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Allicin attenuates calcium oxalate crystal deposition in the rat kidney by regulating gap junction function

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Abstract

Renal calculus is a global common urological disease that is closely related to crystal adhesion and renal tubular epithelial cell impairment. Gap junctions (GJs) and their components (connexins and Cxs) are involved in various pathophysiology processes, but their roles in renal calculi progression are not well defined. Our previous RNA microarray analysis suggests that GJs are one of the key predicted pathways involved in the renal calcium oxalate (CaOx) crystal rat model. In the current study, we found that the Cx43 and Cx32 expression and the GJ function decreased significantly after stimulation with CaOx or sodium oxalate (NaOx) in NRK-52E, MDCK, and HK-2 cells, and Cx43 expression also decreased in renal tissues in renal CaOx crystal model rats. Inhibition of Cx43 in NRK-52E cells by small interference RNA significantly increased the CD44 and androgen receptor expression, and the adhesion between CaOx crystals and cells, which were consistent with the function of GJ inhibitors. On the other hand, after GJ function and Cx43 expression were increased by allicin, diallyl disulfide, or diallyl trisulfide, the impairment of NRK-52E cells by NaOx or other GJ inhibitors and the adhesion between CaOx crystals and renal cells decreased significantly. Furthermore, allicin also increased Cx43 expression and inhibited crystal deposition in rat kidneys. Taken together, our results provide a basis that GJs and Cx43 may participate in renal CaOx stone progression and that allicin, together with its analogues, could be potential drugs for renal calculus precaution.

KEYWORDS
allicin, calcium oxalate, connexin43, gap junction, renal calculus

1 | INTRODUCTION

One of the prerequisites for calculus formation in urine is crystal accumulation into nuclei. Renal tubule epithelial cell injury is one of the conditions for the adhesion of crystals to renal tubules, which is a key early event in the renal calculi formation (Aggarwal, Narula, Kakkar, & Tandon, 2013; Duan et al., 2018). The damage to tight junctions is considered a close factor that is related to the calculus occurrence and development (Peerapen, Chaiyarit, & Thongboonkerd, 2018; Yu, 2015). However, in the process of renal calculi formation, the function of another cell junction, gap junctions (GJs), which is different from tight junctions, and its components (connexins, Cxs) are not well defined.

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GJs are cell-to-cell channels that consist of two connexons on adjacent cells, which permit the intercellular exchange of small molecules (<1 kDa), such as ions (Na^+, K^+, and Ca^{2+}), cyclic adenosine monophosphate, cyclic guanosine monophosphate, inositol 1,4, 5-triphosphate, glutamate, and so on (Donahue, Qu, & Genetos, 2017). The opening and closing of GJ channels can be directly regulated by the membrane potential, intracellular pH, concentration of Ca^{2+}, phosphorylation status, regulatory factors, and exogenous chemicals (Herve & Derangeon, 2013). Connexons are structures composed of six Cxs and various Cx subtypes are found to be expressed in kidney cells (Sala, Badalamenti, & Ponticelli, 2016). There is growing evidence that the expression or channel activity of GJs plays a key role in renal physiopathology and varies along with physiological and pathological situations within distinct renal compartments (Abed, Kavvadas, & Chadjichristos, 2015).

Cxs are involved in several important regulatory processes in the kidney, including the renin angiotensin system, tubuloglomerular feedback, and salt and water reabsorption (Hanner, Sorensen, Holstein-Rathlou, & Peti-Peterdi, 2010). Cx43 is one of the most common Cxs, which has been found in the proximal tubule, collecting duct, nephron vasculature endothelial cells, vascular smooth muscle cells, and podocytes within the kidney (Sala et al., 2016). In diabetic nephropathy conditions, Cx43-mediated cell communication was influenced by chronic exposure to glucose-evoked transforming growth factor-β 1 (TGF-β1; Hills et al., 2018). However, another study demonstrated that Cx43 could attenuate renal fibrosis in diabetes by enhancing the activation of the Nrf2/ARE pathway and reducing fibronectin, intercellular cell adhesion molecule-1, and TGF-β1 expression (Chen et al., 2017). Cx43 may be a novel target for glomerulonephritis and its crosstalk with purinergic signaling contributes to podocyte damage (Kavvadas et al., 2017). Oxalate and calcium oxalate (CaOx) crystals mediate oxidative stress injury and renal tubular epithelial cells apoptosis are important factors in CaOx stone formation. Through regulation of intracellular oxidative status Cx43 hemichannels promote the disassembly of cell junctions (Chi et al., 2016). Cx43 has also been found to be related to other various kidney diseases but the relationship between Cx43 and renal calculi formation is still under investigation.

Inhibition of primary renal tubule epithelium damage may prevent adhesion between the renal tubule epithelium and the crystals. Allicin is a valuable promising Chinese medicine monomer that has a protective effect against drug-induced nephrotoxicity, including acetaminophen (Ko et al., 2017), gentamicin (El-Kashef, El-Kenawi, Suddek, & Salem, 2015), and acrylamide (Zhang, Wang, Chen, Yan, & Yuan, 2013). However, whether allicin and its metabolites could produce promising effects for kidney calculus prevention are still unclear. Our previous Kyoto Encyclopedia of Genes and Genomes pathway analysis of the microRNA microarrays acquired from kidney tissue of renal crystal deposition model rats suggested that the GJs were one of the key predicted pathways (Lan et al., 2017). In this study, we investigated the role of GJs and Cxs (especially Cx43) on renal crystal deposition in vitro and in vivo and explored the potential compounds of GJ enhancers derived from allicin, diallyl disulfide (DADS), or diallyl trisulfide (DATS) for kidney calculus prevention and therapy.

2 | MATERIALS AND METHODS

2.1 | Materials

Dulbecco's modified Eagle's medium (DMEM) and DMEM Nutrient Mixture F-12 (DMEM/F-12) basic, fetal bovine serum (FBS), and trypsin were obtained from Gibco Life Technologies (Grand Island, NY). Dimethyl sulfoxide (DMSO), 18α-glycyrrhetinic acid (18α-GA), carbenoxolone (CBX), 2-aminoethoxydiphenyl-borate (2-APB), Hoechst 33258, anti-Cx32 antibodies, DADS, and lactate dehydrogenase (LDH) assay kits were obtained from Sigma-Aldrich (St. Louis, MO); Simvastatin and tangeretin were from Selleck Chemicals (Houston, TX); DATS was from the Institute for the Control of Pharmaceutical and Biological Products of Chinese (Guangdong, China). Anti-Cx43, anti-CD44, anti-p-P38, anti-androgen receptor (AR), and antitubulin antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-superoxide dismutase 1 (SOD1) was obtained from Abcam (Cambridge, MA) and anti-SLC26A6 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine2000, Lipofectamine® LTX with Plus™ Reagent and calcein-AM (acetoxymethyl ester) were acquired from Invitrogen (Carlsbad, CA). All other commonly used laboratory reagents are in domestic analytical pure grade.

2.2 | Cell lines and cell cultures

NRK-52E, MDCK, and HK-2 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). NRK-52E and MDCK cell lines were cultured in DMEM (HK-2 cell line in DMEM/F-12) supplemented with 10% FBS at 37℃ 5% CO_2 and saturated humidity. The culture medium for the cells was changed every 2 days.

2.3 | GJ functional assay (a "parachute" assay)

This assay was used to evaluate the gap junction intracellular communication (GJIC) function as described previously (Zhao et al., 2017). After cells reached 75-85% density in 12-well plates, approximately 1 μl of calcein-AM (5 μM) was put into donor cells for 30 min. Then the "donor cells" were washed with PBS (phosphate-buffered saline), trypsinized and diluted to 500 cells/ml solution. After the "donor cells" solution was added with the experimental drugs, they were inoculated onto the "recipient cells" and both cell types were cocultured for 4–6 hr at 37°C. "Donor cells" can be dyed and marked by calcein-AM, and the calcein-AM from "donor cells" could be intracellularly delivered into the receiver cells under a normal GJIC function. Through inverted fluorescence microscopy (IX51; Olympus, Tokyo, Japan), the average amount of "recipient cells" containing calcein-AM around a "donor cell" could be calculated and measured as the volume of GJ function.

2.4 | Cx43 small interference RNA (siRNA) interference experiments

After the NRK-52E cells reached 30-50% confluence, nonspecific siRNA (negative control [NC]) or the Cx43 siRNA (50 nM; Ribbon, Guangzhou, China) and Lipofectamine® LTX with Plus™ Reagent were combined and mixed. Next, the siRNA mixture was added to the cells according to the manufacturer's instructions. After 48–72 hr of siRNA treatment, the protein from cells was collected and Western blot analysis was used to verify interference effectiveness. The synthesized Cx43 siRNA sequences are as follows: siCx43_1, 5′-GAACCTACATCATCAGTAT-3′; siCx43_2, 5′-CAGTCTGCCTTTCGTTGTA-3′; and siCx43_3, 5′-GGCTA ATTACAGTGCAGAA-3′. Among them, the inhibitory effects of siCx43_2 and siCx43_3 were significant and were chosen and used in the following experiments.

2.5 | Cell viability detection

First, the cells were inoculated into the 96-well plates at a certain density (approximately 3,000–5,000 per well), and 24 hr later the solution with different drugs at various concentrations was added and incubated for 24 hr. After the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonphenyl)-2*H*-tetrazolium (MTS) was added to cells for a 2–3 hr reaction, the absorbance was detected by a microplate reader (BioTek Instruments, Winooski, VT) at a wavelength of 490 nm. The cell survival rate was determined and converted into the percentage ratio by subtracting the optical density of the corresponding blank group.

2.6 | LDH cytotoxicity test

An LDH assay kit was used to test the LDH activity released from the cytosol of damaged cells according to the manufacturer's protocol. The cells were grown at a concentration of 5,000 cells per well and incubated at 37°C overnight. After the fresh medium containing sodium oxalate (NaOx) or different drugs was changed and treated the cells for 12–24 hr, 50 μl of cell suspension with control, different drugs, or high control samples group were transferred into wells of a new 96-well plate; this set of experiments were performed in triplicate. Before 50 μl of stop solution was added into each well on the 96-well plate, the plate was then shaken for 10 s, and 100 μl reaction mixture (freshly prepared) was added to each well and incubated for up to 30 min at 15–25°C. An enzyme-linked immunosorbent assay (ELISA) reader was used to measure the absorbance of the samples at 490 nm.

2.7 | Crystal-adherence experiment

This assay was used to detect the crystal adhesion ability as described previously (Abd et al., 2018; Duan et al., 2018). Cells were inoculated into six-well plates, and after the cells were grown to 100% confluence, medium with different concentrations of allicin (DATS or DADS) was added; after 2–3 hr NaOx was added to the cells. In Cx43 siRNA interference experiments, the incubation time of siRNAs on NRK-52E was up to 48–72 hr, before NaOx was added, CBX (50 μM) was added to inhibit GJIC function in the corresponding group. After the medium was discarded, the suspension containing 20 μg/cm^2 ponceau S-labeled calcium oxalate monohydrate (COM) crystals (Duan et al., 2018), which were stained red before addition to cells, was added and the cells were incubated at 37°C for 5 min. The cells were then washed for five times with PBS and were placed under a microscope at a perspective of 10^4 to count the number of ponceau S-labeled COM crystals bound to the cell surface. The amount of crystals present at the cell surface represents the ability of the cells to undergo cell adhesion.

2.8 | Western blot analysis

Western blot analysis was carried out as described previously (Lai et al., 2017). After the tissues and cells were washed with PBS three times, they were lysed in radioimmunoprecipitation assay (RIPA) buffer. Before they were collected and lysed by ultrasonication, the cells were scraped and the tissues were cut into pieces. The lysates were centrifuged at 12,000 RCF at 4°C for 30 min, and the supernatants were retained. Before the protein samples were prepared, the protein concentrations were detected with a bicinchoninic acid assay (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of each sample (25–50 μg) were loaded and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. Before the monoclonal antibodies, including Cx32 (1:1,000), Cx43 (1:1,000), CD44 (1:1,000), AR (1:1,000), β-actin (1:5,000), and tubulin (1:2,000) were incubated on the membrane overnight at 4°C, 5% skimmed milk was applied to block the membranes for 1–2 hr. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1–2 hr. The band density data were quantified with respect to the loading control (e.g., tubulin or β-actin) with the ImageJ software (Rawak Software Inc., Germany).

2.9 | Immunofluorescence

After the NRK-52E cells were fixed with 4% paraformaldehyde and blocked with 2% bovine serum albumin (BSA), the cells were incubated with 0.1% Triton X-100 for 10 min to puncture the cell membrane and stain the cytoplasm. Then, the cells were incubated with primary antibody directed against Cx43 (1:200) or CD44 (1:200), at 4°C overnight before incubation with 1:400 DyLight 549-conjugated and DyLight 488-conjugated goat anti-rabbit/anti-mouse secondary antibody (Abbkine, Wuhan, Hubei, China) for 1 hr in the dark. Hoechst 33258 was applied for 10 min to stain nuclei of cells. Then, the cells were washed with PBS and imaged via an Olympus IX73 fluorescence microscope (Tokyo, Japan).

2.10 | Animal experiments

The effect of allicin (DATS) on the formation of CaOx stone and on Cx43 expression was verified in the CaOx urolithiasis model rats

induced by ethylene glycol combined with ammonium chloride (AC; Duan et al., 2018). In this study, male Sprague-Dawley (SD) rats (from Guangdong Medical Laboratory Animal Center, Foshan, Guangdong, China) aged 6–8 weeks were approved by the First Affiliated Hospital of Guangzhou Medical University ethics committee and randomly divided into four groups, six in each group, including the control group (group I), the model group (group II), the model + DATS 8 mg/kg group (group III), and the model + DATS 15 mg/kg group (group IV). In the control group (I), rats were given a normal regular diet and water. In the model group (II), rats were given a gavage of 2% AC (wt/vol) every 2 weeks for the first three days (day/time), and a free drinking of water containing 0.1% (vol/vol) ethylene glycol (EG) every day (28 days). In the DATS group (group III or group IV), the 0.1% EG and 2% AC were given the same as the model group, and simultaneously DATS (8 or 15 mg/kg) was intraperitoneally injected from Days 1 to 21. All the groups of rats were given drinking water ad libitum, and the treatment duration was 28 days. The 24-hr urine and the kidney tissues of rats were collected 28 days later. The tissue was embedded in paraffin and 5-μm slices were made. Hematoxylin-eosin (HE) staining and immunohistochemistry were tested as previously described (Lai et al., 2018). For HE staining results, a polarizing microscope was used to observe the numbers of rat renal tubular cavity CaOx crystals. The kidney calculi crystallization quantity and area were analyzed using Image-Pro Plus 6.0 imaging software (Media Cybernetics, Silver Springs, MD). For immunohistochemistry, after incubations with primary antibodies for Cx43, the sections were incubated with biotinylated secondary antibodies (Maixin Biotech, Fuzhou, China). Subsequently, the slices were stained according to standard immunohistochemistry protocol (Duan et al., 2018), and the stained slide was covered and observed by a PathScope 4S scanner (DigiPath Corp, Wichita, KS).

2.11 | Statistical analysis

All experiments had a minimum of three replicates. The histogram data were displayed with the mean ± *SE* and were analyzed with SPSS 16.0 software (SPSS Inc., IBM, Armonk, NY). Statistical significance ($p < 0.05$) was confirmed by one-way ANOVA (>2 groups) or Student's *t* test (two groups). The histograms and scatter plots were made using GraphPad Prism 6.0 software (La Jolla, CA). In the figures, $^{*}p < 0.05$ in comparison with the corresponding group.

3 | RESULTS

3.1 | Cx43 was suppressed in the CaOx nephrolithiasis model rats and COM- or NaOx-induced renal cells

Similar to the research used for studying the profile of microRNA (miRNA) and messenger RNA (mRNA) in CaOx the nephrolithiasis rat model, we first investigated the expression of SOD1 and Cx43 in kidney tissue in the control group and EG + AC-induced group (model group), each group with six rats marked numbered 1 to 6, respectively. SOD1 is correlated with the removal of the reactive oxygen species, which may participate in the formation of kidney stones. The results showed that both Cx43 and SOD1 expression levels in the model group decreased significantly compared with the expression levels in control group (Figure 1a). To further confirm these variations, the NRK-52E, HK-2, and MDCK cells were incubated with COM (40, 60, and 80 μg/cm^2) or NaOx (0.5, 1, and 2 mM), and Cx43 and Cx32 expression levels were subsequently detected and found to decrease significantly (Figure 1b,c,e). In addition, our "parachute" assay confirmed that NaOx (0.5, 1, and 2 mM) could significantly inhibit GJ function in NRK-52E cells (Figure 1d). Instead of Cx32, Cx43 is a more common type of Cxs, so we focus on its function in nephrolithiasis in the following study.

3.2 | SiRNA knockdown of Cx43 in NRK-52E cells increased the factors related to nephrolithiasis

According to our results, Cx43 expression in NRK-52E cells is significantly higher than that of HK-2 cells. Thus, we chose NRK-52E cells to further explore the role of Cx43 in nephrolithiasis via negative regulation. In this study, we used nonspecific siRNAs as a NC and three Cx43 siRNAs (S1, S2, and S3) to knockdown Cx43 expression, of which S2 and S3 were the most efficient (Figure 2a). Although the expression level of the Cx43 protein was reduced to 50%, which is still a significant expression, it was similar to the variation of Cx43 in the CaOx nephrolithiasis rat model or the COM- and NaOx-induced renal cells. CD44 and AR expression levels are positively correlated to nephrolithiasis formation (Liang et al., 2014; Mulay et al., 2017). Using Western blot analysis, we found that the knockdown of Cx43 expression in NRK-52E cells significantly increased CD44, p-P38, and AR expression compared with that in the NC group (Figure 2b). The variation of the crystal adhesion cytokine CD44 expression and distribution was further confirmed by immunofluorescence (Figure 2c). In addition, the adhesion between COM and the cells damaged by NaOx was tested by crystal-adherence experiment and found to increase compared with that in the NC group (Figure 2e). In addition, consistent with the results of Cx43 knockdown, a GJ inhibitor, CBX (50 μM) also increased the adhesion between COM and the cells compared with the NC group and synergized the effect of Cx43 knockdown on crystal adhesion (Figure 2e). Cx43 siRNA and GJ inhibitors, such as CBX and 2-APB (50 μM; Zhao et al., 2017), also increased the LDH released (LDH cytotoxicity) from the cytosol of NRK-52E and HK-2 cells damaged by NaOx compared with the corresponding group (Figure 2d,f).

3.3 | DATS increased GJ function and Cx43 expression in NRK-52E cells and decreased the factors related to nephrolithiasis

To further investigate whether regulation of GJs or Cx43 changed the factors of nephrolithiasis, we screened allicin (DATS or DADS) for the following study. The promotion effect of DATS and DADS on GJ function was confirmed by a "parachute" dye-coupling test (Figure 3a). The high expression of SLC26A6 may contribute to

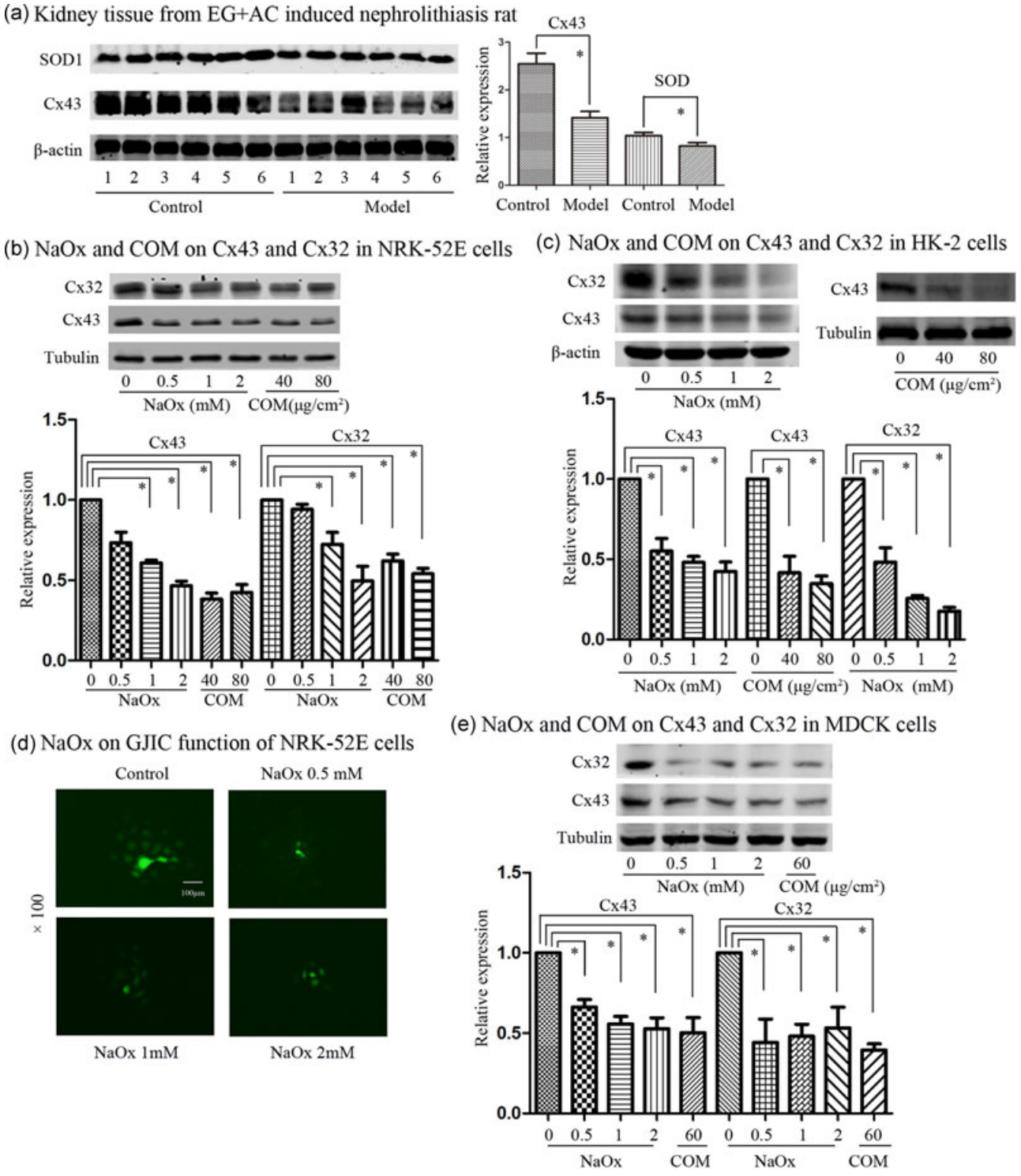

FIGURE 1 Cx43 was suppressed in the CaOx nephrolithiasis model rats and CaOx- or NaOx-induced renal cells. Six different samples marked numbers 1–6 in control group or model group were obtained and detected. Compared with the control group, Cx43 and SOD1 expression in the CaOx nephrolithiasis model group decreased significantly (a). NRK-52E, HK-2, and MDCK cells were incubated with CaOx or NaOx, Cx43, and Cx32 expression were subsequently detected and found to decrease significantly (b, c, e). Our GJ function assay confirmed that NaOx (0.5–2 mM) could significantly inhibit the GJ function of NRK-52E cells (d). The results are presented as the mean ± *SEM*. *$p < 0.05$ in comparison with the corresponding group; $n = 3–6$; $n = 1$ represents an independent cell culture or a different rat. Original magnification: ×100. CaOx: calcium oxalate; COM: calcium oxalate monohydrate; GJ: gap junction; NaOx: sodium oxalate; SOD1: superoxide dismutase 1 [Color figure can be viewed at wileyonlinelibrary.com]

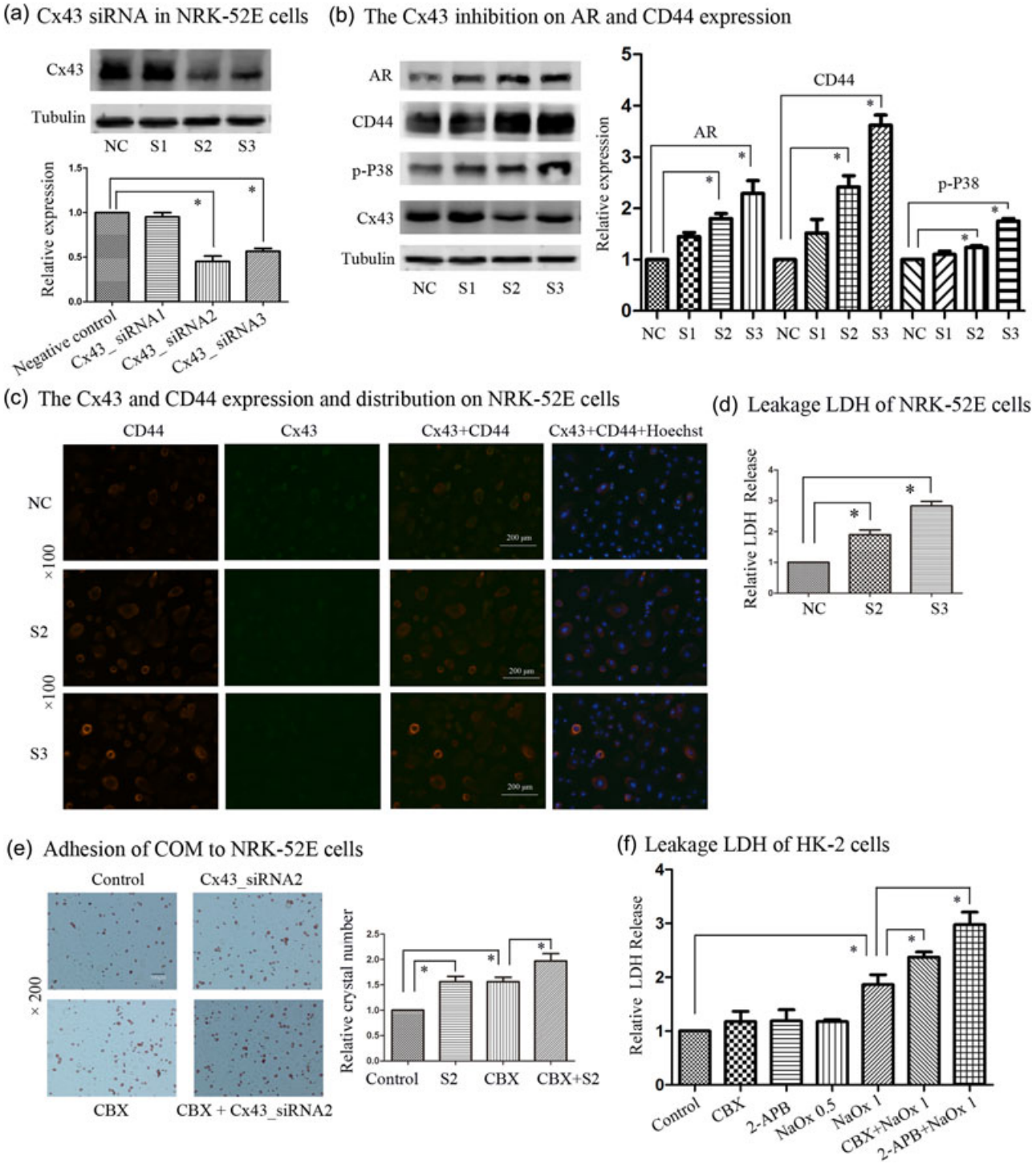

FIGURE 2 SiRNA knockdown of Cx43 in NRK-52E cells increased nephrolithiasis-associated factors. The higher Cx43 expression cells, NRK-52E, were chosen to further explore the role of Cx43 in nephrolithiasis via negative regulation. Nonspecific siRNAs were used as a NC, and three Cx43 siRNAs (S1, S2, and S3) were used to knockdown Cx43 expression, of which S2 and S3 were found to be the most efficient (a). After the knockdown of Cx43 expression in NRK-52E cells, not only CD44, p-P38 and AR expression (by Western blot analysis and immunofluorescence; b, c) but also the number of COMs bound to the cells pretreated with NaOx (by crystal-adherence experiment; e) increased significantly compared with the NC group. A GJ inhibitor (CBX) increased the adhesion between COM and the cells compared with the NC group and synergized the effect of Cx43_siRNA on the number of COM adhesions (e). Cx43 siRNA and GJ inhibitors (CBX and 2-APB) also increased the LDH activity released from the cytosol of cells damaged by NaOx (d, f). The results are presented as the mean ± *SEM*. $^{*}p < 0.05$ in comparison with the corresponding group; $n = 3$; $n = 1$ represents an independent cell culture. Original magnification: ×200. 2-APB: 2-aminoethoxydiphenyl-borate; AR: androgen receptor; COM: calcium oxalate monohydrate; GJ: gap junction; LDH: lactate dehydrogenase; NaOx: sodium oxalate; NC: negative control; siRNAs: small interference RNAs [Color figure can be viewed at wileyonlinelibrary.com]

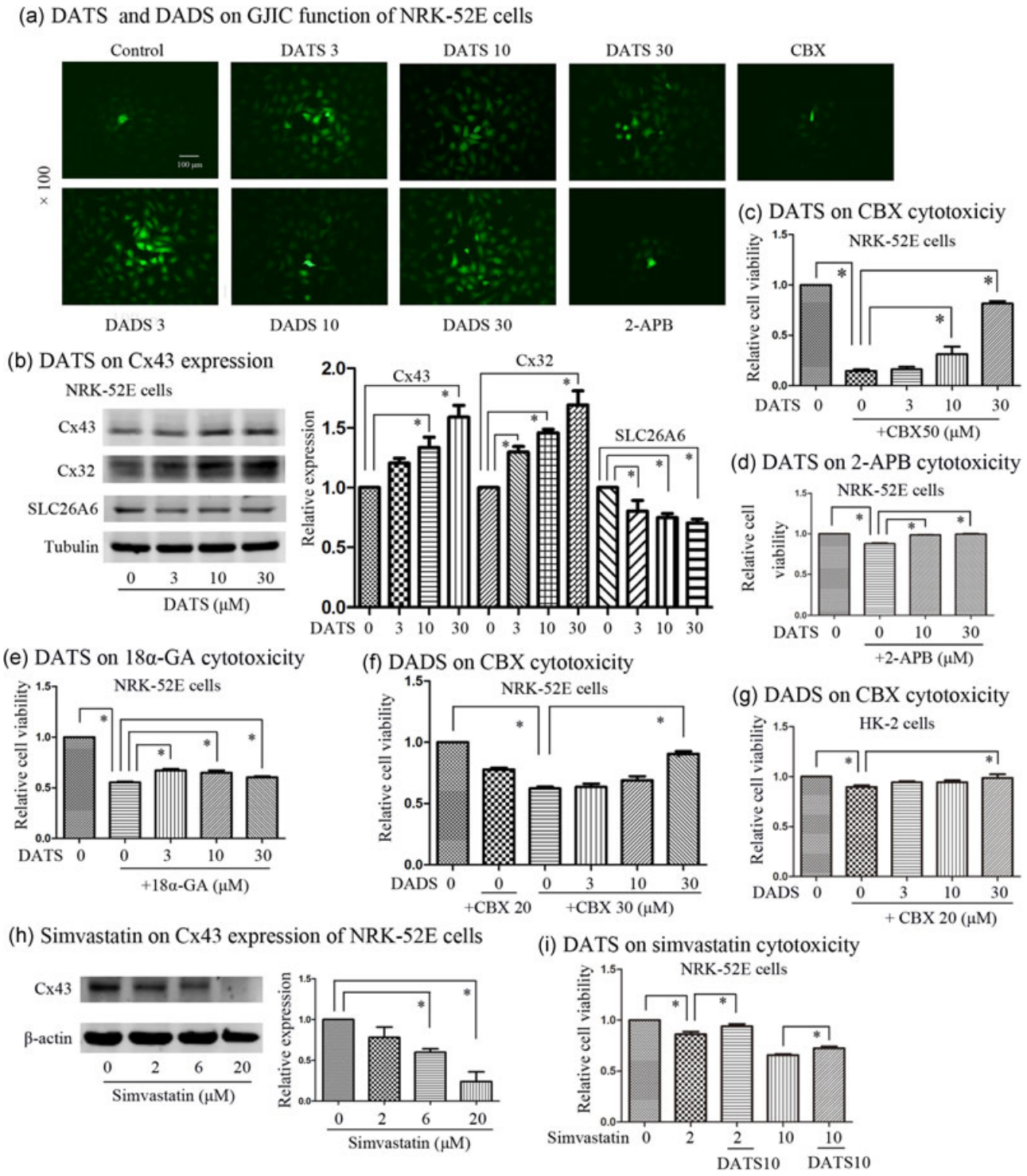

FIGURE 3 DATS increased GJ function and Cx43 expression in NRK-52E cells and decreased the factors associated with nephrolithiasis. The effect of DATS and DADS on GJ function was confirmed by a "parachute" dye-coupling test (a). DATS also increased Cx43 and inhibited the SLC26A6 expression in NRK-52E cells (b). DATS and DADS could also inhibit the cytotoxicity (by cell viability detection) caused by GJ inhibitors, such as 18α-GA (e), 2-APB (d), and CBX (c, f, g). DATS (3, 10, and 30 μM) also inhibited the cytotoxicity on NRK-52E cells caused by simvastatin (i), which significantly decreased Cx43 expression in NRK-52E cells (h). $^*p < 0.05$ in comparison with the corresponding group; $n = 3$; $n = 1$ represents an independent cell culture. The results are presented as the mean ± *SEM*. Original magnification: ×100. DADS: diallyl disulfide; DATS: diallyl trisulfide; 18α-GA: 18α-glycyrrhetinic acid; GJ: gap junction [Color figure can be viewed at wileyonlinelibrary.com]

renal calcification and account for kidney stone formation (Jiang et al., 2018). We found that DATS increased Cx43 and Cx32 expression but inhibited the SLC26A6 expression on NRK-52E cells (Figure 3b). The cell cytotoxicity was measured by the cell viability detection (MTS assay). We found that DATS (3, 10, and 30 μM) and DADS (3, 10, and 30 μM) could also inhibit the cytotoxicity caused by GJ inhibitors, such as 18α-GA (50 μM; Figure 3e), 2-APB (50 μM; Figure 3d) , and CBX (Figure 3c,f,g). DATS (3, 10, and 30 μM) also inhibited the cytotoxicity of NRK-52E cells caused by another inhibitor, simvastatin (Figure 3i), which significantly decreased Cx43 expression in NRK-52E cells (Figure 3h).

3.4 | DATS significantly inhibited the adhesion between COM crystals and renal cells impaired by NaOx

On the other hand, while GJ function and Cx43 expression were increased by DATS or DADS, the adhesion between COM crystals and renal cells decreased significantly after incubation with NaOx (Figure 4a-c). The cell viability of MTS assay results showed that DATS (3, 10, and 30 μM) could also inhibit the cytotoxicity caused by NaOx (0.5, 1, and 2 mM), which formed CaOx crystals when NaOx was added to the cell medium (Figure 4d). As tangeretin (Tan) could also enhance GJIC (Chaumontet et al., 1997), it was also used as a positive control for the function of DATS (Figure 4d).

3.5 | DATS significantly decreased renal crystal deposition in rats and increased Cx43 expression in kidney tissue

After the CaOx stone model of rats was induced by EG combined AC, the effect of DATS on the formation of CaOx nephrolithiasis and on Cx43 expression was detected. After HE staining, the renal crystal deposition in rats were measured by crystallization quantity and area (Figure 5a). The 24-hr urine oxalate and citrate were detected by ion chromatography. As some of the samples were undetectable, each group was up to at least three samples ($n = 3–6$). The results indicated that DATS significantly decreased renal crystal deposition in rats (Figure 5a), the 24-hr urine oxalate levels in rats (Figure 5c), increased Cx43 expression in kidney tissue (Figure 5b), and the 24-hr urine citrate levels in rats (Figure 5c).

Taken together, our results provide a basis that GJs, Cx43, and Cx32 may participate in the development of kidney stones, and DATS or DADS could be potential drugs for renal calculus precaution.

4 | DISCUSSION

CaOx calculus comprises the majority of nephrolithiasis, which may induce inflammation and subsequent renal failure (Mulay et al., 2013). Although the underlying mechanisms for lithogenesis remain elusive, a series of hypothesis, including physicochemical, Randall's plaque, oxidative stress, and renal tubular epithelial injury, were proposed, and the process of urine supersaturation and crystal precipitation, adhesion, aggregation, and growth are proven (Aggarwal et al., 2013). Our research evidence supports a primary role for GJ function, Cx43, and allicin in crystal adhesion in vitro and crystal deposition in vivo. In our study, we found that GJ function and Cx43 expression in the kidneys of nephrolithiasis rats and in HK-2 and NRK-52E cells stimulated by NaOx and CaOx were significantly lower in comparison with those in the control group. By manipulating GJ function or Cx43 with inhibitors, siRNA or enhancers, our results provide a basis that GJ and Cx43 may participate in the development of kidney stones; and targeting GJs or Cx43 may be a promising strategy for nephrolithiasis because of frequent Cx43 loss, which increases CD44 and AR expression (Liang et al., 2014; Mulay et al., 2017). However, the specific mechanisms of Cx43 and GJ on nephrolithiasis require further exploration.

Cxs not only act through a GJ-dependent pathway but also function by channel-independent roles or hemichannel pathways (Carette, Gilleron, Chevallier, Segretain, & Pointis, 2014). In our study, we confirmed that the GJ-dependent pathway was related to the process of nephrolithiasis, but whether GJ-independent or hemichannel pathways are involved in this process requires further confirmation. For HK-2 cells, oxalate-induced activation of the IL-2Rβ and p38 MAPK pathways may lead to a plethora of cellular changes, including the induction of inflammation (Koul et al., 2014). Various crystallopathies, such as gout, pseudogout, atherosclerosis, silicosis, and asbestosis, are accompanied by inflammation and tissue remodeling (Mulay et al., 2013). Recent studies have revealed that aberrant GJs and hemichannels participate in the context of inflammation, including atherosclerosis (Okamoto & Suzuki, 2017), and chronic kidney disease (CKD; Sidaway, 2014). In CKD, Cx43 was upregulated and may represent a new therapeutic target against the progression of CKD (Sidaway, 2014). This indicates that GJs and Cxs may vary and participate in different stages of nephrolithiasis progression. However, whether the variation of GJ and Cx43 in crystal-induced cells or rats gives rise to the pathogenesis of nephrolithiasis or acts as an accompanied consequence is still under investigation. Although Cx43 is one of the most common types among Cxs, Cx32 was also found to be decreased in the NaOx- and CaOx-induced HK-2 and MDCK cells, and the role of Cx32 in nephrolithiasis is also worth further evaluation.

Few drugs were used for renal calculus precaution and treatment because the hysteretic calculus component analysis could usually be finished only after minimally invasive surgery and the multiple influencing factors of nephrolithiasis need to be considered. Diet is important in the maintenance of health and prevention of nephrolithiasis. Garlic has been shown to exert substantial medicinal effects and is considered to be one of the best disease preventative foods (Bradley, Organ, & Lefer, 2016). As naturally Chinese medicine monomers, allicins are found in the bulbs of garlic from *Liliaceaeis*. The potential protection activity of allicin against kidney damage was summarized previously (El-Kashef et al., 2015; Garica-Trejo et al., 2016; Ko et al., 2017), but its role in CaOx nephrolithiasis remains

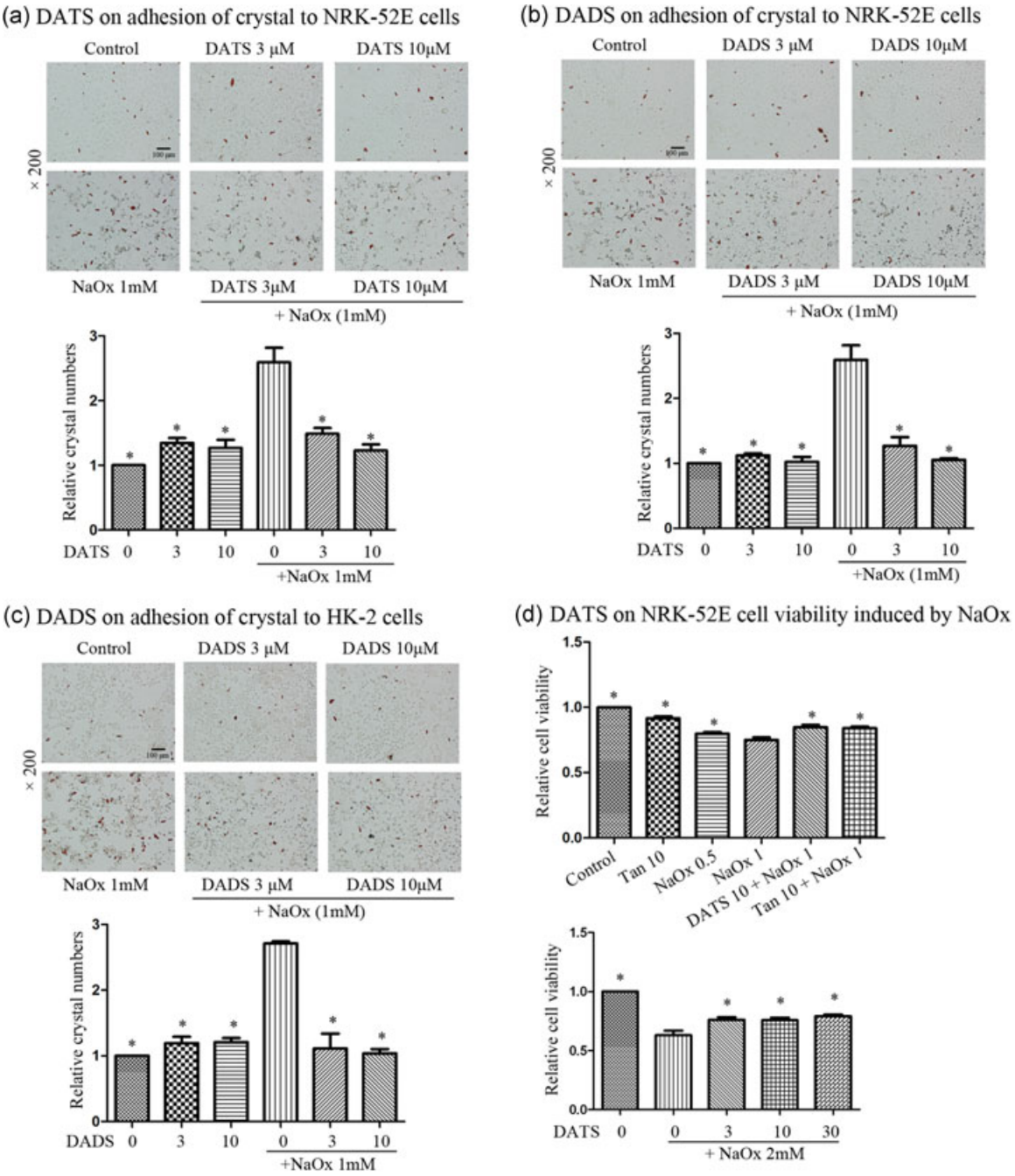

FIGURE 4 DATS significantly inhibited the adhesion between COM and renal cells impaired by NaOx. By crystal-adherence experiment, the effect of DATS or DADS on the adhesion between CaOx crystals and renal cells incubated with NaOx was detected and found to decrease significantly (a, b, c). DATS (3, 10, and 30 μM) also inhibited the cytotoxicity caused by NaOx (0.5, 1, and 2 mM), which formed CaOx crystals when NaOx was added to the cell medium, and Tan was used as a positive control for the GJIC enhancer (d). *$p < 0.05$ in comparison with the unlabeled group; $n = 3–6$; $n = 1$ represents an independent cell culture. The results are presented as the mean ± *SEM*. Original magnification: ×200. CaOx: calcium oxalate; COM: calcium oxalate monohydrate; DADS: diallyl disulfide; DATS: diallyl trisulfide; GJIC: gap junction intracellular communication; NaOx: sodium oxalate; Tan: tangeretin [Color figure can be viewed at wileyonlinelibrary.com]

unclear. This study of allicin may support the targeting of GJs or Cx43 to overcome CaOx nephrolithiasis, and new options are anticipated. However, although we have studied the effectiveness of DATS and DADS on nephrolithiasis, whether their main metabolites, allyl methyl sulfoxide and allyl methyl sulfone, have similar functions and worthy of further exploration. In addition to modulation of GJs or Cx43, allicin may also play roles in nephrolithiasis by its other pharmacological effects, such as anti-inflammation and antioxidation (Garcia et al., 2017; Han et al., 2017; Panyod et al., 2016), which are also key factors associated with the renal stone formation (Khan, 2013). Furthermore, the methods of network pharmacology prediction of the oxalate crystal or other

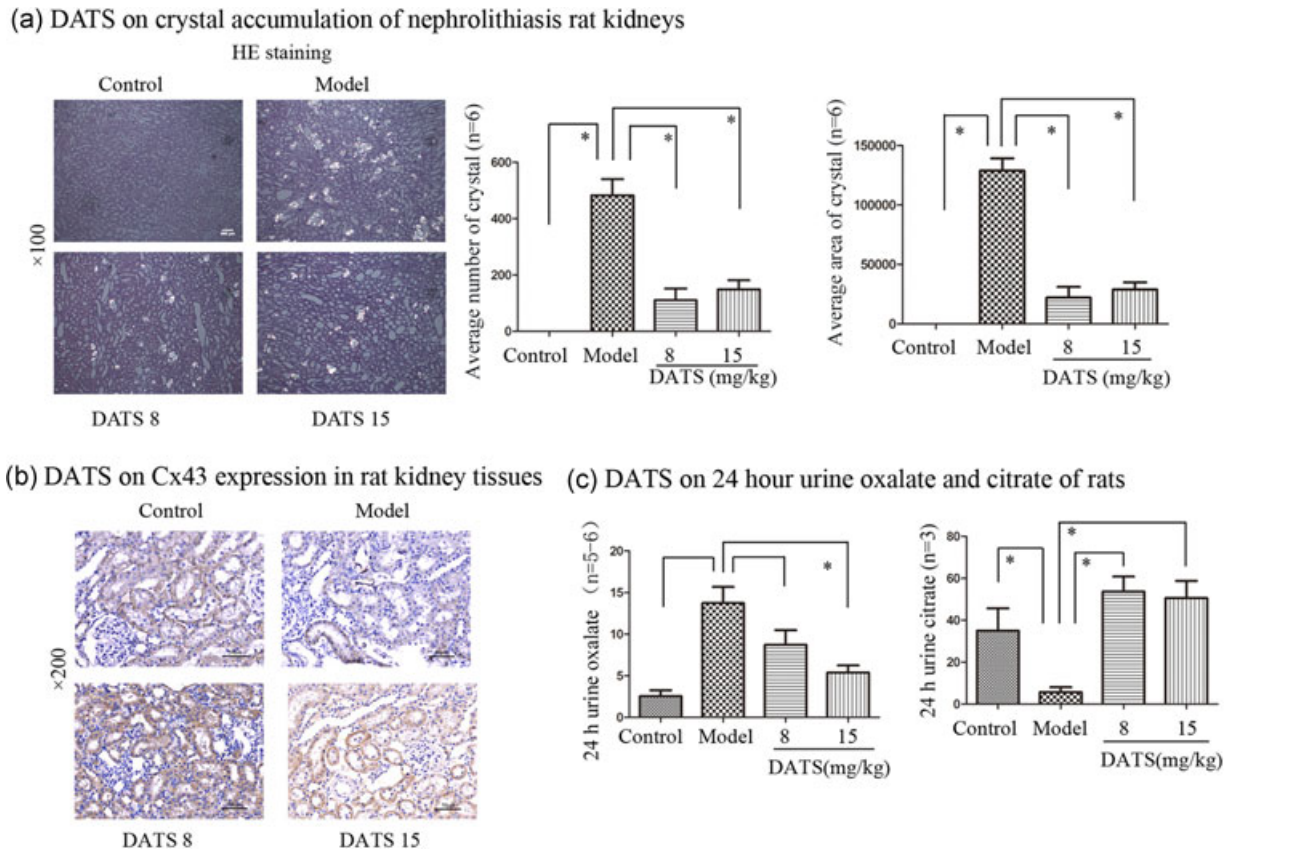

FIGURE 5 DATS significantly decreased renal CaOx crystal deposition in rat kidneys and increased Cx43 expression in kidney tissue. The effect of DATS on the formation of CaOx nephrolithiasis and on Cx43 expression levels was detected in the EG + AC-induced CaOx stone model of rats. DATS significantly decreased renal crystal deposition (by HE staining; a) and 24-hr urine oxalate (by ion chromatography) levels in rats (c). DATS also increased 24-hr urine citrate (by ion chromatography) levels in rats (c), Cx43 expression (by immunohistochemistry) in kidney tissue (b). $^*p < 0.05$ in comparison with the corresponding group; $n = 3–6$; $n = 1$ represents a different rat. The results are presented as the mean ± *SEM*. Original magnification: ×100 (a) and ×200 (b). AC: ammonium chloride; CaOx: calcium oxalate; DATS: diallyl trisulfide; EG: ethylene glycol; HE: hematoxylin-eosin [Color figure can be viewed at wileyonlinelibrary.com]

drugs (e.g., CBX or simvastatin) induced kidney injuries for more Chinese medicinal herbs (Hou et al., 2018), such as allicin, could be used in our future works.

In this study, we used an EG + AC-induced rat model in animal experiments. EG, a precursor of oxalate, could be oxidized, metabolized and excreted from the kidney, resulting in hyperoxalic acid urine, which can cause renal tubule damage and simulate nephrolithiasis, while AC can acidize urine and promote CaOx crystal formation in kidney. However, there are still many limitations to this nephrolithiasis model, as only crystal deposition but not stone was found in the kidneys of rats. More effective animal models, such as EG + vitamin D (Trojan et al., 2017), which could imitate the pathogenesis and consequence of kidney stone, need further exploration, and the knockout of Cx43 alone or its combination with EG + AC for the rat nephrolithiasis model will also be anticipated. More important, more evidence of the nephrolithiasis model and Cxs, especially from clinical research or epidemiological surveys, should be obtained. In addition, DATS was intraperitoneally injected in this study, which might be different from the method of gavage either in dose or in absorption, so the effectiveness of DATS by gavage administration requires our further confirmation in future works, as it is a more convenient method in our daily diet practice.

Taken together, our results provide a basis that GJs and Cx43 may participate in the development of renal CaOx stones. In addition, DATS and DADS and their ramifications or metabolites may be potential drugs for renal calculus and renal impairment precaution by modulating GJs, Cxs, inflammation, or oxidative stress, which may provide a preventive strategy to tackle nephrolithiasis relapse by the addition of garlic in our daily diet.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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