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L-carnitine reverses maternal cigarette smoke exposure induced renal oxidative stress and mitochondrial dysfunction in mouse offspring

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ABSTRACT

Maternal smoking is associated with metabolic disorders, renal underdevelopment and a predisposition to chronic kidney disease in the offspring, yet the underlying mechanisms are unclear. By exposing female Balb/c mice to cigarette smoke for 6 weeks premating, during gestation and lactation, we showed that maternal smoke exposure induced glucose intolerance, renal underdevelopment, inflammation and albuminuria in male offspring. This was associated with increased renal oxidative stress and mitochondrial dysfunction at birth and in adulthood. Importantly, we demonstrated that dietary supplementation of L-carnitine, an amino acid shown to increase antioxidant defenses and mitochondrial function in numerous diseases, in smoke exposed mothers during pregnancy and lactation significantly reversed the detrimental maternal impacts on kidney pathology in these male offspring. It increased superoxide dismutase (SOD)2 and glutathione peroxidase (GPx)1, reduced reactive oxygen species (ROS) accumulation, and normalized levels of mitochondrial preprotein translocases of the outer membrane (TOM20), and oxidative phosphorylation (OXPHOS) complex I–V in the kidney of the mouse progeny following intrauterine cigarette smoke exposure. These findings support the hypothesis that oxidative stress and mitochondrial dysfunction are closely linked to the adverse effects of maternal smoking on male offspring renal pathology. Our studies suggest L-carnitine administration in cigarette smoke exposed mothers mitigates these deleterious renal consequences.

Key words: Chronic kidney disease, Reactive oxidative species, mitochondria.

INTRODUCTION

Maternal cigarette smoking during pregnancy is a well-recognized causative factor for intrauterine growth retardation (2), associated with the underdevelopment of fetal/neonatal tissues (24), including kidney (29). We have recently demonstrated that maternal cigarette smoke exposure (SE) decreased kidney weight, delayed nephron formation and maturation, as well as increased urinary albumin/creatinine ratio at adulthood in the male offspring (1). With the underlying mechanisms not fully understood, we hypothesized that increased oxidative stress and mitochondrial dysfunction are closely involved in these adverse kidney outcomes.

Living organisms are constantly exposed to oxidants from endogenous metabolic processes, such as reactive oxygen species (ROS), a group of oxygen-derived byproducts released during mitochondrial oxidative phosphorylation (OXPHOS) to generate ATP. Oxidative stress occurs when the intracellular antioxidants are unable to counteract the overproduction of ROS, leading to irreversible oxidative modifications to all cellular components, including lipid, protein and DNA, thus affecting cell structure, function, and viability (26). Smoking has been regarded as a major cause of elevated oxidative stress in active and passive smokers (16). Maternal smoking during pregnancy can not only induce severe oxidative stress in the mother, but also the offspring (11, 25), due to the diffusion of free radicals and harmful chemicals within cigarette smoke (e.g. nicotine) through the blood-placental barrier into the fetus (18), This impact, however, has been only scarcely studied in neonatal plasma and urine, and rarely in neonatal organs (such as kidney). We hypothesized that maternal cigarette smoke exposure can increase oxidative stress in new born kidneys, which persists until adulthood.

As the major source of ROS, mitochondrion is the most affected organelle by oxidative stress. As the cellular power house, impaired mitochondria can fatally imperil energy metabolism and cell viability

(26). Therefore, oxidative stress associated mitochondrial damage and dysfunction have been implicated in a number of diseases such as type 2 diabetes (20), cancer and neurodegenerative disease (9). Importantly, such oxidative damage is likely to result in permanent modifications in mitochondrial DNA (mtDNA), which can be maternally inheritable. This potentially increases the risk of these disorders being transmitted to the progeny. Oxidative damage to mtDNA has been found in fetuses and infants whose mothers were exposed to cigarette smoke or nicotine during pregnancy (4, 25), suggesting a possible impact of maternal smoking on mitochondrial function in the offspring. However the effect on kidney function has not yet been explored.

Carnitine, mainly synthesized in the liver and kidney, is essential for mitochondrial fatty acid metabolism (21). Supplementation of L-carnitine, the active form of carnitine, and its derivatives have been shown to attenuate oxidative stress and mitochondrial dysfunction in diverse conditions, such as age-related disorders and chronic heart failure (15, 19). In patients with end-stage kidney disease requiring dialysis, L-carnitine therapy has been shown to restore plasma antioxidant/oxidant homeostasis (13). However, as in most studies in patients with end stage kidney disease, well established pathology is unlikely to be reversed, independent of the inciting mechanism. This study aimed to examine the utility of maternal L-carnitine supplementation post conception to reverse or ameliorate maternal SE-induced renal oxidative stress and mitochondrial dysfunction in male offspring.

MATERIALS AND METHODS

Animal experiments

Female Balb/c mice (8 weeks) were divided into three groups: Sham (exposed to air), SE (cigarette smoke exposure, 2 cigarettes twice daily, 6 weeks before mating, throughout gestation and lactation, previous described (1)); SE + LC (SE mothers supplied with L-carnitine (1.5mM in drinking water) during gestation and lactation). L-carnitine dose and administration were adapted from a previous

study (28). Male breeders and suckling pups stayed in the home cage when the mothers were sham or cigarette smoke exposed. All the offspring studied were males. Offspring were sacrificed at post-natal day 1 (P1), weaning age (P20), and mature age (Week 13). Intra-peritoneal Glucose Tolerance Test (IPGTT) was performed at week 12 as previous described (6). Blood, urine, and kidneys were collected for further analysis.

Kidney histology

Kidney samples from the male offspring were embedded in paraffin and sectioned in 2 μm slices. Kidney structure was examined using hematoxylin and eosin (H&E) and periodic acid Schiff stain (PAS). Glomerular number and size were quantitated as per our previous protocol (1).

Real-time PCR

Kidney total RNA was extracted and purified using TRIzol Reagents (Life Technology, CA, USA), from which cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Real-time PCR was performed using pre-optimized SYBR Green primers (Sigma-Aldrich) and rt-PCR master mix (Life Technology, CA, USA) to assess the mRNA expression level of macrophage chemoattractant protein (MCP-1) in the kidney, with 18S rRNA as the housekeeping gene.

ELISA

The levels of urinary albumin and creatinine, serum insulin and cotinine were measured using Murine Microalbuminuria ELISA kit (Albuwell M, PA, USA), Creatinine Companion Kit (Exocell Inc, PA, USA), Insulin (Mouse) ELISA Kit (Abnova, Taipei, Taiwan), and cotinine ELISA Kit (Abnova, Taipei, Taiwan) respectively as per manufacturer's instructions.

Western Blot analysis SOD assay

Frozen kidneys were homogenized in HEPES buffer (20 mM, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, 70 mM sucrose). The homogenate was centrifuged to isolate cytosolic and mitochondrial fractions. Protein concentrations were determined and stored at -80^{o}C for further analysis.

Proteins were electrophoresed and electro-blotted to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, New Jersey, USA). The membrane was incubated one of the primary antibodies: anti-β-actin (Santa Cruz Biotechnology, California, USA); goat anti-GPx-1 (R&D System, Minneapolis, USA); rabbit anti-MnSOD (Millipore, Massachusetts, USA); rabbit anti-TOM20 (Santa Cruz Biotechnology); and mouse anti-OXPHOS complex I–V cocktail (Abcam, Cambridge, UK), and then a horseradish peroxidase (HRP)-conjugated secondary antibody. The blots were developed with Luminata Western HRP Substrates (Millipore) by ImageQuant^{TM} LAS 4000 (Fujifilm, Tokyo, Japan). The membrane was restored by stripping buffer (Thermo Scientific) afterward. ImageJ (National Institutes of Health) was used for densitometry, and β-actin was used as the house-keeping protein.

Superoxide Dismutase Assay Kit II (Millipore) was used to measure mitochondrial SOD activity in the isolated mitochondrial proteins according to manufacturer's instruction.

Confocal microscopy

For ROS detection, CellROX Deep Red (5μM, Molecular Probes, Australia) was used, and images were collected at 633 nm excitation wavelength and detected in the 640-680 nm emission range. MitoTracker Green FM (200nM, Molecular Probes, Australia) was used to visualize the mitochondria, and images were collected at 458 nm excitation wavelength and detected in the 480-505nm emission range. Multiple images were taken for over 100 cells in each tissue in 3 replicates of three independent samples/ each group. Morphological features were quantified using a confocal laser scanning microscope (Leica TCS SP2 X; Leica, Wetzlar, Germany). All imaging parameters including laser

intensities, Photomultiplier tubes voltage, pinhole were kept constant during imaging. The tissue segmentation method was used for data analysis as described by Bagett et al. (3) and confirmed using a common threshold units for all the images. Data was expressed as mean fluorescent intensity. To calculate the correlation between CellROX and Mitotracker, dual staining using CellRox and Mitotracker was performed and images were taken sequentially using separate confocal channels over a time not greater than 30 seconds. The image pixel intensity value correlation was then calculated using Pearson's correlation for all pixels excluding any pairs containing zero values.

Statistical analysis

One-way ANOVA followed by Fisher Least Significant Difference post hoc tests was used to determine the difference between the groups (Prism 6, GraphPad). Data are expressed as mean ± SEM. $P < 0.05$ was considered as statistically significant.

RESULTS

Maternal L-carnitine (LC) supplementation normalized birth weight and kidney weight in smoke exposed (SE) offspring

Body weight and kidney mass were significantly reduced in the SE offspring at birth (P1; $P < 0.05$, Table 1). This is consistent with human studies (7, 29), and supports the relevance of this mouse model for studying the effect of maternal SE on renal disorders in the offspring. L-carnitine supplementation reversed the phenotype of low birth weight and kidney weight in P1 SE offspring to the control levels (SE + LC group, Table 1).

Maternal L-carnitine supplementation normalized renal phenotype and glucose tolerance in the SE offspring

There were significant delays in the kidney development in the SE offspring until adulthood. The average number of glomeruli was approximately half of the Control ($P < 0.05$), while glomerular size was increased at weaning ($P < 0.05$) but reduced in adulthood ($P < 0.01$) (Figure 1A, B). This was associated with a significant increase in renal MCP-1 mRNA expression in the SE offspring ($P < 0.01$, Figure 1C). Urinary albumin/creatinine ratio (ACR) was also significantly higher in the SE group at week 13. In addition, offspring from SE mothers had normal serum insulin levels (Table 2), but were glucose intolerant (Figure 1D), suggestive of impaired pancreatic insulin secretion.

In contrast, SE offspring of L-carnitine treated mothers showed an improvement in histological and metabolic parameters. Glomerular number and size were normalised (Figure 1A, B); and glucose tolerance returned to the level of the Control group (Figure 1C, D). Renal MCP-1 mRNA expression and urinary albumin/creatinine ratio was no longer significantly different to that observed in the control animals. Serum cotinine levels were increased in both SE and SE+LC groups, confirming cigarette smoke exposure (Table 2).

Maternal L-carnitine supplementation alleviated renal oxidative stress in the SE offspring

Manganese Superoxide dismutase (MnSOD) and Glutathione Peroxidase (GPx)1 were measured as representative markers for antioxidative defense, as each is involved in one of the two-step ROS converting reaction ($O_2^- \rightarrow H_2O_2 \rightarrow H_2O + O_2$). In addition, both mitochondrial (mt-) and cytosolic (ct-) fractions were measured to determine which fraction is more susceptible to damage, and whether the changes are due to altered gene expression or protein translocation between cytoplasm and mitochondria. At P1, all the measured renal antioxidant markers including mt-MnSOD, ct-MnSOD, mt-GPx-1 and ct-GPx-1 were significantly reduced in the SE offspring by 40% ($P < 0.01$), 50%, 60% and 70% ($P < 0.001$) respectively, suggesting a broad adverse effect of maternal SE on renal antioxidant capacity (Figure 2A). However, only ct-MnSOD ($P < 0.05$, Figure 2C) and mt-MnSOD ($P<0.05$, Figure 2E) were significantly lower than the control levels at P20 and week 13 respectively,

without any changes of GPx-1 at either time point, suggesting renal oxidative stress by maternal SE was partially improved as smoke exposure became more remote. The antioxidant activity of renal mt-SOD in the SE offspring was also significantly reduced at P1 ($P < 0.05$, Figure 2B) and Week 13 ($P < 0.01$ Figure 2F), but not at P20 (Figure 2D), confirming the impaired mitochondrial ability of ROS clearance in the SE offspring's kidneys at birth and adulthood. L-carnitine treatment significantly attenuated the reduction of renal MnSOD and GPx-1, in both cytosolic and mitochondrial fractions at P1 ($P<0.05$, Figure 2A), as well as mt-MnSOD at week 13 ($P < 0.05$, Figure 2E) in the SE offspring. Similarly, it also reversed renal mt-SOD activities at both P1 ($P < 0.01$, Figure 2B), and week 13 ($P < 0.05$, Figure 2F). However, in P20 offspring L-carnitine showed no significant effect (Figure 2C, 2D).

The levels of total and mitochondrial ROS were measured as markers of oxidative stress. Kidney tissues were stained with cell-ROX Red and Mitotracker to identify ROS production and localization. There were marked elevations of renal ROS at week 13 ($P < 0.001$, Figure 3A), which were consistent with the observed reductions in MnSOD/GPx-1 expression and activity. The results reflect a dysregulation of renal redox homeostasis in the offspring due to maternal SE. Furthermore, the correlation coefficient of cell-ROX Red and Mitotracker was significantly higher in the kidney of the SE offspring at P1 and week 13 ($P < 0.01$, Figure 3B), suggesting that the majority of excessive ROS is likely derived from the mitochondria. Interestingly, maternal SE had no effect on renal ROS or mitochondrial ROS at P20 (Figure 3A and B). Renal mitochondrial ROS level was significantly reduced by L-carnitine in the SE offspring at P1 and week 13 ($P < 0.001$, Figure 3C). Interestingly, L-carnitine significantly reduced total ROS at P1, P20 and week 13 ($P<0.01$) and mitochondrial ROS at P1 and week 13 compared to control ($P<0.001$ and $P<0.05$ respectively) (Figure 3C).

Maternal L-carnitine supplementation reversed renal mitochondrial dysfunction in the SE offspring

To investigate mitochondrial function, we assessed TOM20, a mitochondrial outer membrane receptor for translocation of cytosolically synthesized mitochondrial preproteins, and OXPHOS complexes I – V, the key components of mitochondrial respiratory chain for ATP synthesis. Renal protein levels of TOM20 and OXPHOS Complex I, III, and V were significantly reduced in the SE offspring at P1 ($P < 0.05$, Figure 4A), suggesting impaired mitochondrial protein and ATP synthesis. These markers were restored by P20 (Figure 4B), but again reduced at week 13 (Figure 4C), mirroring the changes of renal mt-SOD in the SE offspring. Maternal L-carnitine supplementation significantly restored renal levels of mitochondrial TOM20, Complex I, II, III, and V at P1 (Figure 4A). However, no impact was observed at P20 (Figure 4B). At week 13, offspring from L-carnitine treated SE mothers had normalized TOM20, Complex I, II, and V ($P < 0.01$, Figure 4C), suggesting a long-term effect of L-carnitine to prevent mitochondrial dysfunction by maternal SE.

DISCUSSION

We have previously determined that maternal SE prior to, during gestation and lactation induces renal underdevelopment and impaired function in male offspring, although no significant glomerular structural changes and interstitial abnormalities were detected (1). In this study we demonstrated that maternal SE can significantly increase renal oxidative stress and impair mitochondrial function in the offspring at birth and adulthood. Supplementation of L-carnitine from gestation and throughout lactation can effectively restore renal oxidative homeostasis and mitochondrial function in the SE offspring, as well as intrauterine growth retardation.

In this study, SE offspring had reduced body weight and kidney weight at birth, which is consistent with human epidemiology studies (5). In addition, maternal smoke exposure induced glucose intolerance and albuminuria in the offspring from SE mothers. Moreover, SE offspring showed reduced renal levels of MnSOD and GPx-1, two vital enzymes for intracellular antioxidant defense, especially within the mitochondria. Encoded by genomic DNA, MnSOD is uniquely activated in mitochon-

dria and is the only mitochondrial enzyme known to convert O_2^- into H_2O_2, resulting in ROS disposal (27). As such, alternations in MnSOD quantity and activity can directly affect mitochondrial antioxidant capacity. Unlike MnSOD, GPx-1 functions to convert H_2O_2 into H_2O and O_2 can also be modulated by several other enzymes such as catalase or peroxiredoxin. However, the reduction of GPx-1 in these studies is evidence of impaired renal antioxidant capacity in the offspring by maternal SE. In addition, impaired mitochondrial SOD activities and ROS accumulation provided direct evidence for increased renal oxidative stress due to oxidant/antioxidant imbalance in the SE offspring.

The reduction of mitochondrial functional proteins, including TOM20 and OXPHOS respiratory units correlated with increased oxidative stress. In addition, most of the excessive ROS produced were derived from the mitochondria, as determined by dual-staining of ROS and Mitotracker. This suggests an important interplay between redox imbalance and mitochondrial dysfunction in the effector mechanisms of intrauterine SE on the offspring kidney. It is well-established that increased oxidative stress can impair mitochondrial integrity (26), resulting in impaired mitochondrial preprotein import (30), and poor energy metabolism (8). Conversely, mitochondrial dysfunction, such as defects in ATP exportation (12), and/or antioxidant importation may lead to an escalation of oxidative stress. This is supported by the reduction of both mt-MnSOD and TOM20 in SE offspring kidney at both P1 and Week 13. As the result of this dual effect, a cycle of oxidative stress and mitochondrial damage/dysfunction is hypothesized in the SE offspring kidney, which might significantly contribute to kidney underdevelopment and/or the onset/progression of renal-related disorders.

It is surprising that increased renal oxidative stress and mitochondrial dysfunction were detected in the SE offspring both at birth and adulthood yet it was mitigated at weaning. The mechanism of this temporary recovery is unclear, and we can only postulate that it may be due to the protective effect of breast milk, which has been shown to be rich in antioxidants (31). However, this protection was not sustained until adulthood. The persistent impact of maternal SE suggests that the alteration may

be related to epigenetic modifications in the offspring kidney that could not be reversed by the protective effects of breastfeeding. This aspect warrants further investigation.

It is well-reported that L-carnitine supplementation can ameliorate mitochondrial dysfunction and oxidative stress in diverse conditions, including end-stage kidney disease (13). Herein, we showed that this treatment is also able to prevent similar detrimental impacts by maternal SE in the offspring kidney not just immediately at birth, but also in the long term. Several factors could have contributed to this effect. Firstly, maternal plasma L-carnitine levels during pregnancy are lower than normal, which is supposedly linked to inadequate nutrient status (17). Cigarette smoking during pregnancy has been associated with reduced maternal micronutrient intake (22), and hence, is likely to contribute to further reduction of L-carnitine availability in both the mother and fetuses. Thirdly, the kidney being one of the main sites of L-carnitine production is likely to be sensitive to changes in L-carnitine levels. It has been shown that L-carnitine can prevent renal functional deterioration due to ischemic reperfusion injury (23). As L-carnitine is essentially involved in mitochondrial β-oxidation and has important secondary impacts on other metabolic processes, low levels are likely to increase susceptibility to the accumulation of harmful intermediaries (including ROS) and dysregulate energy utilization (21), leading to oxidative stress, and mitochondrial dysfunction. Hence is it unsurprising that maternal L-carnitine supplementation partly reversed the effects of maternal smoke exposure in the offspring's kidney.

Our data demonstrated that L-carnitine significantly reduced total ROS at all time points compared to control, confirming its role as an anti-oxidant. Although L-carnitine significantly improved anti-oxidant defenses in our study, and reduced total and mitochondrial oxidative stress induced by maternal SE in offspring kidney, it is important to note that there is no evidence of its direct effect on ROS scavenging. Unlike its well-studied role in mitochondrial energy metabolism, the underlying mechanism of its secondary antioxidative effect has not been elucidated (14). Given the high correla-

tion between increased oxidative stress and mitochondrial dysfunction in this study, it is likely that L-carnitine increases redox homeostasis through normalizing mitochondrial energy metabolism. The theory is supported by a previous study showing that increasing mitochondrial ATP synthesis is able to normalize ROS production in a diabetic model (10).

In conclusion, our study demonstrates that maternal cigarette smoke exposure leads to glucose intolerance and renal underdevelopment. This was associated with renal oxidative stress and mitochondrial dysfunction in the offspring at birth and adulthood. Importantly, these defects were significantly reversed by the maternal supplementation of L-carnitine during gestation and lactation. This study provides novel insights into abnormalities in mitochondrial function and increased oxidative stress that underpin the adverse effects of maternal SE on renal pathology in the offspring. The studies further suggest the potential for maternal L-carnitine supplementation to limit the pathomechanistic processes that may predispose to the development of kidney disease in the offspring of smoking mothers.

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Figure Captions

Figure 1. Impaired renal development, inflammation, and glucose intolerance in male SE offspring. (A) Average glomerular number and (B) glomerular size of offspring's kidney at P1, P20, and week 13. (C) Renal mRNA expression of MCP-1 at week 13. (D) Intra-peritoneal Glucose Tolerance Test (IPGTT) at week 13. AUC: area under the curve. **P* <0.05; ***P* <0.01.

Figure 2. Renal antioxidant capacity in the offspring. (A, C, E) Renal mitochondrial and cytosolic MnSOD and GPx-1 levels at P1, P20, and Week 13, respectively. (B, D, F) Mitochondrial SOD activity at P1, P20, and Week 13, respectively (B, D, F). n = 4 – 8. **P*<0.05, ***P*<0.01.

Figure 3. Confocal laser scanning microscopy images of total and mitochondrial ROS staining in the offspring kidney. (A) Representative confocal images for cell-Rox staining showing total ROS intensity (B) Representative confocal images for Mitotracker and CellRox co-staining showing that most ROS was localized within or within close proximity to the mitochondria. (C) Quantitative representation of Mean Fluorescent Intensity (MFI) for A and B. n= 3. **P*<0.05; ***P<0.01;* ****P*<0.001 vs control or as indicated.

Figure 4. Renal TOM20 and OXPHOS complex I – V levels in the offspring of Control, SE mothers and SE mothers with L-carnitine treatment (SE+LC) at P1 (A), P20 (B), and Week 13 (C). n = 4 – 8. **P*<0.05, ***P*<0.01, ****P*<0.001.

463 **Table 1.** Body and kidney weight of the offspring

P1	**Control**	**SE**	**SE + LC**
Body weight (g)	1.55 ± 0.05	1.35 ± 0.06*	1.58 ± 0.06#
Kidney weight (g)	0.0081 ± 0.0004	0.0069 ± 0.0004*	0.0086 ± 0.0010#
Kidney/Body (%)	0.52 ± 0.02	0.51 ± 0.04	0.55 ± 0.04

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P20	**Control**	**SE**	**SE + LC**
Body weight (g)	9.97 ±0.16	9.71 ±0.14	9.74 ±0.43
Kidney weight (g)	0.067 ±0.001	0.062 ±0.003	0.067 ±0.002
Kidney/Body (%)	0.67± 0.01	0.062 ±0.03	0.70 ±0.03

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Week 13	**Control**	**SE**	**SE + LC**
Body weight (g)	25.5 ±0.3	25.1 ±0.6	25.3 ±0.3
Kidney weight (g)	0.20 ±0.01	0.19± 0.01	0.19 ±0.01
Kidney/Body (%)	0.77 ±0.01	0.76 ±0.02	0.77 ±0.02

466 Values are means ± SE; * $P < 0.05$ vs Control; # $P < 0.05$ vs SE; n = 6-10

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Table 2. Blood levels of Cotinine, Insulin and urinary Albumin/Creatinine ratio

Week 13	**Control**	**SE**	**SE + LC**
Cotinine (ng/ml)	1.35 ± 0.60	3.90 ± 0.42**	4.48 ± 0.17**
Insulin (ng/ml)	0.53 ± 0.02	0.54 ± 0.01	0.54 ± 0.01
Albumin/Creatinine ratio	43.0 ± 14.0	104.7 ± 19.6*	81.5 ± 32.5

* $P < 0.05$, ** $P < 0.01$ (vs Control), n = 6-10.

Figure 1

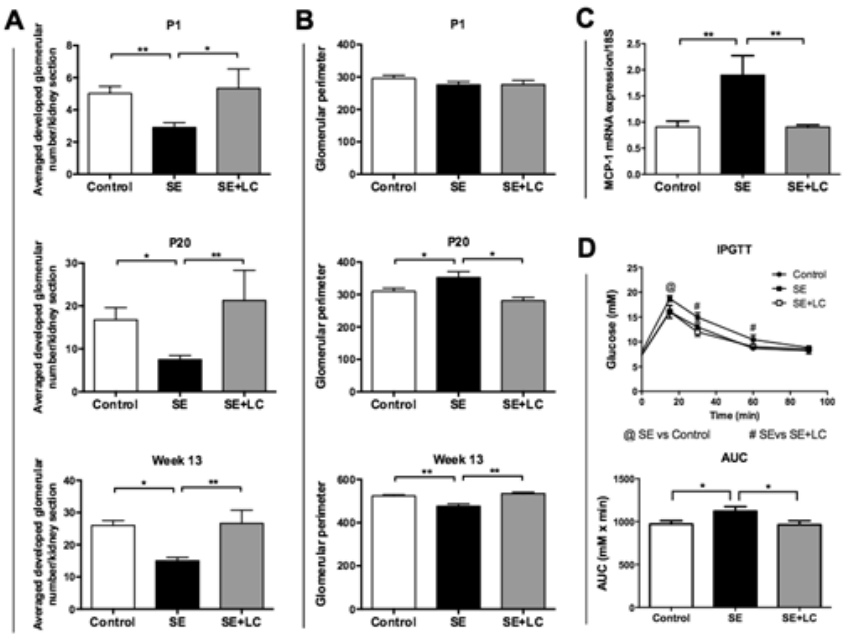

A
P1
Averaged developed glomerular number/kidney section
Control
SE
SE+LC
P20
Week 13
B
Glomerular perimeter
C
MCP-1 mRNA expression/18S
D
IPGTT
Glucose (mM)
Time (min)
@ SE vs Control
# SEvs SE+LC
AUC
AUC (mM x min)

Figure 2

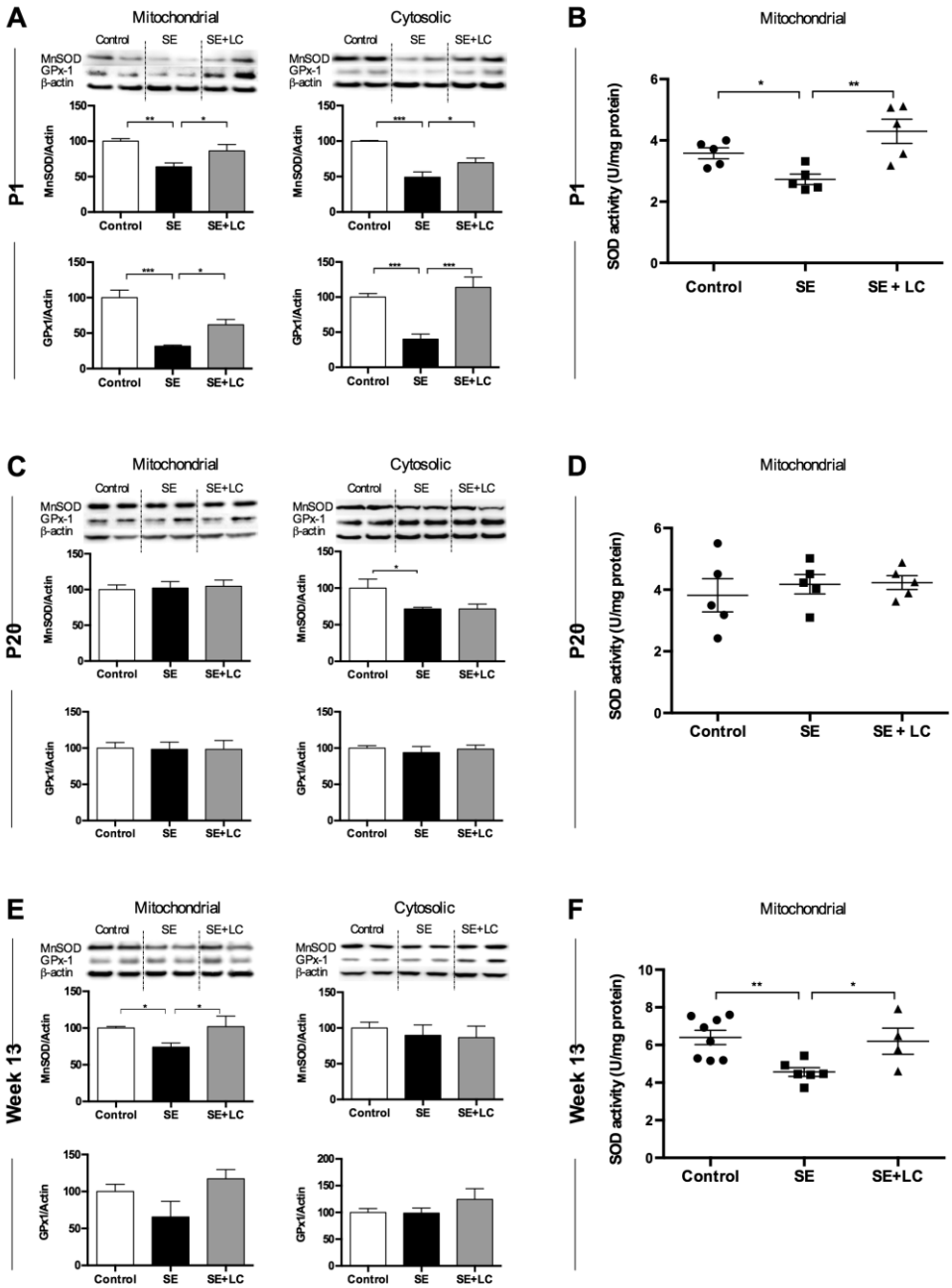

A
Mitochondrial
Cytosolic
Control
SE
SE+LC
MnSOD
GPx-1
β-actin
P1
MnSOD/Actin
GPx1/Actin
B
SOD activity (U/mg protein)
C
P20
D
E
Week 13
F

Figure 3

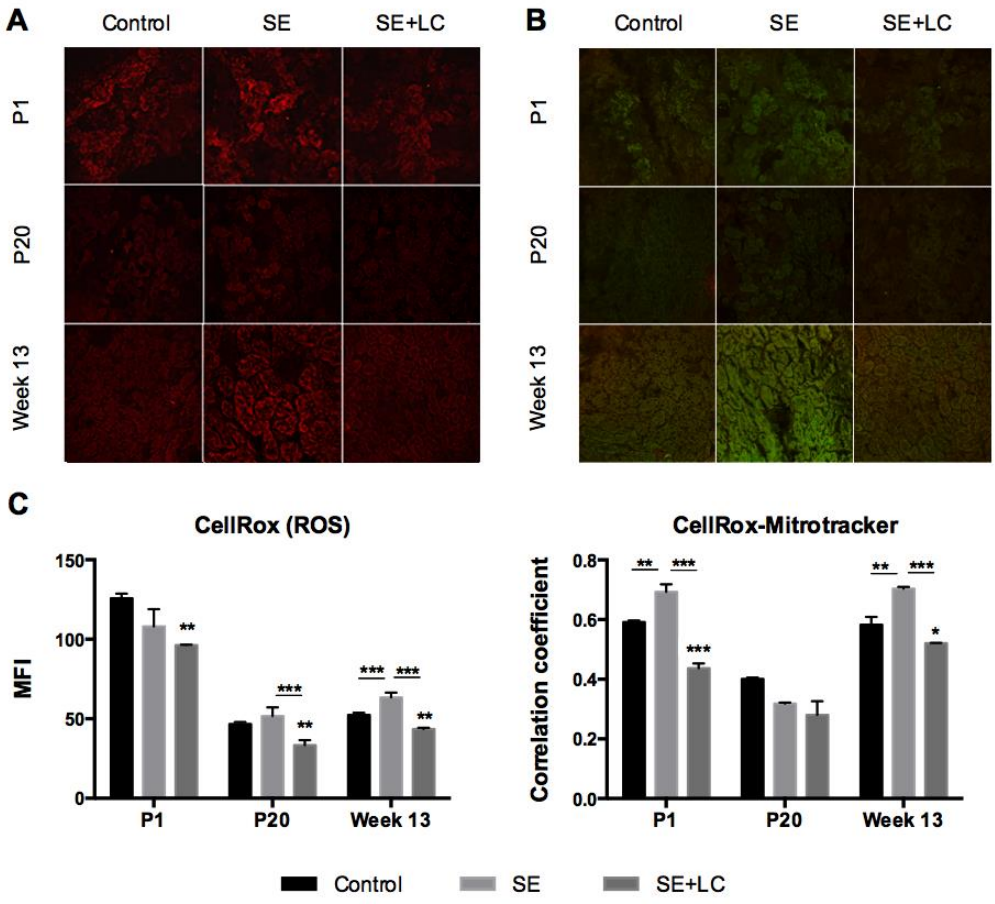
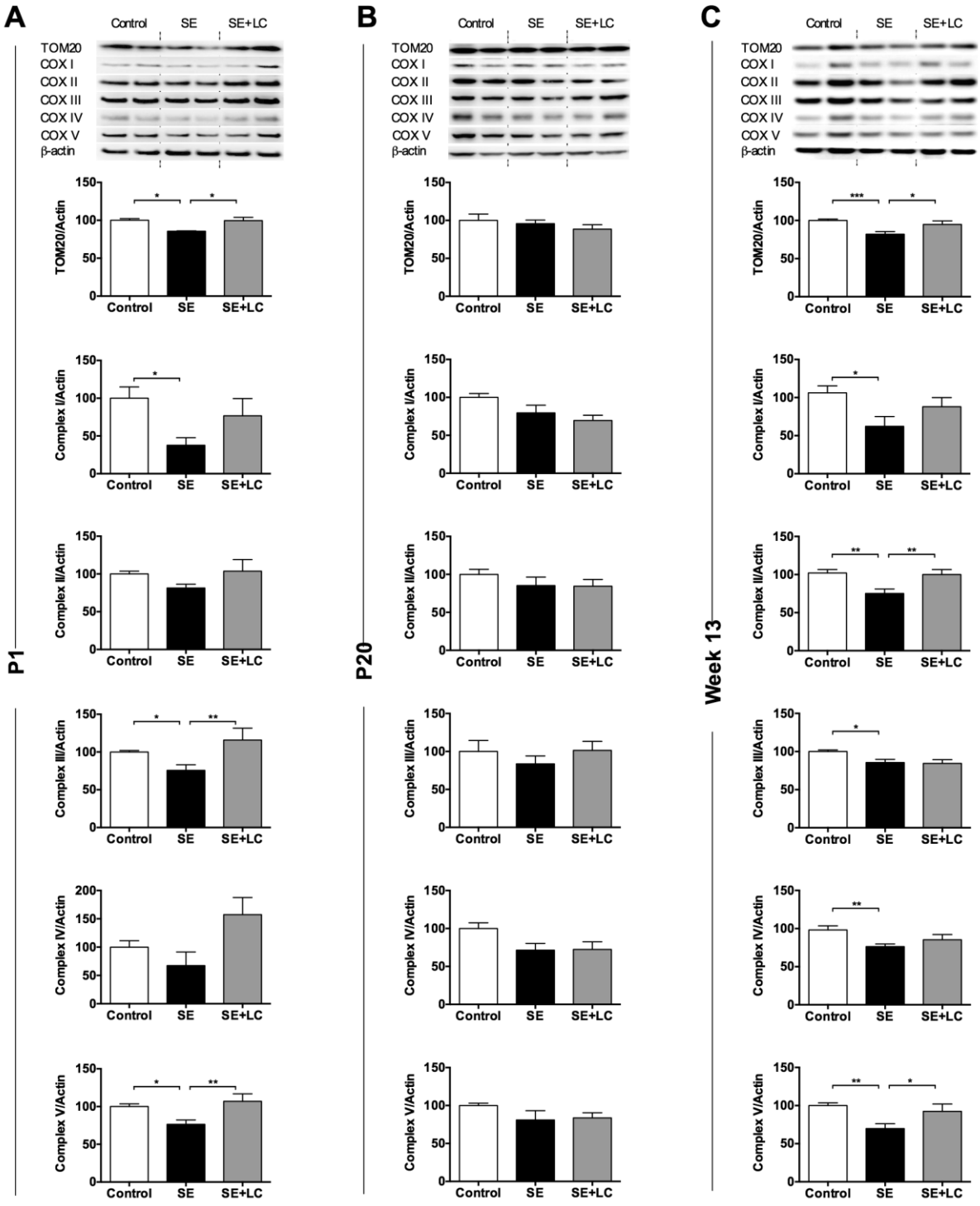

A
Control
SE
SE+LC
P1
P20
Week 13
B
Control
SE
SE+LC
P1
P20
Week 13
C
CellRox (ROS)
MFI
150
100
50
0
**
***
***
***
**
**
P1
P20
Week 13
CellRox-Mitrotracker
Correlation coefficient
0.8
0.6
0.4
0.2
0.0
**
***
***
**
***
*
P1
P20
Week 13
Control
SE
SE+LC

Figure 4


A
B
C
Control
SE
SE+LC
TOM20
COX I
COX II
COX III
COX IV
COX V
β-actin
TOM20/Actin
Complex I/Actin
Complex II/Actin
Complex III/Actin
Complex IV/Actin
Complex V/Actin
P1
P20
Week 13