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Retinoic acid ameliorates blood–brain barrier disruption following ischemic stroke in rats

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ABSTRACT

The intact blood–brain barrier (BBB) is essential in maintaining a stabilized milieu for synaptic and neuronal functions. Disruptions of the BBB have been observed following ischemia and reperfusion, both in patients and in animal models. Retinoic acid (RA), which plays crucial roles during vertebrate organogenesis, has been reported to participate in BBB development. However, it remains unclear whether RA could prevent BBB disruption in ischemic stroke. In this study, we determined that the injection of RA for 4 consecutive days resulted in increases in zonula occludens-1 (ZO-1) and vascular endothelial cadherin (VE-cadherin) expression, which are crucial components of the BBB structure. We demonstrated that RA pretreatment could alleviate the ischemic stroke-induced enlargement of vascular permeability, which is related to the up-regulated expression of ZO-1 and VE-cadherin proteins in rat models of middle cerebral artery occlusion (MCAO). Our findings further corroborated that the RA protective effect on BBB is dependent on RA receptor α *in vitro* oxygen–glucose deprivation (OGD) treatment. Significantly, RA administration immediately after MCAO reduced tissue plasminogen activator (tPA)-induced intracerebral hemorrhage (ICH) and ameliorated neurological deficits 24 h after ischemic stroke. Taken together, our results suggest that RA may become a new therapeutic approach to prevent BBB dysfunction and tPA-induced ICH in ischemic stroke.

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1. Introduction

The blood–brain barrier (BBB) is a specialized interface to separate the blood–brain barrier from neurons and acts as a regulator of nutrient and iron transport and acts as a barrier to harmful factors. The structure of the BBB is primarily composed of brain endothelial cells (BEC), astrocyte end-feet and pericytes [1]. Studies have demonstrated that the intricate junctional complex between BEC, including several crucial proteins that tightly seal adjacent BEC (claudin-5, Occludin, zonula occludens-1 (ZO-1) and vascular endothelial cadherin (VE-cadherin)), is an important structure to maintain BBB integrity and restrict paracellular permeability [2–5]. BBB destruction could directly alter the physiological neuronal milieu, which is accompanied by neuroinflammatory responses, oxidative stress and brain edema [6,7]. BBB destruction ultimately contributes to synaptic and neuronal dysfunction and cognitive changes [8,9]. In both animal and human studies, a compromised BBB has been observed in ischemic stroke, which was caused by thrombosis interrupting the blood–brain barrier flow in the brain vasculature [10–14]. Sufficient evidence has shown that several crucial proteins, including claudin-5, Occludin, ZO-1 and VE-cadherin, were decreased after ischemia and reperfusion, which might cause the disruption of the BBB [15–17].

Tissue plasminogen activator (tPA), an effective thrombolytic agent used to treat acute ischemic stroke, is approved by the U.S. Food and Drug Administration (FDA) [18,19]. A study has shown that tPA could display its fibrinolytic activity and significantly improve the neurological outcome in thromboembolic models [20]. However, clinical trials have indicated that only a few patients are suitable for this drug treatment because of the risk of tPA-induced

Abbreviations: BBB, blood–brain barrier; RA, retinoic acid; ZO-1, zonula occludens-1; PECAM-1, platelet endothelial cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; MCAO, middle cerebral artery occlusion; tPA, tissue plasminogen activator; ICH, intracerebral hemorrhage; TTC, triphenyltetrazolium chloride; BEC, brain endothelial cells; OGD, oxygen and glucose deprivation; RAR, RA receptor; BSS, glucose-free balanced salt solution.

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intracerebral hemorrhage (ICH) complications upon intravenous injection [21]. Recent research has shown that tPA is both necessary and sufficient to enlarge BBB permeability following cerebral ischemia [11,22–24]. TPA activated platelet-derived growth factor α-receptors (PDGFR-α) *via* PDGF-CC, which ultimately lead to ICH. Imatinib, a tyrosine kinase antagonist, inhibited the activity of PDGFR-α and reduced ICH complications associated with tPA administration. However, clinical trials have revealed that imatinib treatment could cause serious side effects, such as cardiotoxicity and peripheral edema [25]. It remains unclear whether the systemic administration of other drugs could directly prevent BBB disruption, minimize potential side effects, and eventually reduce the risk of ICH due to tPA treatment in ischemic stroke.

Retinoic acid (RA) is a bioactive derivative of vitamin A [26] that plays essential and pleiotropic roles in early organogenesis during vertebrate development [27], including having distinct effects on neurogenesis and angiogenesis [28–31]. Furthermore, one intriguing new study has reported that radial glial cell-derived RA is crucial for BBB development in mouse embryos. Stimulation with RA for 48 h increased barrier formation in cultured human adult BEC by inducing barrier-related gene expression, particularly ZO-1 and VE-cadherin [32]. However, the role of RA in BBB disruption in an ischemic stroke remains virtually unknown. In the current study, our purpose is to investigate whether RA treatment could ameliorate BBB destruction and prevent tPA-induced ICH in rat middle cerebral artery occlusion (MCAO) stroke models.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 280–330 g, were used in the experiments. Rats were maintained at 22 °C under 12:12 light/dark cycles with abundant food and water. All procedures complied with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committees of Shandong University.

2.2. Stroke model and in vivo drug administration

Rats were subjected to a 90 min occlusion of the right middle cerebral artery using an intraluminal model with minor modifications [33,34]. In short, the animals were anesthetized with 10% chloral-hydrate and a heating pad was used to maintain the body temperature at 37.5 ± 0.5 °C. The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were then exposed. A 3-0 monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd., Beijing, China) was inserted into the ICA *via* the ECA stump and traveled through the ICA for a distance of 18–19 mm from the bifurcation. Reperfusion was performed by slowly removing the suture from the ECA. Both the ischemia and reperfusion process were monitored by Laser Doppler flowmetry (Perimed, Stockholm, Sweden) to confirm that MCAO was achieved.

Rats were placed in randomized groups that were treated with either vehicle (polyethylene glycol, 0.9% NaCl and ethanol; 70%/20%/10% by volume) or RA (all-*trans*-RA; Sigma–Aldrich) [35]. Before surgery, rats in the RA and vehicle groups received intraperitoneal injections of RA (5 mg/kg/d body weight) or vehicle solution, respectively, for 4 consecutive days. RA was injected after the onset of MCAO and 10 h later to investigate the effect of RA treatment after an ischemic stroke.

To investigate tPA-induced cerebral hemorrhage, human recombinant tPA (Actilyse, Boehringer Ingelheim) or saline was injected intravenously as a 1 mg/kg bolus 10 min before reperfusion, followed by a 9 mg/kg infusion over a 20 min interval using a syringe infusion pump. To enhance the probability of cerebral hemorrhage, rats received intraperitoneal injection of D-glucose (6 ml/kg at 50% wt/vol) 15 min before ischemia, as previous studies have proved that hyperglycemia could increase BBB permeability and the risk of tPA-induced cerebral hemorrhage after ischemic stroke [22,36,37].

2.3. RNA isolation and real-time quantitative PCR

Rat brains were removed immediately after 4 days of RA pretreatment. The dorsolateral cortex from the different groups was dissected using a brain matrix (Braintree Scientific). TRIzol-A$^+$ RNA isolation reagent (Tiangen) was used to isolate total RNA, and then cDNA was synthesized with the Revert Aid First Strand cDNA Synthesis Kit (Fermentas), according to the manufacturer's instructions. The primer sequences used were as follows: ZO-1 forward primer – 5′TCC AGT CCC TTA CCT TTC G 3′, reverse primer – 5′TGG TGC TCC TAA ACA ATC AG 3′; VE-cadherin forward primer – 5′GCG ACG CTT CTA CCA CTT C 3′, reverse primer – 5′TTC CCT TGT TTG GTT ATT CG 3′; Claudin-5 forward primer – 5′GGC ACT CTT TGT TAC CTT GAC C 3′; reverse primer – 5′GGC ACC GTT GGA TCA TAG A 3′; Occludin forward primer – 5′GGA CAG AGC CTA TGG AAC G 3′, reverse primer – 5′CCA AGG AAG CGA TGA AGC3′; PECAM-1 forward primer – 5′ACA GAC AAG CCC ACC AGA G3′, reverse primer – 5′ GTC ATT CAC GGT TTC TTC G3′; β-actin forward primer – 5′CAA CTT GAT GTA TGA AGG CTT TGG T 3′, and reverse primer – 5′ACT TTT ATT GGT CTC AAG TCA GTG TAC AG 3′. Real-time PCR was performed in a Cycler (Bio-Rad), and SYBR-Green (Roche) was used to quantify the content of cDNA. The expression of each gene was normalized to the level of β-actin expression using the $2^{-\Delta\Delta CT}$ method.

2.4. Analysis of cerebrovascular permeability

Alterations in cerebrovascular permeability after MCAO were evaluated by the Evans blue (EB) assay. EB solution (4% in PBS, 2.5 ml/kg; Sigma–Aldrich) was administered intravenously 1 h after reperfusion. Rats were perfused with saline and euthanized 4 h later. Brains were quickly removed and separated into ischemic and contralateral hemispheres. Each hemisphere was homogenized with N,N-dimethylformamide (Sigma–Aldrich) and centrifuged for 40 min at 25,000 × *g*. EB extravasation was quantified from the supernatants by spectrophotometry at 620 nm as previously described [23].

2.5. Quantification of infarct volume

Triphenyltetrazolium chloride (TTC, Sigma–Aldrich) staining was used to measure infarct volume as previously described [38]. Rats were sacrificed 5 h after reperfusion and their brains were removed immediately and then sliced into 2.0-mm-thick coronal sections with the help of a brain matrix. The flash brain slices were stained with 2% TTC for 30 min at 37 °C and then fixed in 4% paraformaldehyde overnight. Infarct tissue was not stained, but visualized as white tissue, whereas the viable tissue was stained red. Digital photographs were taken, and the infarct volume was analyzed using Photoshop CS 5.0. The degree of brain damage was corrected as previous described [39]. In each section, we obtained the infarct area by subtracting the red area of the ipsilateral hemisphere from the total area of the contralateral hemisphere. The total infarct volume for each animal was calculated by adding the damaged area of all brain slices. The results were expressed as the percentage of the total volume of the contralateral hemisphere.

2.6. Western blotting

Rats were euthanized 5 h after MCAO and brains were rapidly removed. Ipsilateral penumbra cortex and matching tissue from

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