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MYO-INOSITOL OXYGENASE EXPRESSION PROFILE MODULATES PATHOGENIC FERROPTOSIS IN THE RENAL PROXIMAL TUBULE

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ABSTRACT

Overexpression of myo-inositol oxygenase (MIOX), a proximal tubular enzyme, exacerbates cellular redox injury in acute kidney injury (AKI). Ferroptosis, a newly coined term associated with lipid hydroperoxidation, plays a critical role in the pathogenesis of AKI. Whether or not MIOX exacerbates tubular damage by accelerating ferroptosis in Cisplatin-induced AKI remains elusive. Cisplatin-treated HK-2 cells exhibited notable cell death, which was reduced by ferroptosis inhibitors. Also, alterations in various ferroptosis metabolic sensors, including lipid hydroperoxidation, glutathione peroxidase 4 (GPX4) activity, NADPH and reduced glutathione (GSH) levels, and ferritinophagy, were observed. These perturbations were accentuated by MIOX overexpression, while ameliorated by MIOX knockdown. Likewise, Cisplatin-treated CD1 mice exhibited tubular damage and derangement of renal physiological parameters, which was alleviated by Ferrostatin-1 (Fer-1), a ferroptosis inhibitor. To investigate the relevance of MIOX to ferroptosis, Wild-type (WT) mice, MIOX-overexpressing transgenic (MIOX-TG) mice and MIOX knockout (MIOX-KO) mice were subjected to Cisplatin treatment. In comparison to Cisplatin-treated WT mice, Cisplatin-treated MIOX-TG mice had more severe renal pathological changes and perturbations in ferroptosis metabolic sensors, which were minimal in Cisplatin-treated MIOX-KO mice. In conclusion, these findings indicate that ferroptosis, an integral process in the pathogenesis of Cisplatin-induced AKI, is modulated by the expression profile of MIOX.
INTRODUCTION

Acute kidney injury (AKI) is characterized by a rapid deterioration of renal functions, and it presents as one of the major causes of morbidity in hospitalized patients (1, 2). It is estimated that more than 2 million deaths a year are related to AKI around the world. Also, in United States patients with AKI are a great burden to the health care system in terms of management and dollar cost (3, 4). Those who survived from AKI have a high risk of progressing to chronic kidney disease (CKD) or even end-stage renal disease (ESRD) (5, 6). Clinically, AKI can occur in a wide variety of clinico-pathologic states, including ischemia-reperfusion injury (IRI), sepsis and the administration of various nephrotoxic agents (7). The pathophysiology of AKI is complex, and it involves various cell types, multiple factors and signaling events related to regulated cells death (7). Morphologically, AKI is characterized by acute tubular necrosis (ATN), renal inflammation, glomerular injury and vascular dysfunction (7). One of the agents that commonly induces AKI is Cisplatin, a nephrotoxic agent that is widely used in the treatment of patients with various forms of neoplasms. Cisplatin-induced nephropathy has been extensively investigated in various animal models to explore the pathogenesis of AKI. Previous studies have shown that Cisplatin is reabsorbed from the glomerular ultrafiltrate in proximal tubules, and it leads to severe ATN due to the DNA damage, cellular redox imbalance and mitochondrial dysfunctions (7, 8). Recently, Cisplatin was noted to induce cell death of cancer cells via different processes, including ferroptosis (9).

In 2012, a new term for regulated cell death was coined, i.e., ferroptosis, and it is characterized by intracellular accumulation of lipid hydroperoxides and overload of free iron.
The process of ferroptosis is independent of molecular pathways that involve caspase and necrosomal complex, while it can be terminated by glutathione peroxidase 4 (GPX4) (9). It has been reported that ferroptosis is incriminated in the pathogenesis of many diseases, such as Huntington’s disease, Parkinson’s disease, hemochromatosis, urinary tract infections and cell death of neoplastic cells following chemotherapy (9 - 11). Due to the abundance of polyunsaturated fatty acids (PUFA) in renal tubular cells (8), it is conceivable that the kidney is readily susceptible to lipid hydroperoxides-induced ferroptosis. In fact, it has been shown that the process of ferroptosis is more relevant than necroptosis and apoptosis in IRI-induce ATN, and it is also the primary cause of folic acid-induce AKI (9, 12), but its role in Cisplatin-induced AKI remains to be explored.

Myo-inositol oxygenase (MIOX), a renal proximal tubular specific enzyme, was identified several years ago (13). It catabolizes myo-inositol to D-glucuronate, and it plays an important role in renal tubular injury (14). The MIOX promoter includes osmotic response elements (OsREs), carbohydrate response elements (ChREs), oxidant response elements (OxREs) and sterol response elements (SREs), and thus MIOX transcription is modulated by oxidant stress, high glucose ambience and the administration of free fatty acids (8, 14, 15). Besides, epigenetic studies have revealed that MIOX expression is dependent on demethylation of MIOX promoter in diabetic state or Cisplatin-induced AKI (8, 14). The up-regulated MIOX expression also promotes the generation of reactive oxygen species (ROS) and thus it conceivably exacerbates renal tubular injury in a variety of pathological states. However, its relevance in the process of ferroptosis remains to be investigated. Our exploratory studies on kidney biopsy tissues of patients with ATN revealed an increased expression of MIOX in
the proximal tubular epithelium (Figures 1C & 1D vs 1A & 1B). Concomitantly, there was a decreased expression of ferritinophagy biomarkers, *i.e.*, nuclear receptor co-activator 4 (NCOA4) and heavy chain of ferritin (FTH1), in the renal tubular epithelia of these patients (Figures 1G & 1H vs 1E & 1F, and 1K & 1L vs 1I & 1J). Because of these changes in the temporal expression of MIOX and ferritinophagy markers in the renal tubular compartment, it led us to investigate the pathobiology of MIOX and ferroptosis in Cisplatin-induced AKI.
RESULTS

The observations described below suggest that one of the important mechanism leading to AKI is mediated by a recently described pathobiological process, known as ferroptosis, besides apoptosis and necroptosis. In this process, a multitude of signaling molecules and factors may be involved that ultimately lead to cell death, for which ferroptosis seems to be an integral part as alluded below in the Cisplatin-induced AKI model.

*Ferroptosis is an essential part in Cisplatin-induced HK-2 cell death:* Normally, the HK-2 cells yielded a flat epithelial morphology in cultures maintained for 48 hours, as indicated by phase contrast microscopy and "direct visualization" of H & E staining (Figures 2A, & 2D, red arrow and arrowhead). Cisplatin treatment led to contraction or shrinkage of HK-2 cells (Figures 2B & 2E, yellow arrows and arrowheads). While co-treatment of Cisplatin and Ferrostatin-1 (Fer-1, inhibitor of ferroptosis) partially reverted the morphological changes (Figures 2C & 2F, blue arrow and arrowhead). TUNEL (terminal deoxynucleotidyl transferase mediated X-dUTP nick labeling) staining revealed notable DNA damage in Cisplatin-treated HK-2 cells compared to the control cells (Figure 2H vs 2G). The co-treatment with Fer-1 remarkably reduced the degree of cell death (Figure 2I). Cell viability, as judged by MTT assay, was substantially decreased with the Cisplatin treatment, which could be rescued by the concomitant treatment with Fer-1 (Figure 2J). Interestingly, deferoxamine (DFO, an iron chelator) and Z-VAD(OMe)-FMK (VAD, an apoptosis inhibitor) treatment also led to an increased survival of the cells (Figures 2J & 2K). However, treatment with Nec-1 (a necroptosis inhibitor) did not restore the viability of cells treated with Cisplatin (Figure 2J),
suggesting that among these three processes, ferroptosis and apoptosis, but not necroptosis, are important modulators in Cisplatin-induced HK-2 cell death. To emphasize the role of ferroptosis, we proceeded to investigate the intracellular dynamics of iron, \textit{i.e.}, ferrum and iron binding protein, \textit{i.e.}, heavy chain of ferritin (FTH1). Normally, FTH1 (FITC-conjugated antibody) was found diffusely distributed in the cytoplasm and minimally localized in the lysosomes (lysotracker red), as evidenced by fluorescent microscopy (\textbf{Figures 2L} – \textbf{2N}). While FTH1 was seen abundantly co-localized in the lysosomes following Cisplatin treatment (\textbf{Figures 2O} – \textbf{2Q}), which could be readily appreciated in the merged photographs (\textbf{Figure 2Q} \textit{vs} \textbf{2N}). Along similar lines, ferrum was found to be mild to moderately co-distributed with lysosomes, however, their co-distribution markedly increased following Cisplatin treatment (\textbf{Figures 2R} – \textbf{2W}). Overall, these initial \textit{in vitro} studies suggested that the pathobiology of ferrum and lysosomes seems to be intertwined during the process of ferroptosis induced by Cisplatin, and this led us to investigate its biology in \textit{in vivo} model systems.

\textit{Ferroptosis inhibition attenuates Cisplatin-induced AKI:} To elucidate the role of ferroptosis in Cisplatin-induced AKI, Fer-1 was administered intraperitoneally into CD1 mice about 45 min prior to Cisplatin treatment. The renal morphological changes in Cisplatin-treated mice included degeneration of tubular epithelia with loss of brush borders and dilatation (\textbf{Figure 3B} \textit{vs} \textbf{3A}). Many of the tubules included casts in the tubular lumina. These tubular changes were considerably attenuated by the administration of Fer-1 (\textbf{Figure 3C} \textit{vs} \textbf{3B}). No discernible changes were observed in the glomerular compartment. PAS staining
also revealed tubular epithelial disruption with sloughing off of the epithelia and shedding of PAS-positive material in the tubular lumina (Figure 3E vs 3D). Interestingly, these changes were largely regressed in mice receiving prior treatment of Fer-1 (Figure 3F vs 3E). Urinary albumin excretion, assessed by SDS-PAGE, increased in Cisplatin-treated mice. The excretion was minimal in the control mice or mice treated with Fer-1, suggesting that Cisplatin led to a compromise in the tubular absorptive capacity with excretion of urinary protein, and it was alleviated by the inhibition of ferroptosis (Figure 3G). Of note, the serum creatinine levels, tubular damage scores and mRNA levels of NGAL and KIM-1 (AKI markers) increased following Cisplatin treatment. Interestingly, these changes were attenuated by the administration of Fer-1 (Figure 3H - 3K), suggesting that Cisplatin induces notable renal functional deterioration by adversely affecting the tubular compartment, and they are alleviated by the inhibition of ferroptosis. Moreover, the perturbation in ferroptosis metabolic sensors, induced by Cisplatin, were partially restored by Fer-1 treatment (Supplemental Figure 1). Besides, Fer-1 treatment two hours prior to the administration of Cisplatin also alleviated the tubular injury (Supplemental Figure 2).

_Cisplatin promotes ROS generation and accentuates MIOX overexpression, leading to lipid hydroperoxidation in Cisplatin-treated HK-2 cells:_ Mitochondrial ROS generation was assessed by Dihydroethidium (DHE) staining. Cisplatin treatment for ~20 hours led to a considerable increase in DHE staining (red fluorescence) in HK-2 cells (Figures 4B vs 4A, & 4D). To confirm the specificity of DHE staining, mitochondrial ROS scavenger Mito Q was used. Cisplatin-induced increased DHE staining was partially quenched by Mito Q treatment.
MIOX expression increased in HK-2 cells after 4 hours of Cisplatin treatment, which was attenuated by Mito Q treatment (Figure 4F, left panel and Supplemental Figure 3). However, no obvious MIOX up-regulation was observed after 20 hours of Cisplatin treatment (Figure 4F, right panel), this may possibly be due to the overwhelming cellular damage that occurred during this prolonged period. To further elucidate the relevance of MIOX in ferroptosis in vitro, MIOX-overexpressing HK-2 cell line, generated in our laboratory, and MIOX-siRNA were employed. MIOX overexpression and gene disruption was confirmed by immuno-blotting procedures (Figure 4F, right panel, lanes 3, 4 & 6). The status of 4-hydroxynonenal (4-HNE), the end product of lipid hydroperoxidation, was evaluated by immunofluorescence microscopy. Normally, there was a minimal background immunofluorescence related to 4-HNE in control untreated cells (Figures 4E & 4G). The MIOX-overexpressing cells or cells treated with Cisplatin showed a moderate degree of fluorescence, while Cisplatin-treated MIOX-overexpression cells yielded the maximal degree of fluorescence (Figures 4E, 4H – 4J). Interestingly, MIOX-siRNA treatment markedly attenuated the fluorescence as compared to the control (Figures 4E & 4L vs 4K). The changes in 4-HNE levels in vitro was further confirmed by immuno-blotting analysis (Figure 4F, right panel).

**MIOX overexpression promotes while its gene disruption alleviates Cisplatin-induced cell death and RSL3-induced ferroptosis in HK-2 cells:** Severe DNA damage, as shown by TUNEL staining, was observed in Cisplatin-treated HK-2 cells (Figure 5B vs 5A). The DNA damage was further accentuated in Cisplatin-treated MIOX-overexpressing cells (Figures 5D
vs 5B & 5C), while it was remarkably reduced by MIOX-siRNA in Cisplatin-treated cells (Figure 5F vs 5E). Both control HK-2 cells and MIOX-overexpressing cells had a cobblestone epithelial morphology in culture at 48 hours (Figures 5G & 5I). Cisplatin treatment led to morphological shrinkage of the cells, and the maximal contraction was seen in Cisplatin-treated MIOX-overexpressing cells (Figure 5J vs 5H). Interestingly, transfection of MIOX-siRNA partially reverted the morphology of HK-2 cells treated with Cisplatin (Figure 5L vs 5K). MTT assay revealed that Cisplatin-induced HK-2 cell death was alleviated by MIOX-siRNA treatment, while it was accentuated by MIOX overexpression (Figures 5M & 5N). To further elucidate the role of MIOX in ferroptosis, RSL3, a GPX4 inhibitor, was used to induce ferroptosis in HK-2 cells. MTT studies showed that RSL3 led to a massive cell death after 6 hours via the process of ferroptosis (Figures 5O & 5P). This ferroptosis-specific cell death, induced by RSL3, was exacerbated by MIOX overexpression while attenuated by MIOX gene disruption (Figures 5O & 5P).

**MIOX overexpression promotes whereas its gene disruption inhibits ferritinophagy in Cisplatin-treated HK-2 cells:** Ferritinophagy, a newly discovered process of selective autophagy, refers to the autophagic degradation of ferritin in the lysosomes, which is guided by a specific cargo receptor, *i.e.*, nuclear receptor co-activator 4 (NCOA4) (16). It is conceivable that intracellular ferritin and NCOA4 are depleted simultaneously by lysosomal degradation in the state of ferritinophagy. In view of this contention, the status of FTH1 and NCOA4 degradation was investigated in the context of ferritinophagy in Cisplatin-treated HK-2 cells. Immunofluorescence microscopy revealed intense fluorescence related to the
expression of NCOA4 in control HK-2 cell (Figures 6A). Notably decreased fluorescence was
observed in Cisplatin-treated HK-2 cells, which was seen maximally in Cisplatin-treated
MIOX-overexpressing cells, as compared to the controls (Figure 6A – 6D). Interestingly,
NCOA4 related fluorescence was partially restored following the transfection of MIOX-siRNA
in Cisplatin-treated cells (Figure 6F vs 6E). Similarly, FTH1, the heavy chain of ferritin,
underwent substantial down-regulation in Cisplatin-treated HK-2 cells (Figure 6H vs 6G).
Further remarkable depletion of FTH1 was observed in Cisplatin-treated MIOX-
overexpressing cells, whereas MIOX-siRNA treatment reverted back the expression quite
similar to that in control cells (Figures 6I-6L). Of note, the decrease of FTH1 in Cisplatin-
treated MIOX-overexpressing cells was partially restored by the administration of NCOA4-
siRNA (Supplemental Figure 4). The results of immuno-blotting studies were similar to the
observations made by immunofluorescence microscopy, and they revealed that MIOX
heavily influenced the degradation of FTH1 and NCOA4 in HK-2 cells following Cisplatin
treatment (Figures 6M – 6O). Interestingly, the depletion of FTH1 was also observed in the
early state (four hours) of Cisplatin treatment, which can be alleviated by Mito-Q treatment
(Supplemental Figure 3).

**MIOX gene disruption attenuates lysosomal ferritin uptake and intracellular free iron
accumulation while MIOX overexpression enhances these processes in Cisplatin-treated
HK-2 cells:** Free iron is required for lipid hydroperoxidation and execution of ferroptosis,
but most of the intracellular iron remains bound to ferritin (9). In the state of ferritinophagy,
ferritin is phagocytosed by lysosomes and it then undergoes enzymatic degradation, leading
to excessive generation of free iron. In view of these inter-related dynamics, lysosome and ferritin were co-stained to elucidate the lysosomal uptake of ferritin in HK-2 cells. Lysosomal associated membrane protein 1 (LAMP1), a commonly used lysosomal marker, was used to stain the lysosomes. Immunofluorescence microscopy revealed considerable co-localization of FTH1 (green) and LAMP1 (red) in Cisplatin-treated HK-2 cells, indicating the translocation of ferritin into the lysosomes (Figures 7C & 7D vs 7A & 7B, and 7M). The translocation was tremendously enhanced in Cisplatin-treated MIOX-overexpressing cells, as can be readily visualized in Figures 7G & 7H, and 7M. Transfection of MIOX-siRNA into Cisplatin-treated cells remarkably disrupted the co-distribution (Figures 7K & 7L vs 7I & 7J, and 7M). To measure intracellular free iron levels, labile iron pool (LIP) assay was performed. The results indicated a marked increase of intracellular free iron concentration in HK-2 cells after Cisplatin treatment, which was accentuated in Cisplatin-treated MIOX-overexpressing cells, and it was attenuated by MIOX gene disruption (Figure 7N).

**MIOX overexpression modulates lysosomal permeability, GPX4 Activity, reduced glutathione (GSH) and NADPH levels in HK-2 Cells following Cisplatin treatment:** Since ferroptosis is akin to autophagic cell death, it is conceivable that the lysosomal permeability of HK-2 cells increases during the ferroptotic process (17). In view of this notion, the status of lysosomal permeability was investigated by Acridine Orange (AO) dye staining. In untreated cells, most of the dye accumulates inside the lysosomes to generate red fluorescence and a small portion of it remains in the cytoplasm to yield green fluorescence. When lysosomal permeability increases, AO leaks from lysosomes into cytoplasm and the
cells yield waxing of green fluorescence while waning of red fluorescence. In this study, we noted that AO-associated green fluorescence increased and red fluorescence decreased in Cisplatin-treated HK-2 cells (Figure 8B vs 8A). These changes in fluorescence were accentuated in Cisplatin-treated MIOX-overexpressing cells (Figures 8D vs 8B & 8C), whereas the changes were minimal in Cisplatin-treated cells transfected with MIOX-siRNA, as compared to the controls (Figures 8F vs 8A, 8C & 8E).

GPX4 is a key antioxidant enzyme, which can block ferroptosis by eliminating lipid hydroperoxides with reduced glutathione, GSH, in states where cells experience excessive ferroptotic pressure (18). Conceivably, ferroptosis can be accelerated when GPX4 is inhibited or GSH is depleted. In view of this, GPX4 activity and GSH concentration were investigated in HK-2 cells. Immuno-blotting analysis revealed that GPX4 expression decreased after Cisplatin treatment in HK-2 cells (Figure 8M). A substantial decline in GPX4 activity was also observed both in Cisplatin-treated HK-2 cells and Cisplatin-treated MIOX-overexpressing cells, and this decrease was not noted in Cisplatin-treated cells transfected with MIOX-siRNA (Figure 8N). The status of intracellular GSH levels was assessed by monobromobimane (MBB) staining. The MBB is taken up by the live cells and binds with GSH to generate blue fluorescence. A considerable decrease in blue fluorescence was observed after Cisplatin treatment, which was further decreased by MIOX overexpression, while partially restored by MIOX-siRNA transfection (Figures 8G – 8L & 8O). In addition, NADPH levels were measured since they modulate ferroptosis sensitivity and also aid in eliminating lipid hydroperoxides (9). The NADPH levels were found to be quite low in MIOX-overexpressing cells (Figure 8P), which may be related to its conversion into NADP+ during
the myo-inositol catabolic pathway (13, 19). Apparently, acceleration of this pathway by MIOX overexpression in Cisplatin-induced injury may have led to the NADPH depletion in HK-2 cells. Understandably, such a depletion of NADPH can be blocked by MIOX gene disruption, as observed in this investigation (Figure 8P).

MIOX overexpression exacerbates while its gene disruption alleviates renal tubular injury, lipid hydroperoxidation and decline in GPX4 activity and NADPH levels in Cisplatin-induced AKI: MIOX-TG mice and MIOX-KO mice were generated, as previously reported (8). The expression of MIOX was confirmed by the immuno-blotting procedures (Figure 9M). Cisplatin (20 mg/kg) or PBS was administered intraperitoneally into WT mice, MIOX-TG mice and MIOX-KO mice. PAS staining demonstrated tubular epithelia disruption, loss of brush borders and casts formation in the tubular lumina in Cisplatin-treated WT mice kidney (Figure 9B vs 9A). These morphological changes were highly accentuated in Cisplatin-treated MIOX-TG mice (Figures 9D vs 9B & 9C). Interestingly, no notable pathological changes were observed in Cisplatin-treated MIOX-KO mice, as compared to the controls (Figures 9F vs 9A, 9C & 9E). Similarly, increased NGAL mRNA levels were observed in Cisplatin-treated WT mice and MIOX-TG mice but not in Cisplatin-treated MIOX-KO mice (Figure 9P). Of note, the tubular injury in Cisplatin-treated MIOX-TG mice was attenuated by the prior administration of Fer-1 (Supplemental Figure 5). To determine the extent of lipid hydroperoxidation during the Cisplatin-induced injury, the status of 4-HNE was assessed by immunofluorescence microscopy. The kidney sections were stained with anti-4HNE (green) and counterstained with TO-PRO-3®-iodide to delineate the nuclei (red). The WT mice
treated with Cisplatin revealed mild greenish cytoplasmic staining compared to the control (Figure 9H vs 9G). The 4-HNE staining was notably increased in kidney sections of Cisplatin-treated MIOX-TG mice (Figures 9J vs 9H & 9I). While minimal 4-HNE staining was observed in Cisplatin-treated MIOX-KO mice, as compared to the controls (Figures 9L vs 9G, 9I & 9K). The changes in 4-HNE levels were confirmed with immuno-blotting studies (Figure 9M).

Then, we measured the GPX4 activity to evaluate ferroptosis termination system in vivo. A decline in GPX4 activity was observed in Cisplatin-treated WT mice and MIOX-TG mice but not in Cisplatin-treated MIOX-KO mice (Figure 9N), although GPX4 expression remained stable after Cisplatin treatment (Figure 9M). Of note, the NADPH levels in untreated MIOX-TG mice were much lower than WT mice (Figure 9O), suggesting that MIOX-TG mice, to begin with, may be relatively more amenable to the process of ferroptosis. Both WT and MIOX-TG mice had markedly decreased NADPH levels following Cisplatin treatment, while a moderate reduction was seen in Cisplatin-treated MIOX-KO mice (Figure 9O).

**MIOX overexpression promotes ferritinophagy in Cisplatin-induced AKI:** Ferritin and its cargo receptor NCOA4 expressions were investigated to assess the status of ferritinophagy in vivo. Immunofluorescence microscopy showed that both ferritin and NCOA4 were mainly expressed in renal tubular epithelia (Figures 10A - L). The immunofluorescence and immuno-blotting studies revealed that NCOA4 expression was decreased in Cisplatin-treated WT mice, however, MIOX-KO mice were unaffected (Figures 10B vs 10A, 10F vs 10E, and 10M). The maximal decrease in the NCOA4 expression was observed in Cisplatin-treated MIOX-TG mice (Figures 10D vs 10B & 10C, and 10M). However, changes in the FTH1 expression in
Cisplatin-treated WT mice were opposite to that observed for NCOA4. There was a marked increase in FTH1 expression in Cisplatin-treated WT mice, as indicated by immunofluorescence and immuno-blotting studies (Figures 10H vs 10G, and 10M). This upregulation might be induced by the accumulated intracellular free iron in the kidney, which can promote FTH1 transcription via a feedback mechanism while inhibiting transferrin transcription at the same time (20, 21). Subsequently, RT-PCR analyses were performed to assess the transcriptional status of FTH1 and transferrin in the kidneys. Increased FTH1 and decreased transferrin mRNA levels were noted in Cisplatin-treated kidneys (Figures 10N & 10O), implying that there may be a high concentration of intracellular free iron in tubular epithelia. Of note, in contrast to NCOA4, the FTH1 expression in Cisplatin-treated MIOX-TG mice was maximally increased while no obvious increase was noted in MIOX-KO mice (Figures 10J vs 10H & 10I, 10L vs 10K, and 10M). Interestingly, MIOX-KO mice had very low basal levels of FTH1 (Figures 10K - 10M), and the processes of ferritinophagy and the feedback mechanism were induced at the very early time period (two hours) following the administration of Cisplatin (Supplemental Figure 6).
DISCUSSION

AKI is closely associated with a variety of cell death processes, including apoptosis, necroptosis and ferroptosis. Most of them have been extensively investigated except ferroptosis, possibly due to its more recent discovery (7, 22). Ferroptosis refers to a subclass of cell death characterized by iron overload and fatal iron-catalyzed lipid damage (23-26). Due to its caspase- and necroosome-independence, ferroptosis differs from apoptosis and necroptosis (9, 27, 28). Previous publications have shown that blockade of autophagy leads to a notable alleviation of ferroptosis, suggesting that it is most likely reflective of an autophagic cell death process (21, 29). Of note, it has been reported that ferroptosis is essential in ischemia-reperfusion injury (IRI)-, Oxalate- and folic acid-induced AKI (12, 27). However, its role in Cisplatin-induced AKI has not been investigated, although this is perhaps the most suitable model since the tubular injury can be reproducibly achieved in vivo and in vitro. Interestingly, it has been reported that there is disruption of iron homeostasis in Cisplatin-treated mice kidneys (30). Besides, Linkermann et al. observed that there was no difference in the survival kinetics among WT mice, caspase-8\(^{-/-}\) mice and mice deficient in receptor-interacting protein kinase 3 (RIPK3\(^{-/-}\), necroptosis) following Cisplatin treatment (27). This would suggest that apart from apoptosis and necroptosis, Cisplatin-induced tubular injury may be induced by some other mechanism(s) or process. In this study, we observed that Cisplatin-induced nephropathy was substantially relieved by Fer-1 (Figure 3). Our in vitro studies also showed that Cisplatin-induced HK-2 cell death was attenuated by Fer-1, DFO, and VAD, but not by Nec-1 (Figures 2A – 2K), indicating that ferroptosis and apoptosis, but not necroptosis, played a key role in the cellular injury. Similar results have
been reported in Cisplatin-induced cancer cell death (31). Our studies also showed an increased ferritin degradation and free iron release from the lysosomes in HK-2 cells following Cisplatin treatment (Figures 2L – 2W). Taken together, these data indicate that ferroptosis is operative in Cisplatin-induced AKI.

An association between ferroptosis and redox perturbations has been described previously (17, 32), it is conceivable that ferroptosis may be modulated by MIOX overexpression in Cisplatin-induced tubular injury since oxygen radicals are generated in MIOX-initiated glucuronate-xylulose pathway (19). MIOX, a proximal tubular specific enzyme, exacerbates kidney redox injury in multiple pathological states (33). Previous publications revealed that transcriptional, translational, epigenetic and post-translational events are involved in the regulation of MIOX overexpression, which can promote ROS generation (14, 15, 33, 34). Our previous work showed that MIOX overexpression accentuates tubular redox injury and apoptosis in Cisplatin-induced AKI (8), but its role in ferroptosis remains enigmatic. Consistent with our previous work (8), our present studies revealed that MIOX overexpression was detrimental to cellular homeostasis in Cisplatin-induced AKI and HK-2 cells injury (Figures 5A - 5N & 9A - 9F). Since both ferroptosis and apoptosis are important in Cisplatin-induced HK-2 cell death and MIOX modulates apoptosis following Cisplatin treatment (8), here the question can be raised if MIOX can modulate the processes of ferroptosis. In view of this, we observed that the significant ferroptosis-specific cell death, induced by RSL3, was modulated by MIOX expression profile (Figures 5O & 5P). Of note, ferroptosis process is somewhat difficult to investigate due to the lack of specific markers. In this regard, pronounced relentless lipid hydroperoxidation and iron overload are
regarded as the key characteristics of ferroptosis (24, 25). Lipid hydroperoxides, the
executioner of ferroptosis, decompose into reactive intermediaries, which then lead to
massive cell death (35), while the increased intracellular free iron acts as a catalyst aiding in
the execution of ferroptosis (36). In this study, 4-HNE, the end product of lipid
hydroperoxides (37), was used to assess the state of lipid hydroperoxidation. Both in vivo
and in vitro studies demonstrated that the increase of 4-HNE levels, induced by Cisplatin
administration, were accentuated by MIOX overexpression while dampened by MIOX gene
disruption (Figures 4E - 4L & 9G – 9M). In addition, Labile Iron Pool (LIP) assay revealed the
increased intracellular free iron levels in HK-2 cells following Cisplatin treatment, which was
regulated by MIOX expression profile (Figure 7N). Taken together, one can conclude that
ferroptosis process in Cisplatin-induced AKI is modulated by MIOX overexpression.

Next, the question addressed was how MIOX overexpression promotes ferroptosis
following Cisplatin treatment. Ferritinophagy, a recently coined term, refers to the process
where massive amount of ferritin undergoes autophagic targeting and lysosomal degradation
(16, 38). It is a selective autophagy that relies on the guidance of NCOA4 to cargo ferritin to
the lysosomes, leading to the generation of intracellular free iron (38, 39). In this study,
Cisplatin treatment led to a simultaneous decrease of FTH1 and NCOA4 protein levels in
HK-2 cells, which was modulated by MIOX expression profile (Figure 6). In in vivo studies,
the expression levels of NOCA4 were decreased following Cisplatin treatment (Figures 10A -
10F & 10M). Interestingly, FTH1 was up-regulated in Cisplatin-treated mice, which may be
due to the modulation by an intricate feedback mechanism (Figures 10G - 10O). In support of
this feedback mechanism are the studies reported by Gao et al., where the transcription and
expression levels of endogenous FTH1 were up-regulated by the increased levels of intracellular free iron during ferroptosis in HT1080 cells (human fibrosarcoma cell line), while there was a simultaneous degradation of the ectopically expressed GFP-FTH1 (21). Intriguingly, MIOX knockout mice had a remarkable baseline decrease in FTH1, possibly due to the lack of MIOX-derived ROS (Figures 10K - 10M). To further confirm the modulation of ferritinophagy by MIOX overexpression, lysosome staining with anti-LAMP1 (40) and ferritin co-staining with anti-FTH1 were performed in HK-2 cells. As shown in Figures 7A - 7D, LAMP1 and FTH1 were co-localized following Cisplatin treatment in HK-2 cells, suggesting that Cisplatin treatment leads to ferritin uptake by the lysosomes. The co-localization was inhibited by MIOX-siRNA while enhanced by MIOX overexpression (Figures 7E - 7M). Since ferritinophagy is a selective autophagy, it is conceivable that lysosomal permeability increases in this process (41, 42). Acridine orange (AO) staining revealed that the lysosomal permeability of HK-2 cells increased following Cisplatin treatment, which was regulated by MIOX expression profile (Figures 8A – 8F). In addition, the intracellular levels of NADPH, the biomarker of ferroptosis sensitivity (43), was lower in MIOX-overexpressing cells and MIOX transgenic (MIOX-TG) mice even without Cisplatin treatment, suggesting that MIOX overexpression alone can sensitize the renal tubular cells to ferroptosis (Figures 8P & 9O).

Besides MIOX overexpression promoting ferroptosis via ferritinophagy and lipid hydroperoxidation, it may also inhibit the “ferroptosis termination system” by down-regulating GPX4 activity and intracellular GSH concentration. Normally, the key antioxidase GPX4, together with GSH, catalyzes noxious lipid hydroperoxides into harmless lipid alcohols, leading to the end of ferroptosis (44). Therefore, it is conceivable that GPX4 inhibitors and
system \( X_c \) antagonists (related to GSH depletion) can serve as ferroptosis inducers (45, 46).

Herein, an obvious decline of “GPX4 activity” was observed in both Cisplatin-treated WT mice and MIOX-TG mice, whereas it was unaffected in Cisplatin-treated MIOX-KO mice (Figure 9N). Similarly, GPX4 activity was reduced in in vitro studies, strengthening the argument that “ferroptosis termination system” is indeed inhibited (Figure 8N). The above findings along with the reduced MBB staining are consistent with our previous observations that the GSH depletion and oxidant stress induced by Cisplatin treatment are accelerated by MIOX overexpression (8) (Figures 8G – 8L & 8O). Taken together, these observations indicate that MIOX overexpression can promote ferroptosis directly or via the inhibition of ferroptosis termination system.

In conclusion, the findings of this study indicate that accentuation of ferroptosis in Cisplatin-induced AKI can be achieved via modulation of multiple mechanisms in states of MIOX overexpression, while its gene disruption reduces such a noxious cellular injury. Lastly, these new insights in the pathogenesis of AKI should give impetus to develop therapeutic strategies (MIOX inhibitors?) to ameliorate renal tubular injury.
CONCISE METHODS

Cell Culture Studies: Human proximal tubular epithelial cell line (HK-2) was purchased from ATCC (Manassas, VA). Cells were maintained in DMEM media, consisting of 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin. They were maintained in a humidified environment with 5% CO₂ at 37°C. In addition, MIOX-overexpressing HK-2 cells, generated in our laboratory, were also employed for various parallel studies. The cells from near confluent cultures were trypsin-dissociated and seeded onto collagen-coated six-well plates at a density of 0.5 -1 X 10⁵ in a culture medium containing 2% FBS. They were allowed to attach overnight, and then treated with 20 μM Cisplatin (Sigma, Cat. P4394) for 4-48 hours. Other chemicals used to treat the cells were as follows: Ferrostatin-1 (Fer-1, Sigma, Cat. SML0583, ferroptosis inhibitor, 0.4 μM), deferoxamine (DFO, Sigma, Cat. D9533, iron chelator, 10-80 μM), necrostatin-1 (Nec-1, Cayman Chemical, Cat. 4311-88-0, necroptosis inhibitor, 50 μM), Z-VAD(OMe)-FMK (VAD, Cayman Chemical, Cat. 187389-52-2, apoptosis inhibitor, 50 μM). Treated cells were harvested and used for various morphological and biochemical studies. Additional knockdown studies included the use of MIOX-siRNA (Origene, Cat. SR310776, 50 nM) and NCOA4-siRNA (Santa Cruz, Cat. sc-61117, 50nM) in cells transfected with Lipofectamine 2000 regent (Invitrogen, Cat. 11668019).

Animal Model System: Eight weeks old male mice in groups of eight of the following strains were used: CD1, wild type (WT) C57Bl/6J, proximal tubular-specific MIOX knockout (MIOX-KO, C57Bl/6J background), and proximal tubular-specific MIOX overexpressing transgenic (MIOX-TG, C57Bl/6J background). The generation of MIOX-TG and -KO mice
has been previously described in our publication (8). Acute kidney injury (AKI) was induced with the administration of a single intraperitoneal injection of Cisplatin (20 mg/kg). Control mice received PBS only. For ferroptosis inhibition studies, Fer-1 was administered 45 minutes to two hours prior to the induction of AKI at a dose of 5 mg/kg. The mice were sacrificed two hours or 3 days later and kidney tissues were harvested for various studies. Prior to sacrifice, the blood and urine samples were collected. Serum creatinine was measured using Quantichrom Creatinine assay kit (BioAssay Systems, Cat. DICT-500). Urine samples were centrifuged at 5,000 x g, and supernatants subjected to 15% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue reagent.

**Morphological Studies and Tubular Damage Scoring:** Four-μm thick tissue sections were heat-deparaffinized and treated with xylene. They were rehydrated with decreasing concentrations of graded series of ethanol. The sections were then successively stained with Hematoxylin (3 min) and Eosin (30 sec). After a brief wash with deionized-distilled water, the sections were dehydrated with ethanol, treated with xylene, coverslip mounted and evaluated. The sections with H & E staining were used to assess tubular damage scoring, as previously reported (47, 48). For each mouse, 100 tubules from 10 different views were evaluated by two investigators. Seven parameters were graded as follows: tubular epithelial cell flattening (0 or 1), loss of brush border (0 or 1), cell membrane bleb formation (0 or 2), cytoplasmic vacuolization (0 or 1), cell necrosis (0 or 2), interstitial edema (0 or 1), and tubular lumen obstruction (0 or 2). The accumulated values ranging for 0 (unaffected) – 10 (severe damage) was considered as indicative of the extent of tubular damage. For PAS
staining, Periodic Acid Schiff Stain kit (Abcam, Cat. Ab150680) was used. Briefly, rehydrated sections were immersed in Periodic Acid Solution for 10 min. They were then rinsed with distilled water and incubated with Schiff Solution for 30 min. After washing with distilled water, the sections were stained with Hematoxylin for 3 min. Following another H₂O wash, Bluing Reagent™ was applied for 30 sec and slides rewashed, dehydrated, treated with xylene and coverslip mounted. The tissue sections were evaluated for cellular morphology of tubules by light microscopy.

**Immunohistochemical (IHC) studies:** Four-μm-thick paraffin tissue sections of kidneys, from renal biopsy tissues of three ATN patients, were de-paraffinized and rehydrated. Following heat-induced epitope (antigen) retrieval, the slide sections were washed twice with TBST containing 0.025% Triton X-100. For blocking the background staining, the sections were immersed in 3% BSA in TBST (137 mM NaCl, 2.7 mM KCl, 16.5 mM TRIS, pH 7.4, containing 0.025% Triton X-100) for 1 hour at 22°C. Sections are then incubated with MIOX antibody (1:200), NCOA4 antibody (Bethyl Laboratories, Cat. A302-272A, 1:100) and FTH1 antibody (Abcam, Cat. ab65080, 1:100) in TBST overnight at 4°C and rinsed with TBST. Endogenous peroxidase was suppressed by incubating the sections with 3% H₂O₂ for 15 minutes. The sections were incubated with HRP conjugated secondary antibody (Vector Laboratories) for 1 hour at 22°C. After washing with PBS, diamino-benzidine (DAB) solution was applied to the sections for 20-35 seconds to develop the peroxidase reaction product. The sections were then immediately washed with deionized distilled water. The sections were counterstained with Hematoxylin, dehydrated in graded series of ethanols, coverslip
mounted and evaluated by light microscopy.

**Immunofluorescence (IMF) Studies:** Initially, the HK-2 cells undergone various treatments were fixed in 4% paraformaldehyde. They were then permeabilized with 0.25% Triton X-100 in PBS buffer. They were immersed in 2% BSA in PBST to block the non-specific background. The cells were then individually incubated with following primary antibodies overnight at 4°C: anti-NCOA4 (Bethyl Laboratories, Cat. A302-272A, 1:100), anti-FTH1 (Abcam, Cat. ab65080, 1:100), anti-4-HNE antibody (Abcam, Cat. ab46545, 1:100) and anti-LAMP1 (Cell Signaling, Cat. 15665S, 1:100). Subsequently, cells were washed with PBS and incubated with secondary antibodies conjugated with fluorescein isothiocynate. The cells were then coverslip mounted and examined by fluorescence microscopy. For kidney tissue immunofluorescence, 4-μm thick cryostat sections were prepared from different group of experiments. They were washed with PBS and immersed in 2% BSA to block the non-specific background. The sections were then incubated with rabbit polyclonal anti-4-HNE, -NCOA4 or -FTH1 primary antibodies for 2 hours at 22°C. Following three washes with PBS, secondary antibodies were applied to the sections and incubation extended for another hour at 22°C. The sections were rewashed with PBS, coverslip mounted and examined with an UV microscope equipped with epi-illumination.

**Protein Expression studies:** Immuno-blottting procedures were employed to evaluate the protein expression in various samples. Briefly, kidney tissues were diced into 1 mm³ and homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 50 mM
TRIS, pH 8.0, containing protease inhibitors). Likewise the cells were lysed with RIPA lysis buffer. The homogenates were centrifuged at 10,000 x g for 5 minutes and supernatants were collected. Protein concentration in the supernatants was measured by using a Pierce™ BCA Protein Assay Kit (Cat. 23225). After adjusting the concentration (100 μg/100 μl) in each of the samples, equal amounts of protein (20 μg) were mixed with the SDS loading buffer, boiled for 5 min, ice-cooled and subjected to 10 or 15% SDS-PAGE. Following the fractionation of proteins by SDS-PAGE, the proteins were transferred to PVDF membranes by electro-blotting procedures. The PVDF membranes were then individually incubated with the following antibodies having respective dilutions overnight at 4°C: anti-MIOX antibody (1:1,000), anti-NCOA4 (Bethyl Laboratories, Cat. A302-272A, 1:1,000), anti-FTH1 (Cell Signaling, Cat. 3998S, 1:1,000), anti-GPX4 (Cayman Chemicals, Cat. 10005258, 1:200), 4-HNE (Abcam, Cat. ab46545, 1:1000) and anti-β-actin (Thermo Fisher Scientific, Cat. A5441, 1:1,000). The membranes were washed with TBST buffer (137 mM NaCl, 2.7 mM KCl, 16.5 mM TRIS, pH 7.4, containing 0.1% Tween-20) and incubated with secondary antibodies at 22°C for 60 min. Following another wash with TBST, the protein bands in the blots were detected by using the ECL chemiluminescence system (Amersham Biosciences).

**Gene Expression Studies:** Real-time PCR was employed to evaluate the gene expression in various samples. Briefly, ~10 mg of kidney tissue was used to isolate RNA, using TRIzol Reagent (Invitrogen, Cat. 15-596-026). The RNA was reverse transcribed into cDNA using GoScript™ Reverse Transcription System (Promega). For quantitative PCR (qPCR), reaction mixture, containing 100 ng cDNA, 1 μmol/L of forward and reverse primer, each, 1X Fast
SYBR Green Master Mix and 2 μl of nuclease-free water, in a total volume of 10 μl was prepared. The reaction mixture was subjected to PCR in an Applied Biosystems Step1Plus™ Real-Time PCR System Thermocycler. Relative CT values compared with GAPDH were used to calculate the mRNA levels in different samples. The primers used in this study were:

- **GAPDH**, Forward: 5’-GAATACGGCTACAGC AACAGG-3’ & Reverse: 5’-GGTCTGGGATGG AAATTGT-G-3’; β-actin, Forward: 5’-AGACCTCTATGCAACAACAGTG-3’ & Reverse: 5’-AC CGATCCACACAGGTACTTG-3’; FTH1, Forward: 5’-GCCCTTTGCAACTTCGTCG-3’ & Reverse: 5’-GTGGTAGTTCTGGCGCACTT-3’; transferrin, Forward: 5’- AACCAGCTCGA AGGCAAGAA-3’ & Reverse: 5’- ACTGCCCCAGAAAGAA ACTGG -3’; NGAL, Forward: 5’- GCCCAGGACTCAACTCGATGACAT-3’; KIM-1, Forward: 5’-GGAAGTAAAGGGGGTAGTGGG-3’ & Reverse: 5’-AAGCAGAAGATGGGCTATT GC-3’.

**Evaluation of Cell Death and Cell morphology in HK-2 Cells:** TUNEL assay was employed to assess the degree of cell death. An *In Situ* Cell Death Detection Kit (Roche, Cat. 11684795910) was used to detect cellular DNA damage. The cells were washed with PBS three times and treated with 4% paraformaldehyde in PBS for 1 hour at 22°C. The cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C. Cells were incubated with TUNEL reagent™ (450 μl Label solution and 50 μl Enzyme solution) for 1 hour at 37°C. Cells were then washed twice with PBS and examined with an UV microscope equipped with epi-illumination. For cell morphology studies, HK-2 cells were examined by phase contrast light microscopy immediately after Cisplatin treatment. In
addition, H & E staining was used to delineate the finer morphological characteristics of the cells as follows. The HK-2 cells were stained with hematoxylin (3 minutes) and eosin (45 seconds) after the fixation with 4% paraformaldehyde (15 minutes). Cells were then washed and examined with light microscopy.

**Assessment of lysosomal permeability, reduced glutathione (GSH) and mitochondrial ROS in HK-2 cells:** The lysosomal permeability in HK-2 cells was determined by Acridine Orange (AO) staining. Cells were incubated in the growth medium, inclusive of AO (4 μg/ml, Sigma, Cat. A6014), and incubation carried out for 20 min at 37°C. The stained cells were washed twice with 3% fetal calf serum in PBS and immediately examined by fluorescence microscopy. For in situ determination of GSH, monobromobimane (MBB, 20 μM, Sigma, Cat. B4380) in PBS was used to stain the cells for 15 min at 37°C following the removal of culture medium. They were coverslip mounted and photographed immediately. For determination of mitochondrial ROS, dihydroethidium (DHE, 20 μM, Sigma, Cat. D7008) was used. It was included in the PBS to stain the cells for 20 min at 37°C. To ensure the specificity of DHE staining, 100 nM mitoquinone (Mito Q, Focus Biomolecules, Cat. 10-1363) was included in the medium to scavenge mitochondrial ROS. Cells were then coverslip mounted and examined by fluorescence microscopy.

**Determination of NADPH Levels in Kidney Tissues and Cells:** About fifteen mg of cortical kidney tissues or \( \sim 2 \times 10^6 \) cells with 70-80% confluency per culture dish (55 cm²) were used to assess the NADPH levels with a NADP/NADPH Assay Kit (Abcam, Cat. Ab65349).
tissues were homogenized in 200 μl NADP/NADPH Extraction Buffer. Likewise the cells were lysed in 200 μl NADP/NADPH Extraction Buffer. The samples were then briefly vortexed and centrifuged at 14,000 x g for 5 min. The supernatants were passed through spin columns provided in the kit and centrifuged at 10,000 x g for 40 min. The filtered samples were then heat-treated at 60°C for 30 min to decompose NADP. 50 μl standards or samples were added into the individual wells of 96 well-plate, which had pre-added 100 μl Reaction Mix (98 μl NADP Cycling Buffer and 2 μl NADP Cycling Enzyme Mix). Following which, 10 μl NADPH Developer was added into each well and the reaction extended for 2 hours. When the reaction period was over, colorimetric reading were made at an OD$_{450}$ nm using a Microplate Reader (BIO-RAD). Finally, the NADPH levels of the samples was calculated by comparing a standard curve.

**Labile Iron Pool (LIP) Assay:** The intracellular free iron levels were measured by LIP assay, as previously described (21). Cells were briefly trypsinized (2 min) and immediately washed with PBS. 0.05 μM calcein-acetoxymethyl ester (AnaSpec, Cat. AS-72126) in PBS was added to the cells and incubated for 15 min at 37°C. After washing with PBS, the cells were divided into 2 aliquots. The cells from the 1st aliquot were treated with 100 μM deferiprone (Sigma, Cat. 379409) for 1 hour at 37°C. The 2nd aliquot of cells was left untreated. Flow cytometric reading was made with excitation of calcein at 488 nm and emission fluorescence at 525 nm. The increase of mean fluorescence in deferiprone- treated cells was compared to untreated cells, which represented the amount of intracellular free iron.
**MTT Assay:** HK-2 cells were seeded onto the 96-well plate (~3,000 cells/well). The cells were treated with 20 μM Cisplatin for 20 hours. The growth medium was removed after treatment and cells were cultured in 1 mg/ml Thiazolyl Blue Tetrazolium Bromide (Sigma, Cat. M5655) in fresh growth medium for 4 hours. Supernatants were carefully removed from the wells. Then, 150 μl Dimethyl Sulfoxide (DMSO, Thermo Fisher Scientific, Cat. BP231-100) was added into each well. The 96-well plates were subjected to Orbital shaking for 10 min to completely dissolve formazan. The absorbance at 490 nm wavelength was then measured by a Microplate Reader.

**Measurement of Glutathione peroxidase 4 (GPX4) Activity:** GPX4 activity was determined as previously described (18). For in vitro studies, ~2×10⁶ cells from culture dishes (55 cm²) were harvested and homogenized in 200 μl of buffer (0.1 M KH₂PO₄/K₂HPO₄, 0.15 M KCl, 0.05% {w/v} CHAPS, 5mM β-mercaptoethanol and protease inhibitors, pH 7.4). For in vivo studies, ~20 mg kidney tissue, frozen in liquid nitrogen, was ground into powder with a pestle and mortar and then homogenized in 200 μl buffer. The homogenates were then centrifuged at 15,000 x g for 20 min at 4°C, and the supernatants were saved. An aliquot was used to measure the protein concentration, using Bradford Reagent™ (BIORAD). For GPX4 activity, 50 μl of supernatant was added into 1 ml of assay buffer (5 mM EDTA, 5 mM reduced glutathione {Sigma, Cat. 1294820}, 0.1% {v/v} Triton X-100, and 180 IU/ml glutathione reductase {Sigma, Cat. G9297}, and 160 mM NADPH/H⁺{Sigma, Cat. 10107824001}, pH 7.8). After 5 min of incubation at 22°C, 5 μl of 30 mM
cumene hydroperoxide (Sigma, Cat. 247502) was added to initiate the reaction. The readings were recorded at an absorbance of 340 nm and measured every 10 seconds until the stability was established, *i.e.*, no detectable change in absorbance. The kinetics representing the rate of change in absorbance, reflective of GPX4 activity, were then calculated.

**Spatial distribution of Ferrum in the Lysosomes:** To delineate the localization of ferrum in Cisplatin-treated HK-2 cells, first lysotracker (Thermo Fisher Scientific, Cat. L7528) in a final concentration of 75 nM was added into the culture medium for 60 min. The live cells were immediately photographed to demarcate the fluorescent lysosomes (red fluorescence). Then, the cells were re-incubated in 1 ml 90% (v/v) ethanol containing 5 μl ferrum solution for 15 min at 37°C. The ferrum solution was prepared by adding 50 μl chloroform into one vial of ferrum (Ursa Bioscience, 520-R). The cells were then re-photographed by fluorescence microscopy to detect ferrum (green fluorescence).

**Statistics:** 2 tailed Student’s t test was used for the difference analysis of two groups, and 1-way ANOVA with Dunn’s multiple comparisons was performed for the difference analysis of three or more groups. *p* less than 0.05 was considered significant, Data were expressed as mean ± SD. GraphPad Prism 7 and Microsoft Excel 2013 were used for all calculations.

**Study approval:** All animal procedures used in this study were approved by the Animal Care and Use Committee of Northwestern University (#2018-2043) and the Second Xiangya
Hospital of Central South University, China (#2018sydw0276). All human studies were approved by the Second Xiangya Hospital of Central South University, China (# 2019-S078).

Written informed consent from participants or their guardians was obtained.
AUTHOR CONTRIBUTIONS

FD contributed to the study design, experiments, data analysis and manuscript preparation. IS contributed to the MIOX overexpression HK-2 cell line preparation and study design. YD contributed to study design and manuscript preparation. MY contributed to collection of human patient samples, clinical data and CD1 mice associated research. YSK contributed to overall study design, data analysis and manuscript preparation.
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Figure 1. Expression of MIOX and ferritinophagy markers in kidneys of patients with acute tubular necrosis (ATN), as assessed by immunohistochemistry (IHC). “Scanning” magnification photomicrographs revealed that MIOX was expressed in the proximal tubular cells, and its expression was increased notably in patients with ATN, as readily seen in high magnification photomicrographs (panels C & D vs A & B). No MIOX expression was seen in the glomerulus. The ferritinophagy biomarkers, i.e., NCOA4 (cargo receptor of ferritin) and FTH1 (heavy chain of ferritin), were seen localized in the renal tubular epithelia, and their expression was notably decreased in patients with ATN (panels G & H vs E & F, and K & L vs I & J). Like MIOX, no significant expression of ferritinophagy markers was noted in the glomerular compartment. Scale bar: 50 μm.
Figure 2. Ferroptosis is an essential part in Cisplatin-induced HK-2 cell death.

Normally, the HK-2 cells exhibited a flat epithelial morphology, demonstrated by phase contrast microscopy and “direct visualization” of H & E staining (panel 2A & 2D, red arrow and arrowhead). Cisplatin (CP) treatment caused shrinkage of HK-2 cells (panel 2B & 2E, yellow arrows and arrowheads). While co-treatment of Cisplatin and Fer-1 led to a reversion of their morphology (panel 2C & 2F, blue arrow and arrowhead). TUNEL staining revealed notable DNA damage in Cisplatin-treated HK-2 cells, and co-treatment with Fer-1 reduced the cell death (panels 2G – 2I). MTT assay revealed reduced cell viability following Cisplatin exposure, which was rescued by Fer-1 treatment (panel 2J) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). Deferoxamine (DFO) and Z-VAD(OMe)-FMK (VAD) treatment also led to an increased survival of cells, while Nec-1 was ineffective (panels 2J & 2K) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). To investigate the intracellular dynamics of iron in HK-2 cells following Cisplatin treatment, distribution of ferrum and heavy chain ferritin (FTH1) were assessed. FTH1 was found diffusely distributed in the cytoplasm (green) and minimally in the lysosomes (red) (panels 2L – 2N). Interestingly, the FTH1 was seen heavily co-localized in the lysosomes following Cisplatin treatment (panels 2O – 2Q). The ferrum was also found to be marginally co-distributed within the lysosomes, and the co-distribution markedly increased following Cisplatin treatment (panels 2R – 2W). Scale bar: 30 μm.
Figure 3. *Ferroptosis inhibition attenuates Cisplatin-induced AKI.* Cisplatin treatment led to a disruption of tubular epithelia, loss of brush borders and cast formation, which were alleviated by the administration of Fer-1 (panels A – C). In addition, PAS staining revealed sloughing off the epithelia and shedding of PAS-positive material in the tubular lumina following Cisplatin treatment (panel E vs D). These changes were attenuated by the prior treatment of Fer-1 (panel F vs E). As assessed by SDS-PAGE, Cisplatin-treatment increased urinary albumin excretion but not in mice pre-treated with Fer-1 (panel G). Similarly, Fer-1 treatment attenuated Cisplatin-induced elevation of serum creatinine levels (panel H) (n=6; *p < 0.05 compared with the control group, #p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). Besides, the increase in tubular damage score and mRNA levels of KIM-1 and NGAL, induced by Cisplatin, were also alleviated by the administration of Fer-1 (panel I-K) (n=4; *p < 0.05 compared with the control group, #p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). Scale bar: 50 μm.
Figure 4. Cisplatin leads to excessive mitochondrial ROS generation and MIOX-overexpression, which in turn accentuates Cisplatin-induced lipid hydroperoxidation in HK-2 cells. Cisplatin treatment increased DHE staining, indicative of mitochondrial ROS, in HK-2 cells, and it was partially reduced by Mito Q-treatment (panels A - D) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). Immuno-blotting studies revealed an increased expression of MIOX following 4 hours of Cisplatin treatment (panel F, left). No obvious MIOX up-regulation was observed after 20 hours of Cisplatin treatment (panel F, right). Besides, the status of MIOX overexpression and gene disruption in HK-2 cells was confirmed by immuno-blotting studies (panel F, right, lanes 3, 4 & 6). Fluorescence microscopy revealed that Cisplatin treatment for 20 hours led to an increased 4-HNE staining, indicative of lipid hydroperoxidation, in HK-2 cells (panels E, G & H) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). The hydroperoxidation was accentuated by the overexpression of MIOX while attenuated by MIOX gene disruption (panels E & I - L). Similar changes in 4-HNE levels in vitro were observed by immuno-blotting analyses (Figure 4F, right panel). Scale bar: 50 μm.
Figure 5. **MIOX overexpression exacerbates cell death while inhibited by its gene disruption in Cisplatin-treated HK-2 cells.** TUNEL staining revealed severe DNA damage in Cisplatin-treated HK-2 cells, which was further promoted by MIOX overexpression, and alleviated by MIOX gene disruption (panels A - F). Cisplatin treatment led to shrinkage of HK-2 cells after 48 hours, and the maximal contraction was seen in MIOX-overexpressing cells, as compared to the controls (panels G - J). MIOX-siRNA treatment partially reverted these changes (panel L vs K). As indicated by MTT assay, Cisplatin-induced cell death was alleviated by MIOX-siRNA treatment, however, it was accentuated by MIOX overexpression (panels M & N) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP + Empty vector group, 1-way ANOVA with Dunn’s multiple comparisons for panel M and 2 tailed Student’s t test for panel N). MTT experiments also revealed that RSL3 (ferroptosis inducer) caused massive ferroptosis-specific cell death in HK-2 cells, which was exacerbated by MIOX overexpression and attenuated by its gene disruption (panels O & P) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the RSL3 + Empty vector group, 1-way ANOVA with Dunn’s multiple comparisons for panel O and 2 tailed Student’s t test for panel P). Scale bar: 30 μm.
Figure 6. MIOX knockdown inhibits while its overexpression accelerates ferritinophagy in Cisplatin-treated HK-2 cells. Immunofluorescence microscopy revealed a decreased fluorescence related to the expression of NCOA4 in Cisplatin-treated HK-2 cells (panels B vs A). The immunofluorescence intensity was maximally reduced in Cisplatin-treated MIOX-overexpressing cells, and it was partially restored following MIOX-siRNA treatment (panel C - F). Similarly, there was a substantial down-regulation of FTH1 in Cisplatin-treated HK-2 cells, and a further decrease in FTH1 related immunofluorescence was observed in Cisplatin-treated MIOX-overexpressing cells, whereas, MIOX-siRNA treatment prevented its down-regulation (panels G - L). The changes in NCOA4 and FTH1 expression levels were confirmed by immuno-blotting studies (panel M - O) (n=4; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). Scale bar: 50 μm.
Figure 7. MIOX overexpression accentuates whereas its gene disruption attenuates ferritin uptake by the lysosomes and accumulation of free iron after Cisplatin treatment. Normally, ferritin (FTH1, green) was seen localized primarily in the cytoplasm and in small amounts in the Lysosome (LAMP1, red) in the untreated HK-2 cells (panels A & B). Immunofluorescence microscopy revealed considerable translocation of FTH1 into lysosomal compartment in Cisplatin-treated HK-2 cells (panels C, D & M) (n=4; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). The translocation was tremendously enhanced in Cisplatin-treated MIOX-overexpressing cells, while remarkably disrupted by the transfection of MIOX-siRNA (panels E - M). To measure intracellular free iron levels, labile iron pool (LIP) assays were performed. The results indicated a marked increase of intracellular free iron concentration in HK-2 cells after Cisplatin treatment (panel N) (n=4; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). The concentration of free iron was seen further increased in Cisplatin-treated MIOX-overexpressing cells, and it was attenuated by MIOX gene disruption (panel N). Scale bar: 50 μm.
Figure 8.  MIOX overexpression promotes lysosomal permeability and decreases GSH concentration, GPX4 activity and NADPH levels in cisplatin-treated HK-2 cells.

Lysosomal permeability was investigated by Acridine Orange (AO) staining. AO-associated green fluorescence in the cytoplasm increased, while red fluorescence in the lysosome decreased, suggesting increased lysosomal permeability of HK-2 cells following Cisplatin treatment (panels B vs A). The changes in fluorescence were accentuated in Cisplatin-treated MIOX-overexpressing cells but attenuated in Cisplatin-treated cells transfected with MIOX siRNA (panels C - F). The expression of GPX4, a key enzyme for ferroptosis inhibition, decreased after Cisplatin treatment (panel M). A substantial decline in GPX4 activity was also observed both in HK-2 cells and MIOX-overexpressing cells, and this decrease was negated by the concomitant transfection with MIOX siRNA (panel N) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn's multiple comparisons). The status of intracellular GSH levels was assessed by monobromobimane (MBB) staining. A decrease in blue fluorescence was observed after Cisplatin treatment, which was further decreased in Cisplatin-treated MIOX-overexpressing
cells, while partially restored by MIOX-siRNA transfection (panels G –L & O) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). The NADPH levels were also found to be low in MIOX-overexpressing cells and Cisplatin-treated cells (panel P). There was further depletion of NADPH in Cisplatin-treated MIOX-overexpressing cell, which was blocked by MIOX gene disruption (panel P) (n=6; * p < 0.05 compared with the control group, # p < 0.05 compared with the CP group, 1-way ANOVA with Dunn’s multiple comparisons). Scale bar: 30 μm.
Figure 9. **MIOX-overexpression exacerbates, while its gene disruption alleviates renal tubular injury, lipid hydroperoxidation and decline in GPX4 activity and NADPH levels in Cisplatin-induced AKI.** The expression profile of MIOX in WT mice, MIOX-TG mice, and MIOX-KO mice was demonstrated by immuno-blotting studies (panel M). Cisplatin treatment led to a severe renal tubular injury in WT mice, which was accentuated in MIOX-TG mice but attenuated in MIOX-KO mice (panels A-F). Similarly, NGAL mRNA levels increased in Cisplatin-treated WT mice and MIOX-TG mice, and a minimal increase was observed in Cisplatin-treated MIOX-KO mice (panel P) (n=4; * p < 0.05 compared with the WT control group, # p < 0.05 compared with the WT CP group, 1-way ANOVA with Dunn’s multiple comparisons). 4-HNE expression levels was used to assess the status of lipid hydroperoxidation. Immunofluorescence and immuno-blotting studies revealed increased 4-HNE levels in Cisplatin-treated WT mice, which were maximal in Cisplatin-treated MIOX-TG mice but minimal in Cisplatin-treated MIOX-KO mice (panels G-M). Besides, a decline in "GPX4 activity" was observed in Cisplatin-treated WT mice and MIOX-TG mice but not in Cisplatin-treated MIOX-KO mice (panel N) (n=6; * p < 0.05 compared with the WT control
group, # p < 0.05 compared with the WT CP group, 1-way ANOVA with Dunn’s multiple comparisons), although “GPX4 expression” remained stable after Cisplatin treatment (panel M). The NADPH levels in untreated MIOX-TG mice were much lower than WT mice (panel O) (n=6; * p < 0.05 compared with the WT control group, # p < 0.05 compared with the WT CP group, 1-way ANOVA with Dunn’s multiple comparisons). Both WT and MIOX-TG mice had markedly decreased NADPH levels following Cisplatin treatment, while a moderate reduction was seen in Cisplatin-treated MIOX-KO mice (panel O). Scale bar: 30 μm.
Figure 10
MIOX-overexpression promotes ferritinophagy in Cisplatin-induced AKI.
Immunofluorescence microscopy showed that both ferritin and NCOA4 were mainly expressed in renal tubular epithelia (panels A - L). The expression of NCOA4 decreased in Cisplatin-treated WT mice, however, MIOX-KO mice were unaffected, as indicated by immuno-fluorescence and -blotting studies (panels B vs A, F vs E, and M). The maximal decrease in the NCOA4 expression was observed in Cisplatin-treated MIOX-TG mice (panels D vs B & C, and M). Intriguingly, the FTH1 expression increased markedly in Cisplatin-treated WT mice (panels H vs G, and M). This up-regulation may be due to the feedback mechanism (Ref # 21), which was substantiated by RT-PCR analyses in the present study. The analyses revealed increased FTH1 and decreased transferrin mRNA levels in Cisplatin-treated kidneys (panels N & O) (n=6; * p < 0.05 compared with the control group, 2 tailed Student's t test). The FTH1 expression in Cisplatin-treated MIOX-TG mice was maximally increased while no obvious changes were noted in Cisplatin-treated MIOX-KO mice (panels J vs H and I, L vs K, and M). Scale bar: 30 μm.