and cortisone were decreased over time due to circadian rhythm rather than testosterone administration. Consistent with previous serum studies, the present salivary findings recommended 1-hour post testosterone administration as the optimal time-lag to measure the effects of testosterone on human behaviors in transdermal testosterone challenge research.

Key words: optimal time-lag; salivary biomarkers; LC-MS/MS; testosterone; transdermal administration

1. Introduction

Testosterone, as a major androgen secreted from the hypothalamus-pituitary-gonadal (HPG) axis, plays an essential role not only in reproduction and sexual differentiation, but also in modulating human behaviors (Bos, Panksepp, Bluthe, & van Honk, 2012; Carre & Robinson, 2020; Eisenegger, Haushofer, & Fehr, 2011; Geniole & Carre, 2018; McCall & Singer, 2012; Zilioli & Bird, 2017). The research examining its effect on psychological behavior has a long history (Carre & Robinson, 2020). Especially in the past two decades, research on the effects of exogenous testosterone administration on human neuropsychological behaviors has become a hot topic (Bos et al., 2012; Carre & Robinson, 2020), driven by the motivation of Challenge Hypothesis (Gray, Straftis, Bird, McHale, & Zilioli, 2020; Wingfield, Hegner, Dufty, & Ball, 1990) and the development of various single-dose testosterone challenge paradigms (Tuiten et al., 2000; Zak et al., 2009). Among them, transdermal testosterone challenge research has received considerable attention because it can rapidly increase systemic testosterone levels and is easily absorbed into the skin without invasion or irritability.

Despite its rapid development, there was no unified transdermal testosterone pharmacological data regarding optimal time-lag between testosterone administration and psychological behavior testing. For instance, Zak and colleagues conducted men's behavioral testing 16 hours after transdermal testosterone administration (Zak et al.). In contrast, some studies recommended 3-4 hours post-administration as the optimal time-lag for behavior measurement (Eisenegger, von Eckardstein, Fehr, & von Eckardstein, 2013; Nave, Nadler, Zava, & Camerer, 2017; Wu et al., 2018; Wu et al., 2019; Wu, Shen, et al., 2020; Wu, Zhang, Ou, Hu, & Zilioli, 2020). Additionally, other studies suggested that transdermal testosterone administration has more rapid effects (within 1-2 hours post-treatment) on human psychological behavior processes (Carre et al., 2017; Carre et al., 2015;

Goetz et al., 2014; Hansen, McAuliffe, Goldfarb, & Carre, 2017; Puiu et al., 2019; Welling, Moreau, Bird, Hansen, & Carre, 2016) via a non-genomic pathway (Filova et al., 2015; Foradori, Weiser, & Handa, 2008). The diversity in the time-lag would hinder the causal understanding of testosterone's time-dependent effects on human psychological behaviors. Thus, a review emphasized that this critical issue needs to be addressed in future testosterone challenge research (Carre & Robinson, 2020).

The study on determining the optimal time-lag for psychological behavior testing requires considering a series of methodological issues. Firstly, samples for determination should be non-invasively collected and show sensitivity to exogenous testosterone administration. Invasive collection may reduce participants' willingness to join the study and induce a selection bias or psychological stress, thereby interfering with their behavioral responses and steroids' levels. In comparison to serum, which requires invasive extraction, saliva showing significant correlation with serum in testosterone measurement (de Wit et al., 2018; Granger, Shirtcliff, Booth, Kivlighan, & Schwartz, 2004) is often collected through non-invasive route. Furthermore, saliva has higher sensitivity in evaluating exogenous testosterone administration. Previous studies reported that elevated testosterone levels in saliva following testosterone administration are many times higher than basal endogenous testosterone levels, such as a 100-fold increase for 1.5 mg/kg transdermal testosterone administration (Schonfelder et al., 2011; Thieme, Rautenberg, Grosse, & Schoenfelder, 2013). However, the increase in serum is only about 60 % (Carre & Robinson, 2020), and that in urine is much smaller (Polet, De Wilde, Van Renterghem, Van Gansbeke, & Van Eenoo, 2018). Secondly, the laboratory contamination of testosterone should not be ignored when dealing with transdermal administration and subsequent determination of the optimal time-lag using oral fluid

samples. The air contamination of testosterone gel in laboratory workspaces could increase potential interference in determining testosterone levels in samples and even the optimal time-lag (Genzen et al., 2019). In our previous study (Wu, Wu, et al., 2020), we found that laboratory testosterone contamination could increase basal testosterone levels dozens of times above the reference range and further drastically elevate testosterone levels after administration. This could postpone the optimal time-lag for behavior testing. Thirdly, it is necessary to use an analysis technique that can precisely determine steroids' levels for investigating the time-lag. Previously, immunoassays were widely used for testosterone determination because they are fast and economical, but they may have lower reliability and specificity compared to liquid chromatography tandem mass spectrometry (LC-MS/MS) (Prasad, Lassetter, Welker, & Mehta, 2019; Wudy, Schuler, Sanchez-Guijo, & Hartmann, 2018). Nowadays, neuroendocrinologists have applied LC-MS/MS to determine steroids' levels in their testosterone challenge research (Nave et al., 2017; Wu et al., 2018; Wu et al., 2019). Finally, a multiple biomarkers system including biomarkers from different classes may comprehensively reflect the elicitation effect of exogenous testosterone administration on high systemic testosterone levels and further facilitate the precise evaluation of optimal time-lag in testosterone challenge research. Previous studies mainly used testosterone and cortisol as biomarkers for determining the optimal time-lag (Eisenegger et al., 2013; Puiu et al., 2019), but they yielded inconsistent results regarding the optimal time-lag. In our previous study (Wu, Wu, et al., 2020), we found that two androgens, androstenedione and DHEA as the upstream precursors of testosterone should be sensitive biomarkers reflecting the elicitation effect of exogenous testosterone administration on systemic testosterone. Moreover, several ratios between gonadal steroids' levels (i.e., the ratios of testosterone to androstenedione, DHEA, estradiol, and of androstenedione to

estrone) might also be suitable biomarkers for assessing the modulated effect of exogenous testosterone administration. In addition, it also found that cortisone, as the main metabolite of cortisol, and the ratio of cortisol to cortisone could be suitable biomarkers for estimating exogenous testosterone's influence on systemic cortisol.

Therefore, building on previous evidences and addressing the aforementioned issues, this study aimed to accurately investigate the optimal time-lag for measuring the effect of transdermal testosterone administration on psychological behavior. To do so, we determined salivary profiles of ten biomarkers using LC-MS/MS after administering both normal testosterone (single-dose) and excess testosterone that mimicked testosterone contamination.

2. Materials and methods

2.1 Participants and testosterone administration

Participants in the study were healthy male undergraduates randomly recruited from a university in China. Among them, smokers, alcoholics, obese individuals (body mass index (BMI) ≥30.0 kg/m^2) and those who had taken medicine (e.g., glucocorticoid and antibiotics drugs) or caffeine or had diseases (e.g., canker sores and inflammation) prior to the experiment were excluded.

Finally, 29 participants joined this double-blind, placebo-controlled study and were randomly assigned to three treatment groups: excess testosterone administration group (n=10; mean age=22.1±1.2 years, age range=20.0-24.0 years; mean BMI=24.7±2.5 kg/m^2, BMI range=21.2-29.8 kg/m^2), normal testosterone administration group (n=10; mean age=21.5±1.0 years, age range=20.0-23.0 years; mean BMI=24.4±2.2 kg/m^2, BMI range=21.5-28.2 kg/m^2) and placebo control group (n=9; mean age=22.1±2.1 years, age range=20.0-25.0 years; mean BMI=25.4±2.5

kg/m^2, BMI range=21.9-29.9 kg/m^2). Participants in the excess testosterone administration group received a dose of 450-mg testosterone in gel [Androgel®], while those in the normal testosterone administration group received a single dose of 150-mg testosterone. In contrast, the placebo control group received a colorless hydro-alcoholic gel. The gels were all applied to participants' shoulders and upper arms following our previously established experimental protocol (Wu et al., 2019; Wu, Wu, et al., 2020). Additionally, the testosterone and placebo administration were conducted in two separated clean laboratory rooms to eliminate potential interference from testosterone contamination in the air (Genzen et al., 2019).

All participants provided written informed consent prior to inclusion. This study was conducted following the Declaration of Helsinki and approved by the Health Science Research Ethics Board of Southeast University.

2.2 Sample collection

Every participant self-reported their demographic information (e.g., height, age and weight) before their experiment session. In order to provide clean and unstimulated saliva samples, they needed to avoid eating and drinking for at least 30 minutes and rinse their mouths gently with clean water several times before collecting samples. The unstimulated sample collection adhered to our previous protocol (Wu et al., 2019; Wu, Wu, et al., 2020).

To minimize the impact of steroids' circadian rhythm, the experiment session was scheduled from 13:00 to 17:30 pm. Baseline saliva samples were collected before testosterone administration (T0), and subsequent samples were collected at 1-hour intervals up to 4 hours post-treatment (T1=1 h, T2=2 h, T3=3 h, and T4=4 h). The collected samples were sealed in clean Eppendorf tubes with numbered tags and stored at -80 ℃ until LC-MS/MS analysis.

2.3 LC-MS/MS assay

Salivary levels of the seven aforementioned steroids were determined with the LC-MS/MS method developed in our previous study (Wu, Wu, et al., 2020). The LC-MS/MS system consisted of an Agilent 1200 high-performance liquid chromatography (Agilent, Germany) and a 3200 QTRAP mass spectrometer (Sciex, USA) that was equipped with an atmospheric pressure chemical ionization source (APCI) and operated in multiple-reaction monitoring and positive mode. Limits of quantification were between 0.010 and 0.030 ng/mL for the seven steroids. Intra-day and inter-day coefficients of variation (n=5) were less than 16.2 % and the recovery (n=5) ranged from 90.0 to 115.7 % for all analytes at three concentrations. The other validation parameters, including selectivity and stability, also met the criteria of FDA guidelines (Wu, Wu, et al., 2020).

2.4 Statistical analysis

IBM SPSS 24.0 for windows was used for data analysis in this study. All data were log-transformed to match the normal distribution before statistical analysis. Repeated measures analysis of variance (RMANOVA) with Greenhouse-Geisser correction was used to examine the impacts of treatment (i.e., excess or normal testosterone administrations and placebo control), time (across five time points, T0-T4) as well as their interaction on salivary levels of the ten biomarkers. One-way analysis of variance (ANOVA) was performed to examine the differences in salivary biomarkers' levels among the three groups at T0-T4. In addition, covariance analysis (COANOVA) with body mass index (BMI) as covariate was performed to confirm the intergroup differences in salivary levels. Post-hoc multiple comparisons basing on least significant difference were used to compare the differences between any two of the five time points or three groups.

3. Results

3.1 Salivary levels of testosterone

Dynamic level changes of testosterone in the excess and normal testosterone administration and placebo control groups were demonstrated in Fig. 1(a). RMANOVA revealed significant main effects of treatment ($F_{2, 26} = 24.525$, $\eta^2 = 0.654$, $p < 0.001$) and time ($F_{2.865, 74.496} = 30.932$, $\eta^2 = 0.543$, $p < 0.001$), as well as their interaction effect ($F_{5.73, 148.992} = 9.023$, $\eta^2 = 0.410$, $p < 0.001$) on salivary testosterone levels. This indicates that testosterone levels in the two testosterone administration groups changed differentially over time relative to the levels in the placebo group. Subsequent one-way ANOVA exhibited that testosterone levels in the two administration groups were significantly higher than those in placebo group after treatment ($F_{2, 26} > 18.535$, ps < 0.001, Table 1). As the intergroup differences might be driven by BMI difference, COANOVA with BMI as a covariate ($F_{2, 25} > 23.240$, ps < 0.001, Table S1) was further examined to corroborate the one-way ANOVA results. However, post-hoc comparisons indicated that the two administration groups showed no significant difference in testosterone levels at T1-T4 (ps > 0.094, Table S2). Besides, the intergroup difference among the three groups for baseline testosterone level was not significant ($F_{2, 26} = 1.805$, $p = 0.184$, Table 1 and $F_{2, 25} = 2.378$, $p = 0.113$, Table S1). Further studies suggested that, compared to baseline levels, salivary testosterone levels in the two administration groups increased significantly 1 hour post-treatment (ps < 0.001). The maximum testosterone level in the excess administration group was observed 2 hours after treatment, which remained stable for the following two hours with no significant difference (ps > 0.484). Besides, testosterone levels in the normal administration group showed significant increase 1 hour post-treatment (ps < 0.001) and kept stable during the following three hours (ps > 0.072). In contrast, the placebo group exhibited no significant

dynamic changes of testosterone levels across T0-T4.

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3.2 Androstenedione and DHEA

Similarly, the significant main effects of treatment ($F_{2, 26} = 10.971$, $\eta^2 = 0.458$, $p < 0.001$ and $F_{2, 26} = 8.683$, $\eta^2 = 0.400$, $p = 0.001$) and time ($F_{4, 104} = 6.660$, $\eta^2 = 0.204$, $p < 0.001$ and $F_{2.408, 62.610} = 10.218$, $\eta^2 = 0.282$, $p < 0.001$) and treatment-by-time interaction effects ($F_{8, 208} = 4.722$, $\eta^2 = 0.266$, $p < 0.001$ and $F_{4.816, 125.220} = 3.596$, $\eta^2 = 0.217$, $p = 0.007$) on salivary androstenedione and DHEA levels were also observed via RMANOVA. It indicated that the androstenedione and DHEA levels in the two testosterone administration groups changed differentially across time relative to placebo as shown in Fig. 1(b) and (c). One-way ANOVA and COANOVA analysis indicated that, relative to placebo, both androstenedione and DHEA levels in the two administration groups increased significantly following testosterone administration (androstenedione: $F_{2, 26} > 7.462$, ps < 0.003 for T1-T4, Table 1 and $F_{2, 25} > 8.398$, ps < 0.002 for T1-T4, Table S1; DHEA: $F_{2, 26} > 9.064$, ps < 0.001 for T2-T4, Table 1 and $F_{2, 25} > 4.129$, ps < 0.028 for T1-T4, Table S1). Neither differences in the two steroids' levels between the two administration groups across T1-T4 (ps > 0.069, Table S2) nor intergroup difference among the three groups at baseline (both $F_{2, 26} < 0.773$, ps > 0.472, Table 1 and $F_{2, 25} < 0.762$, ps > 0.477, Table S1) reached significance. Compared to baseline levels, androstenedione and DHEA levels in the excess administration group were significantly increased 2 h post-treatment (ps < 0.006) and kept stable later (ps > 0.058). Moreover, the levels increased significantly 1 h after normal administration (ps < 0.009) and thereafter showed no difference across

198 T1-T4 (ps > 0.062). It indicated that salivary androstenedione and DHEA showed synchronous time
199 courses with testosterone following testosterone administration.

200 **3.3 Ratio biomarkers among gonadal steroids**

201 As shown in Fig. 2(a-d), salivary levels of the ratios of testosterone to androstenedione, DHEA,
202 and estradiol, and of androstenedione to estrone all increased over time after testosterone
203 administration, which were in line with aforementioned salivary testosterone, androstenedione, and
204 DHEA. The main effects of treatment ($F_{2, 26}$ > 12.739, η^2 > 0.495, ps < 0.001) and time (F > 4.672,
205 η^2 > 0.152, ps < 0.002) and their interaction effect (F > 2.377, η^2 > 0.155, ps < 0.022) were all
206 significant on the levels of these ratio biomarkers. Subsequent analysis verified that their levels in the
207 two administration groups were significant higher across T1-T4 relative to these in the placebo group
208 ($F_{2, 26}$ > 5.853, ps < 0.008, Table 1 and $F_{2, 25}$ > 6.811, ps < 0.004, Table S1) and did not differ
209 significantly at T0 ($F_{2, 26}$ < 1.896, ps > 0.170, Table 1 and $F_{2, 25}$ < 1.762, ps > 0.192, Table S1).
210 Specifically, the four ratios showed significant increases in levels 1 hour post-administration (ps <
211 0.05) and thereafter remained stable (ps > 0.075). Interestingly, compared to the normal
212 administration group, the excess administration group showed significantly higher levels in the ratio
213 of testosterone to androstenedione at T1 and T2 (ps < 0.020, Table S2), and of testosterone to DHEA
214 at T1 (p = 0.037, Table S2).

215

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217

218 **3.4 Cortisol, cortisone and their ratio**

219 As shown in Fig. 3(a), salivary cortisol levels decreased over time in the three groups due to their

well-known secretion rhythm. RMANOVA suggested that the main effect of time was significant on cortisol levels ($F_{2.696, 70.092} = 7.603$, $\eta^2 = 0.226$, $p < 0.001$), but the main effect of treatment or their interaction effect were not ($F_{2, 26} = 1.769$, $\eta^2 = 0.120$, $p = 0.190$ and $F_{5.392, 140.184} = 1.769$, $\eta^2 = 0.061$, $p = 0.533$). Subsequent analysis revealed that there were no significant differences among the three groups in cortisol levels across T0-T3 ($F_{2, 26} < 1.302$, ps > 0.289, Table 1 and $F_{2, 25} < 1.228$, ps > 0.310, Table S1), but there were significant intergroup differences at T4 ($F_{2, 26} = 3.892$, $p = 0.033$, Table 1 and $F_{2, 25} = 3.803$, $p = 0.036$, Table S1) due to significant difference between the excess and normal administration groups (p=0.010, Table S2). Furthermore, compared to baseline, salivary cortisol levels in all three groups showed significant decreases 1 hour post-treatment (ps < 0.043).

Likewise, salivary cortisone levels in the three groups also decreased across time as shown in Fig. 3(b). The main effect of time was also significant on cortisone levels ($F_{2.757, 71.682} = 5.721$, $\eta^2 = 0.180$, $p = 0.002$). But no significant main effect of treatment ($F_{2, 26} = 2.417$, $\eta^2 = 0.157$, $p = 0.109$) and no interaction effect ($F_{5.514, 143.364} = 0.491$, $\eta^2 = 0.036$, $p = 0.799$) were found. Moreover, the three groups showed significantly lower cortisone levels across T1-T4 relative to baseline (ps < 0.017). These results suggested that cortisone levels might also be affected by circadian fluctuation as same as cortisol.

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The ratios of cortisol to cortisone showed a decreasing trend in the three treatment groups as shown in Fig. 3(c). However, no significant main effects of treatment ($F_{2, 26} = 0.993$, $\eta^2 = 0.071$, $p = 0.384$), time ($F_{2.778, 72.227} = 2.491$, $\eta^2 = 0.087$, $p = 0.071$), or the interaction effect ($F_{5.556, 144.454} = 0.832$,

242 $\eta^2 = 0.060$, $p = 0.542$) were found on the ratio of cortisol to cortisone. Subsequent analysis revealed
243 that there were no significant intergroup differences in the ratio of cortisol to cortisone ($F_{2, 26} < 3.248$,
244 ps > 0.055, Table 1 and $F_{2, 25} < 3.120$, ps > 0.062, Table S1).
245
246 ## 4. Discussion
247 This study investigated the optimal time-lag for measuring the effects of testosterone on human
248 psychological behaviors using 4-hours salivary profiles of seven sensitive biomarkers out of ten
249 biomarkers following excess and normal transdermal testosterone administrations (i.e., 450-mg and
250 150-mg [Androgel®] testosterone). Among the seven salivary biomarkers, transdermal testosterone
251 administration significantly and synchronously increased salivary levels of testosterone,
252 androstenedione and DHEA, reaching their maximum levels at 2 hours after 450-mg testosterone
253 administration or 1 hour after 150-mg testosterone administration and thereafter remaining stable up
254 to 4 hours post-treatment. Moreover, the ratios of testosterone to androstenedione, DHEA, estradiol,
255 and of androstenedione to estrone were all significantly increased 1 hour after treatment. To our best
256 knowledge, it was the first time to determine the optimal time-lag based on salivary profiles for ten
257 biomarkers after different dosages of transdermal testosterone administrations. Compared to previous
258 serum studies (Eisenegger et al., 2013; Puiu et al., 2019), it extended six biomarkers to ten
259 biomarkers and complemented the related salivary profiles for further time-lag optimization. In
260 contrast, salivary cortisol and cortisone were significantly decreased over time due to their circadian
261 rhythm, which was consistent with previous serum studies and provided an additional proof for the
262 reliability of the present experimental design.
263 Herein, two different dosages of transdermal testosterone administrations both significantly

elevated salivary testosterone levels but showed different time patterns. The time point at which maximum average level of salivary testosterone reached (t_{max}) was 2 hours after 450-mg [Androgel®] testosterone administration and 1 hour after 150-mg administration (Fig. 1a), respectively. After reaching the maximum level, salivary testosterone in the two administration groups remained significant increase up to 4 hours post-treatment, showing a plateau of 2-3 hours for behavioral measurements. The time pattern observed in the 150-mg administration group was congruent with that in previous studies observing rapid testosterone increases post transdermal administration, such as, salivary t_{max} = 1 hour for 50-mg [Testogel®] testosterone (Thieme et al., 2013), serum t_{max} = 1 hour for 150-mg [Androgel®] testosterone (Carre et al., 2017; Carre et al., 2015; Hansen et al., 2017; Welling et al., 2016), and 1.5-2 hours for 100-mg [Testotop®] testosterone (Puiu et al., 2019). These rapid increases may be attributed to the mechanism by which transdermal testosterone administration disrupts the intrinsic equilibriums between 98% of serum testosterone and its binding proteins (i.e., sexual hormone-binding globulin and albumin), resulting in rapid elevation of serum free testosterone levels (Polet et al., 2018; Thieme et al., 2013). Afterward, the extra free testosterone transfers from serum into saliva via intracellular diffusion (Thieme et al., 2013; Wood, 2009), resulting in rapid increases of salivary testosterone levels. In contrast, the t_{max} was delayed to 2 hours for salivary testosterone in the 450-mg testosterone administration group, which mimicked testosterone contamination (Fig. 1a). The maximum testosterone level in this group was obviously elevated relative to the 150-mg administration group but did not reach statistical significance (*Mean* ±*SEM*, 27226±9904 *vs* 12420±6338 pg/mL; $p = 0.202$, Table S2). This may be due to the fact that the increase of salivary testosterone is positively related to the administration dosages. As a result, the t_{max} for excess testosterone administration was correspondingly delayed for having enough time

to transfer more extra free testosterone from serum into saliva.

Salivary androstenedione showed a similar time-dependent change pattern to testosterone, with a significant increase following both normal and excess administrations in this study (Fig. 1b). These results were consistent with a previous finding that salivary androstenedione levels clearly increased after testosterone administration (Polet et al., 2018). This may result from the reversible conversion between testosterone and androstenedione. The increased free testosterone from exogenous testosterone administration leads to androstenedione elevation through this conversion (Polet et al., 2018). However, serum androstenedione levels showed no significant change after testosterone application (Ponzetto et al., 2016). Therefore, the conversion in reverse from testosterone to androstenedione may only occur in the salivary gland under the catalysis of 17β-HSD enzyme (Blom, Ojanotkoharri, Laine, & Huhtaniemi, 1993; Wood, 2009; Wu, Wu, et al., 2020). This suggests that saliva may be more specific than serum in evaluating optimal time-lag with multiple biomarkers. Additionally, salivary DHEA synchronously increased with testosterone and androstenedione after testosterone administration (Fig. 1c), which is consistent with our previous report (Wu, Wu, et al., 2020). As a precursor androgen of androstenedione, it might be inferred that increased salivary DHEA originates from the metabolism control of elevated salivary androstenedione levels and the reverse conversion of salivary androstenedione just like testosterone to androstenedione in the salivary gland. Certainly, the detailed reverse conversion mechanism of androstenedione to DHEA remains to be explored in a future work showing comprehensive experimental evidence. Notably, the ratio biomarkers that reflect the activity of the HPG axis (i.e., the ratios of testosterone to androstenedione, DHEA, estradiol, and of androstenedione to estrone) were also significantly increased with time, mostly showing the maximum levels at 1 hour post excess or normal

administrations, and thereafter kept stable (Fig. 2). Their time courses were similar to those of salivary testosterone, androstenedione and DHEA, corroborating the time-dependent change pattern of testosterone following transdermal testosterone administration.

Additionally, it was found that transdermal testosterone administration did not elicit decreases of salivary cortisol. Salivary cortisol decreased significantly over time, whether in the two testosterone administration groups or the placebo control group (Fig. 3a). Furthermore, there were mostly no significant differences among the three groups across the five time points (Table 1). Moreover, salivary cortisone showed a similar decrease pattern to cortisol in the three groups (Fig. 3b). The ratios of cortisol to cortisone also showed no significant change after testosterone administration (Fig. 3c). These findings indicate that the time-dependent decrease of salivary cortisol may be due to its well-known circadian rhythm rather than testosterone administration, as previous studies have reported (Eisenegger et al., 2013; Puiu et al., 2019). This may be due to the fact that the present transdermal testosterone dose is insufficient to stimulate adequate levels of systemic testosterone and other androgens to inhibit the activity of the HPA axis (Eisenegger et al., 2013). Furthermore, the suppressive effect of systemic testosterone on cortisol may also be modulated by some psychological variables (Puiu et al., 2019). The present result, in line with previous findings (Eisenegger et al., 2013; Puiu et al., 2019), provide a proof for the reliability of this experimental design.

While the present salivary findings matched a few previous results in serum, this study had several limitations. First, as the study only recruited small-sized healthy male undergraduates from a university, the generalizability of the present results might be impaired in part. Future work should be based on a large-scale cohort where participants show different gender, ages and other variables. Second, saliva samples in this study were collected at 1-hour intervals until 4 hours post transdermal

testosterone administration. Compared to the short interval and long process used in previous serum studies (Eisenegger et al., 2013; Puiu et al., 2019), this method may provide less precise time-lag for measuring the effects of testosterone administration. Third, the present salivary biomarkers did not include metabolites of testosterone, such as dihydrotestosterone and androsterone, as well as sex-hormone binding globulin or albumin. These biomarkers' profiles would further corroborate the time-dependent change pattern of systematic testosterone after exogenous testosterone administration.

5. Conclusion

In summary, the present study successfully estimated the optimal time-lag for measuring testosterone modulation effect in transdermal testosterone challenge research by determining the salivary profiles of ten biomarkers in healthy males following 450- and 150-mg [Androgel®] testosterone administrations. It found that transdermal testosterone administration significantly increased salivary testosterone as well as androstenedione and DHEA post-treatment, reaching their maximum levels 2 hours post 450-mg testosterone administration and 1 hour after 150-mg testosterone administration, respectively. The ratios of testosterone to androstenedione, DHEA, estradiol, and of androstenedione to estrone were all significantly increased 1 hour post-treatment in the two administration groups. In contrast, salivary cortisol was gradually decreased due to circadian rhythm rather than testosterone administration, which was supported by cortisone and their ratio. Combining with our results and previous similar findings, the present study recommended the optimal time-lag as 1 h post-administration in a single-dose transdermal testosterone challenge research with contamination controlled. This would provide some inspirations in time optimization

regarding exploring the effect of testosterone on human psychology and behaviors for future single-dose transdermal testosterone challenge research.

Author Contributions

The study was designed by Yan Wu, Yin Wu and Huihua Deng. Saliva samples were collected by Yin Wu and then were determined by Yan Wu, Liuxi Chu, Haoran Yang and Wei Wang using a developed LC-MS/MS method. The samples data were analyzed by Yan Wu, Yin Wu and Huihua Deng. The first version of the manuscript was written by Yan Wu and the draft was reviewed and edited by Huihua Deng and Yin Wu. All authors approved the final version of the manuscript for submission.

Declaration of Interest Conflict

None interest conflict need to be declared.

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Captions of Tables and Figures

Table 1 Comparisons among the three groups for ten salivary biomarkers' levels across T0-T4.

Figure 1 Salivary level changes of (a) testosterone, (b) androstenedione and (c) dehydroepiandrosterone (DHEA) in the three groups across T0-T4. The salivary level is shown with mean level of each compound at each time point and the error bar is standard error of mean (SEM). Across the timespan that marked with gray, biomarker's levels showed no significant change with time.

Figure 2 Salivary level changes of the ratios of (a) testosterone to androstenedione (T/A4), (b) testosterone to DHEA (T/DHEA), (c) testosterone to estradiol (T/E2) and (d) androstenedione to estrone (A4/E1) in the three groups across T0-T4. The salivary level is shown with mean level of each ratio biomarker at each time point and the error bar is standard error of mean (SEM). Across the timespan that marked with gray, biomarker's levels showed no significant change with time.

Figure 3 Salivary level changes of (a) cortisol, (b) cortisone and (c) the ratio of cortisol to cortisone in the three groups across T0-T4. The salivary level is shown with mean level of each compound at each time point and the error bar is standard error of mean (SEM). Across the timespan that marked with gray, biomarker's levels showed no significant change with time.