Concomitant inhibition of renin angiotensin system and Toll-like receptor 2 attenuates renal injury in unilateral ureteral obstructed mice

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Background/Aims: There has been controversy about the role of Toll-like receptor 2 (TLR2) in renal injury following ureteric obstruction. Although inhibition of the renin angiotensin system (RAS) reduces TLR2 expression in mice, the exact relationship between TLR2 and RAS is not known. The aim of this study was to determine whether the RAS modulates TLR2.

Methods: We used 8-week-old male wild type (WT) and TLR2-knockout (KO) mice on a C57Bl/6 background. Unilateral ureteral obstruction (UUO) was induced by complete ligation of the left ureter. Angiotensin (Ang) II (1,000 ng/kg/min) and the direct renin inhibitor aliskiren (25 mg/kg/day) were administrated to mice using an osmotic minipump. Molecular and histologic evaluations were performed.

Results: Ang II infusion increased mRNA expression of TLR2 in WT mouse kidneys (p < 0.05). The expression of renin mRNA in TLR2-KO UUO kidneys was significantly higher than that in WT UUO kidneys (p < 0.05). There were no differences in tissue injury score or mRNA expression of macrophage chemotactic protein 1 (MCP-1), osteopontin (OPN), or transforming growth factor β (TGF-β) between TLR2-KO UUO and WT UUO kidneys. However, aliskiren decreased the tissue injury score and mRNA expression of TLR2, MCP-1, OPN, and TGF-β in WT UUO kidneys (p < 0.05). Aliskiren-treated TLR2-KO UUO kidneys showed less kidney injury than aliskiren-treated WT UUO kidneys.

Conclusions: TLR2 deletion induced activation of the RAS in UUO kidneys. Moreover, inhibition of both RAS and TLR2 had an additive ameliorative effect on UUO injury of the kidney.

Keywords: Toll-like receptor 2; Renin-angiotensin system; Unilateral ureteral obstruction

INTRODUCTION

Toll-like receptors (TLRs) recognize endogenous danger molecules such as hyaluronan fragments that have been altered from their native state or accumulated in non-physiologic sites or amounts during cellular injury [1-5]. Min et al. [6] reported that TLR inhibition may have renoprotective effects on obesity-related kidney disease through its anti-inflammatory properties. TLR2 and TLR4 play crucial roles in the induction of acute inflammation and early tubular injury in the kidney in a reversible model of acute renal injury [7-9]. Leemans et al.
unilateral ureteral obstruction (UUO) increases renal expression of TLR2. However, Chowdhury et al. [11] reported that TLR2 is not involved in renal injury following ureteric obstruction, and deletion of TLR2 does not have a beneficial effect on kidneys with UUO [10,11]. Although deletion of TLR2 reduces renal injury and infiltration of interstitial myofibroblasts, it does not affect the development of progressive renal fibrosis in UUO mice [10]. It is not clear why deletion of TLR2 does not reduce the renal injury induced by UUO, and the role of TLR2 in renal injury following ureteric obstruction remains controversial.

The renin angiotensin system (RAS) has been reported to play a crucial role in renal inflammation and fibrosis in UUO kidneys [12,13] and inhibition of RAS attenuates renal injury in UUO mice [14,15]. Olmesartan reduces TLR2 in the aortic roots of mice, resulting in a decrease in intimal neovascularization and plaque growth. Losartan decreases the expression of TLR2 mRNA and protein that is upregulated in cyclosporin-induced renal injury [16]. Although these reports suggest that inhibition of RAS reduces TLR2 signaling, it is not clear whether RAS modulates TLR signaling. This study was performed to investigate whether concomitant inhibition of RAS and TLR2 attenuates inflammation and fibrosis in UUO mice and to evaluate the relationship between RAS and TLR2.

METHODS

Animals and drug treatments
All experiments were performed on 8-week-old male C57BL/6 mice (weight 20 to 25 g, Samtako, Osan, Korea) and TLR2 knockout (KO) mice (weight 20 to 25 g, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). The mice were given a standard laboratory diet and water and were cared for under a protocol approved by the Institutional Animal Care and Use Committee of the Chungnam National University Medical School.

Two separate studies were performed. First, we evaluated the association between the RAS and TLR2. Wild type (WT) and TLR2-KO mice (n = 8 per group) were infused with angiotensin (Ang) II to activate RAS. Second, we divided the mice into eight groups: vehicle (Vh)-treated sham (n = 7), aliskiren-treated sham (n = 7), TLR2-KO (n = 8), aliskiren-treated TLR2-KO (n = 8), Vh-treated UUO (n = 8), aliskiren-treated WT UUO (n = 8), TLR2-KO UUO (n = 8), and aliskiren-treated TLR2-KO UUO (n = 8). To establish UUO, complete ligation of the left ureter at the ureteropelvic junction was performed using double silk sutures. Mice were anesthetized with intraperitoneal ketamine (2 mg/kg, Ketalar, Bayer, Leverkusen, Germany) and xylazine (200 µL/kg, Rompun, Bayer). Aliskiren powder was supplied by Novartis Co. (East Hanover, NJ, USA). To continuously deliver solutions over the course of 8 days, an Alzet osmotic minipump (model 2004, Durect Corp., Cupertino, CA, USA) was implanted subcutaneously in each mouse 1 day before UUO or sham operation. The pumps were filled with either normal saline or saline containing aliskiren. Both were administered at a dose of 25 mg/kg/day. Ang II was infused at a rate of 1,000 ng/kg/min via the subcutaneously implanted osmotic minipumps. Mice were sacrificed on day 7 after UUO or on day 12 after Ang II infusion.

Tissue preparation
Tissue preparation was performed as described previously [14]. Briefly, at the end of the study (7 days after UUO and 12 days after Ang II infusion), mice were sacrificed, and both kidneys were excised immediately and cut into three coronal sections. Two pieces were snap-frozen in liquid nitrogen and stored at −70°C for subsequent RNA extraction and protein analysis. The third piece (central section) was fixed in 10% buffered formaldehyde at room temperature and then embedded in Paraplast (Sherwood Medical, St. Louis, MO, USA) for light microscopy and immunohistochemical staining.

Light microscopy
Tissue staining was performed as described previously [14]. Briefly, paraffin-embedded kidney pieces were cut into sections 4-µm thick and mounted on glass slides. The sections were deparaffinized with xylene, stained with hematoxylin and eosin (H&E) and Masson’s trichrome (MT), and examined under an Olympus BX51 microscope (Olympus, Tokyo, Japan). The tubulointerstitial injury score was evaluated based on morphological changes in tubucls, such as dilatation, distortion of tubular basement membranes and atrophy: grade 0, no morphological deformities; grade 1, less than 10%; grade 2, less than 25%; grade 3, less than 50%; grade 4,
Immunohistochemistry

Immunohistochemistry was performed as described previously [14]. Paraffin-embedded tissues were cut into sections 4-µm thick, mounted on glass slides, and stained using indirect immunoperoxidase. The slides were processed for immunodetection of transforming growth factor β (TGF-β) and angiotensin II type 1 receptor (AT1R) with antibodies specific for TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-AT1R (Santa Cruz Biotechnology) respectively. Diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) was used as a chromogen. All samples were evaluated under an Olympus BX51 microscope. The size of the area stained positively for TGF-β (as a percentage of the total area in 10 separate fields of each section under x200 magnification) was determined using a digital camera-based image analyzer (Metamorpho version 6.3, Olympus).

Western blot

Western blot analysis was performed to measure TLR2, renin, and AT1R protein expression. Kidney sections were homogenized in PRO-PREP protein extraction solution (iNtRON, Seongnam, Korea). Total protein (40 µg) was loaded. Samples were wet transferred onto 0.2 µm nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ, USA). Blots were blocked for 1 hour with 5% nonfat dry milk in Tris-buffered saline-Tween buffer composed of 20 mM Tris-HCl (pH 7.6), 0.8% NaCl and 0.05% Tween 20, and incubated overnight at 4°C with anti-TLR2 antibody (Abcam, Cambridge, MA, USA), AT1R (Santa Cruz Biotechnology), anti-renin (Santa Cruz Biotechnology) and anti-β-actin antibody (Santa Cruz Biotechnology). Blots were incubated with hors eradish peroxidase-conjugated secondary anti-rabbit immunoglobulin G antibody (Cell Signaling Technology) for 1 hour. Bands were detected by enhanced chemiluminescence (Millipore, Billerica, MA, USA) and exposed to film. The optical density for quantification was determined using Gel-Pro Analyzer version 3.1 (Media Cybernetics, Bethesda, MD, USA).

RNA extraction, and quantitative real-time polymerase chain reaction

Total RNA was extracted from whole kidney using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2 µg total RNA using an oligo dT primer (Amersham Pharmacia), deoxynucleotide triphosphates (Amersham Pharmacia), moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Grand Island, NY, USA), 0.1 M dithiothreitol, and buffers in a volume of 20 µL. The cDNA reaction mix was diluted to a total volume of 40 µL and polymerase chain reaction (PCR) was performed to amplify the following specific cDNAs: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; primers: sense 5′-CC ACG ACC CCA TAA CAA CAG-3′; antisense 5′-TGA GGG TGC AGC GAA CTT TA-3′); monocyte chemotactic protein 1 (MCP-1; primers: sense 5′-ACT GCA TCT GCC CTA AGG TCT TCA-3′; antisense 5′-AGA GTG CTT TGA GGT GTG TGT GA-3′); osteopontin (OPN; primers: sense 5′-GGC ATT GCC TCC TCC CTC-3′; antisense 5′-CGA GTC TAA GGT TCT CCT CC-3′); TGF-β (primers: sense 5′-AAC TAT TGC TTT AGC TCC AGC TGG AGA-3′; antisense 5′-AGT TGG ATG GTA GCC CTT G-3′); TLR2 (primers: sense 5′-GCC ACC ATT TCC AGC GAC T-3′; antisense 5′-GGC TTC CTC TTT GCC TGG-3′); AT1 receptor (primers: sense 5′-AGA ACA CCA ATA TCA CTG TTT G-3′; antisense 5′-TAG CTG GTA AGA ATG ATT AGG A-3′); and renin (primers: sense 5′-ATG AAG GGG GTG TCT GTG GGG TC-3′; antisense 5′-ATG TCG GGG AGG GTG GCC ACC TG-3′). PCR was performed using SYBR Green PCR mastermix (Qiagen). The amplification reaction volume was 20 µL, consisting of 10 µL iQ SYBR Green PCR mastermix, 2 µL primers, 2 µL cDNA, and 6 µL water. Amplification and detection were performed using a thermal cycler (Rotor-Gene 6000, Corbett Research, Mortlake, Australia). PCR conditions were as follows: denaturation at 95°C for 10 minutes followed by 40 cycles of 10 seconds at 95°C, 15 seconds at the annealing temperature (55°C for GAPDH, TGF-β, MCP-1), and 20 seconds at 72°C. SYBR green fluorescence was measured at the end of each cycle using the comparative threshold.
cycle (Ct) method: $2^{-\Delta\Delta Ct} = 2 - [(\text{Ct of target gene} - \text{Ct of GAPDH in treated mice}) - (\text{Ct of target gene} - \text{Ct of GAPDH in sham mouse})]$. 

**Statistical analysis**

Data are reported as the mean ± SD. Multiple comparisons among groups were performed by one-way analysis of variance with the post hoc Bonferroni test correction (SPSS version 11.0, SPSS Inc., Chicago, IL, USA). The difference between groups was considered statistically significant at $p < 0.05$.

**RESULTS**

**RAS and TLR2 expression in the kidneys of sham-operated and UUO mice**

The kidneys of Ang II-infused WT mice showed increased expression of TLR2 mRNA compared with those of saline-infused WT controls ($p < 0.05$) (Fig. 1A). To evaluate the association between the RAS and TLR2, we evaluated renin and AT1R expression in TLR2-KO mouse kidneys. TLR2-KO mouse kidneys showed little TLR2 expression (Fig. 1B). Renal mRNA expression of renin and AT1R tended to be higher in TLR2-KO mouse kidneys than in WT mice (Fig. 1C and 1D). However, there were no significant differences.

To evaluate RAS activation, we measured renin and AT1R mRNA expression in UUO kidneys. In the TLR2-KO UUO kidney, mRNA expression of renin and AT1 receptor was significantly increased compared with that in WT UUO kidneys ($p < 0.05$) (Fig. 1C and 1D). Protein level of AT1R in TLR2-KO UUO kidneys was significantly increased compared with that in the WT UUO kidneys ($p < 0.05$) (Fig. 1D). Protein level of renin showed increased tendency in TLR2-KO UUO kidneys but there was no statistical significance (Fig. 1C). TLR2 mRNA expression was significantly higher in UUO kidneys than in sham-operated kidneys ($p < 0.05$) (Fig. 1E). Aliskiren treatment reduced renal mRNA expression of TLR2 in UUO kidneys ($p < 0.05$) (Fig. 1E). There was no significant difference in renal mRNA expression of TLR2 between Vh- and aliskiren-treated sham mice.

**Inflammation and fibrosis of kidneys in TLR2-KO UUO mice**

The expression of OPN, MCP-1, and TGF-β mRNA were increased in UUO kidneys compared with sham-operated kidneys. However, there were no significant differences in renal mRNA expression of OPN, MCP-1, and TGF-β between TLR2 KO and WT UUO kidneys (Fig. 2A-2C). Light microscopic examination of WT UUO kidneys showed tissue injury, including infiltration of mononuclear cells into the interstitium and tubules, and desquamation of tubular epithelial cells. There was no significant difference in tissue injury score between WT and TLR2-KO UUO kidneys. TLR2-KO UUO kidneys showed no significant difference in the area positive for MT or TGF-β compared with WT controls (Fig. 2D).

**Inhibition of RAS in TLR2 KO UUO mice**

Aliskiren treatment improved the renal tubulointerstitial injury score in UUO kidneys. Renal tubulointerstitial injury was significantly decreased in aliskiren-treated TLR2-KO UUO kidneys compared to WT UUO kidneys treated with this agent ($p < 0.05$) (Fig. 3). Aliskiren treatment significantly reduced renal mRNA expression of OPN and MCP-1 in UUO kidneys ($p < 0.05$) (Fig. 4). Moreover, renal mRNA expression of OPN and MCP-1 was significantly decreased in aliskiren-treated TLR2-KO UUO kidneys compared to WT UUO kidneys treated with this agent ($p < 0.05$) (Fig. 4).

Aliskiren improved renal interstitial fibrosis in UUO kidneys, as indicated by decreased Masson’s trichrome staining and TFG-β expression (Fig. 5). Aliskiren-treated TLR2-KO UUO kidneys showed significant decreases in areas positive for MT and TGF-β compared with aliskiren-treated WT UUO kidneys ($p < 0.05$) (Fig. 5A and B). Also, renal mRNA expression of TGF-β was significantly decreased in aliskiren-treated TLR2-KO UUO kidneys compared to with WT UUO kidneys treated with this agent ($p < 0.05$) (Fig. 5B). The expression of renin was increased in UUO kidneys ($p < 0.05$) (Fig. 5C). Although not statistically significant, aliskiren treatment showed increase tendency in the renin expression of both WT UUO and TLR2-KO UUO kidneys compared with untreated UUO kidneys (Fig. 5C). The expression of AT1R was increased in UUO kidneys ($p < 0.05$) (Fig. 5D). Aliskiren treatment significantly decreased the AT1R ex-
Figure 1. (A) Representative effects of Ang II infusion on TLR2 mRNA expression. Representative kidneys from Ang II-infused WT sham-operated mice (n = 8) showed higher mRNA expression of TLR2 than saline-infused WT sham-operated mice (n = 7). (B) Representative immunoblots from Ang II-infused WT sham-operated mice (n = 4) and saline-infused WT sham-operated mice (n = 4) TLR2 expression in WT and TLR2-KO mouse kidneys. (C, D) Representative immunoblots from WT mice (n = 4) and TLR2-KO mice (n = 4) renin and AT1R expression. Renin and AT1R mRNA expression levels were significantly higher in UUO kidneys than in sham kidneys (C, D), and in TLR2-KO UUO kidneys compared with WT UUO kidneys (C, D). There was no significant difference in the basal mRNA expression of renin and AT1R between sham and TLR2-KO kidneys. Representative immunoblotting results indicating significantly higher renin and AT1R expression in UUO kidneys (n = 4) compared to sham-operated kidneys (n = 3) (C, D). The protein level of AT1R was significantly increased in TLR2-KO UUO kidneys compared with WT UUO kidneys (D). TLR2 mRNA expression in UUO kidneys was significantly higher than that in sham-operated kidneys (D). Aliskiren treatment reduced the mRNA level of TLR2 in UUO kidneys (E). Ang, angiotensin; TLR2, Toll-like receptor 2; WT, wild type; KO, knockout; AT1R, angiotensin II type 1 receptor; UUO, unilateral ureteral obstruction; S, sham-operated mice (n = 7); A-S, aliskiren treated sham-operated mice (n = 7); TLR2-KO, TLR2-KO mice (n = 8); TLR2-KO UUO, TLR2-KO UUO mice (n = 8); A UUO, aliskiren treated WT UUO mice (n = 8). a,b,p < 0.05.
Figure 2. The effects of TLR2-KO on UUO-induced injury. Renal mRNA expression of (A) MCP-1, (B) OPN, or (C) TGF-β were similar in TLR2-KO and WT UUO mice. There were no significant differences in renal expression of MCP-1 (A, \( p = 0.299 \)), OPN (B, \( p = 0.549 \)), and TGF-β (C, \( p = 0.654 \)) between WT and TLR2-KO UUO kidneys. (D) There was no significant difference in tubulointerstitial injury score, MT stained area, and TGF-β positive area between WT and TLR2-KO UUO kidneys (×200).

UUO: UUO WT mice (\( n = 8 \)), TLR2-KO UUO mice (\( n = 8 \)). TLR2, Toll-like receptor 2; KO, knockout; UUO, unilateral ureteral obstruction; MCP-1, monocyte chemotactic protein 1; OPN, osteopontin; TGF-β, transforming growth factor β; WT, wild type; MT, Masson’s trichrome.
Figure 3. The effects of inhibition of renin angiotensin system and TLR on renal histology 7 days after UUO in (A) S, (B) vehicle-treated WT UUO, (C) aliskiren-treated WT UUO, and (D) aliskiren-treated TLR2-KO UUO mice (×200). Vehicle-treated obstructed kidneys showed marked injury, with mononuclear cells in the interstitium and tubules, and dilatation and desquamation of tubular epithelial cells. In contrast, aliskiren-treated WT UUO mice showed significantly less tubulointerstitial damage. (E) Aliskiren-treated TLR2-KO UUO kidneys showed a significant decrease in tissue injury score compared with aliskiren-treated WT UUO kidneys. TLR, Toll-like receptor; UUO, unilateral ureteral obstruction; WT, wild type; KO, knockout; S, sham; A, aliskiren; S, sham-operated mice (n = 7); UUO, UUO mice (n = 8); A-UUO, aliskiren-treated WT UUO mice (n = 8); A-TLR2-KO UUO, aliskiren-treated TLR2-KO UUO mice (n = 8). *p < 0.05 vs. sham operation, **p < 0.05 vs. UUO, ***p < 0.05 vs. A-UUO.

Figure 4. The effects of inhibition of renin angiotensin system and TLR on MCP-1 and OPN expression 7 days after UUO. (A) MCP-1 and (B) OPN mRNA expression were increased in UUO kidneys compared with sham-operated kidneys. Aliskiren treatment reduced renal mRNA expression of MCP-1 and OPN in UUO kidneys. Aliskiren-treated TLR2-KO UUO kidneys showed a significant decrease in mRNA expression of MCP-1 and OPN compared with aliskiren-treated WT UUO kidneys. TLR, Toll-like receptor; MCP-1, monocyte chemotactic protein 1; OPN, osteopontin; UUO, unilateral ureteral obstruction; KO, knockout; WT, wild type; S, sham-operated mice (n = 7); UUO, UUO operated mice (n = 8); A-UUO, aliskiren-treated UUO WT mice (n = 8); A-TLR2-KO UUO, aliskiren-treated TLR2-KO UUO mice (n = 8). *p < 0.05.
expression in WT UUO kidneys compared with untreated UUO kidneys \( (p < 0.05) \) (Fig. 5D). Although there was no statistical significance, AT1R expression showed the increase tendency in aliskiren-treated TLR2-KO kidneys compared to WT UUO kidneys treated with this agent (Fig. 5D).

**DISCUSSION**

The results of the present study indicated that deletion of TLR2 induces RAS activation in the UUO kidney, and that inhibition of the RAS reduces renal injury in the TLR2-KO UUO kidney.

TLRs are thought to be crucial cellular sentinels that detect “danger” signals released by tissue damage [17], and they may also be important initiators of inflammation and fibrosis. TLR2 is involved in the activation of TGF-\( \beta \) and migration of macrophages and myofibroblasts [10,11]. Deletion of TLR2 reduces apoptosis of tubular cells in renal ischemia reperfusion injury. However, the role of TLR2 in renal injury by UUO is not clear [10,11]. Although TLR2 recruits inflammatory cytokines, macrophages, and fibroblasts in the kidney, most studies have reported that deletion of TLR2 does not reduce the induction of inflammatory cytokines or progression of renal fibrosis [10,11]. Similar to these findings, our study showed no significant difference in renal inflammation or fibrosis induced by UUO between WT and TLR2-KO mice.
The renin and AT1R mRNA expression levels are increased in UUO kidneys [14,18,19]. In general, renin is expressed in the macular densa and AT1R is expressed in tubules and interstitial area. We did not separate the tubular area, but used the whole tissue for immunoblotting. Therefore, we could not determine the precise locations and changes in expression of AT1R. Chronic renal injury results in elevated renin and AT1R expression [20]. In addition, it is well known that activation of RAS also plays an important role in kidney injury induced by UUO. Direct renin inhibition and AT1R KO showed improvement of renal injury in UUO kidneys [12,14].

We showed that deletion of TLR2 tends to increase the basal renal renin and AT1R mRNA expression compared to those in WT mice. Moreover, TLR2-KO UUO mice showed significant increases in renin ren and AT1R mRNA expression compared with WT UUO mice. The enhanced activation of RAS accompanied by inhibition of TLR2 may offset the anti-inflammatory effects of the TLR2 KO and may explain why blocking of TLR2 did not reduce the renal injury induced by UUO. Various kidney injury models have shown that RAS activation is important in the inflammatory process, including increased levels of OPN and MCP-1 expression [21-23]. TGF-β plays a major role in renal fibrosis induced by UUO [24], and RAS activation is involved in TGF-β expression in the obstructed kidney [25].

Although there have been no reports that TLR2 modulates the RAS, there is evidence that the RAS may increase activation of TLRs. It has been reported that Ang II induces activation of TLR4 signaling in cultured fibroblasts and mesangial cells [26,27]. Lim et al. [28] reported that activation of RAS increased renal TLR2 expression whereas inhibition of RAS by an Ang receptor blocker decreased renal TLR2 expression in cyclosporine A-induced renal injury. Our study showed that RAS activation induced by Ang treatment increased TLR2 mRNA expression. Moreover, the induction of renal TLR2 by UUO was reduced by aliskiren treatment. Taken together, these findings indicate that the RAS modulates TLR2 activation. Although the mechanism by which deletion of TLR2 activates RAS is not known, there are a number of possibilities. Deletion of TLR2 may activate a different inflammatory pathway [29]. It was recently revealed that TLR2 plays a role in innate immunity and angiogenesis by sensing the oxidation status and that deletion of TLR2 reduces wound healing in the dermis of mice [30]. TLR2 is expressed at high levels in renal tubules and the vessel endothelium of normal kidneys [31]. It is possible that deletion of TLR2 reduces the healing process of the kidney, thus potentiating other inflammatory processes including activation of RAS.

In summary, the present study indicated that TLR2 deletion results in RAS activation in UUO kidneys. Moreover, concomitant inhibition of RAS and TLR2 has an additive benefit on the amelioration of UUO-induced kidney injury.

**KEY MESSAGE**

1. Toll-like receptor (TLR) 2 deletion results in activation of renin angiotensin system (RAS) in unilateral ureteral obstruction (UUO) kidneys.
2. Concomitant inhibition of RAS and TLR2 has an additive benefit on the amelioration of UUO-induced kidney injury.

**Conflict of interest**

No potential conflict of interest relevant to this article was reported.

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