Original Article

The regulatory role and mechanism of circadian rhythm in hemoglobin co-cultured neurovascular unit*

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Abstract Cerebral hemorrhage, the second most prevalent subtype of stroke, exacerbates blood-brain barrier (BBB) disruption, leading to vasogenic edema, plasma protein extravasation, and infiltration of neurotoxic substances. The brain's clearance capacity plays a crucial role in maintaining BBB homeostasis and facilitating patient recovery post-hemorrhage. This study aims to investigate the impact of circadian rhythms on BBB function, neuronal damage, and clearance capabilities. A Neurovascular Unit (NVU) model was established through a four-cell co-culture of neurons, astrocytes, microglia, and brain microvascular endothelial cells (BMECs). Circadian rhythms were found to play a pivotal role in preserving BBB integrity, reducing oxidative stress-induced neuronal damage and apoptosis, and enhancing the phagocytic capabilities of microglial cells. The study underscores the potential circadian modulation of BBB integrity through key protein expressions, including Occludin, melatonin receptor 1A (MTNR1A), Aquaporin-4 (AQP4), and Low-density lipoprotein receptor-related protein 1 (LRP1). This comprehensive exploration of the NVU under circadian influence provides valuable insights into enhancing brain clearance abilities post-cerebral hemorrhage. The study emphasizes the significance of circadian regulation in neurological disorders, proposing potential therapeutic interventions and highlighting the importance of maintaining circadian rhythms for optimal brain health. The findings lay the foundation for future research targeting circadian-influenced mechanisms within the NVU to improve outcomes for patients post-cerebral hemorrhage.

Key words: Blood-brain barrier; Circadian rhythm; Neurovascular Unit; Melatonin receptor 1A; Aquaporin-4

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Introduction

With the aging of the population, cerebral hemorrhage has emerged as the second most common subtype of stroke, surpassed only by ischemic stroke, and its fatality rate is more than twice that of ischemic stroke[1,2]. Following cerebral hemorrhage, the expansion of hematoma and edema is accompanied by intricate processes involving inflammation, vascular dynamics, and the pathological metabolism clearance. Notably, vasogenic edema arises from the disruption of tight endothelial junctions on the microvascular side of the blood-brain barrier (BBB), leading to an increased accumulation of fluid due to extravasation, posing a serious threat to the health of affected

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individuals[3,4]. Therefore, preserving the blood-brain barrier's ability to clear hematomas metabolites is essential for maintaining brain health and functionality, ensuring an optimal cerebral environment.

In recent years, researchers have discovered that the optimal functioning of a healthy brain depends significantly on the dynamic interactions between neurons and non-neuronal cells. Consequently, the concept of the neurovascular unit (NVU) has been introduced, gaining prominence as a focal point in nervous system research[5]. The NVU comprises neurons, astrocytes, microglia, the blood-brain barrier, and the extracellular matrix bridging these components. Together, they maintain the internal environmental integrity of brain tissue and, through neurovascular coupling (NVC), dynamically regulate local cerebral blood flow and metabolic clearance in real time[6,7].

In the structure of the NVU, the primary function of neurons is to initiate local vascular responses. Neurons regulate cerebral blood flow (CBF) by producing signaling molecules, such as glutamate and adenosine, which act directly or indirectly on local blood vessels, inducing the dilation or contraction of blood vessels[8]. The foot processes of astrocytes are anchored at the vascular interface and serve as the gatekeepers of BBB, controlling and clearing cerebrospinal fluid from the perivascular space[9,10]. Aquaporin 4 (AQP4), expressed in astrocyte foot processes, functions like a water gate, facilitating the entry of cerebrospinal fluid into the brain parenchyma for metabolite exchange[11,12]. Previous research has also demonstrated the involvement of scavenger receptors, such as LRP1 and CD163 on the surfaces of microglia and astrocytes, in the clearance of hematoma following cerebral hemorrhage[13-15].

Considering the vital role of the BBB in preserving central nervous system (CNS) homeostasis and the intricate connections between circadian rhythms and the functional NVU, disruptions in circadian rhythms have been suggested to contribute to the pathology of various chronic brain diseases[16]. In sleep disorders, there is a reduction in cerebrospinal fluid flow within the perivascular space. It is speculated that sleep disorders result in the depolarization of perivascular AQP4 and an increased resistance to fluid outflow between brain tissues. This, in turn, diminishes the efficiency of the cerebral lymphatic drainage system's clearance, potentially impacting circadian regulation of the lymphatic system and clearance function across the blood-brain barrier via AQP4[17]. Moreover, short-wavelength blue light causes biological and psychological internal rhythms to stay in high sync with circadian rhythms. Blue light exposure can inhibit the secretion of melatonin, and exposure to blue light during the day is important for maintaining body's health, alertness and cognitive ability, but direct exposure to low intensity blue light at night may have a serious impact on sleep quality and circadian rhythm[18,19].

In this study, we established an in vitro Neurovascular Unit (NVU) model through co-culturing with hemoglobin and simulated circadian rhythms using short-wavelength blue light exposure. Our aim was to investigate the impact of circadian rhythms on BBB function, neuronal damage, and BBB clearance functionality, along with potential underlying mechanisms.

Materials and Methods

Ethics Statement

Sprague-Dawley (SD) rats were sourced from the Experimental Animal Center, Shanxi Medical University (Shanxi, China). All procedures involving the use of SD rats were ethically approved by the Ethics Committee of the Animal Transformation Center at Shanxi Medical University (No. DW2022029).

Isolation and purification of brain microvascular endothelial cells (BMECs)

Primary BMECs were isolated from 3-day-old SD rats[20,21]. Briefly, large vessels, meninges, and white matter were removed, and the forebrain tissue was minced into small pieces of approximately 1.0 mm^3. After centrifugation at 150 × g for 3 minutes, the precipitate layer was treated with trypsin (2.5 mg/ml, Amresco, USA) and digested at 37°C for 1.5 hours. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium (Gibco, Invitrogen Corporation, USA) with 10% fetal bovine serum (FBS, Hyclone, Thermo Scientific, USA) was added to halt the digestion, followed by centrifugation at 150 × g for 5 minutes. The precipitate was re-suspended in an equal volume of 25% (g/v) bovine serum albumin (BSA, Sigma, USA) and centrifuged at 1600 × g for 5 minutes. The obtained microvessels were digested with type-2 collagenase (1.0 mg/mL, Sigma, USA) for 1 hour and then cultured in DMEM/F12 containing 20% FBS and 1% penicillin/streptomycin. BMECs were collected

and seeded on 25 cm^2 flasks pre-coated with 2% gelatin. Primary BMECs were characterized for von Willebrand factor (vWf) using fluorescence imaging.

Culturing of primary neurons

Neurons were isolated from 1-day-old rats following established protocols[20,21]. Briefly, after the removal of meninges and white matter, purified cerebral cortices were digested with 0.125% trypsin-ethylenediamine tetraacetic acid (Sigma, USA) and then re-suspended in Neurobasal-A medium (Gibco, Grand Island, USA) containing 10% FBS, 2% B27 supplement (Invitrogen, Carlsbad, USA), and 1% penicillin/streptomycin. The cells were seeded at a density of 2 × 10^6 cells/mL into dishes coated with 0.01% poly-L-lysine (Sigma, USA) and cultured at 37°C in a 5% CO2 incubator (Thermo Fisher, Fremont, USA). On the third day, the medium was replaced with new medium supplemented with Ara-C (5.0 mg/ml, Sigma, USA) to inhibit non-neuronal cell growth for 24 hours. Subsequently, the culture medium was changed every 48 hours. Primary neurons were characterized by immunofluorescence labeling for Tubulin.

Primary cultures of cerebral astrocytes.

Primary astrocytes were obtained from 2-day-old rats following established procedures[20]. Briefly, the isolated cells were incubated in flasks pre-coated with poly-L-lysine in DMEM/F12 with 20% FBS and 1% penicillin-streptomycin. Fibroblasts were removed after differential attachment. On day 5, the cultures were shaken overnight to eliminate microglia[21]. Primary astrocytes were characterized by immunofluorescence labeling for glial fibrillary acidic protein (GFAP).

Culturing of primary microglial cells

Rat microvascular microglial cells (Procell Life Science and Technology Co., Ltd., CP-R110) were obtained and cultured following the provided instructions. Primary microglial cells were characterized through immunofluorescence labeling for CD11b.

Identification of four kinds of rat cerebral cells

Cells were seeded in 6-well plates with cell carriers. After adherence, the cell-covered carriers were removed, rinsed with phosphate-buffered solution (PBS), fixed with 4% paraformaldehyde (PFA) for 15 minutes, and permeabilized with 1% X-Triton for 20 minutes. Subsequently, the cell carriers were blocked with 10% goat serum (Sigma, USA) for 1 hour. The cells were then incubated overnight with rabbit anti-von Willebrand factor (1:100; ab6994, Abcam), rabbit anti-Tubulin (1:100; ab18207, Abcam), mouse anti-glial fibrillary acidic protein (1:100; ab10062, Abcam), and mouse anti-CD11b (1:100; ab1211, Abcam) antibodies at 4°C.

The following day, cells were incubated with fluorescently-labeled secondary antibodies (FITC-conjugated goat anti-rabbit IgG, 1:200, ab6717, Abcam; Alexa Fluor 647-conjugated goat anti-mouse IgG, 1:200, ab150115, Abcam) for 1 hour at 37°C. Subsequent imaging was performed on a fluorescence microscope (Fluoview 1000, Olympus, Tokyo, Japan). The number of cell bodies and nuclei were quantified using ImageJ. Purity was calculated as the ratio of the number of cell bodies to the number of nuclei.

Establishment of the in vitro NVU model

As illustrated in Figure 1A, a four-cell co-culture system was established, referencing previous research with modifications. Neurons were seeded at the bottom of Transwell plates (0.4μm, Corning, New York, NY, USA) and maintained in Neurobasal-A medium containing 2% B27 and 1% penicillin/streptomycin for 48 hours. Astrocytes and microglial cells (2 × 10^5 cells/cm^2) were seeded beneath the insert membrane in a petri dish at 48 hours. After 4 hours, the Transwell inserts were inverted and transferred to the plates. Following an additional 48 hours, BMECs (5 × 10^5 cells/cm^2) were seeded on the inner side of the insert membrane coated with gelatin (30.0 mg/ml). For control groups, BMECs, neurons, astrocytes, or microglial cells were individually cultured in the Transwell chambers. BMECs were also cultured with neurons alone, astrocytes alone, microglial cells alone, or without the other three primary cells, designated as the B+N group, B+A group, B+M group, or B group. The day when BMECs were seeded was defined as day zero in vitro (day 0). All cells were cultured for 3-5 days with a daily change of medium before analysis. The procedure for establishing the model is depicted in Figure 1B.

Transendothelial electrical resistance (TEER) measurements

TEER values for different cell co-cultures were assessed using an epithelial-volt-ohm resistance meter (ERS-2, Millipore, USA) following the provided company protocol. The background TEER value was measured in the same well under identical conditions but without cells seeded. The final result

was calculated by subtracting the corresponding background TEER value from the TEER value of different cells and then multiplying by the area of the insert membrane. The values are presented as Ω×cm^2.

Detection of four-hour leakage

The permeability of BMECs was assessed through a 4-hour leakage experiment. After 5 days of co-culture, the insert, serving as the donor pool, was filled with medium, while the medium level in the 12-well plates, acting as the receptor pool, was maintained 0.5 cm lower than the insert. Inserts with no cell culture were employed as controls. The medium level in the donor pool was compared before and after a 4-hour incubation.

Sodium fluorescein (SF) permeability determination

We assessed the apparent permeability (Papp) for the small molecule SF to evaluate the formation of tight junctions by BMECs. SF (100 μg/mL) was introduced into the upper chamber. A 100-μL volume was collected from the lower chamber at 30, 60, 90, and 120 minutes, and subsequently replenished with pre-equilibrated culture media. The absorbances of the samples were measured using a fluorescence spectrophotometer (FLUOstar Omega, BMG Labtech, Offenburg, Germany). The Papp was calculated as (dM/dt)/(A •C), where dM/dt represents the cumulative measured fluorescence intensity in the plate per unit time, A refers to the bottom area of the insert (1.12 cm^2), and C refers to the SF intensity in the upper insert (941).

Measurement of cell viability in the NVU model

Cell viability was assessed using the Cell Counting Kit-8 (CCK8) technique. For the Cell Counting Kit-8 assays (AR1160, Boster), cultures were incubated in culture medium containing 10% (v/v) Cell Counting Kit-8 solution for 2 hours. Absorbance was measured at 450 nm using a microplate reader (Biotek, Winooski, USA).

Phagocytosis assay

The Phagocytosis Assay Kit (ab234054, Abcam) was employed to quantify the phagocytic capacity of astrocytes in the NVU model. Following treatment, cells were incubated with 5 μl of prelabeled zymosan particles for 2 hours and then washed by adding cold phagocytosis assay buffer. Subsequently, cells were analyzed using confocal fluorescent microscopy (SP8 Leica microscope).

Reactive Oxygen Species (ROS) assay

The Reactive Oxygen Species Assay Kit (Yesen, Shanghai, China) was employed to detect oxidative

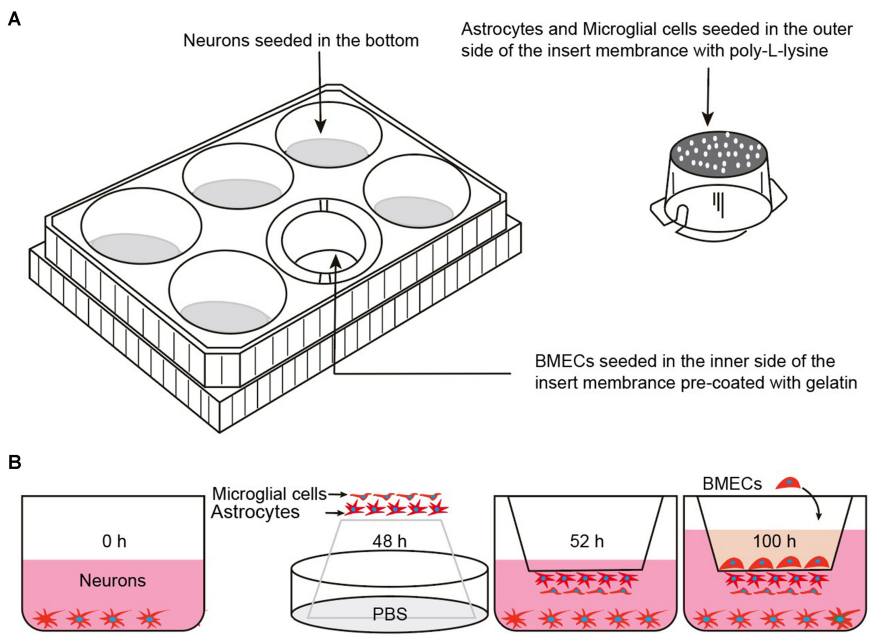

Figure 1. Establishment of in vitro NVU model. (A) Schematic drawing of four-cell co-culture system. (B) The procedure for establishing in vitro four-cell co-culture model.

stress injury in neurons within the NVU model. Following treatment, cells underwent laser irradiation and were subsequently stained with 2,7-dichlorodihydroflfluorescein diacetate (DCFH-DA, in green), followed by an incubation at 37 °C for 1 hour. Finally, cells were analyzed using confocal fluorescent microscopy (SP8 Leica microscope).

Flow Cytometry Analysis

Neuronal apoptosis levels were assessed using the Annexin V-FITC/PI Double Staining Kit (E-CK-A211, Elabscience®). After intervention, neuronal cells were collected with EDTA-free trypsin, rinsed with PBS, and processed following the kit instructions. Briefly, cells were suspended in 100 µL of binding buffer, transferred to a centrifuge tube, 2.5 µL of dye was added, and the mixture was incubated for 30 minutes in the dark. Finally, 400 µL of binding buffer was added to the tube for flow cytometry detection.

Western blotting analysis

Proteins were extracted from cells using RIPA lysis buffer (RIPA, AR0105, Boster), separated by SDS-PAGE (EpiZyme Biotechnology, China), and transferred to polyvinylidene fluoride (PVDF, HVLP09050, Millipore®) membranes. The PVDF membranes were incubated with primary antibodies

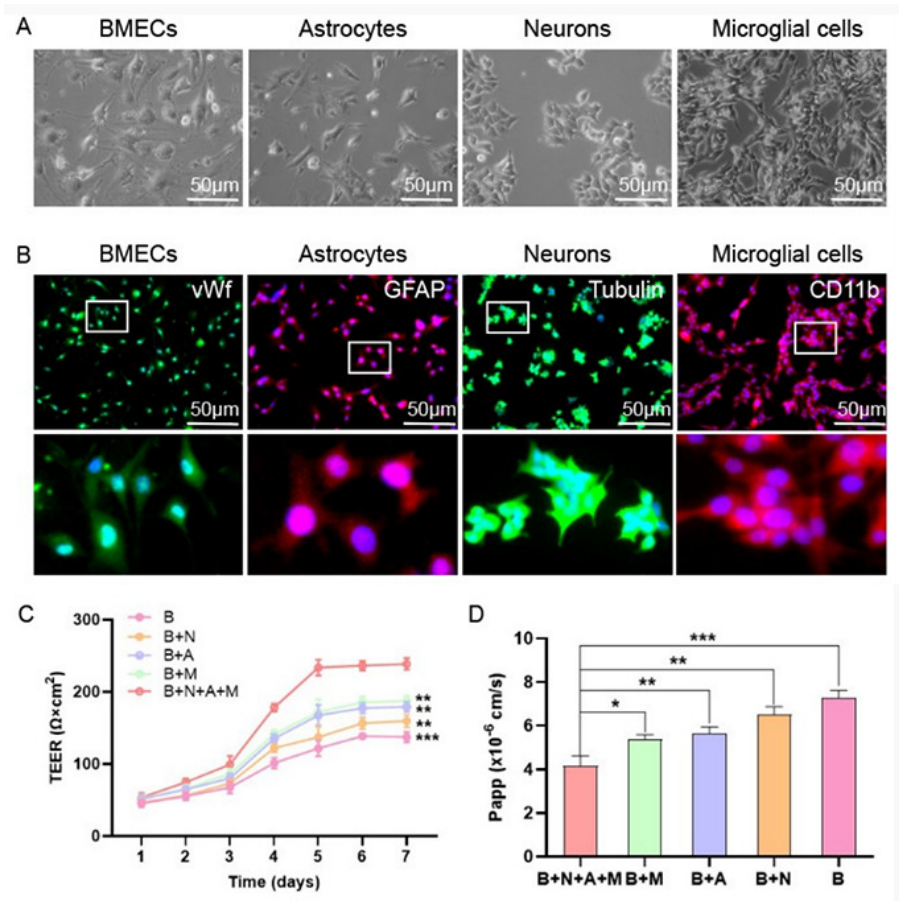

Figure 2. Functional features of the in vitro neurovascular unit (NVU) model. (A) Four cell morphologies for establishment of in vitro NVU model. (B) Immunostaining for all cell types in the NVU model. Brain microvascular endothelial cells (BMECs) stained for von Willebrand factor (vWF), astrocytes stained for glial fibrillary acidic protein (GFAP), neurons stained for Tubulin and microglial cells stained for CD11b. (C) Transendothelial electrical resistance (TEER) values over 7 days. (D) The permeability coefficient of sodium fluorescein in the B + N+A+M, B + M, B + A, B + N and B groups. B + N+A+M group: BMECs cultured with neurons, astrocytes and microglial cells; B + M group: BMECs cultured with microglial cells; B + A group: BMECs cultured with astrocytes; B + N group: BMECs cultured with neurons; B group: BMECs cultured alone in the transwell chamber. Data are expressed as the mean ± SD. **P < 0.01, ***P < 0.001.

at 4 °C overnight, followed by secondary antibodies for 1 hour at room temperature. Subsequently, visualization was performed using the BIO-RAD CHEMIDoc XRS+ system. The primary antibodies used for protein analysis included AQP4 (1:1000; ab259318, Abcam), LRP1 (1:1000; ab92544, Abcam), Occludin (1:1000; ab216327, Abcam), MTNR1A (1:1000; ab158923, Abcam), and β-Actin (1:10000; ab8226, Abcam).

Immunofluorescence analysis

Cells were washed three times with PBS and fixed with 4% PFA at room temperature for 15 minutes. Cell membranes were permeabilized with 1% Triton X-100 for 20 minutes, followed by blocking of non-specific binding sites with 5% BSA (Beyotime, Shanghai) for 1 hour. Cultured cells were then incubated overnight at 4°C with primary antibodies against AQP4 (1:100; ab259318, Abcam), LRP1 (1:100; ab92544, Abcam), Occludin (1:100; ab216327, Abcam), and MTNR1A (1:100; ab158923, Abcam). The next day, fluorescent secondary antibodies were added dropwise to the coverslips in a dark room and incubated for 1 hour at room temperature, followed by staining of the nuclei with 4',6-diamidino-2-phenylindole (DAPI, AR1176, Boster) for 30 minutes. Finally, images were acquired and analyzed under a microscope.

Statistical Analysis

All experiments were conducted at least three times, and data were presented as means ± standard deviations (SD). Data analysis was performed using GraphPad Prism 8 software (San Diego, Calif., USA) or SPSS 18.0 statistical software (IBM Corp). Unpaired two-tailed Student's t-tests (for two groups) or one-way ANOVA (for multiple groups) were employed to assess statistical significance ($P < 0.05$).

Results

Blood-brain barrier (BBB) function in the NVU model

BBB function in the NVU model is depicted in Figure 2. Four types of rat cerebral cells (BMECs, Neurons, Astrocytes, and Microglial cells) were utilized to establish the NVU model (Figure 2A). Cell purity was assessed through immunofluorescence, demonstrating that the purity of BMECs, Astrocytes, Neurons, and Microglial cells from the rat brain exceeded 92%, 96%, 97%, and 98%, respectively (Figure 2B). Subsequently, an in vitro NVU model was established, and BBB properties were measured.

TEER measurements and the endothelial permeability coefficient for sodium fluorescein (SF) were employed to compare the physical properties of the in vitro BBB model across various cultures. The NVU model achieved maximum TEER at 5 days,

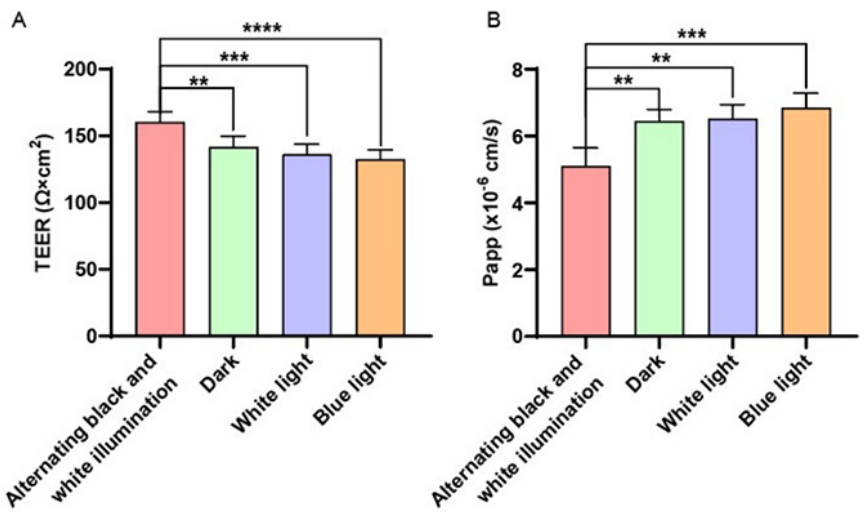

Figure 3. NVU barrier function co-cultured with hemoglobin under different illumination. (A) The TEER values in the alternating black and white light group, dark group, white light group and blue light group. (B) The permeability coefficient of sodium fluorescein in the alternating black and white light group, dark group, white light group and blue light group. Data are expressed as the mean ± SD. **P < 0.01, ***P < 0.001, ****P < 0.0001.

significantly surpassing values in monoculture systems or in BMECs co-cultured with neurons, astrocytes, or microglial cells (Figure 2C). The low paracellular Papp of SF in the co-culture system confirmed the establishment of an impermeable barrier in vitro. The permeability coefficients of SF in the NVU system were notably minimal compared to those in the single culture of BMECs and in BMECs co-cultured with Neurons, Astrocytes, or Microglial cells (Figure 2D).

Protective Effect of Circadian Rhythm on the Barrier Function of NVU Co-cultured with Hemoglobin

To investigate the impact of circadian rhythm on blood-brain barrier function, we established an in vitro NVU model and co-cultured it with hemoglobin. The optimal concentration of hemoglobin for co-culture was determined to be 25μM (Supplementary Figure 1A). Subsequently, NVU co-cultured with hemoglobin was subjected to different illumination conditions (alternating black and white light, dark, white light, and blue light). The TEER value of the alternating black and white light group, simulating circadian rhythm, was significantly higher than that of the other three groups (Figure 3A).

Furthermore, the low paracellular Papp of

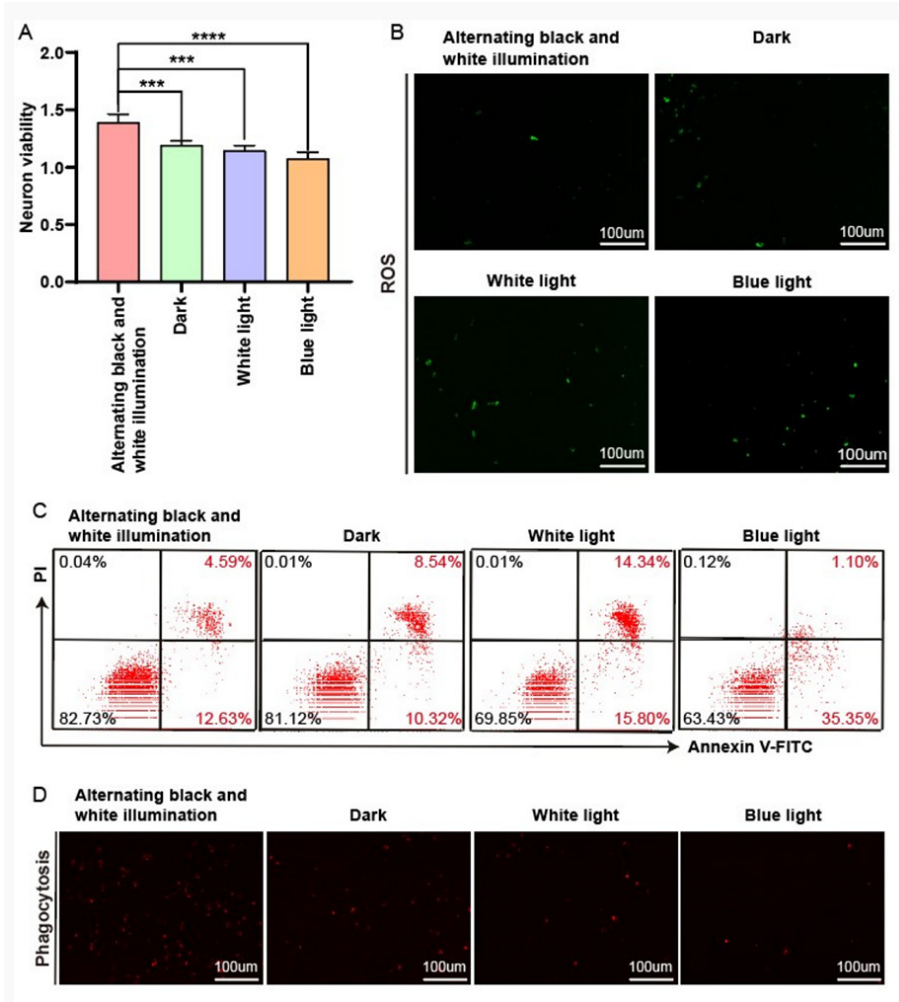

Figure 4. Neuron damage and microglia phagocytosis decreased in NVU co-cultured with hemoglobin under different illumination. (A) Neuron survival rate in the alternating black and white light group, dark group, white light group and blue light group. (B) Neuron oxidative stress injury in the alternating black and white light group, dark group, white light group and blue light group. (C) Apoptosis rate of neurons in the alternating black and white light group, dark group, white light group and blue light group. (D) Microglial phagocytosis in the alternating black and white light group, dark group, white light group and blue light group. Data are expressed as the mean ± SD. **P < 0.01, ***P < 0.001, ****P < 0.0001.

sodium fluorescein (SF) confirmed the establishment of an impermeable barrier in vitro. The permeability coefficients of SF in the alternating black and white light group were minimal compared to those in the dark group, white light group, and blue light group (Figure 3B). These findings suggest that circadian rhythm can preserve the blood-brain barrier function of the nervous system.

Circadian Rhythm Alleviates Neuronal Damage and Decreases Phagocytosis of Glial Cells in NVU Co-cultured with Hemoglobin

Subsequently, we delved into the impact of circadian rhythm on NVU function by treating NVU co-cultured with hemoglobin under different illuminations (alternating black and white light, dark, white light, and blue light). Our findings revealed that the alternating black and white light group exhibited the highest survival rate of neurons, along with the lowest levels of oxidative stress injury and apoptosis compared to the dark group, white light group, and blue light group (Figure 4A-C).

We also investigated the effects of different light irradiations on the phagocytosis of microglia in NVU

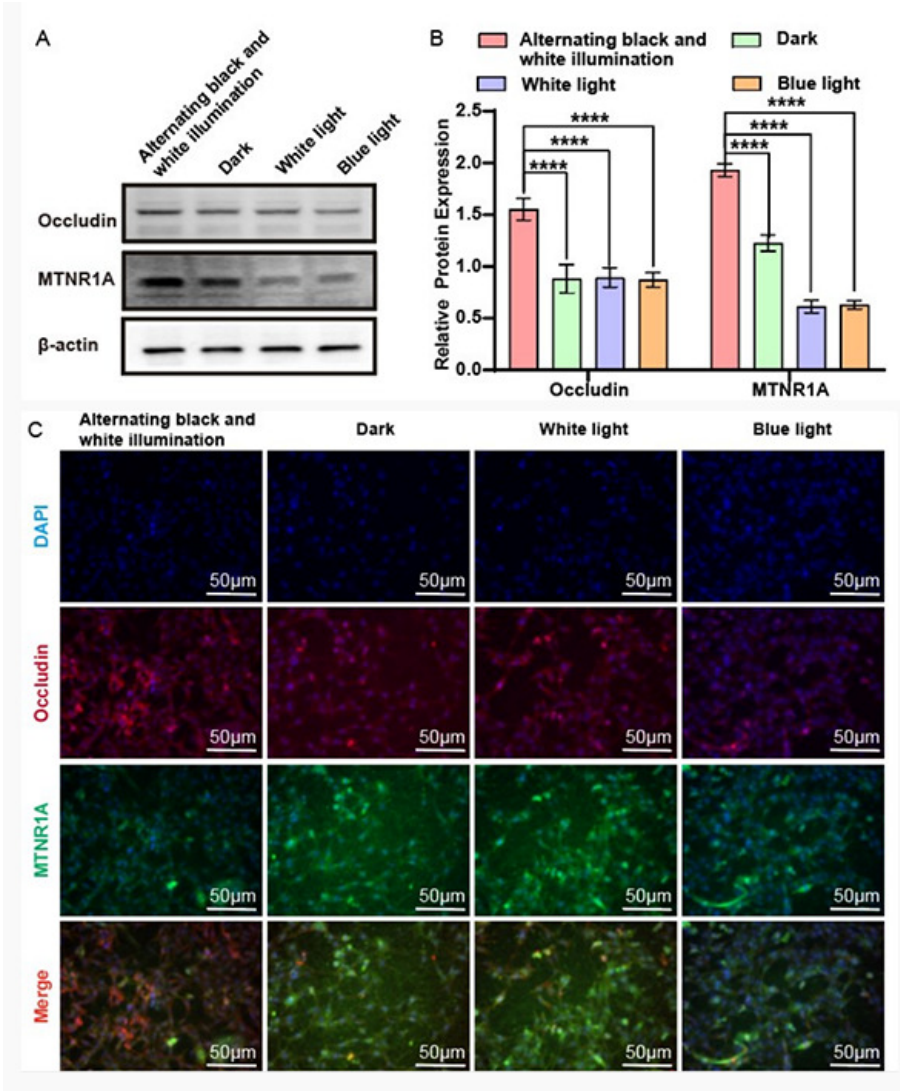

Figure 5. The expression of Occludin and MTNR1A in NVU co-cultured with hemoglobin under different illumination. (A, B) Western blotting and quantification of Occludin and MTNR1A in the alternating black and white light group, dark group, white light group and blue light group, with β-actin as the endogenous control. (C) Immunofluorescent staining of Occludin and MTNR1A in the alternating black and white light group, dark group, white light group and blue light group. Data are expressed as the mean ± SD. **P < 0.01, ***P < 0.001, ****P < 0.0001.

co-cultured with hemoglobin. The results demonstrated a significant inhibition of microglial phagocytosis in the dark group, white light group, and blue light group compared to the alternating black and white light group (Figure 4D). These results highlight that circadian rhythm can mitigate oxidative stress injury and apoptosis of neurons, decelerate the decline in microglial phagocytosis, thereby safeguarding NVU function.

Circadian Rhythm Preserved the Decrease in the Expression of Occludin and MTNR1A in NVU Co-cultured with Hemoglobin

Occludin and MTNR1A are located at the tight junctions of the blood-brain barrier, playing a crucial role in maintaining its physiological function. Subsequently, we examined the expression of Occludin and MTNR1A in NVU co-cultured with hemoglobin under different light irradiations. Western blot results indicated that the expressions of Occludin and MTNR1A in the dark group, white light group, and blue light group were significantly reduced compared to those in the alternating black

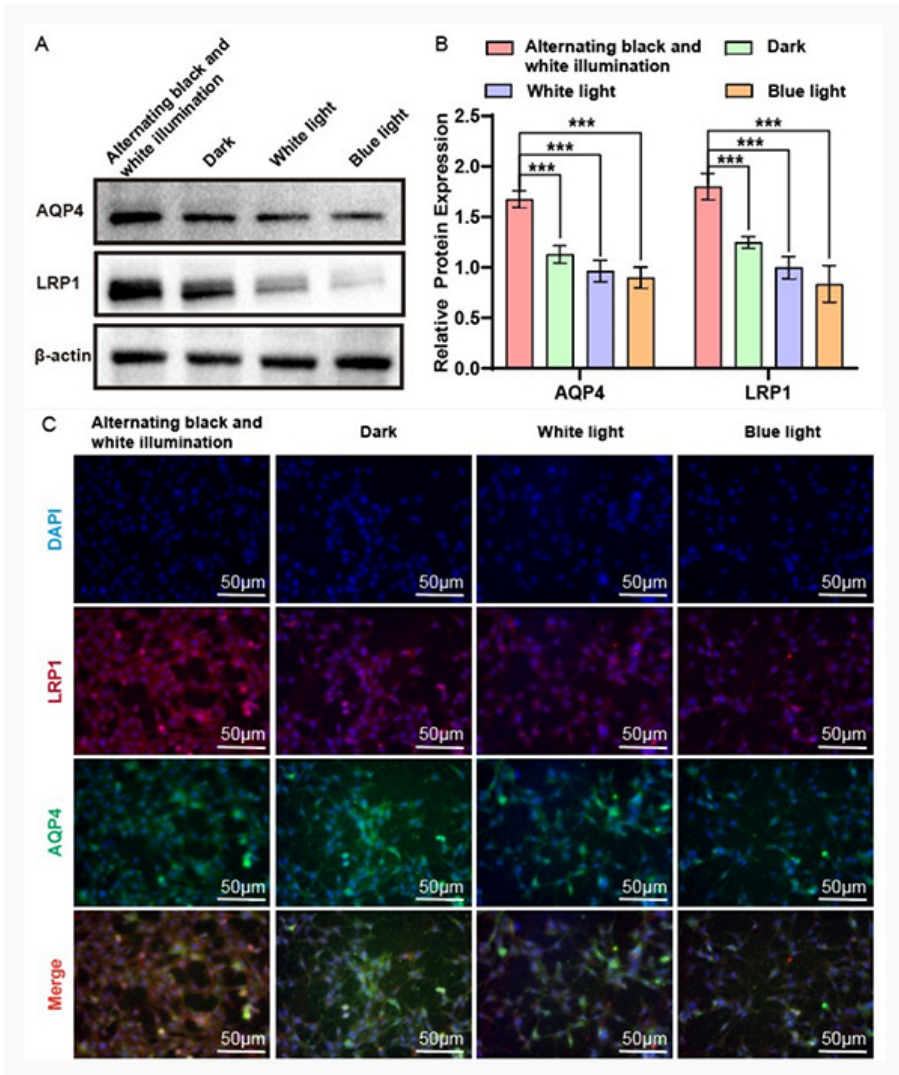

Figure 6. The expression of AQP4 and LRP1 in NVU co-cultured with hemoglobin under different illumination. (A, B) Western blotting and quantification of AQP4 and LRP1 in the alternating black and white light group, dark group, white light group and blue light group, with β-actin as the endogenous control. (C) Immunofluorescent staining of AQP4 and LRP1 in the alternating black and white light group, dark group, white light group and blue light group. Data are expressed as the mean ± SD. **P < 0.01, ***P < 0.001, ****P < 0.0001.

and white light group (Figure 5A, B). Similarly, immunofluorescence staining revealed a substantial decrease in the expressions of Occludin and MTNR1A in the dark group, white light group, and blue light group compared to the alternating black and white light group (Figure 5C). These results suggest that circadian rhythm can mitigate the reduction in the expression of Occludin and MTNR1A in NVU.

Circadian Rhythm Preserved the Decrease in Expression of AQP4 and LRP1 in NVU Co-cultured with Hemoglobin

AQP4 and LRP1 play crucial roles in eliminating metabolic waste and exogenous toxins from the brain, facilitating the removal of hematoma components after cerebral hemorrhage. Finally, we investigated the expression of AQP4 and LRP1 in NVU co-cultured with hemoglobin under different light conditions. The Western blot results demonstrated that the expressions of AQP4 and LRP1 in the dark group, white light group, and blue light group were significantly reduced compared to those in the alternating black and white light group (Figure 6A, B). Similarly, immunofluorescence staining revealed a substantial decrease in the expressions of AQP4 and LRP1 in the dark group, white light group, and blue light group compared to the alternating black and white light group (Figure 6C). These findings suggest that circadian rhythm can mitigate the reduction in expression of AQP4 and LRP1 in NVU.

Discussion

Cerebral hemorrhage is a severe cerebrovascular disorder typically caused by the rupture of blood vessels in the brain. It encompasses intracerebral hemorrhage (ICH), subarachnoid hemorrhage, and intraventricular hemorrhage[22]. The disruption of the blood-brain barrier (BBB) caused by ICH further leads to vasogenic edema, resulting in the extravasation of plasma proteins. This may potentially lead to neurotoxicity and the entry of vasoactive compounds into the brain[23,24]. Therefore, the cerebral clearance process plays a crucial role in maintaining the homeostasis of the brain environment, limiting the excessive release of inflammation, and promoting recovery[25,26].

The neurovascular unit (NVU) is composed of various components, including neurons, astrocytes, microglial cells, the BBB, and the extracellular matrix, which collectively maintain the integrity of the brain tissue environment. Through the mechanism of neurovascular coupling (NVC), the NVU dynamically regulates local cerebral blood flow and metabolic clearance levels in real time[27,28]. In recent years, research focused on neurological disorders using the NVU as the primary investigative unit has become a prominent and influential area of study. The NVU model, established through a co-culture system involving primary brain microvascular endothelial cells (BMECs), neurons and astrocytes, has been employed in research on various cerebrovascular diseases, including ischemic stroke[29,30]. Until now, only a limited number of studies have delved into the use of triple-cell co-culture models for investigating BBB permeability[20]. Building upon the foundation of previous studies, we employed a four-cell co-culture model involving neurons, astrocytes, microglial cells, and BMECs to establish the NVU model. Additionally, compared to the monoculture of BMECs or BMECs co-culture with other three cell types, our four-cell co-culture model exhibited significantly higher TEER values and lower SF permeability. Our results suggest that the four-cell co-culture model established in this study not only recapitulates the diversity of NVU cell types but also effectively simulates *in vivo* conditions.

The circadian rhythm is a biological process with rhythms of 24-hour cycle that could influence the release of neuropeptides, pineal gland melatonin, and adrenal cortex glucocorticoid secretion[31,32]. Irregular eating and sleeping patterns, sudden changes in temperature, and prolonged exposure to blue light could disrupt the circadian rhythm[33]. Certain molecules, such tumor necrosis factor alpha (TNF-α), β-amyloid, and prostaglandin D2 (PGD2), exhibit circadian rhythmic oscillations. These molecules are probably regulated in accordance with the rhythmic changes in BBB transport[34-36]. A study conducted in drosophila revealed that the permeability of BBB is regulated by circadian rhythms, with increased permeability observed during the night[37]. In this study, we employed alternating black-white light to simulate circadian rhythms. The experimental results revealed that, compared to individual treatments with dark light, white light, and blue light, the TEER values of the NVU model subjected to alternating black-white light were significantly higher, with the minimal SF permeability coefficient. Consistently, our findings suggest that circadian rhythms can effectively preserve the BBB function.

Conclusions

In the structure of NVU, neurons play a pivotal role in initiating local vascular responses. Neurons modulate cerebral blood flow (CBF) by generating signaling molecules such as glutamate, adenosine, and others. These signaling molecules exert direct or indirect effects on local blood vessels, inducing vasodilation or vasoconstriction[7]. In the hours following ICH, activated microglia and infiltrating immune cells play a crucial role in protecting surrounding cells and tissues by clearing blood and damaged neurons[38]. Insufficient clearance capability of microglia cells could prolong the damage and further deteriorate the patient's condition[39]. Through our experiments, we observed that the neuronal survival rate was highest in the hemoglobin co-culture NVU model subjected to alternating black-white light treatment, with lower levels of oxidative stress damage and apoptosis. Additionally, compared to the group exposed to alternating black-white light, the phagocytic activity of microglial cells in the dark, white light, and blue light groups was significantly inhibited. These findings suggest that circadian rhythms can alleviate oxidative stress damage and apoptosis in neurons, enhance the phagocytic capability of microglial cells, and thereby protect the normal function of the NVU.

Occludin is present at the tight junctions of BBB and plays a crucial role in maintaining the physiological function of the BBB, serving as a specific indicator for assessing BBB integrity. During ICH, BBB disruption leads to a decrease in occludin expressions [40]. Melatonin, produced by the pineal gland, serves as the effector of the circadian rhythm-biological clock, playing a crucial role in circadian regulation[41]. An animal experimental study revealed that the application of melatonin in transgenic Alzheimer's disease (AD) animal models enhances the glial lymphatic clearance capacity for Aβ, consequently reducing the deposition of Aβ in the brain[42]. Therefore, we hypothesize that circadian rhythms may influence BBB function through the modulation of melatonin secretion. The results of this study reveal that alternating black-white light treatment significantly enhances the expression of Occludin and melatonin receptor 1A (MTNR1A) in NVU, suggesting that circadian rhythms may regulate the integrity of BBB function, possibly through the modulation of melatonin.

Aquaporin-4 (AQP4) is expressed in astrocytes foot processes and functions like a gate to control the entry of cerebrospinal fluid into brain parenchyma for metabolic exchange[11]. Studies have revealed that the inhibition of AQP4 leads to a reduction in the brain's clearance capacity, emphasizing the critical role of AQP4 in the elimination of cerebral metabolic waste and exogenous toxins[43]. Simultaneously, convective activities of the glymphatic system, stimulated by AQP4, enhance the clearance function of the BBB, particularly in astrocytes[44]. Low-density lipoprotein receptor-related protein 1 (LRP1), serving as the primary endocytic receptor in perivascular glial cells of the BBB, is also a principal scavenger receptor facilitating Aβ clearance across the BBB [45]. In the previous studies, we have demonstrated that LRP1 plays a crucial role in promoting the clearance of hematoma components after cerebral hemorrhage[14,46]. This study, for the first time, reveals that alternating black-white light treatment significantly rescued the decreased expression of AQP4 and LRP1 in the NVU co-cultured with hemoglobin. These findings suggest that circadian rhythms can mitigate the reduction in AQP4 and LRP1 expression within the NVU.

In summary, this study established a NVU model through four-cell co-culturing, simulating the BBB environment by co-culturing with hemoglobin. Employing alternating black-white light treatment on the NVU model mimicked circadian rhythms, and it was observed that circadian rhythms could protect BBB integrity and enhance the endogenous brain clearance system's ability to remove hematoma after cerebral hemorrhage. The study focused on the NVU as the research unit, unraveling the role of circadian rhythm on the function of the brain clearance system, and provides new ideas for improving the brain clearance ability of patients after cerebral hemorrhage.

Ethics Statement

Sprague-Dawley (SD) rats were sourced from the Experimental Animal Center, Shanxi Medical University (Shanxi, China). All procedures involving the use of SD rats were ethically approved by the Ethics Committee of the Animal Transformation Center at Shanxi Medical University (No. DW2022029).

Declaration of Interest Statement

The authors declare that they have no competing interests.

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