

# Leaky gut potentially leads to bacterial/endotoxin translocation in a rat model of non-ischemic acute kidney injury

Jiangtao Li

Tongji Hospital,Tongji University School of Medicine

Krishna Rekha Moturi

John H Stroger Hospital of Cook County

Lirui Wang

Tongji Hospital,Tongji University School of Medicine

Kun Zhang

Tongji Hospital,Tongji University School of Medicine

Ying Yu

Tongji Hospital,Tongji University School of Medicine

Xi Liu

Tonji Hospital,Tongji University School of Medicine

Yingying Zhang

Tongji Hospital,Tongji University School of Medicine

Chen Yu (✉ [yuchen@tongji.edu.cn](mailto:yuchen@tongji.edu.cn))

<https://orcid.org/0000-0001-7169-276X>

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## Research article

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## Abstract

Background: Emerging evidence indicates that there is a causal relationship between acute kidney injury (AKI) and gut barrier disruption. The aim of our study was to determine whether the translocation of gut-derived bacteria/endotoxin develops in non-ischemic AKI, and, if so, what is the mechanism behind it?

Methods: SPF male Sprague-Dawley rats were randomly subjected to bilateral nephrectomy (BNx) or sham-operation and observed for 24 hours. Gut permeability was evaluated *in vivo* and *in vitro*. Serum endotoxin and bacterial loads in liver and mesenteric lymph nodes (MLN) were measured. The expression of the key tight junctions (TJs) in ileum, including zonula occluden-1(ZO-1), occludin and claudin-1 were evaluated by Western blot and immunohistochemical staining. The structure of TJs was observed using transmission electron microscopy. Apoptotic changes of ileal mucosa were evaluated by TUNEL staining and ELISA.

Results: Non-ischemic AKI rats (rats subjected to BNx) demonstrated marked blunting and shortening of the gut villi. The gut mucosal permeability was increased in non-ischemic AKI rats, evidenced by the elevated serum levels of D-lactate and the increased amount of FITC-Dextran which passed through the ileum wall. Serum endotoxin was significantly elevated in non-ischemic AKI rats. Non-ischemic AKI rats had relatively higher bacterial loads in liver and MLN than sham-operated rats. For non-ischemic AKI rats, apoptosis of ileal mucosa was significantly accelerated. Neither the protein expression and the distribution pattern, nor the structure of the TJs was altered in non-ischemic AKI rats.

Conclusion: Non-ischemic AKI results in profound gut barrier disruption and thus favors the subsequent episode of bacterial/endotoxin translocation. In this non-ischemic AKI model, the translocation of bacterial/endotoxin was not most likely due to a TJs mediated paracellular pathway.

## Background

Acute kidney injury (AKI) is a frequent and serious event with an increased risk of developing to remote organs dysfunctions [1]. Independent of various etiologies, AKI frequently presents an inflammatory process, which is initially confined to the kidneys but eventually spreads to systemic circulation [2].

The gut barrier has an important role in preventing translocation of bacteria and their products from the gut lumen to the circulation [3]. Disruption of the gut barrier is a critical factor in the development and progression of systemic inflammation, which, in turn, can facilitate multiple organ failure and mortality [4–6]. We and others have previously observed that kidney ischemia-reperfusion injury (IR) can cause a series of intestinal consequences, such as breakdown in mucous membrane barriers and translocation of bacterial/endotoxin, which subsequently triggers systemic inflammation [7–8]. It is well known that remote IR injury may result in systemic inflammatory response [9–10]. Therefore, the contribution of renal failure itself on systemic inflammation may not be determined specifically in models of ischemic AKI.

Bilateral nephrectomy (BNx) is a model of non-ischemic AKI [11–12] that is used to examine the deleterious systemic effects of renal dysfunction without taking into account the confounding effects, such as non-renal organ injury or accelerated systemic oxidative stress, which are commonly involved in ischemic AKI [13–14]. In this study, BNx model was used to determine the unique role of the kidney and AKI in promoting bacterial/endotoxin translocation and the mechanism behind it?

## Methods

### Animals and Surgical protocol

SPF male Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, PR China), weighing 200–250 g, were kept on a 12-hour light-dark cycle with free access to diet and water. All animal experiments were conducted with adherence to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, Public Health Services, National Institutes of Health, NIH publication no. 86 – 23, 1985). The animal protocol was approved by the Experiment Animal Center Committee of Tongji University.

Surgical procedures were performed on two groups of rats (BNx and sham operation), with 6 animals in each group. For all procedures, the rats were fully anesthetized with 2% pentobarbital sodium (50 mg/kg) by intraperitoneal injection. In the BNx model, the kidneys were removed after bilateral renal pedicles were tied off. Sham surgery consisted of the identical procedure except kidneys removal. 24 hours after the surgery, the rats were sacrificed with an overdose of 2% pentobarbital sodium (150 mg/kg, intraperitoneally).

### Biochemistry

Blood was collected by intracardiac puncture, centrifuged (4000 g for 10 min at 4°C) and the serum was collected. Urea and creatinine were measured using quantitative colorimetric assays on an Olympus AU 2700 Analyzer (Olympus Optical Co., Ltd., Tokyo, Japan).

### Endotoxin And D-lactate Measurement

Serum was collected as described above. The levels of endotoxin were determined by a kinetic chromogenic method-based Tachypleus amebocyte lysate assay (Xiamen Limulus Experimental Reagents Factory, Xiamen, China) according to the manufacturer's instruction. D-lactate levels were measured using a D-lactate quantitative colorimetric detection kit (Genmed, Boston, United States).

### In Vitro Gut Permeability

The gut permeability was assessed in an isolated ileum sac using fluorescein-isothiocyanate dextran (FD4), as previously described [15]. 5 cm segment of the terminal ileum was dissected and gently flushed. The bilateral ends of the isolated ileum were tied with silk to prevent FD4 leakage. 200 ul of 40 mg/mL FD4 (Sigma-Aldrich, USA) was injected into the lumen of the isolated ileum. The sac was vibrated carefully in 20 mL of saline under 37 °C for 60 min. The amount of FD4 in saline was used as a measure of gut permeability.

## Bacterial Loads Quantification

Total DNA was isolated from liver and mesenteric lymph nodes (MLN) homogenates using the DNEASY Blood & Tissue Kit (Qiagen, Germany). To quantify microorganisms, we amplified a fragment of the 16SrRNA gene by PCR using universal primers targeting a conserved region as previously described [7].  $\beta$ -actin was applied to normalize the variable mass of the collected tissue samples. Bacterial 16SrDNA gene expression was normalized to  $\beta$ -actin in each sample and calculated by the  $2^{-\Delta\Delta Ct}$  method. The primer sets were listed in Table 1.

Table 1  
List of primers used to quantify bacteria

Table 1. List of primers used to quantify bacteria		
Gene	Forward Primer	Reverse Primer
$\beta$ -actin	5'-TCGTACCACTGGCATTGTGATGGA-3'	5'-ACCGCTCATTGCCGATAGTGATGA-3'
16 s rRNA	5'-TCCTACGGGAGGCAGCAGT-3'	5'-GGACTACCAGGGTATCTAACCTGTT-3'

## Quantitative Determination Of Apoptosis

DNA fragments of cytoplasmic histone were measured to quantify apoptosis. Briefly, ileum samples were lysed and the supernatant (cytoplasmic fraction, 15  $\mu$ g protein) was determined for DNA fragments using a cell death detection ELISA kit (Roche Biochemical) according to the manufacturer's protocol. The quantity of cytoplasmic DNA was expressed as absorbance at 405 nm.

## Western Blot

Protein samples of distal ileum were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated overnight at 4 °C with primary antibodies specific to zonula occluden-1(ZO-1) (ab16502; Abcam, 1:500), claudin-1 (ab37168; Abcam, 1:1000), occludin (ab22048; Abcam, 1:500) and GAPDH (ab26300; Abcam, 1:1000), followed by incubation with the appropriate

secondary antibody (ab97051; Abcam, 1:10000). The immunoreactive bands densities were quantified using NIH Image J software (version 1.47).

## H&e Staining, Microscopy And Image Analysis

Segments of the terminal ileum was fixed with 10% buffered formaldehyde solution and embedded in paraffin. Slices of 4  $\mu\text{m}$  thickness were stained with hematoxylin and eosin (H&E). The stained slices were observed at  $\times 200$  magnification. Villous height was measured using the Image-Pro Plus 6.0 image processing and analysis software, according to the method described previously [16]. For each sample, no less than 10 well-oriented villi were measured and the average value was calculated.

## Terminal Deoxynucleotidyl Transferase Dntp Nick-end Labeling (tunel)

For identifying apoptotic cells *in vivo*, paraffin-embedded sections (thickness, 4  $\mu\text{m}$ ) was stained using a TUNEL kit (Roche In Situ Cell Death Detection Kit) under the instructions from manufacturer. Apoptotic cells were observed using a fluorescence microscopy.

## Immunohistochemical Staining

Ileum tissues embedded in paraffin were cut into 4  $\mu\text{m}$  thick serial sections. The sections were blocked with 5% normal goat serum in PBS containing 0.05% Tween-20 and 0.1% bovine serum albumin for 30 min, and then were incubated with anti-ZO-1 (ab16502; Abcam, 1:100), anti-claudin-1 (ab37168; Abcam, 1:100) or anti-occludin (ab22048; Abcam, 1:100) overnight, followed by goat anti-rabbit IgG antibody(ab97051; Abcam, 1:200). The slides were examined under microscope.

## Transmission Electron Microscopy

Pieces of ileum, 2 mm  $\times$  2 mm, were fixed in 2.5% glutaraldehyde overnight at 4 °C and then post-fixed embedded in 1% osmium tetroxide for 2 h. The fixed tissues were dehydrated through a graded acetone series, embedded in epoxy resin and sectioned (1.5  $\mu\text{m}$ ). After staining with uranyl acetate and lead citrate, the sections were examined under an H-600 Electron Microscope (JEM 1010, Hitachi, Japan) at 80 kV.

## Statistical Analyses

The results of statistical analyses were expressed as means  $\pm$  SD. Differences between two groups were analyzed for statistical significance using two-tailed unpaired t test. Values of  $P < 0.05$  were inferred as

statistically significant. Statistic tests were performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

## Results

### Morphology of Small Intestine

We first sought to develop the non-ischemic AKI model by BNx. As shown in Fig. 1a, BNx evoked AKI with the increase in SCr and blood urea at 24 h. We next examined the histological changes in the mucosal architecture of the ileum. Normal mucosal architecture was observed in the sham operated rats (Fig. 1b). By contrast, severe edema of the mucosal villi was observed in BNx rats. Consistence with the histological changes in the mucosa, the length of villus, which reflects the severity of edema, was significantly shorter in BNx rats than in the sham operated rats ( $p < 0.01$ , Fig. 1. c).

### Evaluation Of Apoptotic Changes Of Small Intestine

In situ nick-end labeling was conducted on the ileum. Small quantities of labeling were viewed on the ileum from sham operated rats. Whereas, significantly increased number of apoptotic nuclei, which localized to the central villi as well as intestinal epithelial cells, were detected on the ileum from BNx rats (Fig. 1.d). DNA fragments of the ileum were significantly increased in BNx rats compared with that from sham operated rats ( $p < 0.001$ ) (Fig. 1.e).

### Increased Gut Mucosal Permeability After Bnx

D-lactate is produced by gut bacteria and later absorbed into circulation. Therefore, D-lactate could be used as an effective marker to monitor an increase of gut permeability [17]. As show in Fig. 1.f, we found higher D-lactate in blood of BNx rats than in sham operated rats. Since the ileum is more susceptible to injury than the colon in the contexts of “severe stress”, we then examined the mucosal permeability in the ileum by a non-everted sac method. As show in Fig. 1.g, the amount of FD4 that passed the wall of small intestine was significantly higher in BNx rats than in sham operated rats ( $p < 0.001$ ).

### Endotoxinemia And Bacterial Translocation

Since a disrupted gut barrier might potentially facilitate translocation of bacteria and their products, we examined the endotoxin levels in peripheral blood and the bacterial loads in liver and MLN. With BNx rats, serum endotoxin levels increased compared to the sham operated rat (Fig. 2.a). In accordance, bacterial loads in liver and MLN were also elevated following BNx (Fig. 2. b).

### Tight Junctions Of Ileum

The disruption of TJs may affect gut mucosal permeability. Hence, we characterized the TJs in ileum by immunohistochemical staining, transmission electron microscopy and Western blot.

Immunohistochemical staining showed that the selected TJs, including ZO-1, claudin-1 and occludin, distributed continuously in BNx rats, consistent with the site of the gut mucosal barrier (Fig. 3.a). A careful analysis of transmission electron microscopy failed to reveal any structural alterations of TJ in BNx rats, such as diffuse TJ abnormalities. (Fig. 3.b). In addition, Western blot analysis showed no change in the levels of ZO-1, claudin-1 and occludin expression between groups (Fig. 3.c).

## Discussion

The major findings of our study are that rats subjected to BNx developed mild gut injury, including decreased intestinal villi height and increased gut permeability. After BNx, bacterial loads in liver and MLN as well as endotoxin in blood increased significantly. Neither the protein expression and the distribution pattern, nor the structure of the TJs was altered in BNx rats.

AKI is a systemic disease and often leads to widespread injury causing multiple organ dysfunctions [18]. Gut is a newly-discovered organ which may be remotely injured after AKI [19]. In mice subjected to BNx, intestinal endothelial and epithelial apoptosis and necrosis develop rapidly with subsequent gut barrier disruption [19–20]. Our study in rats confirms these findings and illustrates the important effects of renal failure on gut, which are independent of renal IR. It is possible that retention of urea as well as other metabolites and hypervolumia related to AKI may impair the gut barrier, resulting in increased permeability.

Inflammation is a prominent component of AKI and patients with AKI frequently suffer from systemic inflammation [21–22]. Currently, our understanding of the mechanisms of AKI-induced systemic inflammation remains incomplete. The gut has long been suspected to play a key role in amplifying the systemic inflammatory response during AKI. In the context of ischemic AKI, emerging evidence indicates that the gut may be an amplifier of systemic inflammation through the translocation of gut bacteria and resultant endotoxinemia [7–8]. Our previously study showed that the gut-derived endotoxin, resulting from an increased gut permeability after severe renal IR, amplifies intrarenal inflammation by activation renal TLR4 signaling [7]. Ding et al. confirmed our findings and demonstrated probiotics can alleviate renal dysfunction caused by renal IR via protecting gut barrier function and preventing endotoxinemia [8]. Of note, data that have been accumulated in ischemic AKI models cannot be expanded to all patients, as ischemia complicates only half of AKI that is encountered in hospitalized patients. Therefore, we used BNx model to test our hypothesis. We show in this study that rats subjected to BNx demonstrated an increased bacterial loads in liver and MLN. We also found a low grade of endotoxinemia in BNx rats. These findings suggest that non-ischemic AKI may share a number of common pictures with ischemic AKI, including gut barrier dysfunction, bacterial/endotoxin translocation and subsequent systemic inflammation. For some patients, BNx is required before renal transplantation due to kidneys that preclude renal allograft placement, high-grade tumor and severe infection [23–24]. In this case, timely and

effective improvement of gut dysfunctions following BNx is of great significance to improve prognosis of the patients.

The changes of TJs in gut epithelial cells are one of the major mechanisms which are involved in transmembrane transfer of gut bacteria and their products [25]. Renal IR rats were reported to be associated with diffuse TJs disintegration in gut epithelial cells [8]. In our study, however, BNx and sham operated rats had a similar protein expression and distribution pattern of ZO-1, claudin-1 and occludin in the ileum. Additionally, TEM did not reveal any structural alterations in TJ with BNx rats. Our findings suggest that the translocation of bacterial/endotoxin in non-ischemic AKI was not most likely due to a TJs mediated paracellular pathway. It has been reported that apoptosis in intestinal epithelial cells was implicated in bacterial translocation [15]. By using TUNEL method, we demonstrated that apoptosis was increased on ileal intestinal epithelial cells in BNx rats. Thus, as a working hypothesis, at least accelerated apoptosis should promote bacterial/endotoxin translocation, and potentially, the shortening of the villi comes as a cofactor.

## Conclusions

In summary, we show that non-ischemic AKI causes gut barrier dysfunction. We provide evidence that gut injury after non-ischemic AKI contributes to increased bacterial/endotoxin translocation. For future research, the causal relationship between bacterial/endotoxin translocation and systemic inflammation in non-ischemic AKI needs to be determined.

## Abbreviations

AKI:acute kidney injury; BNx:bilateral nephrectomy; IR:ischemia-reperfusion injury; FD4:fluorescein-isothiocyanate dextran; MLN:mesenteric lymph nodes; TUNEL:Terminal deoxynucleotidyl transferase dUTP nick-end labeling; ZO-1:zonula occluden-1.

## Declarations

### Acknowledgements

Not applicable.

### Authors' contributions

CY and JL participated in the design of the study. JL and KRM drafted the manuscript. LW and KZ performed animal treatments and samples'collection. YY and YZ performed the experiments and statistical analyses. XL was responsible for creating the images. All authors reviewed and approved the final version of the manuscript.

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## Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

None.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Author details

<sup>1</sup>Department of Nephrology, Tongji Hospital, Tongji University School of Medicine, 389 XinCun Road, Shanghai 200065, China.

<sup>2</sup>Internal Medicine, John H Stroger Jr Hospital of Cook County, Chicago, IL 60612, USA.

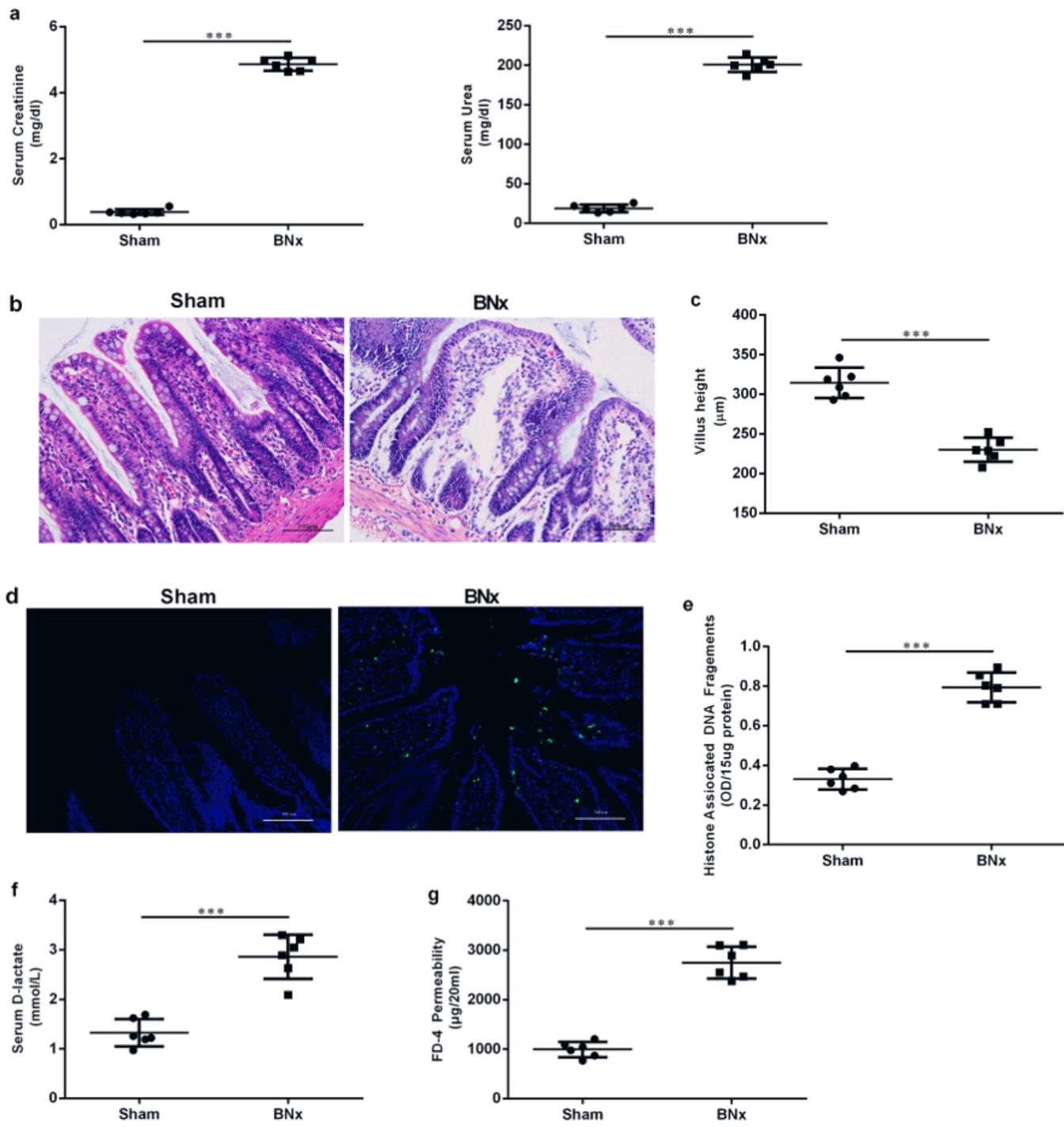
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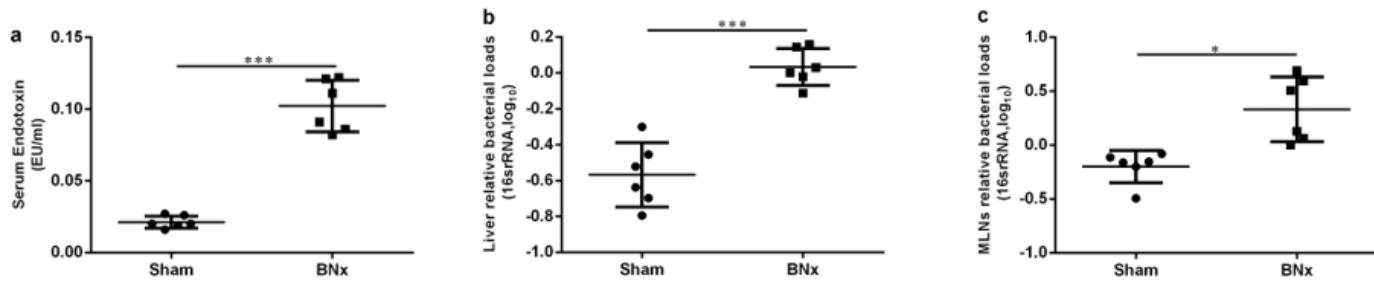
## Figures



**Figure 1**

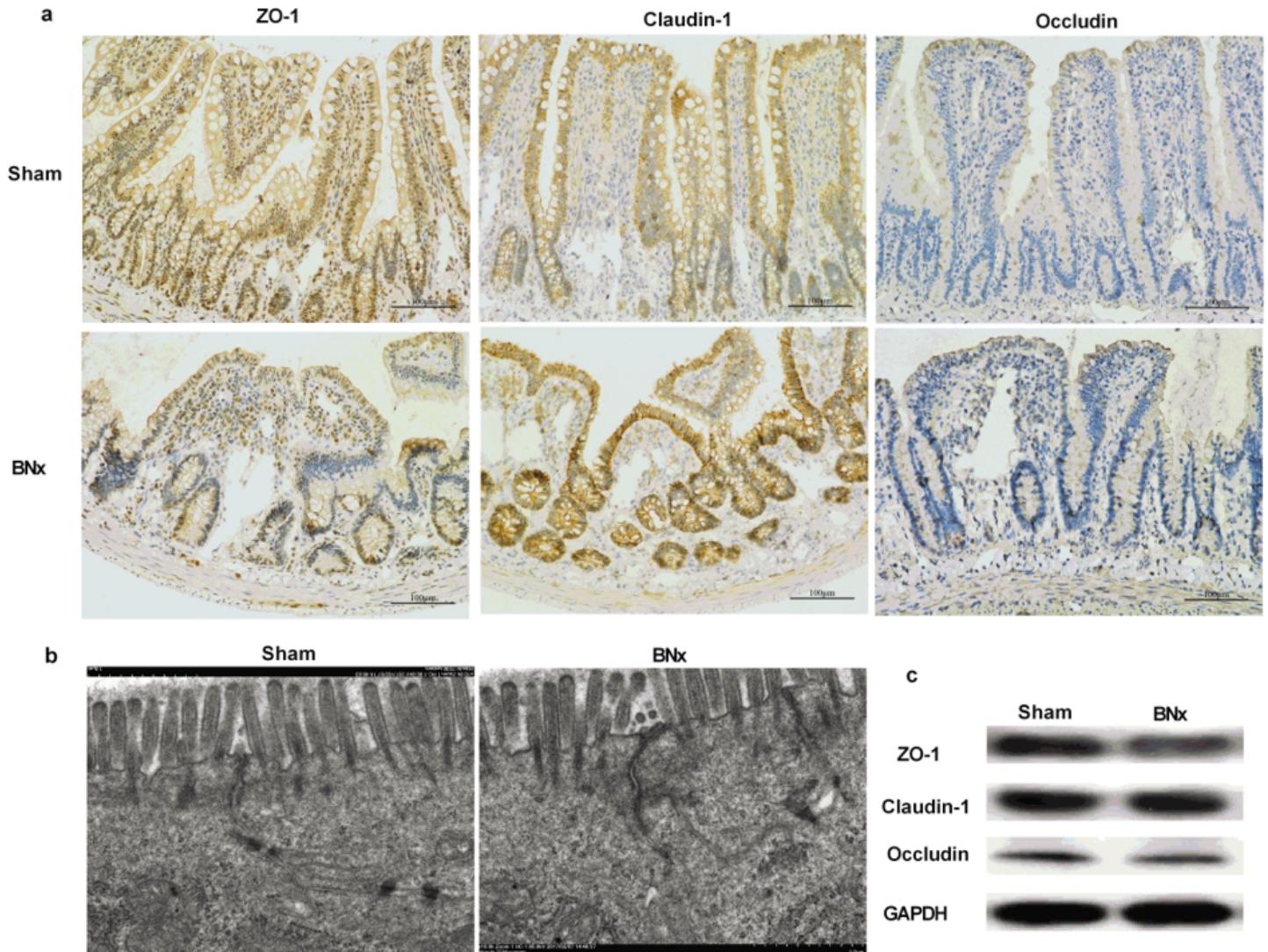
The intestinal consequences of non-ischemic AKI. (a) Renal function was evaluated by serum creatinine and urea levels. (b) Ileum morphological alterations were evaluated by HE stained sections (original magnification $\times 200$ , scale bar=100 $\mu$ m). (c) The villus height was measured and compared. (d) Representative photomicrographs of TUNEL staining in ileum (original magnification $\times 200$ , scale bar=100 $\mu$ m). (e) Apoptosis in the ileum was quantified by Cell Death Detection ELISA. (f) Gut mucosal permeability was evaluated by serum D-lactate. (g) The amount of FD4 that passed the gut wall was

used to evaluate the mucosa permeability of ileum. The two-tailed unpaired test was used ( $n=6$  per group). \*\* $P<0.01$ , \*\*\* $P<0.001$ . BNx: bilateral nephrectomy. FD-4: FITC-Dextran (MW, 4,400 Daltons).



**Figure 2**

Endotoxinemia and bacterial translocation after non-ischemic AKI. (a) The serum level of endotoxin was measured using a Kinetic Turbidimetric LAL method. Bacterial loads at liver (b) and MLN (c) was measured by using 16srDNA analysis. Bacterial loads were represented by relative bacterial loads in log as quantified by qPCR of 16S primer targets normalized to  $\beta$ -actin. Higher values represent more bacteria. Data are expressed by mean $\pm$ SD. The two-tailed unpaired test was used ( $n=6$  per group). \*\* $P<0.01$ , \*\*\* $P<0.001$ . BNx: bilateral nephrectomy. MLN: mesenteric lymph nodes.



**Figure 3**

Non-ischemic AKI had no influence on ileum tight junctions. (a) Immunochemical staining of ZO-1, claudin-1 and occludin in ileum (original magnification $\times$ 200, scale bar=100 $\mu$ m). (b) Transmission electron microscopy of ileal epithelial cells showing tight junction (original magnification $\times$ 30000). (c) Representative Western blot and group data depicting protein abundance of ZO-1, claudin-1, and occludin in the ileum. The two-tailed unpaired t test was used ( $n=6$  per group). BNx: bilateral nephrectomy.

# Supplementary Files

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- TheARRIVEGuidelinesChecklist.pdf