

JPET#92049

## **Opposite Effects of Pre- and Post-Ischemic Treatments with NO Donor on Ischemia/Reperfusion-Induced Renal Injury**

**Atsushi Nakajima, Kyoko Ueda, Masanori Takaoka, Yoshiko Yoshimi,  
and Yasuo Matsumura**

Department of Pharmacology, Osaka University of Pharmaceutical Sciences,  
4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan (A. N., K. U., M. T., Y. Y., Y. M.)

JPET#92049

**Running title:** Nitric Oxide and Acute Renal Failure

**Address correspondence to:**

Yasuo Matsumura, Ph.D.

Department of Pharmacology, Osaka University of Pharmaceutical Sciences,

4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

TEL&FAX: +81-72-690-1051

E-mail: matumrh@gly.oups.ac.jp

Number of text pages: 15

Number of tables: 1

Number of figures: 9

Number of references: 47

Number of words in the Abstract: 241

Number of words in the Introduction: 685

Number of words in the Discussion: 1126

JPET#92049

**ABBREVIATIONS:** NO, nitric oxide; FK409, ( $\pm$ )-(*E*)-4-Ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide, NOS, NO synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS; cNOS, constitutive NOS; ARF, acute renal failure; BUN, blood urea nitrogen; Ccr, creatinine clearance; Pcr, plasma creatinine concentration; UF, urine flow; Uosm, urinary osmolality;  $U_{Na}V$ , urinary excretion of sodium;  $FE_{Na}$ , fractional excretion of sodium.

**Section assignment:** Gastrointestinal, Hepatic, Pulmonary & Renal

JPET#92049

## ABSTRACT

We have previously demonstrated that pre-ischemic treatment with FK409 ( $\pm$ )-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide, a spontaneous NO donor, markedly improves ischemia/reperfusion-induced renal injury. However, there is conflicting information (renoprotective or cytotoxic) as to the contribution of NO to ischemic acute renal failure (ARF). In the present study, we investigated the effect of post-ischemic treatment with FK409 (1, 3, 10 mg/kg i.v.) at 6 h after reperfusion on ischemic ARF, in comparison with the pre-ischemic treatment effect. Ischemic ARF was induced by clamping of the left renal artery and vein for 45 min, followed by reperfusion, 2 weeks after contralateral nephrectomy. Renal function in ARF rats markedly decreased at 24 h after reperfusion. Histopathological examination of the kidney of ARF rats revealed severe renal damage. In contrast to the renoprotective effect by pre-ischemic treatment, post-ischemic treatment with FK409 aggravated the ischemia/reperfusion-induced renal dysfunction and histological damage. Immunohistochemical analysis of renal sections obtained from ARF rats revealed positive staining for nitrotyrosine, a biomarker of peroxynitrite formation, in injured tubular cells, and more intense staining was observed in renal tissues from the animals received post-ischemic treatment with FK409. On the other hand, the formation of nitrotyrosine, neutrophil infiltration into renal tissues and renal superoxide production, all of which were enhanced in ARF rats, were efficiently attenuated by the pre-ischemic treatment with FK409. These results demonstrate that, although pre-ischemic treatment with a NO donor is renoprotective, post-ischemic treatment with the same agent aggravates the ischemia/reperfusion-induced renal injury, probably through peroxynitrite overproduction.

JPET#92049

## Introduction

There is accumulating evidence that, in the kidney, various cells, including vascular endothelial and tubular epithelial cells, can generate nitric oxide (NO), which interacts with vascular smooth muscle, mesangial, and tubular cells to control renal blood flow and glomerular/tubular functions (Kone and Baylis, 1997; Majid and Navar, 2001). In addition to the physiological importance of NO in the regulation of renal hemodynamics and function, recent studies have demonstrated that changes in NO production and/or metabolism in the kidney are closely related to various renal pathological conditions, such as chronic renal failure with renal mass reduction, lipopolysaccharide-provoked renal dysfunction and ischemic acute renal failure (ARF) (Ashab et al., 1995; Caramelo et al., 1996; Schwartz et al., 1997).

ARF is a common clinical complication with an uncertain outcome, ranging from complete restitution to high mortality (Kelly and Molitoris, 2000). Ischemia, followed by reperfusion, is one of the major causes of ARF (Thadhani et al., 1996). Various *in vivo* studies have indicated that NO biosynthesis and its action are closely related to the pathogenesis of ischemia/reperfusion-induced ARF (Conger et al., 1988; Conger et al., 1991; Schramm et al., 1994; Chintala et al., 1993; Pryor and Squadrito, 1995). Conger et al. (1988; 1991) demonstrated that decreased endothelium-dependent vasorelaxation and NO production were related to an impaired renal function observed after ischemia/reperfusion. The NO precursor L-arginine has been reported to ameliorate post-ischemic ARF (Schramm et al., 1994). Furthermore, the inhibition of NO synthase (NOS) was seen to aggravate the post-ischemic ARF (Chintala et al., 1993), thereby suggesting the renoprotective role of endogenous NO in this disease. On the

JPET#92049

other hand, NO may be deleterious because of its reactivity with oxygen free radicals produced during reperfusion of the ischemic kidney to yield toxic products, such as peroxynitrates (Pryor and Squadrito, 1995). Thus, NO seems to have bidirectional effects on the pathogenesis of ischemia/reperfusion-induced ARF, as suggested previously (Goligorsky and Noiri, 1999).

NO is synthesized by different NOS isoforms, which have been cloned and characterized: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Knowles and Moncada, 1994). It has been demonstrated that ischemia/reperfusion-induced renal injury is efficiently attenuated by genetic deficiency or the pharmacological blockade of iNOS (Ling et al., 1999; Walker et al., 2000; Chatterjee et al., 2002). While iNOS-derived NO predominantly elicits pathological effects, eNOS-derived NO is believed to be responsible for maintaining physiological renal hemodynamics and functions (Goligorsky and Noiri, 1999). Most recently, we have observed that there is a marked impairment of renal function in eNOS-deficient mice subjected to 45-minute ischemia, showing a further deterioration of the disease condition compared with wild-type mice (Yamasowa et al., 2005). Although the role of NO in the pathogenesis of post-ischemic ARF is controversial, one interesting investigation has found that a NO donor, sodium nitroprusside, prevents the neutrophil-mediated ischemic ARF, determined using isolated perfused rat kidneys (Linas et al., 1996). We also found that pre-ischemic treatment with FK409((±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide), a spontaneous NO releaser, exerted a remarkable protective effect against the ischemia/reperfusion-induced ARF (Matsumura et al., 1998). The biological actions of FK409, such as vasorelaxation, in isolated blood vessels can be accounted for by spontaneous NO

JPET#92049

release after decomposition of the compound (Kita et al., 1994a; Yamada et al., 1991). In addition, the antiplatelet effects (Kita et al., 1994b) and antianginal effects (Kita et al., 1994c) of FK409 are known to be more potent than those of organic nitrates, such as isosorbide dinitrate, these effects being based on the potential of spontaneous NO generation. Thus, this compound seems to be useful for the evaluation of functional roles of NO in the pathogenesis of ischemic ARF. Although FK409 exerted a beneficial effect by the pre-ischemic treatment (Matsumura et al., 1998), it remains to be determined whether this agent can improve the ischemia/reperfusion-induced renal injury when given after the reperfusion. In general, because ARF cannot be predicted in many clinical cases, it is more important to know whether the post-ischemic treatment is beneficial, at least enhances the recovery process, or is detrimental. Thus, we investigated the effect of post-ischemic treatment with FK409 and the findings were compared with those observed by the pre-ischemic treatment.

## Materials and Methods

**Animals and Experimental Design.** Male Sprague-Dawley rats (10 weeks of age, Japan SLC, Shizuoka) weighing 280-320 g were used. The animals were housed in a light-controlled room with a 12-h light/dark cycle and were allowed *ad libitum* access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study (at 8 weeks of age), the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg/kg, i.p.). After a 2-week recovery period, uninephrectomized rats were divided into seven groups: 1) sham-operated control; 2) vehicle-treated ischemic ARF; 3)

JPET#92049

post-ischemic treatment with FK409 (1 mg/kg, i.v.) in ARF; 4) post-ischemic treatment with FK409 (3 mg/kg, i.v.) in ARF; 5) post-ischemic treatment with FK409 (10 mg/kg, i.v.) in ARF; 6) pre-ischemic treatment with FK409 (3 mg/kg, i.v.) in ARF; 7) pre-ischemic treatment with FK409 (10 mg/kg, i.v.) in ARF. To induce ischemic ARF, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded with a nontraumatic clamp for 45 min. At the end of the ischemic period, the clamp was released for blood reperfusion. FK409 or its vehicle (a mixture of 2.5% ethanol, 30% polyethylene glycol 400 and 67.5% saline) was administered (post-ischemic treatment at 6 h after the reperfusion; pre-ischemic treatment, at 5 min before the ischemia) as a slow bolus injection at a volume of 1 ml/kg into the external jugular vein. In sham-operated control animals, the left kidney was treated identically, except for the clamping. The animals exposed to 45-min ischemia were housed in metabolic cages 24 h after reperfusion; 5-h urine samples were taken and blood samples were drawn from the thoracic 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 excised and examined using a light microscope. In separate experiments, animals were sacrificed at various time points after the start of reperfusion, for evaluation of renal dysfunction and  $O_2^-$  production.

**Renal Functional Parameters.** Blood urea nitrogen (BUN) and creatinine levels in the plasma (Pcr) or urine were determined using a commercial assay kit, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka, Japan), respectively. Urinary osmolality (Uosm) was measured by freezing point depression (Fiske Associates, Norwood, MA, USA). Urine and plasma sodium concentrations



JPET#92049

were determined using a flame photometer (Hitachi, 205D, Hitachinaka, Japan). The fractional excretion of sodium ( $FE_{Na}$ , %) was calculated from the following formula:  $FE_{Na} = U_{Na}V / (P_{Na} \times \text{creatinine clearance, Ccr}) \times 100$ , where  $U_{Na}V$  is the urinary excretion of sodium and  $P_{Na}$  is the plasma sodium concentration.

**Histological Studies.** Excised left kidneys were processed for light microscopic observation, according to standard procedures. The kidneys were then fixed in phosphate-buffered 10% formalin, after which the kidneys were chopped into small pieces, embedded in paraffin wax, cut at 4  $\mu\text{m}$  and stained with hematoxylin and eosin. Histopathological changes were analyzed for tubular necrosis, proteinaceous casts and medullary congestion, as suggested by Solez et al. (1974). Tubular necrosis and proteinaceous casts were graded as follows: no damage (0), mild (1, unicellular, patchy isolated damage), moderate (2, damage less than 25%), severe (3, damage between 25 and 50%) and very severe (4, more than 50% damage). The degree of medullary congestion was defined as: no congestion (0), mild (1, vascular congestion with identification of erythrocytes by x 400 magnification), moderate (2, vascular congestion with identification of erythrocytes by x 200 magnification), severe (3, vascular congestion with identification of erythrocytes by x 100 magnification) and very severe (4, vascular congestion with identification of erythrocytes by x 40 magnification). The scoring of the histological data was performed by independent observers in a double blind manner.

**Measurement of Renal Superoxide ( $O_2^-$ ) Production.** Renal  $O_2^-$  production was measured using a lucigenin-enhanced chemiluminescence assay (Skatchkov et al., 1999). The whole kidney was removed from rats and cut into strips (2-mm pieces). Immediately, renal tissue segments were placed in test tubes containing modified

JPET#92049

Krebs-HEPES buffer (pH 7.4, 99.01 mM NaCl, 4.69 mM KCl, 1.87 mM CaCl<sub>2</sub>, 1.20 mM MgSO<sub>4</sub>, 1.03 mM K<sub>2</sub>HPO<sub>4</sub>, 25 mM Na-HEPES, 11.1 mM glucose) and allowed to equilibrate in the dark for 15 minutes at 37°C before measurement. After the equilibration, lucigenin (5 μM) was added to the tube and then the luminescence was measured using a luminometer (Sirius-2, Berthold Detection Systems, USA). The relative light unit (RLU) was integrated every 3 seconds for 15 minutes and averaged. The renal O<sub>2</sub><sup>-</sup> production was expressed as RLU/min/mg dry tissue weight.

**Immunohistochemical Analysis.** Nitrotyrosine formation, a marker of peroxynitrite formation, in the kidney was determined using immunohistochemical staining. Paraffin-embedded tissue sections (4 μm) were cleared in xylene, ethanol and washed in phosphate-buffered saline. Slides were incubated in methanol with 3% H<sub>2</sub>O<sub>2</sub> for 20 min to block endogenous peroxidase activity. Nonspecific protein binding was blocked by incubation with 10% normal rabbit serum (Histofine SAB-PO kit, Nichirei, Japan). Mouse anti-nitrotyrosine antibody (ZYMED, USA) was incubated with the sections for 1 h at room temperature. After incubation with primary antibody, specific labeling was detected using a biotin-conjugated rabbit anti-mouse immunoglobulin (Histofine SAB-PO kit) and streptavidin-conjugated peroxidase (Histofine SAB-PO kit). Subsequently, the tissue-bound peroxidase was visualized using 3,3'-diaminobenzidine (Histofine DAB kit, Nichirei, Japan). Samples were then viewed under a light microscope.

**Neutrophil Infiltration.** Neutrophil infiltration was evaluated using naphthol AS-D chloroacetate esterase staining (91C, SIGMA-ALDRICH, USA) (Moloney et al., 1960; Chiao et al., 1997) by counting the number of neutrophils present in the outer zone of the medulla of the kidneys. Neutrophils were counted in 50 randomly selected

JPET#92049

high-power fields (x 400) of the outer zone of medulla. Data were expressed as neutrophils per mm<sup>2</sup> of tissue.

**Drug.** FK409, a kind gift from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), was dissolved in a mixture of 2.5% ethanol, 30% polyethylene glycol 400 and 67.5% saline immediately prior to administration.

**Statistical Analysis.** Values are expressed as the mean  $\pm$  S.E.M. Relevant data was processed by InStat (Graph-PAD Software for Science, San Diego, CA). For statistical analysis, we used the unpaired Student's *t*-test for two-group comparison and one-way analysis of variance followed by Dunnett's tests for multiple comparisons. Histological data were analyzed using the Kruskal-Wallis nonparametric test combined with the Steel-type multiple comparison test. For all comparisons, differences were considered significant at  $P < 0.05$ .

## Results

**Time Course of Blood Urea Nitrogen after the Ischemia/Reperfusion.** First, we examined the time course profile of BUN in the ARF rats. As shown in Fig. 1, BUN levels in rats subjected to 45-min ischemia increased gradually after the reperfusion; there was an apparent increase at 6 h after the reperfusion, and thereafter the BUN levels were markedly elevated. Therefore, in the following experiments to evaluate whether the post-ischemic treatment with FK409 is beneficial or detrimental on the ischemic ARF, this agent was administered at 6 h after the start of reperfusion.

**Renal Function after the Ischemia/Reperfusion and Effects of Post-ischemic Treatment with FK409.** The renal function of rats subjected to 45-min ischemia showed a marked deterioration when measured 24 h after the reperfusion (Fig. 2). As

JPET#92049

compared with sham-operated rats, vehicle-treated ARF rats showed significant increases in BUN ( $99.46 \pm 4.9$  versus  $28.85 \pm 1.8$  mg/dl), Pcr ( $2.53 \pm 0.23$  versus  $0.76 \pm 0.08$  mg/dl), urine flow (UF,  $89.0 \pm 4.0$  versus  $24.0 \pm 1.6$   $\mu$ l/min/kg) and  $FE_{Na}$  ( $1.81 \pm 0.22$  versus  $0.35 \pm 0.06\%$ ), and significant decreases in Ccr ( $1.39 \pm 0.14$  versus  $4.18 \pm 0.37$  ml/min/kg) and Uosm ( $480 \pm 28$  versus  $1693 \pm 64$  mOsm/kg). Post-ischemic treatment with FK409 (1, 3, 10 mg/kg, i.v.) at 6 h after reperfusion significantly deteriorated the ischemia/reperfusion-induced renal dysfunction, except for UF and Uosm; these findings were in striking contrast to those observed in our previous study (Matsumura et al., 1998), in which the pre-ischemic treatment with the same agent (1, 3 mg/kg, i.v.) markedly improved the ischemia/reperfusion-induced renal injury. In the present study, a higher dose of FK409 (10 mg/kg) given prior to the 45-min ischemia also exhibited a notable ameliorating effect. The administration of 10 mg/kg FK409 to sham-operated rats produced no significant effects in their renal function (data not shown).

**Histological Renal Damage after the Ischemia/Reperfusion and Effects of Post-ischemic Treatment with FK409.** Histopathological examinations revealed severe lesions in the kidney of vehicle-treated ARF rats (24 h after the ischemia/reperfusion). These changes were characterized by tubular necrosis in the outer zone outer stripe of medulla (Fig. 3B) (scores,  $3.17 \pm 0.17$ , Fig. 4), medullary congestion and hemorrhage in the outer zone inner stripe of medulla (Fig. 3F) (scores,  $2.83 \pm 0.17$ , Fig. 4) and proteinaceous casts in tubuli in the inner zone of medulla (Fig. 3J) (scores,  $2.83 \pm 0.17$ , Fig. 4). Post-ischemic treatment with FK409 worsened the development of all these lesions in a dose-related manner (Fig. 3, Fig. 4).

**Time Course of Renal  $O_2^-$  Production after the Ischemia/Reperfusion and**

JPET#92049

**Effects of Post-ischemic Treatment with FK409.** As shown in Fig. 5, renal  $O_2^-$  production in rats subjected to 45-min ischemia increased gradually after the reperfusion. At 6 h after the reperfusion, there was a significant and marked increase in the renal  $O_2^-$  level, and thereafter its level reached a plateau during the first 24 h after the reperfusion. When 10 mg/kg of FK409 was given at 6 h after the reperfusion, increased renal  $O_2^-$  production was temporarily and markedly reduced from 6.5 to 8 h after the reperfusion, i.e., from 0.5 to 2 h after the administration of FK409, but the reduced level of renal  $O_2^-$  production was restored between 12 and 24 h after the reperfusion. Similar temporal decreases in renal  $O_2^-$  production were observed when a lower dose of FK409 (3 mg/kg) was given (data not shown).

**Nitrotyrosine Formation in the Kidney after the Ischemia/Reperfusion and Effects of Pre- or Post-ischemic Treatment with FK409.** Tyrosine nitration has been used as an index of the nitrosylation of protein by peroxynitrite (Walker et al., 2000). Compared with renal sections of sham-operated rats, immunohistochemical analysis of renal sections obtained from vehicle-treated ARF rats at 24 h after the ischemia/reperfusion revealed positive staining for nitrotyrosine in tubules (Fig. 6C, 6D). Furthermore, more intense staining was observed in renal sections obtained from ARF rats given post-ischemic FK409 treatment (10 mg/kg) (Fig. 6E, 6F). Similar nitrotyrosine-positive staining was increased by the post-ischemic treatment at the lower dose (3 mg/kg) (Fig. 7A). On the other hand, when the same dose of FK409 was administered at 5 min prior to the ischemia, reduced nitrotyrosine staining was observed (Fig. 7B) compared with the case of the vehicle-treated ARF rats. These findings suggest that while the post-ischemic FK409 treatment augments the peroxynitrite

JPET#92049

formation in the kidney subjected to the ischemia/reperfusion, the pre-ischemic treatment with the same agent suppresses it.

**Neutrophil Infiltration in the Kidney after the Ischemia/Reperfusion and Effects of Pre- or Post-ischemic Treatment with FK409.** We evaluated whether pre-ischemic treatment with FK409 suppressed the neutrophil infiltration into renal tissue, an event that has been known to produce  $O_2^-$  (Clancy et al., 1992) and is believed to be one of the main causal factors of the ischemia/reperfusion-induced ARF (Linas et al., 1992). As shown in Fig. 8B, neutrophils were observed in the kidney of vehicle-treated ARF rats 6 h after the reperfusion. The number of infiltrating neutrophils in the vehicle-treated ARF rats was significantly increased compared with that in the sham-operated rats (Fig. 8A, 9A). On the other hand, the neutrophil infiltration was markedly suppressed in the renal tissues of ARF rats given FK409 (3 mg/kg) prior to the 45-min ischemia (Fig. 8C, 9A). When the neutrophil infiltration was determined 24 h after the reperfusion, there was a slight but significant increase, which tended to be suppressed by the pre-ischemic FK409 treatment (Fig. 8D, 8E, 9B), but not by the post-ischemic treatment.

**Effects of Pre-ischemic Treatment with FK409 on Renal  $O_2^-$  Production after the Ischemia/Reperfusion.** Finally, the effect of pre-ischemic treatment with FK409 on renal  $O_2^-$  production in ARF rats. As shown in Fig. 9C, the increased level of renal  $O_2^-$  production at 6 h after the ischemia/reperfusion was markedly suppressed by treatment with FK409 (3 mg/kg) prior to the 45-min ischemia. Similar suppressive effects of the FK409 pretreatment were observed at 24 h after the ischemia/reperfusion (Fig. 9D).

## Discussion

Ischemic ARF is a frequent clinical syndrome with a high morbidity and mortality (Thadhani et al., 1996). Reperfusion of previously ischemic renal tissue initiates a series of complex cellular events that results in injury and the eventual death of renal cells due to a combination of apoptosis and necrosis (Lieberthal and Levine, 1996). The molecular mechanisms underlying the ischemia/reperfusion-induced renal injury are poorly understood, but it has been reported that several causal factors (ATP depletion, reactive oxygen species, phospholipase activation, neutrophil infiltration, vasoactive peptides etc.) are contributive to the pathogenesis of this renal damage (Edelstein et al., 1997). We found that the post-ischemic treatment with FK409 worsened the ischemia/reperfusion-induced renal dysfunction and related tissue injury, in contrast to our previous findings (Matsumura et al., 1998) indicating that FK409 administration prior to the ischemia markedly attenuated the ischemia/reperfusion-induced ARF. Thus, under the same experimental conditions using the same agent and animal species, we demonstrated the duality of the actions of NO in ischemia/reperfusion-induced ARF.

One can speculate that renal and/or systemic hemodynamic effects of FK409 given before or after the ischemia may influence the post-ischemic renal function. In our previous study (Matsumura et al., 1998), the pretreatment with FK409 (1 mg/kg, i.v.) failed to ameliorate the immediate renal hemodynamic changes after the ischemia/reperfusion. In addition, we noted that the blood pressure-lowering effects of FK409 given before or after the ischemia were similar. Moreover, an intravenous administration of hydralazine, at the same hypotensive dose as FK409, had no effect on

JPET#92049

the ischemia/reperfusion-induced renal dysfunction (unpublished observations). Thus, it is reasonable to consider that the contrasting effects obtained by the pre- and post-ischemic treatments with FK409 are independent of the drug-induced transient renal and/or systemic hemodynamic changes.

Several studies have demonstrated that endogenous or exogenous NO protects the kidney against ischemia/reperfusion injury (Schramm et al., 1994; Chintala et al., 1993; Linas et al., 1996). Most recently, we noted that both exogenous and endogenous NO have protective effects against ischemia/reperfusion-induced renal dysfunction and tissue injury, at least in part, through the suppression of endothelin-1 (ET-1) production (Kurata et al., 2004). The overproduction of this peptide in the post-ischemic kidney is known to be one of the major causal factors of this disease (Wilhelm et al., 1999; Matsumura et al., 2000). In addition, we noted that pre-ischemic treatment with FK409 suppressed the enhancement of renal sympathetic nerve activity in the post-ischemic kidney (unpublished observation), which is closely related to the renal dysfunction in the post-ischemic kidney (Fujii et al., 2003). The present study clearly demonstrated that the pre-ischemic treatment with FK409 markedly suppressed the renal  $O_2^-$  production augmented by the ischemia/reperfusion, following the attenuation of neutrophil infiltration. Neutrophil infiltration/migration appears to contribute to the post-ischemic ARF through various mechanisms. Linas et al. (1988; 1992) noted that mild renal ischemia and primed neutrophils synergistically enhanced renal ischemic injury. In addition, monoclonal antibodies to neutrophil adhesion molecules are known to decrease the post-ischemic renal injury (Rabb et al., 1994). Kubes et al. (1991) found that neutrophil adhesion in postcapillary venules was markedly enhanced by a NO synthase inhibitor, and that the inhibitor-induced enhancement was prevented by



JPET#92049

L-arginine, thereby suggesting that NO may be an important endogenous inhibitor of neutrophil adhesion in venules. Moreover, NO is reported to inhibit neutrophil  $O_2^-$  production via direct effects on the membrane components of the NADPH oxidase (Clancy et al., 1992). Taken together, FK409, given prior to the ischemia, exerted a renoprotective effect via multi-functional mechanisms.

The post-ischemic treatment with FK409 was performed 6 h after the start of reperfusion, because the ischemia/reperfusion-induced renal dysfunction was apparent at the same time. Moreover, the enhancement of renal  $O_2^-$  production was also observed. FK409 administration temporarily decreased the renal  $O_2^-$  level, and was followed by intense positive nitrotyrosine staining, suggesting the augmentation of peroxynitrite formation. The ischemia/reperfusion-induced renal dysfunction and tissue injury were much more severe in animals given the post-ischemic treatment with FK409. Such a deteriorating effect of FK409 was not observed when the agent was given immediately after the start of reperfusion (data not shown), and then renal  $O_2^-$  production did not increase. The rate constant for the reaction of  $O_2^-$  with NO is known to be 3-fold higher than that with superoxide dismutase (Crow and Beckman, 1996). Thus, it is most likely that FK409-derived NO reacts with  $O_2^-$  to form peroxynitrite, which causes injury via direct oxidant injury and protein tyrosine nitration (Beckman, 1996; Radi et al., 2001).

Nitrotyrosine formation has been detected in several oxidant-mediated disease models, such as myocardial ischemia/reperfusion injury (Liu et al., 1997), acute pulmonary inflammation (Kooy et al., 1997) and lipopolysaccharide-induced renal injury (Zhang et al., 2000). In ischemia/reperfusion-induced ARF models,

JPET#92049

nitrotyrosine-protein adducts are observed in the tubular epithelium 6~24 h after the ischemia/reperfusion (Walker et al., 2000; Chatterjee et al., 2002; Chiao et al., 1997). Recent studies (Walker et al., 2000; Chatterjee et al., 2002; Chatterjee et al., 2003) demonstrated that iNOS inhibitors could improve the ischemia/reperfusion-induced renal dysfunction and tissue injury, and reduce nitrotyrosine formation, suggesting that iNOS-generated NO mediates the above renal damage through peroxynitrite formation. Most recently, Schneider et al. (2003) demonstrated that iNOS expression in the post-ischemic kidney (24 h after the ischemia/reperfusion) markedly increased to fourfold of the control, in contrast to the moderate down-regulation of eNOS expression. Taken together, iNOS-derived NO seems to contribute to the pathophysiology of ischemia/reperfusion-induced ARF, as suggested (Goligorsky and Noiri, 1999). Selective iNOS inhibitors may be useful against renal dysfunction and injury induced by ischemia/reperfusion of the kidney in humans. Furthermore, this view is strongly supported by the evidence that kidneys of iNOS knockout mice are protected against ischemia/reperfusion-induced ARF (Chatterjee et al., 2003; Ling et al., 1999). Thus, the aggravation of ischemia/reperfusion-induced ARF by post-ischemic treatment with FK409 seems to reflect exaggerated responses to the pathological effect of iNOS-derived NO.

In the present study, FK409 given after reperfusion aggravated the ischemia/reperfusion-induced renal dysfunction and tissue injury, and these lesions were accompanied by enhanced nitrotyrosine formation in the tubular epithelium, suggesting that the increment of peroxynitrite formation is closely related to the above lesions. In contrast, the pre-ischemic administration of FK409 at same dose, which attenuated the ischemia/reperfusion-induced ARF, reduced neutrophil infiltration, renal  $O_2^-$  production

JPET#92049

and nitrotyrosine staining. Thus, our findings proved the diametrically opposed effects of NO donor on the ischemia/reperfusion-induced renal injury. Most recently, we have observed that there is a marked impairment of renal function in eNOS-deficient mice subjected to 45-min ischemia, showing a further deterioration of the disease condition compared with wild-type mice (Yamasowa et al., 2005). Taken together, pre- and post-ischemic treatments with FK409 may reflect the renoprotective effect of eNOS-derived NO and the cytotoxic effect of iNOS-derived NO, respectively, although the functional roles of endogenously generated NO in ischemia/reperfusion-induced ARF remain to be clarified.

### **Acknowledgment**

We thank Daniel Mrozek for reading the manuscript.

JPET#92049

## REFERENCES

- Ashab I, Peer G, Blum M, Wollman Y, Chernihovsky T, Hassner A, Schwartz D, Cabili S, Silverberg D, and Iaina A (1995) Oral administration of L-arginine and captopril in rats prevents chronic renal failure by nitric oxide production. *Kidney Int* **47**:1515–1521.
- Beckman JS (1996) Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* **9**:836–844.
- Caramelo C, Espinosa G, Manzarbeitia F, Cernadas MR, Tejerizo GP, Tan D, Mosquera JR, Digiuni E, Montón M, Millás I, Hernando L, Casado S, and López-Farré A (1996) Role of endothelium-related mechanisms in the pathophysiology of renal ischemia/reperfusion in normal rabbits. *Circ Res* **79**:1031–1038.
- Chatterjee PK, Patel NSA, Kvale EO, Cuzzocrea S, Brown PAJ, Stewart KN, Mota-Filipe H, and Thiernemann C (2002) Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury. *Kidney Int* **61**:862–871.
- Chatterjee PK, Patel NSA, Sivarajah A, Kvale EO, Dugo L, Cuzzocrea S, Brown PAJ, Stewart KN, Mota-Filipe H, Britti D, Yaqoob M, and Thiernemann C (2003) GW274150, a potent and highly selective inhibitor of iNOS, reduces experimental renal ischemia/reperfusion injury. *Kidney Int* **63**:853–865.
- Chiao H, Kohda Y, McLeroy P, Craig L, Housini I, and Star RA (1997)  $\alpha$ -Melanocyte-stimulating hormone protects against renal injury after ischemia in mice and rats. *J Clin Invest* **99**:1165–1172.
- Chintala MS, Chiu PJS, Vemulapalli S, Watkins RW, and Sybertz EJ (1993) Inhibition of endothelial derived relaxing factor (EDRF) aggravates ischemic acute renal failure

JPET#92049

- in anesthetized rats. *Naunyn-Schmiedeberg's Arch Pharmacol* **348**:305–310.
- Clancy RM, Leszczynska-Piziak J, and Abramson SB (1992) Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J Clin Invest* **90**:1116–1121.
- Conger JD, Robinette JB, and Schrier RW (1988) Smooth muscle calcium and endothelium-derived relaxing factor in the abnormal vascular responses of acute renal failure. *J Clin Invest* **82**:532–537.
- Conger JD, Robinette JB, and Hammond WS (1991) Differences in vascular reactivity in models of ischemic acute renal failure. *Kidney Int* **39**:1087–1097.
- Crow JP and Beckman JS (1996) The importance of superoxide in nitric oxide-dependent toxicity. In: *Biological Reactive Intermediates V*, edited by Snyder R, New York, Plenum Press, pp 147–161.
- Edelstein CL, Ling H, and Schrier RW (1997) The nature of renal cell injury. *Kidney Int* **51**:1341–1351.
- Fujii T, Kurata H, Takaoka M, Muraoka T, Fujisawa Y, Shokoji T, Nishiyama A, Abe Y, and Matsumura Y (2003) The role of renal sympathetic nervous system in the pathogenesis of ischemic acute renal failure. *Eur J Pharmacol* **481**:241–248.
- Goligorsky MS and Noiri E (1999) Duality of nitric oxide in acute renal failure. *Semin Nephrol* **19**:263–271.
- Kelly KJ and Molitoris BA (2000) Acute renal failure in the new millennium: Time to consider combination therapy. *Semin Nephrol* **20**:4–19.
- Kita Y, Hirasawa Y, Maeda K, Nishio M, and Yoshida K (1994a) Spontaneous nitric oxide release accounts for the potent pharmacological actions of FK409. *Eur J Pharmacol* **257**:123–130.

JPET#92049

- Kita Y, Hirasawa Y, Yoshida K, and Maeda K (1994b) Antiplatelet activities of FK409, a new spontaneous NO releaser. *Br J Pharmacol* **113**:385–388.
- Kita Y, Ozaki R, Sakai S, Sugimoto T, Hirasawa Y, Ohtsuka M, Senoh H, Yoshida K, and Maeda K (1994c) Antianginal effects of FK409, a new spontaneous NO releaser. *Br J Pharmacol* **113**:1137–1140.
- Knowles RG and Moncada S (1994) Nitric oxide synthase in mammals. *Biochem J* **298**:249–258.
- Kone BC and Baylis C (1997) Biosynthesis and homeostatic roles of nitric oxide in the normal kidney. *Am J Physiol* **272**:F561–F578.
- Kooy NW, Lewis SJ, Royall JA, Ye YZ, Kelly DR, and Beckman JS (1997) Extensive tyrosine nitration in human myocardial inflammation: Evidence for the presence of peroxynitrite. *Crit Care Med* **25**:812–819.
- Kubes P, Suzuki M, and Granger DN (1991) Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* **88**:4651–4655.
- Kurata H, Takaoka M, Kubo Y, Katayama T, Tsutsui H, Takayama J, and Matsumura Y (2004) Nitric oxide protects against ischemic acute renal failure through the suppression of renal endothelin-1 overproduction. *J Cardiovasc Pharmacol* **44** (Suppl 1):S455-S458.
- Lieberthal W and Levine JS (1996) Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. *Am J Physiol* **271**:F477–F488.
- Linas S, Whittenburg D, and Repine JE (1997) Nitric oxide prevents neutrophil-mediated acute renal failure. *Am J Physiol* **272**:F48–F54.
- Linas SL, Shanley PF, Whittenburg D, Berger E, and Repine JE (1988) Neutrophils accentuate ischemia-reperfusion injury in isolated perfused rat kidneys. *Am J Physiol*

JPET#92049

**255:F728–F735.**

- Linan SL, Whittenburg D, Parsons PE, and Repine JE (1992) Mild renal ischemia activates primed neutrophil to cause acute renal failure. *Kidney Int* **42**:610–616.
- Ling H, Edelstein C, Gengaro P, Meng X, Lucia S, Knotek M, Wangsiripaisan A, Shi Y, and Schrier R (1999) Attenuation of renal ischemia-reperfusion injury in inducible nitric oxide synthase knockout mice. *Am J Physiol* **277**:F383–F390.
- Liu P, Hock CE, Nagele R, and Wong PY-K (1997) Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. *Am J Physiol* **272**:H2327–H2336.
- Majid DSA and Navar LG (2001) Nitric oxide in the control of renal hemodynamics and excretory function. *Am J Hypertens* **14**:74S–82S.
- Matsumura Y, Kuro T, Kobayashi Y, Umekawa K, Ohashi N, and Takaoka (2000) Protective effect of SM-19712, a novel and potent endothelin converting enzyme inhibitor, on ischemic acute renal failure in rats. *Jpn J Pharmacol* **84**:16–24.
- Matsumura Y, Nishiura M, Deguchi S, Hashimoto N, Ogawa T, and Seo R (1998) Protective effect of FK409, a spontaneous nitric oxide releaser, on ischemic acute renal failure in rats. *J Pharmacol Exp Ther* **287**:1084–1091.
- Moloney WC, Mcpherson K, and Fliegelman L (1960) Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. *J Histochem Cytochem* **8**:200–207.
- Pryor WA and Squadrito GL (1995) The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am J Physiol* **268**:L699–L722.
- Rabb H, Mendiola CC, Dietz J, Saba SR, Issekutz TB, Abanilla F, Bonventre JV, and Ramirez G (1994) Role of CD11a and CD11b in ischemic acute renal failure in rats.

JPET#92049

*Am J Physiol* **267**:F1052–F1058.

Radi R, Peluffo G, Alvarez MN, Naviliat M, and Cayota A (2001) Unraveling peroxynitrite formation in biological systems. *Free Rad Biol Med* **30**:463–488.

Schneider R, Raff U, Vornberger N, Schmidt M, Freund R, Reber M, Schramm L, Gambaryan S, Wanner C, Schmidt HH, and Galle J (2003) L-Arginine counteracts nitric oxide deficiency and improves the recovery phase of ischemic acute renal failure in rats. *Kidney Int* **64**:216–225.

Schramm L, Heidbreder E, Schmitt A, Kartenbender K, Zimmermann J, Ling H, and Heidland A (1994) Role of L-arginine-derived NO in ischemic acute renal failure in the rat. *Renal Failure* **16**:555–569.

Schwartz D, Mendonca M, Schwartz I, Xia Y, Satriano J, Wilson CB, and Blantz RC (1997) Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in rats. *J Clin Invest* **100**:439–448.

Skatchkov MP, Sperling D, Hink U, Mulsch A, Harrison DG, Sindermann I, Meinertz T, and Munzel T (1999) Validation of lucigenin as a chemiluminescent probe to monitor vascular superoxide as well as basal vascular nitric oxide production. *Biochem Biophys Res Commun* **254**:319–324.

Solez K, Kramer EC, Fox JA, and Heptinstall RH (1974) Medullary plasma flow and intravascular leukocyte accumulation in acute renal failure. *Kidney Int* **6**:24–37.

Thadhani R, Pascual M, and Bonventre JV (1996) Acute renal failure. *N Engl J Med* **334**:1448–1460.

Walker LM, Walker PD, Imam SZ, Ali SF, and Mayeux PR (2000) Evidence for peroxynitrite formation in renal ischemia-reperfusion injury: studies with the



JPET#92049

inducible nitric oxide synthase inhibitor L-N(6)-(1-Iminoethyl)lysine. *J Pharmacol Exp Ther* **295**:417–422.

Wilhelm SM, Simonson MS, Robinson AV, Stowe NT, and Schulak JA (1999) Endothelin up-regulation and localization following renal ischemia and reperfusion. *Kidney Int* **55**:1011–1018.

Yamada H, Yoneyama F, Satoh K, and Taira N (1991) Comparison of the effects of the novel vasodilator FK409 with those of nitroglycerin in isolated coronary artery of the dog. *Br J Pharmacol* **103**:1713–1718.

Yamasawa H, Shimizu S, Inoue T, Takaoka M, and Matsumura Y (2005) Endothelial nitric oxide contributes to the renal protective effects of ischemic preconditioning. *J Pharmacol Exp Ther* **312**:153–159.

Zhang C, Walker LM, and Mayeux PR (2000) Role of nitric oxide in lipopolysaccharide- induced oxidant stress in the rat kidney. *Biochem Pharmacol* **59**:203–209.

JPET#92049

### **Footnotes**

This work was supported by a “High-Tech Research Center” Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2002-2006 and a Grant-in-Aid for Scientific Research 14570092 (to Y.M.) from MEXT.

JPET#92049

## Legends for Figures

**Fig. 1.** Time course of blood urea nitrogen (BUN) in vehicle-treated ARF rats. Each column and bar represents the mean  $\pm$  S.E.M. \*,  $P < 0.01$ , compared with sham rats. ARF, acute renal failure.

**Fig. 2.** Effects of post-ischemic treatment with FK409 on blood urea nitrogen (BUN, A), plasma creatinine concentration (Pcr, B), creatinine clearance (Ccr, C), urine flow (UF, D), urinary osmolality (Uosm, E) and fractional excretion of sodium ( $FE_{Na}$ , F) 24 h after ischemia/reperfusion. FK409 was given intravenously 6 h after reperfusion. Each column and bar represents the mean  $\pm$  S.E.M. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , compared with vehicle-treated ARF rats. ARF, acute renal failure.

**Fig. 3.** Light microscopy of the outer zone outer stripe (A-D), the outer zone inner stripe (E-H) and the inner zone of medulla (I-L) of the kidney in ARF rats treated with vehicle (B, F, J), FK409 3 mg/kg (C, G, K) or FK409 10 mg/kg (D, H, L), 24 h after ischemia/reperfusion, and sham-operated rats (A, F, I). FK409 was given intravenously 6 h after reperfusion. Arrows indicate severe tubular necrosis (B-D), congestion and hemorrhage (F-H) and proteinaceous casts in tubuli (J-L) (hematoxylin-eosin staining, magnification x 200). ARF, acute renal failure.

**Fig. 4.** Effects of post-ischemic treatment with FK409 (3, 10 mg/kg) on histopathological changes in the kidneys of ARF rats. Each column and bar represents

JPET#92049

the mean  $\pm$  S.E.M. of the histopathological score. Grades of score: no change (0), mild (1), moderate (2), severe (3), very severe (4). \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , compared with vehicle-treated ARF rats. ARF, acute renal failure.

**Fig. 5.** Time course of superoxide production in the kidney of vehicle-treated or FK409-treated ARF rats. FK409 (10 mg/kg) was given intravenously 6 h after reperfusion. Each column and bar represents the mean  $\pm$  S.E.M. \*,  $P < 0.01$ , compared with sham rats. †,  $P < 0.05$ , compared with vehicle-treated ARF rats. ARF, acute renal failure.

**Fig. 6.** Immunohistochemical staining for nitrotyrosine in the kidney of ARF rats treated with vehicle (C, D) or FK409 10 mg/kg (E, F) 24 h after the ischemia/reperfusion, and sham-operated rats (A, B). FK409 was given intravenously 6 h after the reperfusion. Arrows indicate nitrotyrosine (A, C, E; magnification x 40, B, D, F; magnification x 200).

**Fig. 7.** Immunohistochemical staining for nitrotyrosine in the kidney of ARF rats given post- (A) or pre- (B) ischemic treatment with FK409 3 mg/kg 24 h after the ischemia/reperfusion. FK409 was given intravenously 5 min before the ischemia or 6 h after the reperfusion. Arrow indicates nitrotyrosine (magnification x 200).

**Fig. 8.** Effect of pre-ischemic treatment with FK409 on neutrophil infiltration 6 h and 24 h after the reperfusion. Neutrophil infiltration was evaluated using naphthol AS-D chloroacetate esterase staining. Light microscopy of the kidney in the ARF rat treated

JPET#92049

with vehicle (B, D) or FK409 3 mg/kg (C, E) 6 h (B, C) and 24 h (D, E) after ischemia/reperfusion, and sham-operated rat (A). Arrows indicate neutrophils (magnification, x 400). FK409 was given intravenously 5 min before the ischemia. ARF, acute renal failure.

**Fig. 9.** Effect of pre-ischemic treatment with FK409 on neutrophil infiltration (A, B) and renal superoxide production (C, D) 6 h (A, C) and 24 h (B, D) after the reperfusion. FK409 was given intravenously 5 min before the ischemia. Each column and bar represents the mean  $\pm$  S.E.M. (n=5~7). \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , compared with vehicle-treated ARF rats. ARF, acute renal failure.

TABLE 1

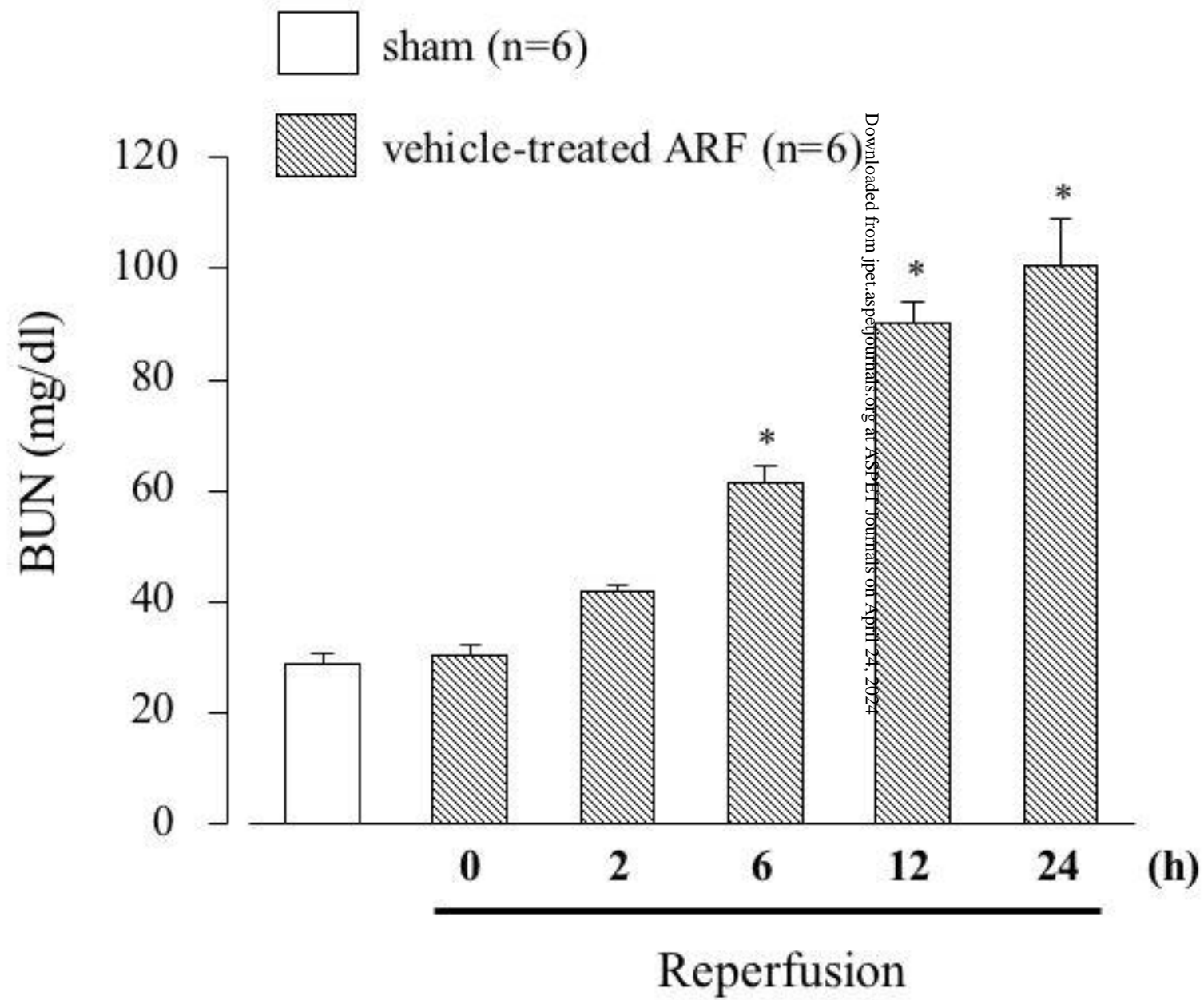
Effects of FK409 on histopathological changes of kidneys in ARF rats

All data are expressed as the number of animals with histopathological changes. Grades: no changes (- , 0), mild ( $\pm$  , 1), moderate (+ , 2), severe ( $++$  , 3), very severe ( $+++$  , 4). FK409 was given intravenously at 6 h after reperfusion.

Each value represents the mean  $\pm$  S.E.M. ARF, acute renal failure

| Experimental group              | ARF + FK409 |       |   |      |       |                           |       |   |      |       |             |       |                |      |       |       |       |   |      |                     |                     |
|---------------------------------|-------------|-------|---|------|-------|---------------------------|-------|---|------|-------|-------------|-------|----------------|------|-------|-------|-------|---|------|---------------------|---------------------|
|                                 | sham (n=6)  |       |   |      |       | vehicle-treated ARF (n=6) |       |   |      |       | ARF + FK409 |       |                |      |       |       |       |   |      |                     |                     |
|                                 |             |       |   |      |       |                           |       |   |      |       | 3 mg/kg     |       | 10 mg/kg (n=6) |      |       | (n=6) |       |   |      |                     |                     |
| Histopathological Changes/grade | -           | $\pm$ | + | $++$ | $+++$ | -                         | $\pm$ | + | $++$ | $+++$ | -           | $\pm$ | +              | $++$ | $+++$ | -     | $\pm$ | + | $++$ | $+++$               |                     |
|                                 | (0          | 1     | 2 | 3    | 4)    | (0                        | 1     | 2 | 3    | 4)    | (0          | 1     | 2              | 3    | 4)    | (0    | 1     | 2 | 3    | 4)                  |                     |
| Tubular necrosis                | 6           | 0     | 0 | 0    | 0     | 0                         | 0     | 0 | 4    | 2     | 0           | 0     | 0              | 2    | 4     | 0     | 0     | 0 | 1    | 5                   | $(3.83 \pm 0.17)^b$ |
| Medullary congestion            | 6           | 0     | 0 | 0    | 0     | 0                         | 0     | 1 | 5    | 0     | 0           | 0     | 2              | 4    | 0     | 0     | 0     | 1 | 5    | $(3.83 \pm 0.17)^a$ | $(3.67 \pm 0.21)^b$ |
| Proteinaceous casts             | 6           | 0     | 0 | 0    | 0     | 0                         | 0     | 1 | 5    | 0     | 0           | 1     | 5              | 0    | 0     | 0     | 0     | 3 | 3    | $(3.50 \pm 0.22)^b$ | $(2.83 \pm 0.17)$   |

<sup>a</sup>  $P < 0.01$ , <sup>b</sup>  $P < 0.05$ , compared with vehicle-treated ARF.



*Fig. 1.*

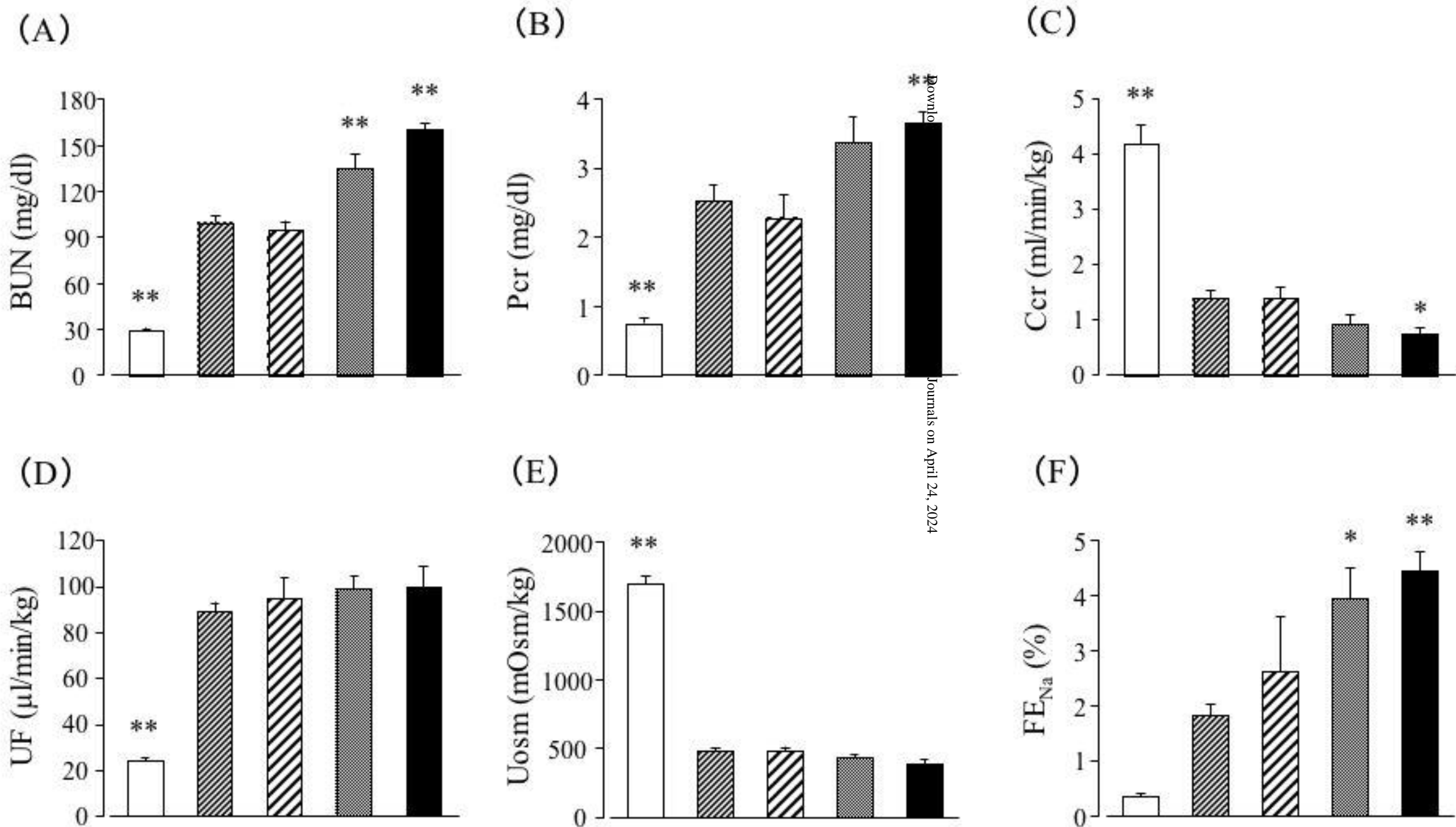
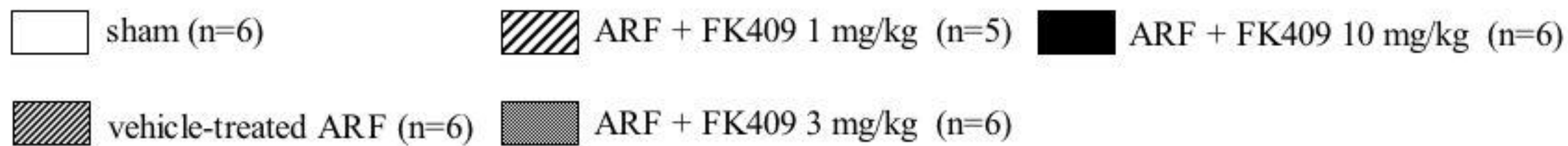


Fig. 2.

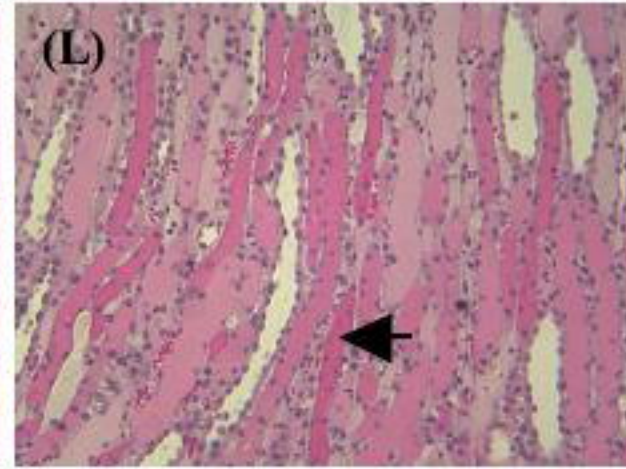
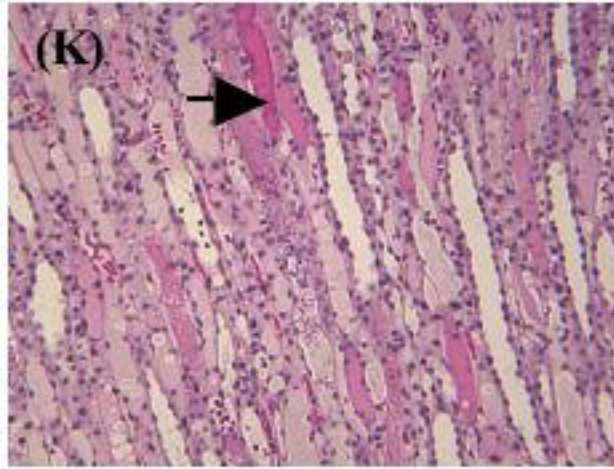
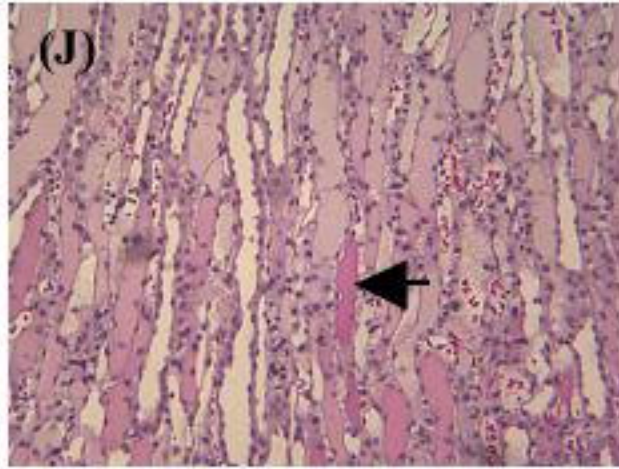
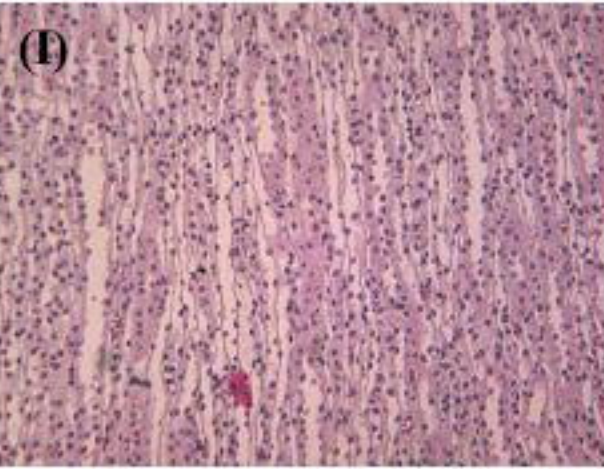
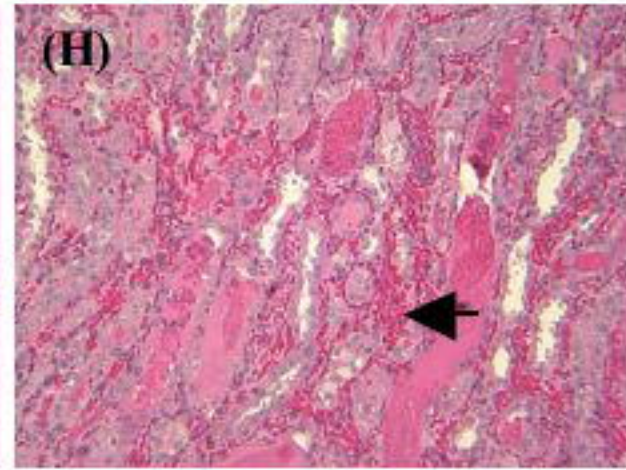
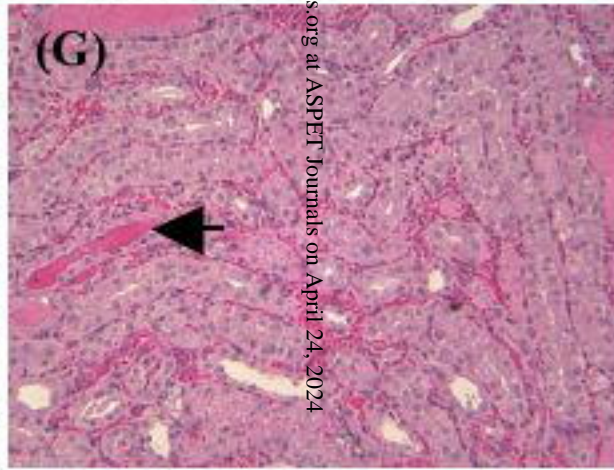
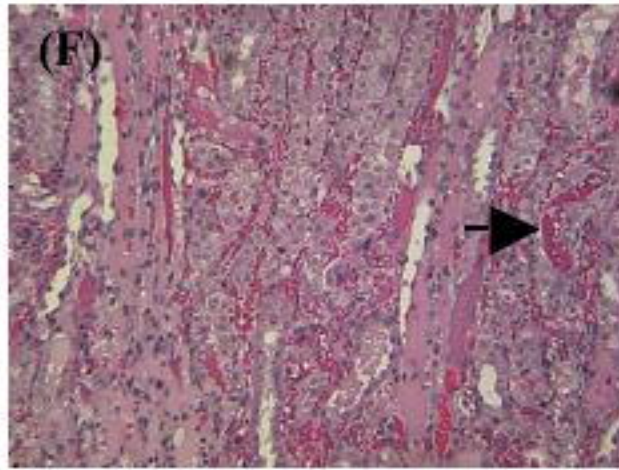
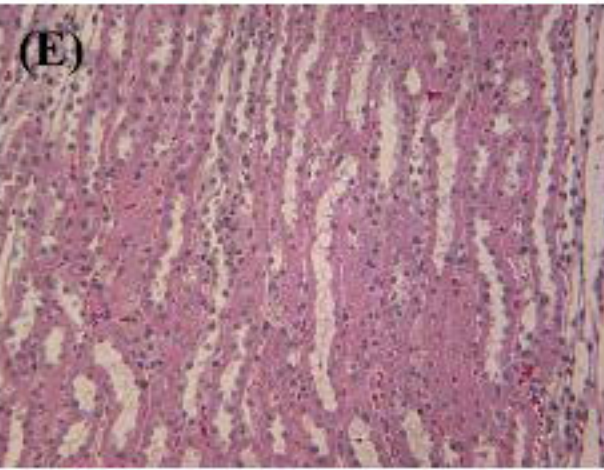
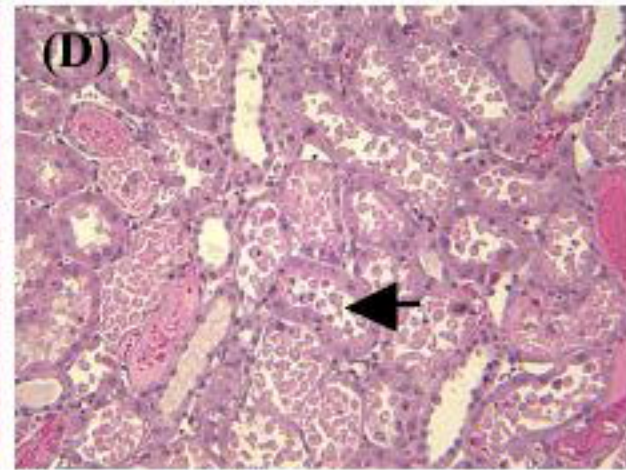
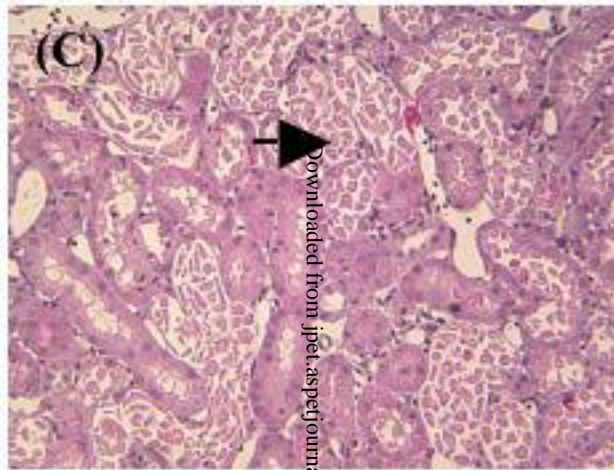
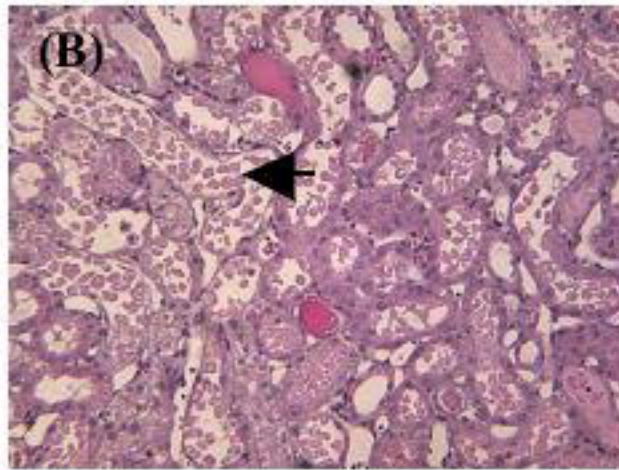
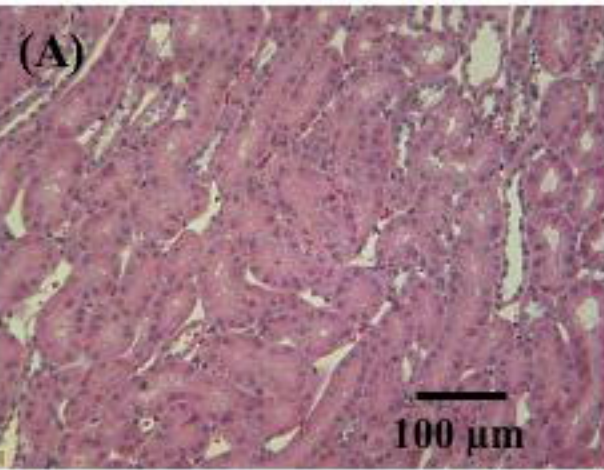


sham

vehicle-treated ARF

ARF  
+  
FK409 3 mg/kg

ARF  
+  
FK409 10 mg/kg



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 24, 2024

Fig. 3.

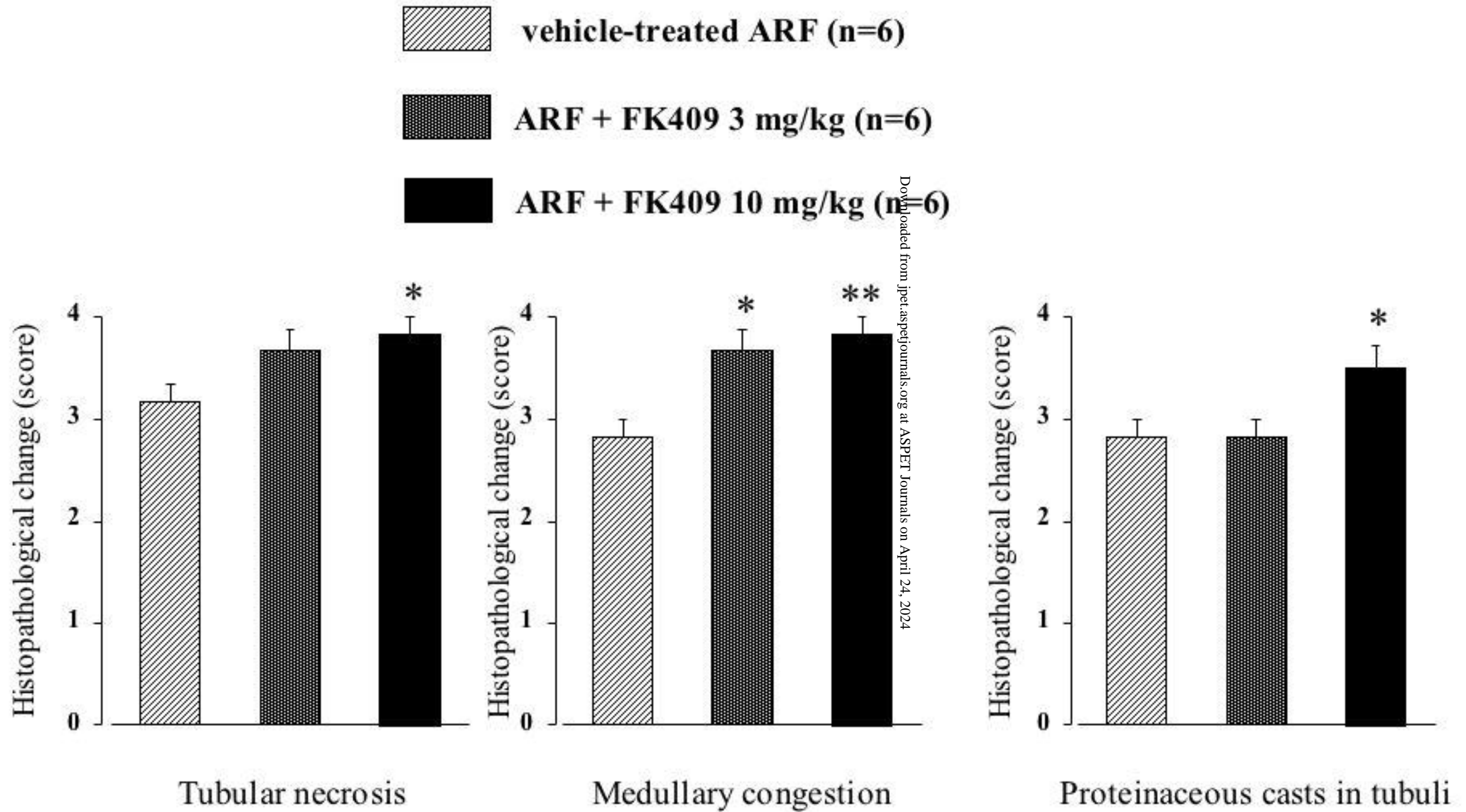


Fig. 4.

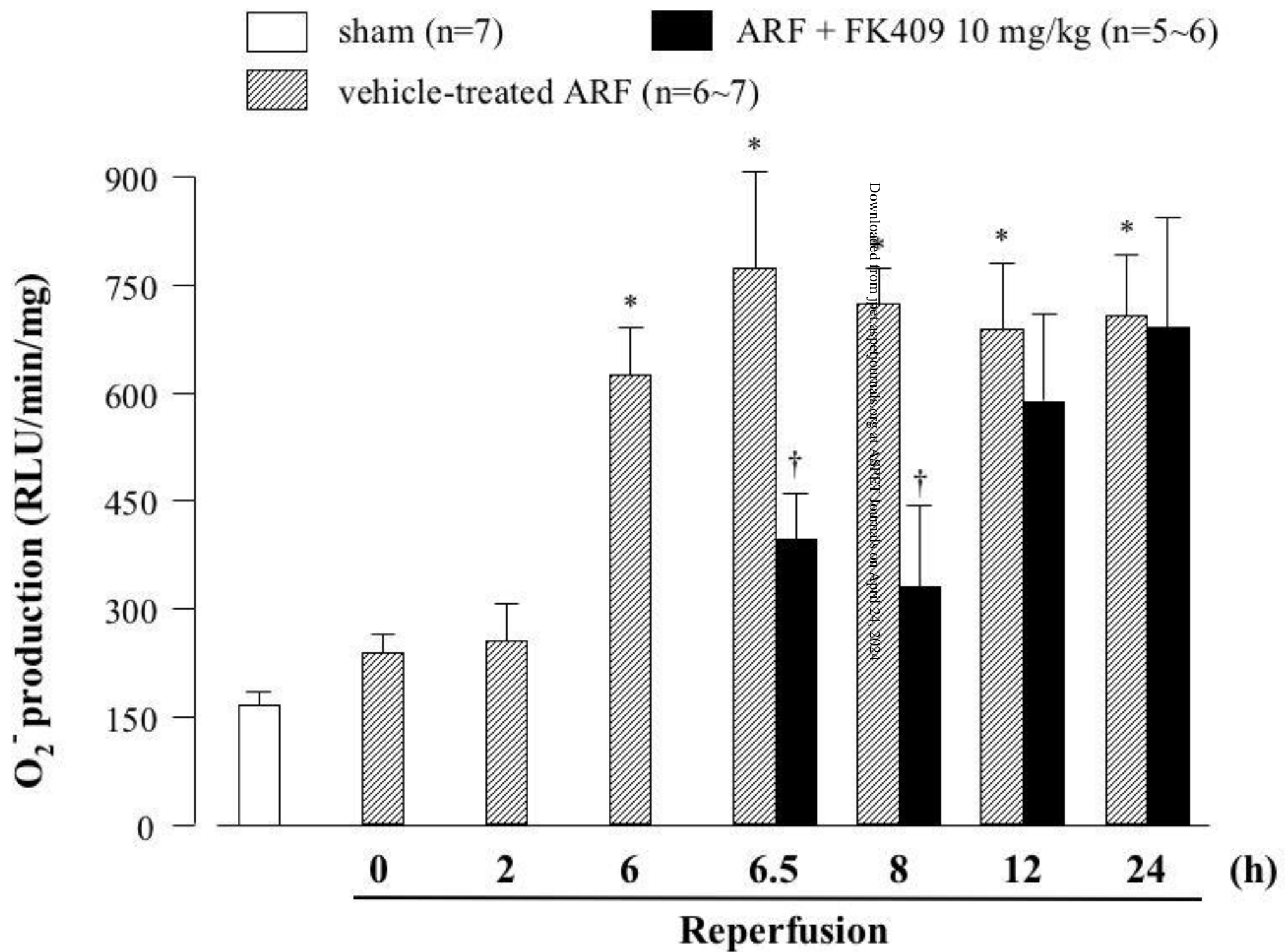
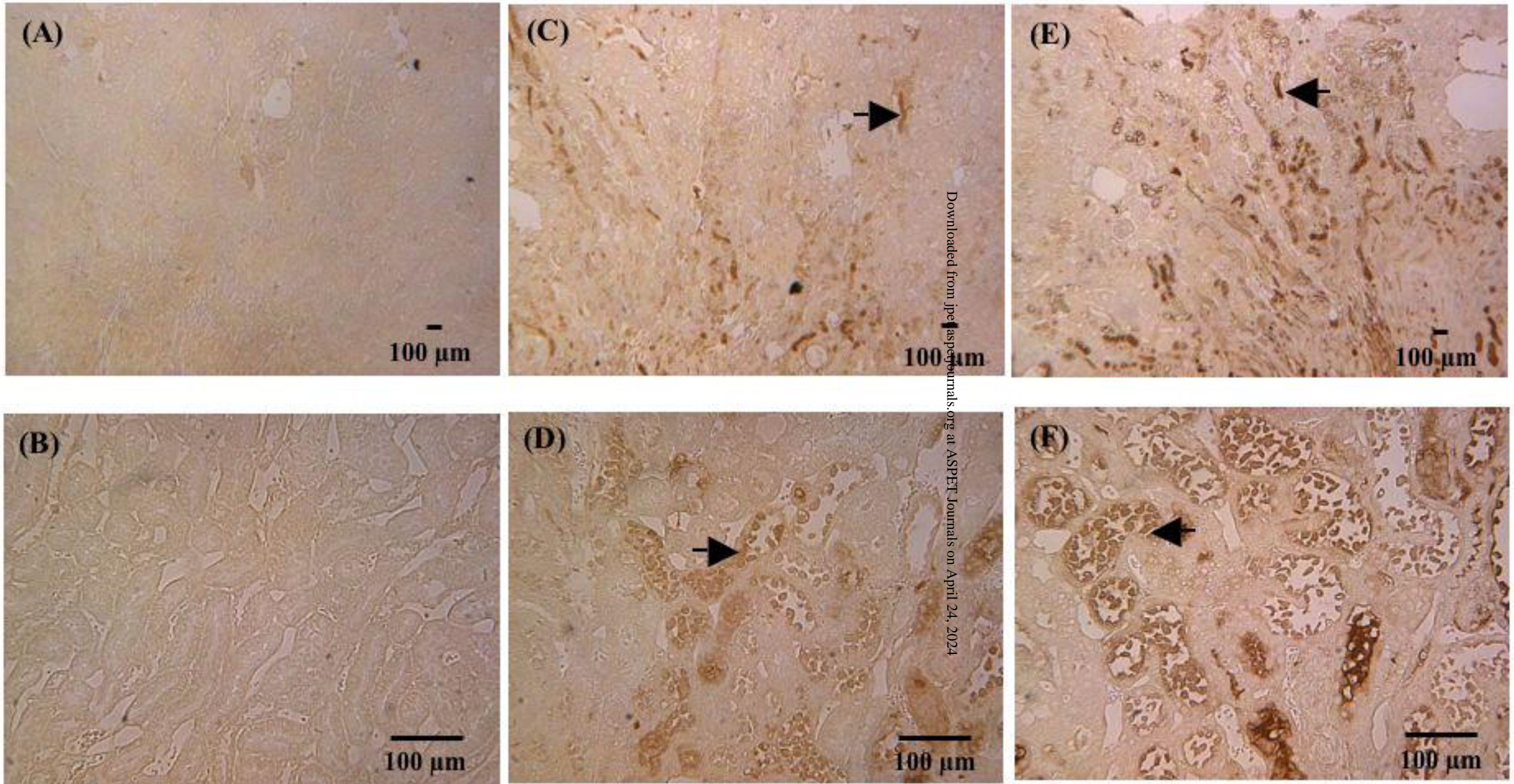
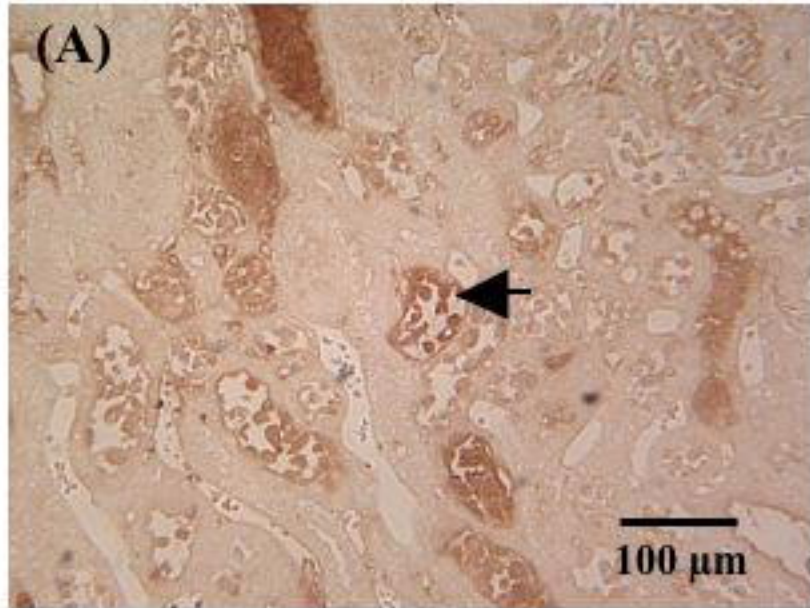


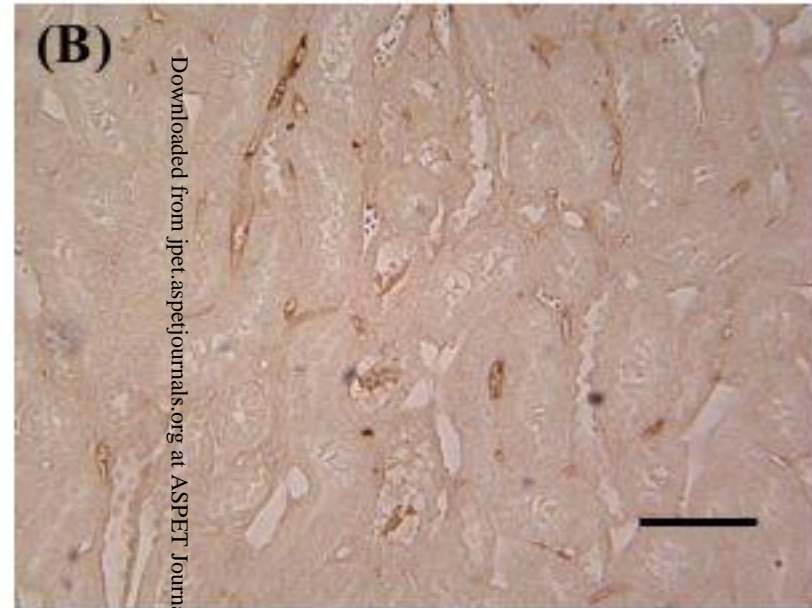
Fig. 5.



*Fig. 6.*

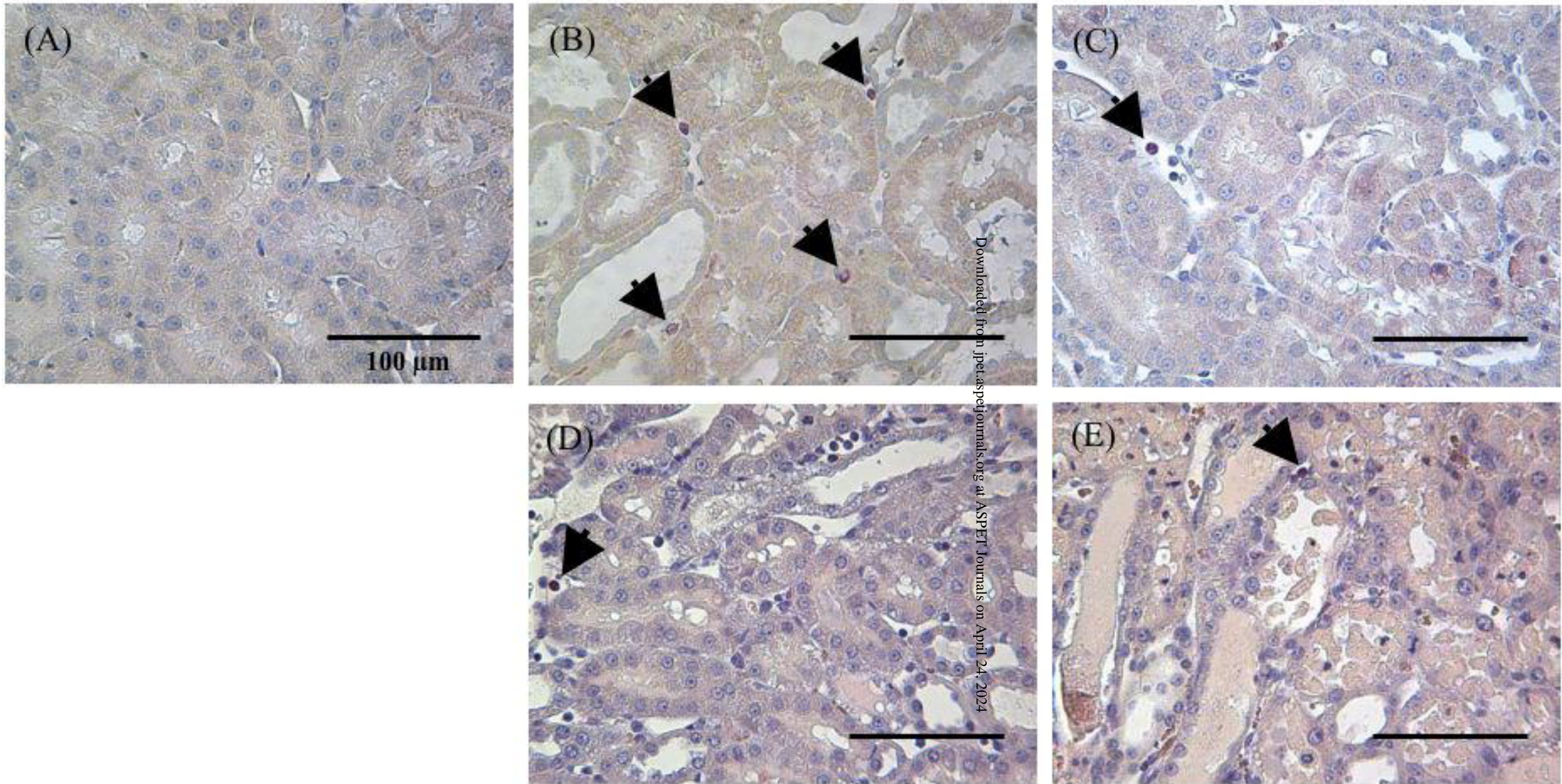


ARF + FK409 3 mg/kg  
**post-ischemic** treatment



ARF + FK409 3 mg/kg  
**pre-ischemic** treatment

Downloaded from [jpet.aspetjournals.org](http://jpet.aspetjournals.org) at ASPET Journals on April 24, 2024



- (A) sham
- (B) vehicle-treated ARF (6 h after reperfusion)
- (C) ARF + FK409 3 mg/kg pre-ischemic treatment (6 h after reperfusion)
- (D) vehicle-treated ARF (24 h after reperfusion)
- (E) ARF + FK409 3 mg/kg pre-ischemic treatment (24 h after reperfusion)

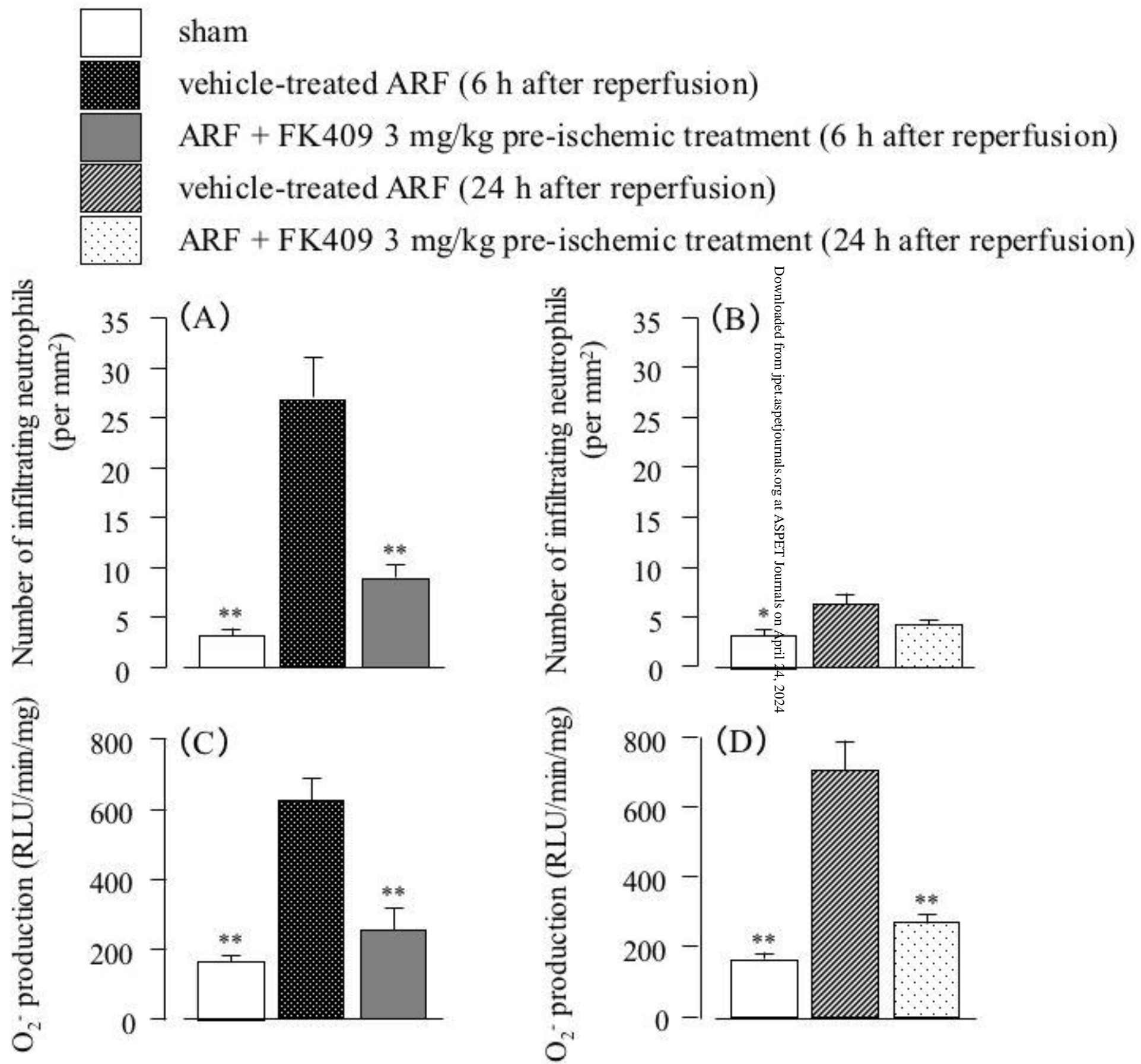


Fig. 9.