Spirogyra neglecta Aqueous Extract Attenuates LPS-Induced Renal Inflammation

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Spirogyra neglecta (SN), commonly named “Tao” in Thai, is a genus of filamentous green macroalgae. SN contains polyphenols such as isoquercetin, catechin, hydroquinone and kaempferol. These constituents exhibit beneficial effects including anti-oxidant, anti-gastric ulcer, anti-hyperglycaemia and anti-hyperlipidaemia in both in vitro and in vivo models. Whether SN extract (SNE) has an anti-inflammatory effect in vivo remains unclear. This study examined the effect of SNE on renal function and renal organic transport in lipopolysaccharide (LPS)-induced renal inflammation in rats. Rats were randomised and divided into normal saline (NS), NS supplemented with 1000 mg/kg body weight (BW) of SNE (NS + SNE), intraperitoneally injected with 12 mg/kg BW of LPS and LPS treated with 1000 mg/kg BW of SNE (LPS + SNE). Biochemical parameters in serum and urine, lipid peroxidation concentration, kidney function and renal organic anion and cation transports were determined. LPS-injected rats developed renal injury and inflammation by increasing urine microalbumin, total malondialdehyde (MDA) and inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β protein expression, respectively. In addition, uptake of renal organic anion, [3H]-oestrone sulphate (ES), was reduced in LPS-injected rats together with increased expression of organic anion transporter 3 (Oat3). However, the renal injury and inflammation, as well as impaired Oat3 function and protein expression, were restored in LPS + SNE rats. Accordingly, SNE could be developed as nutraceutical product to prevent inflammation-induced nephrotoxicity.

Key words anti-inflammatory effect; lipopolysaccharide; renal inflammation; renal organic antion transport; Spirogyra neglecta

INTRODUCTION

Lipopolysaccharide (LPS), a product of Gram-negative bacteria, is a systemic endotoxin which activates through inflammatory responses. LPS administration has shown to associate with overproduction of nitric oxide, tissue injury and organ failure. Moreover, increasing LPS into blood circulation could induce a severe systemic inflammatory reaction implicated in pulmonary, hepatic and renal failures. LPS administration could also cause a reduction of glomerular filtration rate (GFR), as commonly found in septic shock patients. Previous study reported that sepsis is one of the major causes of renal inflammation, which occurs in acute and chronic kidney diseases. For instance, several pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, are released from proximal tubular epithelial cells in acute kidney injury (AKI) patients. In addition, systemic inflammation induced by cyclosporine results in releasing inflammatory cytokines and generating reactive oxygen species, which lead to impair renal function in rats. Sepsis is also a predisposing factor in AKI, as shown by a higher mortality rate compared to non-septic AKI patients.

Kidneys play an important role in the elimination of several xenobiotics, including drugs, toxins and endogenous compounds. The active secretion of anionic substances to the tubular lumen is limited to the basolateral membrane of proximal tubule via several transporters. Organic anion transporters 1 and 3 (Oat1 and 3) and organic cation transporter 2 (Oct2) have been shown to play a major role in the cellular uptake of organic anions and cations across the basolateral membrane of renal proximal tubules. Typical substrates for Oat and Oct include para-aminohippurate (PAH) and 3-methyl-4-phenylpyridinium (MPP+), respectively, while specific substrate for Oat3 is oestrone sulphate (ES). Several compounds have also shown to modulate substrate transport by these transporters. For instance, bradykinin and sn-1,2-dioctanoylglycerol decreased ES and PAH transports via hOAT3 and 1, respectively. In addition, the impairment of organic transporter function associated with inflammation and pathophysiological status. For example, LPS-induced acute renal inflammation reduces Oat1 and 3 expression and function in mice. Similarly, chronic renal failure also decreases mouse renal Oat1 expression and function. Exposure to TNF-α and IL-6 significantly down-regulates mRNA levels of Oat1 and 2 in hepatocytes. Moreover, gentamycin induces myeloperoxidase, an inflammation marker, leads to down-regulate Oat1 and 3 expression in rat kidney. Bilateral urethral obstruction and renal ischemic/reperfusion injury in adult male rats decrease mRNA and basolateral membrane protein expression of both Oat1 and 3. In addition, nitrosative stress induced by sodium nitroprusside (SNP) leads to reduce Oct transport function in isolated hepa.
toocytes. Nevertheless, there is limited information regarding anti-inflammation and renoprotective effects of the remedies on renal organic anion and cation transport that could prevent renal dysfunction.

_Spirogyra neglecta_ (SN) is a genus of filamentous green algae. SN has been widely grown in the Nan River, Northern Thailand. Previous study reported that SN has been found in hot, dry season and before the rainy season. Furthermore, there was no difference in polyphenol content in each season. SN aqueous extract (SNE) contains several phenolic compounds including isoquercetin, catechin, hydroquinone and kaempferol. In vivo studies indicated that this species has several beneficial effects, including anti-gastric ulcer, anti-hyperglycaemia and anti-hyperlipidaemia. However, the information concerning effect of SNE on LPS-induced renal inflammation and its mechanisms remain unclear. This study, therefore, aimed to determine the effect of SNE on renal function, renal organic anion and cation transporter function and its mechanisms involved in experimental LPS-induced inflammation in rats.

MATERIALS AND METHODS

**Chemicals** Lipopolysaccharide and CelLytic™ mammalian tissue lysis/extraction reagent were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Monoclonal TNF-α and polyclonal IL-1β were purchased from R&D systems (Minneapolis, MN, U.S.A.). Monoclonal mouse anti-β-actin was obtained from Abcam (Cambridge, MA, U.S.A.). Polyclonal antibody against Oat3 was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Tritiated para-aminohippurate ([3H]-PAH); specific activity (SA) = 1 Ci/mmol) and tritiated oestron sulphate ([3H]-ES; SA = 50 Ci/mmol) were obtained from PerkinElmer, Inc. Life Sciences (Boston, MA, U.S.A.), while tritiated 1-methyl-4-phenylpyridinium ([3H]-MPP+; SA = 80 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). All other chemicals with high purity were obtained from commercial sources.

**SNE Preparation** Fresh SN was collected from Phrae Province, Thailand. A voucher specimen (number AARL G047) has been deposited at the herbarium of the Applied Algal Research Laboratory Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. SN was rinsed and dried in an oven at 50°C. Dried SN was grinded to a fine powder. Then, the SN extract (SNE) was prepared by soaking 200 g of dry powder in 4 L of distilled water and boiled at 100°C for 1 h. The extract was filtered through a filter paper. Then, it was concentrated using a rotary evaporator and lyophilised by freeze dryer. SNE was stored at 4°C prior to subsequent experiments. The phenolic compounds of this SNE were identified by Duangjai et al. SNE was standardized by determination of total phenolic content to reach a minimum of 77 mg gallic acid equivalent before use in this study.

**Animals and LPS Induction in Rats** Male Wistar rats, each weighing between 170–220 g, were obtained from the National Animal Center, Mahidol University, Salaya, Thailand. The animal facility and protocols were approved by the Laboratory Animal and Use Committee at the Laboratory Animal Research Center, University of Phayao, Phayao, Thailand (Protocol no. 5701040013). All experimental rats were housed under a 12 h light/dark cycle. The 24 rats were randomly divided into two groups: normal saline-injected group, indicated as normal saline (NS), and 12 mg/kg body weight (BW) LPS-injected group, designated as LPS group. A previous study demonstrated that daily injection with LPS at 12 mg/kg BW for 7 d generates mesangial hypercellularity and expansion, and segmental glomerulosclerosis in C57BL/6 mice. In addition, injection with 10, 12 and 15 mg/kg BW of LPS in C57BL/6 mice showed myocardial fibrosis, interstitial edema, erythrocyte leakage and inflammatory cell infiltration in a dose-dependent manner with increasing of mortality rate. Moreover, rats were injected with LPS at 2 mg/kg for 7 d showed the increase in pro-inflammatory cytokines e.g., TNF-α and IL-1β mRNA and protein expression in hippocampus while TNF-α was elevated since the first day of injection and IL-1β was highest detectable at day 7. Thus, establishing LPS induced kidney injury in rats without any dead of the animals, injection of 12 mg/kg LPS was selected in this study. Seven days after daily injection, six rats from NS and LPS group were supplemented with SNE at 1000 mg/kg BW for the next 2 weeks and designated as NS + SNE and LPS + SNE, respectively. SNE was dissolved in sterile water and administered orally by gavage once daily for 14 d as previously report. The protocol of LPS-induced inflammation is summarised in Table 1.

**Renal Function Assessment** Twenty-four-hours urine volume was measured for calculating urine flow rate. Plasma and urine creatinine levels were measured using enzymatic kits obtained from Dialab (Wiener Neudorf, Austria). Creatinine clearance and estimated glomerular filtration rate (eGFR) were subsequently calculated. Urine microalbumin level was detected using an automatic biochemical analyser at the Clinical Laboratory, Maharaj Nakhon Chiang Mai Hospital, Chiang Mai, Thailand.

**Histological Examination** The kidneys were excised and a quarter of the kidney were fixed in 4% paraformaldehyde at 4°C for 12–24 h, washed with PBS, and embedded in a paraffin block. Each slide was cut at 5–7 µm-thick sections and subsequent stained by hematoxylin and eosin (H&E) for renal morphology assessment and periodic acid-Schiff base (PAS) for elucidation of lesions. The tissue morphological changes were determined using bright-field microscopic evaluation.

**Determination of Renal Lipid Peroxidation**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| 1. NS | Normal saline | Normal saline | LPS 12mg/kg BW, i.p. | LPS 12mg/kg BW, i.p. |
| 2. NS + SNE | Water | SNE 1000 mg/kg BW | Water | SNE 1000 mg/kg BW |
malondialdehyde (MDA) levels were analysed according to the manufacturer’s protocol (Cayman Chemical, MI, U.S.A.). In brief, kidney tissues were cut and suspended in CellLytic™ MT cell lysis containing protease inhibitors (Roche, Applied Science, IN, U.S.A.) according to the manufacturer’s protocol (Sigma-Aldrich). The tissues were then homogenized and centrifuged at 1600 × g for 10 min. The supernatant was subsequently collected for MDA measurement. Each sample was expressed as the total MDA level and normalized to the total protein concentration (nmol/mg protein) using the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.).

Transport Study in Renal Cortical Slices To determine effect of SNE on renal organic transport function, the uptake of [3H]-PAH, [3H]-ES and [3H]-MPP+ into renal cortical slices were determined as previously described.⁹⁹ Rat kidneys were removed and placed in oxygenated saline buffer, and renal cortical slices (≤0.5 mm; 5–15 mg wet weight) were cut with a Studie-Riggs microtome and maintained in ice-cold, oxygenated modified Cross and Taggart buffer containing 95 mM NaCl, 80 mM mannitol, 5 mM KCl, 0.74 mM CaCl₂, and 9.5 mM Na₂HPO₄ (pH 7.4). The renal slices were then incubated in modified Cross and Taggart buffer containing 10 µM [3H]-PAH (substrate for Oat1 and 3), 50 nM [3H]-ES (substrate for Oct2) or 1.25 nM [3H]-MPP+ (substrate for Oat3) or 1.25 nM [3H]-MPP+ (substrate for Oat3) or 1.25 nM [3H]-MPP+ (substrate for Oct2) for 30 min at room temperature.

To determine the renoprotective effect of SNE on nitrosative stress, the slices from each rat were pre-incubated in 0.5 mL of buffer in the absence or presence of 5 mM sodium nitroprusside (SNP) for 30 min, and subsequently incubated in buffer containing 10 µM [3H]-PAH, 50 nM [3H]-ES or 1.25 nM [3H]-MPP+ for another 30 min at room temperature. At the end of the experiment, the reaction was stopped by adding 0.1 M MgCl₂. Slices were then washed, blotted, weighed, dissolved in 1 N NaOH and neutralised with 1 N HCl. Scintillation fluid was added, and the radioactivity was subsequently measured using a liquid scintillation analyser (PerkinElmer, Inc., Life Sciences, Boston, MA, U.S.A.). The uptake of these substrates was calculated as tissue to medium (T/M) ratio, that is, (dpm/mg tissue)/(dpm/mL medium).

Subcellular Fractionation and Western Blot Analysis To measure protein expression, subcellular fractions of rat cortical tissues in each animal were prepared using differential centrifugation as previously described.³⁰ Briefly, renal cortical tissues were lysed using lysis buffer containing 1% complete protease inhibitor mixture and centrifuged at 5000 × g for 10 min at 4°C. The supernatant was designated as whole cell lysate. Half of the supernatant was re-centrifuged at 100000 × g for 2 h. The pellet was re-suspended by the same buffer and used as membrane fraction. Protein concentration in each sample was also determined using Bradford assay (Bio-Rad), and the samples were stored at -80°C prior to use. For Western blot analysis, samples were resolved in 4X Laemmli solution, electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, West Milwaukee, WI, U.S.A.). Non-specific binding was eliminated by blocking with 5% (w/v) non-fat dry milk in 0.05% Tween-20 in Tris-buffered saline (TBS-T) for 1 h and incubated overnight with specific primary antibody against TNF-α, IL-1β and Oat3. The PVDF membrane was washed with TBS-T buffer and incubated with horseradish peroxidase-conjugated ImmunoPure secondary goat anti-rabbit or anti-mouse immunoglobulin G (IgG) (Merck, Darmstadt, Germany) for 1 h. Proteins were detected using super signal west pico chemiluminescent substrate (GE Healthcare), and quantitatively analysed using the ImageJ program version 1.44p from Research Services Branch (RSB) of the National Institute of Mental Health (NIMH) (Bethesda, MD, U.S.A.).

Statistical Analysis Data were expressed as mean ± standard error of the mean (S.E.M.) Statistical differences were assessed using one-way ANOVA followed by the LSD post hoc test using SPSS version 23 (IBM Corp., NY, U.S.A.). Differences were considered to be significant when p < 0.05.

RESULTS

Effects of SNE on Plasma Parameters in LPS-Induced Inflammation in Rats As shown in Table 2, kidney index, serum creatinine and eGFR were not significantly different among experimental groups. However, LPS group had an increase in urine microalbumin compared to NS rats (p < 0.05), while SNE normalised this parameter in LPS-treated rats. This data implies that LPS may cause glomerular and/or tubular damage, leading to impaired glomerular and/or tubular function as evident by the presence of proteinuria.

SNE Did Not Alter Kidney Morphology To assess renal morphology, the kidneys were stained by H&E and the lesions were confirmed by periodic acid-Schiff base (PAS). As shown in Fig. 1, there were no differences in the glomerular and tubular structures among experimental groups. Consistently, PAS staining also showed no significant morphological change among groups (Fig. 2), suggesting that dose of LPS in this study had no effect on plasma parameters and kidney morphology. Although there was no significantly change on renal morphology, the urine microalbumin was increased in LPS-induced inflammation group, indicating mild degree of glomerular dysfunction in this study.

Effect of SNE on Renal Lipid Peroxidation Concentration To determine renal lipid peroxidation levels, the total malondialdehyde (MDA) level was determined in renal corti-

Table 2. The Effect of SNE on Plasma Parameters in LPS Induced Inflammation in Rat

| Parameters                              | NS             | NS + SNE          | LPS     | LPS + SNE        |
|-----------------------------------------|----------------|-------------------|---------|------------------|
| Kidney index                            | 6.34 ± 0.38    | 6.44 ± 0.33       | 6.45 ± 0.71 | 6.60 ± 0.15     |
| Serum creatinine (mg/dL)                | 1.45 ± 0.38    | 1.34 ± 0.59       | 1.65 ± 0.40 | 1.54 ± 0.75     |
| Estimate glomerular filtration rate (eGFR; mL/min) | 0.50 ± 0.34    | 0.46 ± 0.23       | 0.33 ± 0.13 | 0.49 ± 0.29     |
| Urine microalbumin                      | 0.50 ± 0.18    | 1.03 ± 0.57       | 1.69 ± 0.22* | 1.50 ± 0.79     |

NS, normal saline; NS + SNE, NS supplemented with SNE at 1000mg/kg BW; LPS, LPS induced renal inflammation; LPS + SNE, LPS supplemented with SNE at 1000mg/kg BW. Values shown are mean ± S.E.M. (n = 6), *p<0.05 represents the significant difference compared to normal control rats.
cal tissues using TBARS assay. As shown in Fig. 3, kidney tissues from LPS-induced inflammation group demonstrated a significant increase in lipid peroxidation, as indicated by a high level of MDA compared with that of NS rats. In contrast, total MDA was significantly decreased in the LPS+SNE group when compared with the LPS group. These data indicate that improvement of renal inflammation by SNE partially reduced lipid peroxidation formation.

SNE Reduces Pro-inflammatory Cytokine Production in LPS-Induced Inflammation in Rats To further investigate the renoprotective effects of SNE in LPS-induced inflammation, protein expression of pro-inflammatory cytokines, TNF-α and IL-1β, in renal cortical tissues were determined using Western blot analysis. As shown in Fig. 4A, TNF-α was increased in LPS rats when compared to the NS group ($p<0.05$), while it was markedly reduced in LPS+SNE rats.
Like TNF-α, renal IL-1β expression was elevated in LPS rats ($p < 0.05$), and SNE supplementation restored this protein to the similar degree as that of NS group ($p < 0.05$) (Fig. 4B). These findings indicate that SNE had an anti-inflammatory effect against renal inflammation.

**SNE Improves Renal Organic Anion Transport Function Mediated by Organic Anion Transporter 3**

To further investigate whether SNE improved renal tubular function mediated by Oat1, 3 and 2, the uptake of $[^3H]$-PAH (substrate for Oat1 and 3), $[^3H]$-ES (specific substrate for Oat3) and $[^3H]$-MPP⁺ (substrate for Oct2) were carried out. As shown in Fig. 5A, renal slices from LPS and LPS + SNE significantly decreased PAH transport mediated by both Oat1 and Oat3 compared to the NS group ($p < 0.05$). The uptake of ES mediated by rat Oat3 ($r$Oat3) was also significantly decreased in LPS-treated rats ($p < 0.05$). On the other hand, LPS + SNE group markedly improved this impairment ($p < 0.05$) (Fig. 5B). Nonetheless, the uptake of MPP⁺ was not statistically significant among experimental groups (Fig. 5C). These data suggest that SNE improved renal transport function mediated by renal Oat3.

**SNE Protects Oat3 Function in Sodium Nitroprusside-Induced Renal Nitric Oxide Production**

Previous study reported that nitric oxide (NO) is a key signalling molecule produced during inflammation in several pathogenesis, including Alzheimer’s disease, diabetes mellitus and rheumatoid arthritis. Thus, this study further investigated whether SNE has renoprotective effect against NO production induced by sodium nitroprusside (SNP) in rat renal cortical tissues. As shown in Figs. 6A and 6C, pre-incubation with SNP still inhibited PAH and MPP⁺ transport mediated by Oat1, Oat3 and Oat3.
and rOct2 in the renal slices from all experimental groups (p < 0.05). This data indicates that SNP-induced NO production led to impairment of renal tubular PAH and MPP+ transport, which could not prevent this impairment by SNE. Interestingly, the slices from NS+SNE and LPS+SNE counteracted the reduction of ES uptake mediated by Oat3 after SNP pre-incubation when compared to either NS or LPS alone (p < 0.05) (Fig. 6B). These suggest that SNE directly protects from nitrosative stress induced by SNP, mainly through Oat3.

SNE Alters Oat3 Expression in LPS-Induced Inflammation in Rats

Previous data in Figs. 5 and 6 suggest that SNE was able to protect renal tubular organic anion transport mainly mediated by Oat3 function. Thus, this study further clarified whether renoprotective effect of SNE involved Oat3 expression. As shown in Fig. 7, membrane Oat3 protein expression was significantly increased in LPS rats and tended to decrease in cytosolic fraction compared to the NS group (p < 0.05). In contrast, SNE was able to normalise membrane expression of Oat3 when compared to LPS alone (p < 0.05). This data suggests that SNE improved renal tubular organic anion transport partly by restoring Oat3 expression and function.

DISCUSSION

Renal inflammation associates with several pathological changes including glomerulonephritis and acute renal failure. In addition, the global burden of disease (GBD) estimated that 1.7 million people worldwide die from kidney failure each year. Thus, the anti-inflammatory effects of drugs and/or natural medicines have raised attention. LPS is an important pro-inflammatory factor that causes renal glomerular and tubular damage. Moreover, it has been widely used as a model for studying inflammation-related renal diseases.

Previous studies reported that the presence of segmental glomerulosclerosis in mice was demonstrated after daily injection of LPS for 7d without change in kidney morphology and GFR. However, microalbuminuria and pro-inflammatory cytokines, notably TNF-α and IL-1β, were increased after LPS-induced systemic inflammation.

In this study, we have shown that 12mg/kg BW LPS induced tubular dysfunction by interrupting renal Oat3 transport function and impairing albumin reabsorption without serum creatinine estimate glomerular filtration rate (eGFR) and kidney morphological changes. Thus, this study clearly showed that LPS at 12mg/kg BW was not only able to induce glomerular dysfunction, but also impaired renal tubular transport function. However, the limitation should be noted. This study used microalbuminuria and renal transporters dysfunction to define renal inflammation. The current study need to use other renal dysfunction marker such as cystatin C to define this condition. Cystatin C recently became a good marker for eGFR measurement. In chronic kidney disease subjects with normal or mild reduction in eGFR increased serum cystatin C without serum creatinine changes. Although plasma creatinine is a predictor for renal function, a previous study also reported that microalbuminuria is considered to be a predictor for clinical acute kidney injury. In addition, this study also shows that LPS-induced impaired renal tubular function and microalbuminuria which was consistent with a recent study suggested that LPS promotes mesangial expansion and glomerulosclerosis. Furthermore, tubular dysfunction induced by proinflammatory cytokines e.g., transforming growth factor (TGF)-β, TNF-α, and IL-8 also caused albuminuria by decreasing albumin reabsorption. This evidence indicated that the significant amounts of albumin fragments in the urine might not only due to glomerular damage, but also renal tubular impairment. Nonetheless, there were several evidences showing MDA appears to be a marker of inflammation. Previous study reported that urinary, glomerular and extracellular MDA levels significantly raised in primary focal segmental glomerulosclerosis. Similarly, plasma MDA levels also increased in chronic renal failure patients. This study demonstrated that
SNE against inflammatory condition by reducing MDA levels. Therefore the improvement of LPS induced kidney injury by SNE partially reduced lipid peroxidation formation.

It has been demonstrated that renal TNF-α gene expression was increased in LPS-induced AKI in rats.\textsuperscript{47} In addition, 1 μg/mL LPS elevated monocyte chemoattractant protein 1 (MCP-1), IL-1β and TNF-α mRNA expression in isolated erythrocyte-perfused rat kidney.\textsuperscript{48} The current study clearly showed that SNE exhibits anti-inflammatory effects in renal cortical tissues by decreasing renal TNF-α and IL-1β protein expression after LPS induction. Consistently, previous study found that the major constituent in SNE, isoquercetin, suppresses inducible nitric oxide synthase expression, thus leading to reduce NO production through \textsuperscript{p44/42} mitogen activated protein kinase (MAPK), \textsuperscript{p38} MAPK and \textsuperscript{c-Jun} N-terminal kinase phosphorylation.\textsuperscript{49} Like SNE and isoquercetin, green tea rich in polyphenols decreases serum TNF-α gene expression in LPS-induced lethality in male BALB/c mice,\textsuperscript{50} while catechin suppresses TNF-α and IL-6 production, as well as nuclear factor-kappaB (NF-κB) activation, in LPS-induced inflammation in BV-2 microglial cells.\textsuperscript{51} Moreover, curcumin reduces IL-1β, IL-6 and TNF-α production, as well as IL-1β gene expression, in hyperosmolality induced by 90 mM NaCl in human corneal epithelial cells.\textsuperscript{52}

The alterations in expression and function of Oats and Octs have been reported in several inflammation-associated renal diseases. For instance, the production of IL-6 in sepsis induced by caecal ligation in mice influences transcription of the genes encoding for hepatic sodium taurocholate cotransporter (Ntcp), multidrug resistant protein 2 (Mrp2) and organic anion transporter protein (Oatp).\textsuperscript{53} Similarly, hyperuricaemic rats reduce Oat1, 3 and 2 transport activities and expression levels.\textsuperscript{54} Moreover, injection of LPS induces inflammation and down-regulates renal mRNA expression of peptide transporter 2 (PEPT2), Oat1, 3 and Mrp4 in mice.\textsuperscript{55} Consistently, the present study showed that LPS reduces Oat1 and 3 functions without changing Oct2 function, whereas SNE was able to improve only organic anion-mediated Oat3 transport function, but not Oat1. Since PAH is a substrate for Oat1 and 3 while ES is specifically transported by Oat3 and Oat3 is the highest expressed on basolateral membrane of renal proximal tubule which is the major segment for tubular transport function.\textsuperscript{15,16} As shown in our results SNE dramatically reduced both renal TNF-α and IL-1β and restore only Oat3 expression and function back to basal level. These results suggesting that the slightly increment of PAH uptake was due to SNE improve only Oat3 function. The possibility of improving Oat3 function by SNE remains unknown. Nonetheless, it might be due to renal Oat1 and Oat3 being differentially regulated under inflammation. Similarly, ochratoxin A, a nephotoxic and carcinogenic substance, decreases Oat1, but not Oat3, expression in ochratoxin A-treated rats.\textsuperscript{55}

Besides, several studies postulated the mechanism and involvement of polyphenols on transporter expression and function. Additionally, curcumin increases protein expression of Oat1 and 3 in cisplatin-induced nephrotoxicity in rats by suppressing inflammatory factors, such as NF-κB and cyclooxygenase-2 (COX-2).\textsuperscript{56} Moreover, red wine polyphenols, like resveratrol, quercetin and myricetin, increase the uptake of MPP+ through human Oat1 and 3 in Caco2 cells, partly involved with anti-oxidative capacity.\textsuperscript{57} As mentioned earlier, there are several polyphenol contents in SNE including isoquercetin, catechin, hydroquinin, rutin, kaempferol, gallic acid, tannic acid and quercetin.\textsuperscript{50} Among these, isoquercetin, a major component found in SNE, decreases COX-2 expression in LPS-stimulated RAW264.7 macrophages.\textsuperscript{58} Similarly, catechin, gallic acid, rutin and quercetin also reduce inflammatory marker in this model.\textsuperscript{58–60} Moreover, kaempferol also inhibits hepatocyte apoptosis in d-GalN/LPS-induced acute liver failure mice\textsuperscript{61} while tannic acid reduces NF-κB expression in LPS-induced BV2 microglial cells.\textsuperscript{52} Furthermore, polyphenol extract also showed anti-inflammatory effect by reducing plasma LPS and IL-6 levels in human volunteers.\textsuperscript{63} Therefore, anti-inflammatory effect of SNE may partially protect renal inflammation through improving the Oat3 transport function via polyphenol activity.

Although LPS induces renal Oat3 function impairment, it dominantly up-regulates membrane Oat3 protein expression (Fig. 7). In this context, an increase in Oat3 expression in LPS treatment could be due to two different mechanisms. Either LPS-induced inflammation may interfere Oat3 protein trafficking or Oat3 has protective mechanism contributing to prevent the accumulation of toxic compounds in renal tissues by up-regulating its expression. Although this study did not directly measure nitrosative stress markers, several evidences also reported that SNP could induce nitrosative stress leading to impaired several transporters. For example, organic cation transport function was decrease in SNP induced nitrosative stress in isolated rat hepatocytes.\textsuperscript{64} In addition, rat brain injected with SNP decrease \textsuperscript{P-glyc} protein (P-gp) function.\textsuperscript{65} Consistently, this study shows that SNP pre-incubation reduced Oat3’s substrate transport, ES, mainly by nitrosative stress induced by SNP. In addition, the slices from NS + SNE and LPS + SNE counteracted the reduction of ES uptake mediated by Oat3 after SNP pre-incubation when compared to either NS or LPS alone (Fig. 6). Again, SNE not only reduces renal inflammation, but also is able to restore Oat3 expression back to basal level, thus leading to partial improvement of renal handling of tubular organic anions. Finally, the current study indicated that SNE reduced renal inflammation by ameliorating inflammatory cytokines. Taken together, SNE has several pharmacological effects which might be a promising therapeutic option for several diseases.

In conclusion, the present study demonstrates the renoprotective effect of SNE involved with the improvement of renal inflammation and organic anion handling, as mediated mainly by Oat3 function and expression. Therefore, SNE has potential as a food supplement to prevent and/or delay progression of acute renal inflammation-induced AKI.

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**Conflict of Interest** The authors declare no conflict of interest.

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