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Impact of maternal e-cigarette vapour exposure on renal health in the offspring

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Abstract

Maternal smoking during pregnancy is a significant risk factor of renal pathology in the offspring. E-cigarettes are perceived to be a safe option and are increasingly used by pregnant women either continuously during pregnancy or as a replacement for tobacco cigarettes. This study aimed to determine the effects of replacing tobacco cigarettes with e-cigarettes during pregnancy, and continuous e-cigarette use during pregnancy on the offspring's kidney. Female Balb/c mice were exposed to either air (Sham) or tobacco cigarette smoke (SE) for 6 weeks prior to mating, during gestation and lactation. A subset of the SE group received e-cigarette vapour (containing nicotine) after mating until pups weaned. Additional female mice were continuously exposed to e-vapour (either with or without nicotine) for 6 weeks prior to mating until pups weaned. Kidneys and urine from the male offspring were assessed at postnatal day 1, day 20 (weaning), and 13 weeks of age (adulthood). E-cigarette replacement was less detrimental to renal development and functional outcomes than continuous SE exposure during pregnancy. However, continuous e-vapour exposure during pregnancy increased markers of oxidative stress, inflammation and fibrosis in the adult offspring, independent of nicotine. E-cigarette use during pregnancy confers future risk to the offspring's kidney.

Key words: oxidative stress; fibrosis; kidney development; inflammation; e-cigarette

Introduction

Cigarette smoking is a risk factor for the development of end stage kidney disease, with nicotine being considered a major contributor as it accumulates within the kidneys and is secreted through the glomeruli and renal tubules [1]. Maternal smoking during pregnancy confers similar risks to the offspring [1-3]. A recent study in children demonstrated an association between maternal smoking during pregnancy and the presence of proteinuria in 3-year old offspring [4], indicating impaired kidney function in early childhood and an increased risk of chronic kidney disease later in life. Maternal

smoking restricts intrauterine growth, leading to reduced birth weight and the underdevelopment of multiple organs, including the kidneys [5-7]. Furthermore, many toxins present in cigarette smoke, such as nicotine, can pass the blood-placenta barrier, directly harming foetal organs. Thus, maternal smoking during pregnancy can affect the kidney health of the unborn child [2]. Previously, we have comprehensively studied the detrimental impacts of prenatal cigarette smoke exposure (SE) on the offspring and discovered that oxidative stress and mitochondrial dysfunction play critical roles in delayed early renal development and renal damage due to maternal SE [6-9]. In addition, oxidative stress injury markers, including nitrotyrosine and 8-hydroxydeoxyguanosin (8-OHdG), were also increased in the kidneys from the SE offspring in line with increased mitochondrial oxidative stress level [7-9].

Nicotine addiction contributes to the failure of smoking cessation especially during pregnancy [10, 11], leading to the pursuit of alternative forms of nicotine administration. E-cigarettes are one such nicotine delivery system and their usage has steadily increased since 2008, especially amongst the younger population [12], with 20% of American middle and high-school students vaping nicotine in 2018 [13]. E-cigarettes are perceived as a safe and effective replacement to tobacco cigarettes, leading to increased usage among pregnant women who feel stigmatised by their tobacco smoking habits [14-16]. Even among obstetricians in 2012, 13.5% believe that e-cigarette vaping during pregnancy poses no adverse health risks to either the user or the unborn child [17]. Thus, e-cigarettes have become the nicotine replacement therapy of choice among pregnant women, being favoured over nicotine patches [18]. Between 2014 and 2016, ever use of e-cigarettes during pregnancy ranged from 13% to 15%, with dual usage (8.54%) being more popular than e-cigarette only vaping (6.52%) and cigarette only smoking (5.62%) [16, 18, 19].

Although e-cigarettes are considered safer than tobacco cigarettes due to less harmful chemicals generated, they have not been established as a completely safe alternative. E-cigarettes heat a liquid (often containing propylene glycol, glycerol, nicotine and flavouring additives), producing many

toxic by-products in the process [20]. We have previously shown increased inflammatory responses in offspring's lungs and brains in response to continuous maternal e-vapour exposure [21-23]. Interestingly, maternal exposure to nicotine-containing and nicotine-free e-vapour differentially altered inflammatory markers, suggesting that nicotine is not solely responsible for the harmful effects to the developing foetus [21]. Furthermore, another study showed that chronic e-vapour exposure in mice impaired glomerular filtration rate and increased kidney fibrosis [24]. However, the impact of maternal e-vapour exposure on the renal health outcome in the offspring is unknown. Similarly, it is unknown whether using e-vapour to replace tobacco smoke (replacement) from conception can protect the offspring's renal health. Therefore, the aims of this study were to determine the effects of, i) replacing tobacco smoke with nicotine containing e-vapour during pregnancy and lactation, and; ii) continuous e-vapour (nicotine containing and nicotine-free) exposure during pregnancy and lactation on renal development and markers of oxidative stress, inflammation and fibrosis in adult kidneys in the offspring. Male offspring were studied because they are more susceptible to kidney damage induced by maternal smoking while female offspring are protected, as elucidated in our previous studies [6, 25].

Materials and Methods

Animals

The animal experiments were approved by the Animal Care and Ethics Committee of the University of Technology Sydney (ACEC#2014-638 and ETH15-0025) and performed according to the Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Female Balb/C mice (7 weeks old, Animal Resource Centre, WA, Australia) had *ad libitum* access to standard laboratory chow and water while housed at 20±2 °C and maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h). Female mice were acclimatised for a week prior to the exposure treatments detailed below.

Modelling maternal smoking and e-cigarette replacement

Female mice were either exposed to room air (Sham) or tobacco smoke (SE) generated by 2 cigarettes (Winfield Red, ⩽16 mg tar, ⩽1.2 mg nicotine, and ⩽15 mg of CO; VIC, Australia) twice daily, 6 weeks before mating and throughout gestation and lactation. This was adopted from our protocol of maternal SE [6, 7, 25], and results in nicotine exposure in the offspring which is equivalent to human babies from light smokers [26]. In a subset of the SE exposed mice, SE exposure was replaced with e-vapour generated from e-liquid containing 18mg/mL nicotine (Replacement) from mating until the pups weaned (Figure 1), as previously described [21, 23]. This nicotine exposure was equivalent to that of 2 tobacco cigarettes as indicated by similar serum cotinine (the major, stable nicotine metabolite) levels in the offspring, as we have previously published in the same cohort of offspring [21].

Modelling maternal e-cigarette vaping

To investigate the impact of continuous e-vapour exposure, another 2 groups of female mice were exposed to either e-vapour generated from e-liquid containing 18mg/mL nicotine (E-cig18), or e-vapour from nicotine free e-liquid (E-cig0) for 6 weeks prior to mating and throughout gestation and lactation (Figure 1). Aerosols were generated by a human-use e-cigarette device (KangerTech NEBOX, 30 Watts, 0.5 Ohms, KangerTech, Shenzen, China) from commercial e-liquids (50% propylene glycol/50% vegetable glycerine, tobacco flavour additives, Vaper Empire, VIC, Australia). Mice were exposed twice daily (10am, 3pm) for 2 x 15 minute (4 x 5 second puffs with 20 second interval between each puff, 60mL), with a 5-minute washout interval.

Dams were removed from their home cages during exposure, while the male breeders and pups were not exposed. Pups were weaned at postnatal day (P) 20 and maintained without additional intervention.

Male offspring were euthanized after deep anaesthesia (2% isoflurane) at P1, P20, and 13 weeks (W13). The animals analysed in this study were a sub-cohort of a previously published study [21]. Kidneys from the male offspring were harvested, weighed and then either snap frozen and stored at -80°C or fixed in 10% formalin for further analyses. Kidney weights (%) were calculated as a fraction of body weight.

Urine from the W13 male offspring was collected from the bladder via puncture and used for albumin and creatinine measurements using Murine Microalbuminuria ELISA kit (Albuwell M) and Creatinine Companion kit (Exocell Inc, PA, USA), respectively as per manufacturer's instructions.

Real-time PCR

Macrophage chemoattractant protein (MCP)-1, NADPH oxidase 4 (Nox4), TNF-α and IL-6 expression were measured in the W13 offspring. These are commonly used markers of kidney inflammation, a key contributor to the development of renal fibrosis and chronic kidney disease. Total mRNA was extracted from the kidneys using TriZol reagent (Thermo Fisher Scientific, MA, USA) and first-strand cDNA generated according to manufacturer's specifications (Promega, Madison, WI, USA). The expression of MCP-1 (forward primer: 5'-GTTGTTCACAGTTGCTGCCT-3', and reverse primer: 5'-CTCTGTCATACTGGTCACTTCTAC-3') and Nox4 (forward primer: 5'CTGGTCTGACGGGTGTCTGCATGGTG-3', and reverse primer: 5'-CTCCGCACAATAAAGGCACAAAGGTCCAG-3') were measured using primers labelled by SYBR green® (Sigma-Aldrich, MO, USA) as we have published previously [9, 27]. TNF-α (CCCTCACACTCAGATCATCTTCTCA, NCBI reference: NM_013693.2, ID: Mm00443259_g1, Thermo Fisher Scientific, MA, USA) and IL-6 (ATGAGAAAAGAGTTGTGCAATGGCA, NCBI reference: NM_031168.1, ID: Mm00446190_m1, Thermo Fisher Scientific, MA, USA) expression were measured using pre-optimised and validated TaqMan® primers and probes. Values are expressed as fold changes to the Sham group and 18S rRNA expression was used as the house-keeping gene.

Glomerular density and perimeter

Formalin fixed, paraffin embedded kidneys were sectioned at 5μm and stained with Periodic Acid-Schiff (PAS). The number of developed glomeruli in a given area was manually counted in the whole

kidney for P1 and from 5 non-overlapping areas on the same slide and averaged for P20 and W13 offspring. Three non-consecutive slides were used for each animal, as previously described [6]. The glomerular perimeter (10/slide) was measured using ImageJ (National Institutes of Health, MD, USA).

Renal histology and morphometric analysis

Glomerulosclerosis and tubulointerstitial fibrosis were assessed using PAS and Picrosirius red staining (PSR). The whole kidney cortex was examined under 400x magnification using a light microscope (Olympus photomicroscope linked to a DFC 480 digital camera). Two independent assessors reviewed histological sections in a blinded manner and scored glomerulosclerosis. The characteristic features of tubulointerstitial fibrosis include tubular atrophy or dilatation, presence of mononuclear inflammatory cells, widening of interstitial spaces with deposition of ECM, interstitial cell proliferation and wrinkling or thickened tubular basement membrane were also reviewed. PSR staining was performed as we have previously demonstrated and quantitated [28]. Ten non-overlapping fields in each animal (5 animals/group) were assessed in total. The average staining area was represented.

Glomerulosclerosis was graded as follows: 0 - normal, 1 - < 25% involvement, 2 < 50% involvement, 3 - < 75%, and 4 - > 75% sclerosis. A semi-quantitative glomerulosclerosis index score was calculated from averaging scores from all counted glomeruli in one section.

Mitochondrial density and ROS level

Frozen kidneys were sectioned to quantify mitochondrial density and ROS levels. Kidney mitochondria were visualised using MitoTracker Green (Thermo Fisher Scientific, Australia) and images were acquired at 488 nm excitation wavelength and detected in the 510-550nm emission range as previously reported [9]. For total reactive oxygen species (ROS), CellROX Deep Red (Thermo Fisher Scientific, Australia) was used and images were acquired at 633 nm excitation wavelength and detected in the 640-680 nm emission range. All imaging parameters including laser intensities, photomultiplier tubes voltage and pinholes were kept constant during imaging. The MitoTracker and

CellRox images were overlayed and the correlation coefficient was calculated to provide information on mitochondrial specific ROS, as we have previously reported [8]. Data was generated using ImageJ (National Institutes of Health, MD, USA).

Immunohistochemistry

Formalin fixed, paraffin embedded kidneys were sectioned at 5µm. Sections were deparaffinised and treated with xylene and decreasing grades of ethanol to distilled water. For antigen retrieval, sections were heated and pressurised in either citrate buffer (pH 6.0) or EDTA buffer (pH 8.0) and then cooled in a water bath. Sections were incubated with the following anti-rabbit primary antibodies: nitrotyrosine (1:600, Merck, NJ, USA), fibronectin (1:100, Abcam, Cambridge, UK), 8-OHdG (1:150, Bioss Antibodies, MA, USA), Collagen IV (1:100, Abcam, Cambridge, UK) or F4/80 (1:250, Abcam, Cambridge, UK) and visualised with the horseradish peroxidase anti-rabbit Envision system (Dako Cytochemistry, Tokyo, Japan). Sections were counterstained with haematoxylin and captured for analysis. Staining was quantified with ImageJ software (National Institutes of Health, MD, USA).

Statistical analysis

Results are expressed as mean ± SD and analysed by one-way ANOVA followed by Fisher's Least Significant (LSD) *post hoc* tests (GraphPad Prism 7, GraphPad Software, Inc, CA, USA). $P<0.05$ was considered the threshold for statistical significance.

Results

Body weight

Maternal SE and e-vapour replacement during gestation

At postnatal day (P)1, the body weight of the male offspring from cigarette smoke exposed dams (SE group) was smaller than those from Sham exposed dams (11.0 ± 2.6% smaller, P=not significant (NS)), and dams with e-vapour replacement from time of conception (Replacement group, $P< 0.05$ vs Sham, Table 1). Kidney development, as assessed by kidney weight, was reduced in the SE and Replacement groups (17 ± 8.7% and 33 ± 16% lighter than the Sham group, respectively, both P =

NS). At P20, both SE and Replacement offspring were significantly smaller than the Sham offspring (both P< 0.05, Table 1). However, kidney mass was not different among the 3 groups. At 13 weeks (W13), SE offspring remained lighter (P< 0.05 vs Sham), with smaller kidneys both as net weight and standardised to body weight as a percentage (both P< 0.05 vs Sham, Table 1). This suggest that e-vapour replacement during pregnancy is unlikely to rescue intrauterine growth restriction caused by maternal SE. Smoking status prior to pregnancy may also play a key role in foetal development.

Continuous e-vapour exposure during pregnancy

At P1, offspring from mothers exposed to e-vapour had similar body weight to the Sham offspring, regardless of nicotine concentration. However, kidney weights as a percentage of body weight were lower in the offspring from dams exposed to nicotine containing e-vapour (E-cig18, 28 ± 10% lower) and nicotine-free e-vapour (E-cig0, 36 ± 15% lower) compared to the Sham offspring (both P=NS). At P20, E-cig18 offspring were significantly smaller (P< 0.05 vs Sham, Table 2), but E-cig0 offspring were significantly larger than the Sham and E-cig18 offspring (P< 0.05 vs Sham and Eicg-18, Table 2). Kidneys were also heavier in the E-cig0 offspring (P< 0.05 vs Sham, Table 2), which was not different from the Sham offspring when standardised by body weight. At W13, maternal e-vapour exposure did not affect adult body weight and kidney mass regardless of nicotine concentration. This confirms the role of nicotine in foetal underdevelopment; however other chemicals in the e-vapour (eg. lipids) may increase early postnatal growth.

Kidney development

Maternal SE and e-vapour replacement during gestation

At P1, glomerular number was significantly reduced in SE offspring (P< 0.05 vs Sham, Figure 2a), which was not reversed by maternal e-vapour replacement (P< 0.05 Replacement vs Sham, Figure 2a). At P20, the number of developed glomeruli remained lower in the SE offspring (P< 0.01 vs Sham, Figure 2b), which was normalised in the Replacement offspring (P< 0.01 vs SE, Figure2b). At W13,

this reduction in glomerular density in the SE group was retained (P< 0.01 vs Sham, Figure 2c), and was partially normalised in the Replacement group (P< 0.05 vs SE). In line with reduced glomerular number, glomerular perimeter at P20 was increased in the SE offspring (P<0.01 vs Sham), which was normalised in the Replacement group (P< 0.01 vs SE, Figure 2d). However, at W13, glomerular perimeter was reduced in the SE offspring (P< 0.05 vs Sham, Figure 2e). Urine albumin to creatinine ratio was increased in the SE offspring (P< 0.05 vs Sham, Table 1) but not in the Replacement offspring compared to the Sham offspring. Although e-vapour replacement is unlikely to rescue intrauterine kidney underdevelopment caused by maternal SE prior to gestation, postnatal catch-up growth may compensate for glomerular development and function.

Continuous e-vapour exposure during pregnancy

At P1, glomerular number was lower in the E-cig18 offspring (14 ± 2.9% lower than the Sham offspring, P = NS) and the E-cig0 offspring (23 ± 3.6% lower than the Sham offspring, P< 0.05, Figure 2a). At P20, E-cig18 and E-cig0 offspring had reduced glomerular density compared to the Sham offspring (P< 0.05 and P< 0.01, respectively, Figure 2b). At W13, glomerular density was reduced in both the E-cig18 and E-cig0 offspring (P< 0.05 vs Sham, Figure 2c). Glomerular perimeter was also significantly reduced in E-cig0 offspring at P20 and 13 W13 (P< 0.05 vs Sham, Figure 2d, e). No differences were observed in the urine albumin to creatinine ratio in the W13 offspring between the groups (Table 2). Thus, continuous e-vapour exposure adversely affects renal development, which is nicotine independent.

Renal oxidative stress and mitochondrial density

Maternal SE and e-vapour replacement during gestation

At W13, renal mitochondrial density, reflected by MitoTracker staining, was significantly increased in the SE offspring (P< 0.01 vs Sham), which was more than doubled in the Replacement offspring (P< 0.01 vs Sham and SE, Figure 3a). Free radical total reactive oxygen species (ROS) level was also

doubled in the Replacement group (P< 0.01 vs Sham and SE, Figure 3b), whereas mitochondrial specific ROS was only increased in the Replacement group (P< 0.05 vs Sham), but not in the SE group (Figure 3c). The expression of a major ROS regulating enzyme, Nox4, was also upregulated in the SE group (P< 0.05 vs Sham), but not in the replacement group (Figure 3e). Protein levels of the oxidative stress marker, nitrotyrosine, was increased in the SE group (P< 0.01 vs Sham), but was reduced in the Replacement group compared to the SE group (P< 0.05, Figure 3f). Furthermore, DNA oxidative damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) was increased in the SE group (P< 0.05 vs Sham) and normalised in the Replacement group (P< 0.05 vs SE, Figure 3g), in line with the oxidative stress marker Nox4. This suggests that maternal e-vapour replacement can reduce oxidative stress-induced damage caused by maternal SE.

Continuous e-vapour exposure during pregnancy

Kidney mitochondrial density was significantly increased in both the E-cig18 and E-cig0 groups (P< 0.05, P< 0.01 vs Sham, respectively, Figure 3a). ROS level was increased to a similar level in the E-cig18 and E-cig0 groups (both P< 0.01 vs Sham, Figure 3b). As a result, mitochondrial ROS level was also increased in the E-cig18 and E-cig0 groups, respectively (both P< 0.01 vs Sham, Figure 3c). Meanwhile, renal Nox4 expression was not significantly different among the three groups (Figure 3e), suggesting Nox4 is not the major regulator of ROS. Kidney nitrotyrosine expression also significantly increased in both the E-cig18 and E-cig0 groups (P< 0.05 vs Sham, Figure 3f), while 8-OHdg staining was increased only in the E-cig0 group (P< 0.05 vs Sham, Figure 3g). Maternal continuous e-vapour exposure increased oxidative stress and related injury in offspring's kidney, which is not nicotine independent.

Inflammatory response and fibrotic change in the kidneys

Maternal SE and e-vapour replacement during gestation

At W13, inflammatory marker, MCP-1 expression was upregulated in the SE offspring (P< 0.05 vs Sham, Figure 4a), which was normalised in the Replacement offspring. The levels of TNF-α and IL-

6 expression was increased in the SE group yet without statistical significance (Figure 4b, c). Renal IL-6 expression was however decreased in the Replacement group compared to the SE group ($P < 0.05$, Figure 4c). Surface macrophage marker, F4/80, staining was significantly increased in the SE group compared to the Sham group at W13 ($P< 0.05$, Figure 4d). Fibrotic markers, fibronectin and collagen IV, were both increased in the kidneys of the SE offspring in adulthood ($P< 0.05$ vs Sham and $P< 0.01$ vs Sham, Figure 4e, f). In the Replacement offspring, renal fibronectin expression was normalised ($P< 0.05$ vs SE, Figure 4e); while Collagen IV expression was still increased compared to the Sham offspring ($P< 0.05$, Figure 4f). However, picrosirius red staining did not demonstrate any significant changes in mature collagen deposition among the three groups (Figure 4h). Furthermore, no significant changes in glomerulosclerosis were observed in PAS stained kidney sections (Figure 4i). The overall results suggest that maternal e-vapour replacement can ameliorate maternal SE induced inflammatory response and fibrotic changes in the offspring's kidney.

Continuous e-vapour exposure during pregnancy

At W13, MCP-1 mRNA expression was nearly doubled in the E-cig18 group only ($P<0.05$ vs Sham and E-cig0, Figure 4a). Renal TNF-α and IL-6 expression were not significantly different among the three groups (Figure 4b, c). F4/80, was increased in the kidneys of both the E-cig18 and E-cig0 offspring compared to the Sham offspring ($P< 0.05$, Figure 4d). Furthermore, both fibronectin and collagen IV staining were increased in the E-cig18 and E-cig0 group compared to the Sham group in the kidneys of adult offspring ($P< 0.05$, Figure 4e, f). However, no changes in picrosirius red staining was observed (Figure 4h), indicating no changes in mature collagen deposition. Glomerulosclerosis index was also not significantly different among the three treatment groups (Figure 4i). Thus, continuous intrauterine e-vapour exposure may predispose the offspring to mild renal fibrotic changes in adulthood.

Discussion

E-cigarettes have emerged as a 'safe' replacement to tobacco cigarettes, delivering nicotine with fewer and lower levels of other harmful compounds compared to tobacco cigarettes [20]. However, nicotine is not solely responsible for impairing foetal development by maternal tobacco cigarette smoking during pregnancy [2, 20]. As such, smoking cessation during gestation is necessary to optimise foetal health outcomes. However, quit rates can be as low as 5% [29], even with nicotine replacement therapy which is no better than placebo in smoking cessation during pregnancy [11]. This makes e-cigarettes an appealing option as a replacement to tobacco cigarettes, especially with the general perception that it is safe during pregnancy. Furthermore, increased usage among the younger generation may inadvertently lead to *in-utero* exposure due to unplanned pregnancies. In this study, we found that although replacing tobacco cigarettes with e-cigarettes resulted in increased total ROS and mitochondrial specific ROS, overall renal development and function was improved compared to continuous cigarette smoke exposure during pregnancy. This beneficial effect was similar to what we have observed in brain metabolic markers [23]. However, continuous e-vapour exposure during pregnancy is still detrimental to foetal development and increased kidney markers of oxidative stress, renal inflammation and fibrosis in the adult offspring, independent of nicotine.

In humans, low birth weight and reduced foetal and infant kidney volumes are commonly observed in smokers' children [30]. Kidney underdevelopment is thought to be due to the vasoconstrictive action of nicotine which leads to placental insufficiency, limiting intrauterine resources to the kidneys and reducing nephron numbers [31]. Here, in the SE group, male offspring weighed less after weaning and had fewer developed glomeruli, indicating intrauterine growth restriction. This is consistent with our previous finding in a different cohort of offspring from SE dams [6, 7], demonstrating the reproducibility of this model. At weaning, SE offspring had glomerulomegaly (increased glomerular size) to compensate for this reduction in the number of developed glomeruli. However, at adulthood, male offspring had fewer developed glomeruli along with a reduction in glomerular size, overall impairing renal function as indicated by an increase in the urine albumin to creatinine ratio. This reduction in

renal function was associated with an increase in the expression of markers of oxidative stress injury, inflammation and fibrosis within the kidneys during adulthood. Similarly, in humans, the progression of chronic kidney disease is associated with increased oxidative stress and inflammation, with the expression of markers being negatively correlated with kidney function [32-34]. Furthermore, antioxidant treatments can reduce cigarette smoke induced kidney damage, supporting the critical role of oxidative stress in the development of kidney disease [7, 9, 35, 36].

E-cigarettes have recently emerged as a replacement for tobacco cigarettes, being more popular than other forms of nicotine replacement therapy among pregnant women [18]. However, their impact on the offspring's health outcomes won't be fully elucidated by human epidemiological studies for at least 50 years. As such, animal models can provide perspectives on the future for this birth cohort. In this study, birth weight and body weight remained low in the Replacement group compared to the SE group likely due to similar levels of nicotine or other chemicals passing the blood-placental barrier and reaching the developing foetus. In the Replacement group, intrauterine kidney development was not improved since renal profile at birth was similar to the SE offspring. However, glomerular size and density were normalised at weaning in the Replacement offspring compared to SE offspring. We speculate that this may be due to different chemical components secreted into the breastmilk, allowing for catch-up growth during the early postnatal period, which requires further investigation. As a result, adult male offspring from the Replacement group did not exhibit a loss of kidney function (as indicated by normalised urine albumin to creatinine ratio) even though the markers of oxidative stress and fibrosis were increased compared to Sham group, although at lower levels compared to the SE group.

Replacing tobacco cigarettes with e-cigarettes during pregnancy seems to improve renal outcomes in the adult male offspring when compared to continuous intrauterine tobacco cigarette smoke exposure. However, this needs to be interpreted with caution, as we only adopted a low dose regime for both tobacco cigarette smoke and e-vapour exposures. It needs to be noted that this study ended when the offspring reached 13 weeks of age, representing young adulthood when mice are just sexually mature.

These adverse changes in oxidative stress and fibrotic markers are still of concern even though renal function and pathology were not altered at this stage, which is consistent with our previous study [6]. Since the risk of chronic kidney disease is much higher in the aging population, it remains to be determined if these early life changes will increase the risk of developing chronic kidney disease given a longer time frame or exposing those offspring to additional postnatal insult, such as smoking or consumption of a 'junk diet', which are known to deteriorate renal function.

Even though e-vapour replacement partially restored renal development and function after weaning, continuous e-vapour exposure during pregnancy was detrimental to the kidneys of the offspring. Although not significant, continuous maternal exposure to nicotine containing e-vapour (E-cig18) still affected intrauterine kidney development, with catch-up growth not prominent during the suckling period. In addition, increased markers of oxidative stress, inflammation and fibrosis were observed in adult offspring's kidneys, although renal function and pathology appear normal . Continuous maternal E-cig0 exposure promoted postnatal growth but simultaneously reduced glomerular size and density after weaning, indicating impaired kidney development which persisted until adulthood. Furthermore, E-cig0 offspring exhibited increased markers of oxidative stress, inflammation and fibrosis compared to Sham offspring. However, these changes were not significant enough to cause a loss of renal function at this age.

Intrauterine e-vapour exposure caused kidney pathological changes in the young adult offspring independent of the presence of nicotine, indicating that nicotine is not solely responsible for the adverse renal outcomes observed. Previously, maternal exposure to high doses of nicotine increased renal fibrosis and reduced glomerular size at P7 and P21 in rats [37]. However, the use of nicotine patches during pregnancy has not been shown to impact birth weight and intrauterine development in humans [38], demonstrating that the observation in the animal study is likely due to unphysiologically high dosages. Thus, it is unsurprising that E-cig18 and E-cig0 offspring exhibited similar impairment in renal development and increased renal oxidative stress and fibrosis. Other components inside the e-vapour could be responsible by damaging mitochondrial wellbeing by increasing mitochondrial

ROS level and oxidative stress. In diabetes, oxidative stress and mitochondrial damage have been implicated as the key mediators in developing nephropathy [39]. Future studies can challenge these offspring with diabetes to examine the implication in the development of kidney disease.

Since this is the first study of its kind, there are some limitations. The first is that a low dose of cigarette smoke and e-vapour was used, representing the situation of light smokers. Although e-cigarette replacement improved foetal outcomes compared to continuous tobacco smoke exposure, this may not apply to heavy smokers. In addition, since the chemical composition in the e-vapour is altered by the heating temperature, using a range of temperature settings can provide more comprehensive information. Furthermore, we only followed up the offspring for 13 weeks, representing young adulthood, whereas chronic kidney disease can take decades to develop in humans. Therefore, further studies can also investigate the offspring at older ages.

In summary, this study reinforces the notion of adverse renal outcomes caused by maternal cigarette smoke exposure during pregnancy. It also provides direct evidence that continuous maternal e-vapour exposure throughout pregnancy is also detrimental to offspring's renal development and induces pathological changes independent of nicotine concentration. Although our data shows that the effects of maternal e-cigarette exposure are no worse than cigarettes, cessation of either tobacco cigarette and/or e-cigarettes should be encouraged during pregnancy to optimise foetal outcomes.

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Disclosure

The authors have nothing to disclose.

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Figure 1: Schematic presentation of the study design. SE: cigarette smoke exposure; Replacement: e-vapour replacing SE during gestation; E-cig18: exposure to nicotine containing e-vapour; E-cig0: exposure to nicotine-free e-vapour.

Figure 2: Glomerulus histological parameters at 1 day (n = 4), 20 days (n = 5) and 13 weeks (n = 5) of age. Results are expressed as mean ± SD. * P< 0.05, ** P< 0.01 vs Sham; # P< 0.05, ## P< 0.01 vs SE. SE: cigarette smoke exposure; Replacement: e-vapour replacing SE during gestation; E-cig18: exposure to nicotine containing e-vapour; E-cig0: exposure to nicotine-free e-vapour.

Figure 3: Mitochondrial density (a, n = 5), total ROS (b, n = 5), mitochondrial ROS (c, n = 5) and markers of oxidative stress Nox4 (g, n=6), nitrotyrosine (f, n = 3) and 8-OHdG (g, n = 3) in W13 male offspring. Representative images for MitoTracker (green), ROS (red), mitochondrial ROS (orange, d) and Nitrotyrosine and 8-OHdG (h) are shown; X40 magnification, scale bar indicates 50µm and applies to all images. Results are expressed as mean ± SD. * P< 0.05, ** P< 0.01 vs Sham; # P< 0.05, vs SE; ‡ P< 0.05 vs E-cig18. SE: cigarette smoke exposure; Replacement: e-vapour replacing SE during gestation; E-cig18: exposure to nicotine containing e-vapour; E-cig0: exposure to nicotine-free e-vapour.

Figure 4: Renal inflammation (MCP-1 (a, n=6), TNF-α (b, n=6), IL-6 (c, n=6), F4/80 (d, n=3)), fibrosis (Fibronectin (e, n=3), Collagen IV (f, n=3), Picrosirius Red (g, n=5)) and glomerularsclerosis (i, n=5) in W13 male offspring. Representative images for F4/80, fibronectin, collagen IV (g), Picrosirius red and Periodic Acid-Schiff are shown (j); X40 magnification, scale bar indicates 50μm and applies to all images. Results are expressed as mean ± SD. *P<0.05, **P<0.01 vs Sham; #P<0.05 vs SE; ‡ P<0.05 vs E-cig18. SE: cigarette smoke exposure; Replacement: e-vapour replacing SE during gestation; E-cig18: exposure to nicotine containing e-vapour; E-cig0: exposure to nicotine-free e-vapour. **Table 1: The body and kidney weights of male offspring in Sham, SE and Replacement groups at 1 day, 20 days and 13 weeks old.**

	Sham	**SE**	**Replacement**
P1	n = 18	n = 19	n = 20
P20 (weaning)	n = 12	n = 17	n = 13
W13 (adulthood)	n = 14	n = 14	n = 16
Body weight (g)			
P1	1.65±0.34	1.47±0.17	1.43±0.22*
P20	10.45±0.90	9.70±0.91*	9.55±0.50*
W13	26.78±1.8	25.50±1.1*	25.92±0.96
Kidney weight (g)			
P1	0.012±0.004	0.010±0.004	0.008±0.003
P20	0.072±0.010	0.068±0.008	0.074±0.011
W13	0.22±0.03	0.19±0.04*	0.22±0.04
Kidney weight (%)			
P1	0.75±0.25	0.60±0.39	0.53±0.22
P20	0.70±0.07	0.73±0.08	0.77±0.1

W13	0.82±0.07	0.78±0.04*	0.87±0.1
UACR (mg/g)			
W13 (n=6)	2.04±0.80	3.70±1.2*	2.52±0.73

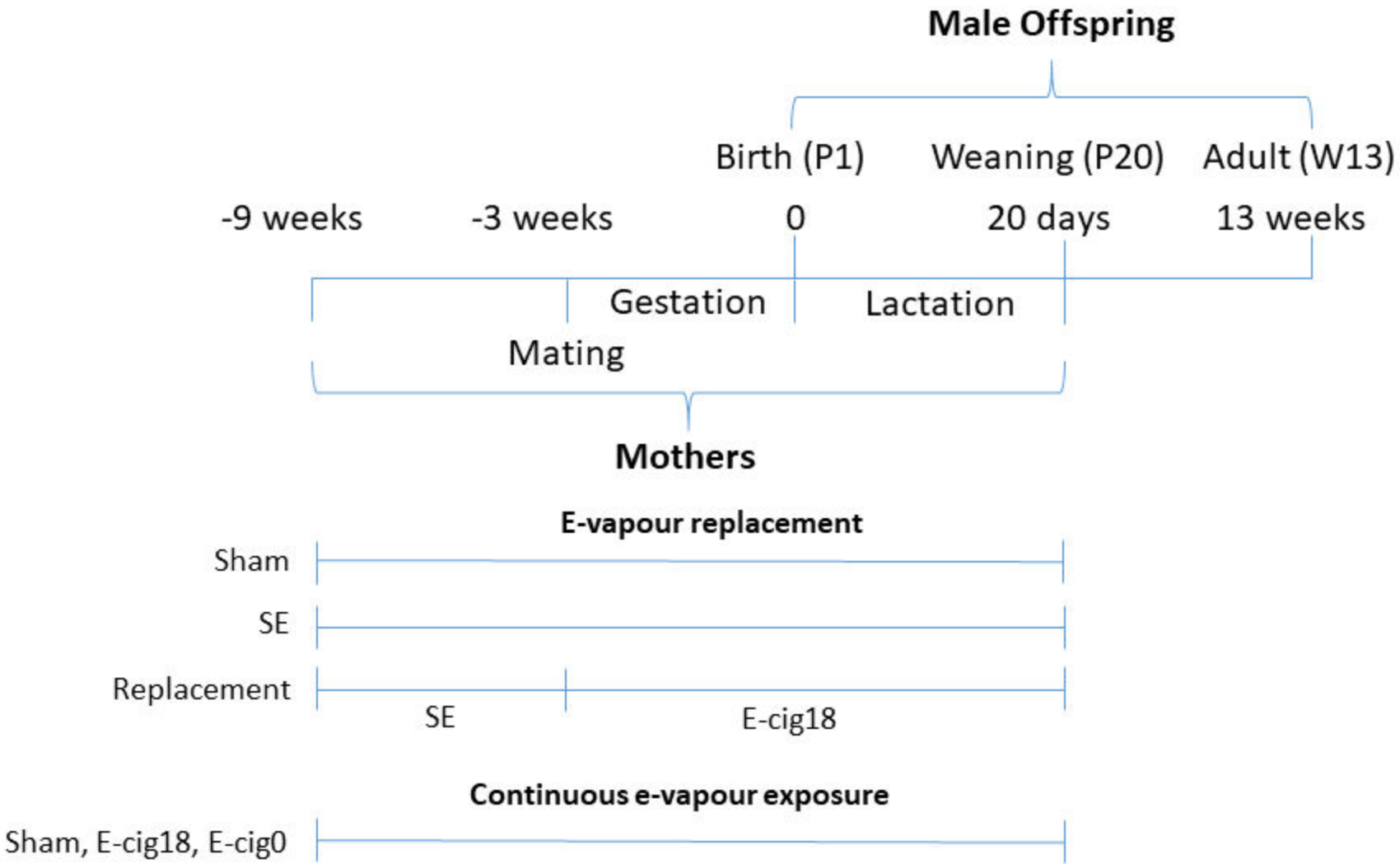
Kidney weights (%) were calculated as a ratio of kidney weight (g) to body weight (g). Results are expressed as mean ± SD. *P<0.05 vs Sham. SE: cigarette smoke exposure; Replacement: e-vapour replacing SE during gestation; UACR: urine albumin to creatinine ratio.

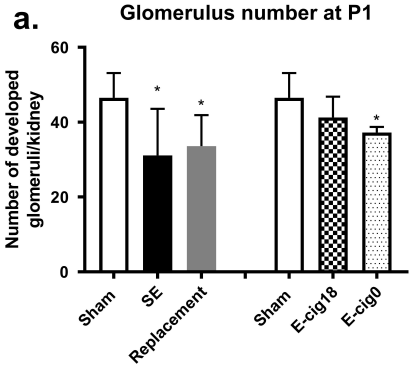
Table 2: The body and kidney weights of male offspring in Sham, E-cig18 and E-cig0 groups at 1 day, 20 days and 13 weeks old.

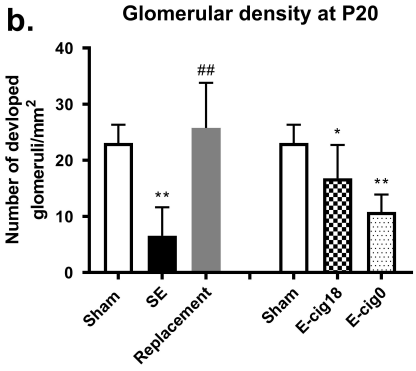
	Sham	**E-cig18**	**E-cig0**
P1	n = 18	n = 16	n = 20
P20 (weaning)	n = 12	n = 15	n = 17
W13 (adulthood)	n = 14	n = 14	n = 14
Body weight (g)			
P1	1.65±0.34	1.54±0.20	1.60±0.22
P20	10.45±0.90	9.80±0.43*	11.10±0.78*‡
W13	26.78±1.8	26.20±1.0	25.82±1.1
Kidney weight (g)			
P1	0.012±0.004	0.008±0.002	0.008±0.002
P20	0.072±0.010	0.071±0.004	0.078±0.004‡
W13	0.22±0.03	0.22±0.04	0.22±0.02
Kidney weight (%)			
P1	0.75±0.25	0.54±0.08	0.49±0.13
P20	0.70±0.07	0.73±0.04	0.71±0.04
W13	0.82±0.07	0.83±0.04	0.84±0.04

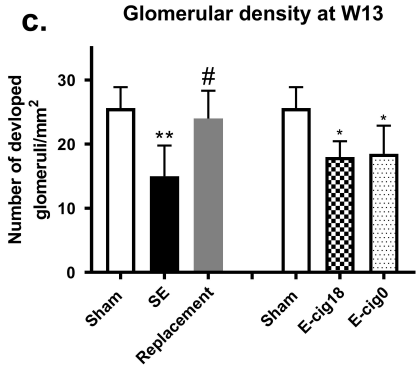
UACR (mg/g)			
W13 (n=6)	2.04±0.80	1.99±0.69	1.90±0.41

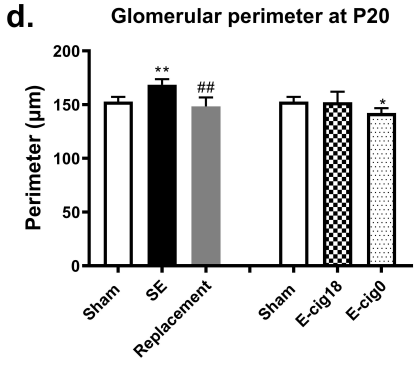
Kidney weights (%) were calculated as a ratio of kidney weight (g) to body weight (g). Results are expressed as mean ± SD. *P<0.05 vs Sham; ‡ P<0.05 vs E-cig18. E-cig18: exposure to nicotine containing e-vapour; E-cig0: exposure to nicotine-free e-vapour; UACR: urine albumin to creatinine ratio.

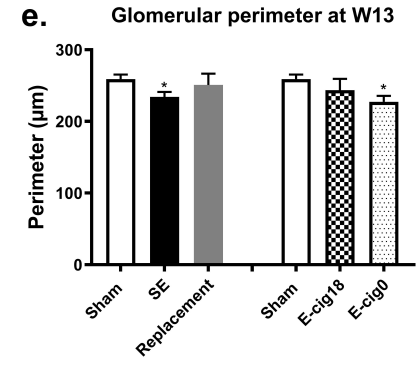

Male Offspring
Birth (P1)
Weaning (P20)
Adult (W13)
-9 weeks
-3 weeks
0
20 days
13 weeks
Gestation
Lactation
Mating
Mothers
E-vapour replacement
Sham
SE
Replacement
SE
E-cig18
Continuous e-vapour exposure
Sham, E-cig18, E-cig0

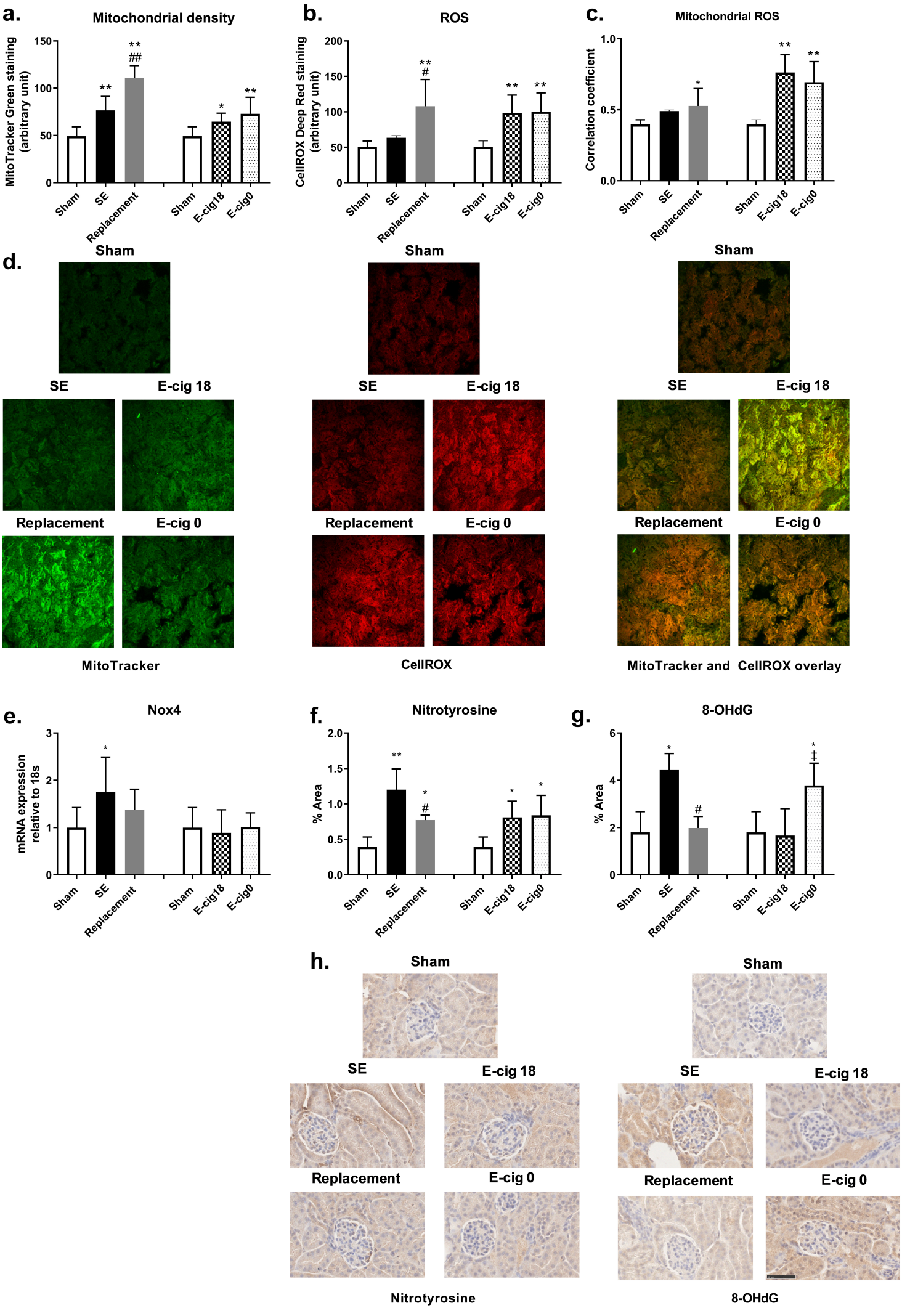

a.
Glomerulus number at P1
Number of developed glomeruli/kidney
60
40
20
0
*
*
*
Sham
SE
Replacement
Sham
E-cig18
E-cig0

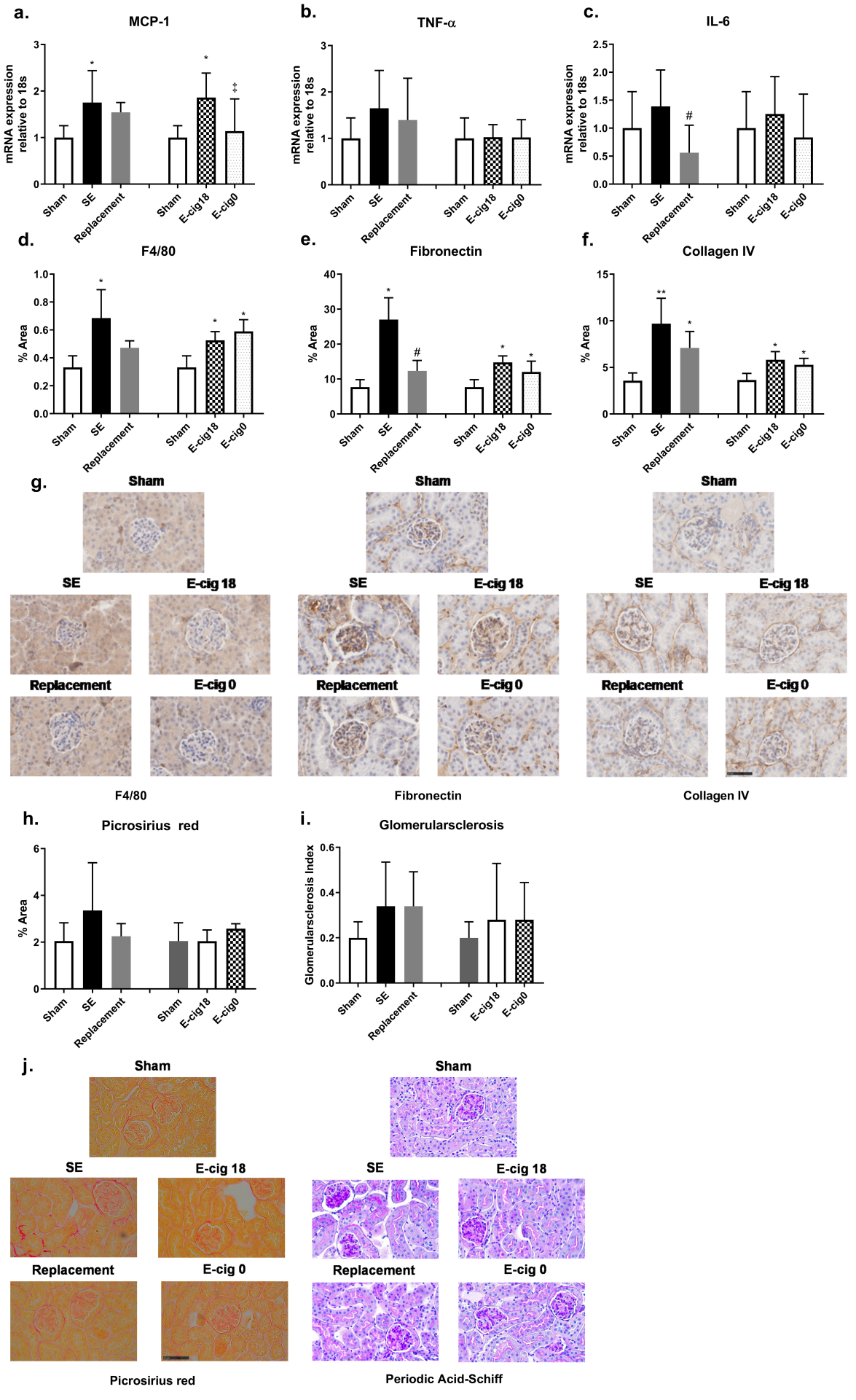

b.
Glomerular density at P20
Number of devloped glomeruli/mm2
40
30
20
10
0
**
##
*
**
Sham
SE
Replacement
Sham
E-cig18
E-cig0


c.
Glomerular density at W13
Number of devloped glomeruli/mm2
30
20
10
0
**
#
*
*
Sham
SE
Replacement
Sham
E-cig18
E-cig0


d.
Glomerular perimeter at P20
Perimeter (µm)
200
150
100
50
0
**
##
*
Sham
SE
Replacement
Sham
E-cig18
E-cig0


e.
Glomerular perimeter at W13
Perimeter (µm)
300
200
100
0
*
*
Sham
SE
Replacement
Sham
E-cig18
E-cig0


a.
Mitochondrial density
MitoTracker Green staining (arbitrary unit)
150
100
50
0
**
**
##
*
**
Sham
SE
Replacement
Sham
E-cig18
E-cig0
b.
ROS
CellROX Deep Red staining (arbitrary unit)
200
150
100
50
0
**
#
**
**
Sham
SE
Replacement
Sham
E-cig18
E-cig0
c.
Mitochondrial ROS
Correlation coefficient
1.0
0.5
0.0
*
**
**
Sham
SE
Replacement
Sham
E-cig18
E-cig0
d.
Sham
Sham
Sham
SE
E-cig 18
SE
E-cig 18
SE
E-cig 18
Replacement
E-cig 0
Replacement
E-cig 0
Replacement
E-cig 0
MitoTracker
CellROX
MitoTracker and CellROX overlay
e.
Nox4
mRNA expression relative to 18s
3
2
1
0
*
Sham
SE
Replacement
Sham
E-cig18
E-cig0
f.
Nitrotyrosine
% Area
2.0
1.5
1.0
0.5
0.0
**
*
#
*
*
Sham
SE
Replacement
Sham
E-cig18
E-cig0
g.
8-OHdG
% Area
6
4
2
0
*
#
*
‡
Sham
SE
Replacement
Sham
E-cig18
E-cig0
h.
Sham
Sham
SE
E-cig 18
SE
E-cig 18
Replacement
E-cig 0
Replacement
E-cig 0
Nitrotyrosine
8-OHdG


a.
MCP-1
mRNA expression relative to 18s
b.
TNF-α
mRNA expression relative to 18s
c.
IL-6
mRNA expression relative to 18s
d.
F4/80
% Area
e.
Fibronectin
% Area
f.
Collagen IV
% Area
Sham
SE
Replacement
E-cig18
E-cig0
g.
Sham
SE
E-cig 18
Replacement
E-cig 0
F4/80
Fibronectin
Collagen IV
h.
Picrosirius red
% Area
i.
Glomerularsclerosis
Glomerularsclerosis Index
j.
Sham
SE
E-cig 18
Replacement
E-cig 0
Picrosirius red
Periodic Acid-Schiff