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The Protective Mechanism of Hydroxysafflor Yellow A on the Destruction of Blood-brain Barrier Induced by β-amyloid

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Abstract:

Objective: The paper firstly investigated the expression and function of LINC00094, MOV10 and SNHG1/SCAMP1 in human brain microvascular endothelial cells incubated with Aβ. The paper explored the effect of Hydroxysafflor yellow A on the destruction of the blood-brain barrier induced by β-amyloid and the regulatory effect on the permeability of the blood-brain barrier in Alzheimer's disease, aiming to provide a new strategy for the treatment of Alzheimer's disease from the perspective of the blood-brain barrier.

Methods: An in vitro BBB model was constructed. qRT-PCR method was applied to detect the expressions of LINC00094, miR-224-5p, and Endophilin-1. Western blot was employed to detect the expressions of Endophilin-1, ZO-1, occludin, and claudin-5. TEER experiment was conducted to test the integrity of BBB. HRP exudation experiment was performed to test BBB permeability. Immunofluorescence was applied to test the expression and distribution of tight junction related proteins.

Results: Hydroxysafflor yellow A can reduce the expression of LINC00094 in ECs incubated with Aβ, the TEER value significantly increased, the amount of HRP exudation decreased, and the expression of related proteins ZO-1, occludin and claudin-5 were all increased, and reduced BBB permeability in the AD microenvironment.

Conclusion: Hydroxysafflor yellow A combined with LINC00094 knockdown can reduce BBB permeability, and regulate BBB permeability in AD microenvironment through the LINC00094/miR-224-5p/Endophilin-1 pathway.

Keywords: Hydroxysafflor yellow A, blood-brain barrier; LINC00094/miR-224-5p/Endophilin-1; Alzheimer's disease

Hydroxysafflor yellow A is a compound with a single chalcone glycoside structure. It is the most effective water-soluble part of safflower with pharmacological effects. Current studies have found that it can play a protective role in lung, brain and myocardium by regulating cell apoptosis, epithelial-mesenchymal transition and oxidative stress.It can inhibit platelet aggregation and release induced by platelet activating factors, and can competitively inhibit the binding of platelet activating factor with platelet receptor. It is the effective component of safflower yellow pigment for promoting blood circulation and removing blood stasis. It is a good raw material for medicine and can also be used for health care products and cosmetics [1-2].

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Methods

1. Establishment of an in vitro blood-brain barrier model

The digested NHA cells were inoculated in the lower part of a 6-well Transwell chamber. 3 ml of DMEM complete medium was added for culture. After the cell density grew to about 80%, ECs were inoculated in the Transwell chamber pretreated with rat tail collagen. An appropriate amount of EBM-2 complete culture medium was added to the upper and lower chambers. After 48 hours, the medium was changed. The blood-brain barrier model in vitro was successfully established after a total of 4 days of culture.

2. Real-time PCR method to detect the expressions of LINC00094, miR-224-5p and Endophilin-1 mRNA respectively

The RNA was extracted from the cells by the Trizol method, then the purity and concentration of the RNA were tested. One Step SYBR®PrimeScriptTM RT-PCR Kit was used to detect

the expressions of LINC00094 and Endophilin-1. A two-step probe kit was used to detect the expression of miR-224-5p. The CT values were determined, using GAPDH and U6 as internal controls, and $2^{-\triangle\triangle CT}$ was used to represent the relative expression levels of LINC00094, Endophilin-1 and miR-497-5p.

3. TEER measurement

A microporous resistance system was used to detect the TEER value. Before the test, the cells were taken out of the CO_2 incubator and placed in an ultraclean bench for 30 minutes. The background resistance of the blank Transwell cell membrane was subtracted from the readings of each set of samples, multiplied by the surface area of the Transwell membrane, and the final TEER value was obtained.

4. Determination of HRP exudation

The serum-free EBM-2 medium containing 0.5 μmol/l HRP was added into the Transwell chamber. The chamber was placed in the incubator for 1 hour. The culture fluid in the lower chamber was collected separately and the HRP content was determined by the TMB color method.

5. Immunofluorescence

Well-growing cells were selected and cultured on coverslips coated with 1% gelatin in advance. After the growth and fusion reached 95%-100%, 4% paraformaldehyde (30 min,25^{0}C), and then penetrated with 0.3% Trixton-100(10 min, 25^{0}C). The cells were sealed with 5% BSA (25^{0}C, 2 h). 1% BSA diluted primary antibodies (ZO-1, occludin and claudin-5) was added and incubated overnight at 4 °C in a humidified chamber. The cells were reheated for 30 minutes, washed with PBST, added with Cy3 labeled fluorescent secondary goat-anti-rabbit antibody diluted with 1% BSA, and incubated for 1.5 hours. The nucleus was washed with PBST in darkness, then stained with DAPI in darkness for 5 minutes. The slides were mounted with anti-fluorescence quenching mounting solution, observed and photographed under a fluorescence microscope.

6. Western blot detection of various proteins

The cell sample was collected, added with an appropriate amount of lysate, then centrifuged to collect the supernatant. The BCA method was employed to detect the protein concentration and determine the sample amount. 8-12% separation gel and concentrated gel were prepared according to the size of the target protein. SDS-PAGE electrophoresis was performed to separate the protein sample, and discontinued after bromophenol blue electrophoresis reached to the bottom. The transfer time was determined according to the molecular weight of the target protein. The film was transferred under the conditions of 100 V, 120 mA, and constant current of 90-180 min. After the transfer, the cells were blocked in a blocking solution at room temperature for 2 hours. Following this, the blocking solution was washed off with TTBS, the primary antibody was diluted according to a certain ratio, and the cells were sealed with membrane at 4 °C overnight. The cells were washed with TTBS, added with HRP-conjugated goat-anti-mouse or goat-anti-rabbit, and incubated at room temperature for 1.5 hours. TTBS cleaning and ECL light were performed. Photographs were taken.

7. Statistical methods

All data in this study were analyzed using the Graphpad 5.0, and the data were expressed as mean ± standard deviation. The t test was used for two groups of data, and the one-way analysis of variance (ANOVA) test was used for multiple groups of data. P value < 0.05 is considered statistically significant.

Results

1. Hydroxysafflor yellow A reduced the expression of LINC00094 in the in vitro BBB model constructed by Aβ incubating ECs

As shown in Figure 1, the expressions of LINC00094 and Endophilin-1 were significantly increased in the ECs incubated with Aβ. The expressions were significantly decreased in the ECs incubated with Hydroxysafflor yellow A. The expression of LINC00094 in the ECs incubated with Aβ + Hydroxysafflor yellow A reversed the increased expression of LINC00094 and Endophilin-1incubated with Aβ, while the expression of miR-224-5p exhibited just the opposite.

2. TEER value, detection of the integrity of the BBB in the ECs incubated with Aβ, and the amount of HRP exudation to detect the permeability of the BBB

ECs were incubated with Aβ. The TEER value of Hydroxysafflor yellow A group was significantly higher than that of the Control group, and the amount of HRP exudation was significantly reduced. Compared with the shNC group, the TEER value of the shLINC00094 group was significantly increased, and the amount of HRP exudation was significantly reduced. The TEER value of Hydroxysafflor yellow A + shLINC00094 group was higher than that of the Hydroxysafflor yellow A, the shLINC00094, and the

PBS + shNC groups, while the amount of HRP exudation was lower than that of the Hydroxysafflor yellow A, the ShLINC00094, and the PBS + shNC groups, as shown in Figure 2.

3. Immunofluorescence to analyze the expressions and distributions of ZO-1, occludin and claudin-5.
Hydroxysafflor yellow A was administrated to increase the expression of ZO-1, occludin and claudin-5, and their distributions on the cell membrane changed from discontinuous to relatively continuous. After the combined application of Hydroxysafflor yellow A and shLINC00094, the expressions of ZO-1, occludin, and claudin-5 were further increased, and their distribution on the cell membrane were more continuous, as shown in Figure 3.

4. The effect of Hydroxysafflor yellow A on various proteins.
The Western blot results are shown in Figure 4. The treatment of Hydroxysafflor yellow A in Aβ-incubated ECs significantly increased the expressions of ZO-1, occludin, and claudin-5. The combined application of Hydroxysafflor yellow A and LINC00094 further increased the expressions of ZO-1, occludin and claudin-5.

Discussion

Alzheimer's disease (AD) is a central nervous system degenerative disease with progressive cognitive dysfunction and behavioral impairment as the main clinical manifestations. It is the most common form of all Alzheimer's. Vascular β-amyloid (Aβ) deposition, endothelial cell dysfunction, vascular and neuronal degeneration, blood brain-barrier (BBB) destruction [3-5] are the main pathological changes of AD. BBB destruction can lead to reduced Aβ clearance, increased deposition in the brain and the formation of pathological blood vessels, thus accelerating the progression of AD [6].

Long noncoding RNA (LncRNA) is a type of transcript with more than 200 nucleotides and represents the most common and new type of noncoding RNA. Studies have shown that LncRNA can be used as a regulator of multiple biological processes, such as pre-mRNA processing and protein translation. The role of LncRNA in the central nervous system has attracted more and more attention from researchers [7].

Endophilin-1 is a member of the endocytic protein family and is mainly expressed in adult brain tissue. Endophilin-1 regulates the expression of tight-junction-related proteins through the EGFR-ERK1/2 and EGFR-JNK pathways [8]. Through Targetscan software analysis, it is found that there is a binding site for miR-224-5p in the 3'-UTR region of Endophilin-1 mRNA, suggesting that Endophilin-1 may be a downstream target gene of miR-224-5p.

The blood-brain barrier is a metabolic barrier that regulates the exchange of substances between the peripheral blood and the central nervous system (CNS), and is of great significance for maintaining the homeostasis and normal functions of the CNS. Some studies suggest that cerebrovascular dysfunction occurs in the early stage of AD, and BBB dysfunction plays a very important role in the accumulation of Aβ and the progression of AD [9-10]. The tight junction protein between endothelial cells is an important structural basis responsible for the integrity of the BBB and plays a key role in regulating the endothelial barrier function. The abnormal expression and distribution of TJs such as ZO-1, occludin and claudin-5 can change the permeability of the blood-brain barrier, and the decrease of tight junction protein expression will lead to the opening of the blood-brain barrier and increase its permeability. Abnormal expression of TJ protein has been reported in many CNS diseases [11-12].

In the bEnd.3 cell line incubated with Aβ1, the expression of TJs decreased, and the redistribution of ZO-1 at the cell boundary caused an increase in the permeability of the blood-brain barrier. In a 5XFAD mouse model of Alzheimer's disease, the internal amyloid plaques were deposited in the brain, blood vessels were destroyed and the shape of TJs was shortened, which increased the permeability of the blood-brain barrier [13]. Consistent with this finding, the exposure of Aβ42, especially in the form of oligomers, significantly reduced the expression levels of ZO-1, occludin and claudin-5, and destroyed the integrity of the mouse brain endothelial cell barrier [14]. Therefore, Aβ may destroy tight junction-related proteins and the adhesion junction of endothelial cells, thereby interfering with the barrier function.

Recently, more and more evidences proved that LncRNA plays an indispensable role in the development of CNS neurons, such as early neuronal differentiation and late synapse formation. LncRNA regulates gene expression by participating in a variety of biological networks, such as transcription, post-transcriptional regulation, and epigenetic regulation. Abnormal expression or mutation of LncRNA is related to various neurological diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease [15].

Whether LINC00094 is expressed in brain microvascular endothelial cells and whether it can

affect the permeability of BBB had not yet been reported. This study provided evidence that LINC00094 is involved in regulating the function of ECs. The results showed that the expression of LINC00094 in ECs incubated with Aβ increased, and the expression decreased after treatment with drugs, suggesting that LINC00094 may be involved in regulating the function of vascular endothelial cells. Further research found that knocking down LINC00094 and giving medications significantly reduced BBB permeability. After silencing LINC00094 and administering drugs simultaneously, it was relatively continuously distributed on the cell membrane. The above results suggest that Hydroxysafflor yellow A increases the expression of ZO-1, occludin and claudin-5 by knocking down the expression of LINC00094, and reduces the permeability of BBB in the AD microenvironment. Many miRNAs are abnormally expressed in CNS or differentially expressed in normal brain tissues and pathological states, while miRNA expression and functional studies in neurodegenerative diseases are getting more and more attention [16]. Our research found that the expression of miR-224-5p was low in ECs incubated with Aβ, and miR-224-5p was expressed after drug treatment. Overexpression of miR-224-5p in Aβ-incubated ECs can reduce the permeability of BBB in the AD microenvironment by promoting the expressions of tight junction related proteins ZO-1, occludin, and claudin-5.

In summary, this study proved that LINC00094 and Endophilin-1 were lowly expressed and miR-224-5p were highly expressed after Hydroxysafflor yellow A treatment. Hydroxysafflor yellow A increased the expression of tight junction related proteins ZO-1, occludin and claudin-5 through the LINC00094/miR-224-5p/Endophilin-1 pathway, and reduced the BBB permeability in the AD microenvironment. The results provide a new basis for Hydroxysafflor yellow A to treat AD from the perspective of BBB.

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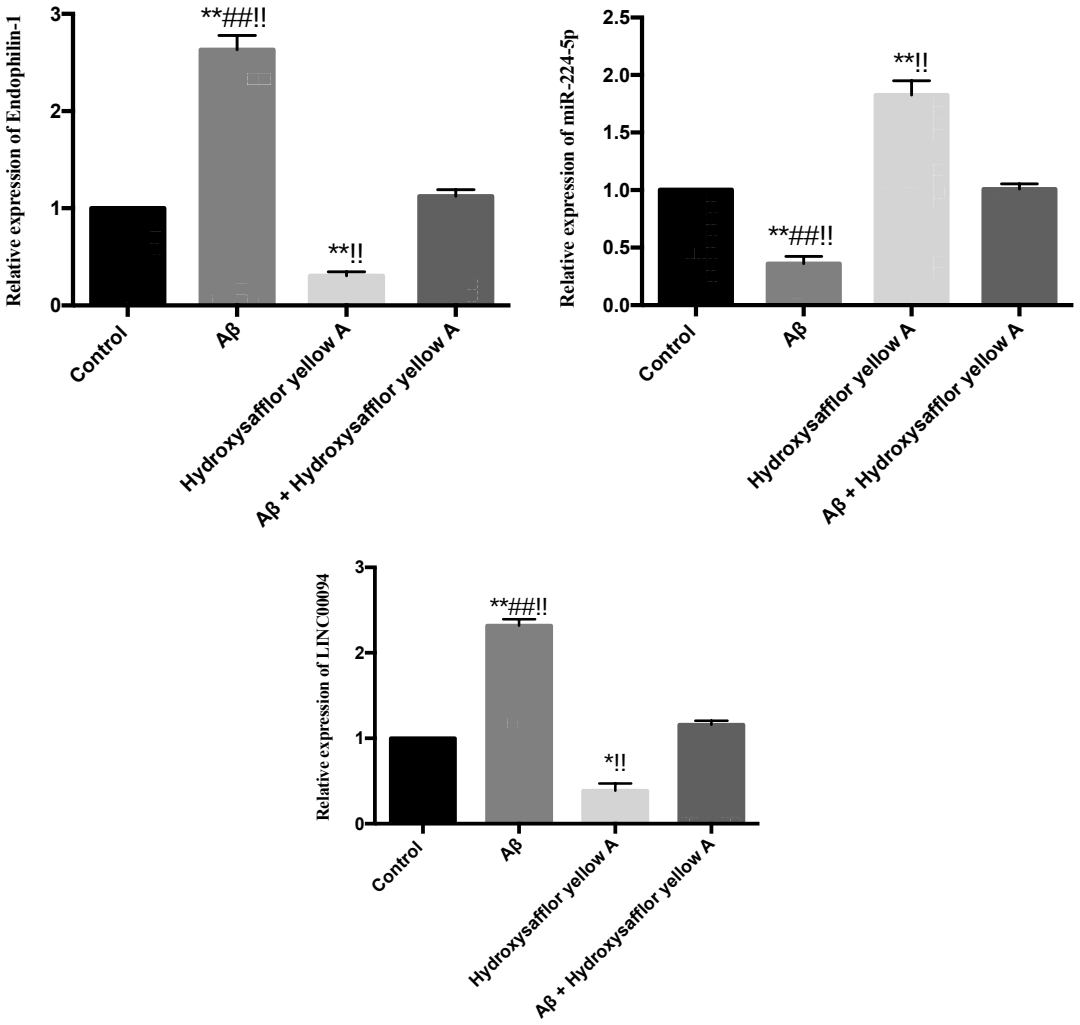

Figure 1. Real-time PCR detecting the expressions of LINC00094, miR-224-5p and Endophilin-1 mRNA.
** p <0.01 vs Control * p <0.05 vs Control ## p <0.01 vs Hydroxysafflor yellow A !! p < 0.01 vs Aβ + Hydroxysafflor yellow A

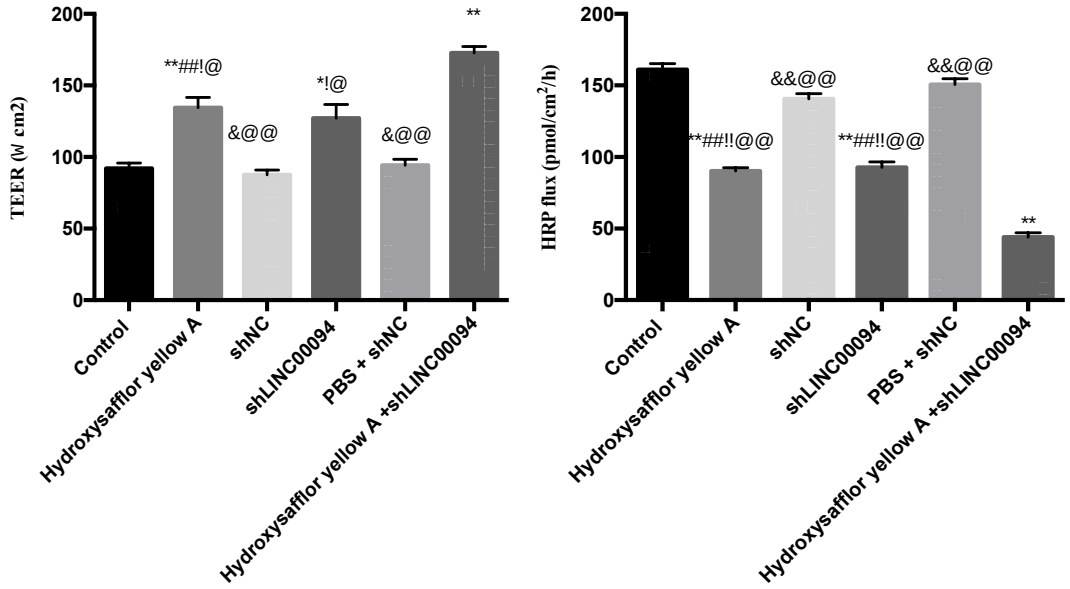

Figure 2. The effect of Hydroxysafflor yellow A on the integrity and permeability of the BBB in ECs incubated with Aβ.

** p < 0.01 vs Control * p < 0.05 vs Control ## p < 0.01 vs shNC # p < 0.05 vs shNC & p < 0.05 vs shLINC00094 !! p < 0.01 vs PBS +shNC

! p < 0.05 vs PBS + shNC @@ p < 0.01 vs Hydroxysafflor yellow A + shLINC00094 @ p < 0.05 vs Hydroxysafflor yellow A + shLINC00094

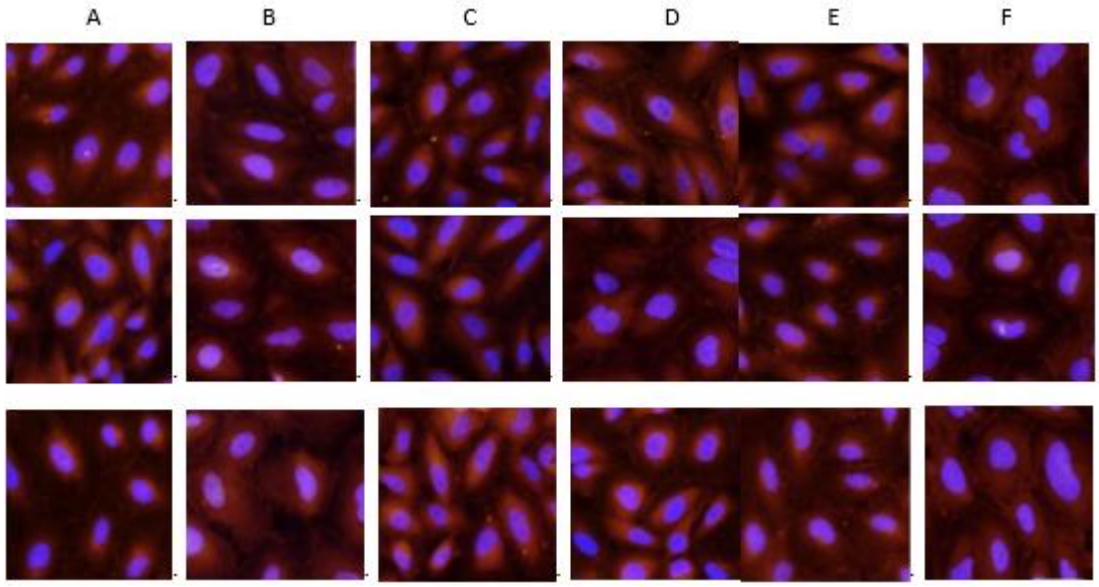

Figure 3. The effect of Hydroxysafflor yellow A on the expressions and distributions of ZO-1, occludin and claudin-5.

A: Control B: Hydroxysafflor yellow A C: shNC D: shLINC00094 E: PBS + shLINC00094 F: Hydroxysafflor yellow A + shLINC00094

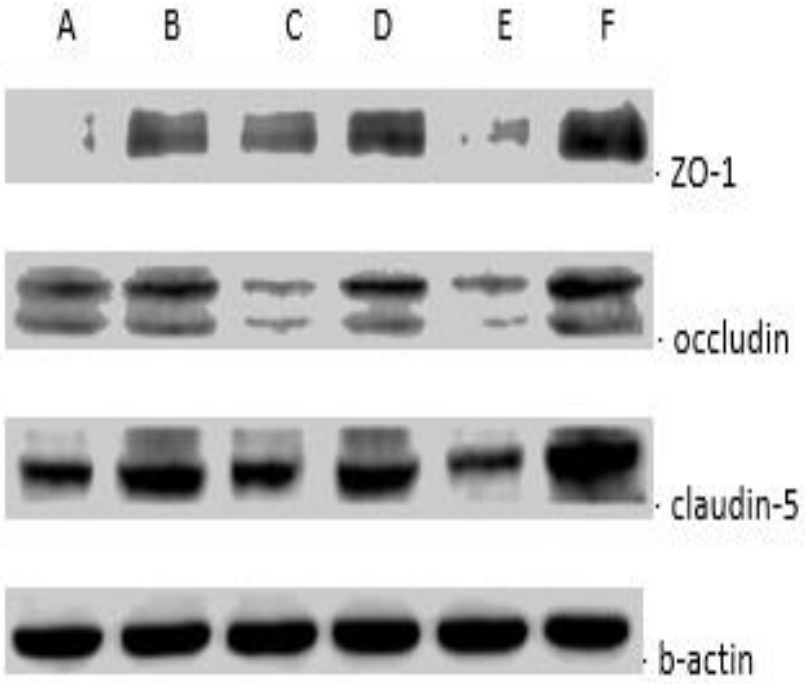

Figure 4. The effect of Hydroxysafflor yellow A on various proteins.

A: Control B: Hydroxysafflor yellow A C: shNC D: shLINC00094 E: PBS + shLINC00094 F: Hydroxysafflor yellow A + shLINC00094