The effect of taurochenodeoxycholic acid on the glucocorticoid receptor-mediated PLC-IP$_3$-calcium pathway in synoviocytes derived from a rat adjuvant arthritis model

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ABSTRACT

Taurochenodeoxycholic acid is one of the main active components of the bile acid pool and has important anti-inflammatory and immunomodulatory properties. This study aimed to explore the therapeutic effects and mechanism of action of taurochenodeoxycholic acid in inflammatory arthritis using a rat adjuvant arthritis model. Fibroblast-like synoviocytes were derived from rats with adjuvant arthritis and cultured using the adherent tissue explant method. Western blotting, enzyme-linked immunosorbent assay, and fluorescent probe-based methods were used to detect the effects of taurochenodeoxycholic acid on glucocorticoid receptor expression, phospholipase C protein levels, and inositol trisphosphate and intracellular free Ca$^{2+}$ concentrations in primary rat fibroblast-like synoviocytes. Taurochenodeoxycholic acid significantly induced the expression of glucocorticoid receptor (P<0.05) and phospholipase C (P<0.05) and enhanced phospholipase C phosphorylation. Moreover, taurochenodeoxycholic acid significantly increased the levels of inositol trisphosphate (P<0.05) and intracellular free Ca$^{2+}$ concentrations. Our results suggest that taurochenodeoxycholic acid activates the phospholipase C-inositol trisphosphate-Ca$^{2+}$ signalling pathway by increasing glucocorticoid receptor expression. These findings provide a theoretical foundation for future studies on the molecular mechanisms underlying taurochenodeoxycholic acid-based treatment of adjuvant arthritis. In conclusion, taurochenodeoxycholic acid exerted anti-inflammatory and immunomodulatory effects by enhancing glucocorticoid receptor expression via non-genomic signalling.

Key words: taurochenodeoxycholic acid; rat adjuvant arthritis model; glucocorticoid receptor; phospholipase C-inositol trisphosphate-Ca$^{2+}$ signalling pathway

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder characterised by several extra-articular manifestations and progressive damage to synovium-lined joints (BRZUSTEWICZ et al., 2015). Animals with RA often experience joint dysfunction and can suffer from a loss of production, which is detrimental to the livestock industry. The incidence of RA can be related to genetic factors, bacterial or viral infections, immunological and endocrine disorders, and other factors (HOLMDAHL et al., 2000). Therefore, establishing an animal model is indispensable for investigating the pathogenesis of RA and the effectiveness of potential therapeutics. Animal models of RA are widely used and are characterised by stable disease signs. Examples include adjuvant arthritis (AA), type-II collagen-induced arthritis, and streptococcal cell wall arthritis (VAN DEN BERG, 2009). The clinical signs of RA in animals are similar to those in human patients; therefore, animal models provide a relevant system for RA research.

Glucocorticoids (GCs) are steroid hormones secreted by the adrenal cortices and are necessary for human survival. Their physiological functions include suppression of immune function and increased glucose production (LECOQ et al., 2009). Glucocorticoids function as stress factors that maintain an internal balance in response to changes in internal and/or external environmental factors (KINO et al., 2011). At the same time, GCs are the most powerful anti-inflammatory drugs that are currently used for the rapid and effective reduction of inflammation in patients with extra-articular or arthritic symptoms. Early treatment with small doses of GCs for short periods of time can reduce RA symptoms by inhibiting inflammation (WANG, 2008); however, GCs do not stop the progression of RA or reduce joint damage, and can even induce undesirable side effects.

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily of transcription factors (DE BOSSCHER, 2007). Complete deletion of the second exon of the GR gene in mice results in severe abnormalities in lung development or death shortly after birth (COLE et al., 1995). The glucocorticoid receptor is responsible for gluconeogenesis in the liver (TRONCHE et al., 2004). In the central nervous system, the absence of GR impairs signalling through the hypothalamic–pituitary–adrenal axis and causes a wide range of disorders, such as physiological and behavioural inhibition (BOYLE et al., 2006).

Taurochenodeoxycholic acid (TCDDA) is one of the main bioactive bile acids in animals. It is formed via a dehydration reaction between taurine and chenodeoxycholic acid (HOFFMAN, 1984). We previously showed that TCDDA significantly suppressed both acute and chronic inflammation (LIU et al., 2011); however, the anti-inflammatory and immunomodulatory mechanisms of TCDDA in the rat AA model remain unclear. We expect that TCDDA can also play a similar role in anti-inflammation in the rat AA model, so as to provide new ideas for the treatment of RA. In the present study, we investigated the mechanism of TCDDA and the involvement of the GR-mediated phospholipase C (PLC)-inositol trisphosphate (IP$_3$)-Ca$^{2+}$ signal transduction pathway in the development of inflammatory arthritis.

Materials and methods

Reagents. Freund’s complete adjuvant (FCA, F5881, Sigma-Aldrich, St. Louis, MO, USA), Sodium TCDDA (purity ≥97%, T0266, Sigma-Aldrich), RU486 (M8046, Sigma-Aldrich), and 2-aminoethyl diphenyl borinate (2-APB, D9754, Sigma-Aldrich) were obtained from Sigma-Aldrich. The latter three were dissolved in DMEM. For western blotting, pre-cast SDS-PAGE gels (P0512, Beyotime Biotechnology, Shanghai, China), BCA Assay Kits (23225, Thermo Fisher Scientific, Waltham, MA, USA), pre-stained protein ladder (26616, Thermo Fisher Scientific), anti-GR rabbit mAb (SAB4501309, Millipore, Billerica, MA, USA), anti-PLC rabbit mAb (5690, Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated PLC rabbit mAb (pPLC, 4510, Cell Signaling Technology), anti-rabbit goat mAb (98164, Cell Signaling Technology), anti-b-actin (66009, Proteintech Group, Rosemont, IL, USA), antibody diluent (P0256, Beyotime
Biotechnology), and SuperSignal West Femto Kit (34094, Thermo Fisher Scientific) were used. For enzyme-linked immunosorbent assay (ELISA) and immunofluorescence analysis, Rat IP₃ ELISA Reagent Kit (CSB-E13004r, Cusabio, Houston, TX, USA) and Fluo-3 AM (S1056, Beyotime Biotechnology, Shanghai, China) were used.

**Isolation and culture of fibroblast-like synoviocytes from AA model rats.** Twenty male Wistar rats, weighing 150±10 g, were obtained from the Experimental Animal Centre of Inner Mongolia Medical University (Approval ID: 20,160,837-6). All animal experiments for this study were approved by the Animal Welfare and Research Ethics Committee of Inner Mongolia Agricultural University (Approval ID: 20,195,917-9), and all efforts were made to minimize animal suffering. All rats were housed at a constant temperature (22±2°C) with a regular light/dark cycle and ad libitum access to food and water. The AA rat model was generated as previously described (HUANG et al., 2008). Briefly, each rat was immunised via an intradermal foot pad injection with 0.1 mL FCA containing 10 mg of the heat-inactivated Bacillus Calmette–Guerin vaccine in 1 mL of paraffin oil. Then, 28 days later, synovial tissues from knee joints were harvested and then washed aseptically 3 times with Dulbecco’s phosphate-buffered saline (DPBS). Next, the tissues were cut into 1–3-mm² pieces and washed with high-sugar Dulbecco’s modified Eagle medium (DMEM) containing 20% foetal bovine serum (FBS). Tissue pieces were placed at the bottom of each cell culture flask. After tightening the cap, the cell culture flask was placed upside down at 37°C in a 5% CO₂ cell culture incubator for adherence. After 3 h, warm high-sugar DMEM containing 20% FBS was slowly added to the flask. Tissues were cultured in the incubator and the medium was replaced every 3 d. After removing the pieces of synovial tissue, adherent cells were further cultured in the same medium. After 3 passages, synoviocytes were collected and used in subsequent experiments. At the time of collection, the majority of synoviocyte cultures contained homogeneous fibroblast-like synoviocytes (FLSs).

**Western blotting.** To investigate the involvement of GR and PLC activity in TCDCA-mediated anti-inflammatory properties, FLSs were divided into the following groups: cultured under normal physiological conditions (negative control group); treated with TCDCA (10⁻⁴ M) for 5, 10, 15, 30, or 60 min; treated with a GR inhibitor (RU486; 10 µM); or treated with TCDCA (10⁻⁴ M) + GR inhibitor (RU486; 10 µM) for 5, 10, 15, 30, or 60 min. The cells were washed twice using DPBS, harvested, and lysed for total cellular protein extraction after being treated. Total protein concentration was determined using the BCA assay kit. For western blotting analysis, protein samples (25 µg) were resolved using SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Rabbit anti-GR , anti-PLC and anti-phosphorylated PLC mAb (1:2,000) were used for protein detection. Proteins were visualized using a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:10,000) and Super Signal West Femto chemiluminescent substrate. The grayscale values of the bands on the immunoblot were measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**ELISA.** On the basis of the preliminary experiments, FLSs were cultured under normal physiological conditions (negative control), washed twice with pre-chilled DPBS, treated with TCDCA (10⁻⁴ M) + RU486 (10 µM) + an IP₃ inhibitor (2-APB; 10⁻⁴ M) for 5, 10, 15, 30, or 60 min, treated with 2-APB alone (10⁻⁴ M), or treated with 10 µM RU486 alone, trypsinised, centrifuged at 5000 × g, and washed three times with DPBS. Next, the cells were re-suspended in DPBS and subjected to repeated freeze–thaw cycles to lyse the cell membranes. Lysates were centrifuged at 5000 × g for 5 min, and supernatants were stored at -20°C until use. IP₃ concentrations were measured using the Rat IP₃ ELISA Reagent Kit according to the manufacturer’s instructions. Three biological replicates were used.

**Fluorescent probe.** The FLSs from AA rats were seeded overnight in 96-well plates (200 µL/well; 1×10⁵/mL) and cultured until the cells reached ~90% confluence. The fluorescence probe Fluo-3 AM was reloaded into 96-well plates at a concentration of 2.5 µM (50 µL) per well. After washing 3–5 times with DPBS, 100 µL of DPBS was added to each
well. FLSs were treated with different TCDCA concentrations (10^{-6}, 10^{-5}, and 10^{-4} M) and the corresponding fluorescence signals were measured for each experimental group for 20 min.

**Statistical analyses.** Statistical analyses were performed using the SAS software version 9.0 (SAS Institute, Cary, NC, USA). The results were expressed as means ± standard deviations of three independent experiments and were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test or two-way ANOVA with Bonferroni’s post-hoc test. Significance was set at P<0.05.

**Results**

*Culture of FLSs from AA rats.* After 3 days of synovial tissue culture, primary FLSs spread out from the edges of the tissues. The cell morphology was relatively uniform, and the cells had a long fusiform shape with protrusions, as well as a few round cells. Cultured cells were passaged on day 7 when cell density was ~80% (Fig. 1).

**Effects of TCDCA on GR expression in AA rat FLSs.** Compared to the negative control, 10^{-4} M TCDCA treatment significantly increased GR expression after 30 and 60 min (P<0.05; Fig. 2); however, no significant differences in GR expression were observed between the negative control group and the groups treated with 10^{-4} M TCDCA for 5, 10, or 15 min. In cells treated with TCDCA for 30 or 60 min, protein levels of GR were significantly decreased in the TCDCA+RU486 group compared to the TCDCA group (P<0.05; Fig. 2). These findings suggested that TCDCA treatment induced GR expression in FLSs from AA rats.

![Fig. 1. Fibroblast-like synoviocytes (FLSs) visualised by light microscopy (200×)](image)

(a) Primary FLSs culture on day 5. (b) Passage 3 FLSs on day 7 following subculture. Bars in (a)-(b): 50 μm

![Fig. 2. Glucocorticoid receptor (GR) protein expression after treatment with taurochenodeoxycholic acid (TCDCA)](image)

GR expression in fibroblast-like synoviocytes (FLSs) treated with TCDCA was evaluated by western blotting; β-actin was used as a loading control. Grayscale values were measured using the ImageJ software. Results are expressed as the means ± standard deviations of three independent experiments and analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test or two-way ANOVA with Bonferroni’s post-hoc test. Compared to negative control, *P<0.05; **P<0.01. Comparing between indicated groups, #P<0.05; ##P<0.01.
Effect of TCDCA on PLC activity in AA rat FLSs. On the basis of the preliminary experiments, we treated FLSs with different concentrations of TCDCA and the GR inhibitor RU486. The treatment of FLSs with TCDCA for 5, 10, or 15 min did not significantly affect PLC levels compared to the negative controls (Fig. 3a); however, PLC levels were significantly increased at 30- and 60-min time points (P<0.05). RU486 significantly reduced PLC levels at 60 min compared to the TCDCA group (P<0.05, Fig. 3a). Furthermore, 10^{-4} M TCDCA treatment only increased pPLC levels in AA rat FLSs at the 60 min time point compared to the negative controls (P<0.05, Fig. 3b). RU486-pre-treated FLSs did not significantly affect pPLC levels; however, the combination of TCDCA with RU486 resulted in significantly higher pPLC levels at the 60 min time point (P<0.05). Collectively, these results suggested that TCDCA increased PLC activity.

Host GR mediated signalling activation in fibroblast-like synoviocytes (FLSs) after treatment with taurochenodeoxycholic acid (TCDCA). Activation of PLC pathways evaluated by western blotting; β-actin was used as a loading control. Grayscale values were measured using the ImageJ software. Results are expressed as means ± standard deviations of three independent experiments and analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test or with two-way ANOVA with Bonferroni’s post-hoc test. Compared to negative control, *P<0.05; **P<0.01. Comparing between indicated groups, #P<0.05; ##P<0.01.

Effect of TCDCA on IP_3 synthesis in AA rat FLSs. The IP_3 concentrations in AA rat FLSs were determined using ELISA. Compared to negative controls, TCDCA treatment for 15, 30, or 60 min significantly increased the IP_3 concentration in AA rat FLSs (Fig. 4). Furthermore, at the 30 min time point, TCDCA treatment in combination with RU486 pre-treatment significantly decreased IP_3 levels compared to TCDCA alone, while the IP_3 concentration was not affected in TCDCA-treated FLSs in the presence or absence of RU486 pre-treatment at other time points. In addition, at the 30 min time point, the IP_3 concentration was significantly decreased in cells pre-treated with 2-APB (P<0.05) compared to that in cells treated with TCDCA alone. However, 2-APB pre-treatment did not affect the IP_3 concentration at all other time points. These findings suggested that TCDCA treatment increased the IP_3 concentration in AA rat FLSs.
Effect of TCDCA on intracellular \([\text{Ca}^{2+}]\) in AA rat FLSs. The fluorescence intensity of the negative control did not change, indicating that the \([\text{Ca}^{2+}]\) did not change (Fig. 5a); however, fluorescence intensity was increased in response to different TCDCA concentrations. The fluorescence signals did not increase after treatment with \(10^{-6}\) or \(10^{-5}\) M TCDCA, but did change in response to \(10^{-4}\) M TCDCA concentration: the signal immediately reached its peak value, then decreased significantly by the 5 min time point, and started to plateau. RU486 pre-treatment significantly increased the fluorescence signal of \(10^{-4}\) M TCDCA-treated cells, and the peak value was reached sooner than in the controls (Fig. 5b). On the other hand, the combination of 2-APB pre-treatment and \(10^{-4}\) M TCDCA treatment decreased fluorescence intensity compared to the controls, while the instantaneous release of \(\text{Ca}^{2+}\) induced by TCDCA in AA rat FLSs was reduced (Fig. 5c). In addition, compared to the negative controls, the fluorescence values of cells treated with \(10^{-6}\), \(10^{-5}\), and \(10^{-4}\) M TCDCA were significantly increased (\(P<0.05\); Fig. 5d). In the presence of GR inhibitor (RU486), the fluorescence intensity values of cells treated with \(10^{-6}\) and \(10^{-5}\) M TCDCA were significantly decreased, while that of the \(10^{-4}\) M TCDCA-treated cells was significantly increased. In the presence of IP_3 inhibitor (2-APB), the fluorescence signal of the TCDCA-treated cells (\(10^{-6}, 10^{-5}\), and \(10^{-4}\) M) was significantly lower compared to that of the controls. Meanwhile, the fluorescence of the negative control FLSs did not change markedly over time (Fig. 5e). In AA rat FLSs treated with \(10^{-4}\) M TCDCA, the fluorescence signal gradually increased, reached a peak at 2 min post-treatment, and then gradually decreased and stabilised. The fluorescence of the RU486-treated FLSs did not change significantly over time; however, TCDCA treatment in combination with RU486 resulted in a rapid increase in fluorescence compared to TCDCA alone, and peak fluorescence was observed sooner. In cells treated with 2-APB alone, fluorescence did not change significantly over time, but decreased compared to that in the blank control, indicating that 2-APB treatment reduced \(\text{Ca}^{2+}\) levels. The fluorescence rapidly increased when FLSs were treated with TCDCA in combination with 2-APB. In these cells, the peak fluorescence was observed sooner, and fluorescence was decreased compared to that of TCDCA-treated.
cells, indicating that 2-APB reduced Ca\(^{2+}\) levels in AA rat FLSs treated with 10\(^{-4}\) M TCDCA. These results suggested that TCDCA increased the [Ca\(^{2+}\)]\(_{i}\) in AA rat FLSs, RU486 inhibited the effect of TCDCA on [Ca\(^{2+}\)]\(_{i}\), while 2-APB blocked the effect of TCDCA on [Ca\(^{2+}\)]\(_{i}\) in AA rat FLSs.

Fig. 5. (a) Effect of taurochenodeoxycholic acid (TCDCA) on intracellular free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{i}\) in fibroblast-like synoviocytes (FLS)). Experimental groups were as follows: i. negative control FLSs, ii. FLSs treated with 10\(^{-6}\) M TCDCA, iii. FLSs treated with 10\(^{-5}\) M TCDCA, and iv. FLSs treated with 10\(^{-4}\) M TCDCA. (b) FLSs were pre-treated with RU486 and then evaluated to determine the effect of TCDCA on [Ca\(^{2+}\)]\(_{i}\) after the following treatments: i. negative control treatment, ii. FLSs + 10\(^{-6}\) M TCDCA, iii. FLSs + 10\(^{-5}\) M TCDCA, and iv. FLSs + 10\(^{-4}\) M TCDCA. (c) FLSs were pre-treated with 2-aminoethyl diphenyl borinate (2-APB) and then evaluated to determine the effect of TCDCA on [Ca\(^{2+}\)]\(_{i}\) after the following treatments: i. negative control treatment, ii. FLSs + 10\(^{-6}\) M TCDCA, iii. FLSs + 10\(^{-5}\) M TCDCA, and iv. FLSs + 10\(^{-4}\) M TCDCA. (d) Dose-dependent effect of TCDCA on [Ca\(^{2+}\)]\(_{i}\) in FLSs. (e) Time-dependent effect of TCDCA on [Ca\(^{2+}\)]\(_{i}\) in FLSs. Compared to negative control, *P<0.05; **P<0.01. Comparing between indicated groups, #P<0.05; ##P<0.01.
Discussion

The FLSs from the rat model of AA share many histological and immunological characteristics with human RA (DENG and LENARDO, 2007). Pathological changes in the AA rat model may result from many factors, such as gene mutations, proto-oncogene activation, synovial cell evolution, inflammatory cell infiltration, crosstalk between pro-inflammatory cytokines, chemokines, and matrix protein-degrading enzymes in the synovial tissue. This causes aberrant signal transduction, impaired proliferation, and apoptosis in synovial cells (BUCKINGHAM and FLOWER, 1997). Currently, GCs are the most effective and widely used drugs for treating RA; however, hormone therapy is often accompanied by numerous side effects, including fat redistribution, diabetes, and osteoporosis (PODGORSKI et al., 1992), thus limiting its long-term use. Therefore, it is important to develop more effective and less toxic anti-inflammatory drugs for the treatment of RA (PODGORSKI et al., 1992; BRICHORY et al., 2001).

We have previously shown that TCDCA has significant anti-inflammatory and immunomodulatory properties. In addition, TCDCA administration to AA rats by gavage for 14 or 31 days significantly alleviated decreased body weight, erythema on limbs and tails, bone deformation, and lower bone density. The joint index and toe-swelling rate of AA rats also decrease significantly following TCDCA treatment (PEIFENG and HONG, 2002). Moreover, TCDCA-induced FLS apoptosis increases the expression and activity of key enzymes, including caspase-3 and caspase-8 (WEI, 2011; HU and SHI, 2001). Furthermore, TCDCA inhibited the binding of fluorescently labelled dexamethasone to GR at low concentrations. It has been shown that TCDCA binds to GR, and activates a GR-associated genomic mechanism to exert anti-inflammatory and immunoregulatory effects (WEI, 2011); however, it is not clear whether TCDCA acts through a non-genomic GR-associated mechanism. Glucocorticoids binding to receptors can activate adenylate cyclase and PLC, thus eliciting a non-genomic effect (WEI, 2011, LEITE-DELOVA et al., 2008). The production of diacylglycerol and IP₃ is induced via PLC, and diacylglycerol activates PKC- and IP₃-mediated mobilization of Ca²⁺ for storage in the endoplasmic reticulum (BENTEN et al., 1997; FALKENSTEIN et al., 2000).

In response to drugs or external stimuli, PLC–IP₃–Ca²⁺ signalling is activated and Ca²⁺ concentration is significantly increased. The PLC–IP₃–Ca²⁺ pathway may play an important regulatory role in the activation of inflammatory cells, as well as the release of related enzymes and inflammatory mediators (LÖWENBERG et al., 2008). For example, it has been shown that the regulation of Ca²⁺ levels in neutrophils affects the inflammatory response (BRICHORY et al., 2001).

Therefore, to determine whether TCDCA-induced anti-inflammatory and immunomodulatory responses occurred through a non-genomic GR route, the molecular mechanism of TCDCA was elucidated in rats with AA. In this study, we evaluated the effects of TCDCA on the levels of GR, PLC, pPLC, IP₃, and Ca²⁺ in AA rat FLSs. Taurochenodeoxycholic acid significantly increased the protein levels of GR, PLC, and pPLC, thereby improving its activity in AA rat FLSs. Taurochenodeoxycholic acid also significantly increased IP₃ and Ca²⁺ concentrations in FLSs. A GR inhibitor (RU486) blocked the effect of TCDCA on [Ca²⁺]i in FLSs and shortened the time required for peak fluorescence to be reached. At the same time, after TCDCA exposure for 60 min, PLC levels in FLSs were significantly lower than those in the pre-treatment group without RU486. Phosphorylated PLC levels in FLSs were higher than those in pre-treated control cells (without RU486), and highest after TCDCA exposure for 60 min. After 60 min, the IP₃ receptor antagonist 2-APB blocked the TCDCA-mediated increase in [Ca²⁺]i. Therefore, the stimulatory effect of TCDCA on [Ca²⁺]i was achieved by increasing the PLC activity and generating intracellular IP₃.

In these studies, the rat model of AA was only an alternative model applied in RA, and the results can only point out the Effect of TCDCA on the GR-Mediated PLC -- IP3 -- calcium pathway. These results can be used to infer that TCDCA has the same effect in other animals, such as humans, but a large
number of validation trials are still needed before TCDCA can be used in clinical treatment of RA.

Conclusions

In conclusion, TCDCA appears to regulate PLC-IP3-Ca2+ signalling through GR. However, whether the anti-inflammatory effect of TCDCA in the AA rat model is mediated via the non-genomic GR mechanism requires further investigation.

Conflicts of Interest

The authors declare no competing interests.

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Author contributions

Conceptualization: Jindi Wu and Fan Bai contributed equally to this work. WM, PL, FB, and YQ; Data curation: SG and WM; Formal analysis: YQ, FB, and JC; Funding acquisition: WM and JC; Investigation: SG and YQ; Methodology: SG and WM; Project administration: JC and YQ; Resources: WM and FB; Software: FB, and SG; Supervision: PL and JC; Validation: PL and JC; Visualization: WM and YQ; Writing - original draft: WM, FB, and SG; Writing - review and editing: WM and FB; All authors reviewed the manuscript.

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