

Specific role of the tight junction proteins occludin and claudin-5 on the blood–brain barrier during *Listeria monocytogenes* infection¹⁾

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Summary

To investigate the blood-brain barrier (BBB) permeability of mice after *Listeria monocytogenes* infection for further study on the mechanism of *L. monocytogenes* crossing the BBB, a mouse model was established and Evans blue assay was performed to assess the BBB disruption. Using relative quantitative real-time PCR, the RNA expression of Zonula occludens-1 (ZO-1), occludin and claudin-5 were detected. In addition, the protein expression level of ZO-1, occludin and claudin-5 were detected by immunohistochemistry and western blot. The extravasation of Evans blue dye was significantly different between 24 h and 96 h ($P < 0.05$). The mRNA expression of occludin and claudin-5 were down-regulated than that of the control group at each sampling point ($P < 0.05$) and ZO-1 showed a significant change at 96 h ($P < 0.05$). In addition, the protein expression level of occludin and claudin-5 decreased significantly at 48 h and 96 h ($P < 0.05$) by immunohistochemistry and western blot, compared with the control, while ZO-1 was almost unchanged ($P > 0.05$). All results indicating that the tight junction integrity of endothelial cells was destroyed and BBB permeability was enhanced in the process of *L. monocytogenes* infection, and this change was related to the decrease of the expression occludin and claudin-5.

Keywords: *Listeria monocytogenes*, blood-brain barrier, tight junction, ZO-1, occludin, claudin-5

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive pathogen that can cause listeriosis, including gastroenteritis, meningitis and encephalitis (20). *L. monocytogenes* is now one of the most important frequent pathogens that have the ability to lead to bacterial meningitis in humans, after *Streptococcus pneumoniae* and *Neisseria meningitidis* (1). As a food-borne pathogen, *L. monocytogenes* can cross the intestinal barrier through intestinal epithelial cells or phagocytes, and reach the liver and spleen along with the lymphatic system and blood circulation system, multiply, and finally spread to the brain through blood circulation. *L. monocytogenes* is known to affect pregnant women, immunocompromised individuals, the young and the elderly via the oral route (3). Although the incidence of human listeriosis is very low, it is recognized as the most severe zoonosis with the highest hospitalization and mortality rate (13.8% among 2480 confirmed cases in the EU) (15). Furthermore, accord-

ing to European surveillance data, encephalitis due to listeriosis is of major veterinary importance because it is associated with high morbidity and mortality in infected ruminants (23).

L. monocytogenes can reach the blood-brain barrier (BBB) causing meningitis and encephalitis. The mechanism of *L. monocytogenes* infection has been studied in both *in vitro* and *in vivo* models (8, 9, 14), but many aspects of the pathogenic mechanism of neurolisteriosis remain elusive. Neurological symptoms appear after artificial infection, and pathological changes include meningeal and cerebral hyperemia, cerebral edema, neuronal degeneration, necrosis and glial cell proliferation. Inflammatory cell infiltration around capillaries in some brain tissues has also been observed, indicating capillary permeability changes. The BBB acts as a physiological barrier to prevent macromolecules and exogenous toxic substances from entering the central nervous system to maintain homeostasis within the central nervous system. The BBB is a low-permeability cell system between the

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central nervous system and circulating blood (14). It mainly comprises brain microvascular endothelial cells (BMECs), basement membrane, and astrocytic foot processes. The BMECs form a dense cell barrier with the tight junctions proteins, which is the structural and functional base of the BBB. Therefore, the tight junctions proteins play an important role in BBB integrity (24). Changes in tight junctions proteins expression or distribution may damage the integrity of the BBB, which can result in changes in the BBB permeability (18, 21).

L. monocytogenes can cross three barriers, of which the intestinal barrier is the first line of defense. In a published study, Drolia et al. (6) have reported that *L. monocytogenes* could cross the intestinal barrier and cause the redistribution of claudin-1, occludin and E-cad in the early stage during infection. The aim of the study was to explore the role of tight junctions proteins in the process of *L. monocytogenes* infection. First, we determined the permeability of the BBB by extravasation of Evans blue. Next, the mRNA and protein expression levels of the tight junctions proteins were detected using qPCR, immunohistochemistry and western blot assays. The results of this study may lay the foundation for further study on the pathogenesis of meningitis caused by *L. monocytogenes*.

Material and methods

Strain and mice. *L. monocytogenes* strain (serotype 4b) used in this experiment was isolated from sheep with encephalitis in Xinjiang Province, China. BALB/c mice (6-8 weeks old) were obtained from the Lab Animal Center of Xinjiang Medical University and assigned to create an infection model *in vivo*. All mice were immunized at the Animal Biosafety Facilities and performed in accordance with requirements of animal use in Shihezi University (Approval number: A2017-150-17). During the experiment every effort was made to minimize animal suffering.

Establishment of infection *in vivo* model. Eight-week-old BALB/c mice were randomly divided into 2 groups, with 30 mice in the test group and 3 mice in the control group. *L. monocytogenes* was inoculated in brain heart infusion (BHI) broth (Difco, USA) and cultured for 16 h at 37°C. A 2/3 LD₅₀ dose of bacteria was injected intraperitoneally into test group mice, while control mice were injected with the same amount of phosphate buffered saline (PBS).

Assessment of the blood-brain barrier integrity. Evans blue assay was performed to assess the BBB disruption based on the procedure of Mikawa et al. (17) and with some developments. In general, the mice were injected with 2% EB dye in PBS (3 mL/kg) through intravenous tail at 2 hours before experimentation. Under anesthesia, the mice were continuously perfused of PBS from the left ventricle until the fluid from the auricle dextra

became colorless. The mice (n = 3 at each time point) were decapitated, and the brain cortices were harvested, weighed, and placed in formamide at 37°C for 72 h, and then centrifuged and the supernatant was harvested. Then, the absorbance of the supernatant was observed at 620 nm using the spectrophotometer. The extravasation of EB dye of each brain cortex was calculated by a standard curve and presented as micrograms per gram of brain tissue.

Relative quantitative real-time PCR (qPCR). qPCR was done according to Kim et al. (12) with modified primers designed by ourselves (Tab. 1) and an anneal temperature of 54°C. Eight-week-old BALB/c mice were randomly divided into 2 groups, with 27 mice in test group and 3 mice in the control group. Each mouse in the test group received 0.2 mL bacteria culture by intraperitoneal injection and was observed for 4 days. The mouse in the control group was intraperitoneally injected with 0.2 mL PBS. The mice (n = 3 at each time point) were anesthetized and then decapitated, and the cortices were carefully separated from the brain tissue on ice, and immediately stored in liquid nitrogen. The RNA was extracted by Trizol reagent (Invitrogen, USA), and cDNA was synthesized with a Prime-Script RT reagent kit (TaKaRa Bio Inc., China). Using the synthesized cDNA as template, the qPCR reaction was performed by the Bio-Rad quantitative PCR system to measure the mRNA expression of target genes. Forty-five cycles of PCR amplification were carried out. The mRNA expression were calculated by the method of $2^{-\Delta\Delta C_t}$.

Immunohistochemistry. Immunohistochemistry assay was done according to Yang, et al. (26). Briefly, eight-week-old BALB/c mice were randomly divided into 2 groups, with 27 mice in the test group and 3 mice in the control group. The mice were intraperitoneally injected with 0.2 mL of sublethal dose of *L. monocytogenes* bacterial solution or PBS buffer. The mice (n = 3 at each time point) were anesthetized, decapitated, and the cortices were carefully separated from the brain tissue on ice, and immediately stored in 4% paraformaldehyde at 4°C until required. The cortices were cut into sections after a series of routinely treatments, then paraffin sections were dewaxed, hydrated, antigens retrieved, and endogenous peroxidase was inactivated for immunohistochemistry. The sections were incubated with anti-ZO-1 (1 : 200, Abcam, ab96587); anti-occludin (1 : 200, Abcam, ab216327); anti-claudin-5 (1 : 200, Abcam, ab15106) at 4°C overnight. Then the paraffin sections were washed with PBS, finally incubated with secondary antibodies for 30 min at 37°C. All above steps included washing with PBS. The sections were then developed in DAB

Tab. 1. Sequences of the primers used for the detection of mRNA specific for ZO-1, occludin, claudin-5 and β -actin

Gene	Sequence (5'-3')	Amplicon size (bp)	GenBank	Temperature
ZO-1	(F) CCACCTCGCAGCATCACAG (R) TGGTCCTTCACCTCTGAGCACTAC	83	NM_013805.4	54°C
Occludin	(F) TTTTGTGGGATAAGGAAC (R) TTGCCATTGGAGGAGTAG	154	NM_001360536.1	54°C
Claudin-5	(F) TGGTGTCTGTCTGGTAGGATGG (R) GTCACGATGTTGTGGTCCAGGAAG	94	NM_009386.2	54°C
β -Actin	(F) CCCATCTACGAGGGCTAT (R) TGTACGCACGATTTC	145	NM_007393.5	54°C

solution (ZSGB-BIO, China) for 3 min and counter-stained with hematoxylin. PBS replaced the primary antibody in the negative controls.

Western blotting. The assay was performed according to Zhan, et al. (27). In brief, eight-week-old BALB/c mice were randomly divided into 2 groups, with 27 mice in the test group and 3 mice in the control group. The mice were intraperitoneally injected with 0.2 mL of sublethal dose of *L. monocytogenes* bacterial solution or PBS buffer. The mice ($n = 3$ at each time point) were anesthetized, decapitated, and the cortices were carefully separated from the brain tissue on ice. The cortices were lysed with RIPA (Solarbio, China), and total protein concentration was measured by the BCA kit (Pierce, USA). The proteins were separated by gel electrophoresis (8%-12%), then electrotransferred onto nitrocellulose membranes. Blots were placed to TBST containing 5% nonfat milk to block for several hours, and incubated with primary antibodies anti-ZO-1 (1 : 1000, Abcam, ab96587); anti-occludin (1 : 1000, Abcam, ab216327); anti-claudin-5 (1 : 1000, Abcam, ab15106) and anti- β -actin (1 : 1000, ZSGB-BIO, China) at 4°C for 14 h, respectively. Then incubated with the HRP-conjugated secondary antibodies. Immunoblots were reacted with SuperSignal West Pico Substrate (ThermoFisher Scientific, USA), and the band intensity was quantified using Image-ProPlus 5.0 software (Media Cybernetics, USA).

Statistics. The data obtained from the test were statistically analyzed by using SPSS20.0 software. Statistical analyses were assessed using one-way ANOVA for differences among multiple groups, followed by Dunnett's multiple comparison tests. P-values < 0.05 were considered statistically significant. Non-significant P-values are shown as n.s., significant P-values for all comparisons are depicted in the figures as follows: * $P < 0.05$; ** $P < 0.01$.

Results and discussion

Establishment of the *L. monocytogenes* infection model. Thirty-six hours after *L. monocytogenes* infection, mice in the experimental group showed depression, and decreased intake of food and drinking water. Forty-eight hours after infection, phenomena such as the mice forming groups and eye secretion increased, followed by death. Four mice died within 48 and 72 h, and three mice died within 72 and 96 h.

Extravasation of Evans blue. The extravasation of EB is an indicator of brain microvascular integrity. Data analysis showed no significant change ($P > 0.05$) in EB content from 0 h to 6 h. At 8 h the EB content in brain tissue formed a minor peak. Then the content of EB began to increase from 10 h to 48 h, peaking at 48 h ($P < 0.05$). Subsequently, the content of EB began to decrease but was much higher than that of the initial stage of *L. monocytogenes* infection (Fig. 1).

Changes in the expression of ZO-1, occludin and claudin-5 after *L. monocytogenes* infection. To study specific role of tight junctions proteins after *L. monocytogenes* infection, the qPCR, immunohistochemistry and western blotting assays were utilized for detecting the expression of ZO-1, occludin and

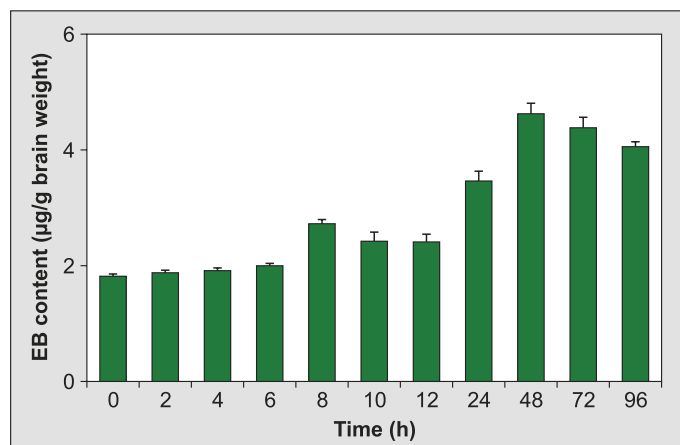


Fig. 1. The BBB permeability following *L. monocytogenes* infection was detected at different time points by Evans blue dye leakage

Explanation: Evans blue content is given as the mean $\pm$ SD ($n = 3$, at each time point)

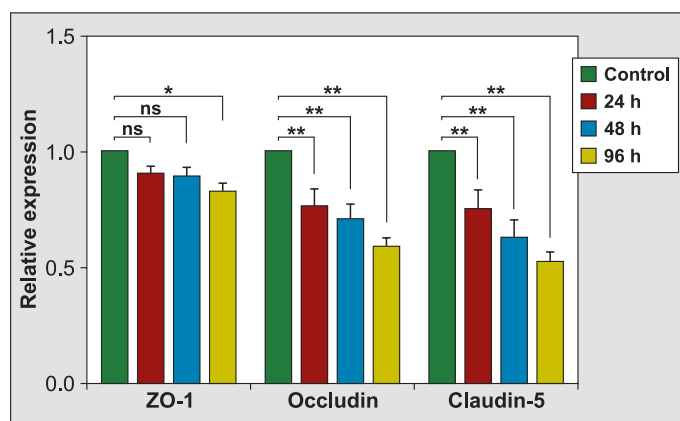


Fig. 2. The mRNA expression of ZO-1, occludin and claudin-5 at different time points after *L. monocytogenes* infection

Explanations: Results are presented as mean $\pm$ SD, $n = 3$ independent experiments per group. The groups were compared by one-way ANOVA and followed by Dunnett's multiple comparison tests. Non-significant P-values are shown as n.s. Significant P-values are shown as follows: * $P < 0.01$; ** $P < 0.05$

claudin-5. As shown by the qPCR results (Fig. 2), the mRNA expression levels of occludin and claudin-5 significantly decreased at each sampling point than that of the control group ($P < 0.05$), while ZO-1 changed significantly only at 96 h ($P < 0.05$).

Immunohistochemical results were shown in Figure 3 and the expression levels of tight junction proteins on cerebral microvascular changed with time after *L. monocytogenes* infection, especially occludin and claudin-5. Specifically, the mean optical density values of occludin and claudin-5 were down-regulated at 48 h and 96 h and showed significantly different than that of control group ($P < 0.05$), while ZO-1 had no statistically significant ($P > 0.05$) (Fig. 4).

To assess the changes in these three proteins, their expression levels were measured using western blotting. The results were consistent with those of qPCR and immunohistochemistry analysis. Compared with the control group, the expression of occludin

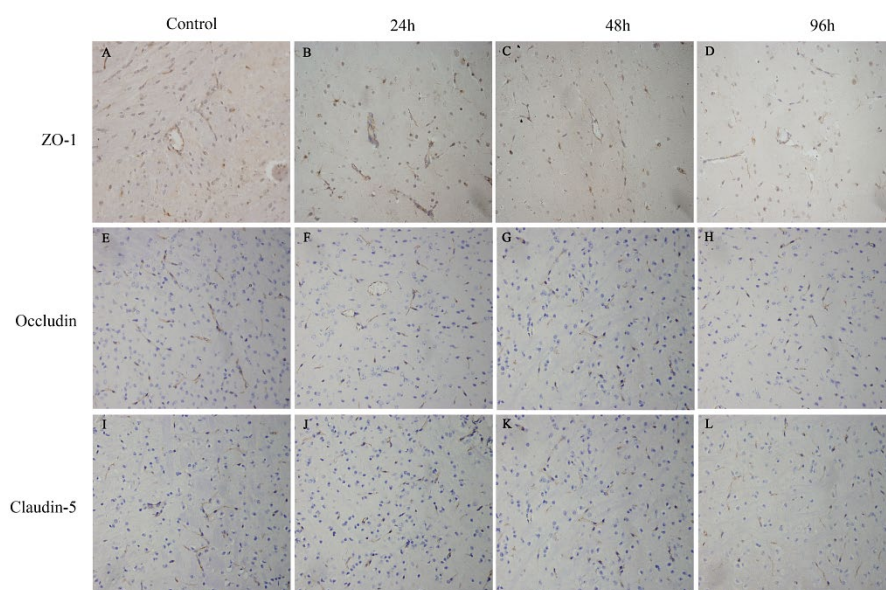


Fig. 3. Immunohistochemical analysis of ZO-1, occludin and claudin-5 expression in brain tissues of mice at different time points after *L. monocytogenes* infection

Explanations: A-D – ZO-1; E-H – occludin; I-L – claudin-5. In the negative controls, PBS replaced the primary antibody. Magnification, $\times 400$

and claudin-5 was significantly decreased at all time points ($P < 0.05$), while ZO-1 was almost unchanged ($P > 0.05$) (Fig. 5, 6).

Here, the *L. monocytogenes*-invasive mouse model was constructed *in vivo* to further investigate the specific role of *L. monocytogenes* proteins on the BBB during *L. monocytogenes* infection. We also found that *L. monocytogenes* entering the mouse's brain induced dynamic changes in BBB permeability and the changes

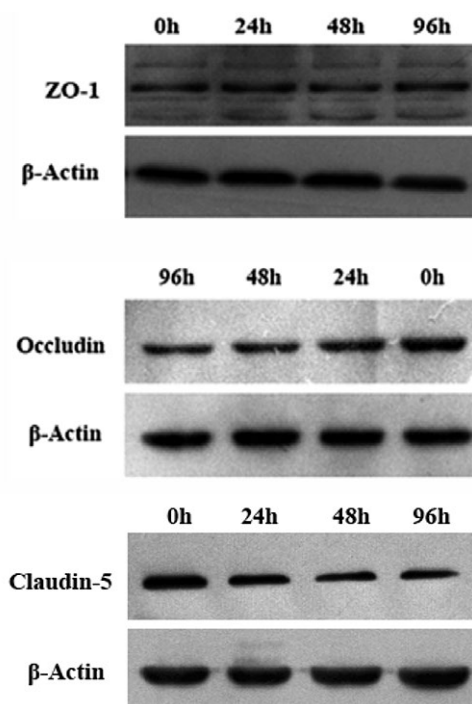


Fig. 5. Immunoblotting for tight junction proteins: ZO-1, occludin, and claudin-5 were assessed in 24 h, 48 h and 96 h groups. β -actin was used as control

were in accordance with changes in the protein expression levels of occludin and claudin-5.

The BBB is the dynamic interface between the central nervous system and blood, and had a highly specialized selective to regulate the movement of material (18). EB is a small molecular dye which has high affinity with plasma albumin and can form the macromolecule which cannot enter into brain under normal conditions. Macromolecules are able to penetrate the BBB when it is damaged and permeability increases. Therefore, the quantitative measurement of EB content in brain tissue by spectrophotometry can accurately reflect the degree of the BBB opening (22). As shown in

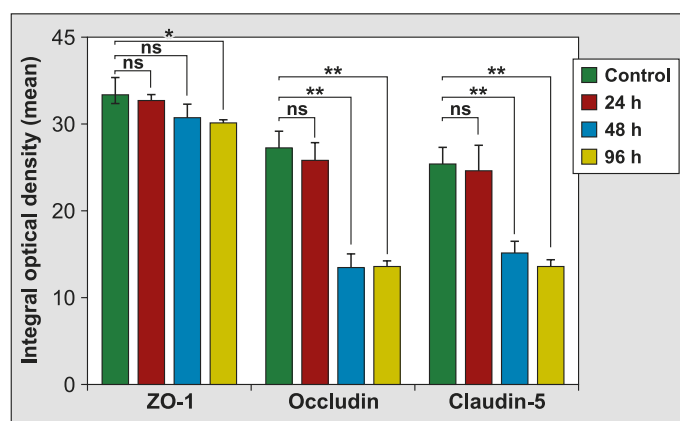


Fig. 4. Quantitative analysis of ZO-1, occludin, and claudin-5

Explanations: Results are presented as mean $\pm$ SD, $n = 3$ independent experiments per group. The groups were compared by one-way ANOVA and followed by Dunnett's multiple comparison tests. Non-significant P-values are shown as n.s. Significant P-values are shown as follows: * $P < 0.01$; ** $P < 0.05$

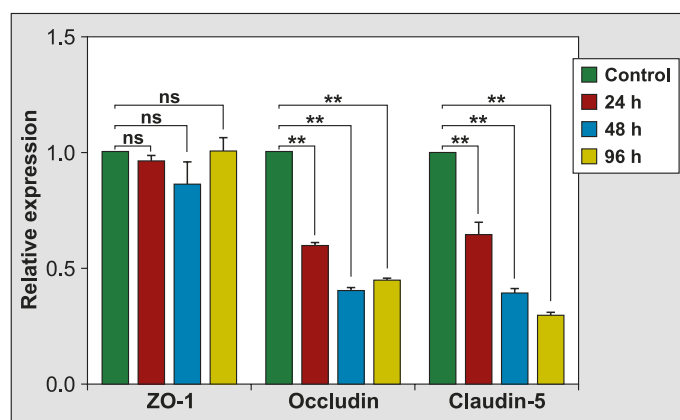


Fig. 6. Quantitative analysis of ZO-1, occludin, and claudin-5. β -actin was used as control

Explanations: Results are presented as mean $\pm$ SD, $n = 3$ independent experiments per group. The groups were compared by one-way ANOVA and followed by Dunnett's multiple comparison tests. Non-significant P-values are shown as n.s. Significant P-values are shown as follows: * $P < 0.01$; ** $P < 0.05$

our quantitative analysis, two peaks related to the BBB permeability were observed: the first small peak at 8 h and the main peak between 48 and 96 h, respectively. The first small peak was thought to be due to the role of cytokines. Many studies have demonstrated that *L. monocytogenes* could locate and replicate in the cytosol, and release multiple cytokines during inflammation. Mantle *et al.* (16) assessed the relationship between inflammation and Alzheimer's disease by constructing the BBB *in vitro* using the BMECs, and found that tumor necrosis factor- α and interleukin-6 have abilities to impair the cells to make the BBB dysfunction with the decrease of transendothelial electric resistance between cells (16). Cohen *et al.* (4) also verified that interleukin-6 modulates the expression of claudin-5 by establishing a model by constructing the BBB model of sheep *in vitro* (4). The second peak may be attributed to a large number of *L. monocytogenes* and blood-derived immune cells entering nerve tissue, based on the increased permeability of the BBB. The tight junctions between BMECs is the basis of the structure and function of BBB, it was reported the change of tight junctions proteins expression destroyed the structure and normal functioning of tight junctions, thus enhanced the permeability of BBB, which is a common occurrence in brain inflammation (10, 28). The tight junctions complex is composed of transmembrane protein, cytoplasmic attachment protein and cytoskeleton protein. ZO-1 belongs to the family of external membrane proteins and is the first confirmed protein. Under normal conditions, ZO-1 connects transmembrane proteins to cytoskeletons to maintain the continuity and integrity of tight junctions, ZO-1 can also identify the locations of tight junctions and transmit a variety of signals (7). Occludin is the first transmembrane protein isolated, and it is the main site of formation of tight junctions between cells. The destruction of BBB around brain tumor tissue is often accompanied by the lack of occludin expression (2). Claudin-5 is another transmembrane protein, which has been considered to be directly involved in the establishment of BBB and the regulation of BBB function (25). In summary, ZO-1, occludin and claudin-5 play a critical role in the integrity of tight junctions and are recognized to be important indicators for maintaining integrity of the BBB.

In order to test whether these three proteins make a difference in the BBB permeability following *L. monocytogenes* infection, qPCR, immunohistochemistry and western blotting assays were used to investigate the time-dependent changes in ZO-1, occludin and claudin-5 after *L. monocytogenes* infection. We found both in mRNA and protein expression of occludin and claudin-5 were significantly decreased between 24 h and 96 h following infection ($P < 0.05$), which was related to the destruction of tight junctions. It is worth noting that the amount of extravasation of EB also increased at the same time. We speculate that

there is a certain correlation between the change of protein expression levels of occludin and claudin-5 and the damage degree of BBB in *L. monocytogenes* infection.

There are increasingly more studies concerning central nervous system infections caused by *L. monocytogenes* that report the mechanisms of how the bacteria enter the central nervous system and disrupt the BBB. Drevets *et al.* (5) showed that *L. monocytogenes* has the possibility to enter the central nervous system by several mechanisms and it can enter and reproduce inside a wide variety of endothelial cells such as the umbilical vein and BMECs, possibly leading to an increase in the permeability and disruption of the BBB. Drolia *et al.* (6) also showed that the redistribution of occludin contributes to *L. monocytogenes* crossing the intestinal barrier, and we guess that occludin lays an important role on the disruption of the BBB during *L. monocytogenes* infection. Occludin and claudin-5 directly participate in regulating paracellular permeability in the BBB to ensure functional stability of the BBB (19). Based on these studies, we used a *L. monocytogenes* strain which was isolated from sheep to construct a mouse infection model to verify the damage of *Listeria* in the process of crossing the BBB. All the results indicated that *L. monocytogenes* could cause the redistribution of tight junctions proteins, especially occludin and claudin-5. And we were also inspired for a new direction in the further study on the mechanisms of how the bacteria enter the central nervous system of sheep.

In conclusion, this paper indicated that *L. monocytogenes* infection increased the BBB permeability in mice, which was related to a decreases in the expression of occludin and claudin-5 in cerebral microvascular endothelial cells. Our results lay the foundation for further study on the mechanisms by which *L. monocytogenes* cross the BBB.

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