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Effects of RNA interference-mediated *E-selectin* gene silencing on cell adhesion molecule expression and cell-cell adhesion in vascular endothelial cells in mice with immunologic contact urticaria

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Abstract

Contact urticaria is recognized as the wheal and flare reaction at a site from direct contact with a chemical or protein agent. Ongoing studies have proposed that gene silencing may have a promising future in finding optimal treatment of a variety of disease; hence, the aim of the study was to investigate the effect of RNA interference-mediated *E-selectin* (*SELE*) gene silencing on cell adhesion molecule expression and on cell-cell adhesion in vascular endothelial cells (VECs) in a mouse model of immunologic contact urticaria (ICU). Following the successful establishment of mouse models of ICU induced by antidinitrophenol immunoglobulin E (IgE) combining 2,4-dinitrofluorobenzene challenge, enzyme-linked immunosorbent assay and immunohistochemistry were used to measure the levels of IgE, interleukin 4 (IL-4), interferon- γ (IFN- γ), and histamine as well as the positive expression rate of *SELE*, respectively. The siRNA-*SELE* vector was constructed and transfection efficiency was estimated prior to performing quantitative reverse-transcription polymerase chain reaction and Western blot assay to determine the relative expression of *SELE*, eosinophil cationic protein (ECP), intercellular adhesion molecule 1 (ICAM-1), L-selectin (CD62L), and the alpha chain of leukocyte function-associated antigen-1 (CD11a). Adhesion assay was then performed to assess the cell adhesion ability in VECs. Elevated levels of IgE, IL-4, IFN- γ , and histamine and increased positive expression rate of *SELE* were indicative of successful establishment of mouse models of ICU. Furthermore, the relative expression levels of *SELE*, ECP, ICAM-1, CD62L, and CD11a were highest in the OE-*SELE* group. Besides, cell adhesion ability of VECs was notably promoted. Collectively, the current study define the potential role of *SELE* silencing as an inhibitor to ICU development by inhibiting cell adhesion ability of VECs.

KEYWORDS

cell adhesion molecule, cell-cell adhesion, *E-selectin*, gene silencing, immunologic contact urticaria, RNA interference, vascular endothelial cells

1 | INTRODUCTION

Contact urticaria, including nonimmunologic contact urticaria (no sensitization required) and immunologic contact urticaria (ICU; sensitization required), is an urticarial wheal and flare reaction at the site of contact sensitization, which can be provoked by a variety of allergens.¹ ICU is primarily caused by low-molecular-weight chemicals (haptens) or proteins and regarded as a type I hypersensitivity reaction associated with the presence of specific immunoglobulin E (IgE).^{2,3} Potential complications of ICU involve organ systems other than skin, and severe complications can lead to anaphylaxis and death.⁴ The complications of ICU may include systemic symptoms (also called “CU syndrome”), which can be classified into four stages as follows: localized urticaria, dermatitis or nonspecific symptoms (itch, tingling, or burning) at the first stage; generalized urticaria at the second stage; extracutaneous involvement (rhinoconjunctivitis, bronchospasm, orolaryngeal, or gastrointestinal) at the third stage; and anaphylactic shock at the fourth stage.⁵ Because of the transient nature of ICU, it remains underreported, underdiagnosed, and undertreated.⁶ Therefore, a therapy targeting for ICU is urgently needed.

The endothelium regulates vascular tone, platelet activity, leukocyte adhesion, and angiogenesis by producing nitric oxide and other regulatory factors.⁷ In humans, endothelial dysfunction is related to an elevated adverse outcome during carotid arterial disease progression.⁸ Selectins are a family of 3 cell adhesion molecules, including E-selectin (SELE), P-selectin, and L-selectin (CD62L), and they greatly affect the leukocyte-endothelial interactions at an early stage of atherogenesis.^{9–11} Also, known as CD62E, SELE is expressed in endothelial cells via cytokine activation and significantly impact metastatic cancer cells.¹² Similarly, a previous study proved that SELE knockdown inhibits the adhesion capability of gastric cancer cells.¹³ Gene silencing refers to a technique used to negatively regulate gene expression and is effectively achieved by RNA interference, which can modulate the expression of specifically targeted genes by small interfering RNA (siRNA) molecules.¹⁴ This technique has been considered as a powerful therapeutic method in finding therapy options for various diseases such as non-small-cell lung cancer, allergic skin disease, and severe acute pancreatitis.^{15–17} By far, only a few studies have discussed the effects of SELE and gene silencing on ICU. Therefore, this study aims to explore the role of SELE silencing in cell adhesion molecule expression and cell-cell adhesion in ICU.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

A total of 60 specific pathogen-free BALB/c mice (30 males and 30 females) weighing 20 ± 2 g were purchased from the Laboratory Animal Center of Southern Medical University (animal license number: SCXK, Guangdong, 2016-0014). All mice were housed in an animal laboratory of Guangdong Pharmaceutical University in barrier environment at a room temperature of $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$, a relative humidity of $60\% \pm 2\%$, and ad libitum access to water and food (particulate standard feed). They were then randomly assigned into the ICU and control groups, with each group comprising of 15 male and 15 female mice. Animal experimentation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The study was performed with the approval of the Animal Care Committee of Renmin Hospital, Hubei University of Medicine.

2.2 | Model establishment

ICU mouse models were established after completion of the 2,4-dinitrofluorobenzene (DNFB) challenge in BALB/c mice passively sensitized with antidinitrophenol IgE antibody. After back depilation ($2\text{ cm} \times 3\text{ cm}$), mice in the ICU group were administered a tail vein injection containing 0.5 mL phosphate-buffered saline (PBS)-diluted monoclonal antidinitrophenol IgE antibody (1:5000, D8406; Sigma-Aldrich, St Louis, MO). After 24 hours, 0.15% DNFB (50 μL) was applied on the ears of each mouse for the induction of ICU, and the mice were observed later. These mice were fed a normal diet during the experiment. The mice showed a wheal reaction following external contact that occurred on and beyond the induction site, indicating successful model establishment. Fourteen days after the model establishment, ICU and control mice were euthanized for the following experiments.

2.3 | Observation of the ICU and control mice after ICU induction

The parameters observed were general conditions (including the spirit, appetite, and sleep), the wheal reaction, scratching behavior, and ear swelling of mice in the ICU and control groups. After administering a tail vein injection containing Evans blue, the mice were photographed using an automatic timing camera with 30-minute intervals. The frequency of wheal reaction at the exposed site on the back of mice was recorded. The mouse activity was captured on a videotape (each time

for 10 minutes) before DNFB challenge, 1 and 12 hours after challenge, and at 10 O'clock daily after the challenge. The videotapes were replayed to record the frequency of the scratching behavior. Vernier caliper was used to detect the swelling of bilateral auricles of mice at 1, 12, and 24 hours, and from the second day to the fourteenth day after DNFB challenge.

2.4 | Enzyme-linked immunosorbent assay

Fourteen days after successful establishment of the model, the blood collected from the excised eyeball was transferred to a ethylenediaminetetraacetic acid tube for centrifugation at 1000 rpm for 10 minutes, after which serum was obtained. According to the instruction in the enzyme-linked immunosorbent assay kit (eBioscience Inc, San Diego, CA), the kits were brought to room temperature for 20 minutes, followed by the preparation of washing buffer. Standard samples (100 μ L) were dissolved and then transferred to the reaction plate for standard curve. Then, the serum (100 μ L) was added into the wells, incubated at 37°C for 90 minutes, rinsed, added with 100 μ L fresh biotinylated antibody working solution, and then cultured for 60 minutes at 37°C. After washing, the samples were allowed to react with fresh enzyme-bound reactant (100 μ L) working solution in the dark, and then incubated at 37°C for 30 minutes. Later, the plate was washed 3 times. Samples were reacted with substrate (100 μ L) in the dark at 37°C for 15 minutes, and then stopping solution was added to terminate the reaction. Next, the Synergy 2 multidetection microplate reader (BioTek, Winooski, VT) was used to measure the optical density (OD) value at 450 nm of each well within 3 minutes. The curve was mapped according to the OD value and then the levels of IgE, interleukin 4 (IL-4), interferon- γ (IFN- γ), and histamine were analyzed.

2.5 | Hematoxylin and eosin staining

Fourteen days after the model establishment, 4 mice from each group were randomly selected and euthanized to collect vascular endothelium. The vascular endothelial cells (VECs) were fixed in 4% formalin for 24 hours, dehydrated using ethanol of grades 80%, 90%, and 100% and *N*-butanol, and embedded in paraffin at 60°C. After slicing the cells into 5- μ m serial sections, the cell samples were allowed to stand at 45°C, collected, and dried at 60°C for 1 hour, followed by dewaxing by xylene. After dehydration, the sections were stained using hematoxylin and eosin. After dewaxing and dehydrating the samples with gradient alcohol, hematoxylin (Beijing Solarbio Science & Technology Co, Ltd, Beijing, China) was used

to stain the samples for 2 minutes. Subsequently, the sections were rinsed under running water for 10 seconds. Next, 1% hydrochloric acid-ethanol was used for color separation for 10 seconds. After rinsing the sections under distilled water for 1 minute, the sections were stained with eosin for 1 minute, rinsed under distilled water for 10 seconds, dehydrated in gradient alcohol, cleared by xylene, and then mounted in neutral balsam. Histological changes in the vascular endothelium of mice were observed under an optical microscope (XP-330; Shanghai Bingyu Optical Instrument Co, Ltd, Shanghai, China).

2.6 | Immunohistochemistry

Fourteen days after the model establishment, the endothelia cells were collected according to the aforementioned procedure. The VECs were fixed in 4% formalin for 24 hours, dehydrated by ethanol of grades 80%, 90%, and 100% and *N*-butanol, and embedded in paraffin in a paraffin box at 60°C. After cutting the cells into 5- μ m serial sections, cell samples were allowed to stand at 45°C, collected, and dried at 60°C for 1 hour, followed by dewaxing by xylene. After dehydration, the sections were immersed in 3% H₂O₂ for 10 minutes and rinsed under distilled water. After antigen repair at high pressure for 90 seconds, the sections were cooled down to room temperature. Next, the sections were washed with PBS 3 times for 3 minutes each. Samples were blocked in 5% bovine serum albumin, incubated at 37°C for 30 minutes, and then added with primary antibody rabbit anti-SELE (100 μ L, 1:1000 to 1:4000, ab93952; Abcam, Cambridge, MA) for incubation at 4°C overnight. After washing the vascular endothelium samples with PBS, the samples were incubated with biotinylated goat anti-rabbit secondary antibody (1:100, HY90046; Shanghai Hengyuan Biological Technology Co, Ltd, Shanghai, China) at 37°C for 30 minutes. The sections were washed with PBS again. Subsequently, streptomycin avidin-peroxidase (Beijing Zhongshan Biotechnology Co, Ltd, Beijing, China) was added and the sections were incubated at 37°C for 30 minutes. Then, the sections were washed with PBS 3 times (each time for 3 minutes) and developed by 3,3'-diaminobenzidine (Beijing Bioss Biotechnology Co, Ltd, Beijing, China) at room temperature. Next, the samples were immersed in hematoxylin for 5 minutes, and rinsed under running water. After rinsing in 1% hydrochloric acid-alcohol for 4 seconds, the sections were rinsed under running water for 20 minutes. Image-Pro Plus software (Media Cybernetics, Bethesda, MD) was used to determine the mean OD value of SELE with positive coloration under high-power lens for quantitative analysis. One pathologist observed 5 high-power fields (100 cells in each field) of

each slice, and positive cells were counted according to different staining conditions. The normal brown cells were regarded as SELE-positive cells.¹⁸ Positive cells per total cells $\geq 10\%$ was recorded as positive and positive cells per total cells $< 10\%$ as negative.

2.7 | Construction of siRNA-SELE vector

The sense and antisense primer of SELE complementary DNA (cDNA) were designed and synthesized according to guidelines of GenBank (accession number: NM_001202). cDNA was used as a template to obtain DNA fragment of SELE via polymerase chain reaction (PCR) amplification. After agarose gel electrophoresis identification, the purified DNA fragment of SELE and pTrack-CMV were digested by restriction endonucleases *Bgl*II and *Sal*I, after which they were recycled using gel extraction kit and were ligated using T4 DNA ligase to construct a recombinant plasmid. Next day, the monoclonal of competent cells was prepared using CaCl_2 method. After extracting the plasmid DNA from transformed competent cells, PCR, double enzyme digestion, and DNA sequencing were performed to identify recombinant plasmid. The empty vector plasmid and recombinant plasmid of pTrack-CMV-SELE-eGFP/pTrack-CMV-siRNA-SELE obtained after sequencing were linearized using restriction enzyme *Pme*I to identify positive clones. Competent cells were transformed to select positive clones for amplification.

2.8 | Cell grouping, transfection, and transfection efficiency detection

The vascular endothelium samples were collected from 8 euthanized mice in the ICU and control group (4 mice from each group) and treated with 0.25% trypsin for 30 minutes at 37°C. After removal of digestive juice, the samples were used to prepare cell suspension using 0.1% collagenase I, screened by a metallic mesh of 100 meshes. Then, the cells were washed with Hank's solution once and cultured at RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum in an incubator of 5% CO_2 at 37°C. The samples were treated with 0.25% trypsin (Gibco), dissociated into single cell suspension with RPMI 1640 medium supplemented with 10% fetal bovine serum, after which routine culture and passage were performed. The subcultured VECs in the logarithmic growth phase were used for the following experiments.

The VECs in the ICU and control groups were allocated into the normal (normal VECs of mice in the control group without any treatment), control (normal VECs of mice in the ICU group without any transfection),

blank (VECs of mice in the ICU group transfected with empty vector pTrack-CMV-eGFP), negative control (NC; VECs of mice in the ICU group transfected with pTrack-CMV-NC sequences-eGFP), si-SELE (VECs of mice in the ICU group transfected with pTrack-CMV-siRNA-SELE-eGFP), and OE-SELE (VECs of mice in the ICU group transfected with pTrack-CMV-SELE-eGFP) groups. One day before transfection, VECs were passaged and seeded in a 6-well plate with 1×10^5 cells in each well, with 70% to 80% cell confluence. Transfection was conducted using Lipofectamine 2000 reagent (11668-019; Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Solutions containing empty vectors (100 ng), NC-siRNA-CAV3 (100 ng), siRNA-CAV3 (100 ng), and CAV3 mimics (100 ng) were diluted using 250 μL of serum-free medium Opti-MEM (51985042; Gibco), mixed with cells to attain the final concentration of 50 nM followed by incubation for 5 minutes at room temperature. Lipofectamine 2000 (5 μL) was diluted with serum-free medium Opti-MEM. The diluted solution was mixed and incubated for 5 minutes. Both of the aforementioned solutions were mixed, cultured for 20 minutes, and then added to cell-culture pore. After transfection, the cells were cultured at 37°C in an incubator with 5% CO_2 at 37°C for 6 to 8 hours and then cultured in complete medium for 24 to 48 hours for subsequent experimentation. After transfection for 8 to 10 hours, the cover glasses with full cell confluence in each group were taken and washed with sterilized PBS 2 times. The nucleus was stained by 4',6-diamidino-2-phenylindole in the dark followed by 3 PBS washes. Then, the cells were placed on a glass slide, fixed with 70% glycerol, and the transfection efficiency was evaluated using a fluorescence microscope (FM-400; Shanghai Pudan Optical Instrument Co, Ltd, Shanghai, China). The experiments were conducted in triplicates.

2.9 | Reverse-transcription quantitative polymerase chain reaction

The vascular endothelium samples were collected, added with liquid nitrogen, and then grounded into uniform fine powder. Total RNA was extracted from tissue samples using Trizol (15596-018; Invitrogen). The concentration and purity of RNA were determined. Total RNA was used as a template for reverse-transcription reaction into cDNA with a total volume of 25 μL using PrimeScript RT reagent kit (RR037A; Takara Biotechnology Co, Ltd, Dalian, China). The reaction conditions were as follows: reverse-transcription 3 times (15 minutes each) at 37°C and reverse transcriptase enzyme inactivation at 85°C for 5 seconds. The cDNA was diluted and mixed with 65 μL of diethyl pyrocarbonate. Quantitative

PCR of cDNA was conducted using SYBR Premix Ex Taq II (Takara Biotechnology Co, Ltd) according to the manufacturer's instructions of the SYBR Premix Ex Taq II kit. The reaction system was as follows: 2× SYBR Premix Ex Taq II (25 µL), forward primer (2 µL), reverse primer (2 µL), 50× ROX reference dye (1 µL), DNA templates (4 µL), and distilled water (16 µL), with a total volume of 50 µL. PCR was performed using an ABI PRISM 7300 System (ABI Company, Oyster Bay, NY), and the reaction conditions were as follows: predenaturation at 95°C for 4 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and final extension at 72°C for 1 minute. β -Actin was selected for internal reference. The primers (Table 1) were designed by Wuhan Bojie Biological Engineering Co, Ltd (Wuhan, China). When cells were in the logarithmic phase of growth, the threshold cycle (C_t) values were set and the expression of the target gene between the ICU and control groups was calculated by the $2^{-\Delta\Delta C_t}$ method. The formula was as follows: $\Delta\Delta C_t = [C_{t \text{ target gene}} - C_{t \text{ reference gene}}]_{\text{ICU group}} - [C_{t \text{ target gene}} - C_{t \text{ reference gene}}]_{\text{control group}}$. β -Actin was used as an internal reference. C_t refers to the amplified cycle conducted 3 times independently.

2.10 | Western blot assay

The VECs of mice were collected, added with liquid nitrogen, and grounded into uniform fine powder. Tissue lysate (1 mL; 50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate [SDS], 1% NP-40, 5 µg/mL

aprotinin, and 2 mmol/L phenylmethylsulphonyl fluoride [PMSF]) was added. After that the proteins were immersed in an ice bath and made into homogenate; protein lysis buffer was added for 30 minutes at 4°C and shaken at 10-minute intervals. The tissue samples were centrifuged at 12 000 rpm at 4°C for 20 minutes. The supernatant was discarded. The protein concentration of each sample was determined using the bicinchoninic acid kit (20201ES76; Yisheng Biotechnology Co, Ltd, Shanghai, China) and was adjusted by deionized water to retain the same loading quantity of a sample. SDS separation gel (10%) and SDS concentration gel (5%) were prepared. The samples were mixed with loading buffer and boiled at 100°C for 5 minutes. After ice bath and centrifugation, the protein samples were equally added into each lane by using a micropipette for electrophoresis, and then transferred onto a nitrocellulose membrane. Then, the membranes were blocked in 5% skimmed milk at 4°C overnight and incubated with primary antibody of SELE (1:1000, ab2497; Abcam), eosinophil cationic protein (ECP; 1:1000, ab20357; Abcam), intercellular adhesion molecule 1 (ICAM-1; 1:2000, ab53013; Abcam), CD62L (1:100 to 1:500, ab135792; Abcam), and the alpha chain of leukocyte function-associated antigen-1 (CD11a; 1:5000, ab52895; Abcam) overnight. After rinsing the samples under PBS 3 times for 5 minutes each time, they were incubated with the second antibody of goat anti-rabbit immunoglobulin G (1:1000; Boster Biological Technology Co, Ltd, Wuhan, China) at 37°C for 1 hour, followed by rinsing with PBS at room temperature 3 times, 5 minutes per time. An enhanced chemiluminescence reagent (Pierce, Rockford, IL) was used to color the membrane for 1 minute at room temperature. After the reagent was extracted, the membranes were covered with a preservative film and observed under X-rays. β -Actin was regarded as the internal reference. The average gray value of target protein band to internal reference band was considered as the relative protein expression. The experiment was conducted in triplicates. The same procedures were also applied on cells after transfection.

2.11 | Adhesion assay

VECs from each group were named in a similar manner as the groups. Cells were seeded into a 24-well plate, and when the cells reached a monolayer confluency of 80% to 85%, THP-1 (human acute monocytic leukemia cell line) cell suspension labeled with calcein-AM (500 µL) was added into each well. Six duplicated wells were set in each group. After culturing for 30 minutes, the suspension from each well was

TABLE 1 Primer sequences of related genes for reverse-transcription quantitative polymerase chain reaction

Gene	Sequence (5'-3')
<i>SELE</i>	F: ATAACGAGACGCCATCATGC R: TGTCCACTGCCCTTGTGC
<i>ECP</i>	F: GAAAGATGGGGCTGTTGAGT R: CTTCTCACCAGGAGGTAGCG
<i>ICAM-1</i>	F: GACCACGGAGCCAATTCTCA R: TCCAGTTCCCAAGCAGTCC
<i>CD62L</i>	F: CATTCTGTAGCCGTCATGG R: AGGAGGAGCTGTTGGTCATG
<i>CD11a</i>	F: TCTGCCTATGACCACAACA R: TCTCCACCTGAAGACAAT
β -actin	F: ATGGGTCAGAAGGACTCTAC R: ACGCTCGGTCAGGATCTTCAT

CD11a, the alpha chain of leukocyte function-associated antigen-1; *CD62L*, L-selectin; *ECP*, eosinophil cationic protein; F, forward; *ICAM-1*, intercellular adhesion molecule 1; R, reverse; *SELE*, E-selectin.

removed and washed with PBS 3 times. The fluorescent expression of cells in each group was detected to estimate the cell-cell adhesion.

2.12 | Statistical analysis

Data were analyzed using SPSS 22.0 software (IBM Corp, Somers, NY). Measurement data were expressed as mean $\pm$ standard deviation. The *t* test was conducted for comparing between two groups; while one-way analysis of variance was conducted for comparing among multiple groups. $P < .05$ was considered to be statistically significant.

3 | RESULTS

3.1 | Comparison of different indexes

Shortly after beginning the experiment, several indexes including general conditions, wheal reaction, scratching behavior, and ear swelling were taken in consideration. Mice in the ICU group presented inferior mentality; messy hair; less activities; and decreased excrement, food intake, and sleep 24 hours after ICU induction compared with the control group. After 6 to 8 days, an obvious decrease in weight gain was observed in mice in the ICU group; moreover, the mice started to lose weight. The control group showed no wheal reaction, while the ICU group showed some wheal reaction at the exposed site on the back. An obvious bump and blue spots were observed on the back of mice treated with tail vein injection containing Evans blue. The diameter of wheal of mice in the ICU group was about 0.1 to 0.5 cm, and disappeared 3 to 4 hours later, with recurrent attacks. The frequency of wheal reaction on the ICU mice reached its peak on 3 days, thereafter it decreased slowly later. In comparison with the control group, it was observed that the scratching behavior frequency significantly increased in the ICU group ($P < .05$), and it reached its peak value 1 hour after ICU induction. On the eighth day after ICU induction,

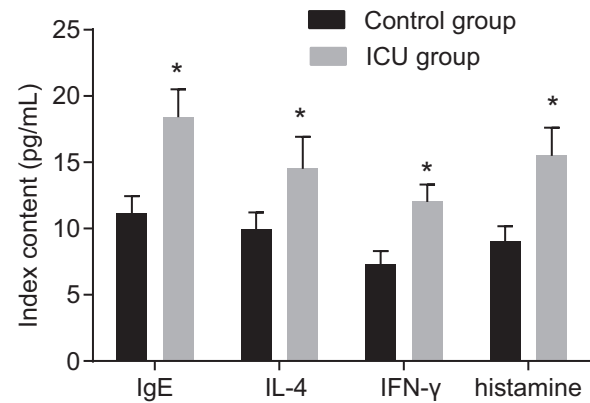


FIGURE 2 Comparison of expression levels of IgE, IL-4, IFN-γ, and histamine in peripheral blood. * $P < .05$ versus the control group. ICU, immunologic contact urticaria; IFN-γ, interferon γ; IgE, immunoglobulin E; IL-4, interleukin 4

another peak value of scratching behavior frequency was observed. After the DNFB challenge on the ear, the ear swelling frequency notably elevated in the ICU group in comparison to the control group ($P < .05$; Figure 1).

3.2 | Serious inflammation in ICU mice

Next, enzyme-linked immunosorbent assay was performed to measure inflammation-related factors IgE, IL-4, IFN-γ, and histamine. As shown in Figure 2, in comparison with the control group, the ICU group presented slightly elevated levels of IgE, IL-4, IFN-γ, and histamine ($P < .05$). These results indicated that ICU led to higher levels of inflammation-related factors.

3.3 | Pathological changes of vascular endothelium

Afterwards, we observed the pathological alterations. The control group showed fine structure of vascular endothelium, and collagen fibers were arranged in neat rows without fracture. In addition, there was no obvious inflammatory cell infiltration (Figure 3, control). The

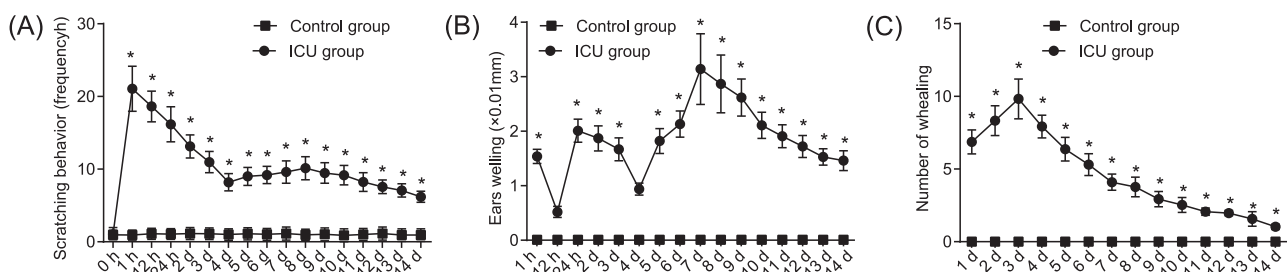


FIGURE 1 Observation of different indexes. (A) Scratching behavior frequency was higher in the ICU group. (B) Ear swelling frequency was higher in the ICU group. (C) Wheal reaction frequency was higher in the ICU group. * $P < .05$ versus the control group. ICU, immunologic contact urticaria

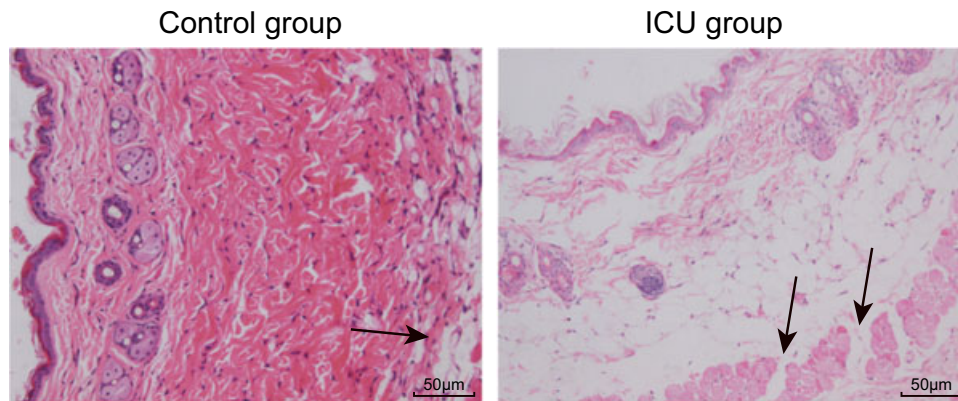


FIGURE 3 Pathological observation of vascular endothelium after hematoxylin and eosin staining ($\times 200$). The scale was $50\ \mu\text{m}$. Four rats from each group were taken, and the arrows point toward the collagen fibers. ICU, immunologic contact urticaria

ICU mice presented normal vascular endothelium without significant changes, edema in dermis, widened collagen bundle distance, and slight inflammatory cell infiltration surrounding blood vessels (Figure 3, model).

3.4 | Positive expression rate of SELE was higher in ICU mice

Subsequently, the positive expression rate of SELE protein was evaluated using immunohistochemistry. As shown in the Figure 4, the positive expression rate of SELE in the ICU group was $79.57\% \pm 8.60\%$ and in the control group was $41.02\% \pm 5.32\%$. In comparison with the control group, it was observed that the positive expression rate of SELE was notably elevated in the ICU group ($P < .05$). Altogether, higher SELE protein-positive rate can be found in the ICU group.

3.5 | Comparison of SELE-related and inflammation-related factors expression levels before transfection

Next, reverse-transcription PCR and Western blot assay were applied to measure the expressions of SELE-related and ICU-related factors in tissues. As shown in Figure 5, the ICU

group showed evidently increased messenger RNA (mRNA) and protein expression of SELE, ECP, ICAM-1, CD62L, and CD11a in comparison to the control group ($P < .05$).

3.6 | Successful transfection of SELE vector

To assess the effects of *SELE* on ICU, Si-*SELE* vector was conducted and transfected. After cell transfection, the normal and control groups did not present a green fluorescence expression under the fluorescence microscope. A significant green fluorescence expression was observed in the blank, NC, si-*SELE*, and OE-*SELE* groups (expression rate reached 80%), indicating that siRNA-*SELE*, NC-siRNA-*SELE*, Ad-CMV-*SELE*-eGFP, and Ad-CMV-eGFP were successfully transfected into the VECs and effectively expressed in the cells (Figure 6).

3.7 | Comparison of SELE-related and ICU-related factors expression levels after transfection

After that reverse-transcription PCR and Western blot assay were used for the determination of the expression levels of SELE and ICU-related factors in cells after

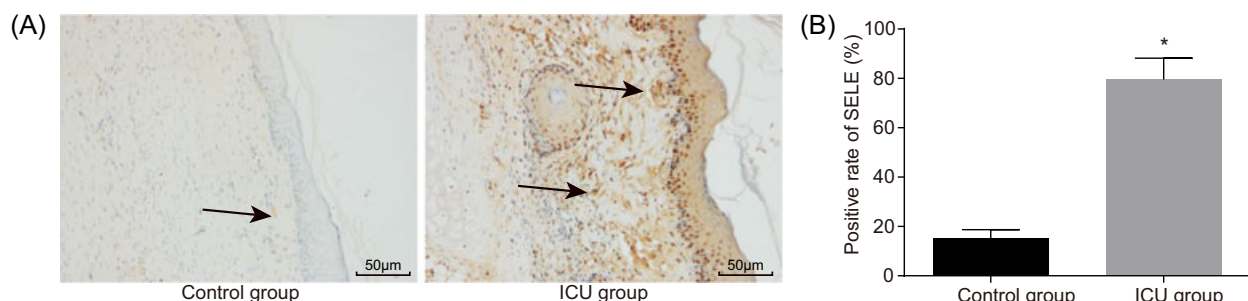


FIGURE 4 Immunohistochemistry proves that ICU group has higher SELE protein-positive rate ($\times 200$). The scale was $50\ \mu\text{m}$. Four rats from each group were taken, and the arrows point toward SELE-positive cells. (A) Results of immunohistochemistry. (B) Positive expression rate of SELE was higher in the ICU group. $*P < .05$ versus the control group. ICU, immunologic contact urticaria; *SELE*, E-selectin

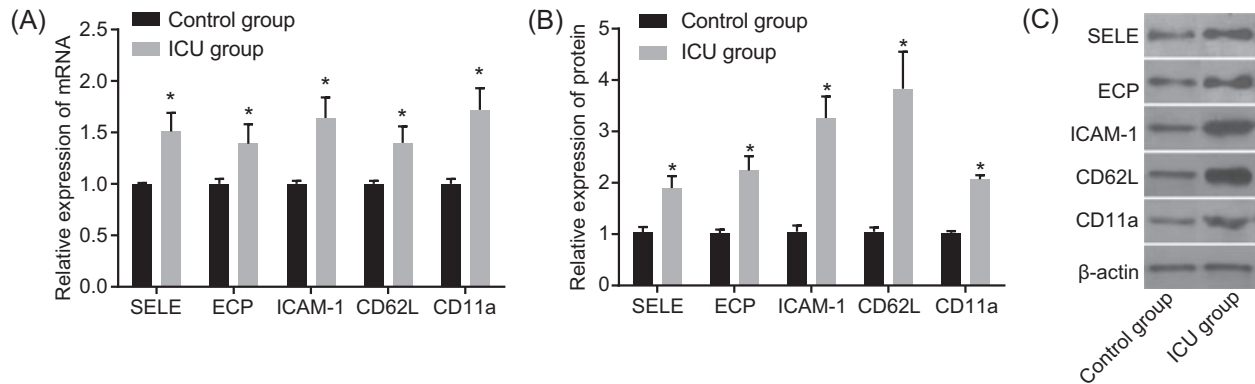


FIGURE 5 Expression of SELE, ECP, ICAM-1, CD62L, and CD11a were higher in the ICU group than in the control group. (A) Relative expression of SELE, ECP, ICAM-1, CD62L, and CD11a using RT-qPCR. (B) Relative expression of SELE, ECP, ICAM-1, CD62L, and CD11a by Western blot assay. (C) Results of Western blot assay. * $P < .05$ versus the control group; $n = 6$; relative mRNA and protein expression were the measurement data; comparison between two groups used t test; the experiment was conducted in triplicates. CD11a, the alpha chain of leukocyte function-associated antigen-1; CD62L, L-selectin; ECP, eosinophil cationic protein; ICAM-1, intercellular adhesion molecule 1; ICU, immunologic contact urticaria; mRNA, messenger RNA; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SELE, E-selectin

transfection. As shown in Figure 7, compared with the normal group, the control, blank, NC, si-SELE, and OE-SELE groups showed an obvious increase in mRNA and protein expressions of SELE, ECP, ICAM-1, CD62L, and CD11a (all $P < .05$). In comparison with the control group, the si-SELE group presented significantly reduced mRNA and protein expression of SELE, ECP, ICAM-1, CD62L, and CD11a, while the OE-SELE group presented completely opposite results (all $P < .05$). No evident differences were observed in the mRNA and protein expressions among the control, blank, and NC groups ($P > .05$).

3.8 | SELE enhanced cell-cell adhesion ability

Finally, adhesion assay was performed to assess the cell-cell adhesion ability in each group. As presented in Figure 8, compared with the normal group, the control,

blank, NC, si-SELE, and OE-SELE groups presented significant elevation in cell-cell adhesion ability (all $P < .05$). On comparing with the control group, it was observed that the si-SELE group had obviously decreased cell-cell adhesion ability, while the OE-SELE group showed increased cell-cell adhesion ability (all $P < .05$). There were no evident differences in the control, blank, and NC groups (all $P > .05$). These findings suggested that SELE might stimulate adhesion ability.

4 | DISCUSSION

ICU is associated with poor quality of life, long-term allergies, and progression from self-limiting skin eruptions to multisystemic.⁶ SELE may be involved in ICU as a beneficial application of siRNA-mediated silencing in vascular endothelium contributing to the successful suppression of leukocyte adhesion.¹⁹ To reduce

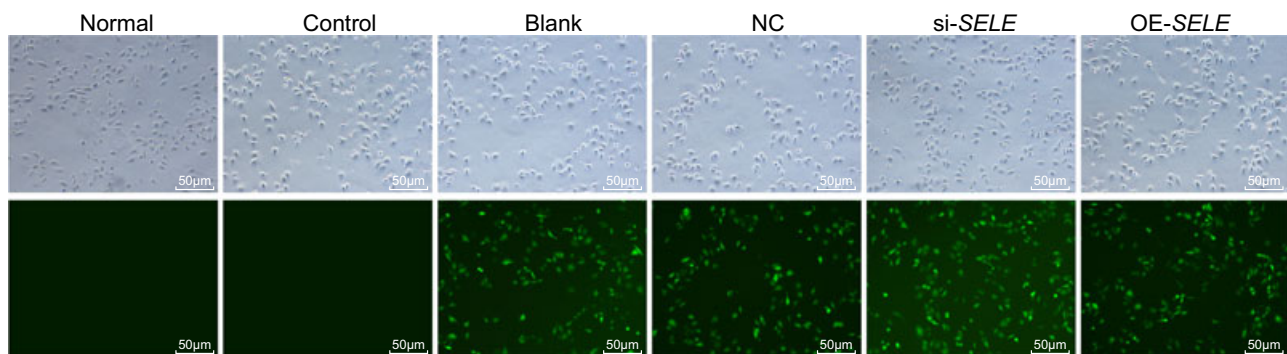


FIGURE 6 Immunohistochemistry indicates successful model establishment ($\times 200$). The scale bar was 50 μm . NC, negative control; SELE, E-selectin; VECs, vascular endothelial cells

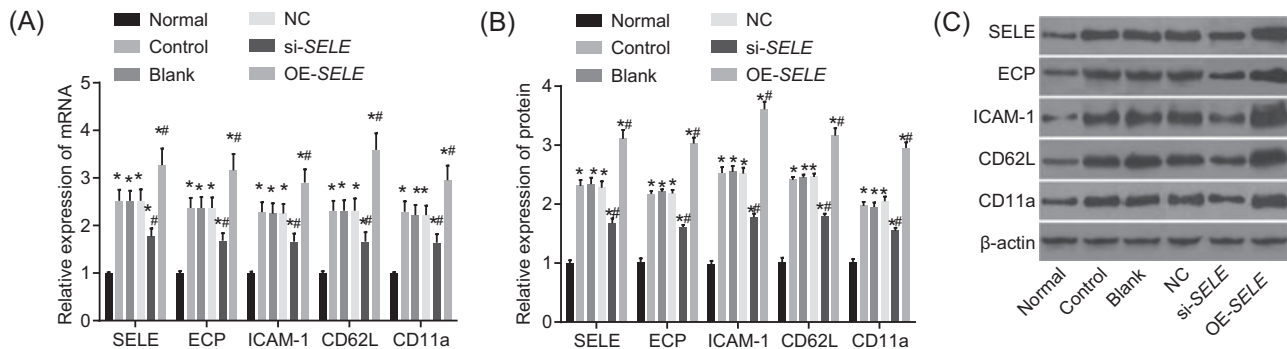


FIGURE 7 Si-SELE vector transfection increases the expression of SELE, ECP, ICAM-1, CD62L, and CD11a. (A) Relative expression of SELE, ECP, ICAM-1, CD62L, and CD11a using RT-qPCR. (B) Relative expression of SELE, ECP, ICAM-1, CD62L, and CD11a by Western blot assay. (C) Gray value analysis by Western blot assay. * $P < .05$ versus the normal group; # $P < .05$ versus the control group; $n = 6$; relative mRNA and protein expression was the measurement data; comparison among groups used one-way analysis of variance; the experiments were conducted in triplicates. CD11a, the alpha chain of leukocyte function-associated antigen-1; CD62L, L-selectin; ECP, eosinophil cationic protein; ICAM-1, intercellular adhesion molecule 1; mRNA, messenger RNA; NC, negative control; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SELE, E-selectin

ICU-induced mortality, this study was conducted to explore the effects of RNA interference-mediated *SELE* gene silencing on endothelial cell adhesion molecule expression and cell-cell adhesion in ICU.

First, the results of this current study revealed that the ICU mice had elevated levels of IgE, IL-4, IFN- γ , and histamine compared with the control mice, which were indicative of serious inflammation in ICU. It has been proposed that the expression of some inflammatory molecules such as IL-4, IL-1 β , and tumor necrosis factor- α is highly active in urticaria.²⁰ It has been reported that ICU is related to the presence of specific IgE, and histamine release had key impacts on ICU pathogenesis.^{3,6}

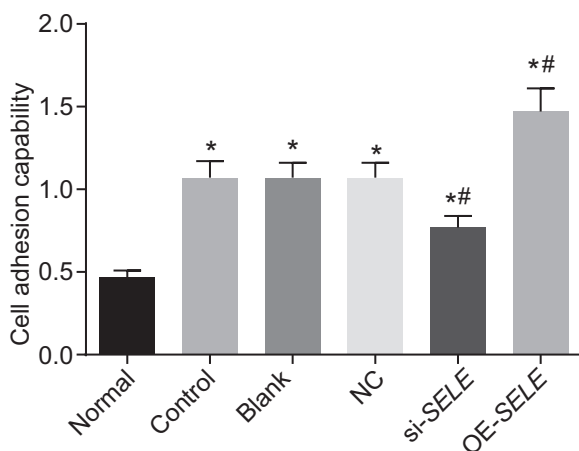


FIGURE 8 Cell-cell adhesion ability was greatly improved by SELE. * $P < .05$ versus the normal group; # $P < .05$ versus the control group; $n = 6$; the data in the figure were the measurement data; comparison among groups used one-way analysis of variance; the experiments were conducted in triplicates. NC, negative control; SELE, E-selectin

Several studies have documented the restorative effect of IL-4 on the development of allergic inflammation, and IFN- γ as an important regulator of immunity and inflammation.^{21,22}

Next, the ICU mice and OE-SELE group showed elevated positive rate of SELE protein, and the elevated expression of SELE, ECP, ICAM-1, CD62L, and CD11a. A prior study by Nimrichter et al²³ stated that selectins in combination with complementary carbohydrates on circulating neutrophils regulate inflammation. Suppression of *SELE* serves as a positive factor during therapy for treating acute or chronic inflammation.²⁴ A previous study observed that the expression of ECP and SELE were significantly higher in patients with allergic rhinitis, suggesting ECP and SELE had significant roles in the pathogenesis of allergic rhinitis,²⁵ which is in consistency with the results of our study. A previous study highlighted the dependence of ECP on the activation of eosinophil, which are bounded by a large number of proinflammatory mediators.²⁶ Meanwhile, Sato et al²⁷ have showed that ECP participates in allergic inflammation. CD62L, known as L-selectin, is a member of selectin family and is of significant importance in regulating the recruitment of monocytes to lymphoid tissue from the blood during an episode of inflammation.^{28,29} In addition, ICAM-1 and CD11a are also factors that are involved in inflammation.^{30,31} ICAM-1, highly active in the areas of inflammation, is considered as the most important adhesion molecule for leukocyte recruitment to inflamed sites.³² CD11a, a component of the heterodimeric $\beta 2$ -integrin LFA-1, is essential in transmigration of leukocytes into tissues during inflammation and homeostasis.³³ Therefore, *SELE* knockdown resulted in decreased levels of ECP, ICAM-1, CD62L, and CD11a.

At last, the expressions of SELE, ICAM-1, and CD11a were notably elevated in ICU mice, suggesting increased cell-cell adhesion strength in ICU mice, and *SELE* obviously enhanced cell-cell adhesion capability. Numerous studies have demonstrated that endothelial cells express several types of adhesion molecules that mediate the recruitment of leukocytes into the inflamed tissue.^{34,35} Adhesion molecules consist of the selectins family and the immunoglobulin group, such as the ICAM-1.¹⁰ *ICAM-1* and *SELE* are implicated in endothelial function in hypertensive individuals.³⁶ CD11a also participates in cell adhesion.³⁷ CD11a and CD11b/CD18 are all members of the leukocyte adhesion molecules β 2-integrin, and CD11b/CD18 expressed by neutrophil is important in mediating neutrophil-endothelial cell interactions and binding to adhesion molecule on the surface of VECs.³⁸ Moreover, *SELE* exerts decisive effects on the beginning of a cell adhesion cascade.²⁴ Smadja et al³⁹ showed that *SELE* stimulates stem cell adhesion in a murine model of infantile hemangioma, which is in consistency with our study. Thus, *SELE* inhibition could decrease the cell adhesion capability of VECs.

5 | CONCLUSIONS

To conclude, the results of this study clarified that siRNA-mediated *SELE* silencing suppresses cell adhesion molecule expression and cell-cell adhesion in VECs in mice with ICU. Further studies are required to explore the underlying mechanism behind siRNA-mediated silencing of *SELE* on cell adhesion molecule expression and cell-cell adhesion in VECs in ICU.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests.

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