



## Research Paper

# The Regulation of Intracellular Ca<sup>2+</sup> in the Pathogenesis of Ischemia/Reperfusion-Induced Acute Kidney Injury in Rats

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### ABSTRACT

The effects of pre- or post-ischemic treatment with verapamil, a Ca<sup>2+</sup> channel antagonist on ischemia/reperfusion (I/R)-induced acute kidney injury (AKI) in rats was investigated and these were compared with the effects of KB-R7943, an inhibitor of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX). I/R-induced AKI was induced by clamping the left renal pedicle for 45-min followed by reperfusion, 2 weeks after contralateral nephrectomy. Renal function markedly decreased 24 h after reperfusion. Pre-ischemic treatment with verapamil or KB-R7943 attenuated the I/R-induced renal dysfunction. On the other hand, the I/R-induced renal dysfunction was overcome by post-ischemic treatment with KB-R7943 but not with verapamil. Histopathological examination of I/R-induced AKI rats revealed severe renal damages such as tubular necrosis and proteinaceous casts in the tubuli. Post-ischemic treatment with KB-R7943 but not with verapamil suppressed these renal damages. These findings suggest that activation of the NCX plays an important role in the regulation of intracellular Ca<sup>2+</sup> in the pathogenesis of I/R-induced AKI.

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**Key words:** Ca<sup>2+</sup> channel antagonist, Na<sup>+</sup>/Ca<sup>2+</sup> exchange inhibitor, acute kidney injury, ischemia/reperfusion.

**Abbreviations:** I/R, ischemia/reperfusion; AKI, acute kidney injury; NCX, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; BUN, blood urea nitrogen; Uosm, Urinary osmolality; FENa, fractional excretion of sodium; UNaV, urinary excretion of sodium; PNa, plasma sodium concentration; Ccr, creatinine clearance; Pcr, plasma creatinine concentration; UF, urine flow.

## INTRODUCTION

Ischemic cell injury in the kidney occurs during cardiovascular, surgery, shock and transplantation, which may lead to acute kidney injury (AKI). It is difficult to determine the pathological roles of many factors involved in cellular injury and resultant organ damage since ischemic AKI is induced not only by the ischemia itself but also by the following reperfusion.

Furthermore, the kidney is a complex tissue comprised of both vascular and tubular networks. One of the major contributors to ischemic cell injury is an increase in intracellular Ca<sup>2+</sup>, which occurs in cases of AKI (Schrier et al., 1987). In the kidney, the protective effect of KB-R7943 (2-[2-[4-nitrobenzyloxy] phenyl] ethyl)

isothioureamethanesulfonate), an inhibitor of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX), on ischemia/reperfusion (I/R)-induced AKI was first demonstrated (Kuro et al., 1999). In addition, I/R-induced renal dysfunction, damage and Ca<sup>2+</sup> accumulation in necrotic tubular epithelium were observed more markedly in NCX1<sup>+/+</sup> wild type than in NCX1<sup>+/-</sup> heterozygous mice (Yamashita et al., 2003), thereby suggesting that Ca<sup>2+</sup> overload through the reverse mode of NCX is a critical factor in the pathology of post-ischemic renal insufficiency. These findings led us to examine whether a Ca<sup>2+</sup> channel antagonist also exerts a protective effect against I/R-induced AKI.

In the present study, whether I/ induced renal

dysfunction would be overcome by pre-ischemic treatment with verapamil, in comparison with KB-R7943 was evaluated (Kuro et al, 1999). Secondly, investigations as to whether or not post-ischemic treatment with verapamil or KB-R7943 can also have a therapeutic effect on the I/R-induced renal dysfunction and damage were carried out since it is more important to examine the effects of drugs administered after reperfusion, and many clinical cases of ischemic AKI cannot be predicted. It is reported here that inhibition of NCX may be considered as a therapeutic approach to protect the post-ischemic AKI.

## MATERIALS AND METHODS

### Animals and Experimental Design

Male Sprague-Dawley rats (280 to 320 g, 10 weeks old, Japan SLC, Shizuoka) were housed in a light-controlled room with a 12-h light/dark cycle and access to food and water was *ad libitum*. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences (Osaka, Japan). Two weeks before the study (rats 8 weeks of age), the right kidney was removed through a small flank incision made following pentobarbital anesthesia (50 mg/kg, i.p.). After a 2-week recovery period, these rats were separated into five groups: 1) vehicle-treated AKI (control); 2) pre-ischemic treatment with verapamil (1 mg/kg, i.v.) in AKI (control+pre-verapamil 1 mg/kg); 3) pre-ischemic treatment with KB-R7943 (2 mg/kg, 10 mg/kg, i.v.) in AKI (control+pre-KB-R7943 2 mg/kg or control+pre-KB-R7943 10 mg/kg); 4) post-ischemic treatment with verapamil (1 mg/kg, i.v.) in AKI (control+post-verapamil 1 mg/kg); 5) post-ischemic treatment with KB-R7943 (2 mg/kg, 10 mg/kg, i.v.) in AKI (control+post-KB-R7943 2 mg/kg or control+post-KB-R7943 10 mg/kg). To induce ischemic AKI, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and the left kidney exposed through a small flank incision. The left renal artery and vein were occluded for 45-min with a non-traumatic clamp. At the end of the ischemic period, the clamp was released and blood reperfused.

Verapamil and KB-R7943 or its vehicle [a mixture of 15% ethanol, 15% polyethylene glycol 400 and 70% saline (0.9%)] was administered (pre-ischemic treatment, 5-min before the ischemia; post-ischemic treatment, immediately after reperfusion) as a slow bolus injection at 1 ml/kg into the external jugular vein. Animals exposed to 45-min ischemia were housed in metabolic cages at 24 h after reperfusion; 5-h later urine samples were taken and blood samples drawn from the aorta at the end of the urine collection period. The plasma was separated by centrifugation. These samples were used for measurements of renal functional parameters. The kidneys were then excised and examined using a light microscope.

### Blood and urine measurements

Blood urea nitrogen (BUN) and creatinine levels in plasma and urine were determined using the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Urinary osmolality (Uosm) was measured by freezing point depression (Fiske, MA). Urine and plasma sodium concentrations were determined using a flame photometer (Hitachi, 205 D, Hitachinaka, Japan). Fractional excretion of sodium (FENa, %) was calculated from the formula;  $FENa = \frac{UNaV}{(PNa \times Ccr)} \times 100$ , where UNaV is urinary excretion of sodium, PNa is the plasma sodium concentration and Ccr is creatinine clearance.

### Histological studies

Excised left kidneys were processed for light microscopic observation according to standard procedures. The kidneys were then preserved in phosphate-buffered 10% formalin, after which the kidneys were chopped into small pieces, embedded in paraffin wax, cut at 3  $\mu$ m and stained with Hematoxylin and Eosin. Histopathological changes were analyzed for tubular necrosis and proteinaceous casts (Caramelo et al, 1996). Tubular necrosis and proteinaceous casts were graded as follows: no damage (- or 0), mild ( $\pm$  or 1, unicellular, patchy isolated damage), moderate (+ or 2, damage less than 25%), severe (++ or 3, damage between 25 and 50%) and very severe (+++ or 4, more than 50% damage). Evaluations were made in a blind manner.

### Drugs

Verapamil was dissolved in 0.9% saline, while KB-R7943 (Kanebo, Ltd., Osaka, Japan) was dissolved in a mixture of 15% ethanol, 15% polyethylene glycol 400 and 70% saline (0.9%) just before administration. Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

### Statistical analysis

Values are mean  $\pm$  SEM. For statistical analysis, one-way analysis of variance followed by Bonferroni's multiple comparison tests was used. Histological data were analyzed using the Kruskal-Wallis non-parametric test combined with the Steel-type multiple comparison test. For all comparisons, differences were considered significant at  $P < 0.05$ .

## RESULTS

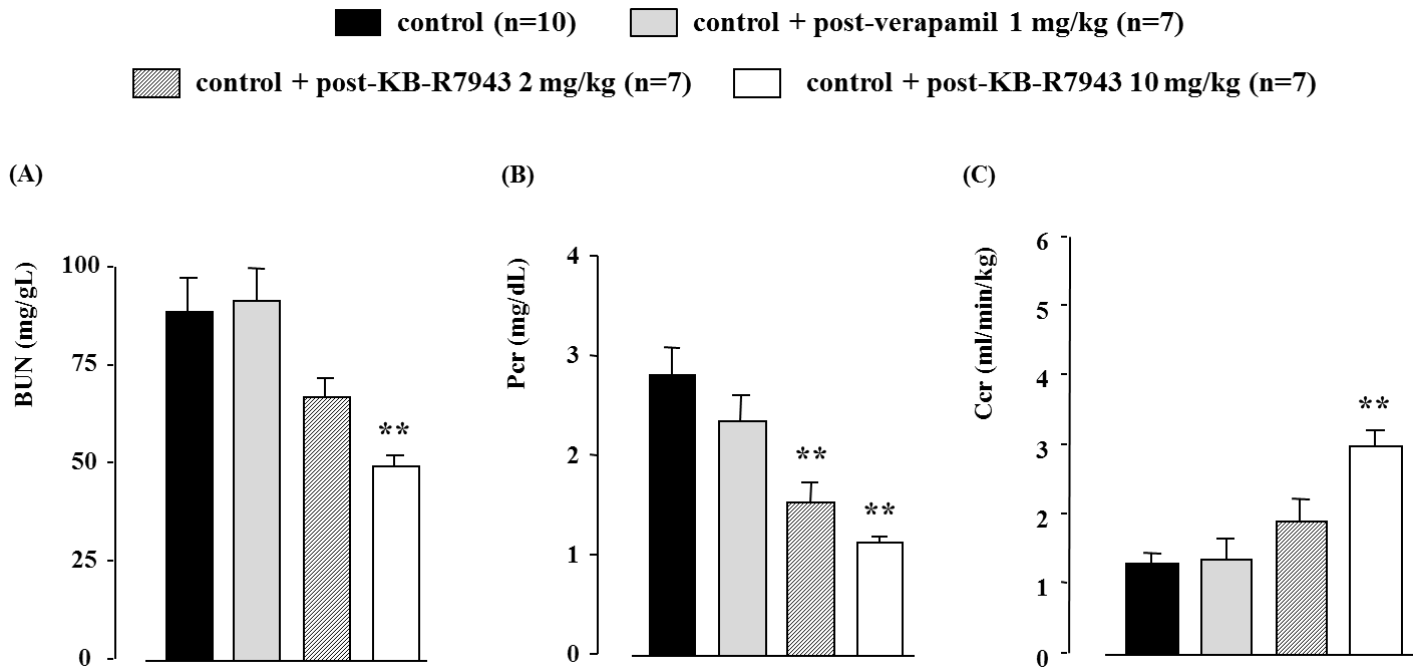
### Renal function after I/R and effects of pre-ischemic treatment with verapamil or KB-R7943

Table 1 shows the effect of pre-ischemic treatment with

**Table 1:** Effects of verapamil and KB-R7943 administered before ischemia/reperfusion on renal function 24 h after reperfusion.

Experimental group	UF	Uosm	FENa	BUN	Pcr	Ccr
	$\mu\text{l}/\text{min}/\text{kg}$	$\text{mOsm}/\text{kg}$	Percentage (%)	$\text{mg}/\text{dl}$	$\text{mg}/\text{dl}$	$\text{ml}/\text{min}/\text{kg}$
Control (n=10)	$88.8 \pm 6.03$	$365 \pm 26$	$1.84 \pm 0.19$	$87.7 \pm 8.89$	$2.80 \pm 0.28$	$1.27 \pm 0.17$
Control + pre-verapamil 1 mg/kg (n=7)	$73.5 \pm 9.71$	$780 \pm 12.7^b$	$0.91 \pm 0.21^a$	$58.1 \pm 6.20$	$1.29 \pm 0.09^a$	$2.87 \pm 0.17^b$
Control + pre-KB-R7943 2 mg/kg (n=10)	$76.2 \pm 7.35$	$595 \pm 55$	$1.02 \pm 0.20^b$	$70.6 \pm 9.52$	$1.31 \pm 0.11^a$	$2.37 \pm 0.30$
Control + pre-KB-R7943 10 mg/kg (n=10)	$42.0 \pm 6.34^a$	$1137 \pm 106^a$	$0.36 \pm 0.08^a$	$35.3 \pm 3.70^b$	$0.83 \pm 0.07^a$	$3.95 \pm 0.45^a$

Each value represents the mean  $\pm$  S.E.M. Drugs were given i.v. 5 min before the ischemia (45 min). At 24 h after reperfusion, 5-h urine was collected. <sup>a</sup> $P < 0.01$ , <sup>b</sup> $P < 0.05$  compared with control rats.



**Figure 1:** Effects of verapamil or KB-R7943 administered after reperfusion on BUN (A), Pcr (B), and Ccr (C) at 24 h after I/R. At 24 h after reperfusion, 5-h urine was collected. Each value represents the mean  $\pm$  S.E.M. **\*\*** $P < 0.01$ , compared with control rats.

verapamil or KB-R7943. Renal functional parameters of rats subjected to 45-min ischemia (control) showed increases in BUN, plasma creatinine concentration (Pcr), urine flow (UF) and FENa and decreases in Ccr and UOsm measured 24 h after reperfusion. As reported (Goldfarb et al., 1983), pre-ischemic treatment with verapamil (1 mg/kg, i.v.) attenuated the I/R-induced renal dysfunction. In addition, I/R-induced renal dysfunction was also dose-relatedly improved by the pre-ischemic treatment with KB-R7943 (2 mg/kg, 10 mg/kg, i.v.). The degree of improvement of verapamil (1 mg/kg, i.v.) is similar to the findings observed with the lower dose (2 mg/kg, i.v.) of KB-R7943.

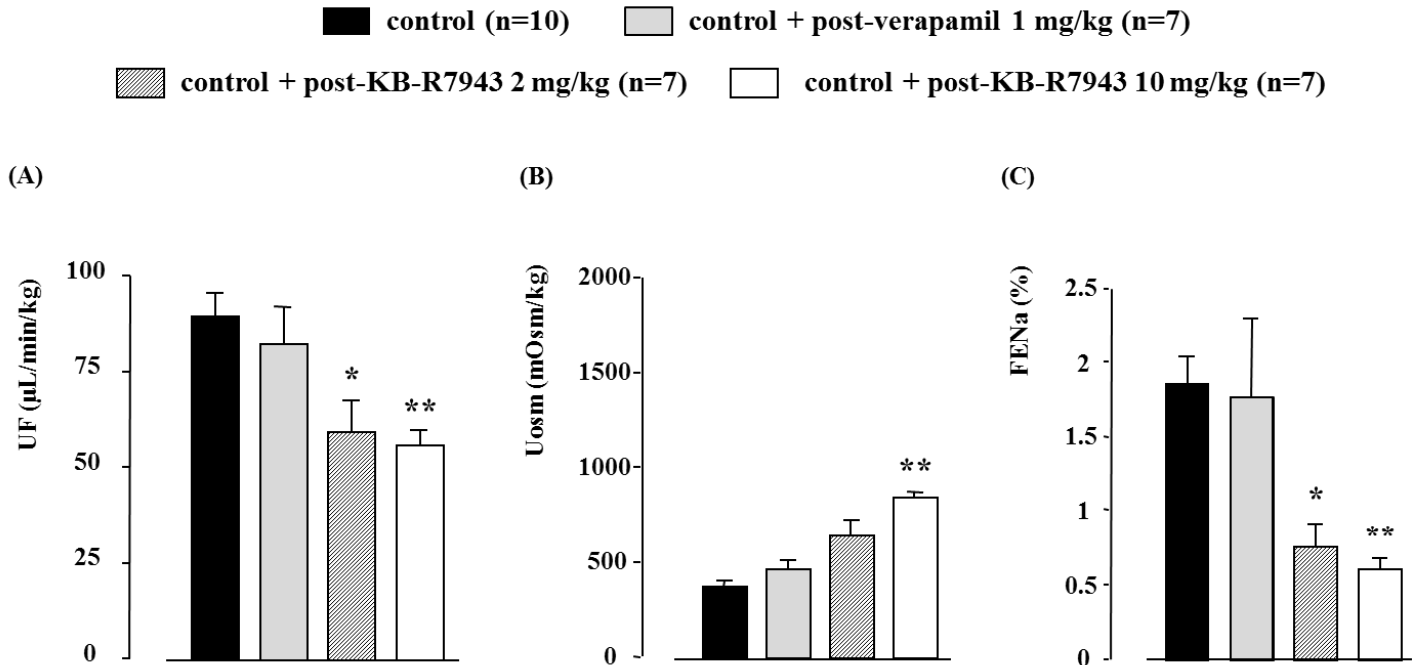
#### Renal function after I/R and effects of post-ischemic treatment with verapamil or KB-R7943

Figures 1 and 2 show the effects of post-ischemic treatment

with verapamil or KB-R7943. In contrast to the findings of the pre-ischemic treatment with verapamil, no significant improvements were observed with post-ischemic treatment with verapamil. On the other hand, the impairment of renal function induced by I/R was dose-relatedly attenuated by KB-R7943, which was administered after reperfusion. At the higher dose of KB-R7943 (10 mg/kg, i.v.), each renal functional parameter was significantly improved.

#### Histological renal damage after I/R and effects of post-ischemic treatment with verapamil or KB-R7943

Histopathological examination revealed severe lesions in the kidney of control rats (1 day after the ischemia and reperfusion). These changes were characterized by tubular necrosis (Figure 3, outer zone outer stripe of medulla) and proteinaceous casts in tubuli (Figure 4, inner zone of medulla). Although post-ischemic treatment with verapamil



**Figure 2:** Effects of verapamil or KB-R7943 administered after reperfusion on UF (A), UOsm (B), and FENa (C) at 24 h after I/R. At 24 h after reperfusion, 5-h urine was collected. Each value represents the mean  $\pm$  S.E.M. \* $P$ <0.05, \*\* $P$ <0.01, compared with control rats.

exerted no protective effects against any of these lesions, the development of all these lesions was dose-relatedly improved by post-ischemic treatment with KB-R7943. In the case of higher dose of KB-R7943 (10 mg/kg, i.v.), there were significant improvements (Table 2).

## DISCUSSION

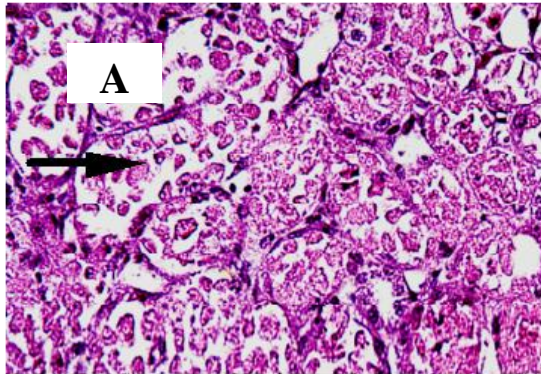
In the present study, it was observed that post-ischemic treatment with verapamil failed to attenuate the renal dysfunction and damage induced by I/R, although pre-ischemic treatment at the same dose of verapamil efficiently suppressed the development of ischemic AKI. In contrast to the findings observed with verapamil, KB-R7943 overcame the I/R-induced renal dysfunction with post-ischemic as well as pre-ischemic treatments.

In addition, the post-ischemic treatment KB-R7943 showed a protective effect against ischemic AKI-induced histological injuries, in the same manner with pre-ischemic treatments. The mechanisms underlying the ischemic renal damage are complex and not fully understood, but it is known that  $Ca^{2+}$  overload to renal epithelial cells may be one of the causal factors of these diseases (Schrier et al, 1984; Wilson et al, 1984; Wong and Chase, 1986). Taken together,  $Ca^{2+}$  influx, mainly through the voltage-dependent  $Ca^{2+}$  channel and through the reverse mode of NCX might occur during the ischemia and after reperfusion, respectively, both of which are likely to be responsible for

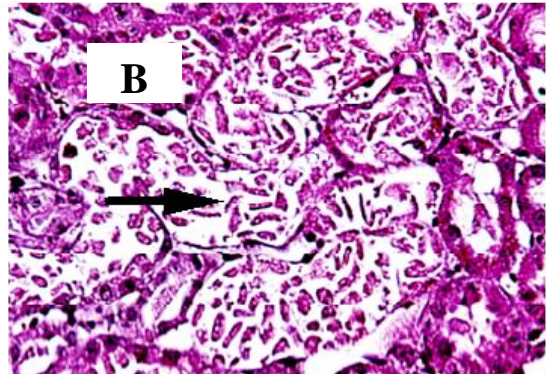
the development and progression of I/R-induced AKI.

$Ca^{2+}$  channel antagonists often have been found to offer protection against ischemic damage. However, the effects of these drugs on post-ischemic AKI are also conflicting. Some papers observed the beneficial effects of verapamil on I/R-induced AKI (Goldfarb et al, 1983; Wait et al, 1983; Dusmez et al, 2014), while others demonstrated no improvements with verapamil on this disease (Malis et al, 1983; Blank et al, 1984). Another  $Ca^{2+}$  channel antagonists, such as azelnidipine or nimodipine, protect tubular cells from apoptosis subsequent to hypoxic injury on post-ischemic AKI (Tanaka et al, 2004; Fröba et al, 2008). Thus, alleviative effect of  $Ca^{2+}$  channel antagonists for I/R-induced AKI is a controversial issue. Although it is difficult to determine the precise roles of  $Ca^{2+}$  influx through the voltage-dependent  $Ca^{2+}$  channel, in the pathogenesis of I/R-induced AKI, the mechanism of action may be the attenuation of renal vasoconstriction for an effect that lessens the ischemic insult and protection of tubular cells from apoptosis subsequent to hypoxic injury by stabilizing cellular and mitochondrial  $Ca^{2+}$  homeostasis.

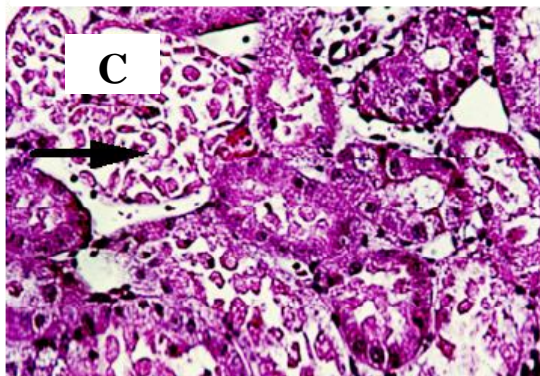
KB-R7943 was reported to selectively inhibit the reverse mode of NCX (Iwamoto et al, 1996) in cardiomyocytes, smooth muscle cells and NCX-1-transfected fibroblasts. Similar inhibitory effects of KB-R7943 on the  $Ca^{2+}$  influx mode of NCX were observed in guinea pig cardiac ventricular cells (Watano et al, 1996). In ischemic cardiac cells, where the intracellular pH is decreased by anaerobic glycolysis and intracellular acidosis, intracellular  $Na^{+}$  concentration rises through increased  $Na^{+}/H^{+}$  exchanger activity (Lazdunski et al, 1985). Moreover,  $Na^{+}/K^{+}$ -ATPase



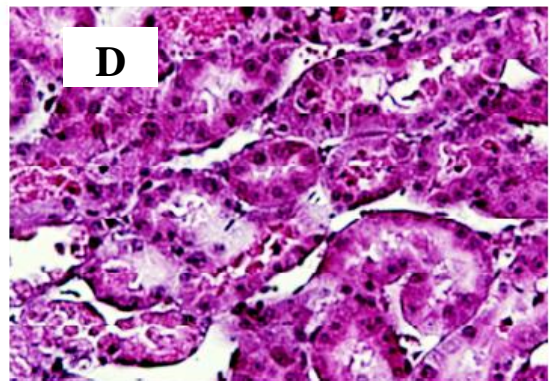
**control**



**control + post-verapamil 1 mg/kg**



**control + post-KB-R7943 2 mg/kg**



**control + post-KB-R7943 10 mg/kg**

**Figure 3:** Light microscopy of the outer zone outer stripe of medulla of the kidney of AKI rats treated with vehicle (A), verapamil (B, 1 mg/kg), KB-R7943 (C, 2 mg/kg) and KB-R7943 (D, 10 mg/kg) at 24 h after ischemia/reperfusion. Drugs were given i.v. after reperfusion. Arrows indicate tubular necrosis (hematoxylin and eosin staining).

activity is inhibited during ischemia (Cross et al, 1995). These phenomena led to an increase in intracellular  $Ca^{2+}$  concentrations through the reverse mode of the NCX system (Dennis et al, 1990). KB-R7943 was reported to reduce the cytosolic  $Ca^{2+}$  overload in isolated rat cardiomyocytes exposed to ischemic condition and to protect against reoxygenation-induced injury in the whole heart (Ladilov et al, 1999).

Nakamura et al (1998) found that KB-R7943 significantly improved I/R-induced injury in the isolated rat perfused heart by post- as well as pre-ischemic treatment, thereby suggesting that the activation of NCX mainly occurs immediately after the reperfusion. In the kidney, it was first demonstrated that pre-ischemic treatment with KB-R7943 shows renal protective effects on I/R-induced AKI (Kuro et al, 1999). In addition, the pathological role of NCX in I/R-induced renal injury, using NCX1<sup>+/-</sup> heterozygous mice was investigated and it was observed that I/R-induced renal dysfunction, histological

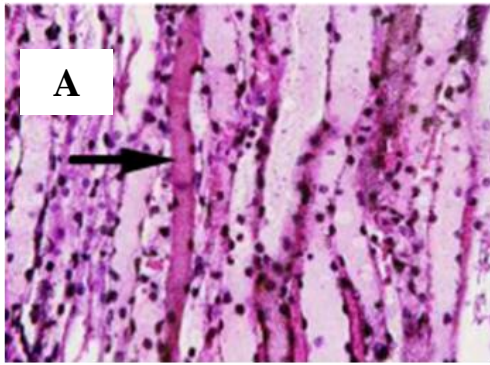
damage and  $Ca^{2+}$  accumulation in necrotic tubular epithelium were more markedly in NCX1<sup>+/-</sup> wild type than in NCX1<sup>+/-</sup> heterozygous mice (Yamashita et al, 2003).

Some reports also indicated the efficacy of KB-R7943 on contrast-induced renal tubular cytotoxicity and AKI (Yang et al, 2013; Yang et al, 2013). These results indicated that  $Ca^{2+}$  overload through the reverse mode of NCX seems to play an important role in the regulation of intracellular  $Ca^{2+}$  in the pathogenesis of I/R-induced AKI.

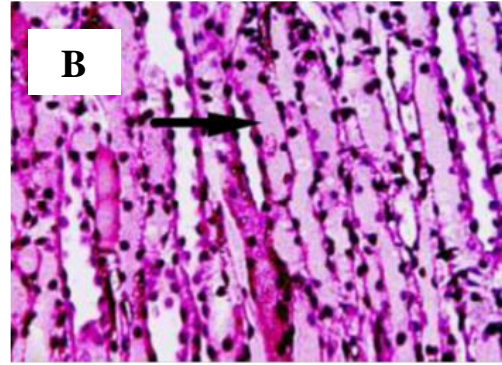
In conclusion, both pre- and post-ischemic treatments with KB-R7943 overcame the I/R-induced renal injury. Thus, inhibition of NCX may be considered as a therapeutic approach to protect the post-ischemic AKI in humans.

#### ACKNOWLEDGMENTS

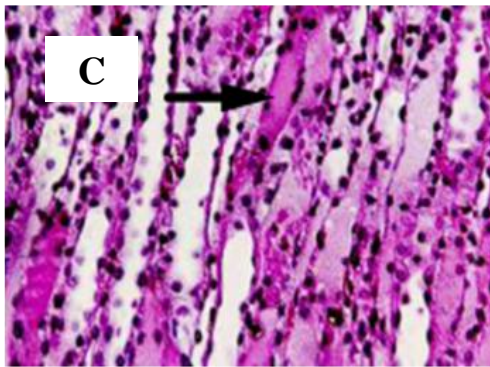
The authors are grateful to Dr. N. Nishimura (New Drug Discovery Research Laboratory, Kanebo, Ltd, Osaka, Japan)



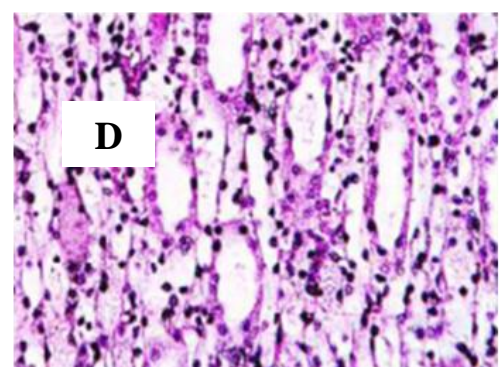
**control**



**control + post-verapamil 1 mg/kg**



**control + post-KB-R7943 2 mg/kg**



**control + post-KB-R7943 10 mg/kg**

**Figure 4:** Light microscopy of the inner zone of medulla of the kidney of AKI rats treated with vehicle (A), verapamil (B, 1 mg/kg), KB-R7943 (C, 2 mg/kg), and KB-R7943 (D, 10 mg/kg) at 24 h after ischemia/reperfusion. Drugs were given i.v. after reperfusion. Arrows indicate tubular necrosis (hematoxylin and eosin staining).

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**Table 2:** Effects of verapamil and KB-R7943 administered after reperfusion on histopathological changes of kidneys 24 h after reperfusion.

Histopathological changes/grade	Tubular necrosis					Protein casts				
	-	±	+	++	+++	-	±	+	++	+++
	0	1	2	3	4	0	1	2	3	4
Control (n=6)	0 (3.67 ± 0.21)	0 (3.67 ± 0.21)	0 (3.67 ± 0.21)	2 (3.67 ± 0.21)	4 (3.67 ± 0.21)	0 (3.83 ± 0.17)	0 (3.83 ± 0.17)	0 (3.83 ± 0.17)	1 (3.83 ± 0.17)	5 (3.83 ± 0.17)
Control + post-verapamil 1 mg/kg (n=6)	0 (3.50 ± 0.22)	0 (3.50 ± 0.22)	0 (3.50 ± 0.22)	3 (3.50 ± 0.22)	3 (3.50 ± 0.22)	0 (3.83 ± 0.17)	0 (3.83 ± 0.17)	0 (3.83 ± 0.17)	1 (3.83 ± 0.17)	5 (3.83 ± 0.17)
Control + post-KB-R7943 2 mg/kg (n=6)	0 (2.83 ± 0.40)	0 (2.83 ± 0.40)	3 (2.83 ± 0.40)	1 (2.83 ± 0.40)	2 (2.83 ± 0.40)	0 (3.17 ± 0.31)	0 (3.17 ± 0.31)	1 (3.17 ± 0.31)	3 (3.17 ± 0.31)	2 (3.17 ± 0.31)
Control + post-KB-R7943 10 mg/kg (n=6)	0 (2.17 ± 0.31 <sup>a</sup> )	0 (2.17 ± 0.31 <sup>a</sup> )	0 (2.17 ± 0.31 <sup>a</sup> )	3 (2.17 ± 0.31 <sup>a</sup> )	3 (2.17 ± 0.31 <sup>a</sup> )	0 (2.33 ± 0.33 <sup>a</sup> )	1 (2.33 ± 0.33 <sup>a</sup> )	2 (2.33 ± 0.33 <sup>a</sup> )	3 (2.33 ± 0.33 <sup>a</sup> )	0 (2.33 ± 0.33 <sup>a</sup> )

Data are expressed as the number of animals with histopathological changes. Values in parenthesis represents the mean ± S.E.M of histopathological change/grade. Drugs were given after reperfusion. Grades: no change (- or 0), mild (± or 1), moderate (+ or 2), severe (++ or 3) and very severe (+++ or 4). <sup>a</sup>*P* < 0.01 compared with control rats.

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