

SGLT2 Inhibition Mitigates Transition from Acute Kidney Injury to Chronic Kidney Disease by Suppressing Ferroptosis

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<https://hdl.handle.net/2324/7363697>

出版情報 : Kyushu University, 2024, 博士 (医学) , 課程博士
バージョン :
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OPEN SGLT2 inhibition mitigates transition from acute kidney injury to chronic kidney disease by suppressing ferroptosis

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Sodium-glucose cotransporter 2 (SGLT2) inhibitors have been shown to be renoprotective in ischemia-reperfusion (I/R) injury, with several proposed mechanisms, though additional mechanisms likely exist. This study investigated the impact of luseogliflozin on kidney fibrosis at 48 h and 1 week post I/R injury in C57BL/6 mice. Luseogliflozin attenuated kidney dysfunction and the acute tubular necrosis score on day 2 post I/R injury, and subsequent fibrosis at 1 week, as determined by Sirius red staining. Metabolomics enrichment analysis of I/R-injured kidneys revealed suppression of the glycolytic system and activation of mitochondrial function under treatment with luseogliflozin. Western blotting showed increased nutrient deprivation signaling with elevated phosphorylated AMP-activated protein kinase and Sirtuin-3 in luseogliflozin-treated kidneys. Luseogliflozin-treated kidneys displayed increased protein levels of carnitine palmitoyl transferase 1 α and decreased triglyceride deposition, as determined by oil red O staining, suggesting activated fatty acid oxidation. Luseogliflozin prevented the I/R injury-induced reduction in nuclear factor erythroid 2-related factor 2 activity. Western blotting revealed increased glutathione peroxidase 4 and decreased transferrin receptor protein 1 expression. Immunostaining showed reduced 4-hydroxynonenal and malondialdehyde levels, especially in renal tubules, indicating suppressed ferroptosis. Luseogliflozin may protect the kidney from I/R injury by inhibiting ferroptosis through oxidative stress reduction.

Keywords Luseogliflozin, Ischemia reperfusion injury, Ferroptosis, Nrf2

There are many causes of acute kidney injury (AKI), including dehydration, hypotension, drug use, nephritis, and obstruction of the urinary tract. Ischemia-reperfusion (I/R) injury is one of the major causes of AKI¹. I/R injury can result from vascular injury related to surgery or trauma, renal vascular disease, cardiac arrest during cardiac surgery, and hypotension, including that associated with sepsis. The mechanism of I/R injury is thought to involve a combination of oxidative stress, inflammation, cellular dysfunction and death, endothelial dysfunction, and activation of the immune response^{2–4}. Nevertheless, effective treatment of I/R injury has not been established and is urgently needed.

Sodium-glucose cotransporter 2 (SGLT2) inhibitors were reported to be renoprotective in diabetes mellitus (DM) patients in the EMPA-REG OUTCOME study⁵. In addition, the DAPA-CKD trial reported that SGLT2 inhibitors also exert renoprotective effects in non-DM patients⁶. Information about the effect of SGLT2 inhibitors on AKI is limited by the small number of relevant clinical trials. However, the results of several small studies and preclinical studies suggest that SGLT2 inhibitors may have protective effects against AKI⁷. In the realm of basic research, the efficacy of sodium-glucose cotransporter 2 (SGLT2) inhibitors in treating AKI has been variably reported, with studies demonstrating both effectiveness and ineffectiveness^{8–11}, leaving their impact a subject of ongoing debate. While some studies have noted improvements in renal reperfusion through enhanced hypoxia-inducible factor 1- α (HIF1 α) reducing apoptosis⁸, elevated levels of vascular endothelial growth factor (VEGF)⁹, and diminished inflammation¹⁰, the underlying mechanisms of SGLT2 inhibitors remain insufficiently elucidated. Consequently, this study aims to comprehensively investigate the renoprotective effects of

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luseogliflozin against ischemia-reperfusion (I/R) injury in non-diabetic mice, focusing particularly on elucidating these effects through metabolome analysis.

Results

Luseogliflozin-mediated inhibition of I/R injury-induced acute tubular necrosis and consequent renal dysfunction

To investigate the effect of luseogliflozin on AKI, 12-week-old male mice were treated with luseogliflozin for 2 weeks prior to 26 min of bilateral renal I/R injury. Serum levels of blood urea nitrogen (BUN) and creatinine (Cr) were clearly elevated at 48 h post I/R injury in both the vehicle and Luseo groups, but BUN levels tended to be lower, and Cr levels were significantly lower, in the Luseo group (Fig. 1a and b). AKI was analyzed histologically by periodic acid-Schiff (PAS) staining (Fig. 1c). Acute tubular necrosis (ATN) scores were attenuated in the Luseo group compared to those in the vehicle group (Fig. 1d). Consistent with the decrease in ATN score, induction of kidney injury molecule 1 (KIM-1), a marker of tubular damage, was significantly suppressed in the Luseo group compared to that in the vehicle group (Fig. 1e and f).

Luseogliflozin-mediated reduction in the transition to chronic kidney disease (CKD) following I/R injury

AKI is an important risk factor for CKD transition. Evaluation of gene expression in the kidney at 1 week post I/R injury showed that the mRNA levels of smooth muscle 22 α (*SM22 α*), transforming growth factor- β (*TGF β*), and collagen I (*COL1A1*), which are involved in fibrosis, were significantly decreased in the Luseo group (Fig. 2a–c). In the I/R-injured Luseo group, the expression levels of smooth muscle actin (SMA), collagen I, and fibronectin, which were increased by I/R in the vehicle group, were reduced to less than half of the levels in the vehicle group (Fig. 2d–i). This corresponded to the decrease in *SM22 α* mRNA in the Luseo group. We also observed decreased expression of α -SMA and fibronectin in I/R-injured kidney tissues in the Luseo group compared to that in the vehicle group (Supplementary Fig. S1a–d). Renal interstitial fibrosis was evaluated at 1 week post I/R injury by Sirius red staining (Fig. 2j and k) and Masson's trichrome staining (Fig. 2l and m). In the I/R-injured Luseo group, renal fibrosis was significantly reduced to about half the level of the vehicle group.

Next, we assessed inflammation, which is closely related to fibrosis of the kidney. The infiltration of F4/80-positive macrophages was significantly reduced in the Luseo group compared to that in the vehicle group (Supplementary Fig. S2a and b). In response to reduced macrophage infiltration, the mRNA expression levels of interleukin-1 β (*IL1B*) tended to decline in the Luseo group compared to those in the vehicle group (Supplementary Fig. S2c). The mRNA levels of *IL6* and tumor necrosis factor α (*TNF*) in the kidney were significantly decreased in the I/R-injured Luseo group compared to those in the vehicle group (Supplementary Fig. S2d and e).

Enhancement of energy sensors and suppression of the glycolytic system through luseogliflozin-mediated inhibition of renal glucose reabsorption

To evaluate SGLT2 inhibitor-induced changes in energy metabolism in the transition from AKI to CKD, kidneys were analyzed 1 week post I/R injury. SGLT2 inhibitors are known to upregulate nutrient deprivation signaling enzymes, such as AMP-activated protein kinase (AMPK) and Sirtuin-3 (*SIRT3*), in the heart and kidneys^{12–14}. Phosphorylation of AMPK protein levels decreased in the I/R-injured vehicle group compared to the sham-operated vehicle group but tended to be restored in the I/R-injured Luseo group (Fig. 3a and b). *SIRT3* is a mitochondria-localized NAD-dependent deacetylase that regulates cellular metabolism and homeostasis¹³. Kidney *Sirt3* protein levels were reduced in the I/R-injured vehicle group compared to those in the sham-operated vehicle group, but were restored to sham levels in the I/R-injured Luseo group (Fig. 3c and d).

Next, to gain a comprehensive understanding of renal metabolic changes during the first week post I/R injury, we performed a metabolomic enrichment analysis. Compared to the vehicle group, the Luseo group exhibited suppression of the glycolytic system and the pentose pathway (Fig. 3e). The Luseo group also showed increases in cardiolipin synthesis pathway activity, triglyceride synthesis, and ketone bodies compared to the vehicle group (Fig. 3f). Based on the increase in cardiolipin biosynthesis, we hypothesize that luseogliflozin facilitates the maintenance of mitochondrial ATP production in response to I/R injury-induced tissue damage and oxidative stress.

Luseogliflozin-mediated improvement in dysfunctional fatty acid oxidation

Because luseogliflozin treatment in I/R-injured kidneys activated ketone body metabolism, but did not reduce the levels of pAMPK and *Sirt3*, which are involved in fatty acid oxidation, we next evaluated the effects of luseogliflozin on fatty acid oxidation. Specifically, we evaluated the expression of peroxisome proliferator-activated receptor (*PPAR*) α , a transcriptional regulator of genes involved in lipid metabolism, and two enzymes involved in fatty acid metabolism, namely acyl-CoA dehydrogenase long chain (*ACADL*) and carnitine palmitoyl transferase 1 α (*CPT1 α*). I/R injury decreased mRNA expression of *PPAR α* , *ACADL*, and *CPT1 α* , but luseogliflozin treatment restored the mRNA levels of each gene to those in the sham-operated vehicle group (Fig. 4a–c). Western blotting results showed that *PPAR α* protein levels and *ACADL* protein levels were also decreased following I/R injury, but were restored to non-I/R injury levels in the Luseo group (Fig. 4d–g). Interestingly, *CPT1 α* protein levels did not differ between I/R-injured kidneys and sham-operated kidneys, but did increase in the I/R-injury Luseo group (Fig. 4h and i). Oil red O staining showed that triglyceride accumulation in I/R-injured kidneys was significantly reduced in the Luseo group (Fig. 4j and k). These results were attributed to an increase in fatty acid oxidation induced by luseogliflozin.

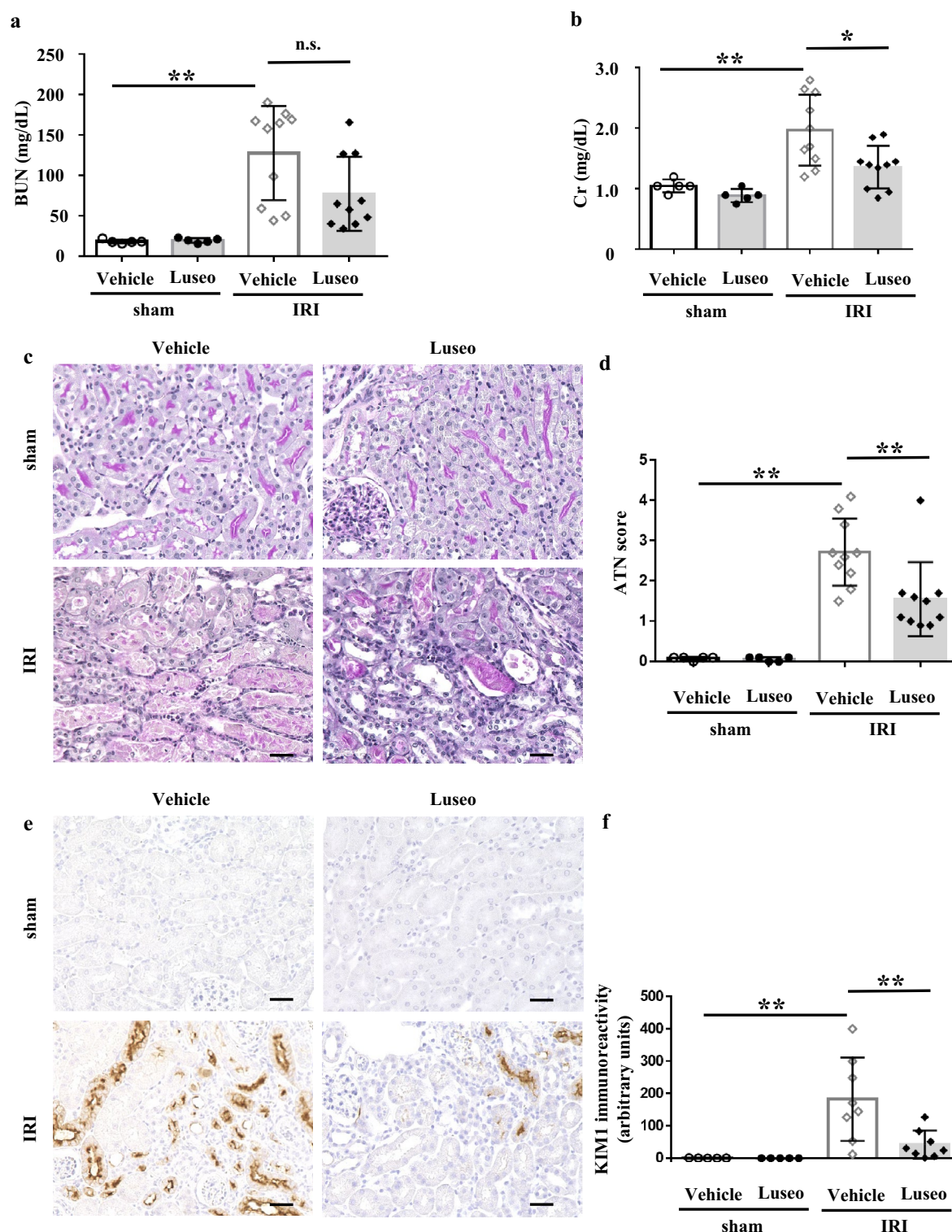


Fig. 1. Luseogliflozin-mediated improvements in renal function and histology at 48 h post ischemia/reperfusion (I/R) injury in mice. (a–c) Serum levels of blood urea nitrogen (BUN; a, and creatinine (Cr; b), and histological findings of kidney sections stained with hematoxylin and eosin (c) at 48 h post I/R injury. (d) Acute tubular necrosis (ATN) scores in the following groups: vehicle-treated + sham-operated (n = 5), luseogliflozin-treated + sham-operated (n = 5), vehicle-treated + I/R-injured (n = 10), and luseogliflozin-treated + I/R-injured (n = 10). (e and f) Histological findings and quantification of kidney sections immunostained for kidney injury molecule 1 (KIM-1) at 48 h post I/R injury; * $P < 0.05$, ** $P < 0.01$, as determined by one-way analysis of variance. n.s., not significant.

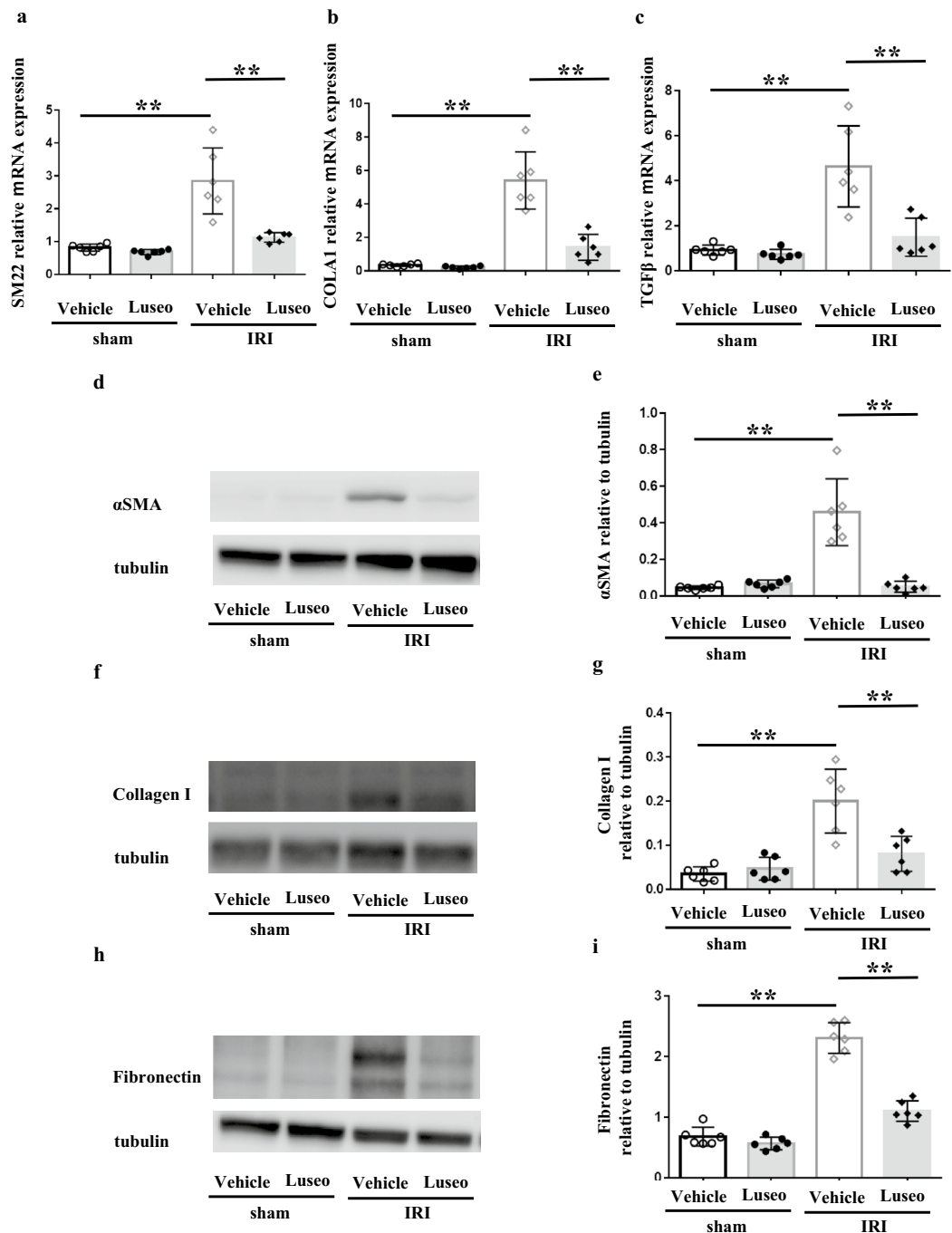


Fig. 2. Luseogliflozin-mediated improvement in renal interstitial fibrosis at 1 week post ischemia/reperfusion (I/R) injury in mice. **(a–c)** Quantification of kidney mRNA levels of *SM22* **(a)**, *COLA1* **(b)**, and *TGF* **(c)** at 1 week post I/R injury. **(d and e)** Western blot analysis and quantification of α SMA expression in the kidney at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 6$), luseogliflozin-treated + sham-operated ($n = 6$), vehicle-treated + I/R-injured ($n = 6$), and luseogliflozin-treated + I/R-injured ($n = 6$). **(f and g)** Western blot analysis and quantification of collagen I expression in the kidney at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 6$), luseogliflozin-treated + sham-operated ($n = 6$), vehicle-treated + I/R-injured ($n = 6$), and luseogliflozin-treated + I/R-injured ($n = 6$). **(h and i)** Western blot analysis and quantification of fibronectin expression in the kidney at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 6$), luseogliflozin-treated + sham-operated ($n = 6$), vehicle-treated + I/R-injured ($n = 6$), and luseogliflozin-treated + I/R-injured ($n = 6$). **(j–m)** Histological findings and quantification of kidney sections stained with Sirius red **(j, k)** and Masson's trichrome staining **(l, m)** at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 5$), luseogliflozin-treated + sham-operated ($n = 5$), vehicle-treated + I/R-injured ($n = 10$), and luseogliflozin-treated + I/R-injured ($n = 10$); * $P < 0.05$, ** $P < 0.01$, as determined by one-way analysis of variance. n.s., not significant.

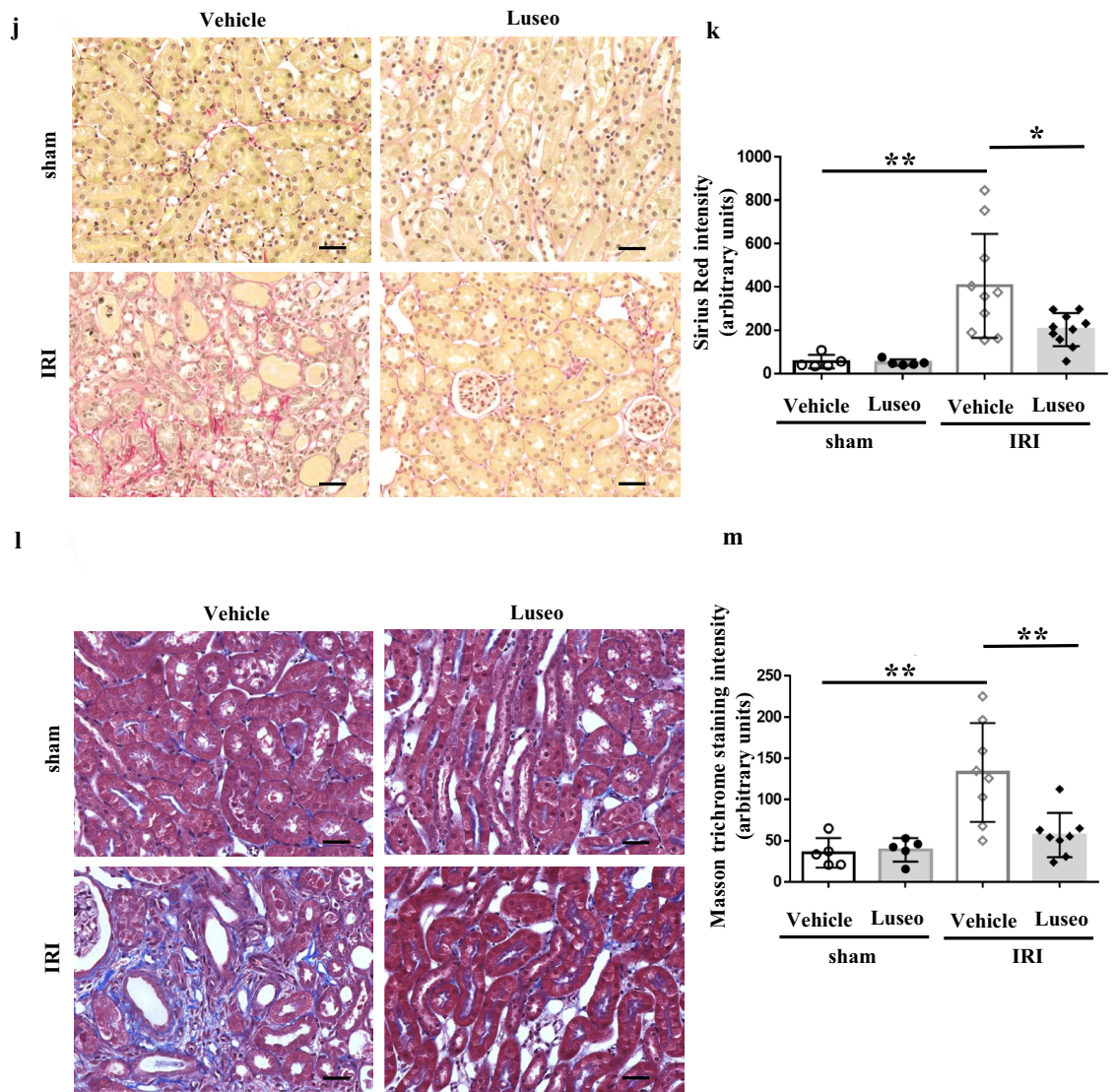


Fig. 2. (continued)

Luseogliflozin-mediated improvement in mitochondrial function and amelioration of oxidative stress through increased Nrf2 expression

Based on the results of metabolomic analysis, we turned our focus toward luseogliflozin-induced changes in mitochondrial function. First, we evaluated the regulator of mitochondrial biosynthesis, PPAR γ coactivator 1 α (PGC-1 α). Western blotting results showed PGC1 α levels were reduced by I/R injury, but were restored to the levels of the sham-operated vehicle group by luseogliflozin treatment (Fig. 5a and b). I/R injury also reduced protein expression of the mitochondrial marker translocase of outer mitochondrial membrane 20 (TOM20), which was restored to the levels of the sham-operated vehicle group by luseogliflozin treatment (Fig. 5c and d). Immunohistochemistry showed that TOM20 expression was especially decreased in the renal tubule in I/R-injured kidneys of the vehicle group but was maintained in the Luseo group (Fig. 5e and f).

Next, we assessed the oxidative stress induced by I/R injury in the kidneys. One week after I/R injury in mice, dihydroethidium (DHE) staining revealed that, although I/R injury increased superoxide levels in kidneys, the effect was mitigated in the Luseo group compared to that in the vehicle group (Fig. 6a and b). In addition, the Luseo group showed lower levels of nitrotyrosine, an indicator of oxidative stress, compared to the vehicle group (Fig. 6c and d). We also evaluated nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional factor of genes involved in antioxidant activity¹⁵ and regulator of oxidative stress that is activated by AMPK^{16,17}. Western blotting revealed an increase in Nrf2 protein levels in response to I/R injury in the Luseo group compared to that in the vehicle group (Fig. 6e and f). The mRNA levels of three Nrf2 target genes, namely glutamate-cysteine ligase modifier subunit (*GCLM*), glutathione-disulfide reductase (*GSR*), and thioredoxin reductase 1 (*TXNRD1*), were decreased following I/R injury, but were restored with luseogliflozin administration (Fig. 6g–i). These findings indicated that luseogliflozin ameliorates oxidative stress in the kidney by improving mitochondrial function and increasing Nrf2 expression downstream of pAMPK.

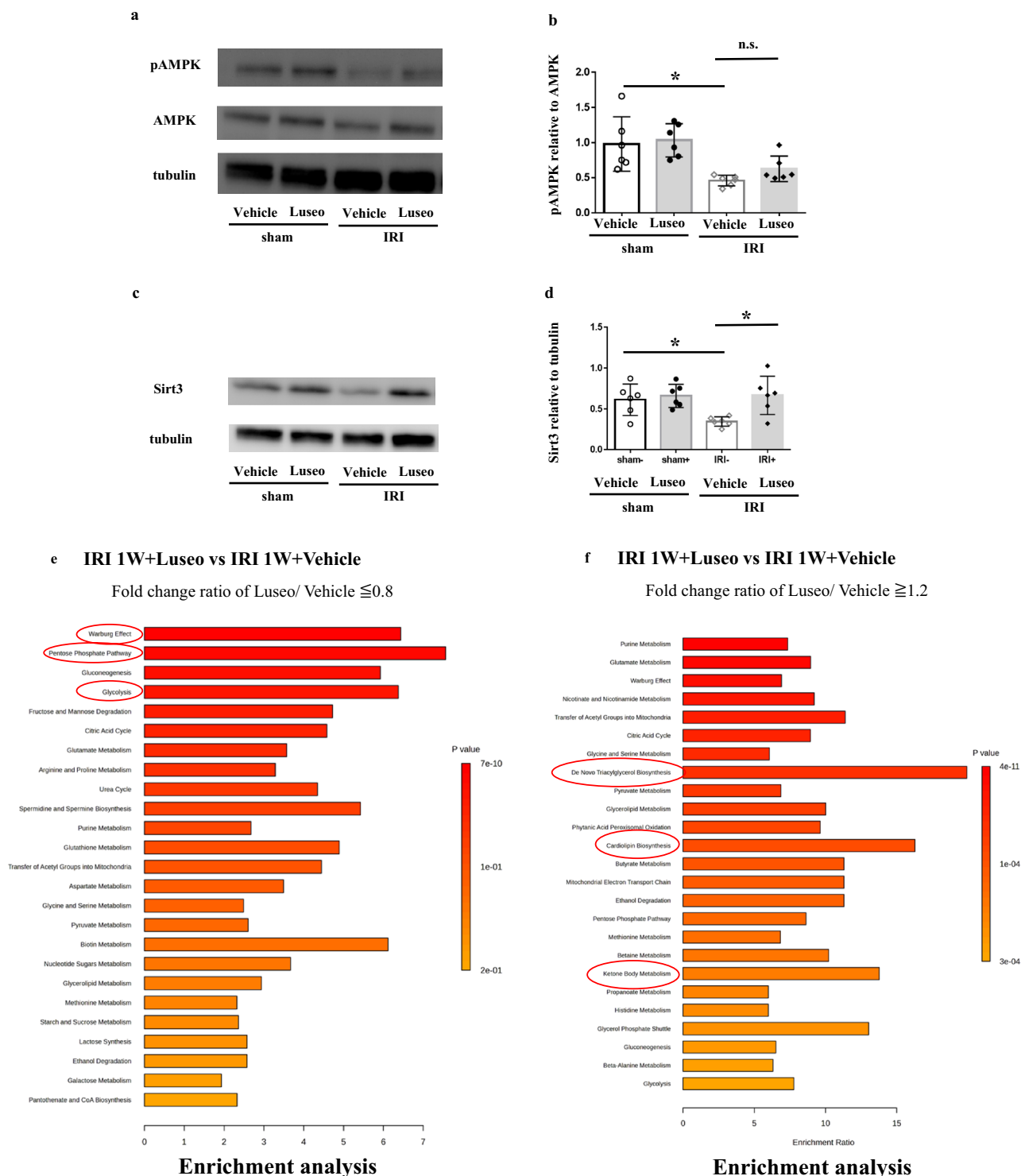


Fig. 3. Luseogliflozin-mediated enhancement of master regulators of starvation and improvement in mitochondrial function, as shown by kidney metabolomic analysis at 1 week post ischemia/reperfusion (I/R) injury. (**a–d**) Western blot analysis and quantification of kidney expression of pAMPK (**a, b**) and Sirt3 (**c, d**) at 1 week post ischemia/reperfusion (I/R) injury in the following groups of mice: vehicle-treated + sham-operated ($n = 6$), luseogliflozin-treated + sham-operated ($n = 6$), vehicle-treated + I/R-injured ($n = 6$), and luseogliflozin-treated + I/R-injured ($n = 6$). (**e** and **f**) Capillary electrophoresis combined with time-of-flight mass spectrometry (CE-TOFMS) and capillary electrophoresis combined with triple quadrupole mass spectrometry (QqQMS) measurements in cation and anion mode were used to generate metabolomics data, which were analyzed using MetaboAnalyst. Of the 116 metabolites identified, enrichment analysis was performed on those with a fold change of ≤ 0.8 (**e**) or ≥ 1.2 (**f**) in the kidneys of luseogliflozin-treated I/R-injured mice ($n = 4$) relative to those in vehicle-treated I/R-injured mice ($n = 4$). * $P < 0.05$, ** $P < 0.01$, as determined by one-way analysis of variance. n.s., not significant.

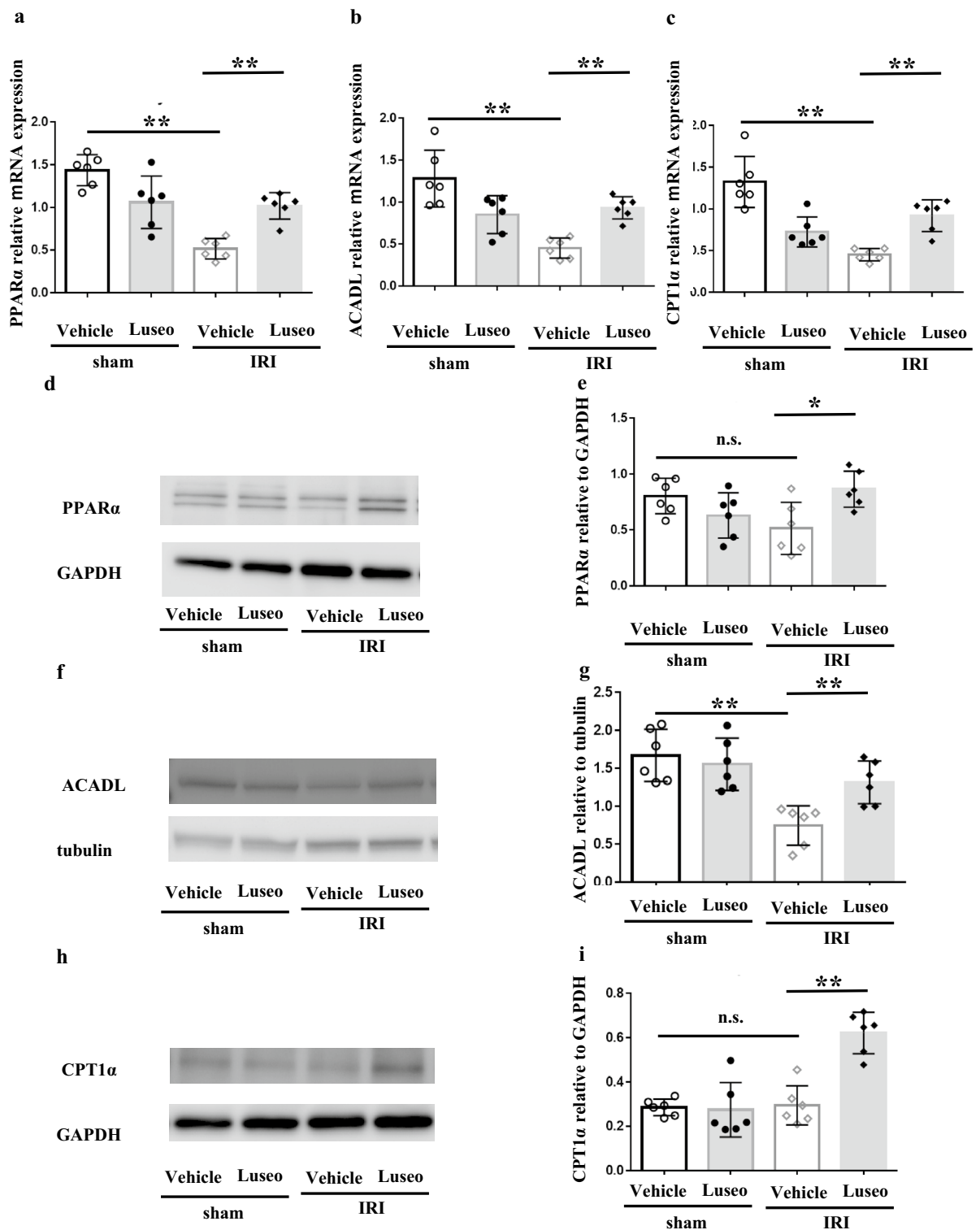


Fig. 4. Luseogliflozin-mediated improvement in dysfunctional fatty acid oxidation at 1 week post ischemia/reperfusion (I/R) injury in mice. (a–c) Quantification of kidney mRNA levels of *PPARα* (a), *ACADL* (b), and *CPT1α* (c) at 1 week post I/R injury. (d–g) Western blot analysis and quantification of kidney protein expression of *PPARα* (d, e), *ACADL* (f, g), and *CPT1α* (h, i) at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 6$), luseogliflozin-treated + sham-operated ($n = 6$), vehicle-treated + I/R-injured ($n = 6$), and luseogliflozin-treated + I/R-injured ($n = 6$). (j and k) Histological findings and quantification Oil red O staining of kidney sections at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 5$), luseogliflozin-treated + sham-operated ($n = 5$), vehicle-treated + I/R-injured ($n = 8$), and luseogliflozin-treated + I/R-injured ($n = 8$); * $P < 0.05$, ** $P < 0.01$, as determined by one-way analysis of variance. n.s., not significant.

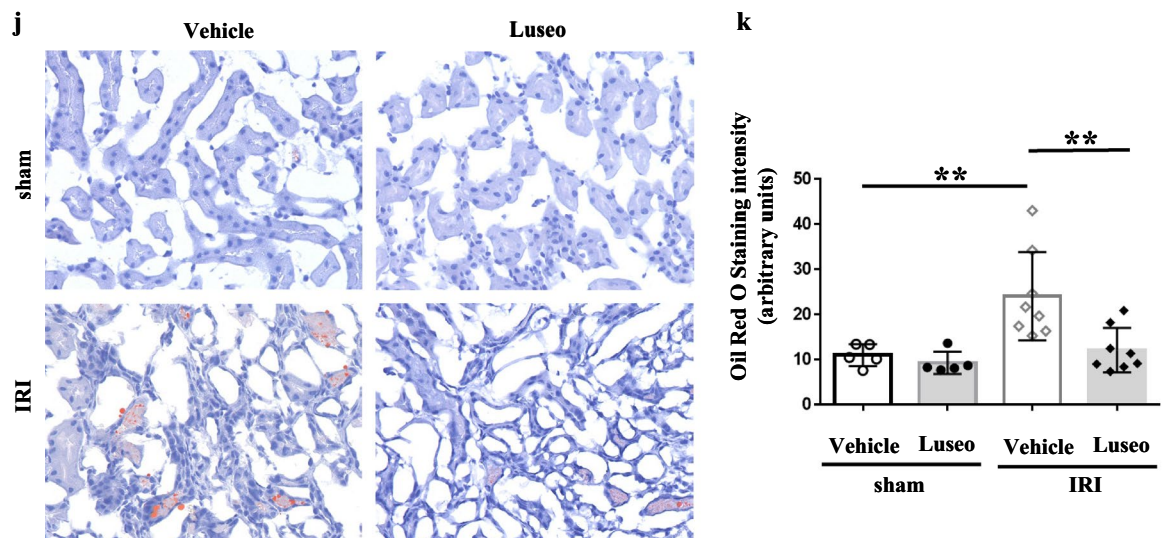


Fig. 4. (continued)

Luseogliflozin-mediated improvement in I/R injury via an anti-ferroptotic effect exerted through the AMPK-Nrf2 pathway

Next, we assessed cell death resulting from oxidative stress. I/R injury increased the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining-positive area in kidneys, but was mitigated in the Luseo group compared to that in the vehicle group (Fig. 7a and b). It has been reported that ferroptosis is closely related to I/R injury¹⁸. Based on our results showing that luseogliflozin treatment increased fatty acid oxidation and decreased oxidative stress in I/R-injured kidneys, we hypothesized that luseogliflozin treatment would reduce ferroptosis. First, we evaluated expression of glutathione peroxidase 4 (Gpx4), an enzyme that plays a crucial role in protecting cells from lipid peroxidation. Western blotting showed that Gpx4 levels were decreased in I/R-injured kidneys, but were restored to the levels found in the vehicle group by luseogliflozin treatment (Fig. 7c and d). Protein expression of transferrin receptor 1 (TfR1), a transmembrane glycoprotein that is involved in cellular uptake of iron and plays an important role in ferroptosis, was increased in I/R-injured kidneys; this effect was mitigated by luseogliflozin treatment (Fig. 7e and f). Immunohistochemistry also showed increased levels of Gpx4 and decreased levels of TfR1 in renal tubules of the Luseo group compared to those in the vehicle group (Supplementary Fig. S3a–d).

Metabolomic analysis of the kidney 1 week post I/R injury showed that luseogliflozin suppressed alpha linolenic acid and linoleic acid metabolism (Supplementary Fig. S4a), and reduced arachidonic acid and adrenic acid levels compared with vehicle (Supplementary Fig. S4b and c). Arachidonic acid and adrenic acid are important polyunsaturated fatty acids (PUFAs) involved in ferroptosis¹⁹; decreased accumulation of PUFAs may contribute to the improvement of ferroptosis. We found that increases in the levels of 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) induced by I/R injury in the kidneys were reduced by luseogliflozin treatment, especially in renal tubules (Fig. 7g–j). In addition, MDA assays revealed that I/R injury increased MDA accumulation in the kidneys, but was mitigated in the Luseo group compared to that in the vehicle group (Fig. 7k).

Discussion

This study demonstrated that luseogliflozin effectively mitigated I/R injury and prevented fibrosis in mice. Luseogliflozin was found to increase Sirt3 and also showed a tendency to increase pAMPK, pivotal energy and stress-response regulators that contribute to reversing the mitochondrial dysfunction and reducing the oxidative stress typically induced by I/R injury. Furthermore, our metabolomic analysis revealed that luseogliflozin inhibited the metabolic shift from fatty acid oxidation to glycolysis commonly observed in I/R injury, thereby decreasing lipid peroxidation and ferroptosis. Figure 8 illustrates the proposed pathways through which luseogliflozin facilitates the transition from AKI to CKD.

Clinical studies, such as the DAPA-CKD trial, have documented the efficacy of SGLT2 inhibitors in enhancing kidney function in non-diabetic patients⁶; however, there are few reports of their impact on AKI⁷. Our results are in line with preliminary research indicating that SGLT2 inhibitors ameliorate I/R injury in mice^{8–10}. Notably, in this study, luseogliflozin improved both AKI and fibrosis in the chronic phase of I/R injury in mice, in contrast to previous findings in which luseogliflozin only ameliorated fibrosis in the chronic phase⁹. These discrepancies may stem from variations in I/R injury models, drug administration methods, or dosing timing. In this study, the degree of improvement of AKI with luseogliflozin was less than the degree of prevention of fibrosis in the chronic phase with luseogliflozin. This finding suggested that luseogliflozin may be more effective in protecting the kidneys during the transition from AKI to CKD than during the acute phase of AKI.

To our knowledge, this is the first study to apply metabolomic analysis to explore how SGLT2 inhibitors influence metabolic pathways in the kidneys of mice with I/R injury. Our analysis indicated that luseogliflozin suppresses the glycolytic system while boosting mitochondrial function and fatty acid oxidation, aligning with

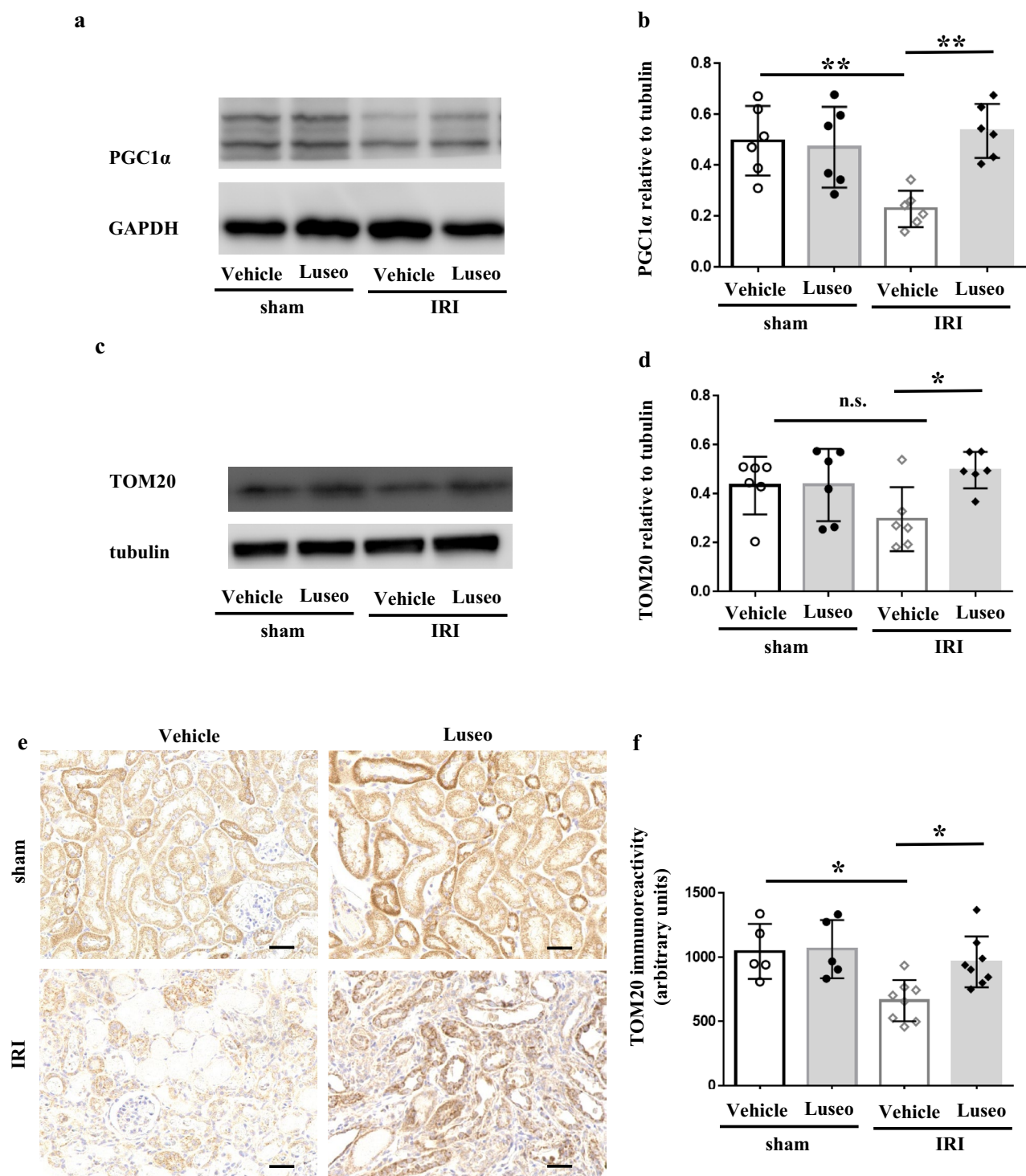


Fig. 5. Luseogliflozin-mediated improvement in mitochondrial function. (a–d) Western blot analysis and quantification of kidney expression of PGC1α (a, b) and TOM20 (c, d) at 1 week post ischemia/reperfusion (I/R) injury in the following groups: vehicle-treated + sham-operated (n = 6), luseogliflozin-treated + sham-operated (n = 6), vehicle-treated + I/R-injured (n = 6), and luseogliflozin-treated + I/R-injured (n = 6). (e and f) Histological findings and quantification of immunostaining for TOM20 in kidney sections at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated (n = 5), luseogliflozin-treated + sham-operated (n = 5), vehicle-treated + I/R-injured (n = 8), and luseogliflozin-treated + I/R-injured (n = 8); * $P < 0.05$, ** $P < 0.01$, as determined by one-way analysis of variance. n.s., not significant.

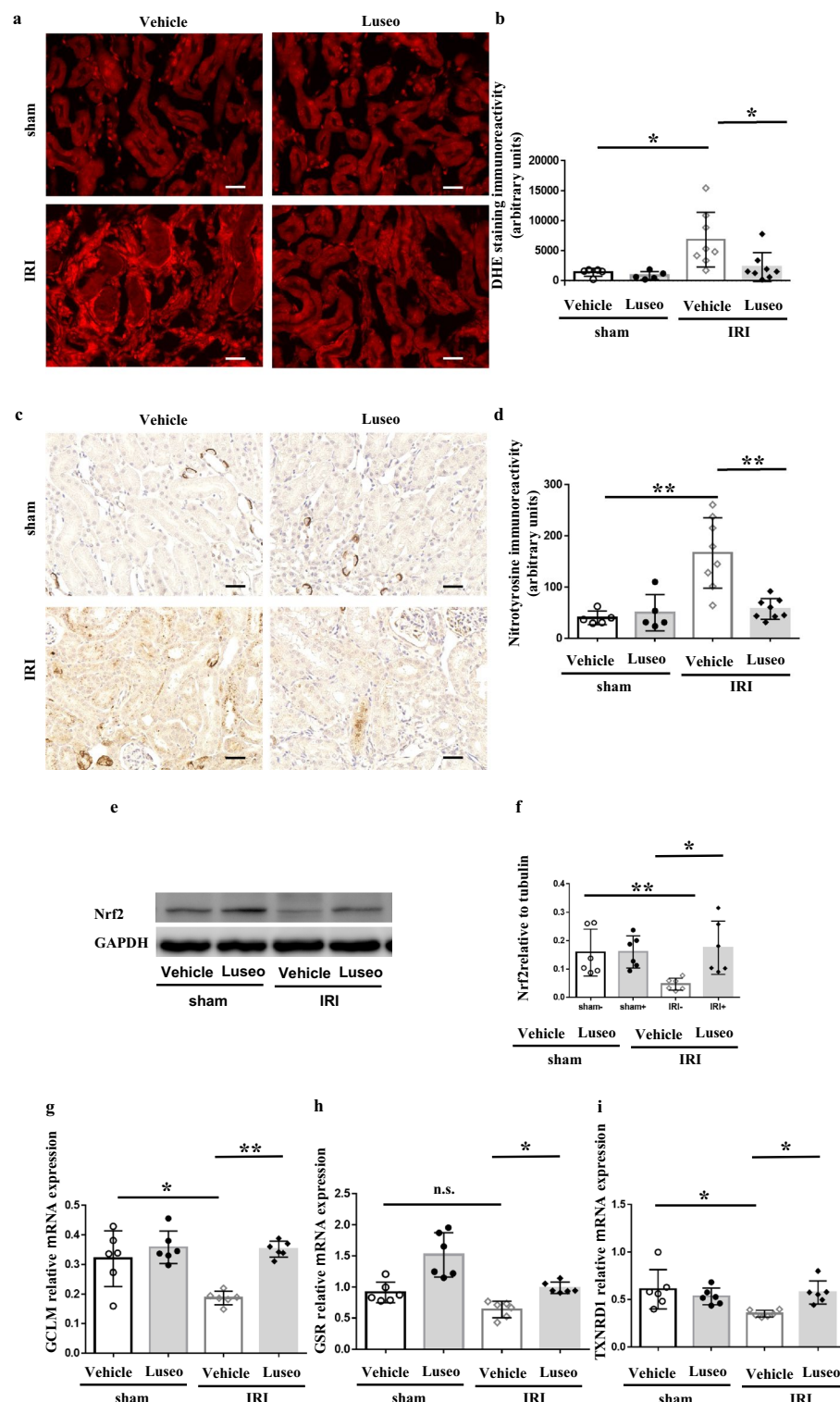


Fig. 6. Luseogliflozin-mediated amelioration of oxidative stress and enhancement of Nrf2, the master regulator of anti-oxidative responses. **(a and b)** Histological findings and quantification of reactive oxygen species in the kidney with dihydroethidium (DHE) staining at 1 week post ischemia/reperfusion (I/R) injury. **(c and d)** Histological findings and quantification of immunostaining for nitrotyrosine in the kidney at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated (n = 5), luseogliflozin-treated + sham-operated (n = 5), vehicle-treated + I/R-injured (n = 8), and luseogliflozin-treated + I/R-injured (n = 8). **(e and f)** Western blot analysis and quantification of kidney expression of Nrf2 at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated (n = 6), luseogliflozin-treated + sham-operated (n = 6), vehicle-treated + I/R-injured (n = 6), and luseogliflozin-treated + I/R-injured (n = 6). **(g–i)** Quantification of mRNA levels of GCLM **(g)**, GSR **(h)**, and TXNRD1 **(i)** in the kidney at 1 week post I/R injury; * $P < 0.05$, ** $P < 0.01$, as determined by one-way analysis of variance. n.s., not significant.

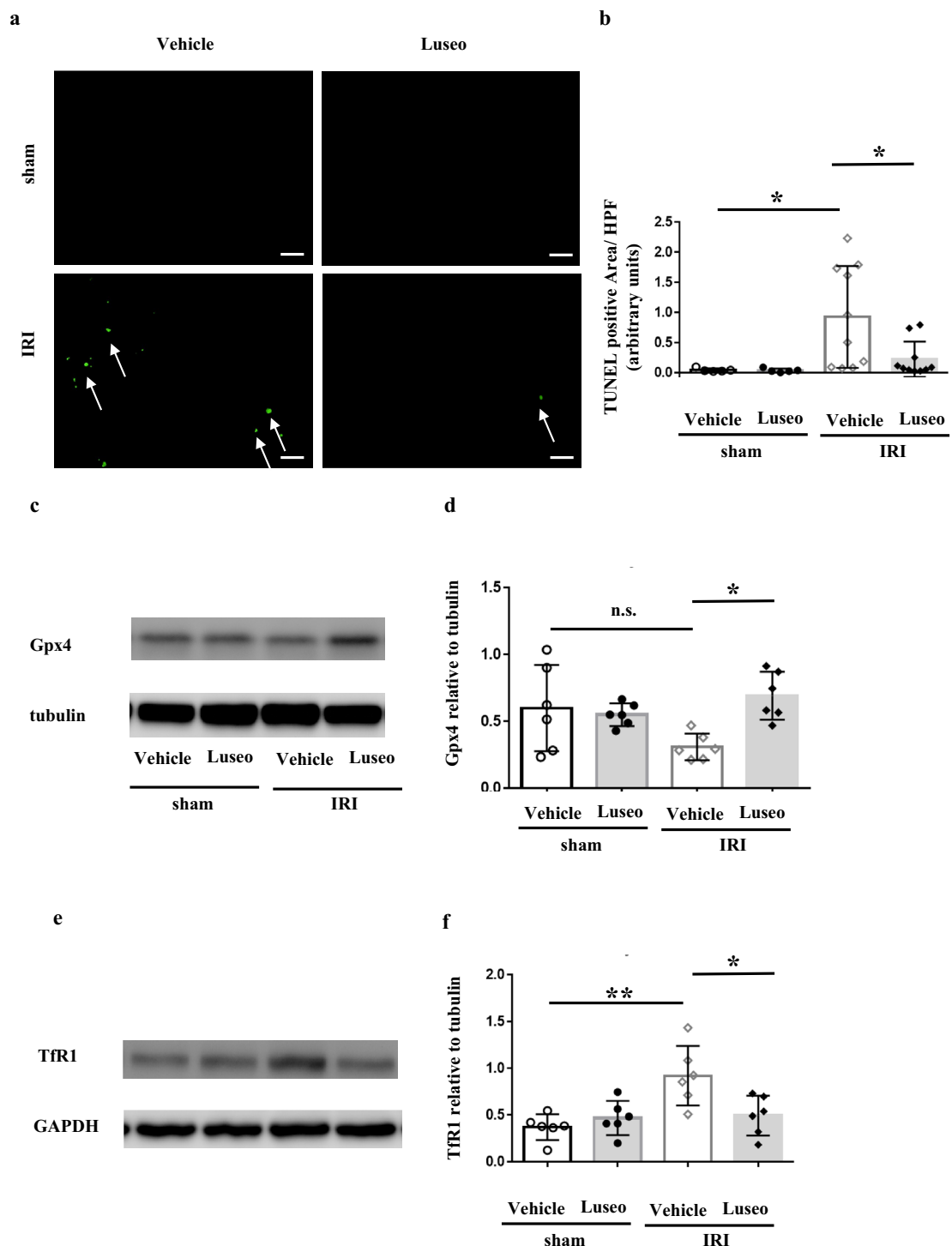


Fig. 7. Luseogliflozin-mediated inhibition of ferroptosis and amelioration of ischemia/reperfusion (I/R) injury. (**a** and **b**) Histological findings and positive area of TUNEL assay staining of the kidney at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 10$), luseogliflozin-treated + sham-operated ($n = 10$), vehicle-treated + I/R-injured ($n = 5$), and luseogliflozin-treated + I/R-injured ($n = 5$). (**c–f**) Western blot analysis and quantification of kidney expression of Gpx4 (**c, d**) and TfR1 (**e, f**) at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 6$), luseogliflozin-treated + sham-operated ($n = 6$), vehicle-treated + I/R-injured ($n = 6$), and luseogliflozin-treated + I/R-injured ($n = 6$). (**g–j**) Histological findings and quantification of kidney immunostaining for 4-hydroxynonenal (4-HNE; **g, h**) and malondialdehyde (MDA; **i, j**) at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 8$), luseogliflozin-treated + sham-operated ($n = 8$), vehicle-treated + I/R-injured ($n = 5$), and luseogliflozin-treated + I/R-injured ($n = 5$). (**k**) MDA concentrations in the kidney at 1 week post I/R injury in the following groups: vehicle-treated + sham-operated ($n = 8$), luseogliflozin-treated + sham-operated ($n = 8$), vehicle-treated + I/R-injured ($n = 5$), and luseogliflozin-treated + I/R-injured ($n = 5$); * $P < 0.05$, ** $P < 0.01$, as determined by one-way analysis of variance. n.s., not significant.

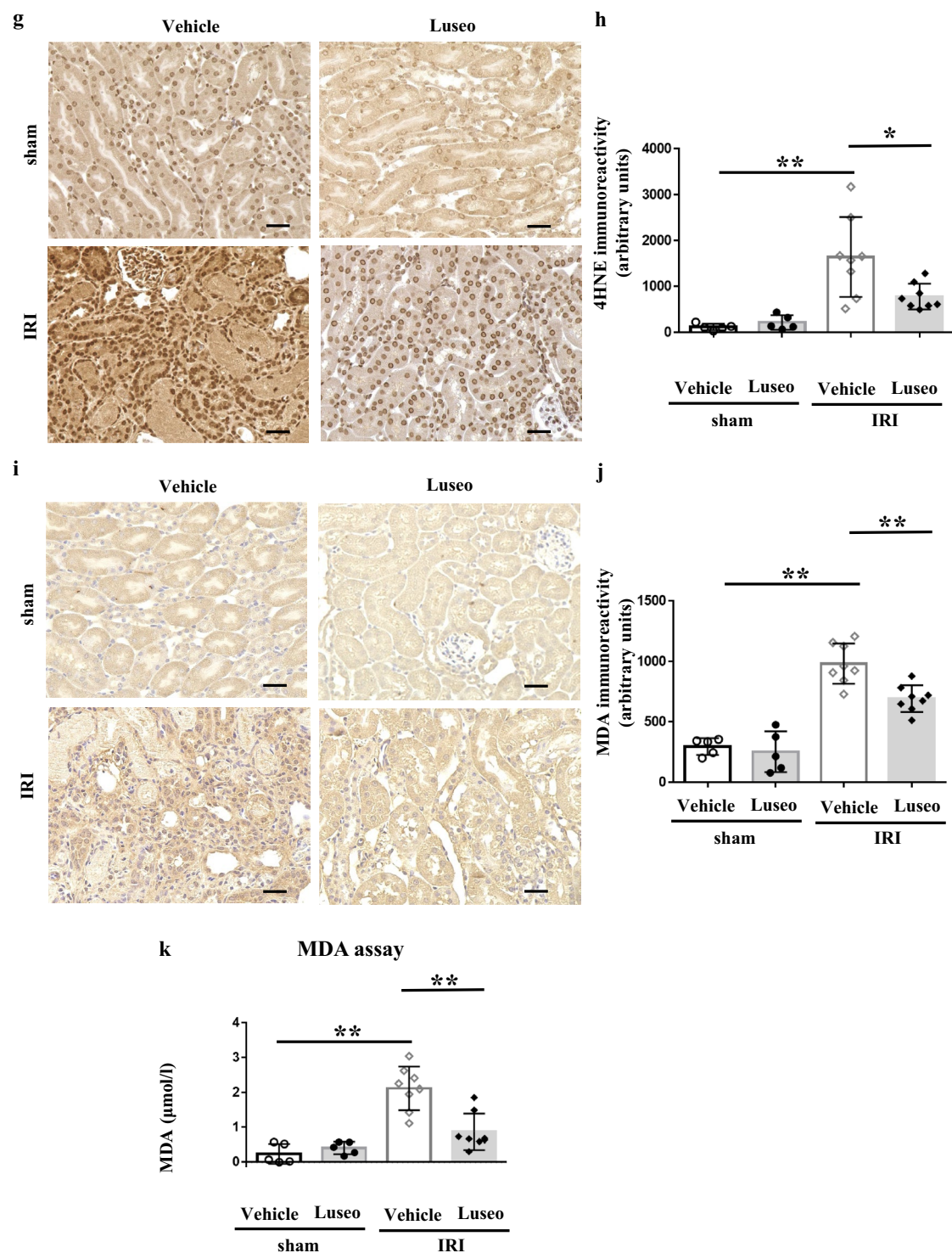


Fig. 7. (continued)

previous studies of SGLT2 inhibitors in murine models of cardiac hypertrophy¹⁹. Building on research involving diabetes models^{12,13}, We demonstrated that luseogliflozin elevated the levels of Sirt3 and showed a tendency to increase PAMPK, enhancing mitochondrial function and fatty acid oxidation.

Previous research has highlighted that SGLT2 inhibitors reduce apoptosis associated with I/R injury^{8,10}. However, it is now recognized that ferroptosis also plays a significant role in cell death during I/R injury¹⁸. Our focus on ferroptosis revealed that luseogliflozin treatment reduced TUNEL-positive cells, increased Gpx4 levels, and decreased the levels of Tfr1, along with 4-HNE and MDA, markers of lipid peroxidation, indicating suppression of ferroptosis. This effect on ferroptosis likely resulted from reduced lipid peroxidation due to diminished oxidative stress and decreased PUFA accumulation, given that increased fatty acid oxidation reportedly inhibits

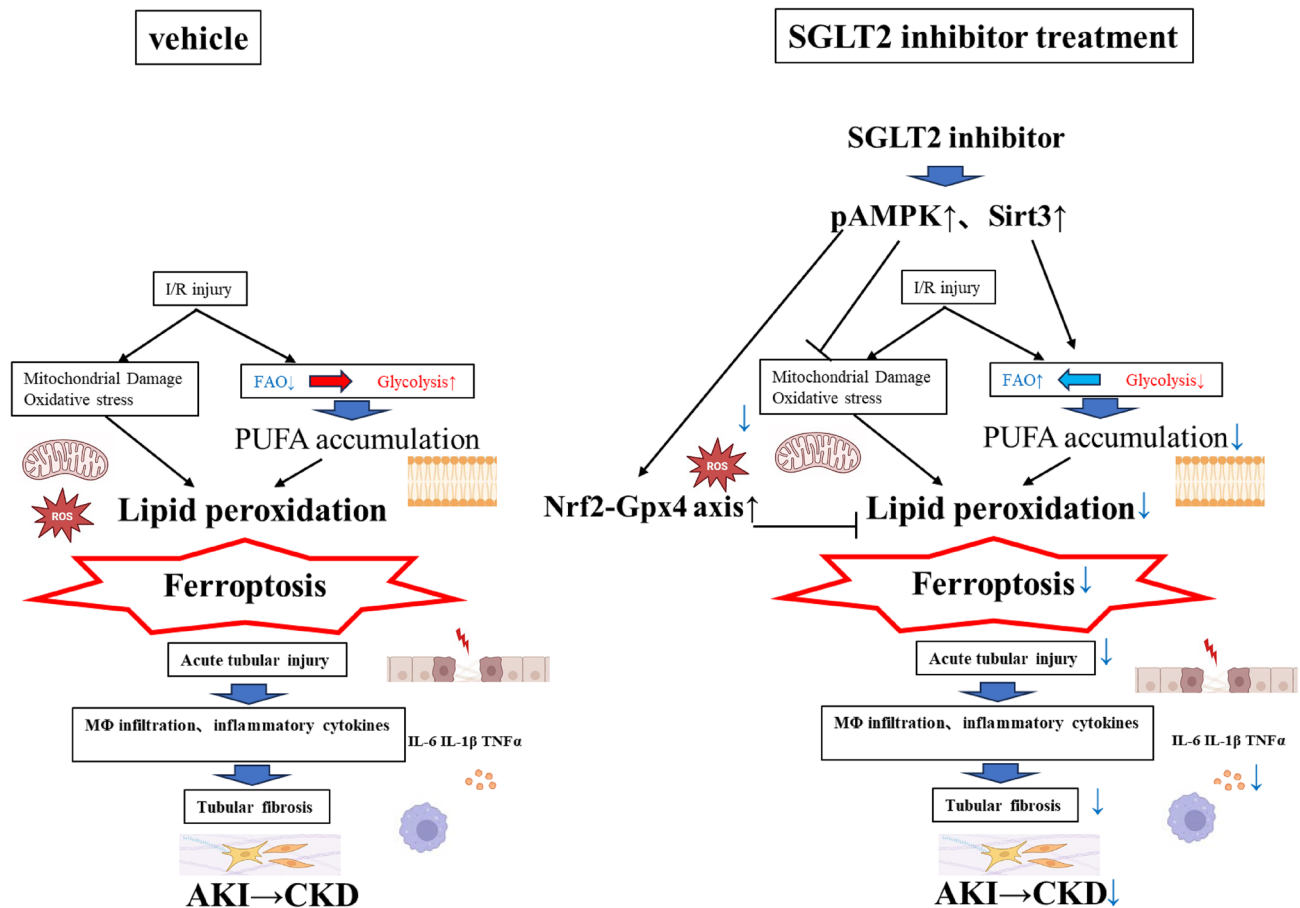


Fig. 8. Schematic diagram of the therapeutic effect of luseogliflozin, showing how it effectively reduces I/R injury and prevents fibrosis in mice. Under normal conditions, I/R injury reduces tubular mitochondrial function and increases oxidative stress. In addition, tubular metabolism shifts from fatty acid oxidation to glycolysis, resulting in accumulation of polyunsaturated fatty acids (PUFAs) and leading to lipid peroxidation and tubular ferroptosis. Next, macrophage infiltration and inflammatory cytokine production occur, leading to tubular fibrosis and the transition from acute kidney injury (AKI) to chronic kidney disease (CKD). Sodium-glucose cotransporter 2 (SGLT2) inhibitors increase the starvation regulator Sirtuin-3 (Sirt3) and also show a tendency to increase pAMPK. Mitochondrial function is improved, oxidative stress is lowered, and PUFA accumulation is prevented by inhibiting the tubular metabolic shift from fatty acid oxidation to the glycolytic system. Consequently, ferroptosis and development of the AKI to CKD transition are prevented.

PUFA accumulation and suppresses ferroptosis^{20–23}. Our metabolomic analysis further showed decreased levels of arachidonic and adrenic acids, PUFAs implicated in ferroptosis¹⁹.

A major limitation of this study was the inability to replicate our *in vivo* findings *in vitro*. Previous *in vitro* studies have shown the efficacy of SGLT2 inhibitors in tubular cells, mesangial cells, and pericytes^{8,24–27}. Nevertheless, we observed no effect of luseogliflozin administration in these cell types under various hypoxic conditions. This discrepancy between *in vitro* and *in vivo* effectiveness might be related to hemodynamic effects or other *in vitro* limitations.

Overall, our findings suggested that luseogliflozin strongly facilitates the management of the transition from AKI to CKD by reducing ferroptosis, offering a promising approach for renal protection.

Methods

Animal experiments and ethics statement

We conducted all animal experiments in accordance with the animal use protocol approved by the Ethics Committee for Animal Experiments of the Graduate School of Medicine, Kyushu University. The approval number is A22-094-0. All experiments were performed in accordance with relevant named guidelines and regulations. All authors complied with the ARRIVE guidelines.

We purchased C57BL/6J mice from CLEA Japan Inc. (Tokyo, Japan), originally sourced from The Jackson Laboratory. Mice were kept in an air-conditioned, specific pathogen-free room at 21 °C and 65% humidity, with a 12:12 h light:dark cycle (lights on at 8 am and off at 8 pm) and free access to food and water. Experiments were reported in accordance with the ARRIVE guidelines.

Experimental procedures for bilateral ischemic AKI in mice

Ischemic AKI was induced in 12-week-old male mice by bilateral renal pedicle clamping, as described previously²⁸. Briefly, mice were anesthetized intraperitoneally with medetomidine hydrochloride (0.3 mg/kg body weight [BW]; Wako, Osaka, Japan), midazolam (4 mg/kg BW; Sandoz, Tokyo, Japan), and butorphanol tartrate (5 mg/kg BW; Wako). Renal pedicles were exposed by flank incisions, and the bilateral renal arteries and veins were exposed through flank incision and then clamped with stainless steel cerebral microaneurysm clips (No. 05, Muromachi, Tokyo, Japan) for 26 min, then released. Mice were randomly assigned to two groups: a control (vehicle) group maintained on a normal rodent diet without drug administration, and a treatment (Luseo) group that received luseoglofloxin (0.01% in chow, 15 mg/kg BW/day) in a normal rodent diet for 2 weeks prior to I/R until sacrifice.

Sample collection

Mice were euthanized by intraperitoneal injection of medetomidine hydrochloride (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg) at 48 h or 1 week post-reperfusion. Blood samples were collected from the inferior vena cava and centrifuged at 3000 × g for 10 min to separate serum for BUN and Cr measurements. After blood collection, 50 mL of ice-cold phosphate buffered saline (PBS, pH 7.4) was perfused, and both kidneys were harvested. One kidney was fixed in 4% formaldehyde in PBS for histological analysis, while the other was frozen in liquid nitrogen for protein and RNA analyses.

ATN scoring, Masson's trichrome and Sirius red staining

Mouse kidneys were fixed in 4% neutral-buffered formaldehyde for 24 h, then immersed in 20% and 30% sucrose for 24 h each. The kidneys were sectioned vertically, with one half embedded in paraffin and the other in OCT compound for frozen sections. Paraffin sections (4 µm) were used for hematoxylin-eosin, Masson's trichrome, and Sirius red staining, following standard methods and a previous report⁹. ATN scoring was based on the outer medulla, assessing tubule necrosis, loss of brush border, cast formation, and dilation: 0 = none; 1 = < 10%; 2 = 11–25%; 3 = 26–45%; 4 = 46–75%; 5 = ≥ 76%. Ten fields per slide were evaluated. For Masson's trichrome and Sirius red staining, 10 fields were randomly selected, and images analyzed with ImageJ software, expressed as mean intensity. Histological images were captured using an Olympus BX53 microscope in a blinded manner.

Immunohistochemistry

Kidney tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 4-µm-thick sections. Immunohistochemistry of KIM1, αSMA, fibronectin, F4/80, nitrotyrosine, 4HNE, MDA, TfR1, and Gpx4 (used for 3,3'-diaminobenzidine [DAB] staining) was examined using the following primary antibodies: polyclonal anti-rabbit KIM1 (1:200 dilution, AF1817; R&D Systems, Minneapolis, MN, USA), monoclonal anti-mouse αSMA (1:200, ab7817; Abcam, Cambridge, United Kingdom), monoclonal anti-rabbit fibronectin (1:200, 610078; BD Biosciences, Franklin Lakes, NJ, USA), monoclonal anti-rabbit F4/80 (1:200, 30325s; Cell Signaling Technology, Danvers, MA, USA), monoclonal anti-mouse CPT1α (1:200, ab128568; Abcam, Cambridge, United Kingdom), polyclonal anti-rabbit nitrotyrosine (1:200, 06-284; Sigma Aldrich, St. Louis, MO, USA), monoclonal anti-rabbit 4HNE (1:200, ab48506; Abcam, Cambridge, United Kingdom), polyclonal anti-rabbit MDA (1:200, ab27642; Abcam, Cambridge, United Kingdom), polyclonal anti-rabbit TfR1 (1:200, sc-32272; Santa Cruz Biotechnology, Dallas, TX, USA), and monoclonal anti-rabbit Gpx4 (1:200, 67763-1-Ig; Proteintech, Rosemont, IL, USA). DAB staining was performed as described in a previous report⁹. The slides were incubated overnight at 4 °C with primary antibody. For DAB color development, an avidin-biotin-horseradish peroxidase detection system (Elite ABC; Vector Laboratories, Burlingame, CA) was utilized. After counterstaining with hematoxylin, the slides were scanned using an Aperio ScanScope AT Turbo (Leica Biosystems, Wetzlar, Germany). Ten fields were randomly selected and images were analyzed with ImageJ software (NIH), expressed as the mean intensity of the positively stained area on the slide. Histological images were captured by an Olympus BX53 microscope in a blinded manner.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from mouse kidney tissues using a MAXWELL®16 LEV simplyRNA Tissue Kit (Promega, Madison, WI, USA). A Promega MAXWELL®16 instrument (Takara Bio Inc., Shiga, Japan) was used to synthesize complementary DNA from 1 µg of total RNA. Real-time PCR was performed using SYBR Premix Ex Taq™ (Takara Bio Inc.) and an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). The mouse 18S rRNA gene (*Rn18s*) was used as an internal control. Expression analysis was performed using the ΔΔCt method with 7500 Software v2.3 (Applied Biosystems). Primers used for PCR reactions are shown in Supplementary Table S1.

Western blot analysis

Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), and samples were adjusted to the same volume. Samples (10 µg) were separated on 5–20% sodium dodecyl sulfate-polyacrylamide gels (#2331830; Atto, Tokyo, Japan) and transferred to polyvinylidene fluoride membranes (BioRad, Hercules, CA, USA) using a Trans-Blot Turbo (BioRad). After blocking the membrane for 30 min at room temperature using Blocking One (#03953-95; Nacalai Tesuque Corporation, Kyoto, Japan), the membrane was washed three times with Tris-buffered saline (TBS-T), soaked in primary antibody, and incubated overnight at 4 °C. The following primary antibodies were used: monoclonal anti-rabbit α/β-tubulin (1:5000, CST#2148; Cell Signaling Technology, Danvers, MA, USA), monoclonal anti-mouse GAPDH

(1:5000, AB8245; Abcam, Cambridge, United Kingdom), monoclonal anti-mouse α SMA (1:1000, ab7817; Abcam, Cambridge, United Kingdom), monoclonal anti-rabbit collagen I, (1:1000, ab21286; Abcam, Cambridge, United Kingdom), monoclonal anti-rabbit fibronectin, (1:1000, ab2413; Abcam, Cambridge, United Kingdom), polyclonal anti-rabbit PPAR α (1:1000, sc-9000; Santa Cruz Biotechnology, Dallas, TX, USA), polyclonal anti-rabbit ACADL (1:1000, 17526-1-AP; Proteintech, Rosemont, IL, USA), monoclonal anti-mouse CPT1 α (1:1000, ab128568; Abcam, Cambridge, United Kingdom), polyclonal anti-rabbit pAMPK α (1:1000, #2531; Cell Signaling Technology, Danvers, MA, USA), monoclonal anti-rabbit SIRT3 (1:1000, #5490S; Cell Signaling Technology, Danvers, MA, USA), polyclonal anti-rabbit PGC1 α (1:1000, NOVUSBIO, Centennial, CO, USA) monoclonal anti-rabbit TOM20 (1:1000, #42406; Cell Signaling Technology, Danvers, MA, USA), polyclonal anti-rabbit Nrf2 (1:1000, ab137550; Abcam, Cambridge, United Kingdom), polyclonal anti-rabbit GCLM (1:1000, 14241-1-AP; Proteintech, Rosemont, IL, USA), polyclonal anti-rabbit NQO1 (1:1000, 11451-1-AP; Proteintech, Rosemont, IL, USA), polyclonal anti-rabbit TXNRD1 (1:1000, 11117-1-AP; Proteintech, Rosemont, IL, USA), polyclonal anti-rabbit TfR1 (1:1000, sc-32272; Santa Cruz Biotechnology, Dallas, TX, USA), and monoclonal anti-rabbit Gpx4 (1:1000, 67763-1-Ig; Proteintech, Rosemont, IL, USA). Membranes were washed three times in TBS-T and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies: anti-rabbit IgG (1:5000, NA934, GE Healthcare, Bucks, UK) and anti-mouse IgG (1:5000, NA931V, GE Healthcare, Bucks, UK). Bands were detected using an enhanced chemiluminescence method (SuperSignal™ West Femto Maximum Sensitivity Substrate; Pierce Thermo Scientific, Rockford, IL, USA), captured using a chemiluminescence imaging system (AE-9300 Ez-capture MG; Atto, Tokyo, Japan), and analyzed using ImageJ software (NIH).

Metabolomic analysis

Metabolomic analysis of kidneys from the vehicle and Luseo groups was performed by Human Metabolome Technologies, Inc. at 1 week post-I/R injury ($n = 4$ mice per group). For CE-TOFMS and CE-QqQMS measurements, kidney samples were crushed with 600 μ L of 50% aqueous acetonitrile solution, centrifuged, and ultrafiltered. The filtrate was dried and dissolved in Milli-Q water for measurement. For LC-TOFMS measurements, samples were crushed with 600 μ L of 1% formic acid-acetonitrile solution, mixed with 200 μ L Milli-Q water, and centrifuged. The supernatant was filtered to remove phospholipids, dried, dissolved in 50% isopropanol, and used for the assay.

TUNEL assay

Kidney apoptosis was detected by TUNEL assays performed in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Kidney tissue samples were mounted in VECTASHIELD Mounting Medium. Images were taken with an Olympus BX53 microscope. Ten fields were randomly selected, and images were analyzed with ImageJ software (NIH), expressed as the mean intensity of the positively stained area on the slide.

DHE staining

To detect intracellular ROS, we performed DHE staining. Hydroethidine dissolved in PBS to synthesize DHE (200 μ M) was incubated with frozen tissue samples at 37 °C for 1 h, shielded from light. The tissue was washed with PBS, then mounted in VECTASHIELD Mounting Medium. Ten fields were randomly selected and images were analyzed with ImageJ software (NIH), expressed as the mean intensity of the positively stained area on the slide.

MDA assay

A 20 mg sample of kidney tissue from each mouse was used for MDA assays of lipid peroxidation. Fluorescence intensity was measured at an excitation wavelength of 540 nm and an emission wavelength of 590 nm, in accordance with the manual provided with the MDA assay kit (Dojindo, Kumamoto, Japan).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0. The significance of differences between two groups was assessed using parametric unpaired two-tailed t tests for normally distributed data or nonparametric two-tailed Mann–Whitney tests for non-normally distributed data. For multiple comparisons, we used two-way analysis of variance followed by Tukey's post hoc test. (* $P < 0.05$; ** $P < 0.01$)

Materials

Luseogliflozin was provided by Taisho Pharmaceutical Co., Ltd. (Tokyo, Japan).

Data availability

The data supporting the findings of this study are included within the article and Supplementary Information. Additionally, further supporting data are available at FigShare (<https://doi.org/10.6084/m9.figshare.25763916>, <https://doi.org/10.6084/m9.figshare.25763283>) and can be provided upon reasonable request.

Received: 5 June 2024; Accepted: 27 August 2024

Published online: 02 September 2024

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Acknowledgements

We thank the Research Support Center for Human Disease Models, Graduate School of Medicine, Kyushu University, for technical assistance. We thank Hiroshi Fujii, Department of Pathophysiology and Experimental Pathophysiology, Graduate School of Medicine, Kyushu University for assisting with the preparation of histological samples, and Masaki Munakata and Mayumi Tanaka, Department of Pathophysiology and Experimental Pathology, Graduate School of Medicine, Kyushu University for assisting with the preparation of histological samples. We also thank Simon Teteris, PhD, Jodi Smith, PhD, and Michelle Kahmeyer-Gabbe, PhD, from Edanz (<https://jp.edanz.com/ac>) for editing a draft of this manuscript.

Author contributions

Y.H. and T.N. developed the study concept. Y.H. and S.A. collected the data. Y.H. analyzed and interpreted the data, conducted the statistical analysis, and wrote the initial draft of the manuscript. M.W. and T.K. contributed intellectual content to the work. K.T., T.N., and K.T. supervised the study, revised the manuscript, and secured research funding. All authors reviewed and approved the final manuscript.

Funding

This work received the research grant by Taisho Pharmaceutical Co., the manufacturer of luseogliflozin.

Competing interests

This work received the research grant by Taisho Pharmaceutical Co., the manufacturer of luseogliflozin. The funding entity did not participate in the study's design, data gathering and analysis, decision to publish, or manuscript preparation, with the sole exception of supplying pharmacokinetic data for luseogliflozin. All the authors declare no conflict of interest.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-71416-0>.

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