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Early Detection of Drug-Induced Renal Hemodynamic Dysfunction Using Sonographic Technology in Rats

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Abstract:	The kidney normally functions to maintain hemodynamic homeostasis and is a major site of damage caused by drug toxicity. Drug-induced nephrotoxicity is estimated to contribute to 19- 25% of all clinical cases of acute kidney injury (AKI) in critically ill patients. AKI detection has historically relied on metrics such as serum creatinine (sCr) or blood urea nitrogen (BUN) which are demonstrably inadequate in full assessment of nephrotoxicity in the early phase of renal dysfunction. Currently, there is no robust diagnostic method to accurately detect hemodynamic alteration in the early phase of AKI while such alterations might actually precede the rise in serum biomarker levels. Such early detection can help clinicians make an accurate diagnosis and help in in decision making for therapeutic strategy. Rats were treated with Cisplatin to induce AKI. Nephrotoxicity was assessed for six days using high-frequency sonography, sCr measurement and upon histopathology of kidney. Hemodynamic evaluation using 2D and Color-Doppler images were used to serially study nephrotoxicity in rats, using the sonography. Our data showed successful drug-induced kidney injury in adult rats by histological examination. Color-Doppler based sonographic assessment of AKI indicated that resistive-index (RI) and pulsatile-index (PI) were increased in the treatment group; and peak-systolic velocity (mm/s), end-diastolic velocity (mm/s) and velocity-time integral (VTI, mm) were decreased in renal arteries in the same group. Importantly, these hemodynamic changes evaluated by sonography preceded the rise of sCr levels. Sonography-based indices such as RI or PI can thus be useful predictive markers of declining renal function in rodents. From our sonography-based

	observations in the kidneys of rats that underwent AKI, we showed that these noninvasive hemodynamic measurements may consider as an accurate, sensitive and robust method in detecting early stage kidney dysfunction. The study also underscores the importance of ethical issues associated with animal use in research.
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Sonography, real-time imaging, non-invasive methodology, renal toxicity assessment, ethical use of animals in research, drug-induced nephrotoxicity, hemodynamics

SHORT ABSTRACT:
Early stage hemodynamic dysfunction is critical to the development of kidney disease. Yet, detection methodologies are limited. Recent advances in sonography provide a noninvasive, accurate option for early detection of kidney injury. This study outlines a step-by-step, sonographic methodology for detecting kidney dysfunction using a drug-induced nephrotoxicity rat model.

LONG ABSTRACT:
The kidney normally functions to maintain hemodynamic homeostasis and is a major site of damage caused by drug toxicity. Drug-induced nephrotoxicity is estimated to contribute to 19-25% of all clinical cases of acute kidney injury (AKI) in critically ill patients. AKI detection has historically relied on metrics such as serum creatinine (sCr) or blood urea nitrogen (BUN) which are demonstrably inadequate in full assessment of nephrotoxicity in the early phase of renal dysfunction. Currently, there is no robust diagnostic method to accurately detect hemodynamic alteration in the early phase of AKI while such alterations might actually precede the rise in serum biomarker levels. Such early detection can help clinicians make an accurate diagnosis and help in in decision making for therapeutic strategy. Rats were treated with Cisplatin to induce AKI. Nephrotoxicity was assessed for six days using high-frequency sonography, sCr measurement and upon histopathology of kidney. Hemodynamic evaluation using 2D and Color-Doppler images were used to serially study nephrotoxicity in rats, using the sonography. Our data showed successful drug-induced kidney injury in adult rats by histological examination. Color-Doppler based sonographic assessment of AKI indicated that resistive-index (RI) and pulsatile-index (PI) were increased in the treatment group; and peak-systolic velocity (mm/s), end-diastolic velocity (mm/s) and velocity-time integral (VTI, mm) were decreased in renal arteries in the same group. Importantly, these hemodynamic changes evaluated by sonography preceded the rise of sCr levels. Sonography-based indices such as RI or PI can thus be useful predictive markers of declining renal function in rodents. From our sonography-based observations in the kidneys of rats that underwent AKI, we showed that these noninvasive hemodynamic measurements may consider as an accurate, sensitive and robust method in detecting early stage kidney dysfunction. The study also underscores the importance of ethical issues associated with animal use in research.

INTRODUCTION:
Serum creatinine (sCr) has been the gold standard metric to assess kidney function for more than two decades. Recently, many studies have reported that renal injury occurs much earlier than the changes in sCr[1]. However, there are no robust methods for detection of hemodynamic changes that occur early in the course of renal injury including drug-induced nephrotoxicity.

Drug-induced acute renal hemodynamic dysfunction leads to renal tissue damage and further progression to renal failure[2,3]. In the past couple decades, studies indicate that imaging tools such as computed tomography (CAT), Functional magnetic resonance imaging (fMRI) and sonography play a role in hemodynamic assessment[4]. In the current imaging tools, gray scale

sonography coupled with Color-Doppler techniques, are the most commonly used to establish and assess anatomical status of kidney[3,5,6]. Sullivan et. al. and Bonnin et. al. recently reported that sonography is an effective, powerful and non-invasive tool in analysis hemodynamic changes in vasoconstriction and hypoxia stress animal models[7,8]. This technique is also commonly used to detect arterial stenosis[9,10].

Latest technical advances in the field of high-resolution ultrasound imaging have allowed investigators to address cardiovascular toxicity using high-frequency (25-80 MHz) and high-resolution (< 0.03 mm resolution) probes, *in vivo*[11]. We hypothesize that using this high-resolution sonography to study kidney will provide an unprecedented opportunity for a non-invasive and sensitive method for early detection of nephrotoxicity.

Cisplatin is used to treat testicular, ovarian, bladder, head, lung, and neck cancers in combination with other drugs[12-14]. Cisplatin has had well-documented nephrotoxicity due to cell necrosis of proximal tubules (PT) and collecting ducts resulted in rising blood urea nitrogen (BUN) and sCr[15]. Herein, we provide a detailed step-by-step methodology of using non-invasive renal sonography to characterize kidney dysfunction using the rat model of Drug (Cisplatin)-induced nephrotoxicity.

PROTOCOL:

Perform all procedures in male Sprague Dawley rats purchased from Charles River Laboratories in accordance with American Veterinary Medical Association (AVMA) guidelines and using approved Institutional Animal Care and Use Committee (IACUC) protocols.

1. Animal preparation and surgical procedures

1.1) Acclimate all animals for one week before any experimental procedure.

1.2) Anesthetize animal using isoflurane (2-3% to induce, and 1.0% to maintain) and apply eye ointment to both eyes to prevent desiccation, irritation or ulceration.

1.3) Remove hair from the animal's chest using #40 blade and depilatory cream as necessary.

2. Nephrotoxicity rat model

2.1) For Cisplatin-induced nephrotoxicity model, administer Cisplatin, using protocol as described previously [15]

2.2) Perform sonography at baseline, 24 hours prior to Cisplatin administration (Day 0). (See step 3. Imaging Protocol)

2.3) Randomize rats (n=6) into two groups. At Day 1, administer Cisplatin (10mg/ml) (10mg/Kg body weight, single dose nephrotoxicity induction), intraperitoneally in study group and normal saline (NS) in control group.

2.4) Anesthetize animal as step 1.2 at 24, 48, 72, 96, 120, 144 hours after Cisplatin administration.

2.5) Take image using high resolution ultrasound system (See Materials and Equipment Table) under stable anesthesia stage of animal. Continue to monitor the animal's basic physiological function during imaging from anesthesia induction through animal fully recovery.

2.6) Monitor animal's vital signs during imaging procedures: rat-temperature: 35.9-37.5, respiratory rate: 66-144/minute, heart rate: 250-600/minute. The optimal vital sign reading in our proposed study is: temperature: 36.5-37.0, respiratory rate: 80-100/minute, heart rate: 450-550/minute.

NOTE: Use Intravenous fluid infusion, and heating lamp to maintain animal's normal physiological condition to minimize the effects of surgery and anesthesia. Assist respiration with mechanical ventilator during the procedure if necessary. However, mechanical ventilation is rarely needed in this experiment.

3. Imaging Protocol

3.1) Transverse image of Kidney (B Mode):

3.1.1) Using MS 250 ultrasound with center frequency of 21MHz connected to the active-port, set the application preset to "General Imaging".

3.1.2) With the animal supine on the platform, position the 21 MHz ultrasound probe using the rail system, midline on animal and isolate the aorta. In this position the probe angle is 90 degrees to the left parasternal line (transverse axis) (Figure 1A.B).

3.1.3) From this position slide the platform with the animal such that the probe is now at the level of the renal artery (either left or right, can image one at a time).

3.1.4) By using the micromanipulators, view either the right or the left renal artery.

3.1.5) Adjust the probe angle by tilting slightly along y axis of the probe to obtain a full kidney view in the center of the screen.

3.1.6) Once the proper landmarks (renal pelvis, renal artery) as illustrated in Figure 1 panels C and D, cine store the image using the highest frame-rate allowed with the probe used.

3.2) Transverse image of Kidney (Color-Doppler view):

3.2.1) Using the Color-Doppler key on the keyboard, turn on Color Doppler acoustic window. This helps to isolate renal artery and renal vein (Figure 1D). (Blue color indicates arterial flow; and red color indicates venous flow).

3.2.2) Ensure that the focus depth (indicated by and yellow arrowhead on the Y axis) lies in the center of kidney. Record the data with cine store.

3.2.3) Make sure record the data at the highest possible frame rate possible (>200 frames/second).

3.3) Transverse image of Kidney (Pulsed-wave or PW view):

3.3.1) Click on the PW key, while in Color-Doppler mode, to bring up a yellow indicator line (Pulsed-wave Doppler sample volume) on the screen (Figure 1F).

3.3.2) Place the yellow line in the renal artery, at an angle that parallels the directionality of the flow through the vessel by using the PW angle key.

3.3.3) For flow assessment in the right renal artery, place the PW yellow indicator line along the renal artery in the direction of the flow (this is shown in blue in Figure 1 panels D and E) ensuring the Doppler angle is 60 degrees or less.

3.3.4) In this mode, the accoustic window splits up into upper and lower sections.

3.3.5) Use Cine store to capture the image of the wave forms that indicate the velocity of the arterial flow at peak systole and diastole.

4. Animal handling after imaging

4.1) From day 0 to day5, placed animal into a clean recovery area (with clean paper towel on bedding) in sternal recumbency position after imaging.

4.2) During anesthetic recovery, keep animal's body temperature with a heating lamp and monitor animal's vital sign with electrophysiological probes until animal fully recover from anesthesia.

4.3) Return recovered animals to the facility housing room when they alert and active.

4.4) Euthanize all rats according to institutional guidelines on Day 6 and harvest kidneys (see step 4.7) for histological assessment as well as step 4.5.

4.5) Collect animal's urine from collection tubes attached in the metabolic cage for creatinine test to check kidney function.

4.6) Perform paraffin section of animal kidney, and do HE (Hematoxylin and Eosin) stain to check Nephrotoxicity (see step 4.7 for detail protocol).

4.7) Sacrifice animals and exsanguinate with 0.9% NaCl solution, followed by 10% buffered formalin fixation through the left ventricle. After exsanguination with 0.9% NaCl solution, the remove the rat kidneys following histological assessment.

4.7.1) Paraffin 6-mm sections to observe the kidney morphology and nephrotoxicity. Dehydrate kidney tissue in 30% sucrose in phosphate-buffered saline (PBS) for 48 hours at 4ºC. Then fixe the sections in 10% buffered formalin for 24–48 hours at 4ºC.

4.7.2) Next, embed the kidney tissue in paraffin, and store the tissue paraffin blocks at room temperature until sectioning. Further section the tissue blocks using a paraffin section machine and place the sections on a coated glass slide.

4.7.3) Deparrafinize the section and rehydrated and stained with Hematoxylin for 10 minutes followed by Eosin for 3 minutes. Mount the sections on a slide and evaluate it by a rodent pathologist.

5. Data Calculation and Analysis

5,1) Calculate renal arterial peak velocities from the Color Doppler images obtained from step 3.2. Select Velocity Time Integral (VTI) tool to trace the peaks of systolic and diastolic velocity.

5.2) Calculate Resistive Index (RI) and Pulsatile Index (PI) using the equations below.

RI= (peak systolic velocity-end diastolic velocity)/peak systolic velocity
PI= (peak systolic velocity-end diastolic velocity)/mean velocity

5.3) Preform statistical analysis of RI and PI results with standard deviations from the average of three cycle measurements. For other standard parameters, please refer to the manuals from the manufacturer to perform data analysis using proprietary software. (see Materials and Equipment Table).

REPRESENTATIVE RESULTS:

The images presented in this study were taken by a single operator. All images were collected using a high frequency ultrasound machine (please see Materials and Equipment Table). All imaging data was analyzed by a single investigator. The results showed that Cisplatin-treated animals had sCr ranging from 0.5 to 2.1 (normal range <1.1) at day 6 (Figure 2A). However, the histological studies demonstrated consistent patterns of acute tubule interstitial injuries when compared to normal saline treated animals.

Using high-resolution ultrasound imaging to measure hemodynamic changes of kidney, data showed that there was no change of morphology in animals treated with NS between day 0 and day 6, while pulsus parvus morphology was detected in animals at day six after Cisplain treatment. The upper limit of normal RI and PI are 0.7 and 1.15, respectively, in rats [16]. Using the above indices to assess hemodynamic changes of kidney, which demonstrated that there is significant increase of RI and PI in Cisplatin-treated animals at day 6.

FIGURE LEGENDS:

Figure 1. Ultrasound apparatus settings for detecting kidney images in rats

Graphical illustrations of imaging system with the setting of animal stage (A) and imaging probe position (B) during the operation of rat kidney sonographic imaging. The sample sonographic images obtained from rat kidney using the high-frequency, high-resolution ultrasound systems (see Material and and Equipment Table). (C-F). The data demonstrate clear kidney anatomic structure and blood flow in the renal vessels with sufficient information for further hemodynamic parameter measurement and analysis.

Figure 2. Histology and Kidney sonographic images of rats under Cisplatin treatment

Serum creatinine (sCr) and histology study presents normal kidney tissue in vehicle treated rat and severe proximal tubular kidney injury (yellow arrow) in Cisplatin treated rat (A, B). sCr increased marginally after Cisplatin treatment, but remained within the normal range (<1.1). Sonographic images of right kidney of rats in Color-Doppler Mode at day 0, 3, and 6 on vehicle

and Cisplatin treated rats (C); hemodynamic parameters, RI and PI, were significant increased, assessed by Color-Doppler ultrasound (D, E). The upper limit of normal RI is 0.7 and 1.15 for PI. Importantly, the above data show those hemodynamic changes preceded the rising of sCr. Pulse wave velocity measurement show a slow and weak pulse (pulsus parvus sign, yellow circle) after Cisplatin treatment which correlate with histology study results. This phenomenon indicates renal artery stenosis, obstruction and further kidney dysfunction. Histological data showed successful drug-induced proximal tubular kidney injury and sonographic assessment showed significant changes in RI, PI and pulse wave velocity using Color-Doppler technology. N=3, *, $p<0.05$.

TABLE LEGENDS:

Table 1. Renal hemodynamic parameters for Drug-induced AKI

DISCUSSION:

In the past decade, many advancements have occurred in sonographic technology including the development of high-frequency mechanical probes, which offer sonographic data with high quality, sensitivity, and accuracy. These probes can provide approximately 50 μm axial resolution at a penetration depth of 5 to 12 mm and high frame rates (greater than 200 frame/sec), thus it can serve as a robust tool to study small animals such as rats and mice[17,18]. Furthermore, it also allows to collect sonographic images on lightly sedated or conscious animals with vital signs at physiological levels. In addition, this non-invasive modality also provides opportunity to perform longitudinal assessment of structural and functional changes during disease progression without sacrificing animals[18].

In 1959, Drs. Rusell and Brush first described the three "R" rules (Replacement, Reduction, and Refinement) to raise awareness of ethical issues in animal use in research. The proposed protocol shows for the first time that non-invasive small animal sonography can utilizes minimal number of animals under least pain, suffering or distress in Nephorotoxicity study. Therefore, it is a potential effective modality to meet the three "R' rules for experimental animals.

Many sonographic studies have focused in cardiac applications; the kidney function assessments were often derived from measurements of cardiac status rather than a direct study of kidney [19-24]. We have established an imaging methodology to visualize anatomical and functional changes in kidney in real time. We used a pre-selected set of complementary acoustic windows, gray-scale/B Mode and Color-Doppler, specific for kidney view. We used the RI and PI indices to evaluate the relationship between these indices and the changes of renal function in the Cisplatin induced toxicity model.

However, there are few challenges and limitations to the proposed imaging methods as follows: 1) Appropriate choice of anesthetic agent and the degree of anesthesia are crucial for cardiac and respiratory stability. Inconsistent physiological phenomenon (including respiratory and heart rate fluctuations) affect renal artery flow, quality of imaging and kidney function assessment. We will use IACUC approved injectable anesthetic agent, Pentobarbital (50mg/kg body weight, i.p.), as our backup anesthetic agent to ensure proper normal physiological function during the imaging and kidney function assessment; 2) Depilation is a critical step, as presence of chest hair affects the quality of the sonographic images; 3) While sonography of kidneys is relatively

straightforward for a trained operator; for the average operator, it is critical to adapt technique to individual animal's unique anatomy and make minor manipulations; 4) If the size of rats is rather large (above 350 grams), a lower frequency probe (less than used in this study 21 MHz) may be required for optimal imaging. It might be prudent to take a training course before the proposed operation of the imaging system.

The novelty in detecting drug-induced nephrotoxicity using the proposed sonographic methodology and derived protocol is its early robust detection of hemodynamic changes in the event of kidney injury. The results indicate that the intra-renal vascular hemodynamic changes in fact precede the rising sCr. These data is benchmarked against the conventional gold standard using histological analysis and demonstrate that small animal sonography is a noninvasive, sensitive, and reproducible modality, which has minimal requirement of animal use. It is thus an effective tool for early detection of drug-induced nephrotoxicity using rat model.

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DISCLOSURES:
No Disclosures

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Figure 1
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Figure 1. Ultrasound apparatus settings for detecting kidney images in rats

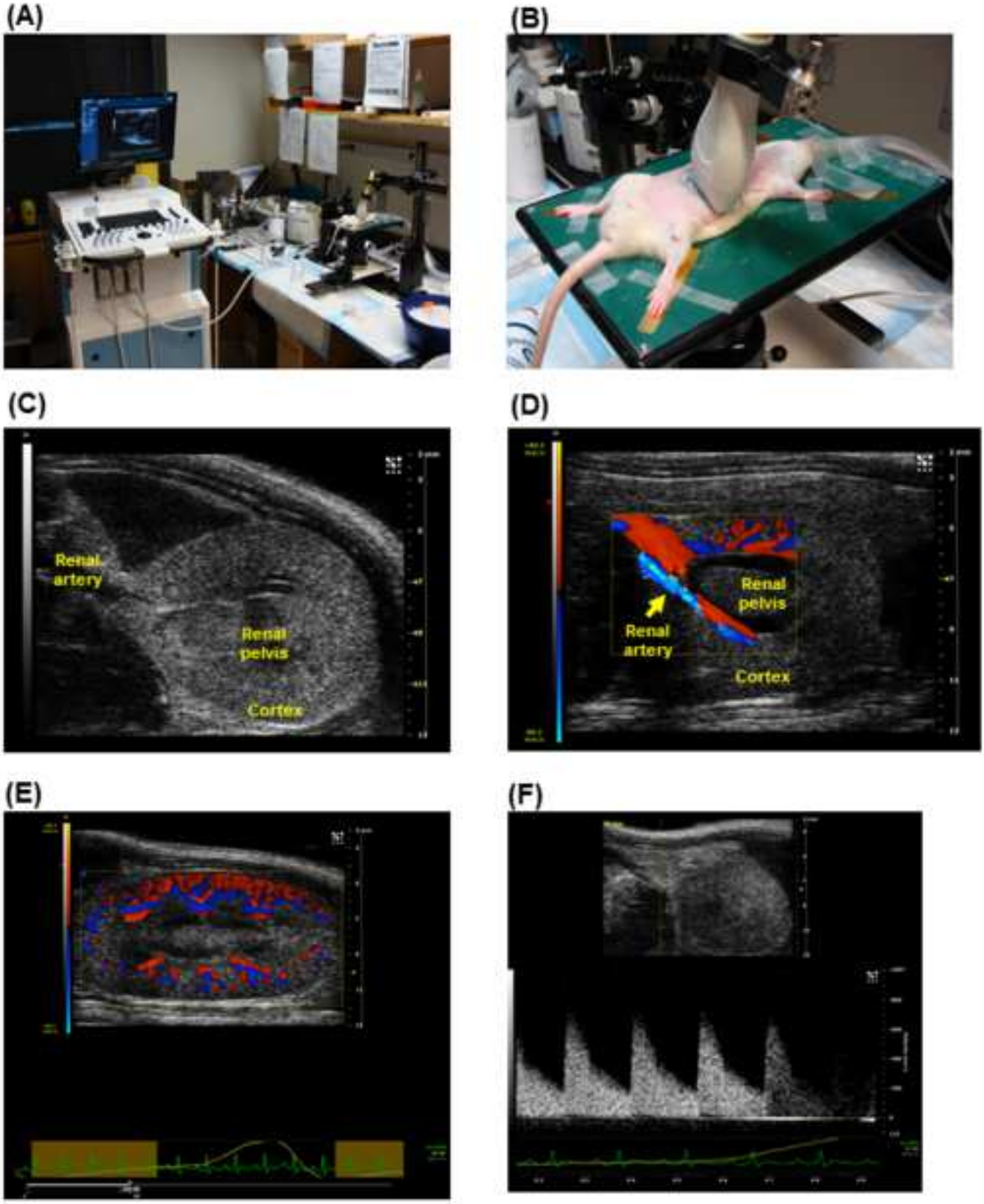

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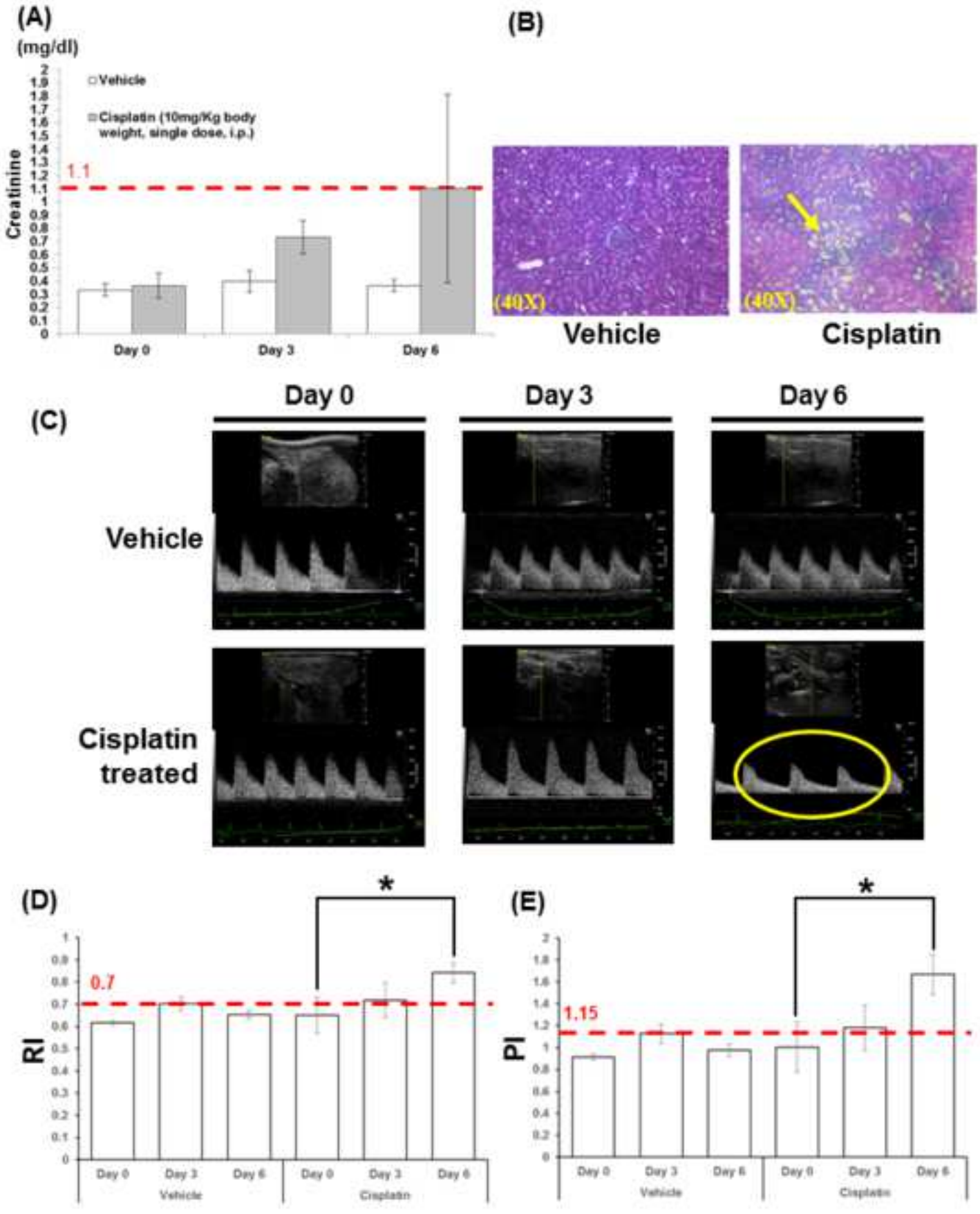
Figure 2. Creatine, Histology and Kidney sonographic images of rats under Cisplatin treatment



Table
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Table 1. Renal hemodynamic parameters for Drug-induced AKI

	Acute Kidney Injury
PI	↑
RI	↑
VTI	↓
Peak-systolic velocity	↓
End-diastolic velocity	↓
Flow volume	↓

Excel Spreadsheet- Table of Materials/Equipment
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Table of reagents

Name of the Reagent	Company	Catalogue Number	Comments
Depilatory cream	Miltex, Inc.	Surgi-Prep	Apply 24 hours prior to imaging
cis-Diamineplatinum(II) dichloride	Sigma	479306	To induce acute kidney injury at small animals.
Isoflurane	Baxter International Inc.	NDC 10019-773-40	2-3% for induction, and 1-1.5 % for maintenance; heart beats will be maintained at above 500 beats per minute

Table of equipments

Material Name	Company	Catalogue Number	Comments
High Frequency Ultrasound	FUJIFILM VisualSonics, Inc.	Vevo 2100	
High-frequency Mechanical Transducer	FUJIFILM VisualSonics, Inc.	MS250, MS550D, MS400	

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March 9th, 2015

Dear Editor-in-chief,

It gives us great pleasure to submit revision version of our manuscript (JoVE52409). We thank editors for their very helpful comments and critique. We have aimed to address all the comments in this letter and incorporated as many suggestions and points into the paper as possible.

List of changes

We thank the editor for their comments and have made all the suggested and required modifications to our manuscript:

I. Editorial comments:
Thanks for reviewer's help to generate the most updated format of our manuscript. We modified following items per reviewer's comments as follow:

1) **In step 2.3 please include injection volume.**
We added requested information in the step 2.3.

2) **Step 2.6 should mention what to do if the vital signs fall outside the ranges listed.**
We modified the paragraph and added requested information at step 2.6.

3) **How are the animal's vital signs monitored with a heating lamp? (step 4.2)**
We have modified and rephrased the protocol section and provided more detail operation steps as suggested at step 4.2.

4) **In step 4.4, more detail is needed to describe how to harvest the kidneys. This can be in the form of additional steps, or a reference.**
We have modified and rephrased the protocol section and provided more detail operation steps as suggested at step 4.4.

5) **In step 4.5, how is the urine collected from the isolated kidneys? What steps are taken?**
We have modified and rephrased the protocol section and provided more detail operation steps as suggested at step 4.2.

6) **Step 4.6 should reference the process of using H&E Slides to check for nephrotoxicity or describe it in detail.**
We have modified and rephrased the protocol section and provided more detail operation steps as suggested at step 4.6.

7) **Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version**.
Thank you very much for your kindly remind, we checked entire manuscript and confirmed no spelling or grammar issues.

8) **If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes**.
All of our figures and tables are original.

II. Reviewers' comments:

Reviewer #1:

1) **The introduction should be changed to mention the existing imaging methods for hemodynamic assessment.**
We thank for reviewer's suggestions, and we have modified and reworded the sentence as suggested. The length of the "Introduction" section is four paragraphs, which covers required information.

2) **Indeed, Doppler US is an important tool to assess changes in renal hemodynamic, yet there are previous publication that used this technique in rodents therefore the authors should cite them in their manuscript (Milman Z et al Nephrol Dial Transplant 2013 28(5):1150-6; Sullivan JC et al Am J Physiol Renal Physiol 2009 297(1):F228-35; Bonnin P et al Ultrasound Med Biol 2008 34(7):1076-84).**
We thank for reviewer's comments and information, we rephrased the paragraph and cite above manuscripts as reviewer's suggestion in the second paragraph at "Introduction" section.

3) **In section 2.6 there is information regarding vital signs measured during imaging procedures from both mice and rats however the results were obtained only from rats,**

please be consistent. Moreover, the huge range of heart rate (250-600/minute) occurs due to different level of anesthetic which would affect Doppler-US parameters.

We thank for reviewer's comments and information. We agree with reviewer's comment and corrected the paragraph to improve consistency of the manuscript. In addition, we provide the information of optimal vital sign reading in our proposed study (temperature: 36.5-37.0, respiratory rate: 80-100/minute, heart rate: 450-550/minute) here for reader's reference.

4) **In section 2.4 the authors mentioned that the rats were analysed at 24, 48, 72, 96, 120, 144 hours after Cisplatin administration, yet they show results obtained only after 6 days. From my experience and also from reading the literature, there are several functional and hemodynamic changes that occur during early time points of renal injury thus it would be highly important to see the results from the entire time kinetics (see- Toxicology and Applied Pharmacology 268(3): 352-361; Experimental and Toxicologic Pathology 59 (3-4): 253-260; etc.)**

We thank for reviewer's comments and information. We agree with reviewer's comment and we add most represented time course data (0, 3 and 6 days) to our data and representative results sections as reviewer's comments.

5) **Results from only 3 rats per group are not enough for statistical comparison! It is important to assess the repeatability of US results from the same rat obtained on several days and also to check the variability between genders and ages.**

We thank for reviewer's comments and information. We agree with reviewer's comments in experimental animal's number, gender and age are important issues. However, our manuscript is aim on providing a cutting-edge methodology using non-invasive sonographic for detecting kidney dysfunction particular at early stage kidney disease using a rat model, we will use this technology to discuss the influences of above risk factors in our following studies. And, in considering of echo current major anima ethical principal at three "R" rules (Replacement, Reduction, and Refinement) to raise awareness of animal use in research, we propose our study with optimal numbers of rats. Our data in deed shows statistical significant difference at RI. PI and Serum creatinine. In addition, Forman et. al. indicated that the heart rate and systolic blood pressure are no significant difference at general adult rats (*J Am Coll Cardiol 1997;30:1872–7*).

Reviewer #2:

1) **Small number of animals. n=3 is not adequate for any scientific report.**

We thank for reviewer's comments. However, our manuscript is aim on providing a cutting-edge methodology using non-invasive sonographic for detecting kidney dysfunction particular at early stage kidney disease using a rat model. And, our data in deed shows statistical significant

difference at RI. PI and Serum creatinine. In addition, in considering of echo current major anima ethical principal at three "R" rules (Replacement, Reduction, and Refinement) to raise awareness of animal use in research, we propose our study with optimal numbers of rats.

2) **Even though data was acquired at several time points between 0 (baseline) and day 6, only data from day 0 and 6 are reported. While it is understandable that histology is available only day 6, imaging and sCr measurements could be reported on a daily basis. Day 6 sCr values are significantly higher compared to baseline measurements. So it is not clear how the authors conclude that the hemodynamic changes precede sCr. I am not sure if the limits of normative values for humans can be translated to rodents. There is also some concern with the use of serum creatinine to evaluate renal function in rodents. BUN is prefered. Also, the sCr values ranged from 0.5 to 2.1 in the three animals. Clearly the limitation of n=3 is evident here. It would also be prudent to include a time immediately following cysplatin administration, unless if the authors have data to support maximum changes occur at day 6.**

We thank for reviewer's comments and information. We agree with reviewer's comment and we add most represented time course data (0, 3 and 6 days) to our data and representative results sections as reviewer's comments. In addition, our manuscript is aim on providing a cutting-edge methodology using non-invasive sonographic for detecting kidney dysfunction particular at early stage kidney disease using a rat model, we will use this technology to discuss the value translation between human and rodents in our future studies.

And, both Creatinine and Blood Urea Nitrogen (BUN) comes from protein intake and the degradation of protein in tissues that may increase by protein catabolism from greater production or kidney dysfunction. However, an increased BUN can be result of either increased or impaired excretion as in kidney disease. Serum Creatinine (sCr) indicates the amount of creatinine in the bloodstream when the kidneys are unable to remove it, which reliably reflects kidney dysfunction. This is the reason we use sCr to evaluate kidney function in our study.

3) **Title should include words: "in Rats".**

We have modified and added "in rats" in the title.

4) **Abstract: "... we showed that these noninvasive hemodynamic measurements can be accurate, sensitive and robust" This is clearly an overstatement. The data at best shows the measurements are sensitive. Accuracy and robustness were not evaluated. n=3 is too small a number to draw any significant conclusions.**

We thank for reviewer's comments and agree with reviewer's suggestion. We have modified and reworded the sentence as suggested.

5) **What is "pulsus parvus morphology"?**
The term of "pulsus parvus morphology" means a weak and slow pattern wave in the sonographic image that indicate slow pulse and hemodynamic changes particular in vascular stenosis.

6) **There are concerns with the use of isoflurane for hemodynamic measurements. The authors seem to recognize this. However they have no provided alternatives. Given the nature of the publication, it will be very useful for the readers in order to duplicate these types of measurements.**
We thank for reviewer's comments and we provide alterative injected anesthetic agents for backup solution.

We hope that you will find favor in all the additional information provided. We would like to express our gratitude for your support to our paper and we look forward to hearing from you.

Yours Sincerely,

Tzongshi Lu, Ph.D.
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