Title Page

E-cigarettes damage the liver and alters nutrient metabolism in pregnant mice and their offspring independent of nicotine

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Abstract

Approximately 15% of pregnant women vape electronic cigarettes (e-cigarettes), exposing the foetus to a range of toxic compounds, including nicotine and by-products of e-cigarette liquid (e-liquid) pyrolysis. Due to the recent emergence of these products, health impacts mainly focus on immediate users, while less emphasis is placed on *in-utero* exposure. Therefore, the aim of this study was to understand the impact of continuous intrauterine e-cigarette vapour (e-vapour) exposure, with and without nicotine, on liver metabolic markers in male offspring. E-vapour was generated using an e-cigarette filled with commercial tobacco flavoured e-liquid (18mg/mL or 0mg/mL nicotine). Female Balb/c mice were exposed to e-vapour for 6 weeks before mating, through gestation and lactation, without direct exposure to the offspring. Livers and plasma from dams and male offspring (13 weeks old) were examined. Maternal e-vapour exposure caused insulin resistance in the dams and impaired glucose tolerance in the offspring, independent of nicotine. Exposure to nicotine-free e-vapour increased liver damage in the dams and offspring associated with nutrient metabolic changes, which were somehow not observed in the offspring exposed to nicotine-free e-vapour. Maternal exposure to e-vapour with nicotine did not cause liver damage but increased liver triglyceride accumulation in the offspring. Therefore, chemicals from the heated humectant may contribute to liver and metabolic disorders; whereas nicotine may partly protect the offspring from the adverse hepatic and metabolic impacts of maternal e-vaping during pregnancy.

Introduction

Electronic cigarettes (e-cigarettes) are marketed as a smoking cessation aid, with claims of safety over tobacco cigarettes and their popularity is derived from safety perceptions, current trends, and reduced costs compared to tobacco cigarette smoking [1]. Over the past decade, e-cigarette vaping (vaping) has become increasingly popular, even amongst individuals without prior tobacco smoking history. Despite recent reports of pulmonary illness in the US, vaping remains a prevalent recreational addiction [2], especially among young adults, with an estimated 3.5 million US middle and high school students using them in 2018 [3]. Given the rates of unplanned pregnancies and perceptions of safety, this may inadvertently increase the number of individuals exposed to e-vapour *in-utero*.

E-cigarette liquid (e-liquid) is composed of nicotine and flavouring compounds suspended in a humectant (normally propylene glycol and/or glycerine) [4]. Heating this liquid produces an aerosol (e-vapour) which is toxic, oxidative, and induces inflammation [1]. In previous studies, intrauterine nicotine administration altered birth weight along with postnatal lung and brain development, leading to respiratory and neurological disorders [5]. Apart from nicotine, e-vapour also contains a complex and diverse mixture of particulate matter, volatile organic compounds, and heavy metals, which can have potential health consequences when inhaled [2]. Previously, we have shown that intrauterine e-vapour exposure increased inflammatory responses in multiple organs, including the lungs [6], brain [7, 8], and kidneys [9], associated with mitochondrial alterations and oxidative stress [9], which are mostly nicotine independent. However, the impacts of intrauterine e-vapour exposure on liver health and nutrient metabolism are unknown.

In mouse models of maternal smoking during pregnancy, intrauterine tobacco smoke exposure impairs foetal development and increases mitochondrial-derived oxidative stress in the liver in adulthood [10]. This is closely related to increased inflammation, fatty liver changes, and

systemic lipid and glucose metabolic disorders in the offspring with no sexual difference [10,11]. Given the inflammatory response and oxidative stress induced by maternal e-vapour exposure in the lungs, brain, and kidneys [6-9], we hypothesised that long-term maternal e-vapour exposure might lead to similar disorders in the liver, resulting in glucose and lipid metabolic changes in both the dams and their offspring. Therefore, the aims of this study were to investigate the impacts of maternal e-vapour exposure from prior to gestation and during pregnancy on hepatic metabolic markers, oxidative stress, inflammation and mitochondrial health in both the mothers and their male offspring. Only male offspring were examined due to similar metabolic responses to the impacts of maternal smoking between male and female offspring.

Methods

Animals

The animal experiments were approved by the Animal Care and Ethics Committee of the University of Technology Sydney (ETH15-0025) and performed according to the Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Virgin 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 breeders were acclimatised for a week prior to the exposure treatments detailed below.

Female breeders were exposed to room air (Sham, n=8), e-vapour generated from e-liquid containing 18mg/mL nicotine (E-cig18, n=8), or e-vapour from nicotine-free e-liquid (E-cig0, n=8) twice daily for 6 weeks prior to mating and throughout gestation and lactation, as previously described [9]. E-vapour was generated by a human-use e-cigarette device (KangerTech NEBOX, 30 Watts, 0.5 Ohms, KangerTech, Shenzen, China) from commercial

e-liquid (50% propylene glycol/50% vegetable glycerine, tobacco flavour, Vaper Empire, VIC, Australia) as we have previously published in the same model [6]. The nicotine dose in the E-cig18 groups represents mothers who are light smokers [6]. Dams were removed from their home cages during exposure, while the male breeders and pups were not exposed. Male offspring were weaned at postnatal day 20 and maintained without additional intervention.

An intraperitoneal glucose tolerance test (IPGTT) was performed as previously described [13] in the male offspring at 12 weeks of age. After 5 hours of fasting, baseline blood glucose levels were measured, followed by glucose injection (2g/kg, IP). Blood glucose was measured at 15, 30, 60, and 90 minutes post-injection. The area under the curve (AUC) of the blood glucose curve was calculated for each mouse.

Dams and male offspring (13 weeks old) were euthanized after deep anaesthesia (2% isoflurane). Livers were harvested, weighed and then either snap-frozen and stored at -80°C or fixed in 10% formalin for further analyses. Liver weights (%) were calculated as a fraction of body weight. Blood was collected via cardiac puncture and glucose levels were measured (Accu-Check$^{(R)}$, Roche, CA, USA). Plasma was separated and stored at -20°C for further analysis.

Bioassays

The plasma activity of Alanine Transaminase (ALT) was measured using the Alanine Transaminase Colorimetric Activity Assay Kit (Cayman Chemical, MI, USA) according to the manufacturer's instructions. Plasma insulin concentration was measured by ELISA (Abnova, Taiwan) according to the manufacturer's instructions. Liver lipids were extracted using the Folch method [14], as previously described [10]. Plasma, liver extracts and glycerol standards (Sigma-Aldrich, MO, USA) were incubated with triacylglycerol reagent (Roche Diagnostics, Basel, Switzerland) using an in-house assay [13]. Plasma non-esterified free fatty acid (NEFA)

concentrations were measured using a NEFA kit (WAKO, Osaka, Japan). The insulin resistance index Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated as insulin (μU/mL) x glucose (mM)/22.5.

Real-time (rt)-PCR

Total mRNA was extracted from frozen liver tissue with TriZol reagent (Life Technologies, CA, USA) and the first-strand cDNA was generated using M-MLV Reverse Transcriptase, RNase H, Point Mutant Kit (Promega, WI, USA). Target gene expression was quantified with manufacturer pre-optimised and validated TaqMan primers and probes (See Table, Supplemental Digital Content 1, which shows details of the primers and probes used, Thermo Fisher, CA, USA) and standardised to 18s RNA. The probes of the target genes were labelled with FAM and those for housekeeping 18s RNA were labelled with VIC. The average of the Sham group was assigned the calibrator against which all other results were expressed as fold changes.

Mitochondrial DNA copy number

DNA was extracted from liver tissue using the DNeasy Blood and Tissue kit (Qiagen). The content of mtDNA was measured by quantifying mRNA expression of mitochondrial-encoded gene cytochrome c oxidase subunit 1 (COX1) (forward primers 5-ACTATACTACTACTAA-CAGACCG-3, reverse primers 5-GGTTCTTTTTTTCCGGAGTA-3) against the nuclear-encoded gene cyclophilin A (forward primers 5-ACACGCCATAATGGCACTGG-3, reverse primers 5-CAGTCTTGGCAGTGCAGAT-3), as we have previously published. COX1 gene expression was standardised to the housekeeping gene, cyclophilin A using ΔΔCt method. The average of the Sham group was assigned the calibrator against which all other results were expressed as fold changes.

Western Blotting

After homogenisation of liver tissue, whole protein and mitochondrial protein samples were extracted via differential centrifugation, as previously described [15]. Protein samples were separated on NuPage Novex 4-12% Bis-Tris gels (Life Technologies, CA, USA) and transferred to PVDF membranes (Pierce, IL, USA). After blocking with 5% skim milk, the membranes were incubated with primary antibodies of interest (mitophagy: microtubule-associated 1A/1B light chain protein 3 (LC3A/B II, 1:2000, Cat# 4108 S, Cell Signalling Technology, MA, USA), dominant optic atrophy (OPA-1, 1:2000, Cat# NB110-55290, Novus Biotechnology, CO, USA), dynamin-related protein 1 (DRP-1, Cat# NB110-55237, 1:2000, Novus Biotechnology, CO, USA); endogenous mitochondrial antioxidants: manganese superoxide dismutase (MnSOD, 1:2000, Cat# 06-984, Millipore, MA, USA), glutathione peroxidase 1 (GPx1, 1:250, Cat# AF3798, R&D Systems, MN, USA); insulin signalling: phosphorylated-protein kinase B (p-Akt (Thr308), Cat#9271, Cell Signalling Technology, MA, USA); lipid metabolism: fatty acid synthase (FASN, Cat# 3180 S, 1:2000, Cell Signalling Technology, MA, USA)), followed by the corresponding secondary antibody (Abcam, Cambridge, UK). Bands were detected with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, CA, USA) and Fujifilm LAS-3000 (Fujifilm, Tokyo, Japan) and then quantified with ImageJ (National Institutes of Health, MD, USA). Results were expressed as a ratio against the housekeeping protein β-actin or cytochrome c oxidase subunit (COX) IV for mitochondrial proteins.

Immunohistochemistry

Formalin-fixed, paraffin-embedded livers were sectioned at 5μm. Sections were deparaffinised and rehydrated in xylene and decreasing grades of ethanol. Antigen retrieval was performed [16] before the sections were incubated with the following anti-rabbit primary antibodies: nitrotyrosine (1:400, Cat# 06-284, Merck, NJ, USA), or F4/80 (1:300, Cat# ab111101, Abcam,

Cambridge, UK) and visualised with the horseradish peroxidase anti-rabbit Envision system (Dako Cytochemistry, Tokyo, Japan). Sections were counterstained with haematoxylin and quantified with ImageJ (National Institutes of Health, MD, USA).

Statistical Analysis

Results are expressed as mean ± SEM and were analysed using one-way ANOVA with Fisher's Least Significant post hoc test if the data were normally distributed. If the data were not normally distributed, they were log transformed to achieve normality of distribution before analysis (GraphPad Prism 7.03, CA, USA). $P<0.05$ was considered the threshold for statistical significance.

Results

Dams

Metabolic markers

In the dams, exposure to nicotine-containing e-vapour (E-cig18) did not alter body or liver weight. Blood glucose concentration was 23% higher in E-cig18 dams, but this was not significant (Table 1). The expression of the insulin-independent glucose transporter (Glut)2 was not different among the groups (Figure 1a), whereas the expression of insulin-dependent Glut4 was increased in the E-cig18 dams ($P<0.05$ vs Sham, Figure 1b). There were no changes in the mRNA expression of markers for glycolysis and gluconeogenesis (phosphofructokinase (PFK)1 and forkhead box protein (FOX)O1 (Figure 1c,d)). Furthermore, there was no difference in plasma insulin levels among the groups (Table 1). However, HOMA-IR index, an indicator of insulin resistance, was increased in the E-cig18 dams ($P<0.01$ vs Sham, Table 1), associated with a decrease in insulin signalling element, p-Akt ($P<0.05$ vs Sham, Figure 1e). Liver and plasma triglyceride (TG) concentrations were not changed in E-cig18 dams

(Table 1, Figure 1f). Thus, direct exposure to nicotine-containing e-vapour caused insulin resistance, but not increased hepatic glucose production.

The dams exposed to nicotine-free e-vapour (E-cig0) were heavier than E-cig18 dams ($P<0.05$), with bigger liver sizes ($P<0.05$ vs Sham, Table 1) but not when expressed as a percentage of the body weight (Table 1). Glut4 expression was also increased in the E-cig0 dams compared to the Sham dams ($P<0.01$, Figure 1b). In the E-cig0 dams, HOMA-IR index was increased ($P<0.05$ vs Sham, Table 1) which was also associated with a decrease in liver p-Akt levels ($P<0.05$ vs Sham, Figure 1e). In addition, liver TG concentrations were increased in the E-cig0 dams ($P<0.05$ vs Sham, Figure 1f), along with increased plasma non-esterified fatty acid (NEFA) concentrations ($P<0.05$ vs Sham, Table 1). Hepatic FASN protein level was significantly increased in the E-cig0 dams ($P<0.05$ vs Sham, Figure 1g). Therefore, direct exposure to nicotine-free e-vapour also induced insulin resistance and impaired insulin receptor pathway activation. In addition, the increase in hepatic de-novo lipogenesis is linked to an increase in hepatic triglyceride and plasma NEFA concentrations.

Markers of liver damage

Plasma alanine aminotransferase (ALT) activity, a clinical marker of liver damage, was not changed in the E-cig18 dams (Table 1). In addition, the hepatic oxidative stress injury marker nitrotyrosine, mitochondrial antioxidants MnSOD and GPx1, and the pro-inflammatory cytokine TNF-α were not different between the E-cig18 dams and the Sham dams (Figure 2). Overall, exposure to nicotine-containing e-vapour did not result in any measurable liver damage in the dams.

In contrast, plasma ALT activity was increased in the E-cig0 dams compared to the E-cig18 dams ($P<0.05$, Table 1). Hepatic nitrotyrosine staining was increased in the E-cig0 dams compared to the Sham dams ($P<0.05$, Figure 2a). Furthermore, MnSOD expression was

decreased in the E-cig0 dams ($P<0.05$ vs Sham, Figure 2b). The E-cig0 dams also had increased F4/80 positive cell number ($P<0.05$ vs Sham, Figure 2d) and increased mRNA expression of IL-1β in the liver ($P<0.05$ vs Sham, Figure 2f). Increased hepatocyte damage in the E-cig0 dams was associated with decreased endogenous mitochondrial antioxidants suggesting increased oxidative stress, along with increased macrophage infiltration and inflammation.

Liver mitochondrial markers

In the E-cig18 dams, there were no changes in mtDNA copy number, mitochondrial fusion marker DRP-1, fission protein OPA-1, and mitochondrial biogenesis marker PGC1α compared to the Sham dams (Figure 3a-c,e). The hepatic protein level of autophagy marker (LC3A/B II) was decreased in the E-cig18 dams compared to the Sham dams ($P<0.01$, Figure 3d). Nicotine-containing e-vapour exposure did not change liver mitochondrial number, mitophagy and mitochondrial biogenesis markers in the dams.

In contrast, among E-cig0 dams the mtDNA copy number was decreased compared to the Sham dams ($P<0.05$, Figure 3a). DRP-1 protein levels were increased in the E-cig0 dams ($P<0.01$ vs E-cig18, Figure 3b). Meanwhile, LC3A/B II protein levels were increased in the E-cig0 dams compared to the E-cig18 dams but were not different from the Sham dams ($P<0.01$, Figure 3d). The expression of PGC1α was decreased in the E-cig0 dams compared to the Sham dams ($P<0.05$, Figure 3e). E-cig0 exposure resulted in hepatic mitochondrial damage as indicated by a reduction in mtDNA copy number, which could be due to reduced mitochondrial biogenesis and increased mitochondrial fission and subsequent recycling. These mitochondrial alterations could be responsible for the oxidative stress, inflammation, and liver damage that was observed.

Offspring

Metabolic markers

Intrauterine exposure to nicotine-containing e-vapour did not alter liver or body weight in 13 weeks old male offspring (Table 2). The area under the curve (AUC) for IPGTT was increased in the E-cig18 offspring ($P<0.05$ vs Sham offspring, Figure 4a). No changes in the blood glucose or plasma insulin levels were observed in E-cig18 offspring (Table 2). In addition, no differences in the glucose metabolic markers, Glut2, Glut4, and PFK1 were observed in the E-cig18 offspring (Figure 4c-e). However, the expression of the gluconeogenesis marker, FOXO1, was increased in the E-cig18 offspring ($P<0.05$ vs Sham, Figure 4f), with a significant increase in p-Akt levels ($P<0.05$ vs Sham; $P<0.01$ vs E-cig0, Figure 4g). Liver TG concentrations were increased ($P<0.01$ vs Sham, Figure 4h), associated with an increase in FASN protein level ($P<0.05$ E-cig18 vs Sham, Figure 4i). Plasma TG and NEFA concentrations were not significantly changed in the E-cig18 offspring (Table 2). Thus, maternal exposure to nicotine-containing e-vapour caused glucose intolerance associated with impaired insulin signalling element and increased hepatic gluconeogenesis, as well as increased hepatic triglyceride accumulation associated with increased de-novo lipogenesis in the offspring.

E-cig0 offspring had smaller liver weights compared to the Sham and E-cig18 offspring ($P<0.05$, Table 2). In addition, the IPGTT AUC value was increased in the E-cig0 offspring compared to the Sham offspring ($P<0.05$, Figure 4a). E-cig0 offspring also had increased mRNA expression of glucose transporters Glut2 ($P<0.01$ vs Sham and $P<0.05$ vs E-cig18, Figure 4b) and Glut4 ($P<0.05$ vs Sham, Figure 4c), glycolysis marker PFK1 ($P<0.05$ vs Sham and E-cig18, Figure 4d), and gluconeogenesis marker FOXO1 ($P<0.01$ vs Sham and $P<0.05$ vs E-cig18, Figure 4e). FASN protein expression was similar to the Sham offspring ($P<0.01$ vs

E-cg18 offspring, Figure 4h). Maternal exposure to nicotine-free e-vapour led to glucose intolerance and altered levels of hepatic glucose metabolic makers in the offspring.

Markers of liver damage

MnSOD and Gpx1 levels were both decreased in the E-cig18 offspring, but without reaching statistical significance (Figure 5a,b). No changes were found in the markers of inflammation in the E-cig18 dams (Figure 5d-f). Thus, maternal exposure to nicotine-containing e-vapour did not cause measurable pathology in offspring's liver.

By contrast, in the E-cig0 offspring, there was an increase in nitrotyrosine staining in the liver ($P<0.05$ vs Sham and E-cig18, Figure 5a). This was associated with a decrease in the levels of the mitochondrial antioxidant GPx1 ($P<0.01$ vs Sham, Figure 5c). In addition, E-cig0 offspring had a 30% increase in mRNA expression of TNF-α compared to the Sham and a 46% increase compared to E-cig18 offspring ($P<0.05$, Figure 5d). No differences in IL-1β expression was observed (Figure 5e). Maternal exposure to nicotine-free e-vapour led to increased oxidative stress and inflammation in the offspring's liver.

Liver mitochondrial markers

In E-cig18 offspring, there were no changes in mitochondrial number as assessed by mtDNA copy number, markers of mitophagy (OPA-1 and DRP-1), autophagy (LC3A/B II), and mitochondrial biogenesis (PGC1α, Figure 6). Thus, maternal exposure to nicotine-containing e-vapour did not affect liver mitochondrial health.

In contrast, E-cig0 offspring had reduced numbers of mitochondria as assessed by mtDNA copy number compared to Sham offspring ($P<0.05$, Figure 6a). There was an increase in autophagy LC3A/B II levels compared to the Sham offspring ($P<0.05$, Figure 6d). There were no changes in mitophagy and mitochondrial biogenesis markers (Figure 6b,c,e). Therefore,

maternal exposure to nicotine-free e-vapour may injure mitochondria resulting in increased autophagy in the E-cig0 offspring.

Discussion

The recent popularity of e-cigarette vaping among young people has affected women of reproductive age, increasing the incidence of intrauterine e-vapour exposure. Identifying the metabolic and hepatic consequences of long-term intrauterine e-vapour exposure is essential, to advise the pregnant women. Such risk information won't be imminently available in humans. Nicotine-free e-vapour (E-cig0) was detrimental to liver health in both dams and male offspring by inducing oxidative stress, injury, and inflammation. Therefore, the chemicals from heated flavouring compounds and humectants could contribute to the adverse health impacts, where the level of nicotine adopted in this study seems to provide a partial protective effect in the offspring. However, exposure to nicotine-containing e-vapour (E-cig 18) still caused intrauterine underdevelopment and promoted hepatic steatosis in adult offspring.

The use of flavouring compounds makes e-cigarettes enticing, resulting in continued use even in the absence of the addictive additive, nicotine. Many flavouring compounds (such as candy and chocolate) are especially appealing to younger individuals [4]. As a result, some states in the US have proposed bans on flavoured e-cigarettes except tobacco flavour. Here we showed that nicotine-free, tobacco flavoured e-vapour still had significant health concerns, not only to the direct user but also their offspring, mostly attribute to heated humectants. Liver mitochondrial health was impaired in the E-cig0 dams, reflected by reduced mtDNA copy number and reduced mitochondrial antioxidants. Mitochondria are intracellular organelles responsible for nutrient metabolism and are also vulnerable to oxidative stress. Upon injury, mitophagy separates the injured fragments to be eliminated by autophagosomes (formed by LC3A/B), and combines healthy fragments via fusion to regenerate a new healthy mitochondrion. If the

damaged mitochondria are not removed, inflammation and oxidative stress are induced [17]. Reduced hepatic mtDNA copy number in the E-cig0 dams could be due to a decrease in mitochondrial biogenesis. Furthermore, mitochondrial antioxidants protect mitochondria from oxidative stress induced damage, which was also reduced, promoting further oxidative stress injury. As a result, plasma ALT activity, a marker of hepatocellular damage, was increased, along with inflammation and macrophage infiltration. The increase in hepatic TG accumulation could arise from increased glucose uptake and conversion to TG through de-novo lipogenesis.

In the E-cig0 offspring, gluconeogenesis was increased, which is linked to impaired systemic glucose clearance during IPGTT, but this does not seem to be due to insulin resistance. Glucose transporters were also increased in the liver, associated with increased glycolysis. This may increase the mitochondrial burden, where glucose is metabolised to ATP. As a result, E-cig0 offspring exhibited a reduction in mitochondria number, whereas the mitochondrial repair mechanism, mitophagy, failed to compensate. The reduced mtDNA number is also consistent in the E-cig0 dams and offspring, indicating that these effects may have been passed on from the mother. The resulting hepatic oxidative stress and inflammation were associated with increased glucose uptake, potentially causing glucose intolerance. Thus, exposure to nicotine-free e-vapour promotes liver disorders and alters nutrient metabolism in both the dams and their offspring.

On the other hand, there were no significant changes in liver markers of glucose and lipid metabolism, as well as inflammation and oxidative stress in both E-cig18 dams and offspring. While unphysiologically high doses of nicotine in animal models is associated with liver damage [18], not all these detrimental effects are observed in humans mostly due to lower nicotine exposure in smokers than laboratory animals [19]. In fact, nicotine has anti-inflammatory properties at physiological doses [20] leading to mostly negative findings in nutrient metabolic markers in E-cig18 dams and offspring. Similarly, exposure to other flavoured e-vapours was

shown to increase airway inflammation, but not when nicotine was also included [21]. Therefore, it is likely that the humectants of the e-liquids are the most detrimental aspect of using e-cigarettes. Thus, nicotine may prevent some of the adverse impacts of maternal e-vapour exposure, but this has yet to be confirmed in humans which may take decades to conclude.

However, E-cig18 offspring still experienced intrauterine underdevelopment similar as those from tobacco cigarette smokers [6]. This may be secondary to nicotine-induced uteroplacental blood vessel constriction and reduced intrauterine resources [22]. Increased liver triglyceride accumulation in E-cig18 offspring was associated with an increase in gluconeogenesis and de-novo lipogenesis. Fatty liver is common in low birth weight infants [23] due to increased de-novo lipogenesis [24]. Furthermore, glucose intolerance could be attributed to increased gluconeogenesis rather than insulin resistance [25].

We need to acknowledge one limitation of this study by only investigating one dose of nicotine exposure. While the dose response effects of maternal e-vaping on offspring's health outcome are unknown in humans, in tobacco cigarette smokers, there is a positive correlation between the daily cigarette number and the risk of miscarriage. Future studies can shed light on this aspect of e-cigarette users.

In conclusion, direct and in-utero e-vapour exposure can lead to hepatic, lipid, and glucose metabolic disorders. The dose used in this study was low (equivalent to light smokers) and future studies can examine higher doses of e-vapour or nicotine. Overall, pregnant women should avoid the use of e-cigarettes, regardless of nicotine concentration, to reduce the risk of hepatic and metabolic disorders.

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Competing interests

The authors declare that they have no conflicts of interest.

References

1. Li, G., S. Saad, B.G. Oliver*, et al.* 2018. Heat or Burn? Impacts of Intrauterine Tobacco Smoke and E-Cigarette Vapor Exposure on the Offspring's Health Outcome. *Toxics*. **6**.
2. Layden, J.E., I. Ghinai, I. Pray*, et al.* 2019. Pulmonary Illness Related to E-Cigarette Use in Illinois and Wisconsin - Preliminary Report. *The New England journal of medicine*.
3. Gentzke, A.S., M. Creamer, K.A. Cullen*, et al.* 2019. Vital Signs: Tobacco Product Use Among Middle and High School Students - United States, 2011-2018. *MMWR Morb Mortal Wkly Rep*. **68**: 157-164.
4. Grana, R., N. Benowitz & S.A. Glantz. 2014. E-cigarettes: a scientific review. *Circulation*. **129**: 1972-1986.
5. Chan, Y.L., B.G. Oliver & H. Chen. 2019. What lessons have we learnt about the impact of maternal cigarette smoking from animal models? *Clinical and experimental pharmacology & physiology*.
6. Chen, H., G. Li, Y.L. Chan*, et al.* 2018. Maternal E-Cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring. *American journal of respiratory cell and molecular biology*. **58**: 366-377.
7. Chen, H., G. Li, Y.L. Chan*, et al.* 2018. Modulation of neural regulators of energy homeostasis, and of inflammation, in the pups of mice exposed to e-cigarettes. *Neuroscience letters*. **684**: 61-66.

8. Nguyen, T., G.E. Li, H. Chen*, et al.* 2018. Maternal E-Cigarette Exposure Results in Cognitive and Epigenetic Alterations in Offspring in a Mouse Model. *Chemical research in toxicology*. **31**: 601-611.
9. Li, G., Y.L. Chan, L.T. Nguyen*, et al.* 2019. Impact of maternal e-cigarette vapor exposure on renal health in the offspring. *Annals of the New York Academy of Sciences*.
10. Li, G., Y.L. Chan, S. Sukjamnong*, et al.* 2019. A Mitochondrial Specific Antioxidant Reverses Metabolic Dysfunction and Fatty Liver Induced by Maternal Cigarette Smoke in Mice. *Nutrients*. **11**: 1669.
11. Saad, S., I. Al-Odat, Y.L. Chan*, et al.* 2018. Maternal L-carnitine supplementation improves glucose and lipid profiles in female offspring of dams exposed to cigarette smoke. *Clinical and experimental pharmacology & physiology*. **45**: 694-703.
12. Al-Odat, I., H. Chen, Y.L. Chan*, et al.* 2014. The impact of maternal cigarette smoke exposure in a rodent model on renal development in the offspring. *PloS one*. **9**: e103443.
13. Chen, H., D. Simar, K. Pegg*, et al.* 2014. Exendin-4 is effective against metabolic disorders induced by intrauterine and postnatal overnutrition in rodents. *Diabetologia*. **57**: 614-622.
14. Folch, J., M. Lees & G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry*. **226**: 497-509.
15. Nguyen, L.T., S. Stangenberg, H. Chen*, et al.* 2015. L-Carnitine reverses maternal cigarette smoke exposure-induced renal oxidative stress and mitochondrial dysfunction in mouse offspring. *American journal of physiology. Renal physiology*. **308**: F689-696.
16. Chan, Y.L., S. Saad, I. Al-Odat*, et al.* 2017. Maternal L-Carnitine Supplementation Improves Brain Health in Offspring from Cigarette Smoke Exposed Mothers. *Frontiers in molecular neuroscience*. **10**: 33.
17. Hu, F. & F. Liu. 2011. Mitochondrial stress: a bridge between mitochondrial dysfunction and metabolic diseases? *Cellular signalling*. **23**: 1528-1533.
18. Salahshoor, M., S. Mohamadian, S. Kakabaraei*, et al.* 2016. Curcumin improves liver damage in male mice exposed to nicotine. *Journal of traditional and complementary medicine*. **6**: 176-183.
19. Moore, D., P. Aveyard, M. Connock*, et al.* 2009. Effectiveness and safety of nicotine replacement therapy assisted reduction to stop smoking: systematic review and meta-analysis. *BMJ (Clinical research ed.)*. **338**: b1024.
20. Kalra, R., S.P. Singh, J.C. Pena-Philippides*, et al.* 2004. Immunosuppressive and anti-inflammatory effects of nicotine administered by patch in an animal model. *Clinical and diagnostic laboratory immunology*. **11**: 563-568.
21. Chapman, D.G., D.T. Casey, J.L. Ather*, et al.* 2019. The Effect of Flavored E-cigarettes on Murine Allergic Airways Disease. *Scientific reports*. **9**: 13671.
22. Suter, M.A., J. Mastrobattista, M. Sachs*, et al.* 2015. Is there evidence for potential harm of electronic cigarette use in pregnancy? *Birth defects research. Part A, Clinical and molecular teratology*. **103**: 186-195.
23. Bugianesi, E., C. Bizzarri, C. Rosso*, et al.* 2017. Low Birthweight Increases the Likelihood of Severe Steatosis in Pediatric Non-Alcoholic Fatty Liver Disease. *The American journal of gastroenterology*. **112**: 1277-1286.
24. Garg, M., S. Bassilian, C. Bell*, et al.* 2005. Hepatic de novo lipogenesis in stable low-birth-weight infants during exclusive breast milk feedings and during parenteral nutrition. *JPEN. Journal of parenteral and enteral nutrition*. **29**: 81-86.
25. Houde, V.P., S. Brûlé, W.T. Festuccia*, et al.* 2010. Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. *Diabetes*. **59**: 1338-1348.

Figure 1. Hepatic mRNA expression of glucose metabolic (Glut2 (a), Glut4 (b), PFK1 (c), FOXO1 (d)), hepatic protein markers of insulin signalling (p-Akt (e)) and lipid synthesis (FASN (g)) and hepatic triglyceride levels (f). Results are expressed as Mean ± SEM (n=6). *P<0.05, **P<0.01 vs Sham. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, FASN: Fatty Acid Synthase, FOXO1: Forkhead box protein O1, Glut: Glucose transporter, NEFA: non-esterified fatty acid, PFK: Phosphofructokinase, TG: triglyceride.

Figure 2. Markers of oxidative stress (nitrotyrosine (a)), mitochondrial antioxidants (MnSOD (b), Gpx1 (d)) and inflammation (F4/80 (d), TNF-α (e), IL-1β (f)) in the Dams. Results are expressed as Mean ± SEM (n=5-8). *P<0.05 vs Sham. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, F4/80: EGF-like module-containing mucin-like hormone receptor-like 1, GPx1: glutathione peroxidase 1, IL-1β: Interleukin 1 beta, MnSOD: manganese superoxide dismutase, TNF-α: Tumor necrosis factor-α.

Figure 3. Liver mitochondrial DNA copy number (a), markers of mitophagy (DRP-1 (b), OPA-1 (c)), autophagy (LC3A/B II (d)), and mitochondrial biogenesis (PGC1α (e)) in the dams. Results are expressed as Mean ± SEM (n=5-8). *P<0.05, **P<0.01 vs Sham, ##P<0.01 vs E-cig18. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, DRP-1: Dynamin related

protein 1, LC3A/B II: Microtubule associated 1A/1B light chain protein 3, OPA-1: Mitochondrial dynamin like GTPase, PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-α.

Figure 4. IPGTT (a) and area under the curve (b, AUC) along with hepatic mRNA expression of glucose metabolic markers (Glut2 (c), Glut4 (d), PFK1 (e), FOXO1 (f)) along with insulin signalling (p-Akt (g)), liver TG (h) and lipid metabolic markers (FASN (i)) in the male offspring. Results are expressed as Mean ± SEM (n=6). *P<0.05, **P<0.01 vs Sham, #P<0.05, ##P<0.01 vs E-cig18. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. AUC: area under the curve, E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, FASN: Fatty Acid Synthase, FOXO1: Forkhead box protein O1, Glut: Glucose transporter, IPGTT: intraperitoneal glucose tolerance test, PFK: Phosphofructokinase, TG: triglyceride.

Figure 5. Markers of oxidative stress (nitrotyrosine (a)), mitochondrial antioxidants (MnSOD (b), Gpx1 (d)) and inflammation (F4/80 (d), TNF-α (e), IL-1β (f)) in the male offspring. Results are expressed as Mean ± SEM (n=5-8). *P<0.05, **P<0.01 vs Sham, # P<0.05 vs E-cig18. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, F4/80: EGF-like module-containing mucin-like hormone receptor-like 1, GPx1: glutathione peroxidase 1, IL-1β: Interleukin 1 beta, MnSOD: manganese superoxide dismutase, TNF-α: Tumor necrosis factor-α.

Figure 6. Liver mitochondrial DNA copy number (a), markers of mitophagy (DRP-1 (b), OPA-1 (c)), autophagy (LC3A/B II (d)), and mitochondrial biogenesis (PGC1α (e)) and in the male

offspring. Results are expressed as Mean ± SEM (n=5-8). *P<0.05 vs Sham. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, DRP-1: Dynamin related protein 1, LC3A/B II: Microtubule associated 1A/1B light chain protein 3, OPA-1: Mitochondrial dynamin like GTPase, PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-α.

Figure 1

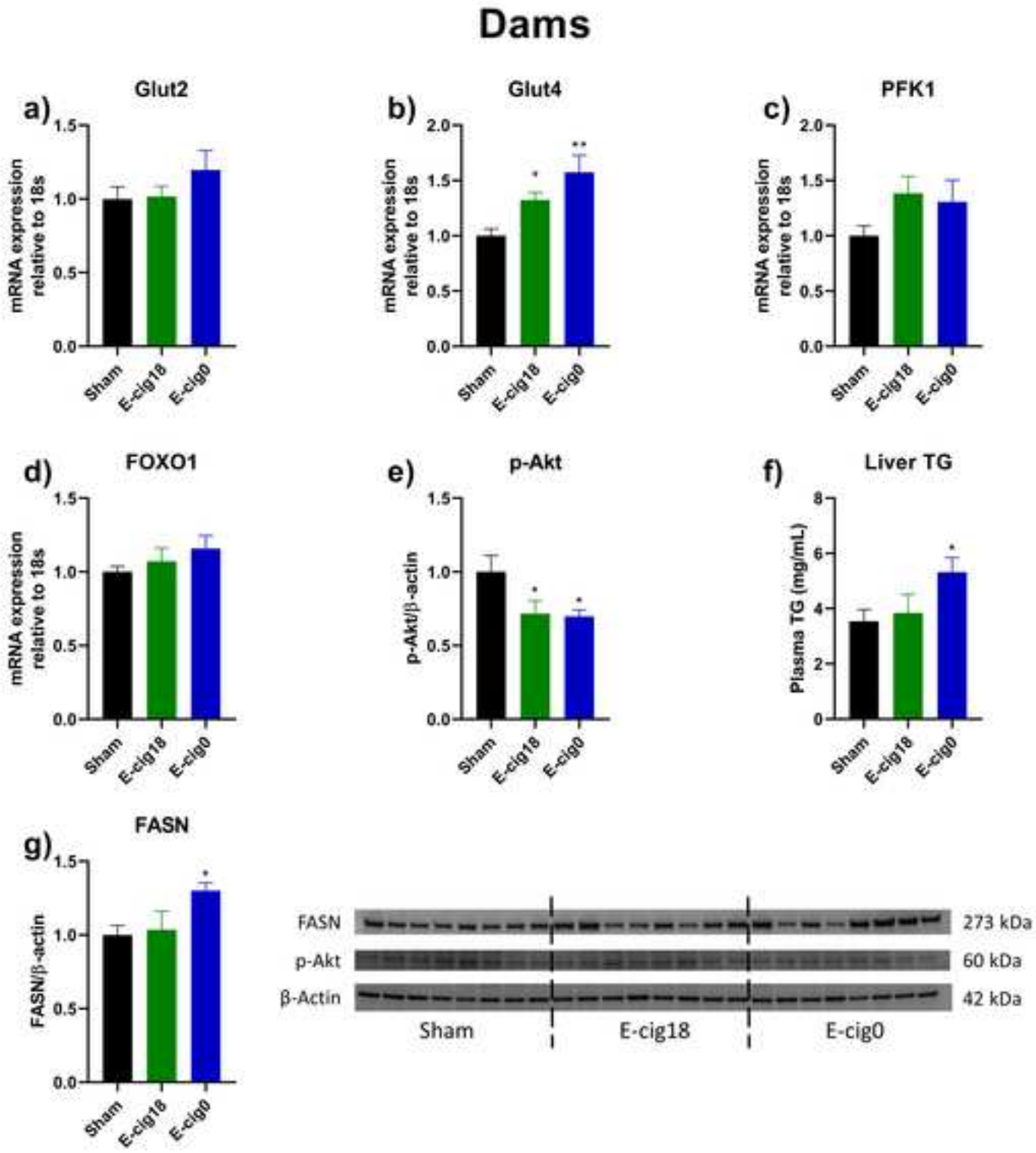
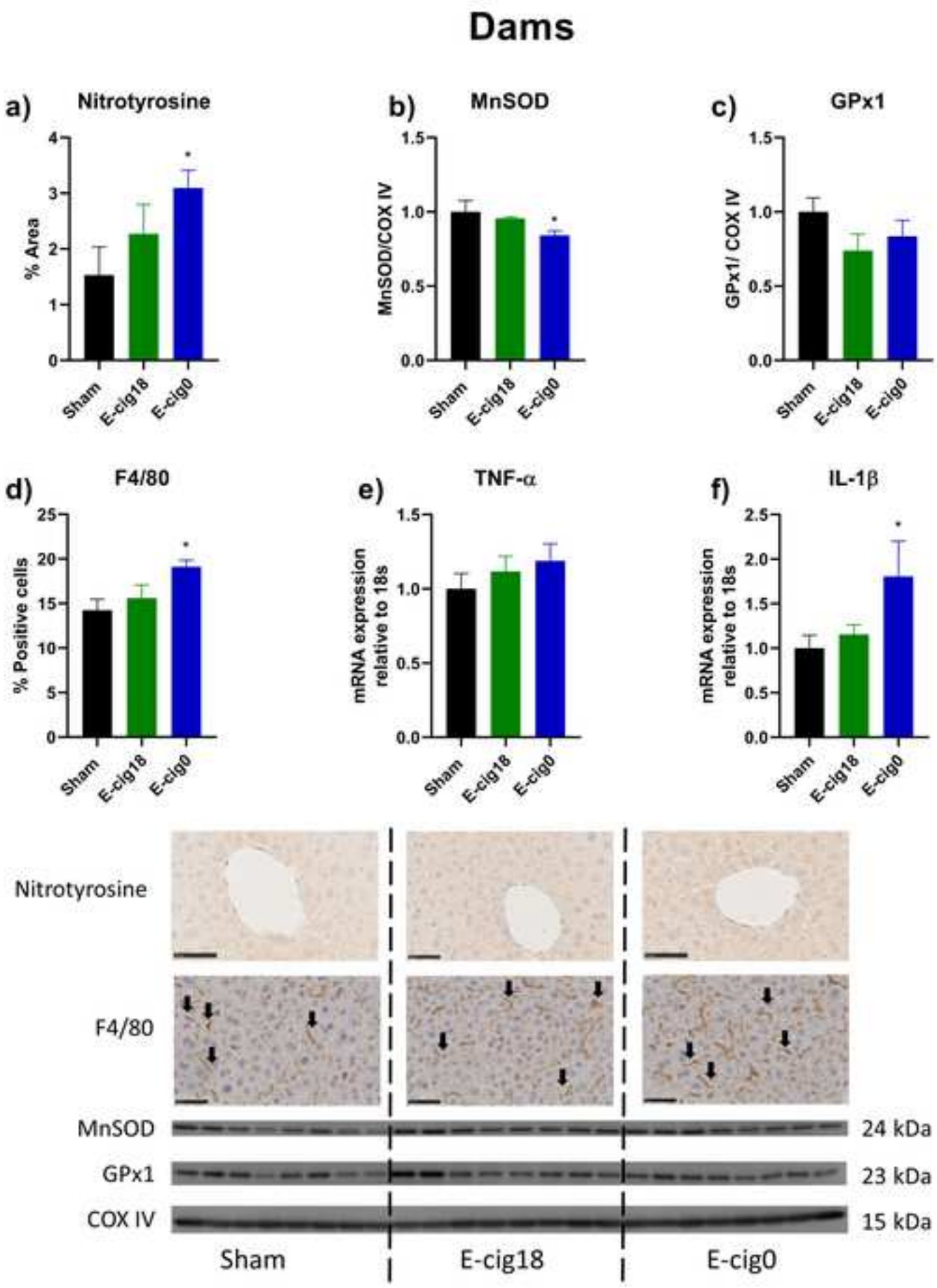


Figure 2

Click here to access/download;Figure;Figure 2.tif



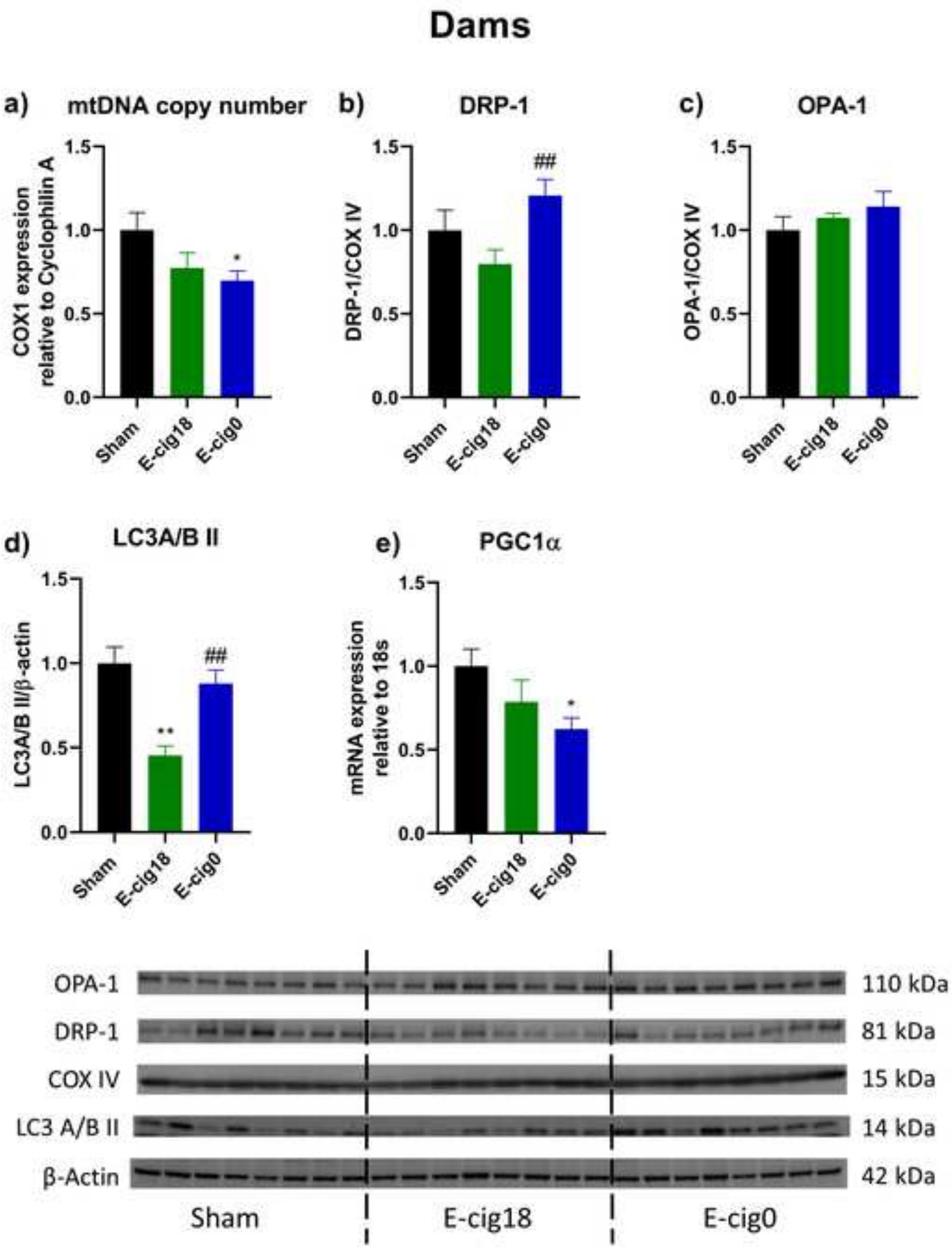


Figure 4

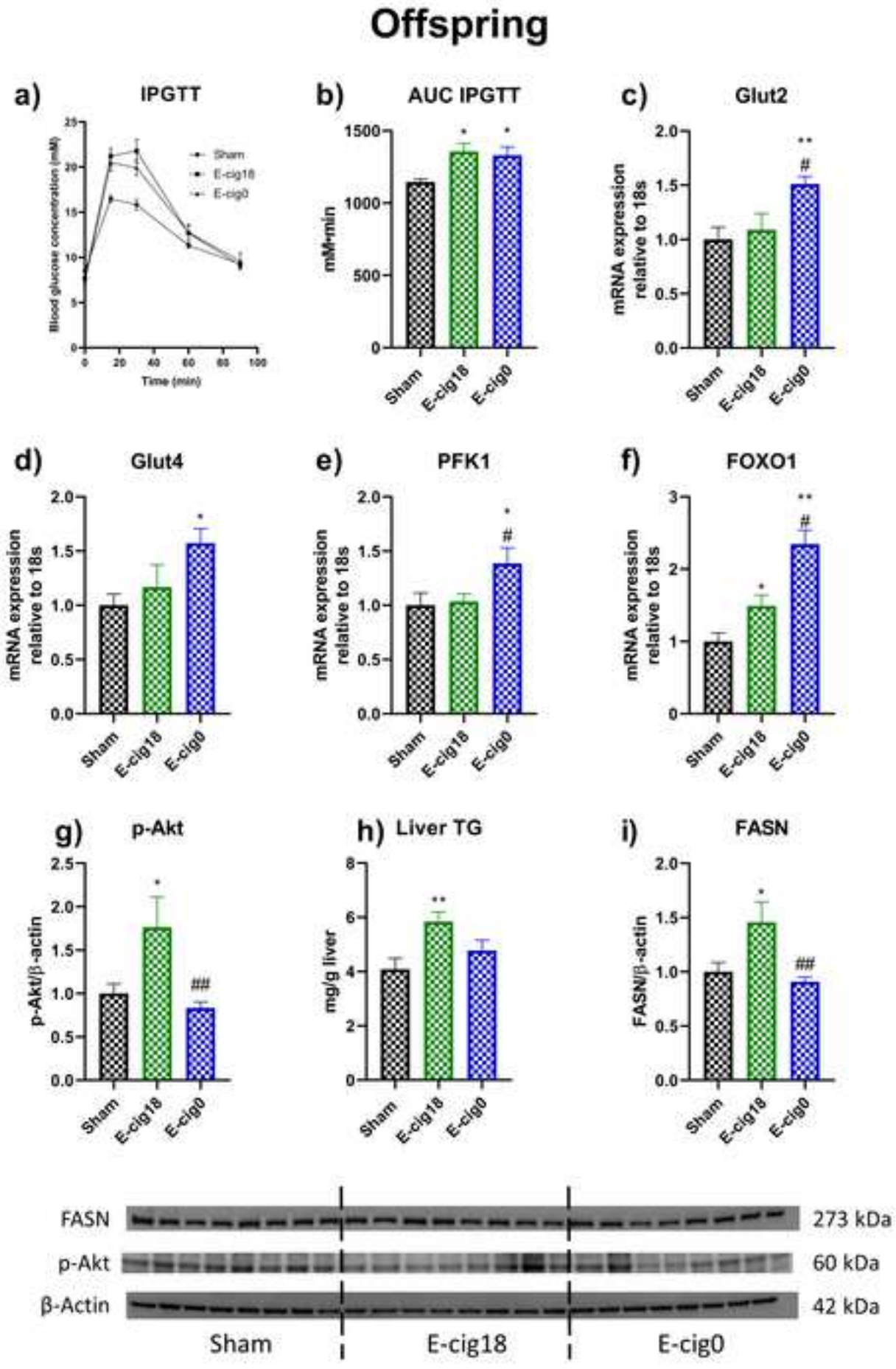


Figure 5

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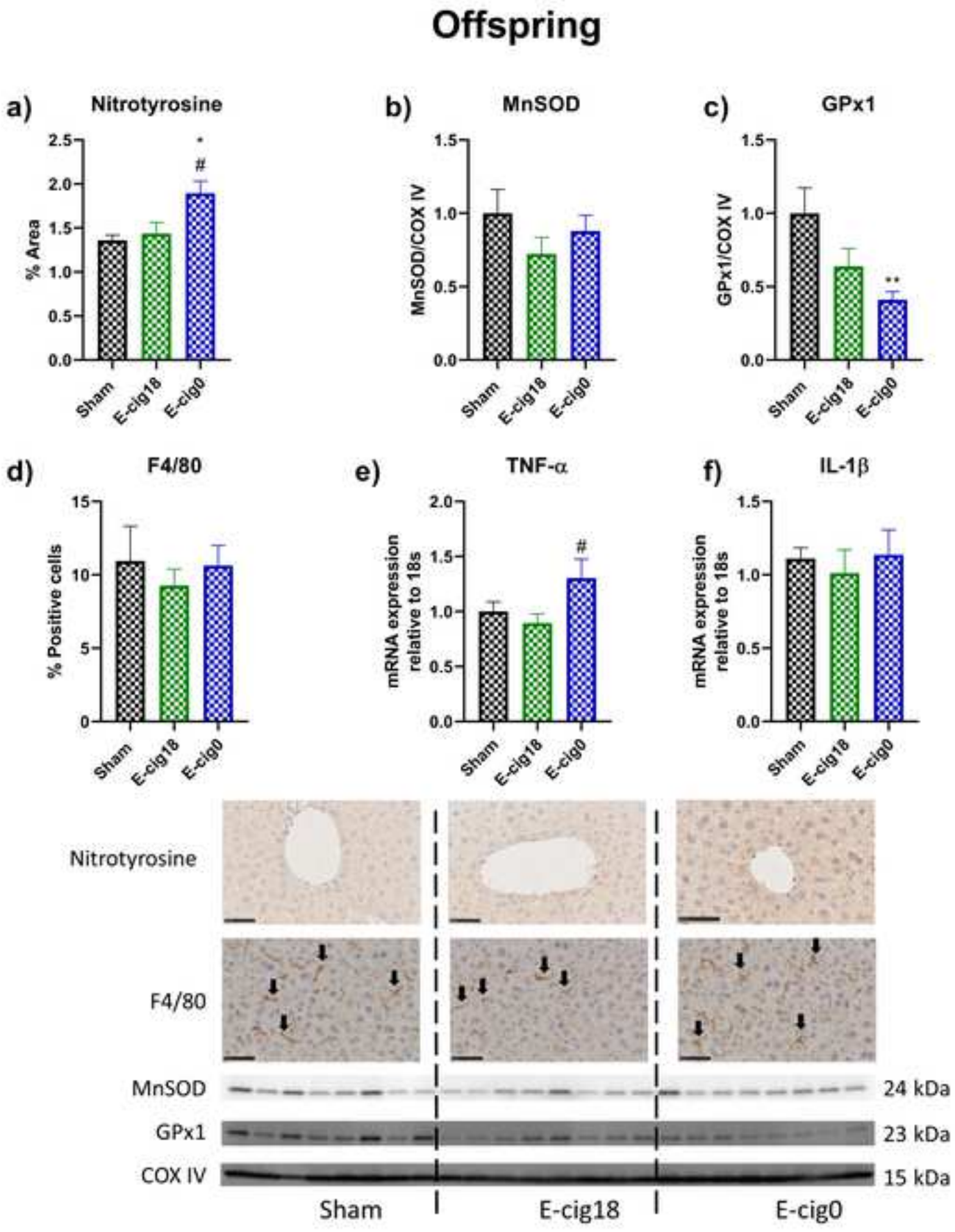

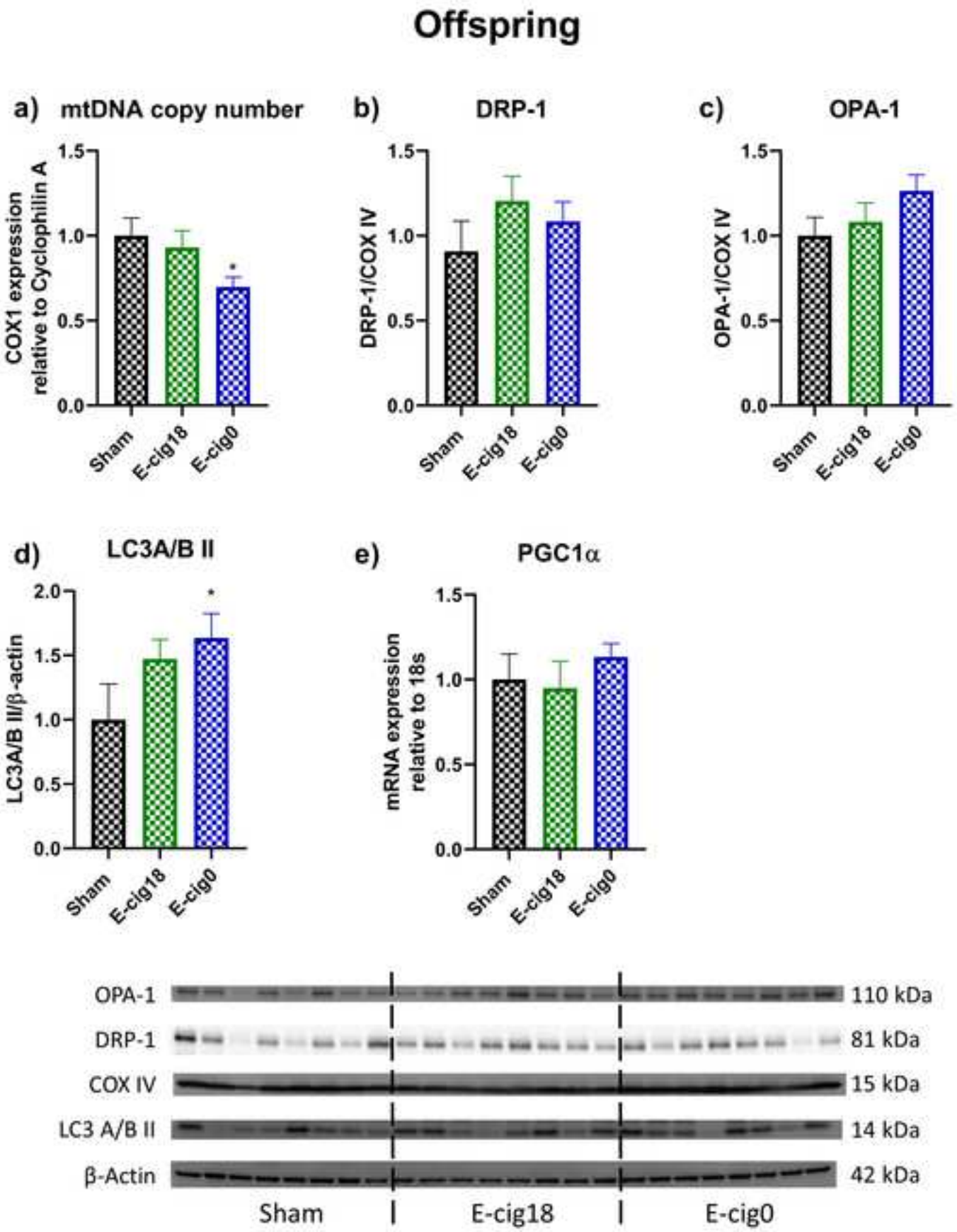

Table 1. Parameters of the dams

	Sham	**E-cig18**	**E-cig0**
Body weight (g)	26.1±0.38	25.6±0.89	27.8±0.34#
Liver weight (g)	1.55 ± 0.07	1.73 ± 0.12	1.87 ± 0.10*
Liver weight (%)	5.93±0.22	6.69±0.28	6.74±0.37
Blood glucose (mM)	9.42 ± 0.83	11.6 ±0.87	10.6 ±0.46
Plasma insulin (ng/mL)	0.70 ±0.047	0.89 ± 0.136	0.85 ± 0.089
HOMA-IR index	6.5±0.620	11.3±1.20**	9.7±0.73*
Liver TG (mg/g liver)	4.0±0.57	3.8±0.67	4.0±0.80
Plasma NEFA (mEq/L)	2.1 ± 0.28	2.82 ± 0.23	3.67 ± 0.29*
Plasma TG (mg/mL)	1.22±0.21	0.92±0.14	1.21±0.29
Plasma ALT (U/L)	13.3±2.6	7.49±1.7	19.3±2.9#

Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 vs Sham, # P<0.05 vs E-cig18. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. ALT: Alanine transaminase, E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, NEFA: non-esterified fatty acid, TG: triglyceride.

Table 2. Parameters in the male offspring (13 weeks old)

	Sham	**E-cig18**	**E-cig0**
Body weight (g)	26.5±0.52	26.01±0.32	26.02±0.33
Liver weight (g)	1.33±0.05	1.31±0.03	1.18±0.02*#
Liver weight (%)	4.99±0.12	5.02±0.09	4.54±0.09*#
Plasma insulin (ng/mL)	0.50±0.015	0.51±0.026	0.56±0.018
Plasma TG (mg/mL)	1.41±0.11	1.13±0.14	1.13±0.04
Plasma NEFA (mEq/L)	4.13±0.47	3.54±0.62	4.20±0.34
Plasma ALT (U/L)	23.0±0.8	24.7±2.9	25.8±2.5

Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 vs Sham, # P<0.05 vs E-cig18. Data were analysed by one-way ANOVA with Fishers LSD post-hoc test. ALT: Alanine transaminase, E-cig0: nicotine-free e-vapour, E-cig18: nicotine-containing e-vapour, NEFA: non-esterified fatty acids, TG: triglyceride.

Table, Supplemental Digital Content 1. TaqMan Probe sequence (Life Technologies, CA, USA) used for rt-PCR.

Gene	NCBI references	Probe Sequence	ID
FOXO1	NM_019739.3	TCGGCGGGCTGGAAGAATTCAATTC	Mm00490671_m1
IL1B	NM_008361.3	TCCTTGTGCAAGTGTCTGAAGCAGC	Mm01336189_m1
PFK1	NM_008826.4	GCGGTGATGCGCAAGGTATGAATGC	Mm00435587_m1
PGC1a	NR_027710.1	CTGGAACTGCAGGCCTAACTCCTCC	Mm01208835_m1
SLC2A2	NM_031197.2	CCGCCTCCCCCGGCGCGCACACACC	Mm00446229_m1
SLC2A4	NM_009204.2	TGGCTCTGCTGCTGCTGGAACGGGT	Mm00436615_m1
TNFα	NM_013693.2	CCCTCACACTCAGATCATCTTCTCA	Mm00443259_g1

Foxo1: Forkhead box protein O1, *PFK1*: Phosphofructokinase 1, *Pgc1a*: Peroxisome proliferator-activated receptor gamma coactivator 1-α, *slc2a2*: Glut2, *Slc2a4*: Glut4, *Tnfα*: Tumor necrosis factor-α.