Tanshinone IIA regulates expression of glucose transporter 1 via activation of the HIF-1α signaling pathway

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Abstract. Tanshinone IIA (Tan 2A) is a lipid-soluble compound extracted from the Chinese herb Danshen (Salvia miltiorrhiza Bunge). It protects neuron and microvascular endothelial cells against hypoxia/ischemia both in vitro and in vivo however the mechanism is not fully known. Glucose transporter 1 (GLUT-1) is ubiquitously expressed in all types of tissue in the human body and serves important physiological functions due to its glucose uptake ability. The present study evaluated the role of Tan 2A in regulating GLUT-1 expression and its potential mechanism. RT-PCR and western Blot were used to detect the expression of GLUT-1. Si RNA mediated knockdown and CHIP assay were used to explore the mechanism of Tan 2A on GLUT-1 expression. Tan 2A treatment induced expression of GLUT-1 and subsequently increased glucose uptake in endothelial cells (ECs). Furthermore, mRNA expression levels of vascular endothelial cell growth factor, BCL2 interacting protein 3 and enolase 2, which are target genes for hypoxia-inducible factor-1α (HIF-1α), were significantly upregulated by Tan 2A. Co-immunoprecipitation demonstrated that Tan 2A markedly increased the association of HIF-1α with recombination signal-binding protein for immunoglobulin kJ region (RBPJkJ). Moreover, knockdown of HIF-1α and RBPs significantly reversed the regulatory effect of Tan 2A on mRNA expression levels of these genes in ECs. The results of the present study suggested that HIF-1α partially mediated the regulatory effect of Tan 2A on GLUT-1 expression in ECs. Therefore, GLUT-1 may be a potential therapeutic target for Tan 2A.

Introduction

The brain utilizes high levels of energy to maintain its physiological function. The mammalian brain depends on glucose as its main source of energy but lacks oxygen and glucose reserves and therefore requires a continuous supply of glucose from blood (1). Two independent groups of transporter proteins, glucose transporters (GLUTs) and solute carrier family members, mediate glucose transport into the brain (2). GLUT protein 1 (GLUT-1), also called solute carrier family member 2 member 1, was first identified in a fetal skeletal muscle cell line (3) and is ubiquitously expressed in all types of tissue (4). It is also reported to be highly expressed in endothelial cells (ECs) of the central nervous system and is considered to be the principal glucose transporter of the blood-brain barrier (5). Functional deficiency of GLUT-1 decreases the amount of glucose available to brain cells, which affects brain function (6).

Danshen (Salvia miltiorrhiza Bunge), a medicinal herb, has been used in traditional Chinese medicine to treat conditions associated with the cardiovascular and cerebrovascular systems, such as stroke (7). Tanshinones are the primary active component of Danshen. Tanshinone IIA (Tan 2A), the most abundant diterpenoid quinone in S. miltiorrhiza, a natural inhibitor of monoacylglycerol lipase, is a fat-soluble component and is reported to exert anti-inflammation, anti-cancer, neuroprotection and hypolipidemic activity (8,9), and have a neuroprotective and cardiovascular protective effect. Tan 2A increases blood flow in the heart and improves myocardial metabolic disorder by increasing the tolerance of cardiomyocytes to hypoxia (10). Tan 2A protects neuron and microvascular ECs against hypoxia/ischemia both in vitro and in vivo (11). Tan 2A sodium sulfonate decreases atherosclerotic lesion area by inhibiting expression of intracellular chloride channel 1 in ECs (12) and activating Kruppel-like factor 4 in macrophages (13,14). The combination of Danshen and Sanqi (a Chinese herbal medicine) prescription has been reported to markedly increase expression of liver glycogen synthesis genes, such as GLUT-1, and improve fat and glucose metabolism (15). To the best of our knowledge, however, whether GLUT-1 expression of ECs is regulated by Tan 2A is not yet known. It was hypothesized that Tan 2A may induce glucose uptake via regulation of GLUT1 expression in vascular ECs.
Previous studies have reported that hypoxia-inducible factor 1α (HIF-1α) may mediate the therapeutic actions of Tan 2A (16,17). For example, Tan 2A is reported to inhibit breast cancer growth by inhibiting HIF-1α expression via the mammalian target of rapamycin signaling pathway (18). Tan 2A also blocks epithelial-mesenchymal transition in breast cancer cell lines by regulating the HIF-1α signaling pathway (19). Tan 2A decreases the inflammatory response in LPS-induced lung injury via the HIF-1α signaling pathway (20). Furthermore, overexpression of GLUT-1 enhances the effect of Tan 2A on the treatment of middle cerebral artery occlusion (21). The present study assessed the regulatory effect of Tan 2A on expression of GLUT-1 in ECs and evaluated whether the effect of Tan 2A was associated with HIF-1α.

Materials and methods

Cell culture and reagents. Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell Research Laboratories, Inc. and cultured in EC medium containing EC growth supplement (ScienCell Research Laboratories, Inc.), 5% fetal bovine serum (FBS; ScienCell Research Laboratories, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin in 37°C. HUVECs at passages 3-5 were used for all experiments. Tan 2A was purchased from Selleck Chemicals (Cat. no. S2365, Lot no. S2767130005001). DMSO (sigma-Aldrich; Merck KGaA) was used as vehicle control. Lipofectamine™ RNAiMAX Transfection Reagent and Lipofectamine™ 3000 Transfection Reagent were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Antibody against GLUT-1 (ab115730, 1:2,000) was purchased from Abcam. Antibodies against HIF-1α (#36169, 1:1,000), RBPJκ (#5313, 1:1,000) and GAPDH (#5174, 1:2,000) and horseradish peroxidase-conjugated goat anti-rabbit (#7074, 1:2,000) or anti-mouse secondary antibodies (#7076, 1:2,000) were purchased from Cell Signaling Technology, Inc.

Transfection and gene silencing. Small interfering (si)RNAs targeting human GLUT-1, HIF-1α and recombination signal-binding protein for immunoglobulin κ region (RBPJκ) were synthesized by Shanghai GenePharma Co., Ltd. siRNA (100 nM) were transfected into cells using Lipofectamine RNAiMAX Transfection Reagent in room temperature for 10 min. About 48 h later, the cells were extracted for RNA or protein detection. Scrambled siRNA was used as a negative control (Shanghai GenePharma Co., Ltd.). siRNA sequences are presented in Table S1.

Reverse transcription-quantitative (RT-q)PCR. Total cellular RNA was extracted using RNAiso Plus (Code No.: 9109, Takara Biotechnology Co., Ltd.). Complementary (c)DNA was synthesized from total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd. TKR-RR047) as follows: 42°C for 2 min, 37°C for 15 min and then 85°C for 5 sec. Primer sequences are presented in Table S1. qPCR was performed using SYBR-Green Master Mix (Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: Pre-denaturation at 94°C for 5 sec; followed by 40 cycles of denaturation, 95°C for 5 sec, annealing at 60°C and extension for 34 sec. The 18s rRNA was used as an internal control (Sangon Biotech Co., Ltd.). The mRNA expression level of each target gene was determined using the 2ΔΔCq method (22).

Plasmid and luciferase activity assay. The DNA fragment containing the hypoxia-responsive element (HRE) sequence (TGTCACTGTCCTGCAGACTCTTAGT) was subclone into the pGL3 basic reporter vector (Promega Corporation). The plasmid luciferase reporters were electroporated into HUVECs using an ECM 839 Electroporation System (Harvard Apparatus) as previously reported (23). Briefly, HUVECs were resuspended in EC medium (without serum; ScienCell Research Laboratories, Inc.), 20 µg plasmid was added and mixed in MicroPulse Cuvettes (0.4 cm, Bio-Rad). The cuvettes are one-piece injection molded chambers with embedded aluminum electrodes and square sealing caps. An electrical pulse (160 mA, 1 ms, 1 pulse) was applied. Cells (2x10⁴) were then seeded in 24 wells plates immediately using EC medium at room temperature for 24 h. The transactivation activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer’s instructions. Briefly, 1X Passive Lysis Buffer was dispensed into each culture vessel for 15 min at room temperature. Then the cell lysis was mixed with Luciferase Assay Substrate and measured firefly luciferase activity in GloMax 20/20 Luminometer (Promega).

Western blotting. HUVECs were lysed with RIPA buffer (Thermo Fisher Scientific, Inc.) with protease inhibitors (Cell Signaling Technology, Inc.). The protein concentration was determined using the bicinchoninic acid protein assay (Thermo Fisher Scientific, Inc.). A total of 20 µg/lane protein sample was resolved on 10% SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes. The immunoblots were blocked with 5% BSA (Sangon Biotech Co., Ltd) for 1 h at room temperature, and probed with the aforementioned antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibodies for 1 h at room temperature. Subsequently, the membranes were washed with TBS-Tween (0.1%) three times, 10 min each. The blots were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.). GAPDH was used as an internal reference. Densitometry analysis was performed using ImageJ (V1.8.0.112; National Institutes of Health).

Glucose uptake assay. Glucose uptake assay was performed using the Colorimetric Glucose Uptake Assay kit (Abcam) according to the manufacturer’s protocol. Briefly, 2-deoxyglucose (2-DG) was added to HUVECs and incubated for 20 min at 37°C. After 2-DG was taken up by glucose transporters and metabolized to 2-DG-6-phosphate, the cells were washed with phosphate-buffered saline (PBS) to remove exogenous 2-DG. Cells were lysed using repeated pipetting, freeze/thaw and heating at 85°C for 40 min. The level of 2-DG-6-phosphate in each sample was determined by enzymatic recycling amplification reaction and analyzed using a microplate reader (optical density, 412 nm) in kinetic mode at 37°C.

Co-immunoprecipitation (Co-IP) assay. HUVEC protein was collected using Pierce™ IP lysis buffer (Thermo Fisher
supernatant including protein were collected. Protein lystate (400 µg) was immunoprecipitated with the RBPjκ antibody (2 µg/well; CST, Inc.; cat. no. #5313) or control Rabbit IgG control Polyclonal antibody (2 µg/well; ProteinTech Group, Inc.; cat. no. 30,000-0-AP) bound to Dynabeads Protein G (50 µl/tube) by using Immunoprecipitation Kit-Dynabeads Protein G (Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. Completes were isolated by addition of 20 µl IP lysis buffer (Thermo Fisher Scientific, Inc.) and 5 µl SDS-PAGE SDS Sample Loading Buffer (5X, Beyotime, cat. no. P0015L) and heat for 5 min at 100˚C, then place the tube on the DynaMag-Spin (Thermo Fisher, cat. 12320D) and load the supernatant/sample onto a gel and assessed using western blotting as per the aforementioned method.

Chromatin IP (ChIP) assay. ChIP assay was performed according to the manufacturer's instructions using Magna ChIP G-Chromatin Immunoprecipitation kit (cat. no. 17-611; Millipore). Briefly, HUVECs were treated with Tan 2A (20 µM) for 16 h in 37˚C. 275 µl 37% formaldehyde (Sangon Biotech Co., Ltd.) was added to 10 ml of growth media to cross-link protein to chromatin for 10 min at room temperature, then quenched with glycine at a final concentration of 125 mM immediately at room temperature for 5 min. Cells were harvested in cold PBS. The cellular nuclear pellets were resuspended with sonication buffer. The resulting cell suspension was sheared by sonication (Misonix Sonicator 3000) on ice using 30/30-sec on/off for 10 min (Output Power: 4). ChIP assay was performed using 1.5 µg Rabbit IgG (1.15 mg/ml, ProteinTech Group, 30000-0-AP) or anti-HIF-1α antibodies (CST, #14179) bounded to the Magnetic Protein G beads. Each IP requires the addition of 500 µl Dilution Buffer. Wash the Protein G bead-antibody/chromatin complex by resuspending beads in 0.5 ml each of the cold buffers in the order listed below [Low Salt Immune Complex Wash Buffer (cat.# 20-1 54), High Salt Immune Complex Wash Buffer (cat.# 20-1 55), High Salt Immune Complex Wash Buffer (cat.# 20-1 55), High Salt Immune Complex Wash Buffer (cat.# 20-1 55)]. The DNA fragment containing the HRE site was amplified by PCR (SYBR-Green Master Mix, Takara Biotechnology Co., Ltd.) using the following primers: forward: 5'-TGG GAA AAG GCA TAG ACT GG-3', reverse: 5'‑ATG CAC GAA TGA GTG CCA TCC AAT CGG TAG TAG CG-3'. PCR conditions were Pre-denaturation, 94˚C for 5 sec; followed by 40 cycles of denaturation, 95˚C for 5 sec, followed by 40 cycles of extension (GO:0006351). transcription pattern. Gene Ontology (GO, http://geneontology.org/) enrichment and Kyoto encyclopedia of genes and genome (KEGG, http://www.genome.jp/kegg/) pathway enrichment analysis of differentially expressed genes were performed using R (R-v3.4.2, r-project.org) based on the hypergeometric distribution. GO datasets used in this research were as follows: response to hypoxia (GO:0001666), regulation of GPCR signaling (GO:001525), negative regulation of cell proliferation (GO:0008285), positive regulation of cell proliferation (GO:0008284), GPCR signaling (GO:0007186), transcription from RNA pol 2 promoter (GO:0045944), transcription, DNA-templated (GO:0006351).

Statistical analysis. Data are presented as the mean ± SEM. Each experiment was independently repeated three times. Statistical significance was calculated using one-way ANOVA and Tukey's multiple comparison test for ≥3 groups. Unpaired Student's t-test was used for comparison between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Tan 2A increases GLUT-1 expression and glucose uptake in HUVECs. To assess the effect of Tan 2A on GLUT-1 expression, HUVECs, a commonly used model for ECs (24), were treated with Tan 2A at 0, 15 and 30 µM for 24 h and 15 µM for 24 and 36 h. RT-qPCR results demonstrated that mRNA expression levels of GLUT-1 in HUVECs exposed to 15 µM Tan 2A increased significantly at 24 and 36 h compared with 0 h control (Fig. 1A). The relative mRNA expression levels of GLUT-1 increased significantly to 3.18±0.32 and 3.65±0.51 following 15 and 30 µM treatment for 24 h, respectively, compared with the 0 h control. (Fig. 1B). Furthermore, GLUT-1 protein expression levels were assessed using western blotting. The protein expression levels were consistent with the mRNA expression levels of GLUT-1, also exhibiting upregulation by Tan 2A (Fig. 1C and D). Moreover, endothelial glucose uptake was assessed using glucose
Figure 1. Tan 2A increases expression of GLUT-1 in HUVECs. (A) HUVECs were treated with Tan 2A or DMSO for 24 and 36 h and GLUT-1 relative mRNA expression levels were examined using RT-qPCR. (B) RT-qPCR of GLUT-1 relative mRNA expression levels in HUVECs treated with Tan 2A at 15 and 30 µM or DMSO for 24 h. ECs were treated with Tan 2A for (C) different durations or (D) increasing concentrations for 24 h and protein expression levels of GLUT-1 were assessed using western blotting. (E) Glucose uptake in HUVECs was assessed following treatment with Tan 2A for 24 h using Colorimetric Glucose Uptake Assay Kit. (F) HUVECs were transfected with siGLUT-1 or scrambled siRNA as a control for 48 h and (G) relative mRNA and protein expression levels of GLUT-1 were detected by RT-qPCR and western blotting, respectively. (H) HUVECs were transfected with siGLUT-1 or control siRNA for 24 h and treated with Tan 2A for 24 h. Data are presented as the mean fold increase ± SEM of three independent experiments. *P<0.05 and **P<0.01. Tan 2A, Tanshinone 2A; HUVEC, human umbilical vein endothelial cell; RT-q, reverse transcription-quantitative; GLUT-1, glucose transporter 1; 2-DG, 2-deoxy-glucose; si, small interfering; NC, negative control.
analog 2-DG. Glucose uptake in HUVECs was significantly enhanced by Tan 2A compared with DMSO control (Fig. 1E). siRNA mediated knockdown of GLUT-1 was evaluated using RT-qPCR and western blotting (Fig. 1F, G), there was a significant decrease compared with the scrambled siRNA. GLUT-1 knockdown significantly decreased the promotive effect of Tan 2A on glucose uptake compared to the scrambled siRNA control (Fig. 1H). These result demonstrated that Tan 2A induced expression of GLUT-1 and thus promoted glucose uptake in HUVECs.

**Tan 2A induces expression of GLUT-1 via HIF-1α in HUVECs.**

RNAseq was used to evaluate the potential mechanisms of the aforementioned effect of Tan 2A in HUVECs. Gene Ontology analysis demonstrated that ‘response to hypoxia’ was markedly induced by Tan 2A (Fig. 2A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs was performed (Fig. 2B). The enriched KEGG pathways were related to biological processes such as glucose metabolism, signal transduction, and cell adhesion. A significant increase in the expression of transcription factor HIF-1α was observed in HUVECs treated with Tan 2A (Fig. 2C, D). The mRNA expression levels of VEGF, BNIP3, ENO2, PGAM1, PKM and ALDOA in control and Tan 2A-treated HUVECs were analyzed using RT-qPCR (n=3). The mRNA expression levels of GLUT-1, VEGF, BNIP3 and ENO2 in control and Tan 2A-treated HUVECs were analyzed using RT-qPCR (n=3). The mRNA expression levels of GLUT-1, VEGF, BNIP3 and ENO2 were analyzed using RT-qPCR (n=3). The mRNA expression levels of GLUT-1, VEGF, BNIP3 and ENO2 in control or HIF-1α-silenced HUVECs treated with Tan 2A for 24 h were analyzed using RT-qPCR (n=3). The HIF-1α silenced HUVECs were treated with Tan 2A for 24 h before western blotting of GLUT-1. Data from 3 independent experiments are presented. *P<0.05 and **P<0.01. Tan 2A, Tanshinone 2A; HUVEC, human umbilical vein endothelial cell; RT-q, reverse transcription-quantitative; GLUT-1, glucose transporter 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; VEGF, vascular endothelial cell growth factor; BNIP3, BCL2 interacting protein 3; ENO2, enolase 2; HIF-1α, hypoxia-inducible factor-1α; PGAM1, phosphoglycerate mutase 1; PKM, pyruvate kinase M1/2; ALDOA, aldolase; CTRL, control; si, small interfering; G Protein-Coupled Receptor, GPCR, RAGE, Receptor for AGE; stem cell (SC).
Genes and Genomes enrichment analysis demonstrated that genes were markedly enriched in ‘HIF-1α signaling pathway’ (Fig. 2B). Furthermore, RNA-seq analysis demonstrated that seven genes were differentially expressed (>1.5 fold) in ‘HIF-1 signaling pathway’, including GLUT-1, vascular endothelial growth factor A (VEGFA), BCL2 interacting protein 3 (BNIP3) and enolase 2 (ENO2) in HUVECs (Fig. 2C). The results of the RNAseq were evaluated using RT-qPCR. There were significant increases in mRNA expression levels of VEGF, BNIP3 and ENO2 following treatment with Tan 2A compared with the control (Fig. 2D-F). Knockdown of HIF-1α significantly inhibited Tan 2A-induced mRNA expression of GLUT-1, VEGFA, BNIP3 and ENO2 in HUVECs (Fig. 2G-K). Western blotting demonstrated that Tan 2A-induced protein expression of GLUT-1 was also significantly reversed by HIF-1α knockdown (Fig. 2L). These results suggested that Tan 2A regulated GLUT-1 expression in HUVECs via the HIF-1α signaling pathway.

**Tan 2A increases HRE activity in HUVECs.** To assess regulation of HIF-1α by Tan 2A in HUVECs, luciferase reporter assay was performed using constructs with the regulatory region of the HRE. The HRE displayed significantly increased luciferase activity in response to Tan 2A compared with the control (Fig. 3A). Chip assay was performed to assess the regulatory effect of Tan 2A on the binding ability of HIF-1α to the potential HRE within the promoter region of the GLUT-1 gene and demonstrated that binding of HIF-1α to the promoter region of GLUT-1 gene was significantly enhanced by Tan 2A in HUVECs (Fig. 3B). HIF-1α is hydroxylated by prolyl hydroxylases (25). RT-qPCR demonstrated that Tan 2A significantly increased expression of EGLN family hypoxia inducible factor 3 (EGLN3), which belongs to prolyl hydroxylase family, compared with the control, these data suggested that EGLN3 mediated Tan 2A-induced activation of HIF-1α in HUVECs (Fig. 3C). These results showed that RBPJκ mediates regulation of GLUT-1 expression in response to Tan 2A.

**Discussion**

Under both normal and pathological conditions, the concentration of glucose in neurons is strictly controlled and relies on sustained blood flow and glucose transport (28). Therefore, altered expression of GLUT-1 at the blood-brain barrier affects the function of neurons. In the present study, Tan 2A regulated GLUT-1 expression and glucose uptake in HUVECs. Furthermore, Tan 2A enhanced the interaction between HIF-1α and RBPJκ and mediates the expression of glucose transporters in neurons (27, 29). These results suggested that Tan 2A may facilitate recovery of brain function via upregulation of the GLUT-1 to increase glucose absorption.

The energy demand of neurons in the brain is served by glucose transported from the blood. Glucose is transported into brain cells via members of the GLUT family. GLUT-3 is primarily expressed in neurons, while GLUT-1 gene expression is limited to ECs in the healthy brain (29) and regulates glucose transport into the brain (30, 31). Two subtypes of GLUT-1, a 55 kDa isoform in brain ECs and a 45 kDa isoform in adjacent astrocytes, have been reported previously (32).

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Previous studies have reported that changes in expression of GLUT affect the function of neurons and that GLUT-1 haploid deficiency induces decreased brain weight, activated astrocytosis, impaired motor performance and diminished brain glucose metabolism in mice (28,33). Our results showed that Tan 2A upregulated GLUT-1 expression and 2-DG uptake in HUVECs. In addition to its role in transporting glucose into the brain (34), GLUT-1 is needed for maintenance of proper brain capillary networks, blood flow and blood-brain barrier integrity (35). Furthermore, inhibition of GLUT-1 decreases EC glucose uptake and glycolysis, which leads to energy depletion, activation of the cellular energy sensor AMPK and decreased EC proliferation (5), as well as decreased nitric oxide-dependent endothelial relaxation (36).

Tan 2A is used in the treatment of cardio- and cerebrovascular disorders, such as coronary heart disease and cerebral infarction (37). Tan 2A exerts anti-atherosclerosis, anti-cardiac hypertrophy and antioxidant effects via regulation of the expression of numerous molecules, including transcription factors, scavenger receptors, ion channels, pro- and anti-apoptotic proteins, growth factors, inflammatory mediators and microRNAs (38). Tan 2A protects against cardiovascular disease via regulation of Akt/glycogen synthase...
levels of GLUT-1 in HUVECs. Furthermore, Tan 2A markedly increased binding of HIF-1α and RBPJκ. It was therefore hypothesized that RBPJκ affects binding of HIF-1α to the HRE in the GLUT-1 promoter and that both RBPJκ and HIF-1α may be involved in regulation of GLUT-1 gene expression induced by Tan 2A. Whether Tan 2A plays the same role under anoxic conditions is still to be studied.

In summary, the present study suggested that Tan 2A induced expression of GLUT-1 via the HIF-1α signaling pathway (Fig. 6). Furthermore, Tan 2A may ameliorate brain glucose metabolism by regulating GLUT-1 mediated glucose transport in HUVECs.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Sequencing Read Archive repository (https://www.ncbi.nlm.nih.gov/sra/PRJNA871126).

Authors' contributions

ZL conceived and designed experiments. YZ and HZ drafted the manuscript. YZ, HZ, YH, SW and ZL performed the experiments and analyzed the data. All authors have read and approved the final manuscript. YZ and ZL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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