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Impact of maternal cigarette smoke exposure on brain and kidney health outcomes in female offspring

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Short title: females protected by maternal smoking

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Abstract

Increased oxidative stress in the brain can lead to increased sympathetic tone that may further induce kidney dysfunction. Previously we have shown that maternal cigarette smoke exposure (SE) leads to significantly increased oxidative stress and inflammation in both brain and kidney, as well as reduced brain and kidney mitochondrial activity. This is closely associated with significant kidney underdevelopment and abnormal function in adulthood in the male offspring. This study aimed to investigate the impact of maternal SE on brain and kidney health in the female offspring. In this study, the mouse dams were exposed to 2 cigarettes, twice daily for 6 weeks prior to gestation, during pregnancy and lactation. Brains and kidneys from the female offspring were collected at 20 days (P20) and 13 weeks (W13) and were subject to further analysis. We found that mRNA expression of brain inflammatory markers interleukin-1 receptor and Toll-like receptor 4 were significantly increased in the SE offspring at both P20 and W13. Their brain mitochondrial activity markers were however increased at W13 with increased antioxidant activity. Kidney development and function in the female SE offspring were not different from the control offspring. We concluded that although brain inflammatory markers were upregulated in the SE female offspring, they were protected from some of the indicators of brain oxidative stress, such as endogenous antioxidant and mitochondrial dysfunction, as well as abnormal kidney development and function in adulthood.

Keywords: brain inflammation, oxidative stress, mitochondrial activity, kidney development
**Introduction**

There is increasing recognition of the gender difference in the physiological processes underpinning disease (1). The impact of gender disparities on the developmental programming of adulthood diseases has been well documented. Prenatal insults can change the physiological development of the foetus and newborn leading to both brain and kidney abnormalities that may only manifest in later life. In the brain, a decrease in hippocampal volume and cortical monocyte infiltration can result in neurological disorders in a gender-dependent manner (2). Under conditions of infection or inflammation, the microglial colonization and activation-induced neuronal loss is more severe in the male offspring compared to the female littermates (2). In contrast, chemical toxicity in humans, such as bone and kidney damage due to cadmium exposure (as found in cigarette smoke) is less severe in males than in females (3).

Cigarette smoke exposure during pregnancy is considered as a leading preventable causes of adverse outcomes in newborn infants, including low birth weight and reduced brain and kidney volumes (4, 5). Although the total number of women smokers has been decreasing worldwide, the rate of decline is slowing, with recent estimates of female smoking rates being 15.3% in the USA (6), 16.3% in Australia, and 23% in Argentina (7). The rate of second-hand smoke exposure is high in developing countries, such as China with 70% of adults exposed to second-hand smoke (8). The World Health Organisation predicts that female smoking will reach an ‘epidemic’ (20% of the population) in developing countries by 2025 (9). Unfortunately, smoking is still common during pregnancy, estimated at up to 15% (10) despite targeted public health education. It has been well documented that maternal smoking is directly linked to adult obesity, type 2 diabetes, chronic kidney disease and psychopathology, as well as childhood asthma (11-15). Our previous studies have shown that maternal cigarette smoke exposure (SE) can increase oxidative stress, mitochondrial dysfunction, and inflammation in both the brains and kidneys in male offspring (15-17). It has been shown that increased oxidative stress in the brain can lead to increased sympathetic tone that may induce hypertension, as well as kidney dysfunction (18, 19). We have also shown that maternal smoking is closely linked to increased oxidative stress, inflammation and mitochondrial dysfunction in both brain and kidney, and renal functional disorders in male offspring at adulthood (15, 16, 20, 21).

There are also numerous studies on the adverse effects of maternal smoking on other organ systems (22-24). However, these studies have mainly focused on male offspring. Such gender bias is common in animal studies. In fact, females are less susceptible to certain diseases than their male...
counterparts (25). Oestrogen is known to inhibit oxidative stress and inflammation (26, 27), and able to protect female offspring against the development of hypertension due to perinatal nicotine exposure (28). In addition, inflammation and oxidative stress also contribute to renal underdevelopment caused by maternal smoking in early life and chronic kidney disease at adulthood (15, 16, 20, 21). Furthermore, there is a gender difference in the development of renal injury where females tend to be protected from renal injury, independent of other health conditions (29-31). Epidemiological studies confirm that the association between low birth weight and adulthood kidney disease is more evident in the males than the females (25) and that maternal smoking is a significant factor leading to low birth weight in humans (32). However, the impact of maternal smoking on oxidative stress, the inflammatory profile and mitochondrial function in the brain and kidney, as well as renal function of female offspring in adulthood is unclear. These uncertainties formed the aims of this study.

Results
Effects of maternal SE on female offspring
Anthropometry
The body weights were significantly lower in the SE female offspring at both P20 and W13 (P<0.05, Table 1). Although the net brain weight was not different between the groups at either P20 or W13, their percentage relative to the body weight was significantly greater than in the SHAM offspring at P20 only (P<0.05, Table 1). Kidney weight was not different between the groups at both ages (Table 1). Plasma cotinine levels in the SE offspring (8.31 ± 1.93 ng/ml) were four times of that in the SHAM offspring (1.98 ± 0.56 ng/ml, P<0.05) at P20.

Brain markers
At P20, brain mRNA expression of inflammatory markers, including IL1 receptor (IL1R), IL6 and toll-like receptor 4 (TLR4) were significantly upregulated in the SE offspring (P<0.05); while the expression of inflammatory cytokines IL1β and tumor necrosis factor (TNF)α was not different between the groups (Figure 1). At W13, mRNA expression of IL1R and TLR4 remained higher in the SE offspring (P<0.05), while the other markers were not different between the groups (Figure 1).

At P20, there was no significant difference in brain protein levels of mitochondrial markers of oxidative stress, including the anti-oxidative marker manganese superoxide dismutase (MnSOD), markers of mitochondrial function. Translocaae of outer membrane (TOM)20 transport proteins from outer membrane into the inner membrane of mitochondria, and oxidative phosphorylation
OXPHOS complexes are the major sites for ATP synthesis with reactive oxygen species (ROS) as the by-products, both of which were not changed in the SE offspring (Figure 2a,b,c). However at W13, the protein levels of MnSOD and the OXPHOS complexes I-IV were all significantly increased in the SE offspring compared with the SHAM offspring (P<0.05, Figure 2d,e,f).

Early growth response protein (EGR)1 - hypoxia induced factor (HIF)-1α pathway is an important protective mechanism during environmental hypoxia to increase cell survival rate (46, 47). At P20, HIF-1α protein levels were significantly decreased in the SE offspring (P<0.05, Figure 3a); and EGR1 mRNA expression was also significantly downregulated at this time point (P<0.05, Figure 3c). At W13, neither HIF-1α protein nor EGR1 mRNA expression was changed (Figure 3c,d).

Kidney markers
mRNA levels of different growth factors involved in renal development were determined in the female offspring of SE and SHAM mothers as we have previously measured in the male offspring (15). However, no difference was observed in the mRNA expression of specific markers associated with kidney development between the two groups at P20 and W13 (Figure 4a,b). Interestingly, in the female offspring, there were no changes in the number of developed glomeruli and size between the groups at P20 or W13 (Figure 5). At W13, fully developed glomeruli were present in the kidneys of both treatment groups (Figure 5). Furthermore, at W13, the markers related to renal injury including fibronectin and collagen IV were not different between the groups (Figure 6). Markers of renal function, such as urinary albumin/creatinine ratio and plasma creatinine, were also shown to be similar between the treatment groups at both P20 and W13 (Table 2).

Discussion
The major finding in this study is that female offspring appear to be protected from some of the expected detrimental effects of maternal cigarette smoke exposure, including: brain inflammation, oxidative stress and mitochondrial dysfunction, as well as renal structural and functional disorders at adulthood, which we have previously demonstrated in male offspring (15, 17).

Maternal smoking is a significant risk to public health. Nicotine, the major addictive substance contained in cigarette smoke has been commonly used to model smoking in previous studies (26, 27). However, these studies have excluded the effects of additional toxic chemicals contained in cigarette smoke (33) that may also affect foetal development and future predisposition to chronic disease. Using a model with direct cigarette smoke exposure more closely approximates the
complexity of exposure to maternal cigarette smoking. Here we have evaluated the levels of cotinine, which is the major metabolic product of nicotine, and we have confirmed that it is increased in P20 female SE offspring, thereby validating the model and hence exposure to multiple toxins contained in cigarette smoke.

Our previous studies have shown that male offspring are vulnerable to maternal cigarette SE-induced underdevelopment of brain and kidney and potential dysfunction in adulthood, which are associated with increased inflammatory markers and oxidative stress(17). In this study, at adulthood, only the receptors of the inflammatory pathway were upregulated in the brains of female offspring. In the brains of male offspring from SE mothers, in addition to those changes also demonstrated in the females, pro-inflammatory cytokine IL6 was also increased. As such, male offspring appear to have higher levels of brain inflammation than the females due to maternal SE. Increases in brain inflammatory cytokines has been shown to predispose individuals to the development of neurodegenerative diseases later in life in both genders (34). It has been found that former male smokers have a higher risk of developing Alzheimer disease (35). We also observed here that adult SE female offspring have normalised expression of pro-inflammatory cytokines. This may be due to potential neuroprotective effects of oestrogen which has been shown to reduce the production of pro-inflammatory cytokines, such as IL-6 and TNFα (36).

Female offspring have been shown to be more resistant to oxidative stress as suggested by previous studies (37-39). Mitochondria are important cellular organelles as they are involved in ATP production; while ROS is a major by-product from OXPHOS complexes I and III during the electron chain transportation during ATP production (40). Excessive ROS accumulation leads to oxidative stress, and resultant cellular toxicity; while the antioxidant enzyme MnSOD can scavenge excessive ROS in the mitochondria to prevent such damage (41). Excessive mitochondrial ROS characterises cerebrovascular pathophysiology (42). As such, MnSOD has been found to reduce lipid peroxidation, protein nitration, and neuronal death after cerebral ischemic injury (43). In humans, maternal smoking was associated with increased oxidative stress in 3 months old babies (44). We have recently demonstrated increased brain oxidative stress in the male SE offspring at W13, adulthood (21), where MnSOD was significantly reduced in the face of increased both TOM20 and OXPHOS protein complexes. TOM20 transports substrates from the outer mitochondrial membrane to OXPHOS complexes for energy production (45), which can generate more free radicals during ATP synthesis. In this study, although increased OXPHOS complexes were observed in the female offspring at W13, which suggests an increased capacity for substrate
metabolism, unchanged TOM20 in the females may indicate unchanged energy metabolism in the brain. The level of brain mitochondrial MnSOD protein was increased in female SE offspring at W13, suggesting an increased ability to scavenge free radicals. As such, female offspring may be better at responding to an environment of higher oxidative stress than the males.

In response to an hypoxic environment, EGR1 is upregulated which in turn stimulates HIF1α to protect tissue from damage and increase survival rate under conditions such as transient focal cerebral ischemia (46, 47). HIF1α can also induce inflammatory responses in the brain to scavenge necrotic tissues (48). EGR1 also appears to have roles in promoting synaptic transmission, plasticity, learning and long term memory (49). Unlike the high level of HIF1α we have recently observed in the adult male offspring of SE mothers, HIF1α protein levels in the females were reduced at P20, but unchanged at W13. This indicates that there may be an adaptive mechanism in early life that protects the females from the impact of a hypoxic intrauterine environment due to maternal SE, which requires further investigation.

Although we have observed some similarities in the effects of smoking on the brain in both genders of offspring, the impact of maternal SE on kidney structure and function is considerably different between the male and female SE offspring. In the male offspring, we recently reported that changes in early growth and developmental factors lead to renal underdevelopment, with resultant renal dysfunction at adulthood following maternal SE (15), similar to what occurs during intrauterine undernutrition where the kidney is one of the organs that are ‘sacrificed’, resulting in renal underdevelopment (50). Here we demonstrate that female offspring are less susceptible to renal underdevelopment and resulting functional disorders due to maternal smoking compared to the male offspring. Either the adaptive changes in growth factors are more successful in the female, or other unknown mechanisms positively contribute. Previously, we have found that reduced mitochondrial activities and increased oxidative stress are closely related to renal underdevelopment and renal functional disorders at adulthood in the male offspring. Renal underdevelopment due to maternal smoking is an independent factor to lead to renal dysfunction in adulthood (25).

Our findings are in agreement with previous studies showing that female offspring are less vulnerable to diseases induced by intrauterine insults (39). It is known that gender is a risk factor for developing kidney disease due to the differences in renal structure, glomerular hemodynamics and hormonal metabolism between the males and females (51). Sex hormones can be an important
factor in affecting renal function, as oestradiol has been shown to inhibit transforming growth factor-β and transcription of downstream collagen IV, to reduce renal injury (52).

In conclusion, our previous papers have reported upregulated inflammatory cytokine IL-6 expression, and reduced anti-oxidative capacity in the brain (17), and renal underdevelopment and abnormal renal function in the kidney (15) of the male offspring by maternal SE. In female offspring reported in this study, we found unchanged IL-6 expression and increased anti-oxidative capacity in the brain, with normal renal development and function at adulthood. Thus, female offspring are more resistant to the detrimental effects of maternal smoking on brain and kidney in comparison to male offspring. Further work is needed to determine the intrauterine factors that differentiate such gender difference.

Materials and Methods

1. Maternal cigarette smoke exposure

Virgin female Balb/c mice (6 weeks, Animal Resources Centre, Perth, Australia) were housed at 20±2°C and maintained on a 12-h light, 12-h dark cycle (lights on at 0600 hours) with ad libitum access to standard laboratory chow and water. After acclimatisation, mice were assigned to SE or sham exposure (SHAM). The SE group was exposed to 2 cigarettes (Winfield Red, nicotine <1.2 mg, CO <15 mg, Philip Morris, VIC, Australia) in a perspex chamber, twice daily for six weeks prior to mating, during gestation and lactation; while the SHAM group was exposed to normal air as previously described (15). They were mated with male Balb/c mice (8 weeks) from the same source. Male breeders and suckling offspring were not exposed to cigarette smoke as we described previously (15). The female offspring were studied at postnatal (P) day 20 (P20, weaning) and week 13 (W13, mature age).

2. Sample Collection

Female offspring were culled after anaesthetic overdose (Pentothal®, 0.1 mg/g, i.p., Abbott Australasia Pty. Ltd., NSW, Australia) as previously described (15). Blood was collected via cardiac puncture and plasma was stored at -20°C. Plasma cotinine concentrations were measured by ELISA (Abnova, Taipei, Taiwan). Urine was collected directly from the bladder. The brain was dissected into the left and right hemisphere. The left brain hemisphere and left kidney were stored at -80°C for mRNA and protein analysis, while the right kidney were fixed with 10% formalin for histological analysis.
3. Quantitative real-time (rt)-PCR

Brain inflammatory markers and renal developmental markers were measured by rt-PCR. Total mRNA was extracted from the brain tissues using TriZol reagent (Life Technologies, CA, USA). The purified total RNA was used as a template to generate first-strand cDNA using M-MLV Reverse Transcriptase, RNase H, Point Mutant Kit (Promega, Madison, WI, USA) as previously described (53). Genes of interest were measured using manufacturer pre-optimized and validated Taqman® primers and probes (Life Technologies, CA, USA). EGR1, probe TGAGCACCTGACCACAGAGTCTTT, NCBI references: NM_007913.5, M20157.1, M19643.1, ID Mm00656724_m1; IL-1β, probe TCCTTGTGCAAGTGCTGAAGCAGC, NCBI references: NM_008361.3, M15131.1, BC011437.1, ID Mm01336189_m1; IL-1R, probe AGCTGACCAGATCAATGATACAA, NCBI references: NM_001123382.1, NM_008362.2, M20658.1, ID Mm00434237_m1; IL-6, probe ATGAGAAAGAGGGTGTCAATGGCA, NCBI references: NM_031168.1, X06203.1, X54542.1, ID Mm00446190_m1; TLR4, probe CCCTGCATAGAGGTAGTTCCTAATA, NCBI references: NM_021297.2, ID Mm00445273_m1; TNFα, probe CCCTCACACTCGATCATCTTCTCA, NCBI references: NM_013693.2, X02611.1, M13049.1, ID Mm00443259_g1) or pre-optimized SYBR Green primers as previously published (15). For the Taqman® probes, target genes were labelled with FAM and the housekeeping 18s rRNA was labelled with VIC. Gene expression was standardized to 18s RNA. Then the average expression of the control group was assigned as the calibrator against which all other samples are expressed as fold difference.

4. Western Blotting

The protein levels of HIF-1α, MnSOD, TOM20 and OXPHOS complexes proteins were measured. The brain was homogenised using cell lysis buffers for whole protein and mitochondrial protein extraction. Protein samples of 40µg were separated on NuPage® Novex® 4-12% Bis-Tris gels (Life Technologies, CA, USA) and then transferred to PVDF membranes (Rockford, IL, USA). Membranes were then blocked with non-fat milk powder and incubated with primary antibodies (HIF-1α (1:1000, Novus Biologicals, Colorado, USA), MnSOD (1:1000) & TOM20 (1:2000, Santa Cruz Biotechnology, Texas, USA) and Mitoprofile Total® OXPHOS complex rodent WB antibody (1:2500, Abcam, Cambridge, UK) for overnight and then goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:2000 for HIF-1α; 1:5000 for MnSOD, TOM20 and OXPHOS complex). Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher, MA, USA) by exposure of the
membrane in FujiFlim (Fujifilm, Tokyo, Japan). Protein band density was determined with Image J software (NIH, MD, USA).

5. Kidney histology
Fixed kidney samples were embedded in paraffin and renal structure was examined using haemotoxylin and eosin (H&E) staining. Glomerular number was estimated by counting the developed glomeruli in 3-4 non-consecutive kidney sections from the same animal, and 6-8 animals were used from each group. Glomerular size for each animal was measured using Image J (Image J, NIH, USA) in an average of 6 different images for the same kidney section then averaged (15). For IHC staining, kidney sections were incubated with rabbit anti-mouse primary antibodies against fibronectin (1:500) and collagen IV (1:500) (Abcam, Cambridge, UK), and Envision & HRP-labelled polymer secondary anti-rabbit antibodies (Dako, CA, USA), followed by horseradish peroxidase enzyme and DAB for colour detection (Dako, CA, USA). On average, 6 different non-overlapping fields of the same kidney section were captured. Quantitation of the positive signals (the intensity of the brown colour) in the captured images was performed using Image J software (NIH, MD, USA) and the percentage of the brown colour of the whole field was determined and averaged.

6. Albumin and creatinine assays
Urine albumin and creatinine were measured using a Murine Microalbuminuria ELISA kit (Albuwell M, Exocell Inc, PA, USA) and a Creatinine Companion kit (Exocell Inc, PA, USA) respectively as previously described (15). Serum enzymatic creatinine levels were measured by an automated analyser (ARCHITECT, Abbott Australasia PTY. LTD, NSW, Australia) (15).

7. Statistical methods
Results are expressed as mean ± S.E.M. Data were analysed for parametric distribution. The difference between groups was analysed using unpaired Student’s t test (Statistica 9, Statsoft, USA).

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References

Table 1. Parameters of female offspring at different ages

<table>
<thead>
<tr>
<th>Offspring</th>
<th>P20</th>
<th>W13</th>
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<tbody>
<tr>
<td></td>
<td>SHAM</td>
<td>SE</td>
</tr>
<tr>
<td>n = 21</td>
<td>n = 17</td>
<td>n = 16</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>9.7 ± 0.24</td>
<td>9.0 ± 0.28*</td>
</tr>
<tr>
<td>Brain (mg)</td>
<td>14 ± 0.6</td>
<td>14 ± 0.4</td>
</tr>
<tr>
<td>Brain%</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.1*</td>
</tr>
<tr>
<td>Kidney (mg)</td>
<td>70.2 ± 6.3</td>
<td>63.9 ± 2.4</td>
</tr>
<tr>
<td>Kidney%</td>
<td>0.73 ± 0.06</td>
<td>0.72 ± 0.02</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. Data were analysed by student’s unpaired t test. * P<0.05, compared to the SHAM offspring at the same age, n=14-21.

Table 2. Markers of renal function at different ages.

<table>
<thead>
<tr>
<th>Offspring</th>
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<th>W13</th>
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<tbody>
<tr>
<td></td>
<td>SHAM</td>
<td>SE</td>
</tr>
<tr>
<td>n = 21</td>
<td>n = 17</td>
<td>n = 16</td>
</tr>
<tr>
<td>Urinary albumin/creatinine ratio (µg/mg)</td>
<td>6.4 ± 0.9</td>
<td>6.2 ± 1.6</td>
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<tr>
<td>Plasma enzymatic creatinine (µmol/l)</td>
<td>18.0 ± 2.9</td>
<td>25.7 ± 5.6</td>
</tr>
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</table>

Results are expressed as mean ± S.E.M. Data were analysed by student’s unpaired t test. * P<0.05, compared to the SHAM offspring at the same age, n=14-21.
**Figure legends**

**Figure 1.** Brain mRNA expression of inflammatory markers in the SHAM and SE offspring (n=8). Results are expressed as mean ± S.E.M. Data were analysed by student’s unpaired t-test. *P<0.05; ** P<0.01. SE: Smoke exposed; TLR: toll-like receptor.

**Figure 2.** Brain mitochondrial protein levels of MnSOD (a and b), TOM20 (b and e), and OXPHOS complexes (CI-V, c and f) in the SHAM and SE offspring at different ages. Results are expressed as mean ± S.E.M. Data were analysed by student’s unpaired t-test. P<0.05; ** P<0.01. MnSOD: manganese superoxide dismutase; OXPHOS: oxidative phosphorylation; SE: smoke exposed; TOM: translocase of the mitochondrial outer membrane.

**Figure 3.** Brain HIF-1α protein and EGR1 mRNA level in the SHAM and SE offspring at P20 and W13 (n=3-8). Results are expressed as mean ± S.E.M. Data were analysed by student’s unpaired t-test. * P<0.05. EGR1: early growth response protein; HIF: hypoxia inducible factor; SE: smoke exposed.

**Figure 4.** Renal mRNA expression of growth and transcription factors in the female offspring mice at P20 (a) and W13 (b). Results are expressed as mean ± SEM, n=6.
BMP, bone morphogenetic proteins; FGF, Fibroblast growth factor; GDNF, glial cell-line derived neurotrophic factor; Pax, paired box; WNT, wingless-type MMTV integration site family member; WT, Wilms tumour inhibitory protein.

**Figure 5.** H&E stained Kidney sections from the sham offspring (Left panel) and SE offspring (Right panel) at P20 (a and b), and W13 (c and d) and glomerular numver and size for the same age. Closed arrows indicate fully developed glomeruli. Results are expressed as mean ± SEM, n=6-8. Mag. 20X.

**Figure 6.** Renal fibronectin (Left panel) and collagen IV (Right panel) protein expression in the Sham (top panel) and SE offspring (lower panel) at W13. Results are expressed as mean ± SEM, n=6. Mag. 40X.