Treatment of Membranous Nephropathy by Disulfiram through Inhibition of Podocyte Pyroptosis

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Keywords
Membranous nephropathy · Disulfiram · Pyroptosis · Podocyte

Abstract

Introduction: Membranous nephropathy (MN) is a common chronic kidney disease in adults and a major challenge of clinical practice for its treatment. Despite major advances, since the discovery of the phospholipase A2 receptor as the major autoantigen of podocytes in MN, the mechanisms leading to glomerular damage remain elusive. Pyroptosis, a newly discovered type of programed necrotic cell death mainly mediated by gasdermin, was found to be responsible for podocyte injury in MN in our recent work. Objectives: The aim of this study was to explore the therapeutic effect of an FDA-approved drug, disulfiram (DSF), in the treatment of MN by inhibiting pyroptosis. Methods and Results: DSF significantly alleviated C3a/C5a-induced podocyte injury in vitro and renal lesions in passive Heymann nephritis (PHN) rats, as reflected by the decreased percentage of propidium iodide staining podocytes, decreased lactate dehydrogenase release from cultured podocytes and improvement in 24-h urine protein, serum albumin, serum creatinine, abnormal alterations of podocyte injury markers Desmin and WT-1 and podocyte foot process fusion in PHN rats. The protective effect of DSF on podocyte injury in vitro and in vivo can be ascribed to its inhibition of the activation and membrane translocation of the pyroptosis executor gasdermin D (GS-DMD) in podocytes. DSF also inhibited the increase and activation of the pyroptosis signaling pathway NLRP3-ASC-Caspase-1/IL-18/GSDMD in C3a/C5a-treated podocytes and renal tissue of PHN rats. Conclusion: DSF is a potential drug for MN treatment, and its clinical application needs to be further investigated.

Introduction

Membranous nephropathy (MN) is one of the most common causes of chronic kidney disease in adults and one of the leading causes of end-stage renal disease [1]. In recent years, the morbidity of MN has increased rapidly in China as well as in other developing countries, imposing a great burden on the medical system and social economy [2–4]. MN is characterized by an accumulation of immune deposits on the subepithelial aspect of the glo-
merular capillary wall by the podocyte. Antibodies that target podocyte or planted antigens accumulate as immune deposits, resulting in activation of the complement system, thus generating the complement-active fragments and finally leading to injury of the podocyte and filtration barrier. Although planted antigens such as cationic BSA or autoantigens of podocytes, including phospholipase A2 receptor, thrombospondin type-1 domain-containing 7A, exostosin 1/2 complex, neural epidermal growth factor-like 1, semaphorin 3 B and protocadherin 7, have been found and the underlying mechanisms of complement-induced podocyte injury in MN, including oxidative stress, endoplasmic reticulum stress, apoptosis, alterations of the cytoskeleton and slit diaphragm, have been illustrated [5–7], the panorama of the process of podocyte damage is far from complete. To date, treatment of MN has focused on nonspecific immunosuppressive therapy, similar to most other autoimmune diseases, and a major dilemma of current treatment is efficacy versus toxicity, especially prevention of MN progression. Therefore, understanding the mechanisms of podocyte injury in MN and exploring more effective targeted drugs may pave the way for better therapy for MN patients.

Pyroptosis is gasdermin-mediated programmed necrotic cell death. It features cell swelling and eventual lysis caused by gasdermin pore formation in the plasma membrane. The oligomerization of the gasdermin-N domain of gasdermin D (GSDMD) after cleavage of GSDMD by canonical inflammasomes activation is a pivotal pathway that drives pyroptosis [8]. Canonical inflammasomes are multimeric complexes that consist of a nucleotide-binding oligomerization domain-like receptor (NLR) or absent in melanoma 2-like receptor pattern recognition receptor, apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1 [9]. Once activated, canonical inflammasomes activate Caspase-1, leading to cleavage of GSDMD and maturation of IL-1β and IL-18, conferring a highly pro-inflammatory phenotype that is different from that of other types of necrosis [10]. Recently, our group first depicted the involvement of pyroptosis, driven by the activation of the canonical inflammasome NLRP3, in the pathogenesis of podocyte injury in MN [11]. Understanding the molecular mechanisms of pyroptosis-mediated podocyte injury in MN not only reveals the pathogenesis of this disease but may also help to develop novel treatments.

The FDA-approved drug tetraethylthiuram disulfide (disulfiram, DSF) is a potent inhibitor of aldehyde dehydrogenase that is responsible for the conversion of alcohol into acetaldehyde and has long been used for alcohol addiction in clinical practice since the 1950s [12]. Recently, DSF was found to inhibit GSDMD pore formation by modifying a conserved cysteine in GSDMD and therefore has an anti-pyroptotic effect [13]. We are interested in this finding and wonder whether the pyroptosis inhibitor DSF has a therapeutic effect on MN by alleviating podocyte injury. In this study, we evaluated the effect and mechanisms of the pyroptosis inhibitor DSF on podocyte injury in MN in vitro and in vivo by using a complement-induced podocyte damage model and the passive Heymann nephritis (PHN) rat model, respectively, to illustrate the therapeutic effect of DSF on MN.

Materials and Methods

Cell Culture and Treatments

A conditionally immortalized human podocyte cell line was kindly provided by Dr. Saleem (University of Bristol, Bristol, UK). The cells were cultured as previously described [14]. Podocytes were treated with 50 nM C3a or C5a for 1 h to establish a complement-induced podocyte damage model. The dosage effect of DSF at gradient concentrations between 50 nM and 1 μM on podocyte protection was explored according to lactate dehydrogenase (LDH) release in our preliminary experiment and 250 nM, the optimal dose, was added 1 h prior to model establishment for further investigation.

Propidium Iodide Staining

Cultured podocytes were stained with propidium iodide (PI) (3.34 μg/mL) at 37°C for 20 min and then incubated with DAPI (15 μg/mL) at 37°C for 20 min for nuclear staining. Photomicrographs were captured with confocal microscopy (LSM710; Carl Zeiss Meditec AG, Oberkochen, Germany).

LDH Release Assay

LDH release from cultured podocytes in the supernatant was detected using an LDH Cytotoxicity Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer’s instructions. Briefly, LDH was assessed based on the chromogenic reaction of 2-p-iodophenyl-3-nitrophenyl tetrazolium chloride catalyzed by diaphorase, and LDH levels were semiquantified via a colorimetric method.

Induction of PHN

Rabbit anti-Fx1A serum was prepared according to Dr. Salant’s protocols and concentrations of anti-Fx1A antibody in the serum were measured by immunofluorescence staining of rat proximal renal tubular epithelial cell brush border [15]. Female SD rats weighing 150–180 g were obtained from Sino-British SIPPR/BK Lab (Shanghai, China). All rats were housed under standard conditions and provided rat chow and water ad libitum in the Experimental Animal Center of Jinling Hospital. Protocols for the animal studies were approved by the Institutional Animal Care and Use Committee of Jinling Hospital, the First School of Clinical Medicine of Southern Medical University, Nanjing, China (Approval Number: SYXK (JS) 2018-0011).
Double immunofluorescence staining, 5-μm thick frozen sections were stained with DAPI (10 μg/mL) at RT for 10 min. For renal Cy3-labeled (1:200 dilution, v/v; Beyotime Biotechnology, Shanghai, China)/ZO-1 (1:200 dilution, v/v; Proteintech Group, Rosemont, IL, USA) at 4°C overnight, followed by incubation with FITC-labeled (1:200 dilution, v/v) secondary antibodies at RT overnight and subsequently incubated with HRP-conjugated secondary antibody (Quanhui Imp & Exp Int'l Co., Ltd., Zhuhai, China) at RT for 40 min and diamino benzidine chromogen for 15 s to 3 min, counterstained with hematoxylin for 10 min and sealed with neutral balsam. The sections were observed, photomicrographs were acquired with a light microscope, and the average IOD/area of all glomeruli per section was regarded as the protein expression level of the sample.

RT-qPCR

Glomeruli were isolated from the fresh renal cortex of rats according to Dr. Salant’s protocol [15]. Glomerular RNA was extracted using a MiniBEST Universal RNA Extraction Kit (Takara Biomedical Technology Co., Ltd., Shiga, Japan) and reverse transcribed with PrimeScript™ RT Master Mix (Takara Biomedical Technology Co., Ltd., Shiga, Japan) according to the manufacturer’s protocols. qPCR was performed according to the protocol of TB Green® Premix Ex Taq™ II (Takara Biomedical Technology Co., Ltd., Shiga, Japan) using the Fast Real-Time PCR System (7900HT, Thermo Fisher Scientific Inc., Waltham, MA, USA). The primers used were as follows: GSDMD (F: 5′-AGATCGGTGGA- CATGCCGT-3′; R: 5′-AGGCCAGATGCGTCTGAA-3′); NLRP3 (F: 5′-CTTCTGAAACGGCGTGA-3′; R: 5′-CCAGAGAAGCGAGTGACCA-3′); ASC (F: 5′-AAAGAGGAAGCGTACAAC-3′; R: 5′-GCTGAGCAGC-3′); Caspase-1 (F: 5′-TAGACTACAGATGCCAACC-3′; R: 5′-GCAATGAGTGCTTGCCTGTG-3′); IL-1β (F: 5′-ATGGGAGATGGTGGTACCA-3′; R: 5′-AGCATTGAGTCTGCTGGA-3′); IL-18 (F: 5′-AGCACTAAGTGATGGATTGG-3′; R: 5′-TATGAGGTTCAACGCTTTG-3′); GAPDH (F: 5′-GCT-CTCTGCTTCCCTGTTCT-3′; R: 5′-GGCAACATGTGCTAAGGTT-3′). The relative quantity of each gene in every sample was calculated and compared via the 2-ΔΔCt method.
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Western Blot

Total protein was extracted from cultured podocytes and rat renal cortex samples with lysis buffer mixed with protease/phosphatase inhibitors (Beyotime Biotechnology, Shanghai, China), while nuclear protein was extracted from rat renal cortex samples with a Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN Biotech, Nanjing, China). Equal amounts of denatured protein samples from different samples were mixed with loading buffer, separated by SDS–PAGE, and transferred to PVDF membranes. After blocking with 5% BSA, the membranes were separately incubated with the following antibodies: GSDMD (1:1,500 dilution, v/v; Affinity Biosciences LTD., Changzhou, China), NF-κB p65 (1:1,000 dilution, v/v), p-NF-κB p65 (Ser536) (1:1,000 dilution, v/v), NLRP3 (1:1,000 dilution, v/v), ASC (1:1,000 dilution, v/v), Caspase-1 (1:1,000 dilution, v/v), IL-1β (1:1,000 dilution, v/v), IL-18 (1:1,000 dilution, v/v), GAPDH (1:3,000 dilution, v/v; Cwbio IT Group, Beijing, China) and Histone H3 (1:3,000 dilution, v/v; Proteintech Group, Rosemont, IL, USA) at 4°C overnight. After incubation with an HRP-conjugated secondary antibody (1:5,000 dilution, v/v; TransGen Biotech, Beijing, China) at RT for 2 h, the protein bands were visualized using Immobilon™ Western Chemiluminescent HRP Substrate (Merck KGaA, Darmstadt, Germany) and photographed using an automatic chemiluminescence/fluorescence image analysis system (5200, Tanon Science & Technology Co., Ltd., Shanghai, China).

ELISA

IL-18 release in the culture supernatant of the podocytes and the serum IL-1β/IL-18 levels of rats were tested using human IL-18 ELISA and rat IL-1β/IL-18 ELISA kits (Boster Biological Technology Co. Ltd., Wuhan, China), respectively. Samples were prepared and measured strictly in accordance with the manufacturers’ protocols.

Statistics

All experiments were carried out at least three times. Statistical analyses were performed with SPSS 20.0 software (IBM Corporation, Armonk, NY, USA). Data are shown as the mean ± standard deviation (SD). ANOVA with LSD-t test (equal variances assumed) or the Welch test with Dunnett’s T3 test (equal variances not assumed) was used for multiple comparisons among groups. All tests were two-sided, and p < 0.05 was considered indicative of statistical significance.

Results

DSF Alleviated C3a/C5a-Induced Podocyte Injury by Inhibiting Pyroptosis

Given that DSF can inhibit pyroptosis by preventing GSDMD pore formation, we first examined the inhibitory effect of DSF on complement-induced podocyte pyroptosis. As shown in Figure 1a, cell immunofluorescence staining showed that signs of activation and membrane translocation of GSDMD, manifested by the colocalization of activated N-terminal fragment of GSDMD (GSDMD(N)) and cytomebrane marker ZO-1 in C3a/C5a-treated podocytes, were significantly suppressed by DSF. Moreover, DSF reduced the increase and activation of the pyroptosis signaling pathway NLRP3-ASC-Caspase-1-IL-18/GSDMD in C3a/C5a-treated podocytes (Fig. 1b). Meanwhile, the increase and activation of one of the key upstream stimulators of NLRP3, NF-κB, were also inhibited by DSF (Fig. 1b). The increased release of the pyroptosis-associated cytokine IL-18, probably derived from the activation of the pyroptosis signaling pathway in C3a/C5a-treated podocytes, was also reduced after DSF treatment (Fig. 1c). On this basis, we further examined the effect of DSF on podocyte injury and found that injury to C3a/C5a-treated podocytes, reflected by the increased percentage of PI staining cells and increased release of LDH, was significantly alleviated by DSF (Fig. 1d, e and f). Taken together, the in vitro experiments validated the protective effect of DSF on C3a/C5a-induced podocyte injury by inhibiting pyroptosis.

DSF Ameliorated Renal Lesions and Podocyte Injury in PHN Rats

On the basis of the in vitro experiments, we continued to explore the effects of DSF on renal lesions and podocyte injury in PHN rats. The rats were randomly divided into 3 groups: NC, PHN and PHN + DSF (n = 6/group). During the 15-day observation period, the rats in different groups showed similar growth trends (Fig. 2a). This result suggested, to some extent, the rats tolerated the current dosage of DSF well. Compared to those in the NC group, rats in the PHN group showed typical nephrotic syndrome with a significant increase in 24 h-UPro and a decrease in ALB after model establishment, while those in the PHN + DSF group showed significant amelioration (Fig. 2b, c). The levels of Scr were elevated in the PHN group and decreased in the DSF treatment group (Fig. 2d). BUN, an indicator of glomerular filtration function, remained largely unchanged in each group of rats throughout the observation period (Fig. 2e). Hyperlipemia, another typical manifestation of nephrotic syndrome that includes increased T-CHOL, TG, HDL and LDL, was also observed after PHN model establishment. Compared with the NC group, the PHN and PHN + DSF groups exhibited gradual increases in the levels of T-CHOL, HDL and LDL during the course of the experiment, and there were no significant differences between the PHN and PHN + DSF groups (Fig. 2f, h and i). However, DSF significantly reduced the increased level of TG in the PHN group on day 15 during the observation period (Fig. 2g). Taken together, these results indicated that DSF could alleviate renal lesions in PHN rats.
Fig. 1. DSF alleviated C3a/C5a-induced podocyte injury by inhibiting pyroptosis. Podocyte injury was induced with C3a (50 nM) and C5a (50 nM). The inhibitory effects of DSF (250 nM) on C3a/C5a-induced pyroptosis were examined. a Representative images of GSDMD(N)/ZO-1/Nucleus (DAPI) triple immunofluorescent staining of treated podocytes. Scale bars = 20 μm. b Representative Western Blot images of GSDMD, NF-κB p65, p-NF-κB p65 (Ser536), NLRP3, ASC, Caspase-1, IL-18 and the internal control (GAPDH) in treated podocytes. c IL-18 release in treated podocytes was detected. d, e Representative images of PI/Nucleus (DAPI) double fluorescent staining of treated podocytes (d) and percentage of PI-positive cells (e); arrows, PI-positive cells; scale bars = 40 μm. f LDH release in treated podocytes was detected. The data above represent three independent experiments in duplicate and are shown as the mean ± SD, and ANOVA with LSD-t test (equal variances assumed) or Welch’s test with Dunnett’s T3 test (equal variances not assumed) was used for multiple comparisons among groups. *, p < 0.05; **, p < 0.01.
We further validated the effect of DSF on podocyte injury in PHN rats. Masson and PASM-Masson staining of the renal tissue in PHN rats revealed pathological features of MN, including subepithelial deposits of fuchsin protein and spike-like changes in the GBM (Fig. 3a). Desmin, one of the most common markers of podocyte injury, increased significantly in the glomeruli of PHN rats while WT-1, another indicator of podocyte injury, manifested decreased expression in the nuclei of podocyte and abnormal distribution in the glomeruli of PHN rats (Fig. 3b, c). Under transmission electron microscopy, characteristic manifestations of MN, including subepithelial dense deposits and widespread foot process fusion of podocytes, delineated by a significant increase in foot process width, were observed in the glomeruli of PHN rats (Fig. 3d, e). Compared to PHN rats, the increased expression of glomerular Desmin, the decreased podocytes nuclear expression and abnormal glomerular distribution of WT-1, as well as foot process fusion of podocytes were all notably alleviated after DSF treatment (Fig. 3b, c, d and e), suggesting the protective effect of DSF on podocyte injury in MN. We also evaluated the effect of DSF on immune complex deposition and complement system activation in the glomeruli of PHN rats; however, no significant difference was found between the PHN and PHN + DSF groups (data not shown).

**DSF Inhibited Podocyte Pyroptosis in PHN Rats**

We proceeded to explore whether the protective effect of DSF on PHN rats could be attributed to its anti-pyro-
Fig. 3. DSF ameliorated glomerular podocyte injury in PHN rats. **a** Representative renal Masson and PASM-Masson staining of the rats (n = 6) in each group; Scale bars = 20 μm. **b, c** Representative renal immunohistochemical staining of Desmin and WT-1 (b) and semiquantification based on the glomerular IOD/area of Desmin (c) of the rats (n = 6) in each group; Scale bars = 20 μm. **d, e** Representative glomerular transmission electron microscopy images (d) and podocyte foot process width (e) of the rats (n = 6) in each group. Arrowheads, subepithelial dense deposit; arrows, podocyte foot process fusion. The data above are shown as the mean ± SD. ANOVA with LSD-t test (equal variances assumed) or Welch’s test with Dunnett’s T3 test (equal variances not assumed) was used for multiple comparisons among groups. *, p < 0.05; **, p < 0.01.

Fig. 4. DSF inhibited the renal pyroptosis signaling pathway in PHN rats. **a** Representative renal GSDMD(N)/Synaptopodin and GSDMD(N)/ZO-1 double immunofluorescent staining of the rats (n = 6) in each group; Scale bars = 20 μm. **b, c** Representative renal immunohistochemical staining (b) and semiquantification based on the glomerular IOD/area of GSDMD(N), NF-κB p65, p-NF-κB p65 (Ser536), NLRP3, ASC, Caspase-1, IL-1β, and IL-18 (c) of the rats (n = 6) in each group; Scale bars = 20 μm. **d** Relative mRNA levels of glomerular GSDMD, NLRP3, ASC, Caspase-1, Caspase-1 p20, IL-1β, IL-1β (mature form), IL-18 and the internal control (GAPDH, Histone H3) of the rats (n = 6) in each group on days 1, 5, 8, 15 after model establishment. The data above are shown as the mean ± SD (c, d, f, g) and were compared to the PHN group (f, g). ANOVA with LSD-t test (equal variances assumed) or Welch’s test with Dunnett’s T3 test (equal variances not assumed) was used for multiple comparisons among groups. *, p < 0.05; **, p < 0.01.

(For figure see next page.)
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GSDMD Synaptopodin Merge
- NC
- PHN
- PHN + DSF

GSDMD (N) ZO-1 Merge
- NC
- PHN
- PHN + DSF

IOD/area
- NC
- PHN
- PHN + DSF

mRNA level (relative to GAPDH)
- GSDMD
- NLRP3
- ASC
- Caspase-1
- IL-1β
- IL-18

IL-1β, pg/mL
- Day 1
- Day 5
- Day 8
- Day 15

IL-18, pg/mL
- Day 1
- Day 5
- Day 8
- Day 15

53 KDa
56 KDa
36 KDa
48 KDa
25 KDa
20 KDa
17 KDa
119 KDa
36 KDa
Discussion

Treatment of MN remains to be explored at present. Although clinical remission rates are improving greatly due to the application of cyclophosphamide, calcineurin inhibitors, and especially CD20-targeted agents, the efficacy of these drugs in preventing MN progression remains to be validated [1, 17]. Therefore, exploring more effective targeted drugs for renal injury in MN is crucial to improve the long-term outcome of patients. As a lytic and highly inflammatory regulated cell death program, pyroptosis occurs downstream of inflammasome activation and is driven by members of the gasdermin family, most notably GSDMD. Recently, a body of evidence has suggested the involvement of pyroptosis in kidney diseases. In a murine model of acute kidney injury (AKI), proteins in the pyroptosis signaling pathway, including Caspase-1, Caspase-11, IL-1β, and GSDMD, were significantly increased, accompanied by elevated renal structural and functional injury [18, 19]. GSDMD also increased and was activated in the kidneys of db/db mice and diabetic nephropathy (DN) patients with the development of tubular injury, demonstrating the close relationship between pyroptosis and DN [20]. Pyroptosis was also associated with lupus nephritis (LN) since an increase and activation of GSDMD can be detected in the kidneys of LN patients and mice [21]. Our previous work also depicted the pivotal role of pyroptosis in mediating podocyte injury in MN [11]. On the other hand, the renal protective effect of inhibitors that target members of the pyroptosis signaling pathway, including NLRP3 and Caspase-1, was also depicted in a murine model of AKI, crystalline nephropathy, and unilateral ureteral obstruction [22–24]. However, to the best of our knowledge, none of the pyroptosis inhibitors used in renal experiments have been applied in clinical practice. A very long journal still exists from bench to bedside. Therefore, in the current study, we validated the therapeutic effect of an FDA-approved drug, DSF, in the treatment of MN through the inhibition of podocyte pyroptosis in vitro and in vivo.

DSF is an inexpensive, accessible and safe drug and has been commonly used for alcohol addiction in clinical practice since the 1950s. It is an inhibitor of aldehyde dehydrogenase that is responsible for the conversion of alcohol into acetaldehyde [12]. DSF was also found to have an antitumor effect on several types of cancer, including non-small-cell lung cancer, liver cancer, breast cancer, prostate cancer, pancreatic cancer, glioblastoma and melanoma, by inhibiting the NF-κB signaling pathway, pro-
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Dr. Lv performed the experiments, collected and analyzed the data, and wrote the manuscript; S. Jiang, M. Zhang, X. Zhu, F. Yang, H. Wang, S. Li, F. Liu, C. Zeng and W. Qin performed the experiments, collected and analyzed the data; L. Li and Z. Liu conceived the study and revised the manuscript. All authors reviewed and approved the manuscript.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by grants from the National Natural Science Foundation of China Special Project (No. 32141004) and the National Key Technology R&D Program of China (No. 2015BAl12B02).

Author Contributions

The overall inhibition of the pyroptosis signaling pathway rather than simply the inhibition of GSDMD pore formation may probably be responsible for the protective effect of DSF in MN.

Conclusion

The FDA-approved drug DSF can alleviate MN by inhibiting podocyte pyroptosis. The findings highlighted the potential role of DSF in the treatment of MN patients in the future.

Statement of Ethics

This study protocol of animals was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Jinling Hospital, the First School of Clinical Medicine of Southern Medical University, Nanjing, China (Approval reference number: 2019JLHGKJDWLS-136).

Conflicts of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by grants from the National Natural Science Foundation of China Special Project (No. 32141004) and the National Key Technology R&D Program of China (No. 2015BAI12B02).

Author Contributions

D. Lv performed the experiments, collected and analyzed the data, and wrote the manuscript; S. Jiang, M. Zhang, X. Zhu, F. Yang, H. Wang, S. Li, F. Liu, C. Zeng and W. Qin performed the experiments, collected and analyzed the data; L. Li and Z. Liu conceived the study and revised the manuscript. All authors reviewed and approved the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.
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