Research Article

Atorvastatin combined with low-dose dexamethasone for vascular endothelial cell dysfunction induced by chronic subdural hematoma

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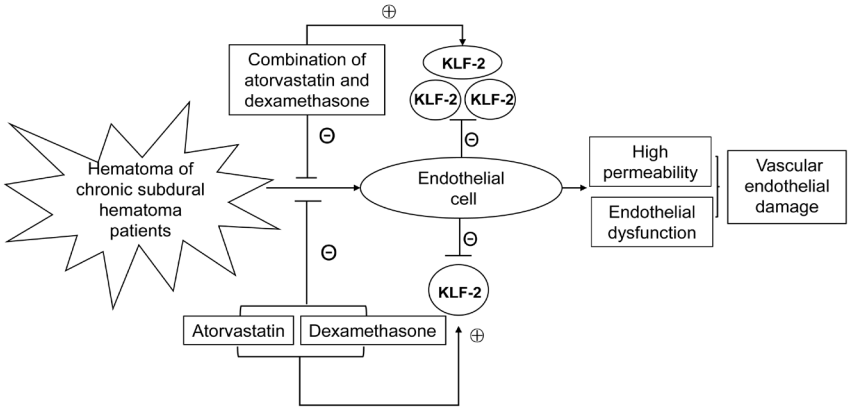
Graphical Abstract *Treatment with atorvastatin and low-dose dexamethasone rescues endothelial cell dysfunction after hematoma injury*



Abstract

Atorvastatin has been shown to be a safe and effective non-surgical treatment option for patients with chronic subdural hematoma. However, treatment with atorvastatin is not effective in some patients, who must undergo further surgical treatment. Dexamethasone has anti-inflammatory and immunomodulatory effects, and low dosages are safe and effective for the treatment of many diseases, such as ankylosing spondylitis and community-acquired pneumonia. However, the effects of atorvastatin and low-dose dexamethasone for the treatment of chronic subdural hematoma remain poorly understood. Hematoma samples of patients with chronic subdural hematoma admitted to the General Hospital of Tianjin Medical University of China were collected and diluted in endothelial cell medium at 1:1 as the hematoma group. Atorvastatin, dexamethasone, or their combination was added to the culture medium. The main results were as follows: hopping probe ion conductance microscopy and permeability detection revealed that the best dosages to improve endothelial cell permeability were 0.1 μM atorvastatin and 0.1 μM dexamethasone. Atorvastatin, dexamethasone, or their combination could markedly improve the recovery of injured endothelial cells. Mice subcutaneously injected with diluted hematoma solution and then treated with atorvastatin, dexamethasone, or their combination exhibited varying levels of rescue of endothelial cell function. Hopping probe ion conductance microscopy, western blot assay, and polymerase chain reaction to evaluate the status of human cerebral endothelial cell status and expression level of tight junction protein indicated that atorvastatin, dexamethasone, or their combination could reduce subcutaneous vascular leakage caused by hematoma fluid. Moreover, the curative effect of the combined treatment was significantly better than that of either single treatment. Expression of Krüppel-like factor 2 protein in human cerebral endothelial cells was significantly increased, as was expression of the tight junction protein and vascular permeability marker vascular endothelial cadherin in each treatment group compared with the hematoma stimulation group. Hematoma fluid in patients with chronic subdural hematoma may damage vascular endothelial cells. However, atorvastatin combined with low-dose dexamethasone could rescue endothelial cell dysfunction by increasing the expression of tight junction proteins after hematoma injury. The effect of combining atorvastatin with low-dose dexamethasone was better than that of atorvastatin alone. Increased expression of Krüppel-like factor 2 may play an important role in the treatment of chronic subdural hematoma. The animal protocols were approved by the Animal Care and Use Committee of Tianjin Medical University of China on July 31, 2016 (approval No. IRB2016-YX-036). The study regarding human hematoma samples was approved by the Ethics Committee of Tianjin Medical University of China on July 31, 2018 (approval No. IRB2018-088-01).

Key Words: brain; brain trauma; cells; central nervous system; inflammation; plasticity; protein; repair

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Introduction

Chronic subdural hematoma (CSDH), a common pathologic condition observed following neurosurgery, occurs primarily in elderly individuals (Wang et al., 2010; Kolias et al., 2014). Complex pathways, such as angiogenesis, inflammation, and coagulopathy, have been implicated in this disorder (Chan et al., 2017). Based on these pathophysiological pathways, some drug therapies, such as antithrombotics (Adelborg et al., 2016) and the angiotensin-converting enzyme inhibitors atorvastatin and dexamethasone (Guha et al., 2016; Poon and Al-Shahi Salman, 2018), have been utilized to prevent surgeries for CSDH.

Atorvastatin is often used to treat patients with high cholesterol and coronary heart disease (Zhang et al., 2018). Atorvastatin may also promote angiogenesis and decrease the effects of inflammation (Kureishi et al., 2000; Youssef et al., 2002; Chen et al., 2020). Previously, we used a subdural hematoma model to demonstrate that atorvastatin could rescue neurological deficiencies of rats by enhancing endothelial renewal and vascular maturation (Wang et al., 2016). Other studies have also demonstrated that statins could improve endothelium repair by promoting endothelial progenitor cells (Miller-Kasprzak and Jagodzinski, 2007; Caporali et al., 2008; Li et al., 2012). Based on a previous randomized clinical trial involving 200 patients, we further demonstrated that atorvastatin is a safe and efficacious nonsurgical alternative for the treatment of CSDH (Jiang et al., 2018). However, in this research, nearly 11.2% of patients in the experimental group showed poor effects and required surgical treatment. Pathophysiological studies of patients treated with statins and in whom statin treatment was ineffective still need to be performed.

Previous studies regarding the effect of atorvastatin on CSDH demonstrated that statins could mediate neovascularization and repair via endothelial progenitor cells, improve vessel maturation by inhibiting vascular endothelial growth factor (VEGF) expression, and reduce the effects of inflammation (Song et al., 2013; Kolias et al., 2014; Sahebkar et al., 2015). However, single statin therapy has many limitations, such as slow action and weak inhibition of inflammation, which lead to the failure of treatment in some patients. Dexamethasone therapy has been evaluated in clinical studies, and many reports have revealed that dexamethasone could block inflammation during hematoma formation by specifically impeding the formation of neo-membranes and neo-capillaries (Drapkin, 1991). However, use of dexamethasone can lead to complications such as hyperglycemia, peptic ulcers, and pulmonary infection (Prud'homme et al., 2016; Zhang et al., 2017). Some studies have reported that low-dose dexamethasone can improve the prognosis of a variety of diseases without increasing the incidence of adverse drug events (Zhang et al., 2015; Fernandez-Codina et al., 2018; Lei et al., 2018).

Lung Krüppel-like factor 2 (KLF2), a member of the zinc finger family of DNA-binding transcription factors, is widely studied in cardiovascular diseases, whereby it reportedly plays an important role in endothelial proinflammatory activation (Gao et al., 2019). Indeed, KLF2 can reduce inflammation of endothelial cells (Jiang et al., 2020).

To continue this research, hematoma fluid samples collected from CSDH patients were used to injure endothelial cells. In this injury model, cells were treated with atorvastatin, dexamethasone, or atorvastatin combined with low-dose dexamethasone after hematoma injury to illustrate the mechanism of CSDH treatment.

Materials and Methods

Cell culture and hematoma stimulation (human samples)

The human cerebral endothelial cell line hCMEC/D3 (Cat# SCC075, Sigma, St. Louis, MO, USA), was cultured in endothelial cell medium (ScienCell Research Laboratories, Carlsbad, CA, USA) and grown on 0.1% collagen type I (Cat# 08-115, Sigma)-coated coverslips until a tight monolayer formed. The medium was replaced every other day. Hematoma samples were obtained from CSDH patients (n = 6) admitted to General Hospital of Tianjin Medical University of China for testing of inflammatory factors or other studies. Informed consent was obtained from all patients.

Inclusion criteria of patients

Patients aged ≥ 18 years and < 90 years, irrespective of sex, were enrolled in this study. Other inclusion criteria included: 1) head computed tomography (CT) examination indicating lesions located in supratentorial and unilateral chronic subdural hematoma, 2) the volume of hematoma was large, 3) the patient needed drilling and drainage operation, and 4) the patient had not previously undergone hematoma surgery.

Exclusion criteria of patients

Patients with known hypersensitivity to the drug components were excluded, as were patients with hematoma accompanied by tumor, hematopathy, tuberculosis, arachnoid cyst, vascular malformation, or ventriculoperitoneal shunt. Patients with severe abnormal heart, lung, liver or kidney functions, which could affect the overall prognosis, were excluded. In addition, patients with poor control of blood glucose (fasting blood glucose of more than 10 mM) or a history of oral statins or steroid hormones within 4 weeks before operation were excluded. Patients who used anticoagulants, such as warfarin, or exhibited obviously abnormal coagulation function were also excluded. Finally, patients who had recently participated in clinical trials for other drugs were excluded.

Preparation of human hematoma samples

The supernatant of each hematoma was collected after centrifugation at 1000 × g for 15 minutes and stored at –80°C. Before use, hematoma samples were diluted with endothelial cell medium at a 1:1 ratio, and then centrifuged at 1000 × g for 15 minutes. Concentrations of interleukin-6, interleukin-8, interleukin-10, and VEGF were tested by enzyme-linked immunosorbent assay after the hematoma was collected, mixed, and diluted at a 1:1 ratio. The results and patients are presented in **Table 1**.

Subsequently, diluted samples were cocultured with hCMEC/D3 cells for 75 minutes. Next, the diluted samples were removed from the incubator and the medium was supplemented with phosphate-buffered saline (PBS), atorvastatin (Cat# PZ0001; Sigma), dexamethasone (Cat# D1756, Sigma), or a combination of atorvastatin and dexamethasone. Samples were then cocultured with hCMEC/D3 cells for 1 hour. The sham group was cultured in normal medium for 135 minutes. Cells were randomly divided into five groups: sham group (n = 3, PBS), hematoma stimulation group (n = 3, diluted hematoma fluid), single atorvastatin treatment group (n = 3, atorvastatin added after hematoma fluid injury), single dexamethasone treatment group (n = 3, dexamethasone added after hematoma fluid injury), and combination treatment group (n = 3, atorvastatin and dexamethasone added after hematoma fluid injury). The experimental method and protocol for the study regarding human hematoma samples were approved by Ethics Committee of Tianjin Medical University of China on July 31, 2018 (approval No. IRB2018-088-01).

Table 1 | **Concentration of factors in hematoma samples of patients with chronic subdural hematoma**

Items	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Mixed patients' hematoma	Diluted with ECM as the ratio of 1:1
Sex	Male	Male	Female	Male	Male	Male		
Age (yr)	65	59	73	76	68	56		
Hematoma volume (mL)	120	90	110	130	120	70		
IL-6 (pg/mL)	37892	18984	7845	6340	5984	34677	17584.21	9083.9
IL-8 (pg/mL)	4589	9803	21569	3598	18950	8790	10849.5	7509.2
IL-10 (pg/mL)	20	10	50	146	90	40	54.61	20.91
VEGF (pg/mL)	7935	17907	9564	5436	28984	37892	19262.19	10982.8

ECM: Endothelial cell medium; IL: interleukin; VEGF: vascular endothelial growth factor.

Hopping probe ion conductance microscopy (HPICM) scanning (human samples)

As previously described (Novak et al., 2009; Liu et al., 2011), an HPICM system composed of an ICnano scanner controller (Ionscope Ltd., Cambridge, UK) and scanning head SH01 (Ionscope Ltd.) was used to detect cell contact without causing damage to cell structures. This technique has nanoscale resolution, and the surface structure of living cells was directly and dynamically observed in the culture medium. After cells were stimulated with hematoma samples, they were scanned for 2 hours. During scanning, PBS, atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone were added. The height of cocultured cells was measured with the HPICM system. Data were obtained after cell images were scanned.

Immunofluorescence (human samples)

hCMEC/D3 cells were collected 1 hour after treatment and washed three times with PBS. The cell membrane was then disrupted with ice-cold acetone for 30 minutes and blocked with goat serum at 37°C for 30 minutes. Cells were incubated overnight with a rabbit anti-vascular endothelial cadherin antibody (1:400; Cat# 2500; Cell Signaling Technology, Danvers, MA, USA), a marker of tight junction proteins to reflect changes of cell permeability, followed by Alexa Fluor-conjugated anti-rabbit IgG (1:500; Cat# 35569; Thermo Fisher, Waltham, MA, USA) for 1 hour at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole for 5 minutes. Immunostaining of cells was visualized under a fluorescence microscope (Olympus, Tokyo, Japan) and captured.

FITC-dextran transport studies (human samples)

Single cell suspensions of hCMEC/D3 cells were placed onto a collagen-coated insert (2×10^5 cells/insert, in 24-well plates; Cat# 3378; Corning, Steuben County, NY, USA) and allowed to grow for 3 days for monolayer formation. Thereafter, the monolayer was treated with atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone in the hematoma sample for 1 hour. FITC-dextran was added to the insert at a ratio of 1:40. Fluorescence intensities (485-nm excitation and 535-nm emission) were then measured with a Cycle 5 fluorometer (BioTek, Winooski, VT, USA). Permeability was determined by measuring the fluorescence intensity of leakage into the lower chamber. Permeability was equal to the ratio of fluorescence intensity of the lower chamber to total fluorescence intensity.

Permeability assays (animal experiment)

Wild-type female athymic nude BALB/c-nu mice aged 6–8 weeks and weighing 17–20 g were obtained from Beijing HFK Bioscience Co., Ltd., Beijing, China (License No. SCXK [Jing] 2019-0008). In total, 50 mice were randomly divided into five groups: sham group (n = 10), hematoma stimulation group (n = 10), single atorvastatin treatment group (n = 10), single dexamethasone treatment group (n = 10), and combination treatment group (n = 10). After administration of Evans blue dye (1%, 3 mL/kg) via the tail vein for 20 minutes, 50 μL of the hematoma (with PBS, atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone) or PBS was subcutaneously injected into the back of the skin. After 30 minutes, animals were euthanatized. The injected skin was dissected, photographed, and extracted by incubation with formamide at 60°C overnight. Absorbance was measured at 620 nm. Animal protocols were approved by the Animal Care and Use Committee of Tianjin Medical University on July 31, 2016 (approval No. IRB2016-YX-036).

Western blot assay (human samples)

Cell samples were collected after drug treatment. Western blot assay was performed as previously described (Rong et al., 2018). Protein samples of hCMEC/D3 cells were incubated overnight with a primary rabbit monoclonal antibody or anti-vascular endothelial cadherin antibody (1:1000; Cat# 2500; Cell Signaling Technology) at 4°C overnight. Subsequently, anti-rabbit horseradish peroxidase-conjugated secondary IgG (1:5000; Beyotime Biotechnology, Beijing, China) was added for 1 hour at room temperature. Membranes were developed with an enhanced chemiluminescence system (Millipore, Billerica, MA, USA). Protein samples were quantified with ImageJ software 1.8.0_112 (National Institutes of Health, Bethesda, MD, USA). β-Actin served as the control.

Quantitative real-time polymerase chain reaction (qRT-PCR) (human samples)

Cell samples were collected after drug treatment. TRIzol™ Reagent (Cat# A33251, Invitrogen, Carlsbad, CA, USA) was used to isolate sample RNAs. A NanoDrop 2000 (ND-2000, Thermo Fisher) was utilized to evaluate the concentration and quality of collected RNA samples. qRT-PCR was performed as previously described (Wang et al., 2016). Rp0 was used as the internal reference standard. Primers used for qRT-PCR are listed in **Table 2**. Data were analyzed by the comparative Ct method. Fold changes were calculated from the $2^{-\Delta\Delta Ct}$ equation for each mRNA.

Gene sequencing arrays (human samples)

mRNA was collected after the rescue treatment. Based on different characteristics of mRNAs and the polyA tail structure on the 3′ end of eukaryotic mRNA, magnetic beads with oligo(dT) were selected for enrichment and purification following total RNA quantification. Fragmentation buffer was added to the purified mRNA to create a short fragment. First-strand cDNA was synthesized with six-base random primers using the mRNA fragment as a template. Second-strand cDNA was synthesized by adding buffer, dNTPs, RNaseH, and DNA Polymerase I; followed by purification with a QIAQuick PCR kit (Cat# 28106; Qiagen, Hilden, Germany) and elution with EB

Table 2 | **Primer sequences used in this study**

Gene	Primer sequence (5'–3')	Product size (bp)
VE-cadherin	Forward: TTC ACC CAG ACC AAG TAC ACA T	22
	Reverse: GCT TGA TGC CCT CGT TG	20
Claudin-5	Forward: AGA TTG AGA GGT CTG GGA AGC C	22
	Reverse: GCT TGA TGA TGC CCT CGT TG	22
ZO-1	Forward: ATA AAG AGA AAG GTG AAA CAC TGC T	25
	Reverse: AGC ACT GTC TCT CTC ATC CCA T	20
Rp0	Forward: TTC ATT GTG GGA GCA GAC	18
	Reverse: TCA CAG TGT GGT AAG CGC AG	18
KLF2	Forward: CAC GCA CAC AGG TGA GAA G	24
	Reverse: CAG CAG TTT CTC CAG AGC	23

KLF2: Krüppel-like factor 2; Rp0: ribosomal protein lateral stalk subunit P0; VE-Cadherin: vascular endothelial cadherin; ZO-1: zonula occludens-1.

buffer. After elution and purification, double-stranded cDNA was treated with terminal repair, base A, and sequencing junctions. Target fragments were then recovered by agarose gel electrophoresis and amplified by PCR to complete preparation of the whole library.

Following complete construction, the library was initially quantified with a Qubit3.0 system and diluted to 1 ng. The insert size of the library was detected with an Agilent 2100 and found to be the expected size. Thereafter, a CFX 96 fluorescence quantitative PCR instrument (Bio-Rad, Hercules, CA, USA) and iQ SYBR GRN kit (Cat# 170-8880, Bio-Rad) were used for qPCR to accurately quantify the effective concentration of the library (library effective concentration > 10 nM) to ensure its quality.

Qualified libraries were sequenced using the Illumina platform (San Diego, CA, USA) and PE150 sequencing strategy (DNA Technologies and Expression Analysis Core, Davis, CA, USA).

Statistical analysis

Data were analyzed with GraphPad Prism software 8.0 (IBM, Armonk, NY, USA) and are presented as the mean ± SD. One-way analysis of variance followed by the least significant difference *post hoc* tests was employed to analyze the results of western blot assay, qPCR, and Evans blue dye tests. Two-way analysis of variance followed by Tukey's multiple comparisons test was used to analyze the results of cell height measurements. A *P*-value less than 0.05 was considered statistically significant.

Results

Detection of optimum timing and dosage of drugs after hematoma injury by HPICM and permeability test (human sample)

Hematoma fluid was added to hCMEC/D3 cells before scanning via HPICM for 2 hours. The results showed that the status of cells began to change at 52 minutes. Images revealed that cell morphology, including cell bodies and the space between cells, began to change following coculture with hematoma samples. The height of cocultured cells was also significantly changed and almost doubled at 75 minutes (**Figure 1A**). All treatment groups were assessed simultaneously to ensure consistency. Based on the images obtained with HPICM, 75 minutes was selected as the injury time.

The effects of atorvastatin and dexamethasone were evaluated by a Transwell assay after the hematoma sample was used applied to hCMEC/D3 cells for 75 minutes. By using different doses of atorvastatin to treat cells with hematoma injury, we found that high-dose atorvastatin (5 or 10 μM) significantly increased cell permeability, indicating that high-dose atorvastatin could induce the injury to endothelial cells. However, when cells were treated with 0.1 μM or 0.5 μM atorvastatin, permeability was substantially decreased compared with cells treated with hematoma alone. No differences were observed when atorvastatin concentrations of 0.5 μM or 0.1 μM were added to cells treated with the hematoma fluid (**Figure 1B**).

In the experiment using dexamethasone to rescue injury to cells induced by hematoma samples, the permeability was not found to be significantly decreased with high-dose treatment (1 μM, $P > 0.05$); however, 0.05, 0.1, and 0.5 μM dexamethasone obviously reduced cell permeability ($P < 0.05$). Moreover, 0.1 μM substantially decreased the leakage between cells (**Figure 1C**). Based on these results, 0.1 μM atorvastatin and 0.1 μM dexamethasone were selected as safe doses for further studies.

Combined treatment maintains the stability of hCMEC/D3 cells in response to injury caused by hematoma fluid (human sample)

After the addition of hematoma samples with PBS, atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone, changes in hCMEC/D3 cells were observed within 2 hours by HPICM. Images revealed that the morphology, including cell bodies and spaces between cells, began to change after cells were cocultured with hematoma samples for 75 minutes. In the treatment groups, these phenomena were not notable and the height of cells decreased, while the cell surface became smooth compared

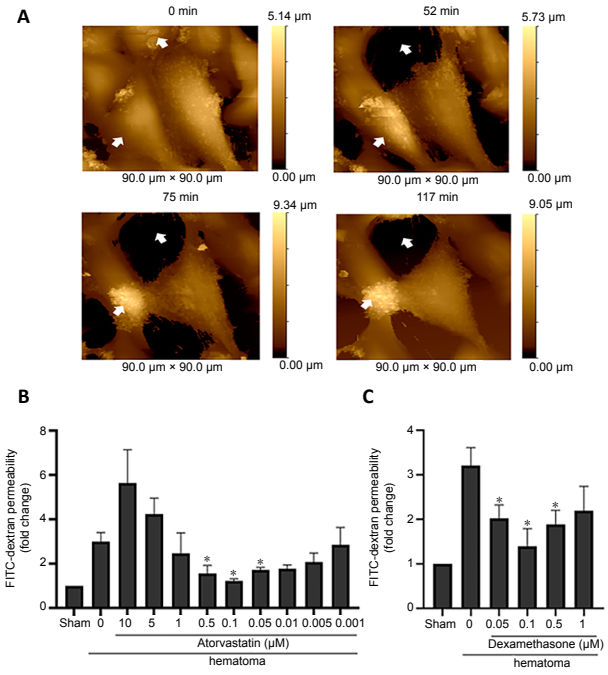

Figure 1 | **The optimum time required for induction of cell damage by hematoma fluid, and optimum drug dosage for treatment of cells after hematoma injury.**

(A) Hopping probe ion conductance microscopy was employed to scan morphological changes of the human cerebral endothelial cell line (hCMEC/D3) following hematoma injury. White arrows indicate intercellular spaces. (B) Relative concentrations of FITC-dextran that passed through hCMEC/D3 monolayers in PBS or atorvastatin treatment groups after hematoma injury. (C) Relative concentrations of FITC-dextran that passed through hCMEC/D3 monolayers in the PBS or dexamethasone treatment groups after hematoma injury. Data are expressed as the mean ± SD (one-way analysis of variance followed by the least significant difference *post hoc* test). *$P < 0.05$, *vs.* hematoma group. (B, C) Independent experiments were conducted twice. FITC: Fluorescein isothiocyanate; PBS: phosphate-buffered saline.

with the hematoma stimulation group. These effects were more significant in the combination treatment group, whereby cells showed slow recovery compared with the single treatment groups over 120 minutes (**Figure 2**).

Atorvastatin and low-dose dexamethasone increase VE-cadherin expression, rescuing vascular permeability after hematoma-induced injury (human sample)

Immunofluorescence staining, western blot assay, and qRT-PCR were performed to investigate changes in intercellular junction protein expression and vascular permeability. The fluorescence of VE-cadherin revealed a significant change in its distribution between control and hematoma-treated cells. In addition, endothelial cells damaged by the hematoma sample had a disordered arrangement. However, such changes were decreased after single or combination treatment. These results indicate that VE-cadherin expression was highest in the combination treatment group compared with other groups (**Figure 3A**).

Changes in VE-cadherin, claudin-5, and ZO-1 expression levels were examined by western blot assay and qRT-PCR (**Figure 3B–E**). Expression of these markers was reduced by hematoma stimulation. However, after single or combination treatment, expression levels were rescued to various degrees. However, VE-cadherin levels were most significantly increased in the combination treatment group.

We further used a monolayer of hCMEC/D3 cells on a Boyden chamber-like porous insert, which was exposed to various treatments (as described above) for 75 minutes after hematoma injury. Thereafter, FITC-dextran (70 kDa) was added to the insert, and the amount of fluorescent dye that infiltrated the endothelial monolayer was measured. Combined treatment strongly prevented the dye from infiltrating the monolayer compared with single or hematoma sample treatment (**Figure 3F**).

These results suggest that atorvastatin combined with low-dose dexamethasone treatment could improve the permeability of endothelial cells after hematoma sample injury.

KLF2 expression is significantly increased in cells treated with low-dose dexamethasone and atorvastatin after hematoma injury (human sample)

Total RNA was collected from different groups of cells, and gene expression was detected using genetic sequencing. In total, 135 genes were screened out by Cluster 3.0 (bonsai.hgc.jp) to cluster differentially expressed genes with a multiple of difference > 1 and $Q \leq 0.05$ between combination treatment (atorvastatin combined with low-dose dexamethasone) and hematoma stimulation groups. The top 50 genes are shown in a heat map (**Figure 4A**). The level of KLF2 was the most apparently increased, and its expression in the combination treatment group was 13 times higher than the hematoma stimulation group. KLF2 expression was also highest in the combination treatment group compared with the single atorvastatin treatment group (**Figure 4B**). A western blot assay was performed to verify KLF2 expression levels (**Figure 4D**) in different treatment groups, and the results were consistent with our RNA-seq results (**Figure 4C**). Thus, our results indicate that KLF2 may have a critical effect on the pathophysiology of CSDH and serve as a good target for further studies.

Atorvastatin and low-dose dexamethasone treatment reduces high permeability *in vivo* (animal experiment)

To investigate the effect of the three different treatments *in vivo*, female athymic nude mice were injected with Evans blue dye via the tail vein. After 20 minutes, hematomas were

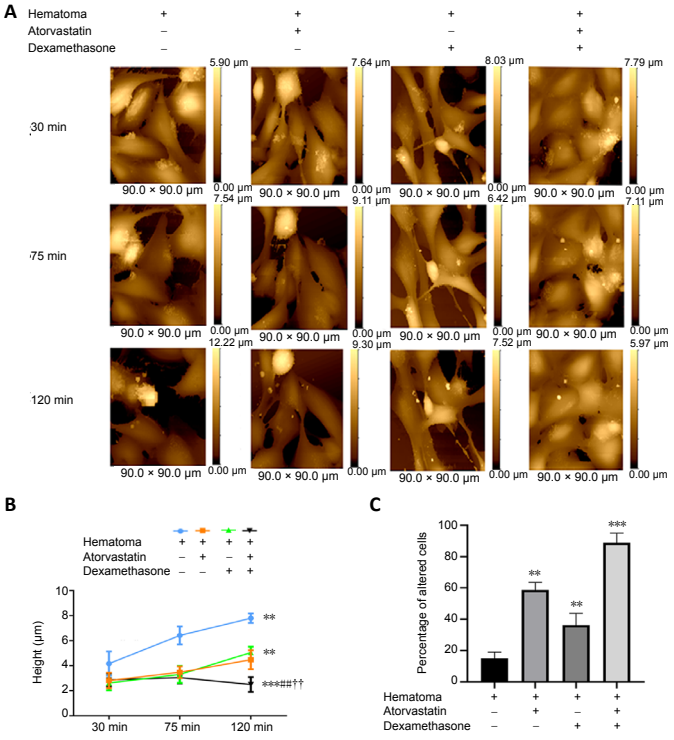

Figure 2 | Atorvastatin and low-dose dexamethasone treatment maintains the stability of human cerebral endothelial cell line (hCMEC/D3) following injury.

(A) Hopping probe ion conductance microscopy analysis of changes in hCMEC/D3 cells with atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone following hematoma-induced injury at various scan times. (B) Changes in cell height. (C) Percentage of altered cells after treatments. Data are expressed as the mean ± SD (n = 3; B, two-way analysis of variance followed by Tukey's *post hoc* test; C, one-way analysis of variance followed by the least significant difference *post hoc* test). $^{**}P < 0.01$, $^{***}P < 0.001$, *vs.* hematoma (+) atorvastatin (–) dexamethasone (–) group; $^{\#\#}P < 0.01$, *vs.* hematoma (+) atorvastatin (+) dexamethasone (–) group; $^{\dagger\dagger}P < 0.01$, *vs.* hematoma (+) atorvastatin (–) dexamethasone (+) group. Experiments were conducted twice.

mixed with PBS, atorvastatin, dexamethasone, or atorvastatin + dexamethasone for subcutaneous injection into the back-skin vessels. Visual inspection revealed that both single and combined treatments (**Figure 5A**) could reduce leakage of the dye. In addition, the effect of atorvastatin combined with low-dose dexamethasone was significantly greater compared with the single treatment (**Figure 5B**).

Discussion

The discovery of a drug therapy to treat hematoma and prevent its recurrence after surgery is important for patients with CSDH. In fact, some studies have demonstrated the effectiveness of treating CSDH with drugs such as statins and hormones. However, the specific mechanism of these treatments has seldom been discussed (Zhang et al., 2017; Jiang et al., 2018; Miah et al., 2018). This study sought to examine the potential mechanism of CSDH drug therapy *in vitro* and *in vivo*. Hematoma fluid samples were used to simulate partial disease status to identify the most effective treatment strategy for CSDHs and obtain data for existing CSDH treatments. Atorvastatin combined with low-dose dexamethasone significantly improved the function and permeability of vascular endothelial cells after injury caused by CSDH hematoma.

For many years, researchers have attempted to elucidate the mechanism of CSDH formation. However, to date, the detailed pathogenesis has not been thoroughly revealed.

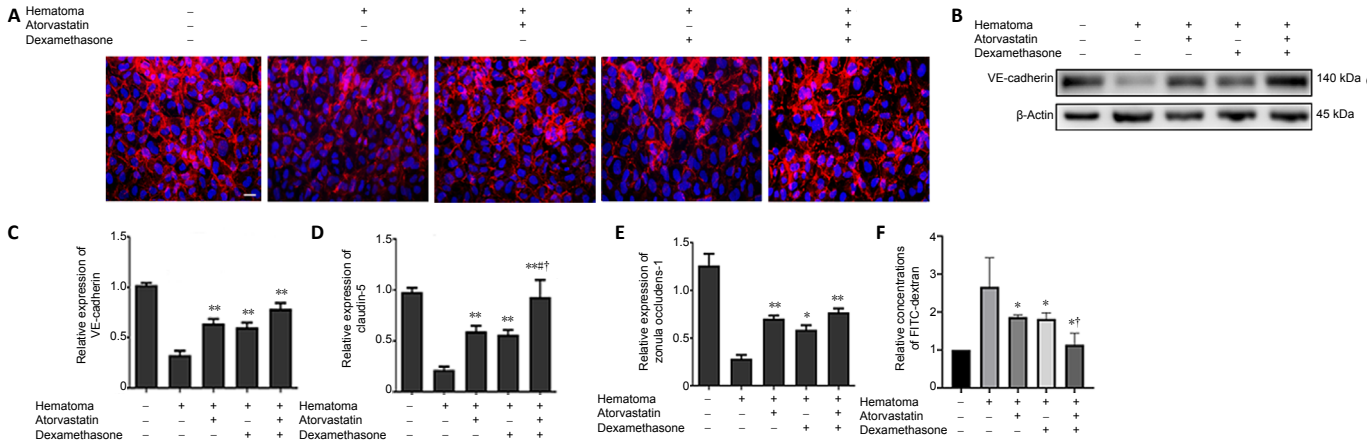

Figure 3 | **Atorvastatin and low-dose dexamethasone treatment increases expression of vascular endothelial cadherin (VE-cadherin) and other tight junction proteins, which rescue vascular permeability after hematoma-induced injury.**
(A) Immunofluorescence photomicrographs of VE-cadherin (red) expressed in the human cerebral endothelial cell line (hCMEC/D3) after treatment. Scale bar: 50 μm. (B) Western blot assay of VE-cadherin in hCMEC/D3 cells after treatment. (C–E) Relative expression of VE-cadherin, claudin-5, and zonula occludens-1 in hCMEC/D3 cells treated with PBS, atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone after hematoma injury, as measured by quantitative real-time polymerase chain reaction. Experiments were independently performed in triplicate. (F) Relative concentrations of FITC-dextran that passed through hCMEC/D3 cell monolayers treated with PBS, atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone after hematoma injury. Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by the least significant difference *post hoc* test). *P < 0.05; **P < 0.01, *vs*. hematoma (+) atorvastatin (–) dexamethasone (–) group. #P < 0.05, *vs*. hematoma (+) atorvastatin (+) dexamethasone (–) group; †P < 0.05, *vs*. hematoma (+) atorvastatin (–) dexamethasone (+) group. Experiments were conducted twice. FITC: Fluorescein isothiocyanate; PBS: phosphate-buffered saline; VE-cadherin: vascular endothelial cadherin.

Many studies have demonstrated that pathological neovascularization and inflammation play important roles in the progression of CSDH (Wang et al., 2010, 2014; Stanisic et al., 2012). Many biomarkers of angiogenesis and inflammation, such as VEGF, interleukin-6, and interleukin-8, are highly expressed in hematoma fluid samples. Moreover, these factors are strongly associated with the prognosis and recurrence of CSDH in post-operation patients (Berghauser Pont et al., 2012; Pripp and Stanisic, 2017). Consistent with the results of this study, a high concentration of VEGF and hyperinflammation caused damage to the tight junctions of neovascular endothelial cells in the hematoma capsule, leading to increased blood vessel permeability. This abnormal increase in vasoactive factors in the hematoma cavity results in increased neovascularization leakage and aggravation of inflammation. The hematoma capsule restricts absorption and diffusion of the hematoma, causing the subdural hematoma cavity to locally form a harmful microenvironment that results in a gradual increase in hematoma.

The results of the injury experiment could explain the leakage associated with neovascularization in the hematoma wall of CSDH patients, which causes exudation to be greater than absorption. Many fresh erythrocytes and hematoma were also found in the hematoma cavity. Therefore, based on vascular repair, stability, and control of local inflammation, the combined treatment is expected to have the strongest therapeutic effect on hematoma.

Combined with the results of our phase II proof-of-concept clinical trial, we demonstrated that atorvastatin combined with low-dose dexamethasone was more effective than atorvastatin alone in reducing hematoma and improving neurological function of patients with CSDH. These data will be published in the near future. In addition to our basic study, we demonstrated that the effect of the combined treatment is better than single treatment. Moreover, RNA-seq analysis identified KLF2 as playing a potentially critical role in both the pathophysiology of CDSH and benefits elicited by combined treatment with atorvastatin and dexamethasone.

To further analyze the mechanism of drug therapy, we performed gene sequencing. KLF2 was found to be significantly altered after combination treatment. Previous studies showed that endothelial KLF2 expression is induced by statins, and some of the multipotent effects of statins in endothelial cells are dependent on KLF2 (Miao et al., 2015). In our experiments, we observed a similar result. Interestingly, however, KLF2 levels in the combination treatment group were almost double those of the single atorvastatin treatment group. Therefore, we speculate that KLF2 plays an important role in combined CSDH therapy.

This study had some limitations. First, dexamethasone and statins act on different pathways and cells, but in this experiment, only the effects of dexamethasone and statins on vascular endothelial cells were observed. Therefore, our results do not fully represent the complete mechanism and target direction of CSDH therapy. In future experiments, multicellular cocultures should be established based on the results of gene detection to comprehensively elucidate the causes of disease development and evaluate drug mechanisms and targets.

In summary, the hematoma fluid of patients with CSDH could damage the vascular endothelium; however, treatment with atorvastatin and low-dose dexamethasone could rescue endothelial cell dysfunction by increasing the expression of tight junction proteins after hematoma injury. The effect of combined treatment was also significantly enhanced compared with single atorvastatin treatment. KLF2 may play an important role in the treatment of CSDH.

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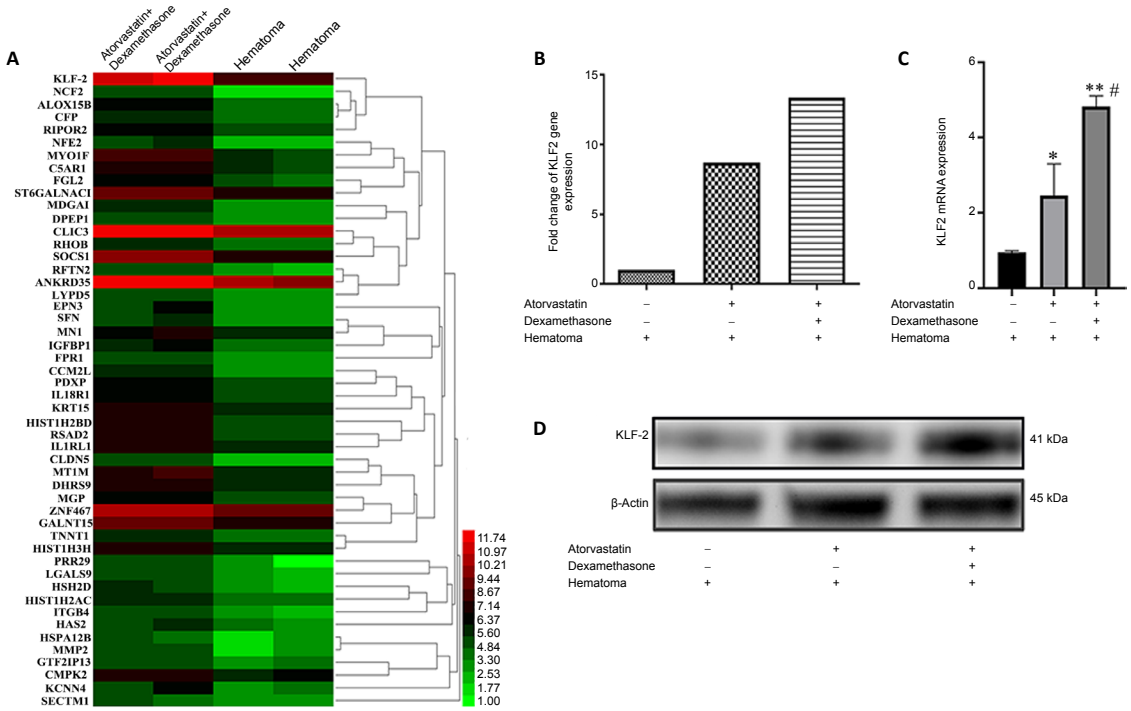

Figure 4 | **KLF2 expression is significantly increased in the combination treatment group after hematoma injury.**
(A) Heat map representation of gene sequencing data for altered expression levels of the top 50 genes in cell lines subjected to combination treatment after hematoma injury (red indicates high expression, green indicates low expression). (B) Fold change of KLF2 gene expression in different treatment groups (qRT-PCR), compared with the hematoma stimulation group. (C) qRT-PCR analysis of KLF2 mRNA expression in different treatment groups. (D) Western blot assay of changes in KLF2 expression in various treatment groups, compared with the hematoma stimulation group. Data are expressed as the mean ± SD (n = 3; one-way analysis of variance followed by the least significant difference *post hoc* test). *P < 0.05, **P < 0.01, *vs*. hematoma (+) atorvastatin (–) dexamethasone (–) group; #P < 0.05, *vs*. hematoma (+) atorvastatin (+) dexamethasone (–) group. KLF2: Krüppel-like factor 2; qRT-PCR: quantitative real-time polymerase chain reaction.

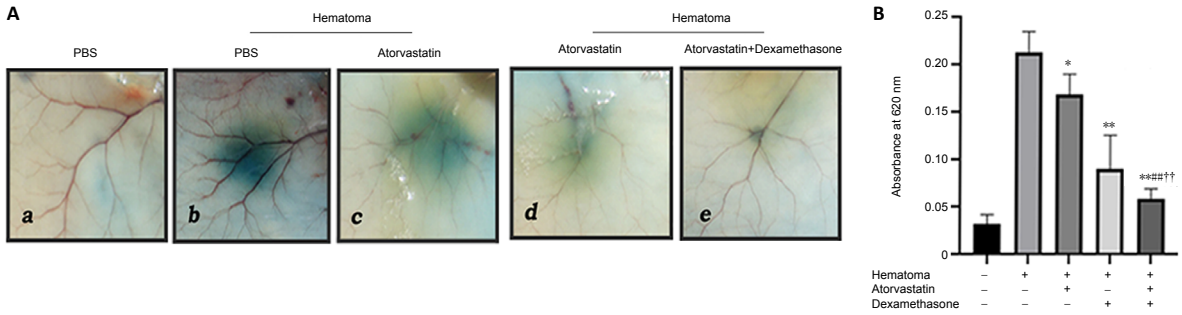

Figure 5 | **Atorvastatin and low-dose dexamethasone treatment reduces high permeability *in vivo*.**
(A) Evans blue images of hematoma-induced leakage into the skin with PBS, atorvastatin, dexamethasone, or a combination of atorvastatin and dexamethasone. (B) Quantitative analysis of dye extracted from sham-, hematoma-, atorvastatin-, dexamethasone-, or combination-treated skin samples retrieved from experimental animals. Data are expressed as the mean ± SD (n = 10; one-way analysis of variance followed by the least significant difference *post hoc* test). *P < 0.05; **P < 0.01, *vs*. hematoma (+) atorvastatin (–) dexamethasone (–) group. ##P < 0.01, *vs*. hematoma (+) atorvastatin (+) dexamethasone (–) group. ††P < 0.01, *vs*. hematoma (+) atorvastatin (–) dexamethasone (+) group. PBS: Phosphate-buffered saline.

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Biostatistics statement: *The statistical methods of this study were reviewed by the biostatistician of General Hospital of Tianjin Medical University.*

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