Therapeutic Effect of Extracellular Vesicles Derived from HIF Prolyl Hydroxylase Domain Enzyme Inhibitor-Treated Cells on Renal Ischemia/Reperfusion Injury

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Abstract
Introduction: Acute kidney injury (AKI) is a major public health problem worldwide. However, there is no definitive therapies to treat established AKI. In this study, we used FG-4592 to induce hypoxia inducible factor (HIF) expression in cells and then explored whether the extracellular vesicles (EVs) secreted by HIF-upregulated cells could alleviate ischemia/reperfusion injury (IRI)-induced AKI. Methods: FG-4592/HK2-EVs and FG-4592/HEK293-EVs were prepared by treating HK2 or HEK293 cells with FG-4592 for 24 h, respectively. HK2 cells under hypoxia were treated with FG-4592/HK2-EVs or FG-4592/HEK293-EVs to observe the therapeutic effect of EVs on H/R-induced apoptosis and inflammation. Mice were treated with FG-4592/HEK293-EVs after IRI to observe whether FG-4592/HEK293-EVs treatment could alleviate ischemic AKI. Results: The expression of HIF was induced by FG-4592 in a dose-dependent manner in HK2 and HEK293 cells under normoxia. In vitro, FG-4592/HK2-EVs and FG-4592/HEK293-EVs inhibited apoptosis and inflammation induced by H/R. In vivo, treatment with FG-4592/HEK293-EVs significantly ameliorated renal tubular injury and inflammation caused by IRI. In addition, the expression of HIF-1α in cells and kidneys was significantly downregulated by FG-4592/HK2-EVs and FG-4592/HEK293-EVs treatment. Conclusion: This study demonstrated that EVs derived from HK2 or HEK293 cells after FG-4592 treatment could alleviate renal tubular injury and inflammation, suggesting a novel therapeutic role of FG-4592/EVs in the treatment of AKI.

Introduction

Acute kidney injury (AKI) is a common clinical syndrome associated with high in-hospital morbidity, mortality, and cost [1]. Long-term outcomes of AKI can make complete recovery of kidney function difficult. Studies showed that 41.2% of AKI patients did not fully recover renal function before discharge, which could lead to chronic kidney disease (CKD) and end-stage renal disease [2]. However, there is no definitive therapies to treat established AKI.
The hypoxia inducible factor (HIF) is a protein heterodimer consisting of an inducible α subunit, HIF-α, and a constitutively expressed subunit, HIF-β. Under normoxia, the two critical prolyl residues of HIF-α are hydroxylated by a specific prolyl hydroxylase domain (PHD). HIF-α binds to the von Hippel-Lindau protein (pVHL)-E3-Ubiquitin ligase complex, which can be ubiquitinated and rapidly degraded by the proteasome. Under hypoxia, the function of PHD is inhibited, which causes the stabilization of HIF-α, leading to the transcription of HIF target genes [3, 4]. The target genes of HIF, including EPO, VEGF, and some glycolysis-related genes, are involved in the regulation of erythropoiesis, angiogenesis, glycolysis, and iron metabolism, which can improve the adaptability of cells to hypoxia [5–7]. Studies show that HIF activation not only alleviates apoptosis, but also inhibits the activation of inflammatory pathways and the release of pro-inflammatory cytokines by regulating the transcription of target genes, thereby protecting against AKI [8].

Extracellular vesicles (EVs) are lipid bilayer-enclosed structures secreted by cells and are divided into three categories according to their size and biogenesis: exosomes, microvesicles, and apoptotic bodies. EVs contain proteins, lipids, RNA, and DNA and participate in the transmission of information between cells [9]. HIF activation can regulate the transcription of target genes, thereby promoting the expression of certain proteins, lipids, and RNA which reduce inflammation and apoptosis [8]. Furthermore, studies have found that some of these renoprotective molecules upregulated by HIF can be encapsulated into EVs, making EVs protect against AKI. For example, Jia et al. [10] have found that HIF-1α activation induced by hypoxia could make exosomes carry more miR-21, thereby affording EVs with a therapeutic effect on alleviating sepsis-induced AKI. This indicated that EVs secreted by HIF-activated cells may become a new type of nano-therapy for AKI [10, 11]. However, it is noted that hypoxia not only activates HIF but also increases pro-inflammatory exosomal contents [12]. Thus, for better application of EVs released by HIF-activated cells in AKI treatment, it is necessary to find a better way to upregulate HIF expression. HIF PHD enzyme inhibitor (PHI) is a new class of oral small-molecule drug used for renal anemia in CKD patients, which upregulates the expression of HIF by inhibiting PHD. Recent studies found that HIF-PHI could inhibit apoptosis, promote cell proliferation, and reduce interstitial inflammation and fibrosis by upregulating the expression of HIF, thereby exerting a protective effect on AKI and CKD [8, 13].

In this study, we used FG-4592, a type of HIF-PHI, to induce HIF expression in HK2 and HEK293 cells and explored whether the EVs released by HIF-upregulated cells could alleviate ischemia/reperfusion-induced (IRI) AKI. Our research may provide a new application of HIF-PHI and a new nano-therapy for AKI.

### Materials and Methods

#### Cell Culture

HK2 and HEK293 cells (American Type Culture Collection) were cultured in DMEM/F12 (Gibco) with 10% fetal bovine serum (Gibco) in a humidified incubator with 5% CO2/95% air. HK2 cells were seeded into 35-mm dishes and allowed to reach 80–90% confluence before the experiment. For hypoxia, the cell culture medium was replaced with DMEM/F12 without glucose and fetal bovine serum. The cell supernatants were centrifuged at 3,000 rpm for 20 min and 10,000 g for 20 min to remove cells and debris. Afterward, the supernatants were concentrated using a 100-kDa molecular-weight-cutoff centrifugal filter unit (Millipore) at 4,000 g for 20 min and then were ultracentrifuged at 200,000 g for 2 h. The pellet was resuspended with PBS and stored at −80°C.

#### Preparation of FG-4592/HK2-EVs and FG-4592/HEK293-EVs

The concentrations of FG-4592 used to treat HK2 and HEK293 cells under normoxia were 10 and 30 μM. DMF, the solvent of FG-4592, was used as a control. After 24 h of stimulation, the cell supernatants were centrifuged at 3,000 rpm for 20 min and 10,000 g for 20 min to remove cells and debris. Afterward, the supernatants were concentrated using a 100-kDa molecular-weight-cutoff centrifugal filter unit (Millipore) at 4,000 g for 20 min and then were ultracentrifuged at 200,000 g for 2 h. The pellet was resuspended with PBS and stored at −80°C.

#### Characterization of FG-4592/HK2-EVs and FG-4592/HEK293-EVs

The size distribution of EVs was measured using nanoparticle tracking analysis (NTA) (ZetaView PMX 110; Particle Metrix). The morphology of EVs was observed under a transmission electron microscope (H-7650; Hitachi). Surface markers of EVs (Alix, CD63, and TSG101) were detected by western blotting.

#### Animals and Treatment with EVs

All animal experiments were approved by the Institutional Animal Care and Use Committee, in accordance with the standards for the care and use of laboratory animals. IRI surgery was performed as previously described [14]. Male C57BL/6 mice (6–8 weeks old) were anesthetized via intraperitoneal injection of pentobarbital sodium (50 mg/kg; Sigma–Aldrich). Both renal pedicles were clamped using microaneurysm clamps for 30 min to induce renal IRI. For sham operations, both renal pedicles were exposed but not clamped. The body temperature was maintained at 37°C during the entire surgery. FG-4592/HEK293-EVs (200 μg) or PBS were intravenously injected into mice at 0 and 24 h after reperfusion. The mice were sacrificed at 48 h after reperfusion. For assessing the survival rate, the time of death of each of the mice was recorded by one researcher who was blinded to the assignment.
### Kidney Pathology

Kidneys were fixed with 4% formaldehyde overnight, then embedded in paraffin, and sectioned to a thickness of 4 μm. The sections were stained with periodic acid-Schiff, and 10 random fields were chosen to evaluate the average kidney injury scores as follows: 0, no damage; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%.

### Labeling of FG-4592/HK2-EVs and FG-4592/HEK293-EVs

To obtain DIO-labeled FG-4592/HK2-EVs and FG-4592/HEK293-EVs, HK2 and HEK293 cells were treated with DIO (Invitrogen) for 2 h before FG-4592 stimulation and then washed with PBS to remove the free dye. After FG-4592 stimulation for 24 h, DIO-labeled FG-4592/HK2-EVs and FG-4592/HEK293-EVs were isolated as described above.

### Cellular Uptake of FG-4592/HK2-EVs and FG-4592/HEK293-EVs in vitro

FG-4592/HK2-EVs or FG-4592/HEK293-EVs (20 μg) were incubated with HK2 cells under H/R condition for 12 h. Cell nuclei were stained with DAPI, and DIO-positive puncta in the cytoplasm were observed by confocal microscopy (FV1000; Olympus).

### Flow Cytometry

The rate of apoptosis of HK2 cells was determined by flow cytometric analysis using an Annexin V/PI kit (BD Biosciences, USA) following the manufacturer’s instructions. The HK2 cells were digested by trypsin without EDTA (C0205, Beyotime) and washed twice with cold PBS. Then the cells were resuspended by 500-μL 1X Binding Buffer (BD Biosciences, San Jose, CA, USA) following the manufacturer’s instructions. The HK2 cells were incubated with HK2 cells under H/R condition for 12 h. Cell nuclei were stained with DAPI, and DIO-positive puncta in the cytoplasm were observed by confocal microscopy (FV1000; Olympus).

### Immunofluorescence Staining

For immunofluorescence analysis, live cells in confocal dishes were washed twice with PBS. Then 200-μL 1X Binding Buffer (BD Biosciences) and one drop of Hoechst (Thermo Fisher Scientific) were added into each dish. After incubated in dark for 10 min at 37°C, the cells were washed with PBS. Then 200-μL 1X Binding Buffer (BD Biosciences) and 5-μL PI (BD Biosciences) were added into each dish. After incubated in dark for 10 min at 37°C, the cells were washed again with PBS. Then cells were covered by 200-μL RPMI 1640 (without phenol red) and visualized under a confocal microscope (FV1000; Olympus).

### ELISAs

The levels of interleukin-6 (IL-6) in the supernatants and kidney tissue homogenates were measured using ELISA kits (Absin, China). 100 μL different concentrations of standard and experimental samples were added to each well of IL-6 microplate before incubated for 2 h in RT (25°C). Then the plate was washed with the washing buffer for 3 times. 100-μL antibody was then added and incubated for 2 h in RT (25°C). Then the plate was washed for 3 times again. After that, 100-μL SA-HRP was added and incubated in the dark in RT (25°C) for 20 min. Then we washed the plate and added 100-μL chromogen solution. After putting in the dark in RT (25°C) for 20 min, each well was added with 50-μL stop solution. The value of OD was detected at 450 nm. The concentration of each sample was calculated referring to the standard curve.

### qPCR Assay

Total RNA was extracted from cells or kidney tissues using TRIzol (Takara). Next, cDNA was synthesized from the RNA using a PrimeScript RT reagent kit (Takara). RT-qPCR was performed using a 7,300 real-time PCR system (Applied Biosystems). The primer sequences are listed as below (Table 1).

### Western Blotting

Western blotting was performed as previously described [15]. Cells or tissues were lysed by the RIPA lysis buffer (Thermo Scientific). The protein content was detected by a BCA protein assay kit (Thermo Fisher Scientific). Then, the protein samples were separated by SDS-PAGE (Genscript) and transferred onto polyvinylidene difluoride membranes (Millipore). Western Quick Block Kit (Genscript) was used for blocking. The membranes were incubated with primary antibodies overnight in 4°C, then washed and incubated with secondary HRP-conjugated antibodies for 1 h in RT (25°C). The signals were detected using an enhanced chemiluminescence advanced system (GE Healthcare). The following primary antibodies were used: anti-Alix (sc-53540; Santa Cruz Biotechnology), anti-CD63 (sc5275; Santa Cruz Biotechnology), anti-TSG101 (ab125011; Abcam), anti-HIF-1a (ab2185; Abcam), anti-HIF-1a (ab179483; Abcam), anti-NF-kB p-p65 (3033; Cell Signaling Technology), anti-KIM-1 (AF1817; R&D Systems), and anti-β-actin (ZF2001; ZFanti). HRP-conjugated anti-mouse immunoglobulin G (IgG), anti-rabbit IgG, and anti-goat IgG (Abcam) were used as secondary antibodies.

### Table 1. The primer sequences

| Gene     | Forward                          | Reverse                          |
|----------|----------------------------------|----------------------------------|
| β-Actin-HOMO | CTCATCGCTAGTGAAGATGCTCCACCGA    | TCCCTTAATTGCAGCAGCATGGATT       |
| CCL-2-HOMO  | CTTGGGTGTGTTGAGTGAGGTT          | AGCAGAAAGTGGTGTCCTGAGATT        |
| TNF-α-HOMO | CGAAGTGGTGTGCTGTTGCTT           | CCCGACTTCTCATGCATTGGCC          |
| IL-6-HOMO  | GCTCTGGCTTGTTCCTCCTCA           | AATCTACCTCGTCTTTTGAGG           |
| β-Actin-MUS | GGGAAATCTCGTGCTGTCGTA           | AGGGCTGAAAGAAGCGCT              |
| CCL-2-MUS  | TGTAGGGTGGTGTTGAAAAAGG          | GTGCTGACCCCCCAAGAGAAAT          |
| TNF-α-MUS  | AGACAGCGAGCGAATCGTGCA           | GCACCCACATACGAGCTCAA           |
| IL-6-MUS   | GTACCAGCAGCAGTTCAGCG          | CCACCAAGAGACGATGCAA            |

CCL-2, chemokine (C C motif) ligand 2; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.
Fig. 1. Characterization of FG-4592/EVs. a Western blotting analysis of HIF-1α (n = 3). b Western blotting analysis of HIF-1α (n = 3). c Western blotting analysis of EV-associated markers (Alix, CD63, and TSG101). d Representative transmission electron microscopy images of EVs (scale bar, 200 nm). e Size distribution analysis of EVs by NTA. f Representative images of DIO-labeled EVs in HK2 cells under confocal microscopy (scale bar, 50 μm). Data are presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA. ANOVA, analysis of variance.
Results

Preparation and Characterization of FG-4592/EVs

Western blotting showed that the expression of HIF-1α in HK2 cells was increased in a dose-dependent manner after treatment with FG-4592 for 24 h under normoxia (shown in Fig. 1a). The cell supernatant was concentrated and ultracentrifuged to isolate FG-4592/HK2-EVs. Western blotting confirmed the expression of EV markers such as Alix, CD63, and Tsg101 in FG-4592/HK2-EVs (shown in Fig. 1c). Double-layer cup-shaped membrane vesicles were observed by transmission electron microscopy (shown in Fig. 1d). The mean diameter of FG-4592/HK2-EVs was 128.6 nm, as detected by NTA (shown in Fig. 1e). Substantial FG-4592/HK2-EVs labeled by DIO were observed in the cytoplasm of HK2 cells treated with H/R under a confocal laser scanning microscope, which proved FG-4592/HK2-EVs could be taken into cells effectively (shown in Fig. 1f). To verify the universality of FG-4592/EVs function, we used FG-4592 to treat HEK293 cells for 24 h to obtain FG-4592/HEK293-EVs. Similarly, FG-4592 induced HIF-1α expression in HEK293 cells in a dose-dependent manner, as shown by western blotting (shown in Fig. 1b). The expression of Alix, CD63, and Tsg101 was also detected (shown in Fig. 1c). The saucer-like double-layer membrane structure was observed by transmission electron microscopy (shown in Fig. 1d). The average particle size of FG-4592/HEK293-EVs was 130.2 nm, as measured by NTA (shown in Fig. 1e). Using a confocal laser scanning microscope, we observed that HK2 cells could effectively take up FG-4592/HEK293-EVs (shown in Fig. 1f).

FG-4592/HK2-EVs Alleviate H/R-Induced HK2 Damage

We showed that FG-4592 could induce the expression of HIF-1α under normoxia (shown in Fig. 1a, b) and hypoxia (shown in online suppl. Fig. 1a; for all online suppl. material, see www.karger.com/doi/10.1159/000522584) in a dose-dependent manner in HK2 cells. Western blotting showed that FG-4592/HK2-EVs could downregulate the expression of pp65 when the concentration of FG-4592 was 30 μM (shown in Fig. 2a). FG-4592/HK2-EVs also significantly inhibited the release of IL-6, as evaluated by ELISA (shown in Fig. 2b). Furthermore, the cell morphology under light microscopy in the FG-4592/HK2-EV treated group was well preserved compared to that in the H/R group (shown in Fig. 2c). PI fluorescence staining showed that apoptosis induced by H/R was ameliorated by FG-4592/HK2-EVs (shown in Fig. 2d), which was also confirmed by Annexin V/PI flow cytometry (shown in Fig. 2e). In addition, FG-4592/HK2-EVs significantly downregulated the expression of HIF-1α induced by H/R, indicating that hypoxia was ameliorated (shown in Fig. 2f). Our findings indicated that FG-4592/HK2-EVs could inhibit apoptosis and inflammation induced by H/R. Furthermore, it is worth noticing that FG-4592 alone showed no significant effects on the mRNA expression of pro-inflammatory factors in H/R-treated cells (shown in online suppl. Fig 1b).

FG-4592/HEK293-EVs Alleviate HK2 Damage Induced by H/R

Furthermore, we used FG-4592 to treat HEK293 cells for 24 h to obtain FG-4592/HEK293-EVs to verify the universality of FG-4592/EV function. The morphology of cells treated with FG-4592/HEK293-EVs viewed under a light microscope was well preserved compared to the H/R group, which was similar to FG-4592/HK2-EVs (shown in Fig. 3a). PI fluorescence staining showed that treatment with FG-4592/HEK293-EVs reduced apoptosis (shown in Fig. 3b, c). Furthermore, FG-4592/HEK293-EVs significantly downregulated the expression of chemokine (C C motif) ligand 2, IL-6, and tumor necrosis factor-α at the mRNA level (shown in Fig. 3d) and inhibited the activation of pp65 (shown in Fig. 3e) and ameliorated the secretion of IL-6 (shown in Fig. 3f). FG-4592/HEK293-EVs also inhibited the expression of HIF-1α induced by H/R, indicating that hypoxia was ameliorated (shown in Fig. 3g). This demonstrated that FG-4592/HEK293-EVs could also inhibit H/R-induced apoptosis and inflammation.

Fig. 2. FG-4592/HK2-EVs alleviate HK2 damage induced by H/R.

a Western blot analysis of p-p65 (n = 3).
b ELISA analysis of IL-6 in supernatant (n = 3).
c Morphology of HK2 under light microscope (scale bar, 50 μm).
d Immunofluorescence staining analysis of PI under confocal microscope (n = 3).
e Flow cytometry analysis of Annexin V/PI (n = 3).
f Western blotting analysis of HIF-1α (n = 3). Data are presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus H/R group, one-way ANOVA, ANOVA, analysis of variance. (For figure see next page.)
Therapeutic Effect of EVs Simulated by HIF-PHI on AKI

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FG-4592/HEK293-EVs Protect against Renal IRI in Mice

A murine model of IRI was established to evaluate the therapeutic efficacy of FG-4592/HEK293-EVs. FG-4592/HEK293-EVs were administered intravenously at 0 h and 24 h after reperfusion (shown in Fig. 4a). We observed that FG-4592/HEK293-EVs treatment significantly raised the survival rate (shown in Fig. 4b), and alleviated the serum creatinine of AKI mice (shown in Fig. 4c), as well as reduced pathological damage, including tubular epithelial cell (TEC) necrosis, shedding, accumulation of cell debris, and cast formation in kidneys (shown in Fig. 4d), and downregulated the expression of the tubular injury marker KIM-1 (shown in Fig. 4e). FG-4592/HEK293-EVs treatment significantly reduced the mRNA levels of chemokine (C C motif) ligand 2, IL-6, and tumor necrosis factor-α (shown in Fig. 4f). Also, FG-4592/HEK293-EVs inhibited IRI-induced HIF-1α expression in vivo (shown in Fig. 4h). These results indicated that FG-4592/HEK293-EVs could significantly protect against IRI-induced kidney damage and inflammation.

Discussion

In the present study, we demonstrated that treatment with FG-4592/EVs alleviated apoptosis, inhibited the expression of pp65, and reduced the release of IL-6, exerting a remarkable therapeutic effect on H/R-induced damage in vitro. In addition, treatment with FG-4592/EVs after IRI significantly improved the survival rate and renal function of AKI mice, and alleviated kidney injury and inflammation, suggesting that FG-4592/EVs could be used as a new and safe type of nano-therapy for AKI.

Studies have found that activation of HIF-1α in early stage of AKI could promote kidney repair [16]. In addition, the activation of HIF-1α induced by hypoxia could promote the secretion of exosomes in tubular cells. And those exosomes could reduce H/R-induced TEC apoptosis, which may become a new type of nano-therapy for AKI [17]. Our study showed that the expression of HIF was upregulated by FG-4592 in a dose-dependent manner in HK2 and HEK293 cells under normoxia, and EVs secreted by HIF-activated cells had a therapeutic effect on AKI.

Renal TECs make up a significant portion of the kidney and are vulnerable to diverse injuries during AKI [18]. Recent research studies have shown that after injury, TEC can produce various bioactive molecules that drive interstitial inflammation and fibrosis, thereby becoming the core driver in the transition of AKI to CKD [19–21]. Our research found that FG-4592/EVs treatment could alleviate apoptosis of tubular cells, as well as inhibit the activation of NF-kb pathway induced by hypoxia, which reduced the release of pro-inflammatory factors such as IL-6 from injury tubular cells, thereby exerting a therapeutic effect on AKI.

FG-4592 can reduce HIF degradation by inhibiting PHD activity [22]. Studies showed that pretreatment of FG-4592 before injury could ameliorate AKI by upregulating the expression of HIF-1α in TEC, suggesting that HIF-PHI could be used to prevent AKI [23, 24]. However, when the damage has occurred, activation of HIF-1α by HIF-PHI after injury did not show the protection effect on AKI [23, 25]. In addition, HIF has multiple target

Fig. 3. FG-4592/HEK293-EVs alleviate HK2 damage induced by H/R. a Morphology of HK2 under light microscope (scale bar, 50 μm). Immunofluorescence staining analysis of PI under confocal microscope (n = 3) (b, c). d Real-time PCR analysis of inflammatory cytokine mRNA levels (n = 3). e Western blotting analysis of p-p65 (n = 3). f ELISA analysis of IL-6 in supernatant (n = 3). g Western blotting analysis of HIF-1α (n = 3). Data are presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus H/R group, one-way ANOVA. ANOVA, analysis of variance; TNF-α, tumor necrosis factor-α; CCL2, chemokine (C C motif) ligand 2.

Fig. 4. FG-4592/HEK293-EVs protect against renal IRI injury. a Schematic diagram of the experimental design. In brief, mice were treated with FG-4592/HEK293-EVs (200 μg) or vehicle at 0 and 24 h after IRI and were sacrificed at 48 h after disease induction. b Effects of FG-4592/HEK293-EVs on survival rate (n = 7). c Effects of FG-4592/HEK293-EVs on serum creatinine at 24 and 48 h after IRI (n = 6). d Representative images of PAS staining of renal cortex and the quantification of tubular injury based on PAS staining (n = 6). Scale bar, 50 μm and 100 μm. e Western blotting analysis of KIM-1 in kidney tissues (n = 5). f Real-time PCR analysis of inflammatory cytokine mRNA levels in kidney (n = 6). g ELISA analysis of IL-6 in kidney tissues (n = 6). h Western blotting analysis of HIF-1α in kidney tissues (n = 3). Data are presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001 versus IRI group, one-way ANOVA. PAS, periodic acid-Schiff; ANOVA, analysis of variance; TNF-α, tumor necrosis factor-α; CCL2, chemokine (C C motif) ligand 2.

(For figure see next page.)
**Figure 1. A:** FG-4592/HEK239-EVs: 200 μg Tail vein injection

**Figure 2.**

**A:** Shown is the survival curve of mice after IRI and treatment with FG-4592/HEK293-EVs. **B:** The probability of survival for mice after IRI and treatment with FG-4592/HEK293-EVs. **C:** Serum creatinine levels in different groups. **D:** Light microscopy images of kidney sections from different groups. **E:** Western blot analysis of Kim-1 and β-Actin expression. **F:** mRNA levels of CCL2, IL-6, and TNF-α in different groups. **G:** IL-6 protein levels in different groups. **H:** Western blot analysis of HIF-1α and β-Actin expression.
genes, some of which are detrimental to the recovery of AKI. According to our previous study, high-dose HIF-PHI treatment could promote tubulointerstitial fibrosis by activating HIF-1α-KLF5-TGF-β1 signaling [13]. We proved that FG-4592/EVs have significant advantages compared to HIF-PHI. Even if the injury has occurred, FG-4592/EVs treatment could still effectively alleviate AKI. Furthermore, compared with the H/R and IRI groups, FG-4592/EVs could downregulate the expression of HIF-1α, which may reduce the possible side effects caused by extensive transcription of target genes of HIF.

Notably, like a double-edged sword, EVs derived from HIF-activated cells may have a complicated influence on AKI. There is evidence that hypoxic preconditioning triggers HIF-1α expression in tubular cells and promotes the secretion of EVs, which have the potential to protect against ischemic AKI and septic AKI [11, 17]. On the other side, hypoxia-induced EVs have been shown containing miR-23a and TGF-β mRNA [26, 27], leading to tubulointerstitial inflammation and fibrosis by activating macrophages and fibroblasts, respectively, which gives rise to the safety concern in therapeutic application of these vesicles. In the present study, we found that in addition to hypoxia stimulation, FG-4592 could effectively upregulate HIF-1α expression in tubular cells under normoxia, and EVs derived from FG-4592-treated cells significantly alleviated IRI-induced renal injury and inflammation. Our previous study has confirmed that a gentle HIF activation is beneficial in improving renal function and fibrosis [8, 28, 29]. Our previous study has confirmed that a gentle HIF activation is beneficial in improving renal function and fibrosis. However, when HIF is excessively activated in TECs, HIF-1α-KLF5-TGF-β1 signaling would be activated, thereby promoting tubulointerstitial fibrosis [13]. This indicates that a low activation level of HIF may increase protective effect in cells, while over-activation of HIF may cause extensive transcription of pro-inflammatory or pro-fibrosis genes, which could be shuttled into EVs and consequently influence EVs’ function. Our study suggests that FG-4592 can serve as a feasible way to activate HIF in an appropriate degree to generate EVs with renoprotective effects.

This study demonstrated that EVs derived from HK2 or 293 cells after FG-4592 treatment could alleviate H/R-induced apoptosis and inflammation in vitro. In addition, treatment with FG-4592/EVs significantly improved the survival rate and renal function of mice with IRI by ameliorated renal tubular injury and inflammation. Our study may provide a novel insight about FG-4592 in alleviating AKI.

**Statement of Ethics**

The animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of Jiangsu Province (Jiangsu Government Publication No. 45, revised, 2008).

**Conflict of Interest Statement**

Bi-Cheng Liu is one of Editorial Board Member of Kidney Diseases. Other authors have no conflicts of interest to declare.

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**Author Contributions**

Z.-Y.D., T.-T.T., L.-L.L., and B.-C.L. designed the study; Z.-Y.D. and T.-T.T. carried out experiments; Z.-Y.D., Z.-L.L., and J.-Y.C. analyzed the data; Z.-Y.D. and T.-T.T. made the fig.; Z.-Y.D., T.-T.T., Y.W., B.W., and B.-C.L. drafted and revised the paper; all authors approved the final version of the manuscript.

**Data Availability Statement**

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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