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# Endothelial Nitric Oxide Contributes to the Renal Protective Effects of Ischemic Preconditioning

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**ABBREVATIONS:** I/R, ischemia/reperfusion; IP, ischemic preconditioning; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS; cNOS, constitutive NOS; ARF, acute renal failure; NO-ARG, $N^G$-nitro-L-arginine; BUN, blood urea nitrogen; Pcr, plasma creatinine concentration; UF, urine flow; Uosm, urinary osomolality; PKC, protein kinase C; $K^+_{ATP}$ channel, ATP-sensitive potassium channel.

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## ABSTRACT

We determined whether endothelial nitric oxide synthase (eNOS) plays an important role in the renal protective effect of ischemic preconditioning (IP) against the ischemia/reperfusion-induced acute renal failure (ARF), by using eNOS deficient ($eNOS^{-/-}$) and wild type ($eNOS^{+/+}$) mice. Ischemic ARF was induced by occlusion of the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after contralateral nephrectomy. IP, which consists of three cycles of 2-min ischemia followed by 5-min reperfusion, was performed prior to 45-min ischemia. In $eNOS^{+/+}$ mice, IP treatment markedly attenuated the ischemia/reperfusion-induced renal dysfunction and significantly improved histological renal damage such as tubular necrosis, proteinaceous casts in tubuli, and medullary congestion. Constitutive nitric oxide synthase (cNOS) activity in the kidney without IP was markedly decreased 6 h after reperfusion, but this decreased response was not observed in $eNOS^{+/+}$ mice with IP treatment. The improvement of renal dysfunction in $eNOS^{+/+}$ mice with IP treatment was abolished by pretreatment with $N^{G}$-nitro-L-arginine, a non-selective NOS inhibitor, whereas aminoguanidine, an inducible NOS inhibitor, had no effect. Finally, no protective effects of IP on ischemia/reperfusion-induced renal dysfunction and histological damage were observed in $eNOS^{-/-}$ mice. These findings strongly support the view that eNOS-mediated NO production plays a pivotal role in the protective effect of IP on ischemia/reperfusion-induced ARF.

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Prior exposure to brief periods of tissue ischemia leads to a state of increased tolerance to the effects of subsequent ischemia/reperfusion (I/R)-induced injury. This phenomenon was referred to as ischemic preconditioning (IP) by Murry et al. (1986), who first demonstrated this benefit in the dog heart. Thereafter, IP has been extended to several organs including the brain (Heurteaux et al., 1995), liver (Peralta et al., 1999), skeletal muscle (Schroeder et al., 1996), and kidney (Lee and Emala, 2000). Several studies on cardiac IP have demonstrated that myocardial protection by IP does not exceed 3 h (called early preconditioning or 1st window), but reappears 12-24 h after IP treatment and lasts about 72 h (known as delayed preconditioning or 2nd window) (Kuzuya et al., 1993; Yellon and Baxter, 1995; Bolli, 2000). Although the precise mechanisms by which IP reduces the I/R-injury remain obscure, several factors have been reported to contribute to IP-mediated tissue protection. In cardiac IP, the translocation and activation of myocardial protein kinase C (PKC), which is caused by increased production of adenosine and A1 receptor activation, play an important role in myocardial protection (Dawney et al., 1993). Activated PKC is known to lead to the activation of ATP-sensitive potassium ($K^+_{ATP}$) channels, which appears to be closely related to IP-induced tissue protection (Auchampach and Gross, 1993). Recent studies indicated the important role of mitochondrial $K^+_{ATP}$ channels in the cardioprotective effect of IP (Gross and Fryer, 1999; Miura et al., 2000). In addition to the PKC-$K^+_{ATP}$ channel pathway, there is accumulating evidence that nitric oxide (NO) production is involved in IP-mediated cardioprotection (Bolli et al., 1997; Takano et al., 1998).

Endogenous NO is synthesized by the enzyme NO synthase (NOS), catalyzing the substrate, L-arginine, to L-citrulline and NO. Three different NOS isoforms have been cloned and characterized: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Both eNOS and nNOS are constitutively expressed NOS isoforms whose

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activity is regulated by the cytosolic concentration of calcium and by the presence of cofactors such as FAD/FMD, NADPH, and tetrahydrobiopterin, therefore eNOS and nNOS are known as constitutive NOS (cNOS) (Knowles and Moncada, 1994). On the other hand, iNOS is a calcium-independent synthase whose activity appears to rely on its protein expression induced by transcription factors such as nuclear factor-κB (Knowles and Moncada, 1994). In the heart, it has been reported that iNOS and/or eNOS are involved in the cardioprotective effect of IP, both in early and delayed preconditioning (Bolli et al., 1997; Takano et al., 1998; Bell and Yellon, 2001; Bell et al., 2002; Shinmura et al., 2002). Although there is some information on the possible involvement of NO in renal IP (Torras et al., 2002), it remains obscure which type of NOS isoforms is involved in the renal protective effect of IP. One available piece of evidence has been reported by Ogawa et al. (2001), who obtained findings that the renal protective effect of IP against the I/R-induced injury was abolished by $N^G$-nitro-L-arginine, a nonselective NOS inhibitor, and was enhanced by L-arginine treatment. Furthermore, they found that IP alone does not lead to iNOS protein expression whereas the I/R with or without IP treatment enhanced the protein expression, suggesting that NO production mediated by iNOS activity may contribute to the renal protective effect of IP. However, the above study was evaluated on the renal hemodynamic function in the acute phase of reperfusion injury (30-60 min after the reperfusion). Thus, the role of NO/NOS system in delayed preconditioning in the kidney remains to be elucidated.

Most recently, we have found that the protective effect of IP on I/R-induced acute renal failure (ARF), which is observed 24 h after reperfusion, is associated with renal NO production following the increase in eNOS protein expression after reperfusion (Yamashita et al., 2003). However, there is no direct evidence that eNOS is responsible for the renal protective effect of IP. To confirm this, we evaluated the effects of pharmacological

blockade and the genetic deficiency of eNOS in IP-mediated renal protection from I/R-induced injury.

## Materials and Methods

**Animals and Experimental Design.** C57bl/6J wild-type and eNOS$^{-/-}$ mice (20-25g, 11-13 weeks old, Jackson Laboratories, Bar Harbor, ME) were used. 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 Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study, the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg/kg, i.p.). After a 2-week recovery period, these mice were separated into three groups: (1) sham-operated control, (2) I/R group (untreated ARF): 45-min ischemia followed by 24-h reperfusion, and (3) IP treatment group (IP + ARF): 3 cycles of 2-min ischemia followed by 5-min reperfusion prior to I/R. The mice 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 non-traumatic clamp. At the end of the ischemic period, the clamp was released to allow reperfusion. In sham-operated control mice, the kidney was treated identically, except for clamping. Animals were housed in metabolic cages at 24 h after reperfusion; 24-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 function parameters. The kidneys were excised and examined using a light microscope.

In separate experiments, left kidneys were obtained just after IP treatment and at 0, 6,

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and 24 h after reperfusion, and NOS activities were measured. To evaluate the effects of the pharmacological blockade of NOS activities on IP-mediated renal protection, $N^{G}$-nitro-L-arginine (NO-ARG, 10 mg/kg, i.v.), a non-selective NOS inhibitor or aminoguanidine (10 mg/kg, i.v.), an iNOS inhibitor was pretreated 5 min before starting IP, as a slow bolus injection (1 mL/kg) into the external jugular vein, and renal functional parameters were determined as described. The doses of these drugs were determined based on previous studies (Toda et al., 1993; Ortiz et al., 1996).

**Histological Studies.** The excised kidneys were preserved in phosphate-buffered 10% formalin, embedded in paraffin wax, cut into thin sections (4 μm) according to conventional techniques. The sections were 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 by: no congestion (0), mild (+1, vascular congestion with identification of erythrocytes by x400 magnification), moderate (+2, vascular congestion with identification of erythrocytes by x200 magnification), severe (+3, vascular congestion with identification of erythrocytes by x100 magnification), and very severe (+4, vascular congestion with identification of erythrocytes by x40 magnification). Evaluations were made in a blind manner.

**Renal Functional Parameters.** Blood urea nitrogen (BUN) and creatinine levels in plasma (Pcr) were determined using a commercial assay kit, BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Urinary osmolality (Uosm) was measured by freezing-point depression (Fiske Associates, Uxbridge,

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MA).

**Measurement of NOS Activity.** $Ca^{2+}$-dependent and $Ca^{2+}$-independent NOS activities (cNOS and iNOS activities, respectively) were determined by measuring the conversion of L-[$^3$H]arginine to L-[$^3$H] citrulline using a NOS detection kit (NOS detection kit BOX-2, Sigma, St. Louis, MO). Briefly, whole frozen kidneys were homogenized in ice-cold homogenization buffer containing protease inhibitors. Homogenates were centrifuged (30 min at 5,000 x g) to remove tissue debris and the supernatant was utilized for the measurement of NOS activities. The samples were incubated in assay buffer containing 0.323 μM L-[$^3$H]arginine, 600 μM $CaCl_2$, 1 mM NADPH, in the presence of an excess amount of calmodulin, at 37℃ for 30 min. After stopping the reaction, the samples were centrifuged (3 min at 3,000 rpm) and radioactive activities of L-[$^3$H]citrulline were measured using a liquid scintillation counter (TRI-CARB, Packard, Tokyo, Japan). To determine $Ca^{2+}$-independent NOS (iNOS) activity, the assay was conducted in the presence of 10 mM EDTA without $CaCl_2$. $Ca^{2+}$-dependent NOS (cNOS) activity was calculated by subtracting iNOS activity from total NOS activity.

**Drugs.** NO-ARG (Peptide Insitute Inc, Osaka, Japan) and aminoguanidine (Tocris Cookson, Bristol, UK) were dissolved in 0.9% saline.

**Statistical Analysis.** Values are expressed as the mean ± S.D. 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 comparison. Histological data were analyzed using the Mann-Whitney test. For all comparisons, differences were considered significant at $P < 0.05$.

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## Results

**Effects of IP Treatment on I/R-Induced Renal Dysfunction.** As shown in Fig. 1, the renal functional parameters of mice subjected to 45-min ischamia showed marked deterioration, as measured 24 to 48 h after reperfusion. As compared with sham-operated control mice, I/R (untreated ARF) mice exhibited significant increases in BUN, Pcr, and urine flow (UF), and significant decreases in Uosm. However, I/R-induced changes in renal functional parameters were markedly attenuated by IP treatment (IP + ARF).

**Effects of IP Treatment on I/R-Induced Histological Renal Damage.** Histological examination revealed severe lesions in the kidney of untreated ARF mice (48 h after the 45-min ischemia and reperfusion). These changes were characterized by tubular necrosis (outer zone outer stripe of medulla), proteinaceous casts in tubuli (inner zone of medulla) and medullary congestion and hemorrhage (outer zone inner stripe of medulla). IP treatment markedly improved the development of all these lesions (Table 1). Typical photographs are shown in Fig. 2.

**Measurement of NOS Activity in the Kidney.** Changes of NOS activity were evaluated in kidneys exposed to I/R with or without IP treatment. As shown in Fig. 3A, IP treatment alone and 45-min ischemia with or without IP treatment exhibited no changes in cNOS activity compared with sham-operated mice. cNOS activity was significantly reduced at 6 h after reperfusion in I/R mice without IP, but the reduced level recovered at 24 h after reperfusion. On the other hand, the I/R-induced reduction of cNOS activity at 6 h after reperfusion was not observed in the kidney with IP treatment. As shown in Fig. 3B, iNOS activities were not detected in kidneys of the sham-operated control, IP treatment alone and 45-min ischemia with or without IP treatment. However, at 6 and 24 h after reperfusion, there were notable increases in iNOS activities, which were markedly

suppressed by the IP treatment.

**Effects of NOS inhibitors on Renal Protection by IP Treatment.** We next examined the effects of the pharmacological blockade of NOS activities on IP-mediated renal protection. As shown in Fig. 4, the pretreatment with NO-ARG, a nonselective NOS inhibitor, almost completely abolished the renal protective effects of IP against I/R-induced renal dysfunction. On the other hand, aminoguanidine, a preferential inhibitor of iNOS, had no effect on the IP-induced improvement of renal dysfunction.

**I/R-Induced Renal Dysfunction and Effects of IP Treatment in eNOS$^{-/-}$ Mice.** As shown in Fig. 5, there was marked impairment of renal function in eNOS$^{-/-}$ mice subjected to 45-min ischemia, as measured 24 to 48 h after reperfusion, showing a tendency to further deterioration compared with wild-type mice. In contrast to the case in wild-type animals (Fig. 1), IP treatment failed to improve I/R-induced renal dysfunction. In addition, some mice exposed to 45-min ischemia and reperfusion died between 24 to 48 h (2 of 8 mice in both groups, respectively).

**I/R-induced Histological Renal Damage and Effects of IP Treatment in eNOS$^{-/-}$ Mice.** Histological examination revealed severe lesions in the kidney of untreated eNOS$^{-/-}$ mice (48 h after 45-min ischemia and reperfusion), as seen in wild-type animals. However, in contrast to the findings observed in wild-type animals, IP treatment of eNOS$^{-/-}$ mice did not attenuate I/R-induced histological damage (Fig. 6, Table 2).

## Discussion

Our recent study using rats demonstrated that the protective effect of IP on I/R-induced renal dysfunction and tissue injury correlated with eNOS protein expression and NO production in the kidney (Yamashita et al., 2003). This study was performed to determine

whether the eNOS/NO system is responsible for IP-mediated renal protection. We obtained evidence that an IP-mediated improvement on post-ischemic renal injury at 24-48 h after reperfusion was observed in wild-type mice, but not in $eNOS^{-/-}$ mice, in which there was somewhat augmented renal dysfunction in the post-ischemic kidney. Pretreatment with NO-ARG, a nonselective NOS inhibitor, abolished the protective effect of IP, whereas aminoguanidine, an iNOS inhibitor, failed to affect the IP-mediated renal protection. Thus, it seems likely that the eNOS/NO system plays a crucial role in the IP effect on I/R-induced renal injury.

Lieberthal et al. (1991) found that impairment of renal hemodynamics in rats with hypovolemic shock induced by hemorrhage, was to some extent overcome by the inhibition of NO production. The NO synthase inhibitor was reported to prevent hypoxia/reoxigenation injury in rat proximal tubules, thereby suggesting that NO is synthetized in proximal tubules and is involved in tubular hypoxia/reoxigenation injury (Yu et al., 1994). In contrast, Chintala et al. (1993) noted that the inhibition of NO production with a NO synthase inhibitor significantly deteriorated the renal function of the post-ischemic kidney in anesthetized rats, whereas pretreatment with the NO precursor L-arginine abolished the NO synthase inhibitor-induced deterioration of renal function. Similar improvement by L-arginine against the decreased renal function in ischemic ARF was noted by Schramm et al. (1994), although they observed no detrimental effect of the NO synthase inhibitor. We found that I/R-induced renal dysfunction and tissue injury were markedly attenuated by pre-ischemic treatment with FK-409, a spontaneous NO releaser (Matsumura et al., 1998). Thus, the pathophysiological roles of NO in ischemic ARF are controversial. Recent studies clearly demonstrated that renal I/R injury was efficiently attenuated by genetic deficiency or the pharmacological blockade of iNOS (Ling et al., 1999; Walker et al., 2000; Chatterjee et al., 2002), hypothetizing that iNOS-generated NO mediates

I/R-induced renal damage. One possibility is that a toxic peroxynitrite, a reactive substance of NO with superoxide anion, may cause renal damage via direct oxidant injury and protein tyrosine nitration (Walker et al., 2000; Chatterjee et al., 2002). While iNOS-derived NO predominantly elicits pathologic effects, eNOS-derived NO is believed to be responsible for maintaining physiologic renal hemodynamics and functions (Goligorsky and Noiri, 1999). Thus, the heterogeneity of the effects of NO in the pathophysiology of renal I/R injury seems to be closely related to the alterations of iNOS and eNOS (or cNOS) expression and/or activity.

In this study, cNOS activity in the kidney was decreased 6 h after reperfusion, whereas iNOS activity, which was not detected before and immediately after ischemia, gradually increased after reperfusion. IP treatment completely restored the decreased cNOS activity to the basal level and suppressed the elevation of iNOS activity. Thus, the I/R injury seems to be associated with the imbalance of cNOS and iNOS activities, and the improvement of this imbalance may be involved in the IP-mediated renal protective effects.

The precise mechanisms by which IP treatment prevents the loss of cNOS activity after reperfusion, are unclear. Changes in eNOS protein expression seem to be at least partly related to those of cNOS activities. Most recently, Muscari et al. (2004) demonstrated that cardiac IP inhibits the loss of eNOS protein expression and enhances its activity in rat hearts exposed to I/R. We also observed that eNOS protein expression was increased in the post-ischemic rat kidney with IP treatment (Yamashita et al., 2003). Further studies are required to clarify the mechanisms underlying the IP-induced increase in eNOS protein expression.

The signaling mechanisms underlying the NO-mediated IP effect have been discussed. In isolated rat hearts, Lochner et al. (2000) indicated the importance of the NO-guanylyl cyclase-cyclic GMP pathway in the cardioprotective effects of IP, by using NO donors and

inhibitors of NOS and guanylyl cyclase. It has been demonstrated that the activation and translocation of PKC during cardiac IP is NO dependent (Ping et al., 1999). The mitochondrial $K^+_{ATP}$ channel, which is contributive to IP-mediated myocardial protection (Auchampach et al., 1993), could be activated by an NO donor in ventricular myocytes (Sasaki et al., 2000). In addition, it has been reported that hepatic IP is mediated by the inhibitory action of NO in endothelin-1 overproduction induced by I/R (Peralta et al., 1996). There is growing evidence that endothelin-1 is closely related to the development of I/R-induced ARF (Takaoka et al., 2000). In a recent study, we obtained evidence that renal endothelin-1 overproduction induced by I/R was markedly suppressed by IP treatment (Yamashita et al., 2003). Taken together with the view that exogenous and endogenous NO could suppress endothelial endothelin-1 production (Mitsutomi et al., 1999), the down-regulation of endothelin-1 biosynthesis may be involved at least partly in NO-mediated renal protection by IP.

We propose that eNOS-mediated NO production plays a crucial role in delayed preconditioning of the mouse kidney. Moreover, the attenuation of iNOS activity and/or the improvement of cNOS/iNOS imbalance may be involved in the IP-mediated renal protective effects.

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## Footnotes

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## Figure legends

**Fig. 1.** Effects of I/R with or without IP treatment on blood urea nitrogen (BUN) (A), plasma creatinine concentration (Pcr) (B) , urine flow (UF) (C), and urinary osmolality (Uosm) (D). At 24 h after reperfusion, 24-h urine was collected. Each value represents the mean ± S.D. *$P$<0.01, compared with I/R mice without IP treatment (untreated ARF). I/R, ischemia/reperfusion; IP, ischemic preconditioning; ARF, acute renal failure.

**Fig. 2.** Light microscopy of the inner zone (A-C), outer zone inner stripe (D-F) and outer zone outer stripe (G-I) of the kidney of sham-operated control and I/R mice with or without (untreated ARF) IP treatment. Arrows indicate proteinaceous casts in tubuli (B), congestion and hemorrhage (E) and tubular necrosis (H). I/R, ischemia/reperfusion; IP, ischemic preconditioning; ARF, acute renal failure. (hematoxylin and eosin staining).

**Fig. 3.** Renal cNOS (A) and iNOS (B) activities in a sham-operated control, IP alone, and I/R mice with or without (untreated ARF) IP treatment. Each value represents the mean ± S.D. *$P$<0.01, compared with sham-operated mice. †$P$<0.01, compared with I/R mice without IP pretreatment (untreated ARF). I/R, ischemia/reperfusion; IP, ischemic preconditioning; ARF, acute renal failure; N.D., not detected.

**Fig. 4.** Effects of NO-ARG or aminoguanidine on blood urea nitrogen (BUN) (A), plasma creatinine concentration (Pcr) (B), urine flow (UF) (C), and urinary osmolality (Uosm) (D) of I/R mice with IP treatment (IP + ARF). At 24 h after reperfusion, 24-h urine was

collected. Each value represents the mean ± S.D. $^{*}P<0.05$, $^{**}P<0.01$, compared with IP + ARF. I/R, ischemia/reperfusion; ARF, acute renal failure; IP, ischemic preconditioning; NO-ARG, $N^{G}$-nitro-L-arginine.

**Fig. 5.** Effects of I/R with or without IP treatment on blood urea nitrogen (BUN) (A), plasma creatinine concentration (Pcr) (B), urine flow (UF) (C), and urinary osmolality (Uosm) (D) in $eNOS^{-/-}$ mice. At 24 h after reperfusion, 24-h urine was collected. Each value represents the mean ± S.D. $^{*}P<0.01$, compared with $eNOS^{-/-}$ I/R mice without IP treatment (untreated ARF). I/R, ischemia/reperfusion; IP, ischemic preconditioning; ARF, acute renal failure.

**Fig. 6.** Light microscopy of the inner zone (A-C), outer zone inner stripe (D-F) and outer zone outer stripe (G-I) of the kidney of $eNOS^{-/-}$ sham-operated control and I/R mice with or without (untreated ARF) IP treatment. Arrows indicate proteinaceous casts in tubuli (B, C), congestion and hemorrhage (E, F) and tubular necrosis (H, I). I/R, ischemia/reperfusion; IP, ischemic preconditioning; ARF, acute renal failure. (hematoxylin and eosin staining).

TABLE 1

Histopathological changes in the kidneys of wild-type ARF mice with or without IP treatment

| Experimental group | Proteinaceous casts in tubuli | Medullary congestion | Tubular necrosis |
|---|---|---|---|
| Untreated ARF (*n*=6) | 3.00 ± 0.91 | 3.33 ± 0.51 | 3.33 ± 0.81 |
| IP + ARF (*n*=6) | 2.17 ± 0.76[a] | 2.33 ± 0.51[a] | 2.50 ± 0.83[a] |

Values represent the mean ± S.D. of histopathological change/grade. Grade: no change (0), mild (1), moderate (2), severe (3), very severe (4). ARF, acute renal failure. IP, ischemic preconditioning. [a]$P<0.05$, compared with untreated ARF mice.

TABLE 2

Histopathological changes in the kidneys of eNOS$^{-/-}$ ARF mice with or without IP treatment

| Experimental group | Proteinaceous casts In tubuli | Medullary congestion | Tubular necrosis |
|---|---|---|---|
| eNOS$^{-/-}$ untreated ARF (*n*=6) | 3.33 ± 0.81 | 3.17 ± 0.76 | 3.00 ± 0.64 |
| eNOS$^{-/-}$ IP + ARF (*n*=6) | 2.83 ± 0.76 | 3.33 ± 0.81 | 3.17 ± 0.76 |

Values represent the mean ± S.D. of histopathological change/grade. Grade: no change (0), mild (1), moderate (2), severe (3), very severe (4). ARF, acute renal failure. IP, ischemic preconditioning.

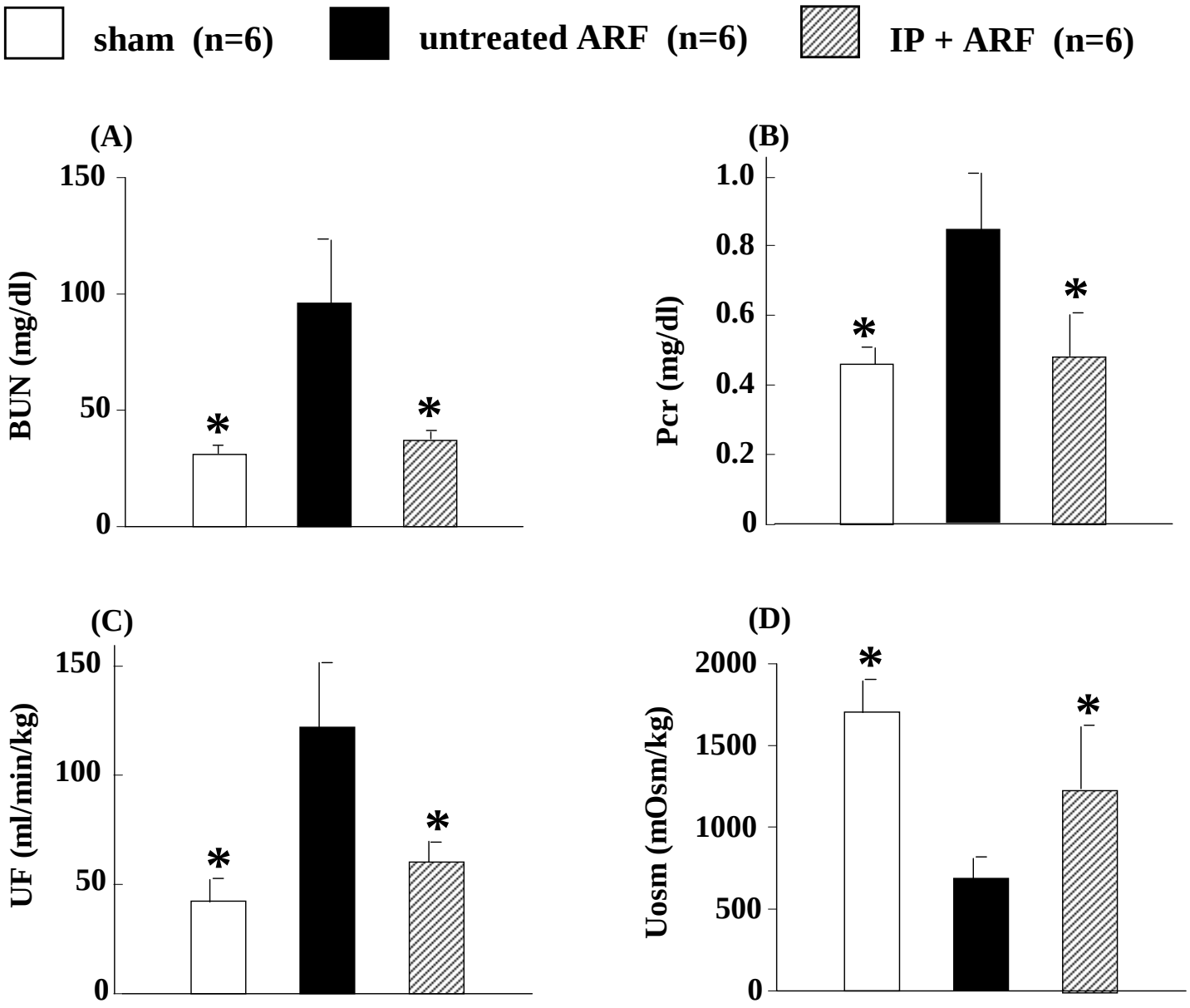


**Fig.1. Yasasowa et al.**

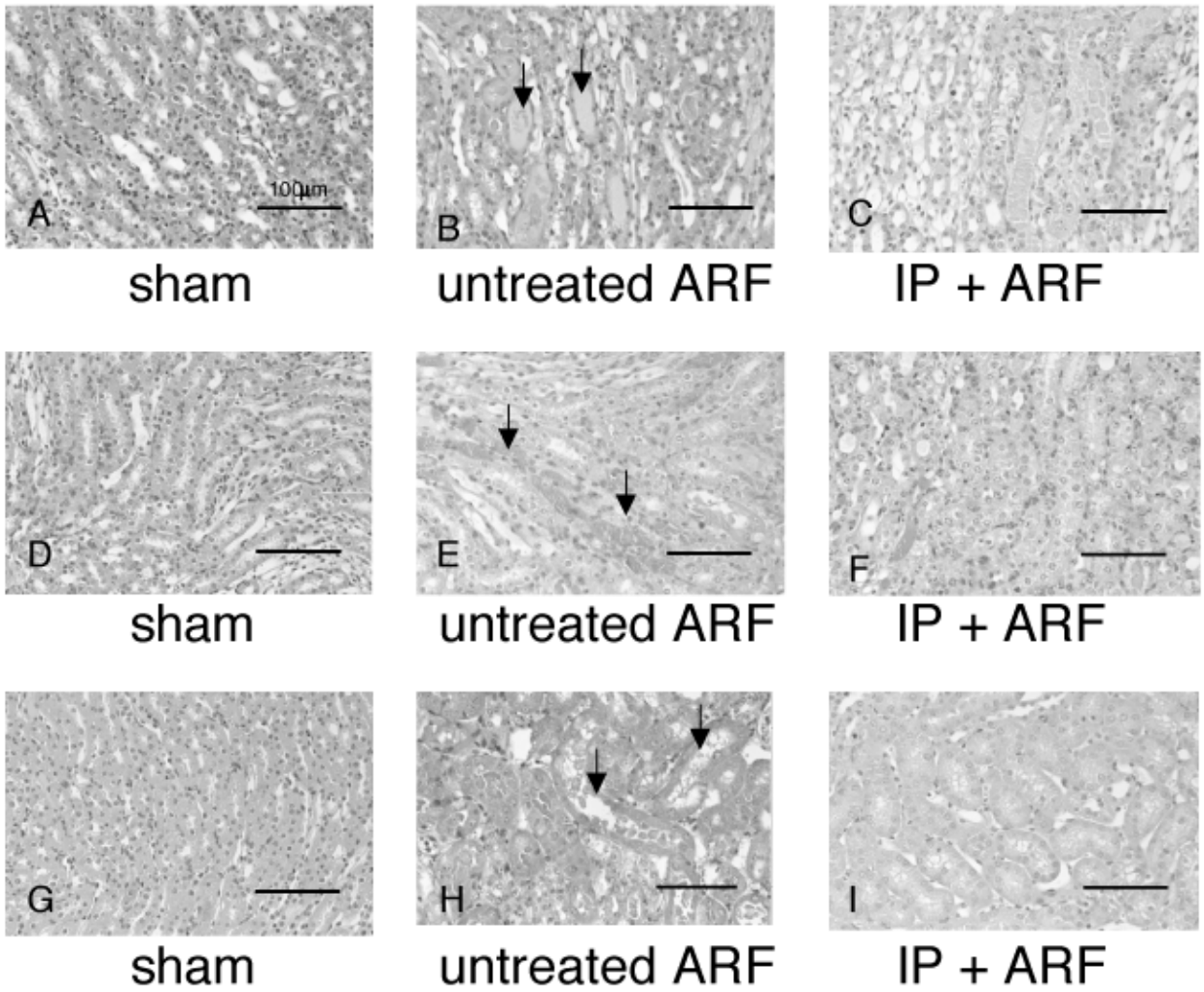


**Fig.2. Yasasowa et al.**

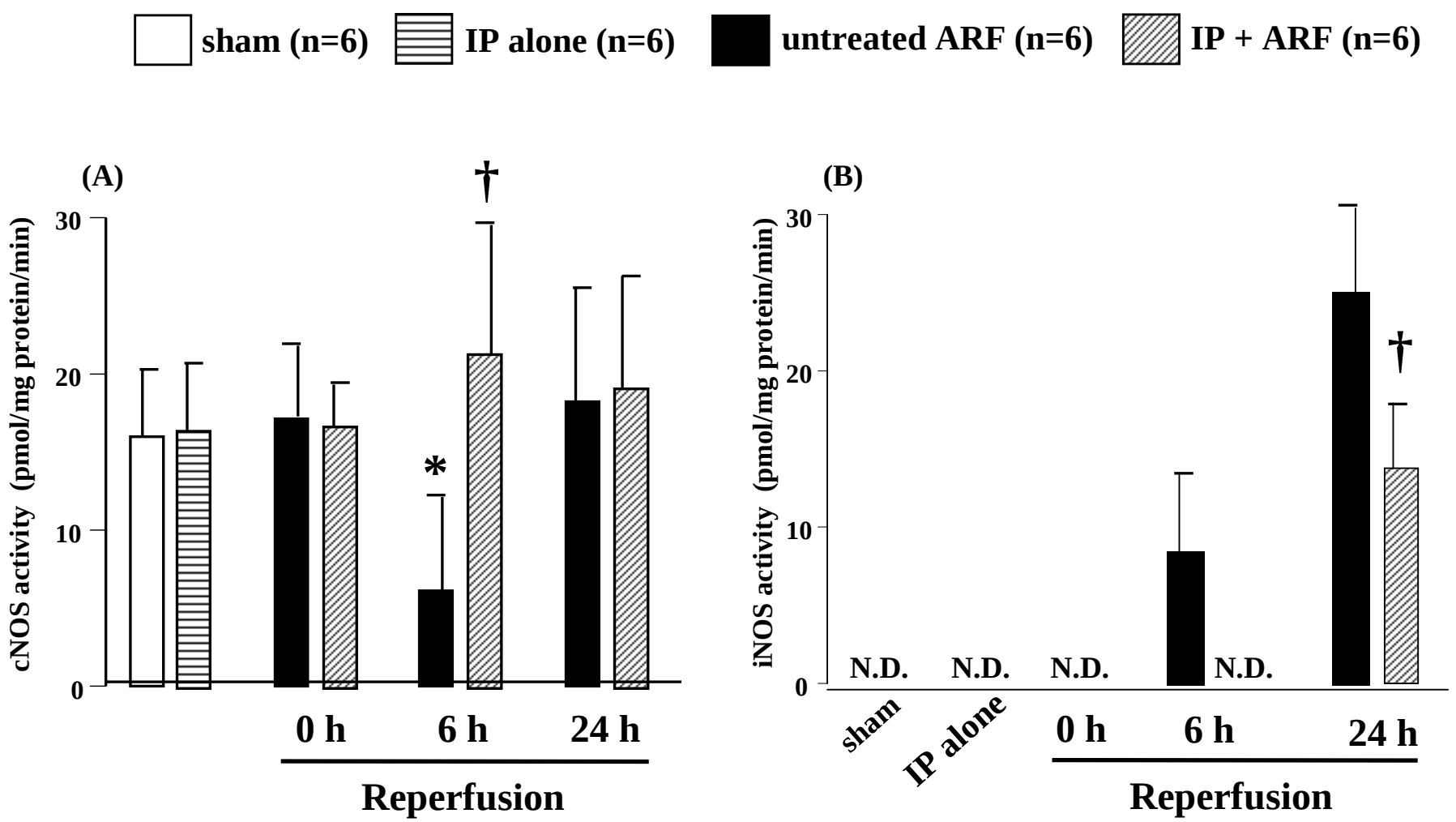


**Fig.3. Yasasowa et al.**

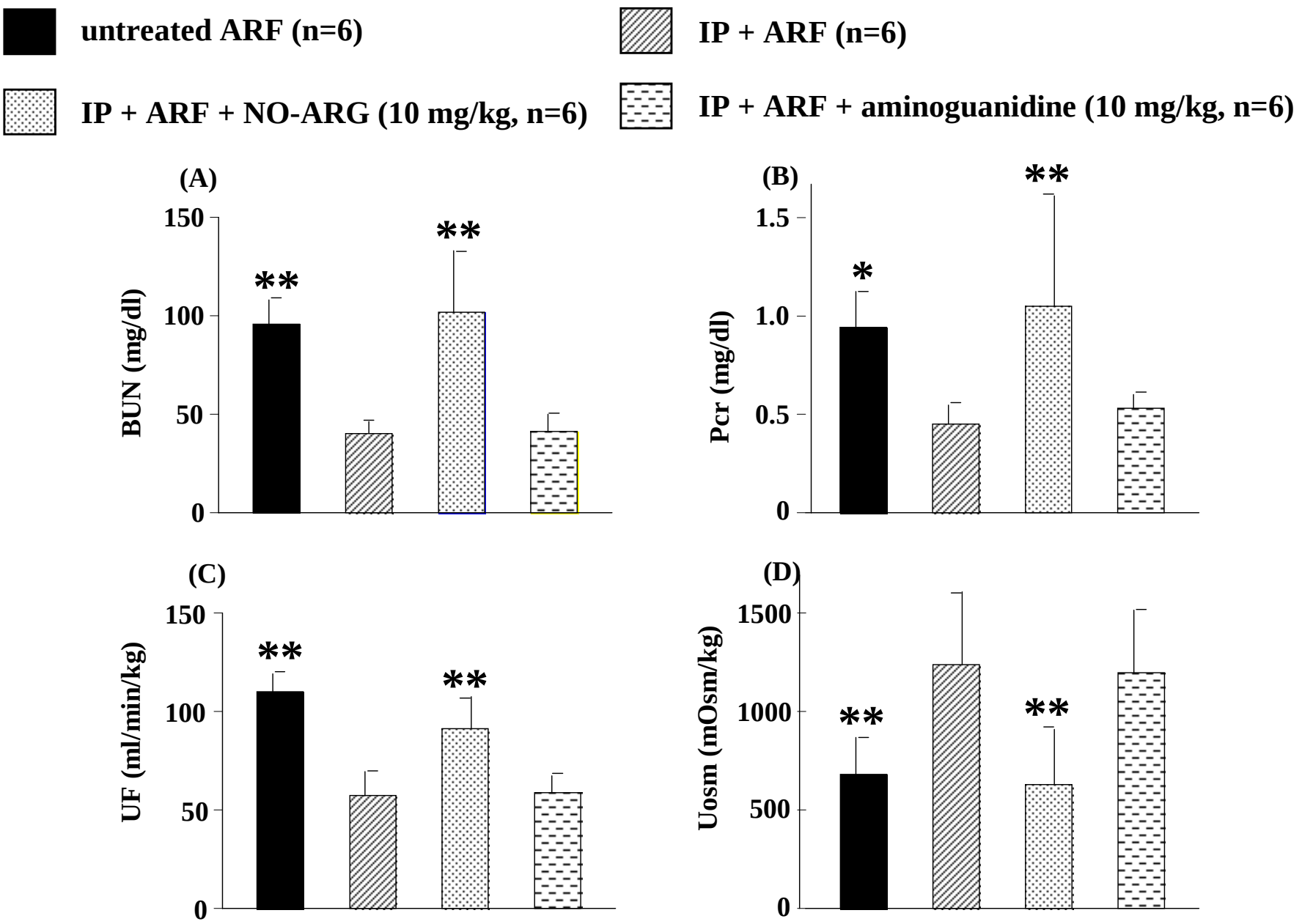


**Fig.4. Yasasowa et al.**

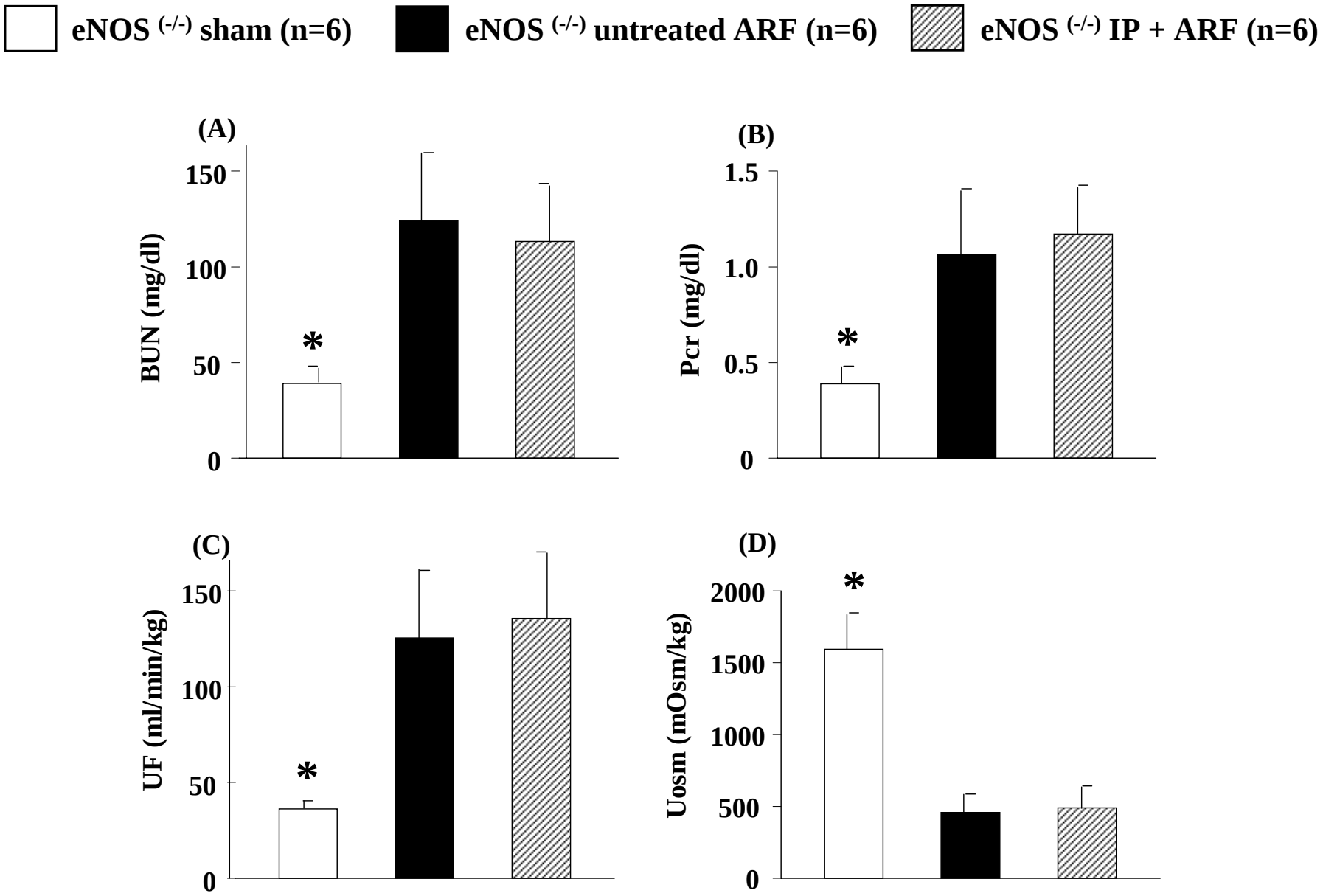


**Fig.5. Yasasowa et al.**

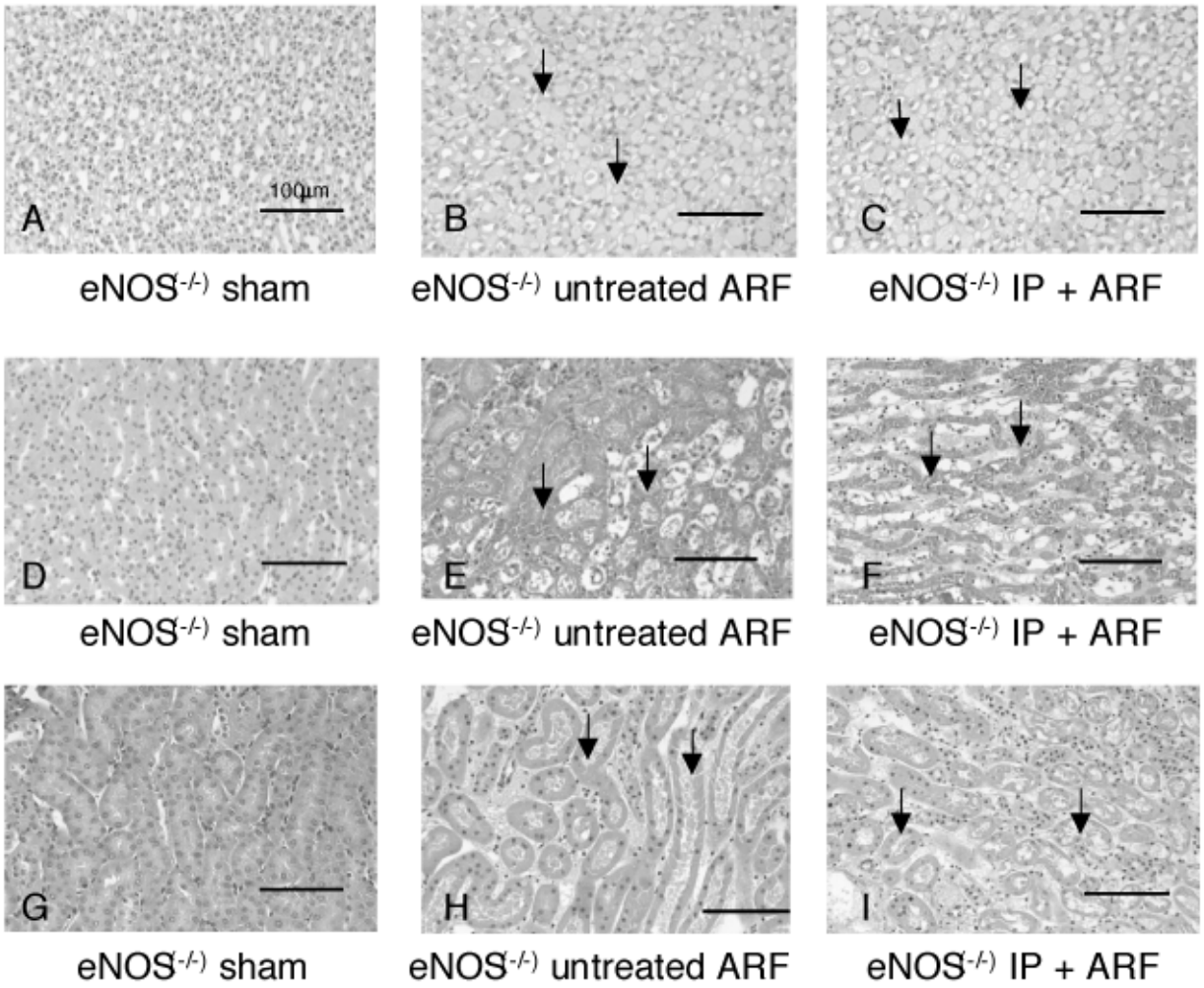


**Fig.6. Yasasowa et al.**