Supplementary Materials for

Reducing Ischemia Kidney Injury Through the Application of a Synchronization Modulation Electric Field to Maintain Na^+/K^+-ATPase Functions

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Materials and Methods

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Materials and Methods

Composition of the solutions

Relaxing solution: 120mM Potassium Glutamate, 5mM K_2PIPES, 1mM MgSO4, 0.1mM K_2EGTA.

External solution: 3.5mM 3,4-Diaminopyridine (3,4-DAP), 86mM NaCl, 10mM CsCl, 4mM KCl, 2.15mM Na_2HPO_4, 0.85mM $NaH_2PO_4 \cdot H_2O$, 1.8mM $CaCl_2$,1.5mM $BaCl_2 \cdot 2H_2O$, 1μM Tetrodotoxin (TTX). The pH of the solution was adjusted to 7.10 via HCl at room temperature. Final $[K]_o$ was ~4mM and $[Na]_o$~90mM.

Internal solution (at physiological condition): 58mM K-glutamate, 6.8mM $MgSO_4 \cdot 7H_2O$, 5mM MOPS, 20mM EGTA, 10mM CsOH, 4mM Na_2-Creatine phosphate, 5mM 5'-ATP-K_2. The pH of the solution was adjusted to 7.30 via KOH at room temperature.

Washing-out solution (Internal solution lack of ATP molecules): 68mM K-glutamate, 6.8mM MgSO4, 5mM MOPS, 20mM EGTA, 4mM K2HPO4, 10mM CsOH, 200nM Antimycin A. The pH of the solution was adjusted to 7.30 via KOH at room temperature.

MOPS: 3-(N-Morpholino) propanesulfonic acid; EGTA: Ethylene glycol-bis (2-aminoethylether)- N,N,N′,N′-tetraacetic acid.

Urinary flow and Na+ excretion under the physiological conditions

Sprague-Dawley rats were anesthetized with 70 mg/kg inactin (i.p.) and 50 mg/kg ketamine (i.m.) and then placed on a temperature-controlled table with the body temperature maintained at 37 °C throughout the experiment. The surgical procedure was similar as described previously (*65, 66*). Both the left and right ureters were catheterized

with PE-50 tubing to collect urine from each kidney separately. For the urinary flow and Na^+ excretion in response to 3rdgen-SMEF under physiological conditions, the decelerating 3rdgen-SMEF was applied to one kidney using the electrode arrays and the other kidney as control. After a 30-min equilibration period, the urine was collected in Eppendorf tubes for 30 minutes as baseline. Then, the decelerating 3rdgen-SMEF was turned on and urine was collected for another 30 minutes. The decelerating 3rdgen-SMEF was then switched off and the urine was collected for another 30 minutes from both kidneys. Sodium concentrations were measured with a Flame Photometer (BWB Technologies).

Urinary flow in response to acute volume challenge

Acute volume expansion was performed as we previously reported (*57, 58*). Briefly, SD rats were anesthetized with inactin (70 mg/kg, i.p.) and ketamine (50 mg/kg, i.m.). The carotid artery was cannulated to monitor the mean arterial pressure (MAP). Saline (0.9%NaCl) with 2% bovine serum albumin (BSA) was intravenously infused at a rate of 6 ml/hour through the jugular vein. The accelerating-3rdgen-SMEF was applied to both kidneys throughout the experiment with the frequency increased from 50 Hz to 300Hz. The potential difference applied between each pair of electrodes was 100 mV.

After 30-min equilibrium, the urine samples (collected from both ureter catheters) were collected continuously for 30 minutes before acute volume expansion as the baseline. Then the rats were given an acute volume challenge by an intravenous bolus of 0.9% normal saline (0.9%NaCl, 5% body weight, i.v.) using an infusion pump. For the treatment group, the accelerating-3rdgen-SMEF was simultaneously turned on. The group of rats without the application of electric field served as controls. Urine samples were collected at 30 min intervals for 90 minutes after acute volume expansion for both control and treatment groups. Then the accelerating-3rdgen-SMEF was turned off and the urine samples were collected for additional 60 minutes. At the end of the experiment, both kidneys were removed, drained, and weighed.

Plasma creatinine (Pcr) and Neutrophil gelatinase-associated lipocalin (NGAL) measurement

For Pcr concentration measurements, blood (20μl) was collected into a heparinized micro capillary tube from the tail vein and was immediately centrifuged to separate blood

cells from plasma. Plasma (10µl) was collected into an Eppendorf tube and stored in a -80°C freezer until analyzed. Pcr concentrations were measured with HPLC-mass spectrometry at O'Brien Center at the University of Alabama at Birmingham.

Plasma NGAL levels were measured with an ELISA kit (R&D Systems, MLCN20) following the manufacturer's instructions.

Glomerular filtration rate (GFR) measurement in conscious mice

GFR was measured in conscious C57BL/6J mice using a single bolus intravenous injection of fluorescein isothiocyanate-sinistrin (FITC-sinistrin) (Fresenius Kabi Austria GmbH) as described recently with a modification (*62, 63*). Briefly, the FITC-sinistrin solution (4 µl/g body weight) was injected via retro orbital sinus of the mice under light anesthesia with isoflurane. Blood was collected (about 5 µl/each) from a small nick at the end of the tail into a heparinized microcapillary tube at 3, 7, 10, 15, 35, 55, 75 and 90 min after injection. The blood samples were centrifuged, and plasma fractions (2 µl/each) were collected. FITC-sinistrin fluorescent intensities of the plasma samples were measured using a plate reader (Cytation5, BioTek) with 485-nm excitation and 538-nm emission. GFR was calculated using a two-compartment model of two-phase exponential decay (GraphPad Prism) and was presented as microliters per minute (µl.min-1).

Histology, Immunofluorescence and TUNEL staining on kidney tissue sections

Paraffin embedded kidney samples were sectioned into 4-µm slices. Histological morphology and the degree of renal tubular injury were evaluated with periodic acid-Schiff (PAS) staining and interstitial fibrosis was assessed with Masson Trichrome staining as previously described (*62, 63*). For statistical analysis, at least 10 visual fields of each specimen were randomly selected and photographed under the microscope (200×, Olympus BX53), and the positive areas were statistically analyzed with Fiji/ImageJ.

Tubular apoptosis was assessed by Terminal dUTP Nick-End Labeling (TUNEL) staining with *in Situ* Cell Death Detection Kit (Roche, catalog no. 11684795910) according to manufacturer's instructions. TUNEL-positive cells/nuclei are quantified as the percentage (%) of TUNEL and DAPI double-positive cells relative to total cells (DAPI-positive cells). For statistical analysis, 10 visual fields of each specimen were randomly selected and photographed with fluorescence microscope (Keyence BZ-X710) and analyzed with Fiji/ImageJ.

For the immunofluorescence, the kidney slices were subjected to antigen retrieval with a sodium citrate buffer and permeabilized with 0.3% Triton-X 100 in PBS, blocked with 5% normal goat serum, and probed with the primary antibody CD31 (Abcam, ab182981). After washing, the tissue sections were probed with a fluorophore-conjugated secondary antibody Donkey Anti-Rabbit IgG H&L (Abcam, ab150075). Then, the tissue sections were photographed with fluorescence microscope (Keyence BZ-X710) and analyzed with Fiji/ImageJ. Five images per kidney were acquired and the CD31 density (vessel density) index was calculated by dividing the CD31-positively stained area by the kidney area.

All morphometric analyses were performed by an experienced renal pathologist (L. Fu) blinded to the experimental procedures.

Western blotting

Western blotting was used to confirm the purity of isolated mitochondria protein. Mitochondria protein extracts from kidney tissue (30 μg per lane) were separated on a 7.5 % SDS-PAGE gel as described previously (*66, 67*). After blocking for 1 hour at room temperature with Odyssey Blocking buffer-TBS, the membranes were incubated overnight with GAPDH and ATP5A antibodies (ab8245 and ab14748, Abcam,

respectively) at 4°C. The membranes were then incubated with Goat anti-Mouse IgG H&L (IRDye® 800CW) secondary antibody (ab216772, Abcam) and imaged on an Odyssey Imager (Li-Cor).

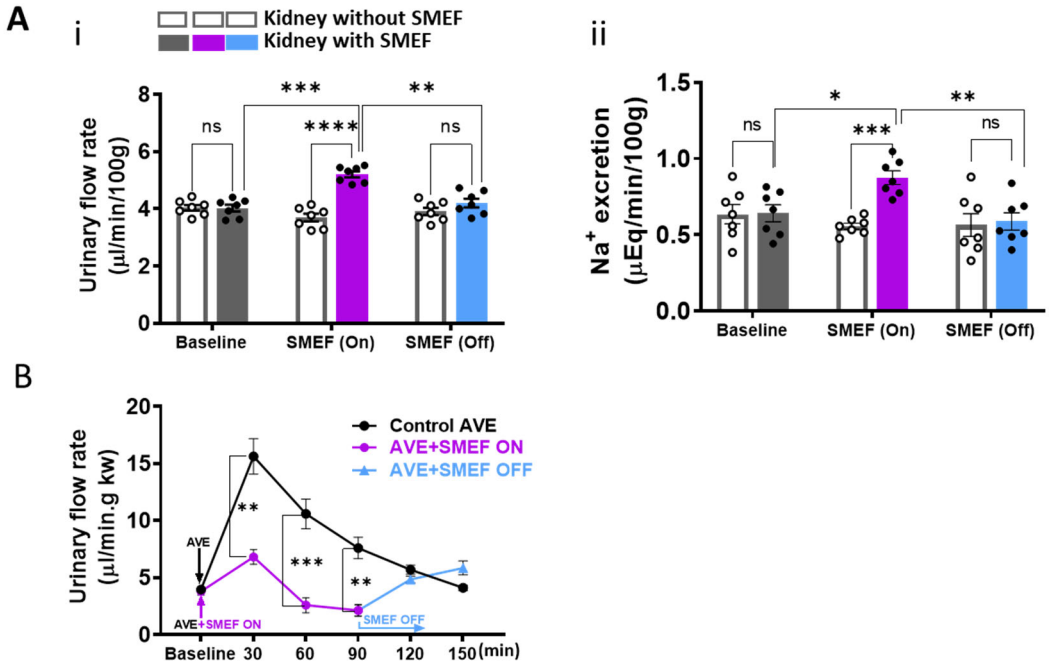

Figure S1. Renal tubular sodium handling is modulated by the application of *decelerating-* and *accelerating-3rdgen-SMEF in vivo.* The effects of *3rdgen-SMEF* modulation of Na/K Pump activity on sodium handling under basal conditions and during a volume challenge were evaluated *in vivo*. **A** shows the urinary flow and sodium excretion rates before, during, and after application of *decelerating-3rdgen-SMEF*, which was only applied to the left kidney (the right served as a control). **A.i** represents urinary flow rates, while **A.ii** represents urinary sodium excretion rates. Prior to applying *decelerating-3rdgen-SMEF*, urine flow and sodium excretion rates were essentially the same in each kidney. Application of the *decelerating-3rdgen-SMEF* was associated with an increase in both parameters only in the treated kidney. When the *deaccelerating-3rdgen-SMEF* was turned off, all the measured factors returned to basal levels. Data are shown as mean ± s.e.m. Repeated measures ANOVA was performed (Asterisks represent an analysis with Bonferroni's multiple comparison test. n=7 rats, *p<0.1; **P < 0.01; ***P < 0.001; ****P<0.0001;). **B** illustrates renal responses to acute volume expansion in the presence and absence of *accelerating-3rdgen-SMEF* (note that 3rdgen-SMEF was applied to both kidneys in these experiments). Basal urinary flow rates were similar in both groups. Acute volume expansion caused a rapid and profound increase in urine flow that peaked within 30 minutes. The flow gradually decreased over the next 120 minutes as the volume load from the saline bolus was excreted. The animals in which

accelerating-3rdgen-SMEF was applied did not see this increase. Urine flow rates had dipped returned to baseline after 30 minutes. Upon turning off the *accelerating-3rdgen-SMEF,* the urine flow rate rapidly rose to above basal values and remained elevated for the duration of the experiments. Data are shown as mean ± s.e.m. The urinary flow rates measured when the accelerating-3rdgen-SMEF was on were compared to the control group by paired t-test (**P < 0.01; ***P < 0.001; ****P<0.0001; n=5/group)

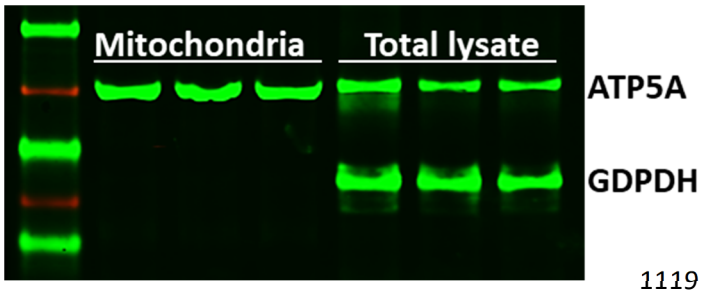

Fig. S2. Isolated mitochondria. A representative western blot of isolated mitochondria and total tissue lysate. Mitochondrial membrane ATP synthase 5antibody was used as a marker for mitochondria. For the detection of potential contaminations, antibody against GAPDH was also used.

Figure. S3 Representative image for the application of the *accelerating-3rdgen-SMEF* on discarded human donor kidneys. The paired control and treated kidneys were from the same kidney donor. As showed in the image, the left kidney was applied with *accelerating-3rdgen-SMEF* and the right kidney from the same donor was as untreated control (n=5 pairs of kidney grafts from 5 kidney donors). The total storage time was 24 hours.

Data file S1. Raw data for all figures where n is less than or equal to 20, including data for Fig. 1D, Fig. 2Cii-iii and Diii, Fig. 3A, B and C i-iv, Fig. 4A, B, C, D and F, Fig. 5A, B, C and E, Fig. 6B and fig. S1 A and B.

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