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GC-MS-based metabolic signatures reveal comparative steroidogenic pathways between fetal and adult mouse testes

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Abstract

Background: Previous studies on gonadal steroidogenesis have not compared metabolic pathways between fetal and adult mouse testes to date.

Objectives: To evaluate comparative metabolic signatures of testicular steroids between fetus and adult mice using gas chromatography-mass spectrometry (GC-MS)-based steroid profiling.

Materials and methods: GC-MS with molecular-specific scan modes was optimized for selective and sensitive detection of 23 androgens, 7 estrogens, 14 progestogens, and 13 corticoids from mouse testes with a quantification limit of 0.1-5.0 ng/mL and reproducibility (coefficient of variation: 0.3%-19.9%). Based on 26 steroids quantitatively detected in testes, comparative steroid signatures were analyzed for mouse testes of 8 fetuses on embryonic day 16.5 and 8 adults on postnatal days 56-60.

Results: In contrast to large amounts of steroids in adult testes ($P < .0002$), all testicular levels per weight unit of protein were significantly increased in fetal testes ($P < .002$, except 6β-hydroxytestosterone of $P = .065$). Both 11β-hydroxyandrostenedione and 7α-hydroxytestosterone were only measurable in fetal testes, and metabolic ratios of testosterone to androstenediol and androstenedione were also increased in fetal testes ($P < .05$ for both).

Discussion and conclusion: Testicular steroid signatures showed that both steroidogenic Δ^4 and Δ^5 pathways in the production of testosterone were activated more during prenatal development. Both 7α- and 11β-hydroxylations were predominant, while hydroxylations at C-6, C-15, and C-16 of testosterone and androstenedione were decreased in the fetus. The present GC-MS-based steroid profiling may facilitate understanding of the development of testicular steroidogenesis.

KEYWORDS

androgen, testosterone biosynthesis, GC-MS, steroid profiling, mouse testis

1 | INTRODUCTION

Androgens and their precursors are key factors influencing sexual differentiation during fetal development. Testicular differentiation occurs during the fifth week of pregnancy in humans, and Leydig cells start to secrete testosterone from the sixth week. Testosterone is then converted into dihydrotestosterone (DHT), a more potent androgen, by 5α-reductase in genital skin to induce the formation of male external genitalia.[1] DHT can also be produced via an alternative "backdoor" pathway without testosterone intermediacy if the classic Δ^5 pathway is disrupted by disorders of sex development[2,3] as initially reported in testes of the tammar wallaby young.[4] Mutations associated with the backdoor pathway can lead to undermasculinization of XY individuals.[5] This alternative pathway may also be functional under normal conditions.

There are two distinct types of Leydig cells between mammalian prenatal and postnatal stages, representing fetal and adult Leydig cells.[6] In addition to morphological differences,[7] expression levels of 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) are also different.[8] Adult Leydig cells then replace fetal ones after birth and before puberty.[6,9,10] Before 1990s, in vitro studies have clearly indicated that the testicular steroidogenic metabolism is significantly changed through both Δ^4 and Δ^5 pathways (Figure 1) in different stages of postnatal development.[11,12] However, these studies have resulted in some discrepant findings caused by the use of different substrates. Comparative metabolism based on naturally occurring endogenous steroids between fetal and adult mouse testes remains unreported. Testicular 7α-hydroxylation of testosterone and androstenedione is known to regulate testosterone production.[13] Other types of steroid hydroxylation in testes have not been identified to date.

In this study, gas chromatography-mass spectrometry (GC-MS)-based quantitative profiling[14,15] was further developed and applied to measure localized concentrations of 23 androgens, 7 estrogens, 14 progestogens, and 13 corticoids in both fetal and adult mouse testes. These quantitative results may reveal testicular metabolic signatures including steroidogenic and oxidation pathways along with male development. In subsidiary analysis, metabolic ratios of metabolites to corresponding precursors were also assessed to imply related enzymatic activities.

2 | MATERIALS AND METHODS

2.1 | Materials

Reference standards of 23 androgens, 7 estrogens, 14 progestogens, and 13 corticoids were obtained from Steraloids, Avanti Polar Lipids, or Sigma-Aldrich. Internal standards (ISs) including 2,2,3,4,4,6,-d_6-dehydroepiandrosterone and 16,16,17-d_3-testosterone for androgens, 2,4,16,16-d_4-17β-estradiol for estrogens, 2,2,4,6,6,17α,21,21,21-d_9-progesterone and 2,2,4,6,6,21,21,21,-d_8-17α-hydroxyprogesterone for progestogens, and 9,11,12,12-d_4-cortisol for corticoids were purchased from Avanti Polar Lipids and C/D/N Isotopes. Sodium phosphate monobasic, sodium phosphate dibasic, sodium acetate (ReagentPlus, ≥99.0%), acetic acid (glacial, ≥99.0%), and L-ascorbic acid were purchased from Sigma-Aldrich. All organic solvents and deionized water were of analytical or high-performance liquid chromatography (HPLC) grade. They were purchased from Burdick & Jackson. Pulverization of testis samples was performed using a TissueLyser with zirconia beads (3.0 mm ID; Toray Industries). Oasis HLB cartridge (3 mL, 60 mg; Wasters) was used for solid-phase extraction (SPE). All steroids were derivatized using trimethylsilylating (TMS) agents, *N*-methyl-*N*-trifluorotrimethylsilylacetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) (Sigma-Aldrich).

2.2 | Calibration and quality control samples

Stock solutions of individual steroids were prepared in methanol/chloroform (9:1, v/v) to obtain final concentration of 1000 μg/mL. Stock solutions of four catechol estrogens (2-OH-E1, 2-OH-E2, 4-OH-E1, and 4-OH-E2) were mixed with 1 mg/mL L-ascorbic acid to prevent oxidation. All working solutions were prepared using methanol/chloroform (9:1, v/v) at 0.001-5 μg/mL. All standard solutions were stored at −20°C until use.

Steroid-free tissue samples were prepared 1 day before the experiment for calibration. Mouse liver samples (50 mg) were pulverized in 1 mL methanol/chloroform (1:1, v/v) with 4 zirconia beads at 25 Hz for 5 minutes to isolate steroids followed by centrifugation twice at 12,000 rpm for 3 minutes. Supernatants were discarded. The remaining tissue sample was washed with l mL of chloroform/0.6 mol/L methanolic HCl (1:1, v/v), sonicated for 5 minutes, and centrifuged three times at 15 800 *g* for 3 minutes. Supernatants were discarded. To eliminate residual methanolic HCl, 1 mL of 20% ethanol was added for washing five times. Tissue samples were then frozen at −80°C. No steroid was detectable in GC-MS chromatogram. For quality control (QC) analysis, steroid-free serum was purchased from BBI solutions (Pen-Y-Fan Industrial Estate) and additional centrifugation was carried out with an Amicon Ultrafilter (MWCO 3000; Millipore) as previously reported.[16]

2.3 | Testis sampling

For quantitative steroid profiling, fetal testes of 8 mice from C57BL/6JJmsSlc pregnant individuals (Japan SLC Inc) were obtained on embryonic day 16.5 (E16.5). Adult testes were obtained from C57BL/6JJmsSlc male mice (n = 8) on postnatal days 56-60. One testis from each adult mouse and two testes from each fetal mouse were collected. One-tenth the weight of an adult testis and one of two fetal testes were used for calculating total amount of protein, which were determined using a BCA Protein Assay Kit (Pierce Biotechnology). All protocols for animal experiments were approved by the Animal Care and Use Committee of Kyushu

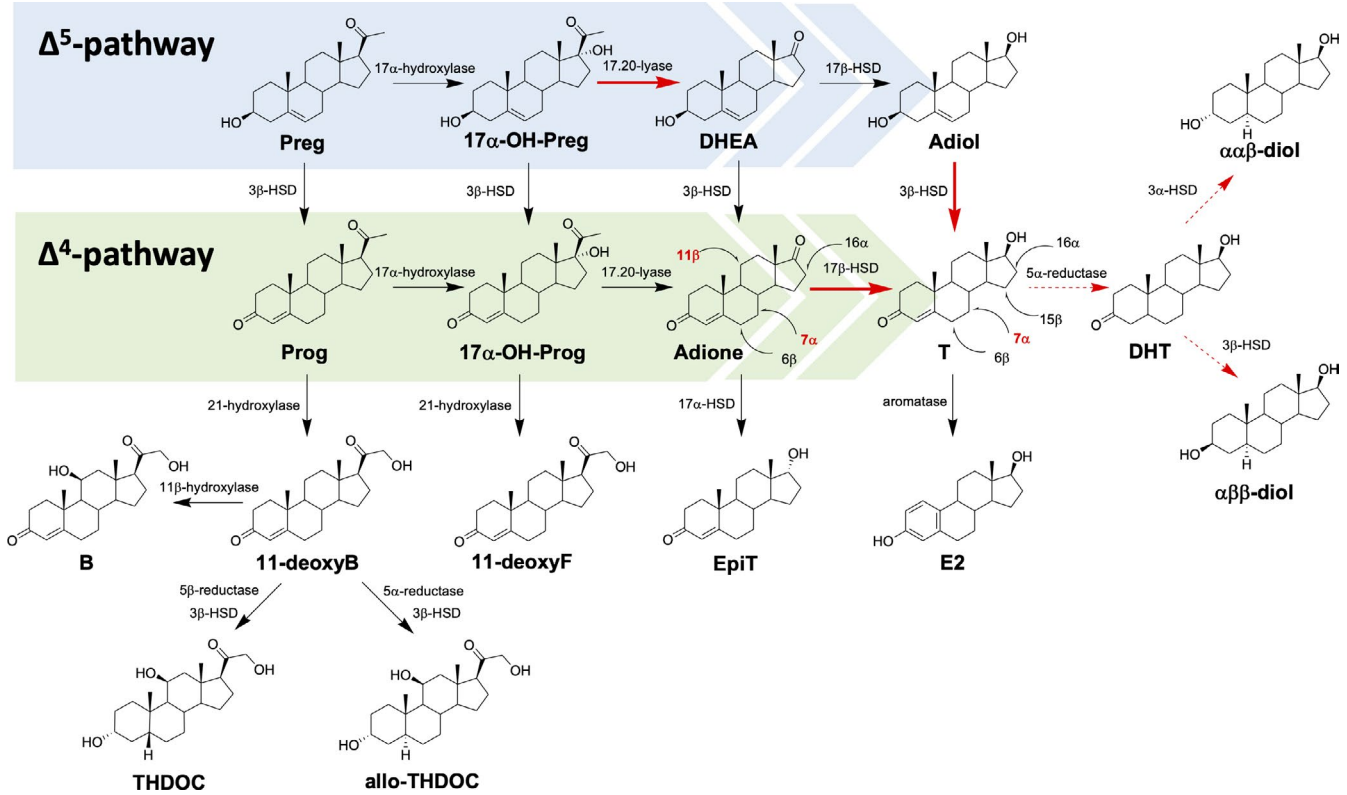

FIGURE 1 Comparative androgen metabolisms via Δ^4 and Δ^5 steroidogenic and hydroxylation pathways between fetal and adult mouse testes. Fetal testicular metabolic ratio of 17α-OH-Preg to DHEA known to indicate 17,20-lyase activity in the Δ^5 pathway is increased (red solid line). Two different metabolic ratios representing 3β-hydroxysteroid dehydrogenase (HSD) (Adiol → T) in the Δ^5 pathway and 17β-HSD (Adione → T) in the Δ^4 pathway are predominant in fetal mouse testes (red solid line), while further metabolic pathways of testosterone to produce ααβ-diol and αββ-diol via dihydrotestosterone (DHT) are decreased in the fetus (red dotted line). Androgen hydroxylations at 7α and 11β are significantly elevated in prenatal development (red colored)

University. All experiments were performed in accordance with guidelines.

2.4 | Instrumental conditions

GC-MS analysis was performed using a Shimadzu GC 2010 Plus Gas Chromatograph interfaced with a triple-quadrupole GCMS-TQ8050 (Shimadzu Corporation). The ion source temperature was 230°C, and the electron energy was 70 eV. Each sample (2 μL) was injected in split mode (10:1) at 280°C and separated through an MXT-1 capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, Silcosteel-treated stainless steel; Restek, Bellefonte, PA, USA). The flow rate of ultra-high-purity helium as carrier gas was 1.3 mL/min. Transfer line temperature was set at 300°C. Oven temperature was initially 235°C. It was ramped to 250°C at 1°C/min (3-min hold). It was then ramped to 270°C at 2°C/min (3-minutes hold), further ramped to 310°C at 40°C/min, and finally increased to 325°C at 0.55°C/min. For quantitative analysis, characteristic ions and retention time of all steroids and their TMS derivatives were determined in full-scan mode at *m/z* 100-700. Both selected-ion and multiple-reaction monitoring (SIM and MRM) modes were used (Table S1).

2.5 | Sample pretreatment

The lyophilized mouse testis was spiked with 20 μL of IS mixture (2,2,4,6,6,21,21,21-d_8-17α-hydroxyprogesterone, 1 μg/mL; 2,2,3,4,4,6-d_6-dehydroepiandrosterone and 2,2,4,6,6,17α,21,21,21-d_9-progesterone, 0.5 μg/mL; 16,16,17-d_3-testosterone and 9,11,12,12-d_4-cortisol, 0.25 μg/mL; 2,4,16,16-d_4-17β-estradiol, 0.2 μg/mL) and mixed with 1 mL of 0.2 mol/L phosphate buffer (pH 7.2). The sample was pulverized using a TissueLyser at 25 Hz for 10 minutes with three zirconia beads and centrifuged at 15 800 *g* for 10 minutes. After transferring the supernatant into a glass tube, the residual sample was extracted one more time. Supernatants were then combined. After loading the combined supernatant onto a preconditioned Oasis HLB cartridge, the SPE cartridge was washed with 1 mL of water twice and eluted with 2 mL of methanol and 2 mL of 90% methanol. The combined eluate was evaporated under a nitrogen stream at 40°C. The sample was dissolved in 1 mL of 0.2 mol/L acetate buffer (pH 5.2) and 100 μL of 0.2% ascorbic acid solution. It was then extracted with 2.5 mL of methyl *tert*-butyl ether twice. The organic solvent was evaporated under a nitrogen stream at 35°C and further dried in a vacuum desiccator with P_2O_5/KOH for at least 30 minutes. Finally, the dried residue was derivatized with 50 μL of MSTFA/NH_4I/DTE (500:4:2, v/w/w) at 60°C for

20 minutes. Then, 2 μL of the final mixture was injected into the GC-MS system.

2.6 | Method validation

Quality control (QC) samples were prepared at four different concentrations (limit of quantification [LOQ], threefold or fivefold of LOQ, medium at 5 or 25 ng/mL, and high at 50 ng/mL) for each steroid according to individual sensitivity with steroid-free serum and quantified with mass spectrum peak height ratios relative to those of ISs. Calibration samples for method validation were prepared at 10 different concentrations ranging from LOQ to 100 ng/mL based on 200 μL of serum. Validation results were expressed as LOQ, calibration linearity (r^2), precisions as coefficients of variation (%CV), and accuracies as percent relative errors (%bias) in triplicates. Extraction recovery was assessed using QC samples at three different concentrations (low, 1 ng/mL; medium, 10 ng/mL; and high, 50 ng/mL) in triplicates for each steroid by spiking steroid-free serum samples with known amounts of working solutions. Absolute recoveries were determined by comparing analytical results of samples to those of non-extracted standard samples.

2.7 | Statistical analysis

Data were analyzed using SPSS (v 22.0; SPSS Inc) and GraphPad Prism (v 8; GraphPad Software). Comparative results between fetal and adult testes were evaluated using non-parametric Mann-Whitney *U* test. Differences with *P*-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Optimized GC-MS conditions

Although all steroids were detectable within 32 minutes, an additional run was undertaken to eliminate untargeted endogenous steroids including cholesterol esters and other lipophilic steroids for subsequent sample runs. Using this GC condition, all 57 steroids were analyzed as their trimethylsilyl (TMS) derivatives. Characteristic fragment ions from their mass spectra were identified (Table S1). Collision energy was set at 3-45 V at 3-V intervals. The most intensive or selective ions were used as quantitative ions. Analytical selectivity and sensitivity of most steroids were improved in MRM mode rather than SIM mode. However, THF, allo-THF, 21-deoxyF, 11-deoxyF, E, B, F, 6β-OHE, and 6β-OHF showed better detectability in the SIM mode because of extensive fragmentation during electron-impact ionization of GC-MS.[17] A combined MRM-SIM method was therefore processed. Under these analytical parameters, ββα-diol, ααα-diol, ααβ-diol, and βββ-diol could not be completely separated. Thus, quantitative results of ββα-diol and ααβ-diol might include those of ααα-diol and βββ-diol, respectively.

3.2 | Method validation

Method validation was carried out with calibration and QC samples prepared using both steroid-free serum and tissue samples. LOQ, linearity as correlation coefficient (r^2), precision, and accuracy were evaluated. LOQ ranges were as follows: androgens and estrogens, 0.1-0.5 ng/mL; progestogens, 0.25-1.0 ng/mL; and corticoids, 0.25-5.0 ng/mL. The calibration curve including a double blank sample without ISs, blank samples including matrix and ISs, and 10 spiked samples from the LOQ to 100 ng/mL showed good linearity (0.9801-0.9995) for all analytes except for E1 (r^2 = .9766) and 7β-OH-Preg (r^2 = .9738). Precision (%CV) in triplicate was 1.3-19.9 for LOQ level, 1.5-19.8 at low, 0.8-19.0 at medium, and 0.3-17.9 at high analyte concentrations. The accuracy (% bias) was 92.5 ± 18.5 (mean ± SD) for androgens, 80.7 ± 19.4 for estrogens, 56.0 ± 26.4 for progestogens, and 91.3 ± 16.4 for corticoids. Absolute recovery rates (%) of androgens, estrogens, progestogens, and corticoids were 78.5 ± 14.4, 73.0 ± 19.5, 56.9 ± 19.8, and 61.8 ± 16.5, respectively (Table S2). Results of intra-day (n = 4) repeatability obtained from steroid-free tissue samples were 2.6-13.5%CV and 80.7%-107.7% bias, which revealed no significant matrix effects in these tissue samples.

3.3 | Altered steroid metabolism in fetal and adult mouse testes

One testis from each adult (n = 8) and two testes from each fetal individual mouse (n = 8) were used for this study. The average weight of adult testes was 93.9 mg, while weights of fetal testes were immeasurable because of their trace amounts (<1 mg). Among 57 steroids, 26 were measured in fetal and adult mouse testes. Two androgens, 11β-OH-Adione and 7α-OHT, were only detected in fetal testes, whereas 6β-OH-Adione, 16α-OH-Adione, 15β-OHT, 16α-OHT, DHT, ααβ-diol, E2, 17α,20α-DHP, iso-Pone, B, and allo-THDOC were undetectable in fetal testes. When levels of other 13 steroids were quantitatively determined, they were found to be significantly increased in adult mice ($P < .0002$ all; Figure S1).

As absolute amounts of individual steroids in testes can be affected by testicular size, normalization is needed for testicular concentrations of steroids. To compare concentrations of testicular steroids between fetal and adult mice, absolute amounts of testicular steroids were corrected for the total amount of extracted protein (Table 1). The levels of all steroids comparable were significantly increased in fetal testes ($P < .002$, 6β-hydroxytestosterone of $P = .065$). Based on levels of individual steroids, metabolic ratios of precursors to their corresponding metabolites were also compared (Table S3). Metabolic ratios of T/Adiol, 11-deoxyF/17α-OH-Prog, DHEA/17α-OH-Preg, and T/Adione known to be associated with steroidogenic pathways were higher in fetal testes than in adult testes ($P < .05$ for all). In contrast, metabolic

TABLE 1 Levels of testicular steroids from fetal and adult mice

	Concentration (pg/mg protein)		
	Fetus	Adult	
Compounds (abbreviation)	Median (Range)	Median (range)	*P*-value[a]
DHEA	128.0 (100.0-257.2)	16.6 (15.0-19.8)	<.001
Adiol	684.8 (512.9-828.1)	68.9 (26.5-184.4)	<.001
Adione	4651.1 (626.0-6465.7)	536.9 (410.6-1027.6)	<.001
6β-OH-Adione	ND	35.7 (28.0-79.4)	NA[b]
7α-OH-Adione	4015.4 (928.7-5548.9)	163.3 (106.4-198.5)	<.001
11β-OH-Adione	1173.0 (725.3-1280.9)	ND	NA
16α-OH-Adione	ND	14.5 (8.7-38.4)	NA
T	17 748.3 (6292.1-20 249.9)	718.0 (395.1-5283.1)	<.001
6β-OHT	167.4 (39.3-336.0)	43.7 (17.2-222.1)	.065
7α-OHT	547.8 (208.4-1305.0)	ND	NA
15β-OHT	ND	6.6 (4.4-9.1)	NA
16α-OHT	ND	215.5 (32.8-914.8)	NA
EpiT	96.1 (68.3-197.9)	10.3 (5.8-48.0)	<.001
DHT	ND	23.0 (11.3-49.2)	NA
ααβ-diol [βαβ-diol]	ND	58.3 (13.8-338.4)	NA
αββ-diol	714.8 (197.5-1528.8)	64.0 (29.1-351.2)	<.002
E2	ND	3.8 (1.9-7.8)	NA
Preg	502.8 (175.1-847.1)	119.2 (47.7-221.3)	<.002
17α-OH-Preg	339.6 (249.9-381.3)	55.7 (40.1-115.3)	<.001
Prog	332.4 (94.5-547.5)	40.4 (21.2-197.6)	<.002
17α-OH-Prog	476.8 (105.3-2164.4)	66.7 (52.7-523.6)	<.002
17α,20α-DHP	ND	7.5 (2.3-49.8)	NA
iso-Pone	ND	30.8 (9.0-52.6)	NA
11-deoxyF	790.5 (107.7-1006.3)	15.5 (8.5-39.1)	<.001
B	ND	53.4 (10.0-296.7)	NA
allo-THDOC	ND	11.3 (7.8-19.1)	NA

Abbreviation: ND, not detected.

[a]Statistical significance was determined using the Mann-Whitney *U* test.

[b]Not applicable because analyte was not detected in neither fetal testes nor adult mouse testes.

ratios of 6β-OHT/T and 7α-OH-Adione/Adione associated with steroid hydroxylation were altered based on different hydroxylation sites ($P < .001$ and $P < .02$, respectively).

4 | DISCUSSION

Here, we developed a quantitative profiling assay for 57 steroids based on selective detection in both MRM and SIM modes of GC-MS. Large amounts of steroid sulfates could contribute to local production of steroids via their hydrolysis in Sertoli cells.[18,19] Free steroids without deconjugation steps were also evaluated in this study. Analytical sensitivities were improved for most androgens and estrogens (4 to 10 times), while those of progestogens and corticoids were similar or 2-4 times more sensitive than results obtained from our previous method.[15] However, several progestogens and corticoids including Preg and F and their hydroxylated metabolites show better analytical sensitivities in a method coupled with supported liquid extraction purification.[17]

Although a lineage association between fetal and adult Leydig cells has not been fully identified,[20] fetal Leydig cells are known to persist up to 30% of total Leydig cells in adult testes.[21,22] However, 11β-OH-Adione and 7α-OHT were undetectable in adult testes, although adult testes are larger and contained more Leydig cells than those of fetal testes.[23] In general, 11β-OH-Adione does not have any androgenic activity,[24] while 11-oxygenated androgens have been recently focused for clinical applications.[25] However, studies are required to uncover physiological roles of 11-oxygenated metabolites in fetal testes.

Androgenic inactive 7α-OHT has testicular autoregulatory function. Its testicular production is inversely correlated with

5α-reduced androgens.[26] Here, no 7α-OHT was found in adult testes, while 5α-DHT was undetectable in fetal testes (Table 1). The lack of fetal testicular DHT is concurrent with a study indicating the absence of DHT in fetal plasma.[27] Metabolic activities of both 3α-HSD and 5α-reductase are inhibited by 7α-OHT and 7α-Adione in adult rat testes,[28] which might explain ααβ-diol catalyzed by 3α-HSD was not detected here. In addition, 7α-Adione in a Leydig cell was significantly higher in fetus than in adults ($P < .04$). Such results imply that androgenic 7α-hydroxylase activity is predominant in fetal testes rather than in adult testes (Table S3). Other oxidative metabolisms at C-6, C-15, and C-16 of T and Adione were inferior in fetal testes.

Cytochrome P450c17 (CYP17A1) mediates both 17α-hydroxylase and 17,20-lyase activities for androgen biosynthesis in both the adrenal cortex and testes.[29] It can mediate the Δ^5 pathway involving conversion of Preg to 17α-OH-Preg and then to DHEA. It can also mediate the Δ^4 pathway involving the conversion of Prog to 17α-OH-Prog and further to Adione (Figure 1). A preference for either Δ^4 or Δ^5 pathways in Leydig cells is dependent on CYP17A1 substrate. It could also be affected by species and age.[30] cDNA cloning revealed that the amino acid sequence of CYP17A1 isolated from rodent testes was 69% similar to that of humans. It is predominantly active in the Δ^4-androgenic pathway,[31] while the Δ^5 pathway is strongly preferred for 17,20-lyase activity in human fetal testes.[32] Metabolic ratios of 17α-OH-Preg to Preg and 17α-OH-Prog to Prog corresponding to 17α-hydroxylase were not significantly different between the two testicular groups, whereas DHEA/17α-OH-Preg, one of metabolic ratios related to 17,20-lyase, was higher in fetal Leydig cells (Table S3). However, comparative evidences in direct measurement of enzyme activities would be conducted to verify previous findings.[31,32]

In general, fetal Leydig cells do not express 17β-HSD, an enzyme catalyzing the final step to produce testosterone via Δ^4 pathway (Figure 1), and Adione is therefore major androgen in fetal testes.[8] Testicular testosterone in fetus is presented because Adione is transferred to Sertoli cells, which express 17β-HSD type 3 only in the fetal period, and then converted to testosterone.[33] Most abundant steroid testosterone in fetal testes (Table 1) may, therefore, reflect its amount in fetus Sertoli cells. Increased metabolic ratio of T/Adione in fetal testes was observed (Table S3). In contrast to the Δ^4 pathway, 17β-HSD in the Δ^5 pathway catalyzes DHEA to produce Adiol. This was similar between fetal and adult mouse testes, whereas the T/Adiol ratio corresponding to 3β-HSD activity was increased in fetal testes. These results are in accordance with that the production of testicular testosterone is a dominant reaction for masculinization of fetal mouse.[34]

Metabolic ratio of 11-deoxyF to 17α-OH-Prog known to indicate 21-hydroxylase activity was remarkably elevated in fetal testes ($P < .001$; Table S3). CYP21A1 was predominantly expressed in fetal mouse testis, but not in adult testes. It declined significantly after birth.[35] The decreased expression of CYP21A1 in adult mouse testes might have derived from the persistence of fetal Leydig cells in adults or its low expression in adult cell population. CYP21A1 can also convert Prog to 11-deoxycorticosterone (11-deoxyB) as the pivotal step in mineralocorticoid metabolism. However, 11-deoxyB was not measured in this study.

Despite interesting findings for mouse testicular steroidogenesis, this study did not provide longitudinal data with more sampling at different timepoints along with aging. No tracing experiments with direct measurement of enzyme activities were followed. In summary, GC-MS-based steroid analysis coupled with molecular-specific MS scan modes was developed and validated in this study to evaluate steroidogenic and oxidation pathways simultaneously. Among 57 analytes monitored, 26 were quantitatively detected in fetal and adult mouse testes. Present testicular steroid signatures revealed that both steroidogenic Δ^4 and Δ^5 pathways are activated to produce testosterone at prenatal and postnatal stages of the mouse. In particular, 11β-OH-Adione and 7α-OHT were only detected in fetal testes, indicating that CYP11B1 is expressed in fetal Leydig cells.[36] Based on the higher metabolic ratio of 7α-OH-Adione/Adione and the presence of 7α-OHT in only fetal testis, 7α-hydroxylase might be a dominant steroidogenic enzyme in prenatal development.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. Funding sources had no role in the study design, data collection, data analysis, or data interpretation. They did not contribute to the writing of this manuscript or the decision to submit it for publication either.

AUTHORS' CONTRIBUTIONS

SH developed the analytical assay, analyzed the samples, and wrote the manuscript. TB and SY prepared mouse testis specimens. DJB, KM, and JHK interpreted quantitative data and participated in designing the experimental protocol. MHC conceived the study, supervised all experiments, and wrote the manuscript. All authors reviewed the manuscript and approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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