〈Regular Article〉

Anti-inflammatory effect of tadalafil, a phosphodiesterase 5 (PDE5) inhibitor, in autoimmune prostatitis

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ABSTRACT Prostatitis is one of the most common diseases in urology. The pathology involves intricate interactions among multiple factors, and this has limited the establishment of evidence-based treatment for prostatitis. An autoimmune prostatitis mouse model has recently been suggested to be appropriate as a model of human prostatitis pathology. PDE5 inhibitors are indicated as therapy for benign prostatic hyperplasia with lower urinary tract symptoms, pulmonary arterial hypertension and erectile dysfunction, and have also been shown to have an anti-inflammatory effect against tissue inflammation. Thus, we hypothesized that a PDE5 inhibitor may have a preventive effect on prostatitis. To verify this hypothesis, the anti-inflammatory effects of a PDE5 inhibitor, tadalafil, were examined in an autoimmune prostatitis mouse model.

C57BL/6 male mice aged 15 weeks were used in the study. Prostate glands from Wistar rats aged 5 weeks were used as a source of prostate antigen (protein concentration 10 mg/ml).

Prostate antigen (100 μg in 100 μl of adjuvant) was subcutaneously injected into C57BL/6 mice. Tadalafil (25 μg in 50 μl of water) was orally administered every day for up to 10 weeks after establishment of the model. Control mice received water only (50 μl). Inflammatory changes were investigated using histological, biochemical and immunohistochemical analyses.

Histological analysis using hematoxylin-eosin and Masson-Trichrome staining showed inhibitory effects on tissue fibrosis following invasion by inflammatory cells in tadalafil-treated mice compared with control mice. Biochemical and immunohistochemical analyses using an inflammation-related proteome assay and immunostaining showed decreased levels of M-CSF, TREM-1, TIMP-1, CCL2, CCL3 and CXCL2 in tadalafil-treated mice compared with control mice.

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Tadalafil has an inhibitory effect on tissue fibrosis and decreases cytokine levels after an inflammatory response. These results suggest that PDE5 inhibitors might be effective as therapy for prostatitis.

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Key words：Phosphodiesterase 5 inhibitor, Tadalafil, Autoimmune prostatitis, Anti-inflammation

INTRODUCTION

Prostatitis is a common disease in urology, with a population-based prevalence of 1.0 to 14.2%[1, 2]. Multiple factors including autoimmune response, hormonal environment, chronic pelvic ischemia, high pressure urination (reflex) and infection are involved in the pathology of prostatitis[3, 4]. This complexity has made it difficult to establish evidence-based treatment for prostatitis, and has also prompted development of numerous animal models to elucidate the pathology[5]. An autoimmune prostatitis model has been proposed to be appropriate as a reflection of human prostatitis pathology[5, 6].

Phosphodiesterase (PDE) is an enzyme that regulates the intracellular level of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), and plays a central role in multiple cellular functions[7]. There are 11 isoenzymes (PDE1-11) in the PDE family, and these isozymes have different expression and function in different tissues[8]. PDE5, one of the best-studied PDEs, specifically targets cGMP, which is typically generated by nitric oxide (NO)-mediated activation of soluble guanylate cyclase[9]. PDE5 inhibitors increase the level of intracellular cGMP, an activator of cGMP-dependent protein kinase and subsequent phosphorylation of specific substrate proteins[10]. cGMP plays a central role in signal transduction and regulates physiological responses such as smooth muscle relaxation following a decrease in the intracellular calcium concentration[10].

PDE5 inhibitors are indicated for benign prostatic hyperplasia with lower urinary tract symptoms (BPH/LUTS), pulmonary arterial hypertension (PAH), and erectile dysfunction (ED)[11], and a PDE5 inhibitor has also been shown to have an anti-inflammatory effect against tissue inflammation[6]. Therefore, we hypothesized that a PDE5 inhibitor may also have this effect in prostatitis. To verify this hypothesis, we investigated the anti-inflammatory effect of tadalafil, a well-known PDE5 inhibitor, in an autoimmune prostatitis mouse model established by Rivero *et al*.[5, 12].

MATERIALS AND METHODS

Research animals and approval

C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME, USA) aged 15 weeks were used in the study. Wistar male rats (Jackson Laboratory) aged 5 weeks were used as a source of prostate antigen (PAg) for C57BL/6 male mice. The study was approved by the animal research committee of Kawasaki Medical School, Kurashiki, Japan (approval numbers 18-075 and 20-090) and was conducted according to the guidelines for the care and use of laboratory animals of Kawasaki Medical School.

Autoimmune prostatitis mouse model

PAg for C57BL/6 mouse was extracted from Wister rat prostate that was resected under inhalational anesthesia using sevoflurane (introduction 5%, maintenance 3%, Pfizer Japan Inc. Tokyo, Japan). The resected prostate was homogenized with Cell Lysis Buffer (including phosphatase and protease inhibitor) and PMSF (1 mM) (Cell Signaling Technology, Danvers, MA, USA) using a homogenizer. Tissue fragments were removed after centrifugation (15000 × g, 5 min, 5℃). The

remaining liquid was used as PAg after adjusting the protein concentration to 10 mg/ml. C57BL/6 mice were subcutaneously injected with PAg at 100 μg / 100 μl of adjuvant (Titer MAX® Gold Adjuvant: Sigma-Aldrich Japan G.K. Tokyo, Japan) to establish the autoimmune prostatitis mouse model. By the preliminary examination, we confirmed the validity of autoimmune prostatitis model using an inflammation-related proteome assay (Mouse Cytokine Antibody Array Panel A®: R&D Systems, Minneapolis, MN, USA), and systemic changes in model mouse were not observed.

Tadalafil administration

Mice orally received tadalafil (Toronto Research Chemicals, Toronto, ON, Canada) at 25 μg / 50 μl of pure water every day for up to 10 weeks after establishment of the autoimmune prostatitis model. Control mice received pure water only (50 μl). The prostate was resected under inhalational anesthesia at 1, 5 and 10 weeks, and the anti-inflammatory effect of tadalafil was investigated using histological, biochemical and immunohistochemical analyses. By the preliminary examination, inflammatory changes in autoimmune prostatitis model were locally occurred in prostate. For this reason, we evaluated the efficacy of tadalafil limit in the prostate only.

Histological analysis

Prostate tissues resected from model mice were fixed in Carnoy fixative fluid (Wako Pure Chemical, Osaka, Japan) for 3 h. Fixed tissues were dehydrated in an ethanol series and finally in absolute ethanol. Dehydrated tissues were embedded in paraffin wax, cut to a thickness of 5 μm, mounted on slides and dried for 45 min at 45℃. Tissue slides were de-waxed and hydrated through ethanol graded solutions to water. Histological analysis using hematoxylin-eosin (HE) and Masson-Trichrome (MT) staining was performed using an Olympus BX-53 microscope (Olympus, Tokyo, Japan).

Biochemical analysis

Using a similar procedure to that described above for rat prostate, prostate tissues from mice were homogenized with Cell Lysis Buffer and PMSF, tissue fragments were removed after centrifugation (15000 × g, 5 min, 5℃). Biochemical changes in the prostate were investigated using an inflammation-related proteome assay (Mouse Cytokine Antibody Array Panel A®: R&D Systems, Minneapolis, MN, USA). The protein concentration of each sample was 300 μg / ml. The pixel density was calculated using an image analyzer (Image Quant LAS4000 mini®) and software (Image Quant TL®) GE Healthcare Japan, Tokyo, Japan). Calculated pixel density was corrected using endogenous control, negative and positive control were set as pixel density 0 and 100. Significantly lower level ($p < 0.0001$, Welch test) of cytokine was set as representative candidate for the effect of tadalafil in autoimmune prostatitis.

Immunohistochemical analysis

Immunohistochemical analysis was performed using immunostaining (ImmPRESS Reagent HRP, Vector Laboratories, Burlingame, CA, USA). Blocking was performed for 10 min at room temperature with BLOXALL™ blocking solution (Vector Laboratories) after de-waxing and hydration. Based on the results of biochemical analysis, primary antibodies against M-CSF, TREM-1, TIMP-1, CCL2, CCL3 and CXCL2 were used, with all diluted in 2.5% normal horse serum (Vector Laboratories): anti-M-CSF antibody (ab234259, Abcam plc, Cambridge, UK; diluted 1000-fold); anti-TREM-1 antibody (bs-4886R, Bioss Inc., MA, USA; diluted 100-fold); anti-TIMP-1 antibody (ab240504, Abcam plc; diluted 1000-fold; anti-CCL2 (MCP-1) antibody (ab25124, Abcam plc; diluted 100-fold); anti-CCL3 (MIP-1a) antibody (sc-2357, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 100-fold); and anti-CXCL2 antibody (GTX74085, Gene Tex Inc., Irvine, CA,

USA; diluted 100-fold). Negative control (NC) was used Rabbit IgG Control Antibody (Vector Laboratories). The primary antibody reaction was performed overnight at 4℃. The secondary antibody reaction against M-CSF, TREM-1, TIMP-1, CCL2, CCL3 and CXCL2 were performed using an ImmPRESS Reagent Anti-Rabbit IgG Polymer Kit (Vector Laboratories) for 30 min at room temperature. The immune substrates were ImmPACT DAB Substrate, Peroxidase (HRP) (Vector Laboratories) for M-CSF, TREM-1, TIMP-1, CCL2, CCL3 and CXCL2. Counterstain was performed using a Hematoxylin Counterstain (Vector Laboratories). Immunohistochemical analysis using immunostaining was examined using an Olympus BX-53 microscope (Olympus, Tokyo, Japan).

Statistical analysis

The pixel density range was defined as 0 (negative control) to 100 (positive control), and expressed as the mean and standard error (SE). Excel Statistics (Microsoft Corp., Redmond, WA, USA) was used for statistical analysis. Differences between treated and control mice were analyzed by Welch test, with $p < 0.05$ considered to indicate a significant difference.

RESULTS

Tadalafil inhibited prostatic fibrosis after inflammation

HE staining showed greater invasion of inflammatory cells in control mice compared with tadalafil-treated mice at weeks 1 and 5 after establishment of the autoimmune prostatitis model. Inflammatory cells were mainly present in interstitial tissue (Fig. 1). MT staining showed more tissue fibrosis in control mice compared with tadalafil-treated mice at weeks 5 and 10, with fibrosis mainly seen in interstitial tissue (Fig. 1). Thus, tadalafil-treated mice showed inhibition of tissue fibrosis after invasion of inflammatory cells in the autoimmune prostatitis model.

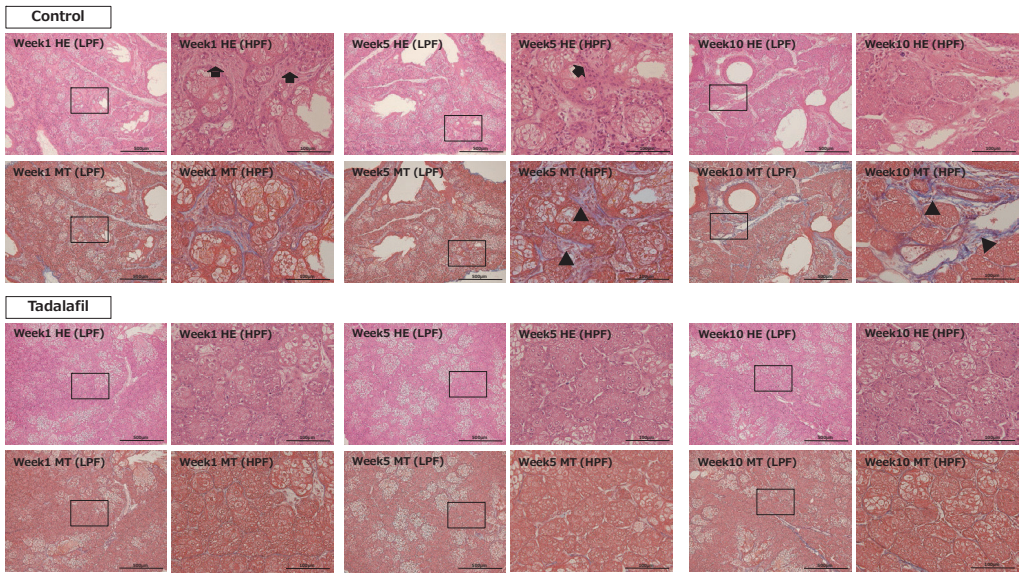

Fig. 1. Histological analysis
Histological analysis showed greater interstitial tissue fibrosis (▲) after inflammatory cell invasion (➡) in control mice compared with tadalafil-treated mice. Tadalafil-treated mice had inhibition of these inflammatory changes. Scale bar: low power field (LPF) 500 μm, high power field (HPF) 100 μm. Magnification: LPF 100×, HPF 200×. □ : magnification area from LPF toHPF. n = 4 (each week).

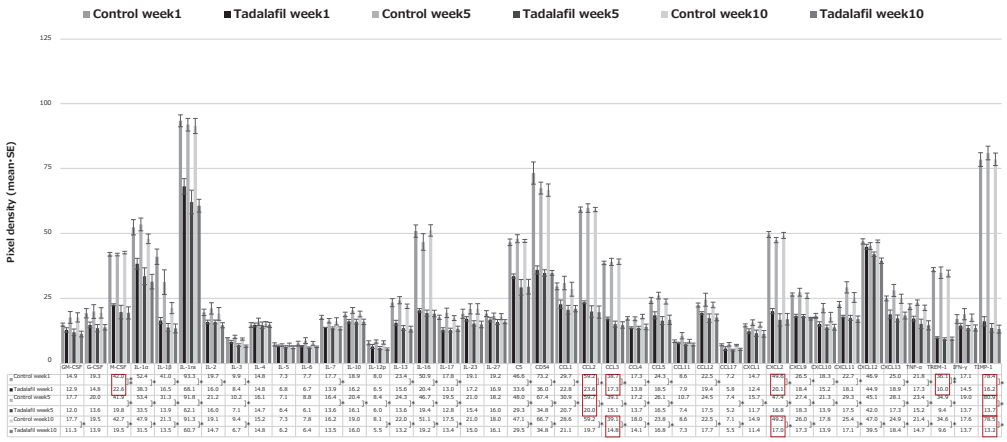

Fig. 2. Biochemical analysisn
An inflammation-related proteome assay showed decreased production of many cytokines in tadalafil-treated mice compared with control mice. Significantly decreased levels of M-CSF, TREM-1, TIMP-1, CCL2, CCL3 and CXCL2 were observed in tadalafil-treated mice. Indicated data was showed mean and standard error (SE). $^{*}p < 0.05$, $^{**}p < 0.0001$ for tadalafil-treated vs. control mice (Welch test). n = 4 (each week).

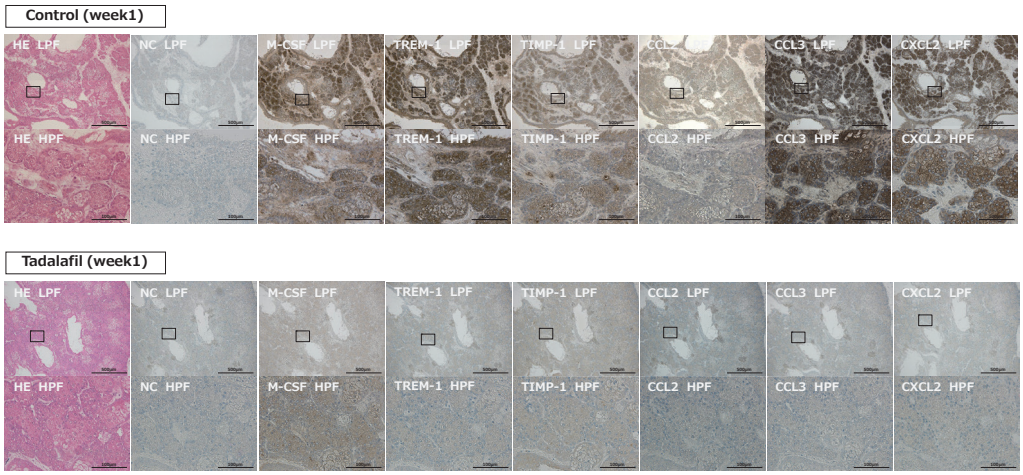

Fig. 3. Immunohistochemical analysis
Immunostaining showed decreased production of M-CSF, TREM-1, TIMP-1, CCL2, CCL3 and CXCL2 in tadalafil-treated mice compared with control mice. NC (negative control): Rabbit IgG (Control Antibody). Scale bar: low power field (LPF) was 500 μm, high power field (HPF) was 100 μm. Magnification: LPF 100×, HPF 200×. □: magnification area from LPF to HPF. n = 4 (each week)

Tadalafil decreased cytokine levels in autoimmune prostatitis

The inflammation-related proteome assay showed decreases in many cytokines in tadalafil-treated mice compared with control mice at different time points after establishment of the autoimmune prostatitis model (Fig. 2). Significantly lower levels of M-CSF, TREM-1, TIMP-1, CCL2, CCL3 and CXCL2 were observed in tadalafil-treated mice ($p < 0.0001$, Welch test). Decreases in M-CSF and TREM-1 mainly occurred at week 1, whereas decreases in TIMP-1, CCL2, CCL3 and CXCL2 were seen over the course of the study.

Therapeutic effect of Tadalafil and localization of cytokines

M-CSF, TREM-1, TIMP-1, CCL2, CCL3, CXCL2 were selected from the biochemical data for investigation of immunohistochemical changes using immunostaining. This analysis showed decreased levels of these proteins in tadalafil-treated mice compared with controls. Decreases in M-CSF, TREM-1, TIMP-1 and CCL3 mainly occurred in ductal tissue and interstitial tissue, while those for CCL2 and CXCL2 mainly occurred in ductal tissue.

DISCUSSION

Prostatitis involves intricate interactions among many factors, and there is a lack of appropriate and effective therapies[3]. Paulis[13] suggested that macrophages, T-lymphocytes, and production of cytokines by these cells play key roles in prostatitis pathophysiology (Fig. 4). Macrophages have a dominant role in tissue inflammation by promoting an inflammatory reaction through production of cytokines and activation of other cells[13]. Macrophages are divided into M1 and M2 macrophages. M1 macrophages are involved in promotion of inflammatory reactions, whereas M2 macrophages suppress these reactions[13]. Tissue inflammation changes the macrophage type from M2 to M1, and the M1 cells then produce large amounts of cytokines, including CCL3 (MIP-1 α), TNF- α, IL-1 β, and IL-6[13]. Desireddi *et al.*[14] have shown that CCL3 (MIP-1 α) levels are positively correlated with the severity of prostatitis in humans. T-lymphocytes also contribute to inflammatory reactions by producing cytokines and activating other cells. Th1 T-cells produce INF- γ, which activates M1 macrophages (promotion type)[13], while Th2 T-cells produce IL-4, IL-5, and IL-13, which cause activation of M2 macrophages (suppression type)[13]. A subgroup of Th cells, which are referred to as Th17 T-cells, produce IL-17, which then induces production of CCL2 (MCP-1)[13]. Murphy *et al.*[15] found a positive correlation between CCL2 (MCP-1) levels and the severity of prostatitis. Thus, CCL3 (MIP-1 α) and CCL2 (MCP-1) may be novel target biomarkers for the pathology of prostatitis. Our results showed decreased production of CCL3 (MIP-1 α) and CCL2 (MCP-1) in tadalafil-treated mice compared with

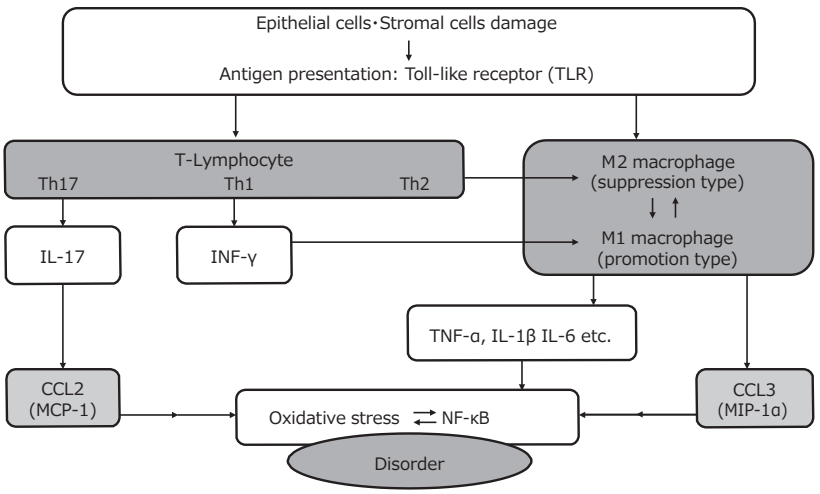

Fig. 4. Pathophysiology of prostatitis
Macrophages, T-lymphocytes, and production of cytokines by these cells play key roles in the pathophysiology of prostatitis.

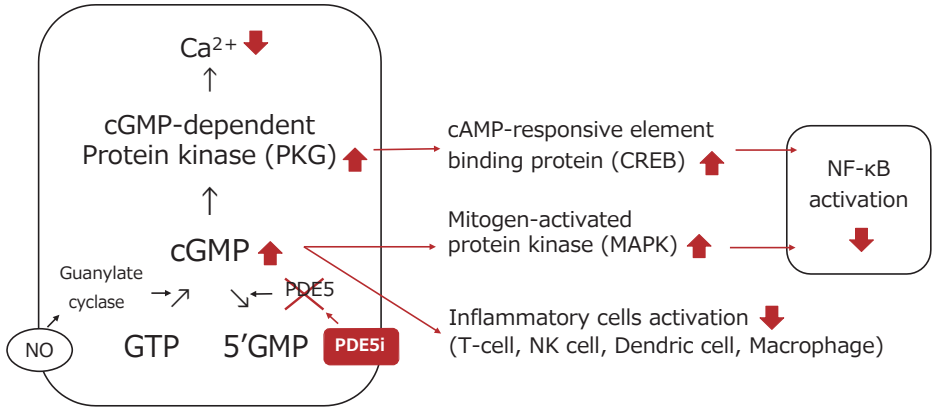

Fig. 5. Anti-inflammatory effect of PDE5 inhibitor in autoimmune inflammation
PDE5 inhibitors elevate intracellular cGMP and PKG. Elevated intracellular cGMP inhibits inflammatory cells and activates MAPK. Furthermore, elevated intracellular PKG activates CREB. Elevated MAPK and CREB regulate the NF-κB activation and inhibit NF-κB related cytokines in autoimmune inflammation. PKG: cGMP-dependent protein kinase, MAPK: cGMP activates mitogen-activated protein kinase, CREB: cAMP-responsive element binding protein.

controls, which suggests that these cytokines may also be useful as target biomarkers of a therapeutic effect.

PDE5 inhibitors are indicated for treatment of BPH/LUTS, PAH, and ED[11], and may also be effective for other conditions (e.g. inflammation)[16]. Several reports have also discussed the potential of PDE5 inhibitors for treating other conditions[9], such as hormonal deficiency[17], vascular endothelial dysfunction[18], tissue ischemia[19, 20], and inflammatory disease including infection[21], cancer[20], and diabetes[20]. Cyclic nucleotides (cAMP and cGMP) control a network of pro-inflammatory and anti-inflammatory mediators and inflammatory cells are sensitive to alterations of cyclic nucleotides[22]. PDE5 inhibitors elevate intracellular cGMP and cGMP-dependent protein kinase (PKG)[23]. Elevated intracellular cGMP activates mitogen-activated protein kinase (MAPK) and inhibits T-cells, NK cells, Dendric cells and Macrophage infiltration[16, 23](Fig. 5). Furthermore, elevated intracellular PKG activates cAMP-responsive element binding protein (CREB)[16]. Elevated MAPK and CREB regulate the NF-κB activation and inhibit NF-κB related cytokines in autoimmune inflammation[16]. Our results showed an inhibitory effect on tissue fibrosis following inflammatory cell invasion and NF-κB related upstream inflammatory cytokines (CCL2, CCL3, etc.) activation in tadalafil-treated mice compared with control mice. These findings indicate that PDE5 inhibitors may be a novel therapy for prostatitis.

CONCLUSION

Tadalafil has an inhibitory effect on tissue fibrosis and decreases production of cytokines following an inflammatory response. These findings suggest that PDE5 inhibitors might be effective for treatment of prostatitis.

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CONFLICT OF INTEREST

The authors declare no financial or commercial

conflict of interest

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