Relaxin protects against renal ischemia-reperfusion injury

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1Department of Clinical Nutrition, School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan;
2Department of Applied Biological Sciences, Shizuoka University, Shizuoka, Japan; and 3Department of Medicine, Yaizu Municipal General Hospital, Yaizu, Japan

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Yoshida T, Kumagai H, Kohsaka T, Ikegaya N. Relaxin protects against renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 305: F1169–F1176, 2013. First published August 14, 2013; doi:10.1152/ajprenal.00654.2012.—Relaxin, a pregnancy hormone, has antiapoptotic and anti-inflammatory properties. The aim of this study was to determine the effects of relaxin on ischemia-reperfusion (IR)-induced acute kidney injury. Male rats underwent unilateral nephrectomy and contralateral renal IR (45 min of renal pedicle clamping). Rats were divided into three groups: 1) sham group, 2) IR group, and 3) IR-RLX group (rats treated with relaxin before ischemia). In this group, relaxin was infused at 500 ng/h via subcutaneous osmotic minipump for 24 h beginning 2 h before renal ischemia. At 24 h after reperfusion, renal function was assessed and kidneys were removed for analysis. There was no significant difference in blood pressure among the three groups. IR increased plasma levels of creatinine and urea nitrogen, and relaxin provided protection against the increases in these two parameters. Relaxin significantly decreased plasma TNF-α levels and renal TNF receptor 1 mRNA expression, compared with the IR group. Semiquantitative analysis of caspase-3 activity in the IR kidneys was reduced in the IR-RLX group. The results demonstrated that relaxin provided protection against IR-induced renal injury by reducing apoptosis and inflammation.

Acute kidney injury (AKI) is a common complication in critically ill patients with an incidence of 30–60% as defined by the RIFLE classification (9, 31, 32). Renal ischemia/reperfusion (IR) injury is one of the most common causes of AKI and IR-induced AKI is associated with high morbidity and mortality in the intensive care unit (3, 13, 14). Moreover, IR also plays a pathogenic role in the development of delayed graft function after kidney transplantation. The pathogenesis of AKI is complex, and the role of apoptosis, endothelial dysfunction, and inflammation have been elucidated. Apoptotic cells are found in both distal and proximal tubular cells in AKI and various experimental antiapoptotic treatment modalities ameliorate IR-induced AKI. Although previous studies shed light on the mechanisms of AKI, effective therapeutic interventions, with the exception of dialysis, are not available.

Females are known to suffer less severe renal IR injury than males (11, 35). Estrogen improves survival in females compared with normal males in experimental renal IR injury (23). In general, the gender disparity has been interpreted primarily to reflect female hormone-mediated protection against pathological conditions (21). The polypeptide hormone relaxin, a well-known pregnancy hormone, is a member of the insulin/relaxin superfamily. Relaxin has gained attention recently based on its antiapoptotic and anti-inflammatory properties in various clinical settings. Recent studies have demonstrated that relaxin reduces myocardial injury and preserves ventricular function in animal models of IR by reducing the number of apoptotic cells and caspase-3 activity (2, 27). Relaxin is also reported to ameliorate IR injury in other organs such as lungs (1) and intestines (17).

The main hypothesis tested in the present study was that relaxin is therapeutically useful for ischemic AKI. To test the hypothesis, we investigated the effects of relaxin on IR-induced AKI in rats.

Materials and Methods

Effects of relaxin on IR injury. Male Wistar rats (SLC, Hamamatsu, Japan) weighing 150 g were randomly allocated into the following groups: 1) sham + saline group (sham group, n = 8); 2) IR + saline group (IR group, n = 8); and 3) treatment with relaxin 2 h before the onset of reperfusion (IR-RLX, n = 8). Relaxin was purified from porcine ovaries by the method of Sherwood and O’Byrne (28). For continuous administration of relaxin (500 ng/h) or saline, animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and surgically implanted with an osmotic minipump (model 2001D; Alzet, Cupertino, CA) under the skin. The osmotic minipump was warmed to 37°C for 2 h before implantation as start-up time. The selected dose of relaxin was chosen based on previous studies in which injection of the same dose resulted in serum relaxin levels comparable to those recorded at midgestation in female rats, when renal plasma flow and glomerular filtration rate were maximal during pregnancy (4). In addition, we performed another experiment in which relaxin was infused via osmotic minipump just after IR (n = 5), and renal function (plasma creatinine and urea nitrogen) was assessed and compared with the IR group (n = 6) at 24 h of reperfusion.

Kidney IR injury animal model. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and placed on a thermoregulated table to maintain rectal temperature at 37°C throughout the experiment. Then, the right kidney was removed, and an atrumatic vascular clamp was placed across the left renal artery and vein for 45 min. After release of the clamp, the abdominal incision was closed in two layers with 2–0 sutures. Sham-operated animals underwent anesthesia, laparotomy, and renal pedicle dissection only. All rats were fed standard diet and had free access to tap water. At 24 h after reperfusion, the rats were killed and kidney and plasma samples were taken. Some parts of the kidney was frozen in liquid nitrogen and stored at −80°C for protein and mRNA analyses while others parts were processed for histological examination by fixation with 4% paraformaldehyde and embedding in paraffin. All animal procedures followed the Guide for Animal Experimentation, School of Food and Nutritional Sciences, University of Shizuoka.

Analytical procedures. Plasma and urine creatinine and urea nitrogen (UN) levels were measured by the enzymatic creatinine assay and urea-ultraviolet method, respectively. Plasma TNF-α concentration was determined by ELISA using the rat TNF-α ELISA kit (R&D Systems, Minneapolis, MN). Plasma creatinine was measured by the urase-ultraviolet method, respectively. Plasma TNF-α concentration was determined by ELISA using the rat TNF-α ELISA kit (R&D Systems, Minneapolis, MN).
Plasma relaxin concentrations were measured by ELISA using relaxin antibody (12).

**Histology and immunohistochemistry.** The paraffin-embedded kidney samples (3-μm thick sections) were stained by periodic acid-Schiff (PAS) method for microscopic examination. Tubular epithelial damage was evaluated by semiquantitative grading (grade 0; normal, grade 1; tubular epithelial injury <25% of the total number of tubules, grade 2; 26–50%, grade 3; 51 to ≤75%, grade 4; >75%, as described previously; Ref. 30) in 20 randomly selected cortical and outer medullary areas per sample. Immunohistochemistry was performed by using the Dako EnVision-HRP Detection Kit (Dako, Kyoto, Japan), according to the method described previously (36). After deparaffinization, endogenous peroxidase activity was blocked with 3% H2O2 solution in methanol. Then, the tissues were blocked with 10% normal goat serum and incubated overnight with several primary antibodies at 4°C. The primary antibodies were thrombomodulin (TM) antibody (kindly provided by Dr. M. Nakano, Mitsubishi Gas Chemical, Nihonbashi, Japan), Calbindin D-28k antibody (Sigma-Aldrich, St Louis, MO) to localize distal tubules, anti-rabbit RXFP1 antibody (12), to detect relaxin receptor, and single-stranded DNA (ssDNA) antibody (Dako). The primary antibody was localized by labeled polymer reagent and color development with 3,3′-diaminobenzidine tetrahydro-

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<th>Sham (n = 8)</th>
<th>IR (n = 8)</th>
<th>IR-RLX (n = 8)</th>
<th>P Valuea</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>171 ± 1.7</td>
<td>164 ± 2.5</td>
<td>158 ± 2.8d</td>
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<tr>
<td>Kidney weight, g</td>
<td>0.813 ± 0.028</td>
<td>0.989 ± 0.023</td>
<td>0.932 ± 0.059</td>
<td>NS</td>
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<tr>
<td>Mean blood pressure, mmHg</td>
<td>86 ± 5.6</td>
<td>90 ± 3.2</td>
<td>100 ± 3.6</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dl</td>
<td>0.17 ± 0.01a</td>
<td>3.08 ± 0.15</td>
<td>1.70 ± 0.33b</td>
<td>&lt;0.001</td>
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<tr>
<td>Plasma urea nitrogen, mg/dl</td>
<td>11.9 ± 0.4a</td>
<td>131.5 ± 6.3</td>
<td>86.1 ± 12.7b</td>
<td>&lt;0.001</td>
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<tr>
<td>Plasma TNF-α, pg/ml</td>
<td>11.7 ± 2.4a</td>
<td>28.9 ± 3.9</td>
<td>15.1 ± 2.7c</td>
<td>&lt;0.005</td>
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Data are means ± SE. IR, ischemia-reperfusion; IR-RLX, rats treated with relaxin before ischemia. *P < 0.01 vs. other groups; bP < 0.01 vs. IR; *P < 0.05 vs. IR; **P < 0.05 vs. sham; *P value for ANOVA, including all 3 groups.

![Fig. 1. Kidney sections were stained with periodic acid-Schiff (PAS). Ischemia-reperfusion (IR) increased tubular injury (B and E) compared with the sham group (A and D). Relaxin reduced tubular injury after ischemia (C and F). Magnification: ×50 in A–C (bar = 200 μm); ×200 in D–F (bar = 50 μm).](image-url)
Table 2. Effects of relaxin on indexes of histological and immunohistochemical renal damage

<table>
<thead>
<tr>
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<th>Sham (n = 8)</th>
<th>IR (n = 8)</th>
<th>IR-RLX (n = 8)</th>
<th>P Valueb</th>
</tr>
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<td>Renal tubular damage score</td>
<td>0.00 ± 0.00</td>
<td>3.02 ± 0.24</td>
<td>1.76 ± 0.23</td>
<td>&lt;0.001</td>
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<tr>
<td>Loss of thrombomodulin staining (endothelial cell damage score)</td>
<td>1.45 ± 0.08</td>
<td>2.21 ± 0.16</td>
<td>1.66 ± 0.13</td>
<td>&lt;0.005</td>
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<tr>
<td>TUNEL-stained cells/field</td>
<td>0.20 ± 0.04</td>
<td>12.73 ± 1.41</td>
<td>3.48 ± 0.44</td>
<td>&lt;0.001</td>
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<tr>
<td>Single-stranded DNA-positive cells/field</td>
<td>0.13 ± 0.05</td>
<td>7.75 ± 1.30</td>
<td>3.30 ± 1.16</td>
<td>&lt;0.001</td>
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Data are means ± SE. TUNEL, terminal transferase-dUTP nick-end labeling. *P < 0.01 vs. other groups; bP < 0.01 vs. IR; cP < 0.05 vs. IR; dP value for ANOVA, including all 3 groups.

Fig. 2. RXFP1 expression in the kidneys. Colocalization of RXFP1 (A–C) and calbindin D-28K (E–G) was evident in serial immunohistochemical sections. RXFP1 expression was identified in calbindin-D28K-positive distal tubular epithelium of sham (A and E), IR (B and F), and IR-RLX (C and G) groups. Negative control slides of RXFP1 and Calbindin-D28K are shown in D and H, respectively. Bar = 50 μm.
measured semiquantitatively by counting 30 fields under ×200 magnification. The number of apoptotic cells in the outer medulla and cortex was calculated as well as by terminal transferase-dUTP-nick-end labeling method (7). The number of ssDNA-positive cells was determined by examination of 30 randomly selected renal outer medullary areas under ×200 magnification. Thrombomodulin is expressed on the surface of endothelial cells, and loss of TM immunostaining was considered to represent endothelial cell damage. Endothelial damage was evaluated semiquantitatively (grade 0, normal; grade 1, <10%; grade 2, 11–25%; grade 3, 26–50%; grade 4, 51 to ≤75%; grade 5, >75% of TM-negative endothelial cells; Ref. 10).

Terminal transferase-dUTP-nick-end labeling assay. As described above, apoptosis was also determined by terminal transferase-dUTP nick-end labeling (TUNEL) assay using paraffin-embedded kidney tissue sections (3 μm) and ApoTag in situ apoptosis detection kit (S7101; Millipore, Temecula, CA), according to the protocol supplied by the manufacturer. Briefly, kidney sections were dewaxed and incubated in 3% H2O2 to eliminate endogenous peroxidase activity. Then, the sections were washed in distilled water followed by PBS for 5 min. Proteinase K (Dako, Glostrup, Denmark) was applied on the sections that were incubated for 5 min at room temperature. After being washed, equilibration buffer was applied to the sections for facilitating the penetration of terminal dUTP transferase; the TdT enzyme. Then, TdT enzyme reaction mixture was applied to the sections, which were incubated in a humidified chamber for 1 h at 37°C to allow extension of the nick ends of the DNA fragments with digoxigenin-dUTP. The sections were washed in a stop buffer and incubated in 3% H2O2 to eliminate endogenous peroxidase activity. After being rinsed the sections with PBS, color was developed using horseradish peroxidase-conjugated secondary antibody (dilution, 1:3,000). The ECL Western blotting system (GE Healthcare Life Sciences, Buckinghamshire, UK) was used for detection. Bands were visualized with a luminoanalyzer (LAS-3,000 mini; Fuji Photo Film, Tokyo). β-Actin (Sigma-Aldrich; dilution, 1:3,000) was also used to evaluate the evenness of the protein loading. Band intensity was estimated using an Image software (Quantity One; Bio-Rad Laboratories, Hercules, CA) and expressed as percentage of sham.

Expression of TNF-α receptor 1 mRNA by real-time RT-PCR. TNF-α receptor 1 (TNFR1) mRNA expression was examined by real-time RT-PCR. Total RNA was extracted from snap-frozen kidney cortex tissue using the Isogen II (Nippon gene, Toyama, Japan). Reverse transcription was performed using PrimeScript RT reagent kit (Takara, Shiga, Japan). Real-time PCR amplification was performed using SYBR Premix Ex Taq (Takara) with specific primers sets, prepared using the Perfect real-time supporting system of Takara for TNFR1 (primer set ID: RA044490) and GAPDH (primer set ID: RA015380). To quantify target mRNA abundance, differences in threshold cycles between the gene target (TNFR1) and GAPDH were calculated and then relative mRNA abundance was calculated using the 2−ΔΔCt method. The mRNA level was expressed relative to the mean value of sham rats.

Statistical analysis. Values are expressed as means ± SE. ANOVA was used for multiple-group comparisons, followed by the Turkey honestly significant difference test. Differences were considered significant when the P < 0.05. All statistical tests were based on the use of Bonferroni correction.
RESULTS

Relaxin improves renal function and attenuates post-IR increase in TNF-α. There were no significant differences in mean blood pressure among the three groups (Table 1). Plasma relaxin concentrations were 32.8 ± 2.44 ng/ml at death. Compared with the sham-operated rats, plasma creatinine and UN were significantly higher at 24 h after IR injury in both IR and IR-RLX groups. However, the increases in creatinine and UN in the IR-RLX group were significantly lower than those of the IR group. At 24 h after IR, plasma TNF-α was significantly higher in the IR group compared with the sham group. However, the extent of such increase was reduced in the IR-RLX group, relative to the IR group (Table 1). In the relaxin treatment after IR experiment, the relaxin-treated groups also showed significantly lower serum creatinine (IR, 3.12 ± 0.48; RLX after IR, 1.37 ± 0.48 mg/dl) and UN (IR, 134.3 ± 7.2; RLX after IR, 68.0 ± 16.9 mg/dl).

Fig. 4. Kidneys were stained for apoptosis using terminal transferase-dUTP nick-end labeling (TUNEL) technique (A–C) and immunohistochemistry of single-stranded DNA (E–G). Compared with the sham controls (A and E), apoptotic nuclei (black arrows) are more evident in the IR group (B and F). Relaxin treatment decreased apoptotic cells compared with IR group (C and G). Negative control slides of TUNEL and single-stranded DNA are shown in D and H, respectively. Bar = 50 μm.
Relaxin ameliorates histological damage after IR. The renal tubular damage score correlated with plasma creatinine ($r^2 = 0.9234$; $P < 0.001$) and UN levels ($r^2 = 0.9324$; $P < 0.001$). Examination of kidneys harvested 24 h after IR injury showed loss of the brush border, vacuolation, and desquamation of epithelial cells in the renal tubular epithelium (Fig. 1). Consistent with preserved renal function, tubular damage was significantly less in the kidneys of the IR-RLX group than the IR group (Table 2).

Staining for RXFP1 was localized to distal tubular cells, which immunostained positively for calbindin D 28K. However, damaged cells did not show positive staining for RXFP1 or calbindin D 28K in both the IR and IR-RLX groups (Fig. 2). Thrombomodulin was expressed on the surface of normal endothelial cells but not in injured endothelial cells (Fig. 3). Endothelial cell damage score (loss of immunostaining for thrombomodulin) was higher in the IR group than the sham group but was significantly lower in the IR-RLX compared with the IR group, but similar to that of the sham group (Table 2).

Antiapoptotic effects of relaxin in IR injury. The extent of apoptosis in kidney sections was assessed at 24 h after IR by TUNEL, ssDNA staining, and analysis of procaspase-3 expression level. IR resulted in a significant increase in the number of TUNEL-positive cells in the outer medulla compared with the sham group (Fig. 4, A–C). Treatment with relaxin before IR significantly reduced the number of TUNEL-positive cells compared with the IR group (Table 2). A similar trend was noted in experiments involving immunostaining for ssDNA (Fig. 4, D–F). Interestingly, the number of ssDNA-positive cells was smaller than that of TUNEL-positive cells (Table 2). Similar results were also observed in Western blot for procaspase-3. Procaspase-3 expression was significantly increased in the IR group, relative to the sham group (Fig. 5), and treatment with relaxin prevented overexpression of procaspase-3. There results indicate that relaxin inhibits apoptosis in acute kidney injury after IR.

Expression of TNFR1 mRNA in the kidney. At 24 h after IR, TNFR1 mRNA in the kidney was significantly higher in the IR group compared with the sham group. However, the extent of such increase was reduced in the IR-RLX group, relative to the IR group (Fig. 6).

**DISCUSSION**

To our knowledge, this is the first study to investigate the effect of relaxin in IR renal injury. IR-induced AKI is not only associated with high morbidity and mortality, but also increased risk of development of chronic kidney disease (CKD) and progression to end-stage renal disease (15). IR injury is also an important problem affecting the outcome of organ transplantation. At present, relaxin is known to provide protection against IR injury in the myocardium, lung, intestine, and brain (1, 17, 24, 34). The present study showed that treatment with relaxin decreased not only plasma UN and Cr levels but also prevented histological damage and reduced the severity of apoptosis.

The pathogenesis of AKI is complex and involves the direct effects of IR on vascular endothelial cells, tubular epithelial cells, and immune cells. In this study, we examined the protective effects of relaxin on apoptosis and inflammation in the kidneys. Inflammation plays a major role in the progression of AKI. Pro-inflammatory cytokines including TNF-$\alpha$ and IL-6 are generated by injured renal tubular cells (6). After reperfusion, cytokine generation is stimulated rapidly in other organs, leading to increased levels of circulating cytokines (26). In the present study, relaxin significantly reduced plasma TNF-$\alpha$ compared with IR rats. Furthermore, relaxin also reduced TNFR1 mRNA expression in the kidney of IR rats. These findings suggest that relaxin reduces renal inflammation after AKI. TNF-$\alpha$ is known to be a cytotoxic cytokine that induces apoptosis of renal tubular epithelial cells, through interactions with TNFR1 (20). The TNF-$\alpha$-TNFR1 complex stimulates the activity of caspase-8 and its downstream effector, caspase-3, by forming a complex with death domain containing adaptor proteins such as TNF receptor 1-associated death domain.
AKI, it may also prevent the progression from AKI to CKD. Anti-TNF-α treatment is reported to reduce postchemic renal injury, inflammation, and apoptosis (5, 19). In this present study, relaxin inhibited TNFR1 mRNA and caspase-3 expression in the kidney. These results suggest that inhibition of TNF-α pathway by relaxin is involved in antiapoptosis effects. Distal tubular epithelial cells are more resistant to IR injury via antiapoptotic factors than proximal cells (8). It is reported that the majority of apoptotic tubular cells are found in the distal and proximal tubules at 1 day after IR injury (25). Other studies showed no changes in Bax expression levels in the distal tubules, whereas the levels of Bcl-2, its heterodimeric partner, were increased (33). Bcl-2 inhibits apoptosis induced by etiological factors that promote ischemia and IR injury (18). Suzuki et al. (29) reported the therapeutic effect of Bcl-2 overexpression on tubulointerstitial injury in a mouse model of IR injury. In cardiomyocites, relaxin increased the Bcl-2/Bax protein ratio and decreased apoptotic cells under the condition of excess oxidative stress (22). In the present study, pretreatment with relaxin before IR injury provided protection against IR-induced damage of both proximal and distal tubules. Our study also identified the expression of relaxin receptor mainly in the distal tubules. Considered together, these results suggest that relaxin, acting through its receptor, provides protection against cell death and is a potentially useful agent for the prevention of ischemia-related tissue damage. In this regard, distal tubules protected by Bcl-2 also functions as a reservoir for the production of growth factors that are critical to the maintenance, regeneration and/or preservation of existing distal tubules (8). Since the maintenance of distal tubular viability may allow time for repair from reperfusion-induced damage, the effect of relaxin on tubular cells was also considered to contribute not only to protection but recovery of tubular epithelial injury. It is noteworthy that damaged tubules did not express relaxin receptor. This means diminished effect of relaxin through renal receptors after the development of AKI and that early treatment with relaxin is potentially more effective and desirable.

IR is reported to cause endothelial injury in the peritubular microcirculation with subsequent renal dysfunction. Based on the finding of thrombomodulin expression in the peritubular region, damage of peritubular endothelial cells was prevented by pretreatment with relaxin. Growth factors from less injured distal tubules may be more effective under preserved capillaries than damaged microcirculation.

In the present study, relaxin was continuously administered 24 h from 2 h before ischemia. Other researchers have used intravenous relaxin at one or two doses in short-term animal experiments (16, 17). Administration of relaxin in advance of the ischemic insult demonstrated its beneficial effect against ischemia-reperfusion injury. Furthermore, the additional experiment showed that administration of relaxin after ischemia attenuated also AKI induced by IR. Further studies are necessary to test the clinical effects of relaxin in IR injury.

AKI is currently considered an independent risk factor for CKD and end-stage renal disease. Interstitial fibrosis is histologically characteristic of CKD and ESRD. Since relaxin has a potent anti-inflammatory and antifibrotic effects in many organs including kidneys (36) and prevents the progression of AKI, it may also prevent the progression from AKI to CKD. Further studies are needed to investigate the long-term consequences of relaxin in AKI model.

In conclusion, the present study demonstrated that relaxin administered before IR renal injury protected against such injury in rats, and this effect probably maintained the integrity of endothelial cells and renal tubular epithelium by suppressing the regulatory mechanism of apoptosis and inflammation. These findings suggest the therapeutic potential of relaxin against renal IR injury.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: T.Y., H.K., and N.I. performed experiments; T.Y., H.K., T.K., and N.I. analyzed data; T.Y., H.K., T.K., and N.I. interpreted results of experiments; T.Y. and T.K. prepared figures; T.Y. and H.K. drafted manuscript; T.Y., H.K., and N.I. edited and revised manuscript; H.K., T.K., and N.I. approved final version of manuscript; H.K. and N.I. conception and design of research.

REFERENCES


