Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Expression of NKCC2, NCC and Na^+-K^+-ATPase Genes Encoding during Acute Renal Failure in Dogs

Hui Zhang[1,§], Zhenyu Chang[1,2,§], Meixian Bao[1], Mujeeb Ur Rehman[1], Khalid Mehmood[1,3], Fazul Nabi[1,4], Xiaoxing Wu[1], Xinxin Tian[1], Xiaodan Yuan[1], Muhammad Ijaz[5] and Donghai Zhou[1,*]

[1]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, People's Republic of China; [2]Key Laboratory of Clinical Veterinary Medicine in Tibet, Tibet Agriculture and Animal Husbandry College, Linzhi 860000 Tibet, China; [3]University College of Veterinary and Animal Sciences, Islamia University of Bahawalpur-63100, Pakistan; [4]Lasbela University of Agriculture, Water and Marine Science Uthal, Pakistan; [5]Department of Clinical Medicine & Surgery, University of Veterinary and Animal Sciences Lahore-54000, Pakistan
*Corresponding author: bigdefoot@163.com

ARTICLE HISTORY (16-313)

Received: November 25, 2016
Revised: February 22, 2017
Accepted: June 30, 2017
Published online: August 08, 2017

Key words:
Acute renal failure
Dogs
Kidney
Na^+-K^+-ATPase
NCC
NKCC2

ABSTRACT

Acute renal failure is a condition in which kidneys are unable to eliminate all the waste from the body. Renal failure results in acid-base imbalance and electrolyte homeostasis. Na^+-K^+-$2Cl^-$ Cotransporter (NKCC2), Na^+-Cl^- Cotransporter (NCC) and Na^+-K^+-ATPase Cotransporter (Na^+-K^+-ATPase) are the important water and sodium channels proteins in the kidney. The aim of this study was to determine the gene expression level in the kidney of dogs suffering with the acute renal failure. A total four dogs with acute renal failure and four healthy dogs were used in this study. The biochemical analysis was done via semi-automatic biochemical machine, while the urine and urinary sediment examination was observed via microscopic study. The acute renal failure was diagnosed and confirmed via necropsy and histopathological examinations and finally the protein expression was analyzed by immunohistochemistry. Results showed that the concentration of sodium, potassium, creatinine and urea nitrogen were significantly different from control group. The level of pH and specific gravity (SG) were decreased with urobilinogen (URO) and were increased during acute renal failure. The urinary sedimentation results revealed the presence of RBCs and WBCs in urine samples. The kidney volume was increased with renal capsule due to hyperplasia and renal pelvis expansion. The hydronephrosis was also observed along with hemorrhages, shrinkage, necrosis, and inflammation in acute renal failure groups by histopathology. The immunohistochemical results revealed that the NKCC2, NCC and Na^+-K^+-ATPase were highly expressed in acute renal failure dogs. Therefore, it was concluded that sodium channels proteins possibly play an important role in dog's acute renal failure cases.

To Cite This Article: Zhang H, Chang Z, Bao M, Rehman MU, Mehmood K, Nabi F, Wu X, Tian X, Yuan X, Ijaz M and Zhou D, 2017. Expression of NKCC2, NCC and Na^+-K^+-ATPase genes encoding during acute renal failure in dogs. Pak Vet J, 37(4): 460-464.

INTRODUCTION

Acute renal failure (ARF) which is also known as acute kidney injury (AKI) is the deterioration of renal functions over a period of hours to days (Wada *et al.*, 1999; Mehta *et al.*, 2003). It results in failure of kidneys to excrete nitrogenous waste products and unable to maintain fluid and acid-base balance along with electrolyte homeostasis dysfunction. In recent years, urinary system problems have become more serious in dogs due to chronic kidney diseases and common in the old age that leads to high mortality and morbidity rate. Approximately 10% of dogs develop chronic kidney disease, which is the leading cause of death in dogs and cats (Bartges *et al.*, 2012).

The kidney diseases can lead to water and electrolyte metabolic disorders, therefore previous studies showed the expression of genes encoding NKCC2, NCC and Na^+-K^+-ATPase transport proteins with renal disorders in animals. In addition, these proteins play major role in sodium-chloride absorption and secretion in wide variety of

[§]These authors contributed equally to this work.

animals (Kaplan *et al.*, 1996; Albert-Quan *et al.*, 1997; Mccormick *et al.*, 2003; Hu *et al.*, 2013). Bumetanide-sensitive NKCC2 is the member of the cation cotransporter family, it is distributed in thick ascending limb (TAL) that can reabsorb about 20-25% NaCl (Musselman *et al.*, 2010; Bartges, 2012). NKCC2 is essential for normal kidney function to regulate the ions movement throughout the kidney cells and control sodium balance. The renal expressions of NKCC2 decreased in acute renal failure infected animals, which lead to polyuria, nocturia and low density of urine (Spichler *et al.*, 2007). Thiazide-sensitive NCC expresses in distal convoluted tubule (DCT) and it can reabsorb about 5-10% NaCl (Dimke *et al.*, 2011). It is a target protein of diuretics during hypertonic or hypotonic low-chloride condition and other clinical featuresactivate NCC receptor, which induce the expression and activity of NCC (Albert-Quan *et al.*, 1997). Na^+-K^+-ATPase is an electrogenic transmembrane ATPase, it is found in the plasma membrane of eukaryotes cells. This enzyme transport sodium and potassium across the cells and is essential for cell physiology. The Na^+-K^+-ATPase can maintain resting potential, facilitate active transport, signal transducer/integrator and regulate cellular volume in animals (Chen *et al.*, 2011; Talsma *et al.*, 2014). In case of acute renal failure, the activity of Na^+-K^+-ATPase reduces, which lead to homeostasis damage. Recent empirical studies suggested that different degree of water, electrolyte metabolic disorders are associated with all kinds of kidney diseases, mainly with the protein levels of NKCC2, NCC and Na^+-K^+-ATPase located in distal nephron that causes the abnormal water re-absorption of sodium (Masilamani *et al.*, 2002; Hu *et al.*, 2011; Schiessl *et al.*, 2013). The expression of genes encoding NKCC2, NCC and Na^+-K^+-ATPase during the acute renal failure of dogs has not been studied yet.

Current study evaluated the renal NKCC2, NCC and Na^+-K^+-ATPase protein level and expression behavior to understand the mechanism of water and salt metabolism. Moreover, the acute renal failure was diagnosed through necropsy and histopathological examinations in dogs. This study provided a reference for comprehensive treatment and control of acute renal failure in dogs.

MATERIALS AND METHODS

Animal treatment and experimental groups: A total eight dogs (6-months old), were selected from the animal hospital of Huazhong Agricultural University (Wuhan, China), it consist of half healthy and half acute renal failure dogs and divided into control and ARF groups respectively; All dogs were raised under recommended temperature and standard hygienic conditions and fed *ad libitum* normal diet. After 2 days, about 1.5mL blood and 3mL urine samples was collected from both groups, each sample was centrifuged at 3500×g for 20 min to separate the serum, and stored at -20°C, until subsequent use and further analysis. After the treatment, all the dogs were killed using KCl (%), and the kidney samples were collected and fixed in 4% paraformaldehyde, while some of the kidney tissues were harvested and kept in -80°C for later use. This study was approved by the Animal Welfare and Research (Huazhong Agricultural University Wuhan, China).

Biochemical criterion determination of serum: The concentration of sodium, potassium, chlorine, creatinine and urea nitrogen in the serum samples were measured via semi-automatic biochemical machine (COULTER®LH 750, Guangdong). (Zhang *et al.*, 2009).

Urine routine and urinary sediment examination: All the urine samples were collected and prepared according to a standardized method described in previous study (Hokamp *et al.*, 2016). Briefly, 10ml urine samples were centrifuged at 2000 rpm for 10 min; then the supernatant was removed, and the sediment was resuspended with a Pasteur pipette in the remaining urine; the re-suspended sediments were transferred into a glass slide, which was covered with a cover slip. The glass slides were observed in light microscope. In both institutions, the samples were examined within 3 h of urine collection.

Histological staining (H&E): The kidney tissue samples were fixed overnight in 10% paraformaldehyde. After 72h, the tissues were dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin wax. Sections were incised with 4μm thickness to prepare the slides. These paraffin-embedded sections were dew axed in xylene and stained with H&E stain. Pathological changes in the kidney tissues were observed in light microscope.

Immunohistochemistry (IHC): NKCC2, NCC and Na^+-K^+-ATPase were detected and localized at cellular level by IHC according to previous study (He *et al.*, 2006). Briefly, formalin-fixed kidney tissues were embedded in paraffin wax, and histological section were cut into 4 μm thickness and placed on poly lysine-coated slides. After deparaffin and antigen recovery, the slides were washed thrice in peroxidase blocking solution (Dako Cytomation, Carpentaria, CA, USA). The slides were incubated with rabbit anti-goat polyclonal antibodies (KPL) in 1:600 dilutions at 4°C overnight (Tuojie biological technology Co., LTD, Wuhan). After washing with PBS, the sections were incubated at 37°C for 1.5 h with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Tuojie biological technology Co., LTD, Wuhan). The immune labeled slides were examined using the microscope (Olympus CX31; Olympus, Japan). Alongside no primary antibodies were as negative controls.

Statistical analysis: The data were analyzed with one way ANOVA by T-test to compare the differences between mean values of control and acute renal failure groups. All statistical were analyzed with a chi-squared test using SPSS (release 18.0 standard version, Statistical Analysis System). The values of $P<0.05$ were considered as statistically significant.

RESULTS

The biochemical criterion analysis of serum: The results of biochemical criterion analysis are presented in table 1. The concentration of sodium, potassium, creatinine and urea nitrogen in the serum samples found significant different ($P<0.05$) between control and acute renal failure groups; while chloride ion value found statistically non-significant between groups.

Table 1: Biochemical criterion analysis of urine between control and acute renal failure group

Group	No	Na^+ (mmol/l)	K^+ (mmol/l)	Cl^- (mmol/l)	BUN (mmol/l)	Cre (μmol/l)
ARFG	3	138.5±5.85*	5.2±1.18*	106.8±5.47	20.4±14.37**	447.8±346.02**
CG	3	144.8±1.29	4.3±2.42	111.3±2.42	4.7±0.98	61.62±11.50

(Acute renal failure group: ARFG; control group: CG).

Table 2: The different of urine routine between control and acute renal failure group

Items	CG	ARFG
URO	0	1.05±0.73**
PRO	0	0.73±0.21**
PH	6.25±0.16	5.33±0.45*
SG	1.02±0.02	1.01±0.03
RBC/μL	0	287±11.02**
WBC/μL	0	10.23±1.14**

(Acute renal failure group: ARFG; control group: CG).

The change of urine routine and urinary sediment: The results of urine output and urinary sediment are presented in table 2 and figure 1. The level of the pH and SG were decreased, and URO was increased in acute renal failure group, while protein (PRO), ketone body (KET), bilirubinic acid (BIL) and glucose (GLU) were not changed significantly. The urinary sediment testing presented the RBC and WBC in acute renal failure groups.

Renal anatomy examination: The kidneys of ARF group were soft in texture and surface of kidney was smooth while it was normal in control groups (Fig. 2A and 2B). However, the kidney volume increased about 2 times of the control group; the renal capsule showed hyperplasia with vascular filling, and visible to white point necrosis in the kidneys (Fig. 2C). The renal pelvis expansion (Fig. 2a), hydronephrosis (Fig. 2b) were also observed in acute renal failure group.

Histological staining (H&E) and immunohistochemical (IHC) evaluation of kidney: Tissue samples were subjected to histopathological examination by H&E staining. The results are presented in figure 3. The control group showed a normal structure with no histopathological changes (Fig. 3A). Histopathological, hemorrhages, shrinkage, necrosis, and inflammation were observed in acute renal failure group. Glomerulus showed bleeding with renal capsule rupture (Fig. 3a), broken or shrinking (Fig. 3d). Moreover, distal convoluted tube wall rupture (Fig. 3d), proximal convoluted tubule epithelial cell degeneration (Fig. 3e), and collection tube interstitial hemorrhage (Fig. 3c) was found in acute renal failure group. To confirm the expression of NKCC2 in TAL epithelial cells, NCC and Na^+-K^+-ATPase in distal convoluted tubule epithelial cells, the kidney tissue samples were detected using immunohistochemical technique and observed with a light microscope. The immunohistochemical results are presented in figure 4. NKCC2, NCC and Na^+-K^+-ATPase were highly expressed in acute renal failure dogs as compared to control group. The negative control has no expression in this study.

DISCUSSION

Acute renal failure is the alteration in kidney function including abnormal filtration, loss of drainage and maintaining blood of some part or all of the original function, which suffers the body with metabolic product aggregation. Furthermore, it affects the humoral regulation, acid-base and electrolyte balance in the blood (Wada *et al.*, 1999; Mehta *et al.*, 2003). Clinical symptoms caused by acute renal failure include acute diarrhea, abdominal pain, vomiting, pale mucosa, polyuria, oliguria, acid-base balance disorders and uremia. Moreover, the glomerular filtration function rapidly dropped to less than 50% of the normal function with increase in serum creatinine and blood urea nitrogen concentration in ARF (Hladunewich *et al.*, 2004; Lassnigg *et al.*, 2004; Yoshida *et al.*, 2004; Levin *et al.*, 2007; Kellum *et al.*, 2015) ARF caused by a variety of reasons and is also secondary to other diseases (Borrego *et al.*, 2003). There are various factors associated with development of ARF in dogs. Electrolyte and mineral concentration is a key concern in renal failure disorder; however, involvement between imbalances or alteration in electrolytes and minerals in ARF is unclear, but such abnormalities are linked with various renal disorders including ARF. Therefore, the aim of this study was to investigate the level of these markers and we found significantly altered levels of sodium, potassium, creatinine and urea nitrogen in ARF group. Electrolytes biomarkers largely correlated with various renal abnormalities and may increase morbidity rate (Hokamp *et al.*, 2016). Elevated blood urea nitrogen and creatinine are indicators of decreased renal function resulting acute renal failure or acute renal injury, and this is an indicator for defect in clearance of waste products (Thadhani *et al.*, 1996). Additionally, in the current study the level of the pH and SG were decreased and URO was increased, while the urinary sediment testing presented the RBCs and WBCs in acute renal failure groups. Concentration and quantity of urine vitally depends on water re-absorption by water channels in the renal collecting system. Defects water channel signaling and regulation are associated with severe abnormalities in renal water handling which is observed in various animal species (Kortenoeven *et al.*, 2013).

The physical examination of ARF dogs in this study revealed depressed and poor body condition of dogs with weakness and retarded growth. The gross lesions of the kidney revealed the enlarged kidney and the hyperplasia of renal capsule with vascular filling, and visible to white point necrosis, renal pelvis expansion and hydro-nephritis. We examined the protein expression of NKCC2, NCC and Na^+-K^+-ATPase via immunohistochemical staining. The results showed that renal NKCC2, NCC and Na^+-K^+-ATPase were markedly up-regulated in ARF affected dogs. In animals including humans, NCC co-expressed in the short end portion of distal convoluted tubule (DCT). Activation of NCC and NKCC2 may imitate linkage to elevated Na^+-K^+-ATPase activity interacellularly (Arystarkhova *et al.*, 2014). The kidney plays an essential role for the regulation of blood pressure through controlling the water and sodium chloride balance in human, NKCC2 is significant in NaCl reabsorption. The activity of NKCC2 in the TAL directly or indirectly alters NaCl reabsorption and several hormones control the NaCl reabsorption through a mechanism of action. In general, renal NKCC2 is significant in physiology of kidney, however regulation and molecular mechanism of NKCC2 is unknown (Ares *et al.*, 2011).

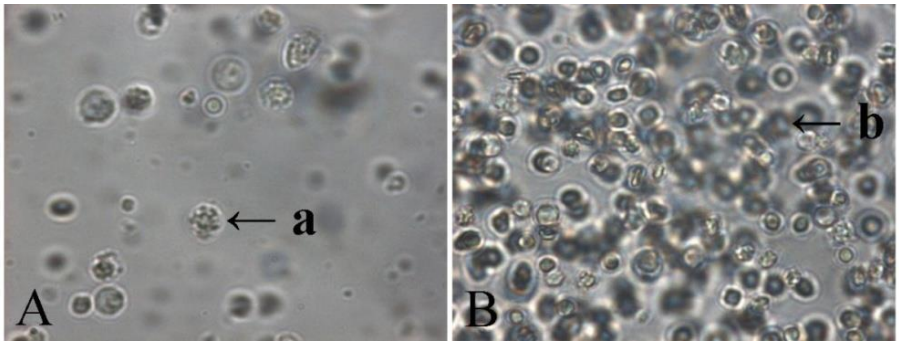

Fig. 1: Urine sediment analysis of acute renal failure in dogs.

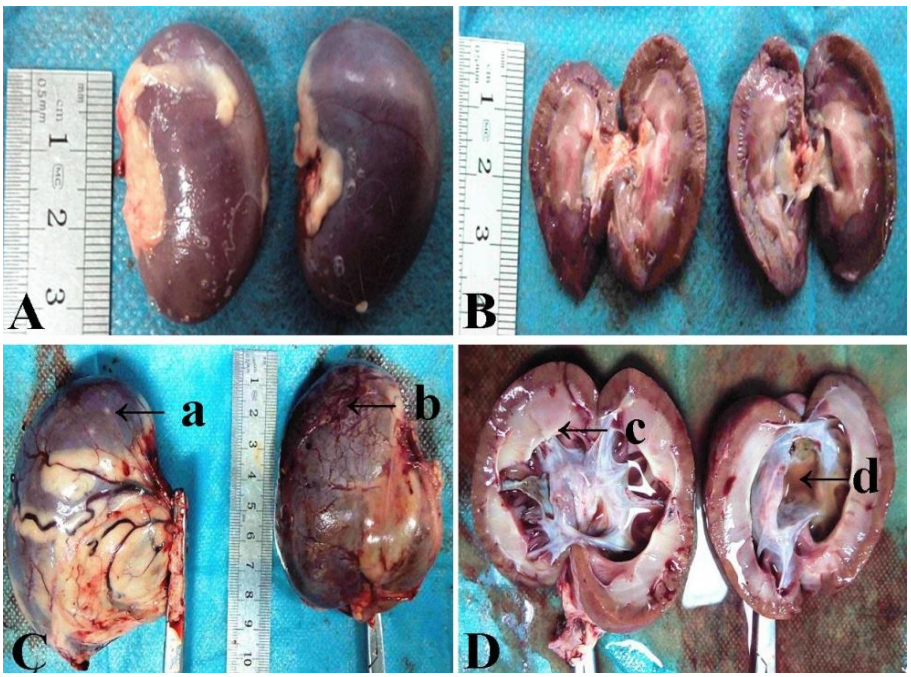

Fig. 2: Gross photographs of autopsy in kidney from dogs between control and acute renal failure group. (control group: A, B; acute renal failure group: C, D) (a) (b) Whole kidney swelling with necrosis and hemorrhage; (c)(d) renal parenchyma thin and calyx thickening with hydronephrosis.

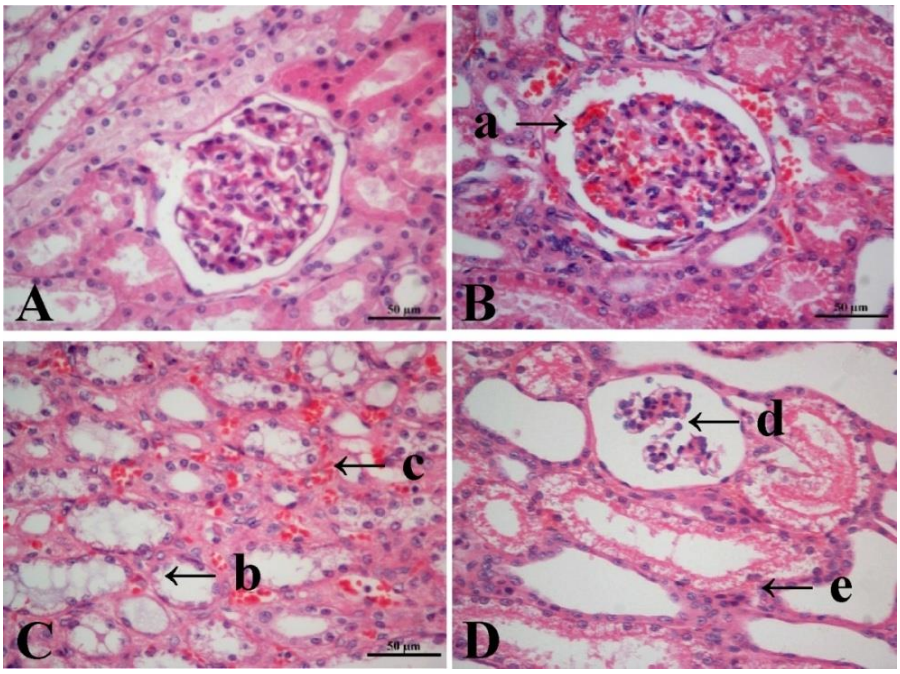

Fig. 3: Histological examination of the kidney tissues in acute renal failure (H&E). (A) Control group; (B, C, D) acute renal failure groups. (a) Glomerular bleeding and renal capsule rupture; (b) Distal convoluted tube wall rupture; (c) Collection tube interstitial hemorrhage; (d) Glomerulus broken and shrinking; (e) Proximal convoluted tubule epithelial cell degeneration.

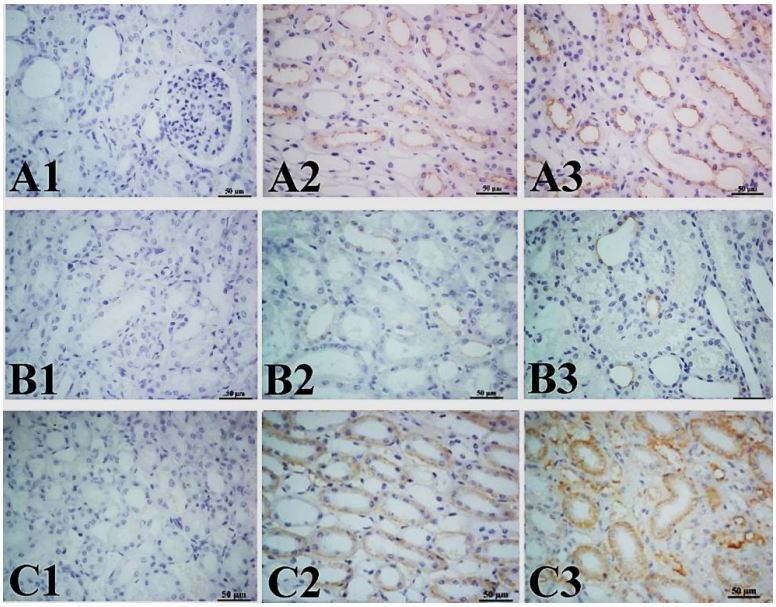

Fig. 4: Expression of genes encoding NKCC2, NCC and Na^+-K^+-ATPase during the acute renal failure of dogs. (A, B, C) IHC of NKCC2, NCC and Na^+-K^+-ATPase in acute renal failure samples were seen at 3100 magnification. (A1, B1, C1) Negative control has no positive response in this study; (A2, B2, C2) Normal control, a few antibodies can be observed in kidney; (C3, C3, C3), Significant antibodies can be observed in acute renal failure groups.

Conclusions: Enhanced expression of NKCC2, NCC and Na^+-K^+-ATPase are associated with ARF in dogs. Water and electrolyte disorders in acute renal failure may be due to change in the expression of these protein levels.

Acknowledgments: This study was supported by Hubei Provincial Natural Science Foundation of China (Grant No: 2014CFB244), the Fundamental Research Funds for the Central Universities (Program No: 2011PYO78) and Chinese Agricultural Research Systems (CARS-38).

Authors contribution: DHZ, ZYC and MXB conceived and designed the experiments; ZYC, MXB and HZ performed the experiments; MUR, KM, FN, XXW, MI, XXT and XDY contributed reagents, materials, and analysis tools; HZ and DHZ wrote the manuscript.

REFERENCES

Albert-Quan MD, Richard-Adams MD, Cssnp EEMR, *et al*., 1997. Serum creatinine is a poor marker of glomerular filtration rate in patients with spina bifida. Dev Med Child Neuro 39:808-10.

Ares GR, Caceres PS and Ortiz PA, 2011. Molecular regulation of NKCC2 in the thick ascending limb. Am J Physiol-Renal 301:1143-59.

Arystarkhova E, Ralph DL, Liu YB, *et al*., 2014. Paradoxical activation of the sodium chloride cotransporter (NCC) without hypertension in kidney deficient in a regulatory subunit of Na^+-K^+-ATPase, FXYD2. Physiol Rep 2:433-43.

Bartges JW, 2012. Chronic kidney disease in dogs and cats. Vet Clin N Am-Small 42:669-92.

Borrego FJ, Viedma G, Pérez DBP, *et al*., 2003. Acute renal failure secondary to hepatic veno-occlusive disease in a bone marrow transplant patient. Nefrologia Publicacion Oficial De La Sociedad Espanola Nefrologia 23:50-4.

Chen R, Jinn TY, Chen YC, *et al*., 2011. Active ingredients in Chinese medicines promoting blood circulation as Na^+-K^+-ATPase inhibitors. Acta Pharmacol Sin 32:141-51.

Dimke H, San Cristobal P, de Graaf M, *et al*., 2011. γ-adducin stimulates the thiazide-sensitive NaCl cotransporter. J Am Soc Nephrol 22:508-17.

He RH, Sheng JZ, Luo Q, *et al*., 2006. Aquaporin-2 expression in human endometrium correlates with serum ovarian steroid hormones. Life Sci 79:423-9.

Hladunewich MA, Lafayette RA, Derby GC, *et al*., 2004. The dynamics of glomerular filtration in the puerperium. Am J Physiol-Renal 286:496-503.

Hokamp JA, Cianciolo RE, Boggess M, *et al*., 2016. Correlation of urine and serum biomarkers with renal damage and survival in dogs with naturally occurring proteinuric chronic kidney disease. J Vet Intern Med 24:1283-8.

Hu LF, Li Y, Neo KL, *et al*., 2011. Hydrogen sulfide regulates Na^+/H^+ exchanger activity via stimulation of phosphoinositide 3-kinase/akt and protein kinase G pathways. J Pharmacol Exp Ther 339:726-35.

Kaplan MR, Mount DB and Delpire E, 1996. Molecular mechanisms of NaCl contrsport. Annu Rev Physiol 58:649-68.

Kellum JA, Sileanu FE, Murugan R, *et al*., 2015. Classifying AKI by urine output versus serum creatinine level. J Am Soc Nephrol 26:2231.

Kortenoeven MLA, Pedersen NB, Miller RL, *et al*., 2013. Genetic ablation of aquaporin-2 in the mouse connecting tubules results in defective renal water handling. J Physiol 591:2205-19.

Lassnigg A, Schmidlin D, Mouhieddine M, *et al*., 2004. Minimal changes of serum creatinine predict prognosis in patients after cardiothoracic surgery: a prospective cohort study. J Am Soc Nephrol 15:1597.

Levin A, Warnock DG, Mehta RL, *et al*., 2007. Improving outcomes from acute kidney injury: report of an initiative. Am J Kidney Dis 50:1.

Masilamani S, Wang X, Kim GH, *et al*., 2002. Time course of renal Na^+-K^+-ATPase, NHE_3, NKCC2, NCC, and ENAC abundance changes with dietary NaCl restriction. Am J Physiol Renal Physiol 283:648-57.

Mccormick SD, Sundell KB, Jornsson BT, *et al*., 2003. Influence of salinity on the localization of Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$ Cotransporter (NKCC2) and CFTR anion channel in chloride cells of the hawaiian goby (stenogobius hawaiiensis). J Exp Biol 206:4575-83.

Mehta RL and Chertow GM, 2003. Acute renal failure definitions and classification: time for change? J Am Soc Nephrol 14:2178.

Musselman TM, Zhang Z and Masilamani SM, 2010. Differential regulation of the bumetanide-sensitive cotransporter (NKCC2) by ovarianhormones. Steroids 75:760-5.

Schiessl IM, Rosenauer A, Kattler V, *et al*., 2013. Dietary salt intake modulates differential splicing of the Na^+-K^+-$2Cl^-$Cotransporter NKCC2. Am J Physiol Renal Physiol 305:1139-48.

Spichler A, Ko AI, Silva EF, *et al*., 2007. Reversal of renal tubule transporter downregulation during severe leptospirosis with antimicrobial therapy. Am J Trop Med Hyg 77:1111-9.

Talsma AD, Chaves JF, Lamonaca A, *et al*., 2014. Genome-wide screen for modifiers of Na^+-K^+-ATPase, alleles identifies critical genetic loci. Molecular Brain 7:1-16.

Thadhani R, Pascual M and Bonventre JV, 1996. Acute renal failure. N Engl J Med 334:1448-60.

Wada Y, Saeki T, Miyamura S, *et al*., 1999. Successful treatment of acute renal failure in a patient with essential mixed cryoglobulinemia using prednisolone and cryofiltration. Nippon Jinzo Gakkai Shi 41:29-33.

Yoshida S, Miyakawa H and Uchida S, 2004. Abnormalities in electrolyte and acid-base homeostasis in uremic patients. Nippon Rinsho Japanese. J Clin Med 62:96-100.

Zhang D, Wang L, Dong ZN, *et al*., 2009. Evaluation of performance of Cobas6000 automatic biochemical analyzer in clinical test. Analytical Instrumentation 1:14-19. (in Chinese).