

Maternal smoking during pregnancy specifically reduces human fetal Desert hedgehog gene expression during testis development.

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Supplementary file for Methodology

Testosterone extraction and LH/hCG validation

Spin-columns (GE Healthcare Bucks, UK) filled with 250 µl of C18 (binding capacity 10 µg/µl, C18 Self Pack POROS 10 R2, Applied Biosystems Ltd, Warrington, UK) and washed with 6 column volumes of acetonitrile followed by 6 column volumes of milliQ water. Aliquots of 50 µl of fetal plasma and normal adult male non-haemolysed plasma (repeated x4) were loaded onto the columns which were then washed with 6 column volumes of milliQ water. Bound steroid hormone was eluted with 2 ml methanol, which was collected and evaporated at room temperature. The dried samples were reconstituted with 50 µl of testosterone assay buffer (Perkin-Elmer Life Sciences) and 25 µl aliquots used for testosterone assay. The recovery of testosterone was 122±9.7%.

To control for hCG cross-reactivity in the LH assay, additional aliquots of fetal plasma were mixed 1:1 with 0, 102 and 10,100 U/l hCG and included in the LH assay. The difference between LH values obtained when the plasma was combined with 10,100 compared with 0 U hCG was +1.9 U LH/l, indicating a 0.02% cross-reaction of intact hCG in the LH assay, which agrees with values given in the kit inserts.

Real-time quantitative PCR (qPCR)

Primers used for real-time PCR were:

Gene	Genbank	Forward Primer	Reverse Primer
Wnt2b (WNT2B)	nm_004185	atgggtccaactcaccgcag	ccgtctgttccctttgatgtcttgct
Wnt4 (WNT4)	nm_030761	gcctcctcgtcttcgccgtc	catgactccagggtccgctgctc
Wnt5a (WNT5A)	nm_003392	gagaaggcgcaagacaggca	caccaccctgctgctgct
LH-receptor (LHR)	nm_000233	gctgtgcttttagaaactgccaaca	ttcatagtcccagccactcagttcact
FSH-receptor (FSHR)	nm_000145	aagccagcctcacctatcccagc	ctcattgtctctgccagagaggatctct
Cytochrome P450 11a1 (CYP11A1)	nm_000781	ctcttaagtccgagggcccca	gcctctggagccatcacctctc
Cytochrome P450 17a1 (CYP17A1)	nm_000102	ccatttctgaacgcaccgg	agagaggccaaggaacagggtc
Hydroxysteroid (17-beta) dehydrogenase 3 (HSD17B3)	nm_000197	cccattctattcggttcgatgggc	gccagagtcagcgaaggcga
GATA-4	nm_002052	tctgtgccaactgccagacc	ttgggcttccgttttctggttg
ckit receptor (CKIT)	X06182	tgtgtgtaaggaaacgctcgactacc	aatcgaccggcattccaggatag
Transcription factor AP-2 gamma (TFAP2C)	nm_003222	ccggctctgctgggagaagt	ctggttactaggaattcggctcaca
Sox9	nm_000346	acttgacacacgccgagctca	gctggtactgtaatccgggtggtc
Kit ligand (KITL)	nm_000899	ccagaacagctaaacggagtcgcc	cagattcctgcagatccctca
Patched1 (PTC1)	nm_000264	aaaccggcagccgcgataag	ccggatgttgctgggag
Insulin-like growth factor 3 (INSL3)	nm_005543	tgttccaccgaagccagga	tggcggatggtgagaggtc
Fibroblast growth factor 9 (FGF9)	nm_002010	tcaggcggaggcagctatactgc	gagtccactgtccacgcctcga
Cytochrome P450 21 (CYP21)	nm_000500	cggacctgtcctgggagactactcc	ctgggctctcatgcgctcaca
Melanocortin 2 receptor (MC2R)	nm_000529	tcattttgccagaaagtctctgc	cctgctgtggttaaggcggg
Cytochrome P450 11B1 (CYP11B1)	nm_000497	tgtgtgatgctccggagga	cgcaatcgggtgaagcgcc
X-chromosome open reading frame 6 (CXORF6)	nm_005491	catgcttccccatttcgccat	cttctgtctccttaacagttccctgatg
Wilms tumour1 (WT1)	nm_024425	tcccggtccgaccaccta	tggagtttggtcatgtttctctgatgc
GATA-6	nm_005257	aataattccattcccactgactccaacttc	aatacttgagctcgtgttctcggg

AhR	nm_001621	aatacagagttggaccgtttgctagc	tggcctccgtttcttcagtaggg
PrIR	nm_000949	ccctggcaaaactaaagaactctcctattc	aagtagcagagtgaaaacggttcagat
SF-1 (Nr5a1)	nm_004959	tcccttctgcccgttccagaaat	tgaagccattggcccgaatct
ADRBK1	nm_001619	agatactgtgccccgagccca	aattccaccaagggcctggc
Desert hedgehog (Dhh)	nm_021044	acaacccccgacatcatcttcaagg	gtggccgtcctcgtccca
StAR	nm_000349	ggctggcatggccacagact	ttgggcagccacccttga
POR	nm_000941	tttctagcatgacggacatgattctgt	tttctcatctttccacaaagctgctc
AR	nm_001011645	cttctgggtgcactatggagctctca	aacatttccgaagacgacaagatggac

Polycyclic aromatic hydrocarbon (PAH) determinations

Seven internal standards were added at 0.05 µg to freeze-dried livers and PAHs extracted into ethanoic potassium hydroxide (90°C, 8 hours) followed by 3x10 ml of isohexane and evaporation to 3 ml. Sample clean-up involved absorption chromatography to remove lipids. Samples were loaded onto 10 g silica columns (Merck, Nottingham, UK) previously conditioned with 40 ml iso-hexane (Rathburn Chemicals, Walkerburn, UK), and 75 nM iso-hexane: DCM (1:1v) fractions collected and concentrated under nitrogen. PAH content was determined using gas chromatography linked to mass spectrometry (GC/MS) operated in the single ion monitoring (SIM) mode (Thermo Electron Trace MS (Hemel Hempstead, UK) linked to a Trace 2000 GC fitted with an AS2000 auto-sampler). Separations were effected on a Zebtron ZB5 fused silica capillary column coated with 95% dimethylpolysiloxane/5% phenyl with a phase thickness of 0.25 mm (Phenomenex, Macclesfield, UK). The operating temperature for each PAH analysis started at 70°C for 3 minutes, was then ramped at 5°C/min to 250°C and held for 1 minute, then ramped to 300°C at 6°C/min and held for 6 minutes, then ramped to 325°C at 10°C/min and held for 5 minutes. The carrier gas was helium and samples were injected in splitless mode with a surge. The mass spectrometer was operated in the electron ionization (EI+) mode at 70 electron volts and a source temperature of 250°C. The ions monitored for each compound were between 128-278 m/z (see Supplementary file). Response factors were calculated relative to the 7 internal standards. Contamination was minimized by muffling glassware at 450°C prior to use and residual contamination was accounted for by including blanks in the protocol. Certified reference materials (CRM) could not be found for sample matrix under investigation and reproducibility was therefore monitored by repeated analysis of spiked liver tissue. Quality control samples were included with each batch of experimental samples analyzed. Experimental blanks were included with each batch of experimental samples and results corrected accordingly.

The 7 internal standards used were:

d-8 naphthalene, d-10 acenaphthene, d-10 fluorene, d-10 phenanthrene, d-10 anthracene, d-10 pyrene, d-12 chrysene (Greyhound Chromatography, Birkenhead, UK).

The ions monitored for each compound were:

m/z 128 (naphthalene), m/z 152 (acenaphthalene), m/z 153 (acenaphthene), m/z 166 (fluorene), m/z 178 (phenanthrene), m/z 178 (anthracene), m/z 202 (fluoranthene), m/z 202 (pyrene), m/z 228 (benzo[a]anthracene), m/z 228 (chrysene), m/z 252 (benzo[b]fluoranthene), m/z 252 (benzo[k]fluoranthene), m/z 252 (benzo[a]pyrene), m/z 276 (indeno[1,2,3-cd]pyrene), m/z 278 (dibenzo[a,h]anthracene) and m/z 276 (benzo[ghi]perylene).

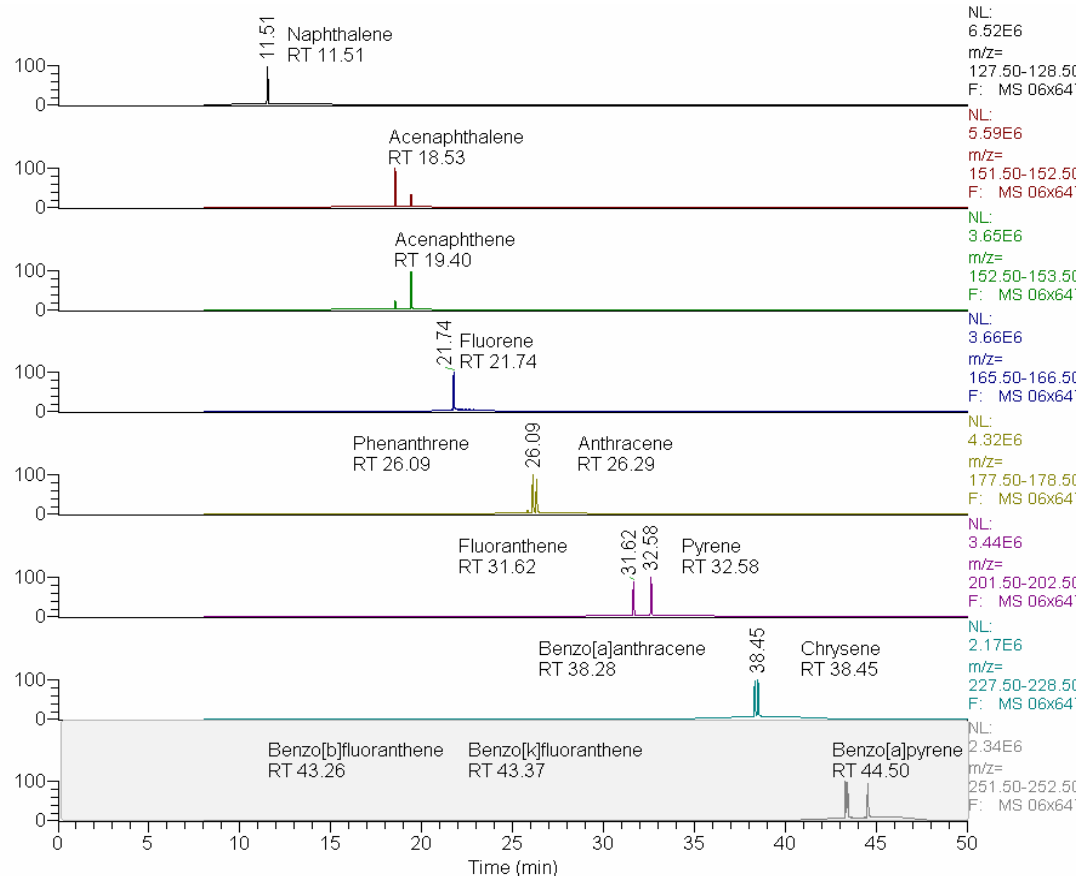
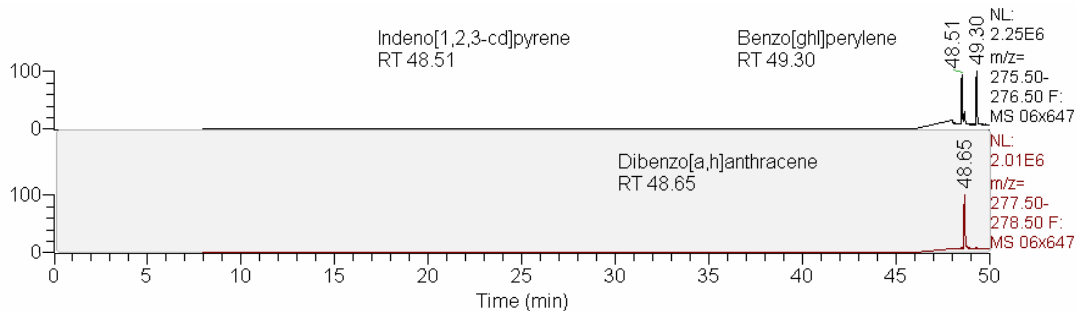
The ions monitored for the internal standards were:

m/z 136 (d-8 naphthalene), m/z 164 (d-10 acenaphthene), m/z 176 (d-10 fluorene), m/z 188 (d-10 phenanthrene), m/z 188 (d-10 anthracene), m/z 212 (d-10 pyrene) and m/z 240 (d-12 chrysene).

The recovery values for PAH analysis were (mean±sd%, n=9-20):

80.98±16.64% (naphthalene), 88.21±13.82% (acenaphthalene), 116.15±25.16% (acenaphthene), 91.91±9.94% (fluorene), 115.41±25.90% (phenanthrene), 107.28±12.45% (anthracene), 107.20±14.90% (fluoranthene), 104.10±13.18% (pyrene), 105.68±7.58% (benzo[a]anthracene), 93.35±11.12% (chrysene), 96.02±9.87% (benzo[b]fluoranthene), 95.79±11.04% (benzo[k]fluoranthene), 96.04±12.79% (benzo[a]pyrene), 99.69±18.36% (indeno[1,2,3-cd]pyrene), 96.71±8.14% (dibenzo[a,h]anthracene) and 92.24±12.79% (benzo[ghi]perylene).

Representative chromatograms of PAH response factor standards: 16 PAH analytes.



Representative chromatograms of PAH response factor standards: 7 deuterated internal standards.

