https://doi.org/10.31925/farmacia.2023.1.23 **ORIGINAL ARTICLE**

ACORUS TATARINOWII SCHOTT INCREASES THE PERMEABILITY OF BLOOD-RETINAL BARRIER: EVIDENCE FROM AN *IN VITRO* STUDY

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Manuscript received: December 2022

Abstract

Blood-retinal barrier (BRB) restricts systemically administered drugs from entering the retina. In this study, we aimed to investigate the effects of the *Acorus tatarinowii Schott* (Shi Chang Pu (SCP)) extract on the permeability of BRB and clarify the underlying mechanisms. Rat retinal vascular endothelial cells (RVECs) were grown on transwell inserts to model the tight junctions of inner BRB *in vitro*. The results showed that after SCP treatment, the junctions between RVECs gradually disappeared, and the transepithelial electrical resistance (TEER) value of the cell monolayer was markedly decreased in time, indicating damage to the barrier integrity. Moreover, the expression levels of tight-junction proteins ZO-1 and occludin were significantly decreased after SCP treatment. In conclusion, this study provides promising evidence that the integrity and permeability of inner BRB could be obviously changed by SCP treatment.

Rezumat

Bariera hemato-retiniană (BHR) împiedică medicamentele administrate sistemic să pătrundă în retină. În acest studiu, ne-am propus investigarea efectelor și a mecanismelor de acțiune ale extractului de *Acorus tatarinowii Schott* (Shi Chang Pu (SCP)) asupra permeabilității BHR. Au fost cultivate celule endoteliale vasculare retiniene de șobolan (CEVR) pe inserții *transwell* pentru a modela joncțiunile interne strânse ale BRB *in vitro*. Rezultatele au arătat că după tratamentul SCP, joncțiunile dintre CEVR au dispărut treptat, iar valoarea rezistenței electrice transepiteliale (RETE) a monostratului celular a scăzut semnificativ în timp, indicând deteriorarea integrității barierei. Mai mult, nivelurile de expresie ale proteinelor cu joncțiune strânsă ZO-1 și ocludină au fost semnificativ scăzute după tratamentul SCP. În concluzie, acest studiu oferă dovezi promițătoare că integritatea și permeabilitatea BHR interioare ar putea fi modificate în mod evident prin tratamentul SCP.

Keywords: *Acorus tatarinowii Schott*, retinal vascular endothelial cells, blood-retinal barrier, tight junctions

Introduction

The retina is a light-sensing ocular tissue. The blood-retinal barrier (BRB) regulates fluids and molecular movement between the circulating blood and neural retina. The inner BRB is created by complex tight junctions of retinal capillary endothelial cells [1]. Disruption of the inner BRB, featured by retinal oedema, is an early and typical event in a number of eye diseases, such as diabetic retinopathy [2]. However, the presence of tight barriers also restricts systemically administered drugs from entering the retina [3]. Therefore, it is of critical importance to modulate the BRB permeability to facilitate the entry of therapeutic drugs into the retina.

Natural products possess a vast chemical diversity, and are appealing as valuable starting points for drug discovery since the ancient times [4, 5]. *Acorus tatarinowii Schott* (*Acoraceae* family) (Shi Chang Pu in Chinese (SCP)), first recorded in the Shennong Materia Medica, is a well-known Chinese traditional herb. The main biologically active ingredients of SCP include flavonoid glycosides, phenylpropane derivatives, amides and lignans [6]. SCP exerts a broad range of pharmacological effects such as protection of PC12 cells from amyloid-beta induced neurotoxicity [7], prevention against cardiovascular diseases[8], as well as increasing the permeability of blood-brain barrier in rats [9]. In this study, we established an *in vitro* model to investigate the effects of SCP extract on the permeability of BRB and clarify the underlying mechanisms.

Materials and Methods

Preparation for SCP extract

Dry material of SCP (773.2 g) was put into 7 litres of distilled water and distilled for 30 h. The yield of oil extract was 12.2 g. Then, the dregs were discarded,

and the decoction was condensed to 1 g/mL. The SCP extract was a blend of oil and decoction.

Cell culture and treatments

Rat retinal vascular endothelial cells (RVECs), obtained from PriCells Biotechnological Co., Ltd. (Wuhan, China), were cultured in RPMI-1640 Medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin–streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

When the cells reached confluence, they were transferred to Transwell inserts (0.4 μm pore size; Corning Inc., Corning, NY, USA), and grown for 15 days before experiments.

The cells were divided into three groups: Group A: RVECs without SCP treatment; Group B: RVECs treated with SCP extract for 12 h; Group C: RVECs treated with SCP extract for 24 h.

Transmission electron microscopy (TEM) analysis

The membrane along with the cells was removed, fixed with 2.5% glutaraldehyde (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 4 h at 4°C, rinsed with PBS (phosphate buffered saline) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then fixed in 1% osmium tetroxide (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at room temperature. After dehydration with graded ethanol solutions, thin sections were stained with uranyl acetate and lead citrate, and observed under a JEM-1200 EX microscope (JEOL, Ltd., Tokyo, Japan).

Measurement of transepithelial electrical resistance (TEER)

The barrier integrity was evaluated by TEER measurement using an endothelial volt/ohm meter (EVOM2, World Precision Instruments, Sarasota, FL, USA) in cell monolayers grown on transwell system. TEER values were calculated by subtracting the background resistance of cell-free inserts, and expressed as $\Omega \cdot cm^2$.

Cell viability assay

Cell viability was detected by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Cells were plated into 96-well plates, and 20 μL of MTT solution (5 mg/L; Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After incubation for additional 4 h, 150 μL of DMSO (dimethyl sulfoxide) was added to dissolve the formazan crystals (Sigma-Aldrich, St. Louis, MO, USA). The absorbance of each well was read at 570 nm on a microplate reader (Multiskan EX, Lab systems, Helsinki, Finland).

RT-qPCR (reverse transcription quantitative real-time Polymerase Chain Reaction) analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Waltham, Massachusetts, USA), and cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). qPCR reactions were then carried out using the SYBR Premix Ex Taq II kit (TaKaRa, Dalian, China) on a 7500 Fast Real-Time Sequence detection system (Applied Biosystems, Foster City, CA, USA). The target gene expression was calculated using the $2(-\Delta\Delta C(T))$ method [10], and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control.

Western blot analysis

Total protein was extracted using RIPA (radioimmunoprecipitation assay buffer) protein extraction reagent (Beyotime, Shanghai, China). Equal amounts of protein *per* lane were separated by SDS–polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated with specific primary antibodies overnight at 4°C: ZO-1 (1:1000; Invitrogen, cat. no. 33-9100, CA, USA) or occludin (1:1,000; Invitrogen, cat. no. 71-1500, CA, USA), followed by incubation with HRP-conjugated secondary antibody (Southern Biotech, Birmingham, AL, USA) for 1 h at room temperature. The immunoreactive bands were visualized with the enhanced chemiluminescence reagents (Bio-Rad Laboratories, Hercules, CA, USA), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a protein-loading control.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA). The results were presented as mean ± standard deviation (SD), and comparisons between groups were made by using Student's *t*-test or one-way ANOVA as appropriate. A p value below 0.05 was considered to indicate a statistically significant difference.

Results and Discussion

The examination of cells' tight junctions structure and cells' integrity by REM and TEER

RVECs were grown on a transwell culture system to model the inner BRB *in vitro*, and the structure of tight junctions was examined by TEM. The results showed that in Group A, the cell junction gap was tightly connected, but after SCP treatment, the junctions gradually became disappeared, and an enlarged cleft was observed, the effect increasing with the time of exposure (Figure 1).

The barrier integrity was then evaluated by TEER measurement. As shown in Figure 2, after SCP treatment, the TEER value of the cell monolayer was markedly decreased with time, and the difference between Group A and Group C was statistically significant.

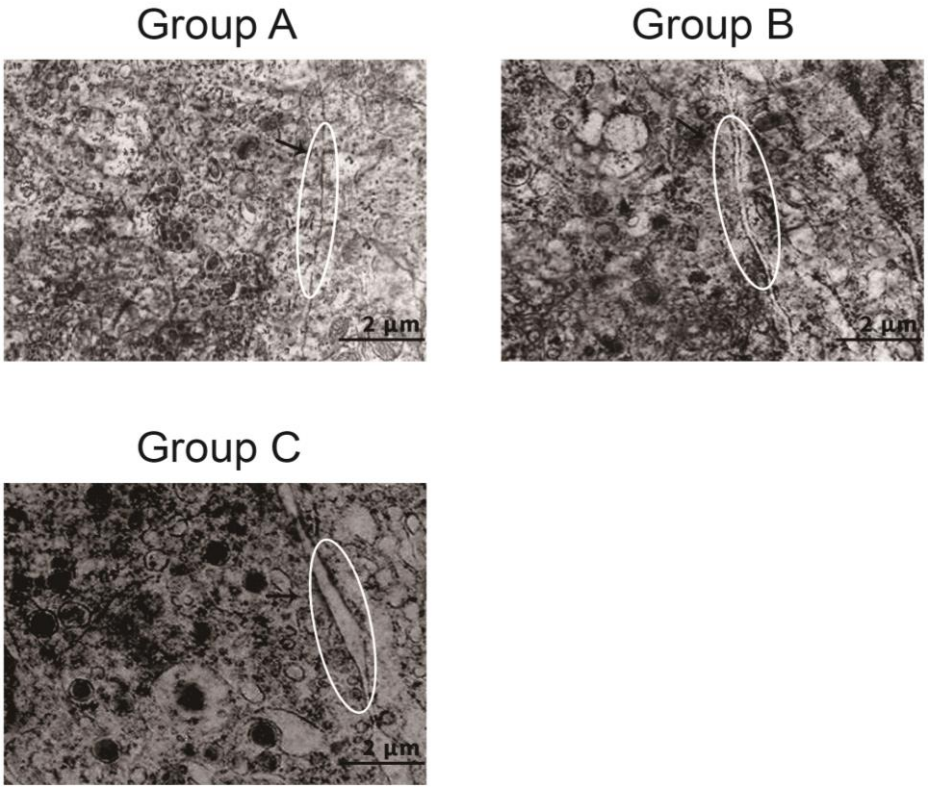

Figure 1.
Effect of SCP extract on the structure of tight junctions, as detected by transmission electron microscopy

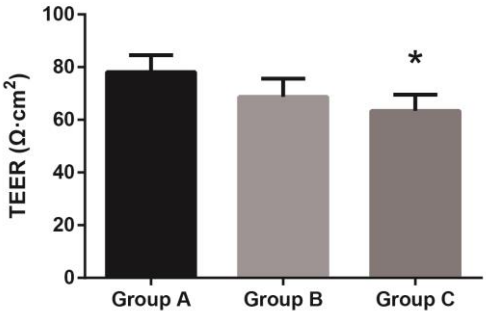

Figure 2.
Effect of SCP extract on the barrier integrity between RVECs, as evaluated by TEER measurement. The results were presented as the mean ± SD.
*p < 0.05 compared with group A

Cell viability evaluation by MTT assay
Cell viability was detected by MTT assay, and the results confirmed that treatment with SCP extract for 12 h or 24 h had no obvious effect on the viability of RVECs (Figure 3).

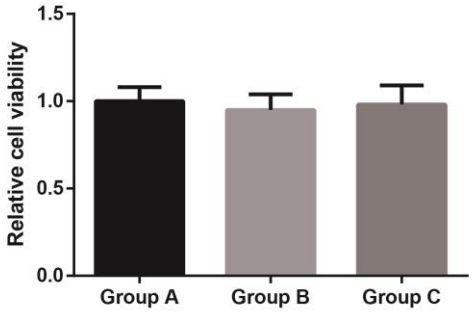

Figure 3.
Effect of SCP extract on the viability of RVECs, as detected by MTT assay. The results were presented as the mean ± SD

ZO-1 and occludin gene expression and protein levels
We further carried out RT-qPCR analysis, and observed that SCP treatment led to the decreased expression of ZO-1 and occludin mRNA in RVECs (Figure 4).
In addition, through western blot analysis, we also observed that the protein levels of ZO-1 and occludin in RVECs were notably reduced by SCP treatment (Figure 5).

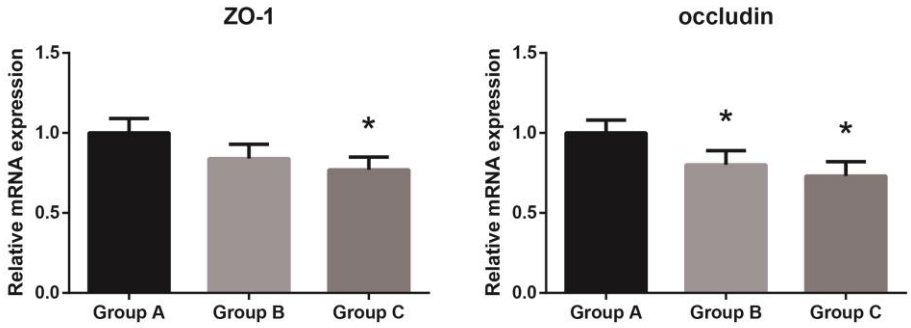

Figure 4.
Effect of SCP extract on the mRNA expression of ZO-1 and occludin in RVECs, as detected by RT-qPCR analysis. The results were presented as the mean ± SD. *p < 0.05 *vs.* Group A

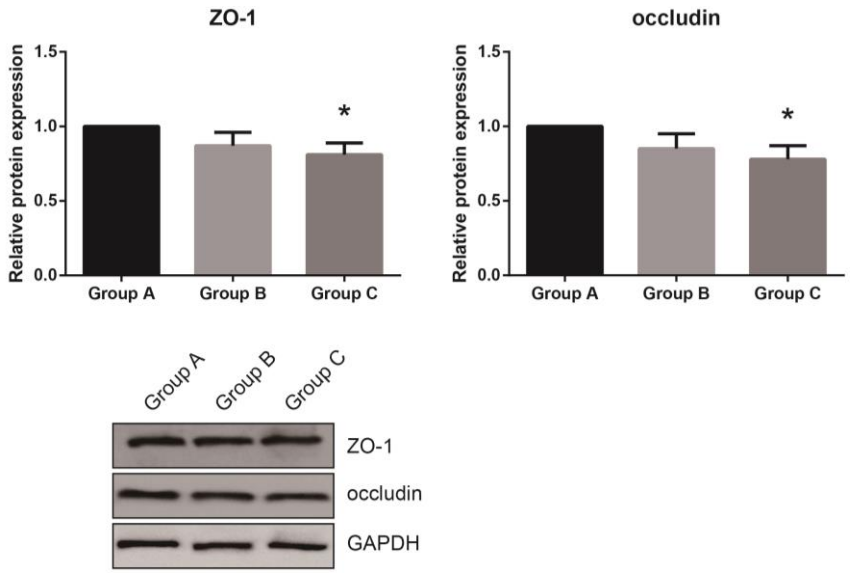

Figure 5.
Effect of SCP extract on the protein expression of ZO-1 and occludin in RVECs, as detected by western blot analysis. The results were presented as the mean ± SD. *p < 0.05 compared with Group A

There is an ever-increasing demand for managing eye diseases, which have a significant effect on vision and life quality. The structure of the human eye is very complex in nature, and retinal transfer of therapeutic drugs from the circulating blood is constrained by the existence of BRB [11, 12]. Thus, BRB modulation can increase the efficacy of various therapies. The inner BRB is similar to the canonical barrier of the CNS [13], and SCP was previously reported to increase the permeability of blood-brain barrier [9]. In this research, we successfully developed an *in vitro* model of inner BRB. Based on this model, we further evaluated the effects of SCP extract on the permeability of BRB and clarify the underlying mechanisms.

BRB modulation should be transient and selective in a controlled manner, and we confirmed that SCP extract was not harmful for RVECs. Through TEM analysis, we noted that SCP treatment caused a loss of the physiological barrier between RVECs. TEER is a widely accepted method to confirm the integrity and permeability of the cellular barriers, and TEER values are promising indicators of the integrity of cellular barriers [14]. This study demonstrated that TEER value of the cell monolayer was markedly decreased after SCP treatment, indicating the damage of the barrier integrity. Tight junctions (TJs) are sites of cell-cell adhesion composed of a series of transmembrane and cytoplasmic proteins, being the main structure of the inner BRB [15]. Increasing of inner BRB permeability is closely related with the degradation of TJs. For example, in diabetic retinopathy, growth factor-stimulated alterations in TJs contribute to vascular permeability [16]. Zonular occluden-1 (ZO-1) and occludin are identified as TJ marker proteins, and they play a critical role in TJ formation [17, 18]. Studies showed that the disfunction of ZO-1 determine a loss of barrier function as well as in the reorganization of apical actin and myosin[19, 20]. Our results showed that the expression levels of

these two proteins were markedly decreased by SCP treatment, indicating the degradation of TJs.

Conclusions

The present study provides promising evidence that the integrity and permeability of inner BRB could be obviously changed by SCP treatment. We therefore believe that collaborative use with SCP may increase the effectiveness of therapeutic drugs for eye diseases.

Acknowledgement

This work was supported by the scientific research projects of Heilongjiang Provincial Health Commission (No.20210707020308).

Conflict of interest

The authors declare no conflict of interest.

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