MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE

SUMY NATIONAL AGRARIAN UNIVERSITY

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UDC 23.4:633.854.78

PHD THESIS

EXPERIMENTAL RESEARCH ON PATHOGENESIS OF STREPTOCOCCUS SUIS INFECTION

Field of knowledge: 21 – veterinary

Specialty: 211 – veterinary medicine

Submitted for a scientific degree of Doctor of philosophy

The dissertation contains the results of own research. The use of other authors' ideas, results and texts link to the source

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Sumy - 2023

ANNOTATION

Liu Mingcheng **«Experimental research on pathogenesis of *Streptococcus suis* infection»** - Qualifying Educational and Scientific Work on the Rights of the Manuscript. Dissertation for the degree of the Doctor of Philosophy in the specialty 211 «Veterinary medicine» − Sumy National Agrarian University, Sumy, 2023.

The dissertation presents an experimental research on the pathogenic mechanism of meningitis of the streptococcal infection of pigs based on study of the pathogenetic processes of pyroptosis through the expression of mRAN of pyroptosis-related genes and proteins and changes in morphologyof endothelial cells of brain microvessels of white mice infected with *Streptococcus suis serotype 2.*

Streptococcus suis (S. suis) is an important zoonotic pathogen that can cause many diseases in pigs, such as sepsis, arthritis, endocarditis and meningitis, of which meningitis is the most serious. There are 35 serotypes of, and serotype 2 is the most virulent. At the same time, *Streptococcus suis* serotype 2 (*S. suis 2*) can also infect humans, causing serious public health problems. Although *S. suis 2* has attracted great attention worldwide, the research on its pathogenesis is still limited. The adhesion of pathogenic bacteria to the surface of host cells or tissues and its subsequent invasion and diffusion are the key steps of pathogenic bacteria. And the interaction between pathogen and host is involved in all of these processes. Therefore, to study the pathogenic mechanism of pathogenic bacteria is to study the interaction between pathogenic bacteria and host. This paper described several common virulence factors, such as CPS, SLY, MRP, EF, SAO, Srt, FBPS, SadP and Eno. Under the actions of

virulence factors, *S. suis 2* adheres and colonize to the mucosal and epithelial surface of host cells. Then *S. suis 2* invades into deeper tissues and bloodstream. If *S. suis 2* in the blood doesn't cause fatal sepsis, it can go to the third stage. The third stage is to cross the Blood brain barrier (BBB) and to get access to the central nervous system (CNS), and ultimately causes meningitis. During pathogenesis, *S. suis 2* interacts with multiple cells of the host, such as neutrophils, macrophages, epithelial cells, and microvascular endothelial cells, to evade the innate or adaptive immunity of the host.

Cells are the basic unit of life, and cell death plays an important role in the body's metabolism, the occurrence and development of diseases. Pyroptosis is a form of programmed cell death. Pyroptosis is significantly different from other cell death methods (such as apoptosis, necrosis, etc.) in morphological characteristics, occurrence mechanism, and mechanism of action. When a cell undergoes pyroptosis, the nucleus condenses to form a pyroptotic body, numerous pores appear in the cell membrane, the cell swells and ruptures, releasing its contents. Caspase family is a homologous and structurally similar proteolytic enzyme in cytoplasm, which selectively recognizes and cleaves peptide bonds behind downstream target aspartic acid residues. Caspase-1,4,5,11 can induce pyroptosis through different pathways. Besides caspases, gasdermin also plays an important role in pyroptosis. Gasdermins (GSDMs) are a family of functionally diverse proteins expressed in a variety of cell types and tissues. The Gasdermin family includes 6 members, of which gasdermin D is the executor of pyroptosis. Upon cleavage by activated capsese, gasdermen D can be divided into N and C segments. Among them, the N fragment can form pores in the cell membrane, leading to cell swelling, rupture, outflow of cytokines and other contents, triggering the body's

immune response, and leading to pyroptosis. The occurrence of pyroptosis can be divided into the classical pathway and the non-classical pathway. The classical pathway mainly depends on caspase-1, while the non-canonical pathway depends on the activation of caspase-4/5/11. In addition, there are uncommon caspase-3/8-mediated pathway and granzyme-mediated pathway. As a way of cell death, pyroptosis is inextricably linked to disease. Inflammasomes and cytokines produced in the process of pyroptosis can trigger an inflammatory response in the body, and an excessive inflammatory response can lead to diseases, such as infectious diseases, neurological diseases, and tumors. In infectious diseases, pyroptosis is closely related to the infection of a variety of bacteria, fungi and viruses, and Pathogen-associated molecular patterns (PAMPs) and Lipopolysaccharide (LPS) can be recognized by corresponding inflammasomes and caspases, respectively, and activate the downstream pyroptotic pathways. Pathogen infection is the main way to induce pyroptosis. In cardiovascular diseases, a high-fat environment can induce an increase in reactive oxygen species (ROS), trigger endothelial cell pyroptosis, and exacerbate the development of atherosclerosis (AS). In the nervous system, cell death is involved in the pathogenesis of the progression of degenerative diseases of the central nervous system, such as Alzheimer's disease (AD), Parkinson's disease (PD), and stroke. In terms of tumors, pyroptosis can inhibit the occurrence and development of tumors, and at the same time, as a pro-inflammatory death, pyroptosis can form a microenvironment suitable for tumor cell growth, thereby promoting tumor growth.

In this study, *S. suis 2* was used to infect mouse brain microvascular endothelial cell (bEnd.3). First, the infection conditions were screened. The results showed that the

optimal infection number was 100:1 and the optimal infection time was 12 hours. Brain microvascular endothelial cells were divided into 4 groups: control group, LPS+ Adenosine triphosphate (ATP) infection group, *S. suis 2* infection group and *S. suis 2*+ Ac-YVAD-CMK (CMK, caspase-1 inhibitor) infection group. The following three groups were infected with *Streptococcus suis* type 2 according to the above conditions, and multiple replicates were performed simultaneously. After 12 hours, the cells and cell supernatants were collected for different tests. Total RNA was extracted, RNA concentration was measured, and cDNA was obtained by reverse transcription. cDNA was detected by qPCR for mRNA expression of cytokines. Compared with the control group, the mRNA expression levels of caspase-1, il-18 and il-1beta in LPS+ATP group and *S. suis 2* group were higher than those in the control group, indicating that a large number of cytokines related to pyroptosis were secreted. At the same time, mRNA expression levels of caspase-1, il-18 and il-1beta in *S. suis 2* +CMK group were significantly lower than those in *S. suis 2* +CMK group, indicating that CMK inhibitor played an inhibitory role. At the same time, protein was extracted from the collected cells, the protein concentration was measured, and then WB test was carried out to detect the protein expression level of related genes. Compared with the control group, the protein expressions of IL-18, IL-1β,caspase-1,GSDMD and GSDME in LPS+ATP group were significantly up-regulated, increasing by 0.477, 0.088, 0.378, 1.118 and 3.05 times, respectively. The protein expressions of IL-18, IL-1β,caspase-1,GSDMD and GSDME in S. SUIS 2 group were significantly up-regulated, increasing by 1.024, 0.066, 0.376, 0.453 and 1.654 times, respectively. Compared with S. SUIS 2 group, protein expression in *S. suis 2*+CMK group was significantly decreased by 0.6, 0.396, 0.298,

0.743 and 0.586 times, respectively. The results showed that *S. suis 2* infection of brain microvascular endothelial cells caused intense inflammatory reaction and even pyroptosis of cells. At the same time, the inhibitor CMK played a good inhibitory effect. In addition, the protein content of IL-6, IL-10, IL-18, IL-1beta, caspase-1 in the cell culture supernatant was also detected.

Compared with control group, the relative expression of TNF-α, IL-6, IL-10, IL-18, IL-1β in cell supernatant of SS group and LPS+ATP group was significantly increased, and the difference was statistically significant ($P < 0.01$). Compared with *S. suis 2* group and LPS+ATP group, the relative expression level of TNF-α, IL-6, IL-10, IL-18, IL-1β in *S. suis 2* + CMK group was significantly decreased, and the difference was statistically significant ($P < 0.01$), indicating that coke death caused by *S. suis 2* and LPS+ATP could be inhibited by CMK. At the same time, At the same time, the release rate of lactate dehydrogenase in the cell supernatant was also measured, and the results showed that the release rate of lactate dehydrogenase in LPS+ATP group and *S. suis 2* group was much higher than that in the control group, and the difference was extremely significant. In *S. suis 2* + CMK group, the release rate of lactate dehydrogenase decreased sharply under the action of the inhibitor. In order to observe the morphology of infected cells, the collected cells were also observed by transmission electron microscopy. The results of electron microscopy showed that the cell membrane of LPS+ATP group and *S. suis 2* group was broken and the cell contents were leaked out. Combined with the above other results, it can be concluded that under the infection of *S. suis 2*, brain microvascular endothelial cells produced an inflammatory response and pyroptosis occurred.

In the dissertation work on the basis of research are substantiated the pathogenic mechanism of *Streptococcus suis,* inhibitor protection to develop target drugs and vaccine for meningitis, reduce losses of pig industry and promote the healthy development of animal husbandry.

Key words: pigs, infection, diagnosis, etiology, bacterias, Streptococcus suis, virulence factors, pathogenesis, meningitis, pyroptosis.

АНОТАЦІЯ

Люй Мінчень **«Експериментальне дослідження патогенезу стрептококової інфекції свиней»** – Кваліфікаційна наукова праця на правах рукопису. Кваліфікаційна наукова робота на правах рукопису. Дисертація на здобуття наукового ступеня доктора філософії за спеціальністю 211 «Ветеринарна медицина» − Сумський національний аграрний університет, м. Суми, 2023.

У дисертаційній роботі представлено експериментальне дослідження патогенезу менінгіту за стрептококової інфекції свиней на основі дослідження патогенетичних процесів піроптозу через експресію mRAN пов'язаних з піроптозом генів і білків та змін морфології ендотеліальних клітин мікросудин головного мозку білих мишей при інфікуванні *Streptococcus suis серотипу 2.*

Streptococcus suis (S. suis) є зоонозним патогеном, який може спричинити у свиней сепсис, артрит, ендокардит і менінгіт, з яких менінгіт є найнебезпечнішим. Існує 35 серотипів даного патогена, а серотип 2 є найбільш вірулентним. У той же час *Streptococcus suis* серотипу 2 (*S. suis 2*) також може інфікувати людей,

викликаючи серйозні проблеми зі здоров'ям. Інфекції, спричинені бактеріальним патогеном *S. suis 2,* поширені у більшості країн світу. Проте дані про патогенетичні процеси розвитку стрептококової інфекції все ще обмежені. Адгезія патогенних бактерій до поверхні клітин або тканин господаря та подальша їх інвазія та дифузія є ключовими етапами патогенетичних механізмів розвитку хвороби. Взаємодія між патогеном і чутливим організмом є ключовим аспектом у розвитку всіх патогенетичних процесів. Тому вивчення патогенезу за інфекційних хвороб означає вивчення взаємодії між патогенними бактеріями та чутливим організмом тварин.

У дисертаційній роботі представлено дані про декілька поширених факторів вірулентності збудника *S. suis 2*, таких як CPS, SLY, MRP, EF, SAO, Srt, FBPS, SadP та Eno. Під дією факторів вірулентності *S. suis 2* прикріплюється та колонізується на поверхні епітеліальних клітин слизової оболонки господаря. Потім *S. suis 2* проникає в глибші тканини та кровотік. Якщо *S. suis 2* в крові не викликає смертельного сепсису, патологічний процес переходить в третю стадію розвитку хвороби. Третій етап характеризується в проходженні гематоенцефалічного бар'єру (ГЕБ) і ураженні центральної нервової системи. Інфекційно-запальний процес охоплює мозкові оболонки, що характеризується розвитком менінгіту. Щоб уникнути впливу вродженого або адаптованого імунітету господаря бактеріальний патоген *S. suis 2* взаємодіє з кількома клітинами господаря, такими як нейтрофіли, макрофаги, епітеліальні клітини та ендотеліальні клітини мікросудин.

Клітини є основною одиницею життя, а смерть клітини відіграє важливу

роль у метаболізмі організму, виникненні та розвитку захворювань. Піроптоз є формою запрограмованої некротичної загибелі клітини. Піроптоз суттєво відрізняється від інших видів загибелі клітин (таких як апоптоз, некроз тощо) за морфологічними характеристиками, механізмом виникнення та механізмом дії. Коли клітина піддається піроптозу, ядро конденсується з утворенням піроптотичного тіла, у клітинній мембрані з'являються численні пори, клітина набухає і розривається, вивільняючи свій вміст.

Сімейство каспаз є гомологічним і структурно подібним протеолітичним ферментом у цитоплазмі, який вибірково розпізнає та розщеплює пептидні зв'язки за цільовими залишками аспарагінової кислоти. Каспаза-1,4,5,11 може індукувати піроптоз різними шляхами. Окрім каспаз, газдермін також відіграє важливу роль у розвитку піроптозу. Газдерміни (GSD-M) – це група функціонально різноманітніих білків, що експресуються в різних типах клітин і тканин в тому числі і в епітеліальних. Родина гасдермінів включає 6 видів білків, з яких лише гасдермін D (GSDM-D) приймає участь у патогенетичному процесі розвитку піроптозу клітин. Каспаза розщеплює гасдермін D на сегменти N і C. Потім вивільнений N-сегмент може формувати в плазматичній мембрані клітин пори, що призводить до набряку клітини, розриву, відтоку цитокінів та іншого вмісту, запускаючи імунну відповідь організму та призводячи до піроптозу.

Виникнення піроптозу розділяють на класичний шлях і некласичний шлях. Класичний шлях в основному залежить від каспази-1, тоді як некласичний процес розвитку піропотзу залежить від активації каспази-4/5/11.

Крім того, існує процес розвитку піроптозу, що обумовлений активацією

каспази-3/8, а також патологічний процес, опосередкований гранзимом.

Як спосіб загибелі клітин, піроптоз нерозривно пов’язаний із захворюваннями. Інфламасоми та цитокіни, що утворюються в процесі піроптозу, можуть викликати запальну реакцію в організмі, що може призвести до прояву інфекційних, неврологічних та онкологічних захворювань. За інфекційних захворювань піроптоз тісно пов’язаний з інфекційними процесами, етіологічним чинником яких є бактерії, мікроскопічні гриби та віруси. Асоційовані з патогенами молекулярні структури і ліпополісахариди можуть бути розпізнані відповідними інфламасомами та каспазами і , відповідно, в клітинах організму активізуються піроптичні процеси.

Збудники інфекційних захворювань є основним етіологічним фактором індукції піроптозу. При серцево-судинних захворюваннях високий вміст жиру може викликати збільшення активних форм кисню, що спричинює піроптоз ендотеліальних клітин, а також активізує процес розвитоку атеросклерозу та інсульту. Піроптоз нервових клітин бере участь у патогенезі прогресування дегенеративних захворювань центральної нервової системи, таких як хвороба Альцгеймера та хвороба Паркінсона. Піроптоз може як пригнічувати появу пухлин, так і створювати оптимальні умови для їх росту і розвитку.

У результаті проведених досліджень було відпрацьовано застосування культури клітин ендотелію мозку Bend3 за різних схем зараження *Streptococcus suis* 2-го типу, підібрано дозу для інфікування з метою отримання оптимального процесу експресії РНК та виділенням інтерлейкінів.

У результаті проведених досліджень вперше визначено рівнів білків та генів,

пов’язаних з піроптозом. Під час досліджень для інфікування ендотеліальних клітин мікросудин головного мозку миші (bEnd.3) використовували ізолят *S. suis 2*. На першому етапі було проведено перевірку умов зараження. Результати показали, що оптимальне число зараження ендотеліальних клітин становило 100:1, а оптимальний час зараження становив 12 годин. Ендотеліальні клітини мікросудин головного мозку миші (bEnd.3) були розділені на 4 групи: контрольна група, інфікована група клітин LPS+ аденозинтрифосфатом (АТФ), інфікована група клітин *S. suis 2* і інфікована група клітин *S. suis 2* + Ac-YVAD-CMK (CMK, інгібітор каспази-1). три групи були інфіковані *Streptococcus suis* типу 2 відповідно до вищевказаних умов, і кілька повторень були виконані одночасно. Через 12 годин клітини та супернатанти клітин збирали для проведення тестів.

Під час проведення досліджень екстрагували загальну РНК, визначено концентрацію РНК, та кДНК отримували шляхом зворотної транскрипції. У результаті проведених досліджень кДНК була виділена за допомогою полімеразної ланцюгової реакції (ПЛР) для експресії мРНК цитокінів.

За результатами досліджень встановлено, що рівні експресії мРНК каспази-1, il-18 та il-1beta у групі LPS + ATP і *S. suis 2* були вищими, ніж у контрольній групі. Отримані дані вказують на те, що виділялася велика кількість цитокінів, пов’язаних з піроптозом. У той же час рівні експресії мРНК каспази-1, il-18 та il-1beta у групі *S. suis 2* + CMK були значно нижчими, ніж у групі *S. suis 2* + CMK, що свідчить про те, що інгібітор каспази-1 CMK проявляв пригнічуючу дію.

Під час проведення досліджень із зібраних клітин екстрагували білок. Також було проведено визначення концентрації білка, а потім проводили тест для

виявлення рівня експресії білка споріднених генів. Встановлено, що в порівнянні з контрольною групою експресія білків IL-18, IL-1β, каспази-1, GSDM-D і GSDM-E у групі LPS + ATP була значно підвищеною, збільшуючись у 0,477, 0,088, 0,378, 1,118 і 3,05 рази відповідно. Експресія білків IL-18, IL-1β, каспази-1, GSDM-D і GSDM-E у культурі клітин інфіковіаних *S. suis 2* була значно підвищена, а саме у 1,024, 0,066, 0,376, 0,453 і 1,654 рази відповідно.

Результати проведених досліджень показали, що порівняно з групою культури клітин інфікованих *S. suis 2*, експресія білка в групі культури клітин *S. suis 2* + СМК значно знижувалася в 0,6, 0,396, 0,298, 0,743 і 0,586 раза відповідно. Отримані результати показали, що інфікування збудником *S. suis 2* ендотеліальних клітин мікросудин (bEnd.3) викликало інтенсивну запальну реакцію і навіть піроптоз клітин. У той же час інгібітор каспази-1 СМК зіграв досить гарний інгібуючий ефект. У результаті проведених досліджень було встановлено, що у супернатанті клітинної культури також виявлено вміст білка IL-6, IL-10, IL-18, IL-1бета, каспази-1.

У подальшому дослідженні з’ясовано, що порівняно з контрольною групою відносна експресія TNF-α, IL-6, IL-10, IL-18, IL-1β у супернатанті клітин групи *S. suis* та групи LPS + ATP була значно збільшена, і різниця була статистично достовірною ($P < 0,01$).

Було встановлено, що порівняно з групою культур клітин інфікованих *S. suis* та групою культур клітин LPS+ATP відносний рівень експресії TNF-α, IL-6, IL-10, IL-18, IL-1β у групі *S. suis* + СМК був значно знижений, і різниця була статистично достовірною ($P < 0,01$). Отримані результати досліджень вказують на

те, що загибель клітин, викликана *S. suis 2* і LPS+ATP, може пригнічуватися Ac-YVAD-cmk (CMK), який є селективним інгібітором фермента каспази-1 і має нейропротекторну та протизапальну дію.

Наступним етапом досліджень було проведення дослідження за зміною морфології інфікованих клітин. Дослідження морфологічних змін в культурі клітин і фіксацію етапів піроптозу проводили за допомогою трансмісійної електронної мікроскопії. Результати електронної мікроскопії показали, що цілісність клітинної мембрани в дослідній групі культури клітин LPS+ATP і групі *S. suis 2* була порушена, реєстрували швидке вивільнення назовні вмісту клітин.

Дані отримані в ході дослідження доводять, що при інфікуванні бактеріальним патогеном *S. suis 2* ендотеліальних клітин мікросудин головного мозку білих мишей виникає запальна реакція та патогенетичні процеси розвитку піроптозу.

На основі отриманих результатів досліджень обґрунтовано патогенез менінгіту за стрептококової інфекції свиней, спричиненого *S.suis cepomuny 2,* що є основою для розробки таргетних терапевтичних засобів та розробки вакцини проти стрептококової інфекції свиней, що в свою чергу забезпечать зменшенню втрат у свинарстві і сприяє розвитку тваринництва.

Ключові слова: свині, інфекція, діагностика, етіологія, бактерії, Streptococcus suis, фактори вірулентності, патогенез, менінгіт, піроптоз.

LIST OF WORKS PUBLISHED ON THE THEME OF THE DISSERTATION

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1. **Liu Mingcheng,** Kasianenko Oksana (2022). Screening of infection conditions for brain microvascular endothelial cells infected by Streptococcus suis. *Ukrainian Journal of Veterinary and Agricultural Sciences,* 5, № 2, 28–31. DOI: https://doi.org/10.32718/ujvas5-2.04

(PhD participant in carrying out of experimental researches, processing of results , preparation of article for printing).

2. **Liu Mingcheng,** Xiaojing Xia, Xingyou Liu, Kasianenko Oksana (2021). Research Progress on the pathogenic mechanism of Streptococcus suis 2. *Scientific Messenger of LNU of Veterinary Medicine and Biotechnologies. Series Veterinary Science,* 23, № 104, 30–35. DOI: https://doi.org/*10.32718/nvlvet10405*

(The applicant participated in research, analysis of the results and writing the article).

3. Kasianenko O., **Liu Mingcheng** (2022). The molecular mechanism of pyroptosis and its related diseases. *Bulletin of Sumy National Agrarian University*, 2 (57), 16–26. DOI: https://doi.org/10.32845/bsnau.vet.2022.2.3

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CONTENTS

LIST OF CONDITIONAL ABBREVIATIONS

S.suis - *Streptococcus suis*

S. suis 2 - *Streptococcus suis serotype 2*

bEnd.3 - Brain Microvascular Endothelial Cell

ELISA - Enzyme linked immunosorbent assay

QPCR - Quantitative polymerase chain reaction

GSDMD - Gasdermin D

PAMPs - Pathogen-associated molecular patterns

DAMP - Damage-associated molecular pattern

CNS - Central nervous system

BBB - Blood brain barrier

FBS - Fetal bovine serum

TNF - Tumor necrosis factor

LPS - Lipopolysaccharide

ATP - Adenosine triphosphate

WB - Western blot

RIPA - Radio Immunoprecipitation Assay

PMSF - Phenylmethanesulfonyl fluoride

HRP - Horse Radish Peroxidase

IL - Interlukine

BMEC - Brain microvascular endothelial cell

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

MOI - Multiplicity of infection

CMK - Ac-YVAD-cmk

CPS - Capsular polysaccharide

MRP - Muramidase-released protein

EF - Extracellular factor

FBPS - Fibronectin- and fibrinogen-binding protein

SadP - Streptococcus suis adhesin P

Eno - Enolase

SAO - Surface antigen I

EF-Tu - Elongation factor thermo unstable

MCP-1 - Monocyte Chemoattractant Protein-1

NLRP3 - Nod-liker Recptor Protein 3

PD-L1 - Programmed death-ligand 1

TNF-α - Tumor necrosis factor-α

HDL - High density lipoprotein

NLRC4 - NOD-like receptor containing protein 4

AIM2 - Absent In Melanoma 2

DMEM - Dulbecco's modified eagle medium

FITC - Financial institutions training centre

DMSO - Dimethyl Sulphoxide

PVDF - Polyvinylidene fluoride

INTRODUCTION

Revalence of the topic. *Streptococcus suis (S.suis)* is an important zoonotic pathogen, which can cause serious diseases such as meningitis, pneumonia, endocarditis, polyserositis, arthritis, septicemia and abortion in pigs, and can also cause infection and morbidity in related practitioners. In recent years, the incidence rate of streptococcal meningitis has shown a significant upward trend. For humans, the threat of *S.suis* is also increasing. In 1998 and 2005, two outbreaks of human infection with *S. suis* occurred in China, with a total of 230 people infected and 52 people died. Therefore, strengthening the prevention and control of the disease has become an urgent task.

The premise of inducing meningitis is that *S.suis* invades the central nervous system (CNS) and breaches the blood brain barrier (BBB). Due to the presence of BBB, even though bacteria can enter the blood through the skin mucosa and other parts, a large number of bacteria in the blood cannot enter the brain through BBB. The body relies on this barrier to protect the brain tissue from damage and maintain the homeostasis of the central nervous system. Brain microvascular endothelial cell (BMEC) is the basic component of BBB, and a variety of neurological diseases are related to the dysfunction of BBB, and *S.suis* can interact with BMEC and then cross the BBB to cause CNS infection. However, the antibiotics used to treat the infection cannot pass through the barrier to reach the therapeutic target site, which is the key to the difficulty in the control of bacterial meningitis. Therefore, elucidating the mechanism of *S.suis* breaking through BBB into CNS is an important breakthrough in developing *S.suis* meningitis control strategy.

Connection of work with scientific programs, plans, topics. The research work was carried out in accordance with the priority direction defined by the Cabinet of Ministers of Ukraine Resolution № 942 of 09/07/2011 «Life sciences, new technologies for the prevention and treatment of the most common diseases».

The dissertation work is carried out in accordance with the programs of research work of Sumy National Agrarian University «Optimization of the complex measures to prevent the occurrence and spread of infectious animal diseases in the farms of the North-Eastern region of Ukraine» (№ 0122U001254) The work was carried out for the period from 2019 to 2023 at the departments of the Department of Epizootology and Parasitology of Sumy National Agrarian University. In addition, veterinary studies were conducted at Henan, China.

The purpose and objectives of the study.

The purpose of the research was to experimentally substantiate the pathogenesis of meningitis during streptococcal infection caused by *S.suis serotype 2* (*S. suis 2*) based on the study in vitro pathogenetic processes of the pyroptosis.

In order to achieve the main research goal, it was necessary to solve a number of interrelated tasks:

– to experimentally substantiate the use of the brain microvascular endothelial cell (bEnd.3) for infection with *S.suis serotype 2 (S. suis 2);*

– to analyze the infection pathway of the brain microvascular endothelial cell (bEnd.3) by *S.suis serotype 2;*

– to investigate of the mRNA levels and the protein levels of cytokines secreted by the infected cells (bEnd.3);

– to determined of the protein content of cell supernatants

— to study morphological changes in infected cells (bEnd.3) after to determine if pyroptosis occurred during infection by *S. suis 2;*

— to confirm the pathogenesis of meningitis of the streptococcal infection caused by *S.suis serotype 2* (*S. suis 2*).

Object research - streptococcal infection, pathogenetic processes of the pyroptosis.

Subject of research – of the brain microvascular endothelial cell (bEnd.3), *S. suis2,* virulence factors of *S. suis2,* mRNA, cytokines, the mechanism of pyroptosis, morphological changes in infected cells, pathogenesis of *S. suis* infection.

Research methods. Scratch culture was used to cultured and count *S. suis 2*, immunofluorescence was used to to identify mouse brain microvascular endothelial cells, RT-PCR was used to detect the mRNA expression of individual genes in brain microvascular endothelial cells, WB was used to detect the expression of individual proteins in cells, ELISA was used to detect the expression of individual genes in cell supernatants, electron microscopy was used to show the morphology of pyroptosis cell.

Scientific novelty of the obtained results.

According to the research results, it was theoretically and experimentally established for the first time that pyroptosis takes part in the pathogenetic process of developing meningitis due to streptococcal infection of pigs.

In this study, brain microvascular endothelial cells were prepared and identified, and the optimal multiplicity of infection (MOI) and infection time of *S. suis* infection brain microvascular endothelial cells were screened. Different methods were developed

to detect the cytokines secreted by infected cells from the perspective of gene and protein. At the same time, pyroptosis of the cells was observed by electron microscopy.

The practical significance of the results. The results of theoretical and experimental studies provide a scientific basis for the *S. suis 2* to break through the BBB and escape host immunity. It is helpful to carry out accurate and targeted drug screening and vaccine development for meningitis.

The results of experimental studies were used in the preparation of scientific and practical recommendations «Streptococcus suis infection (Etiology, Epidemiology, Laboratory diagnosis, Prevention and Treatment)», authors: Mincheng Lui, Kasianenko Oksana, approved at the meeting of the Academic Council of the Sumy National Agrarian University, protocol № 18, from 29.05. 2023. The main results of the dissertation work are presented in the scientific and practical recommendations «Streptococcus suis infection (Etiology, Epidemiology, Laboratory diagnosis, Prevention and Treatment)», protocol № 18, dated 29.05. 2023, authors: Mincheng Lui, Kasianenko Oksana, the materials of which were introduced into the educational process during the teaching of the disciplines: «Veterinary technologies for the prevention of infectious diseases of animals», «Epizootology and infectious diseases».

The applicant's personal contribution is to design experiments, carry out experimental studies in laboratory and production environments, analyse and process experimental results, draw conclusions and recommendations, and prepare material for publication. The author, with the Scientific supervisor (consultant) - doctor of veterinary sciences, professor O Kasianenko - determined the goal and task of the work, substantiated the scientific direction and research program, analyzed the obtained results.

The ideas, hypotheses, and experimental data included in the dissertation research are not duplicated, planned, executed, and belong to the dissertation student. Independently developed scientific provisions, carried out laboratory research, analysis and interpretation of the obtained results, processing of literary sources of foreign authors, statistical processing of materials and formulated conclusions.

An analysis of the plagiarism check report for the presence of textual borrowings (Strike plagiarism program) was carried out. The reviewers came to the conclusion that the dissertation work of Mingcheng Liu on the topic: «Experimentalresearch on pathogenesis of Streptococcus suis infection» is the result of independent research of the acquirer and does not contain elements of plagiarism and borrowing in accordance with the resolution of the CMU dated 12.01.2022 № 44, paragraph 9. The used ideas, results and texts of other authors have a link to the corresponding source.

Publications. According to the results of research, 13 scientific papers were published, including: 5 journal articles in professional editions of Ukraine, 7 conference articles, one is scientific and practical recommendations.

Structure and scope of the dissertation. The dissertation includes an annotation, introduction, 3 sections, conclusion and a list of sources used, which including 222 foreign ones. The main part of the dissertation is 129 pages of printed text, containg 5 tables and 30 figures.

CHAPTER 1.

LITERATURE REVIEW ON THE TOPIC AND CHOICE OF RESEARCH DIRECTIONS

1 *S.suis* and it's role

1.1 Introduction of *S. suis*

In the swine industry, *S. suis* is regarded as a major infectious disease that frequently co-occurs with meningitis, septicemia, or arthritis. The pig business suffers significant financial losses due to these diseases, which are also significant factors in the need to treat huge herds of pigs with antibiotics as a preventative measure. It has been established that the 1954-reported *S. suis* is the etiological agent for this type of persistent bacterial illness [1, 2]. *S. suis* can also infect humans, and it has become a serious endemic public health threat.

S.suis is gram-positive bacteria, aerobic or facultative anaerobic bacteria. The bacteria were 1 ~ 2μm in diameter, single or double, oval in shape, and presented long chains in liquid medium. The longer the chain, the stronger the pathogenicity [3]. Most streptococci have capsules visible in young cultures, and most have no flagellates, do not form spores, and cannot move. The colonies were small, pale, transparent and slightly sticky. Streptococcus epizoose produced obvious β-type hemolysis on the blood plate. The colonies were round, moist, smooth and translucent after being cultured at 37℃ on the blood Martin AGAR plate for 24h. *S. suis* produces α or β hemolysis on the blood plate, which is usually α hemolysis first, and β hemolysis after delayed culture. Or there is no hemolysis around the colony, scraping the colony to see alpha or beta

hemolysis. *Streptococcus suis type 2* showed alpha hemolysis in sheep and beta hemolysis in horse blood plate.

The structure of streptococcus antigen is complex, with three main components, namely group specific antigen, type specific antigen and nuclear protein antigen [4]. (1) Group specific antigen (C antigen), which is a polysaccharide component in the cell wall of the hammer bulb, has group specificity and is haptens, whose antigenic determinant is amino carbohydrate. Based on this, the Lancefield method divided streptococcus into 20 sero groups according to the different antigens. (2) Type specific antigen, a protein component of streptococcus cell wall, is located on the surface of C antigen, so it is also called surface antigen [5]. The antigen can be divided into four components, that is, M, T, R and S, among which the M component is related to bacterial virulence, has anti-phagocytic activity and is related to immunity. According to the different M proteins, the bacteria can be classified into their respective groups. For example, group C can be divided into more than 20 types, group D into 10 types, and group E into 6 types. (3) The nucleoprotein antigen, also known as P antigen, is a non-specific antigen. The P antigen of various streptococcus has the same property and is the main component of bacteria, without group or type specificity.

According to the hemolysis phenomenon can be divided into α streptococcus, β, γ streptococcus, α type hemolytic streptococcus virulence is not strong, more for the conditional pathogenic bacteria. β-type streptococcus hemolyticus is highly pathogenic, while γ-type streptococcus hemolyticus is generally not pathogenic. According to the different characteristics of the capsule antigen, the bacteria can be divided into 35 serotypes (type 1-34 and 1/2) [6]. Serotype 2 of S. suis is typically isolated from

clinically ill piglets and is thought to be the most virulent subtype of the organism. Streptococcus biochemical reaction is relatively active, can ferment lactose, sucrose, trehalose, heptoxin, raffinose, do not ferment mannose, arabinose and so on. The pathogenicity of this bacterium to animals is related to its main pathogenic factors such as capsule, toxin and enzymes [7].

1.2 Virulence factors of *S.suis*

Major virulence fact

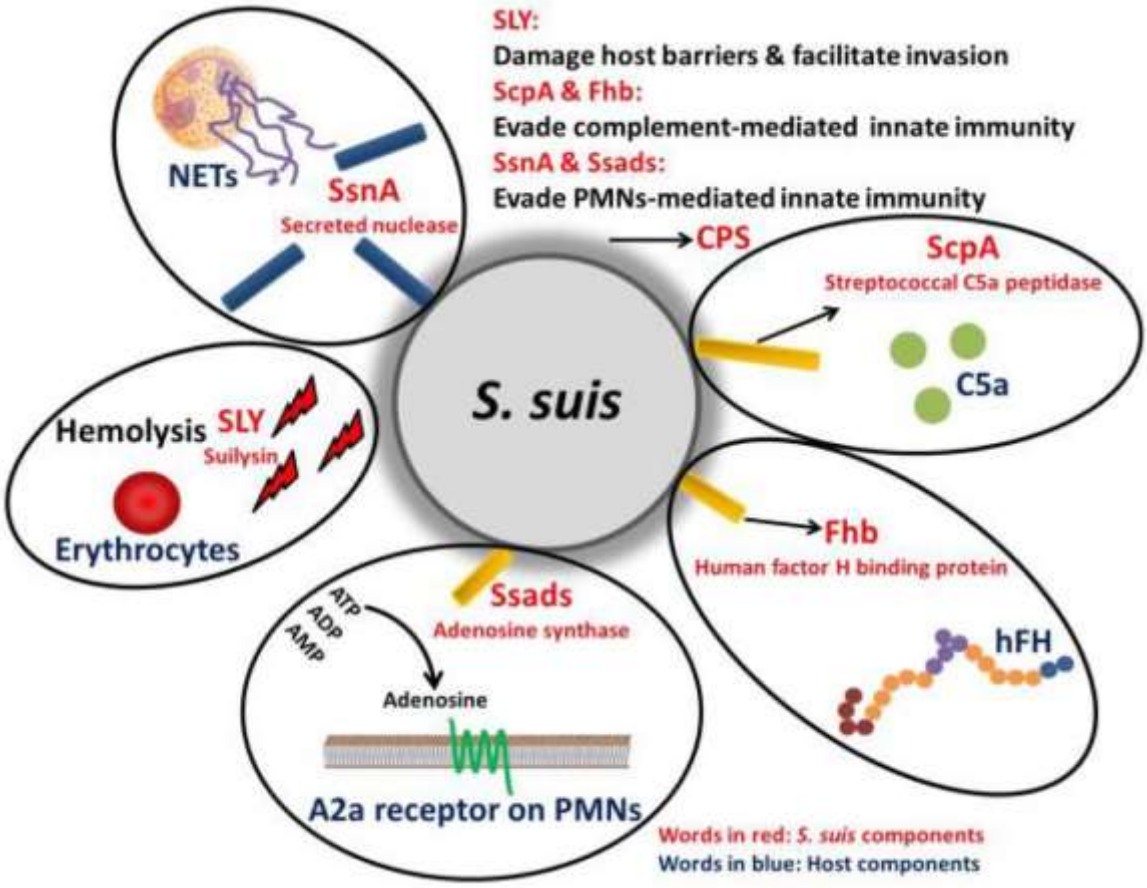

Fig 1.1 Part of virulence and their roles [8]

Capsular polysaccharide (CPS)

First reported by Smith H E et al. [9], CPS is one of the typing markers of *S. suis 2*, and it is also an important protein and virulence factor for *S. suis 2* to resist

phagocytosis by macrophages. The polysaccharide is mainly composed of five monosaccharides, N-acetylneuraminine, galactose, glucose, rhamnose and N-acetylglucosamine [3]. The composition and structure of the polysaccharide in the capsule are different due to the different types of monosaccharides contained and the way they are connected. The composition and structure of capsular polysaccharide are related to the pathogenicity and serum type of bacteria, and are essential components of pathogenic isolates. Capsular polysaccharides play an important role in evading the body's immune system and protecting bacteria from phagocytosis [10]. Houde M et al. [11] studied the molecular mechanism of anti-phagocytosis of *S. suis 2* capsular polysaccharide. Wild and missing strains of *S. suis 2* capsular polysaccharide were incubated with macrophages after heat inactivation, and observed under confocal laser microscope 1, 6 and 24 h later, respectively. It was found that the wild strain delayed and inhibited the production of nitric oxide during infection compared with the absent strain, indicating that the capsular polysaccharide had an inhibitory effect on the nitric oxide signaling pathway during infection, thus destroying the lipid microregion on the cell surface, preventing the dependent recognition of lactosidine ceramide, and protecting the fine bacteria against macrophage phagocytosis.

Hu Dan et al. compared the biological characteristics of the wild strain 05ZYH33 with the deficient strain of capsule polysaccharide, and found that the deficient strain was prone to be cleared by the whole blood due to the loss of surface mucosaccharides and the loss of the ability to agglutinate with *S. suis 2* capsule specific antiserum, but the adhesion ability to epithelial cells HEP-2 was enhanced. The results indicated that the capsule played an important role in the process of bacterial resistance to

phagocytosis and bacterial adhesion.

Suilysin(Sly)

Sly is a cholesterol-dependent pore-forming cytotoxin, a protein secreted during *S. suis 2* growth and present in most virulent *S. suis 2* strains, expressed in the medium and secreted in the supernatant of the medium. Hemolysins are encoded by the sly gene into two types, oxygen-sensitive Streptohemolysin O (SLO) and oxygen-stable Streptohemolysin S (SLS), with molecular weights of 54 kD and 62 kD, respectively. Sly will lose its hemolytic activity after oxidation, and cholesterol can also inhibit its hemolytic activity [12]. Sly can lyse and damage a variety of cells, and plays an important role in the process of *S. suis 2* invading the central system and destroying the BBB. Hemolysin can also damage the coagulation system and immune system of the body through the damage of blood plates and lymphocytes. Hemolysin positive SS can destroy chorioidal plexus epithelial cells, thus breaking through the cerebrospinal fluid barrier and causing meningitis symptoms [13].

In addition, Sly can induce the changes of host cytoskeleton [14] and the release of pro-inflammatory and immunomodulatory cytokines and chemokines, such as interlukin-6(IL-6), interlukin-8(IL-8), tumor necrosis factor-α(TNF-α), and interlukin-8(IL-10) [15]. Hemolysin is also a good immunoprotective antigen [16]. Based on the analysis of the whole genome sequence of S. SUIS 2 strain 05ZY H33, Liu L et al [17]. cloned and expressed the hemolysin protein and conducted an immunoprotective evaluation of this protein, and the results showed that hemolysin had obvious immunoprotective effects.

Muramidase-released protein(MRP) and Extracellular factor(EF)

MRP, a 136 kDa cell wall-anchored surface protein, is an important virulence factor and is released into the culture supernatant of virulent *S. suis 2* strains after treatment with cytoplasmic enzymes [18]. Studies have also shown that MRP is a major fibrinogen binding protein [19]. The binding of MRP to human fibrinogen increases the activity of *S. suis 2* in human blood, helps *S. suis 2* to cross BBB and bind to host cells, and promotes the development of meningitis.

EF is an extracellular protein with a molecular weight of approximately 110kDa encoded by the epf gene. It is a secreted protein and can only be detected in the supernatant of the culture medium.

MRP and EF are related to the virulence of *S. suis 2*, however, natural strains lacking MRP and EF are still pathogenic. It may be that the strains expressing MRP and EF are more virulent than the strains without MRP and EF [20]. Both MRP and EF can be expressed in *S. suis 2* strains isolated from diseased pigs in China, while the non-pathogenic strainS did not express MRP and EF [21].

Fibronectin- and fibrinogen-binding protein(FBPS)

FBPS is a bacterial surface protein that exists in various serotypes and is widely distributed in virulent, virulent, and nonvirulent strains of *S. suis 2* [22]. FBPS are composed of two domains with unique folds. The C-terminus of FBPS binds to host cell by fibronectin. and the N-terminus is attached to the bacterial surface which can promotes the adhesion of *S. suis 2* to host cells [23]. It is an atypical MSCRAMM because it lacks the typical “Leu- Pro- X- Thr- Gly” (LPXTG) surface anchoring motif. Previous studies have shown that FBPS is not necessary for tonsil colonization, but it

may play a role in the colonization of specific organs involved in SS infection. Compared with the wild strain, FBPS deletion significantly reduced the virulence of the strain. FBPS can be used as adhesins to promote the adhesion of *S. suis* to host cells, and can also be used as bacterial factors to activate signaling pathway through β1 integrin receptor to induce the production of chemokine [24]. FBPS has also been identified as a major H-factor binding protein by 2D-Far-western blotting [25].

***S. suis* adhesin P(SadP)**

SadP is a cell wall adhesin that binds Galabiose galA1-4Gal-oligosaccharide as a terminal epitope of the erythrocyte epithelium series of glycolipid (Gbs) receptors [26]. SadP can be divided into PN subtypes and PO subtypes based on the specificity of oligosaccharides [27]. The PN subtype is found in systemic strains that cause meningitis, while the Po subtype is distributed in asymptomatic carrier and respiratory strains. Galabiose-binding SadP was identified as Leu- Pro- N- Thr- Gly (LPNTG)-anchored cell wall protein [28]. The binding specificity analysis of SadP for galactose present in Gbs receptors shows that SADP has the highest specificity for triosaccharide Gb3/CD77 receptors [29]. The Gb3/CD77 receptor precursor (Gb3/CD77), or mature form, is present in a variety of host tissues, including the small intestine of humans and pigs. In addition, Gb3/CD77 is an adhesin-mediated receptor that binds to host tissues of a variety of human pathogens and acts as a receptor for bacterial toxins.

Enolase (Eno)

Eno is a glycolytic enzyme that catalyzes the conversion of phosphoglycerol to phosphoenolpyruvate and is considered to be a multifunctional protein on the surface of most streptococcal populations and serotypes [30, 31]. This enzyme can locate on the

cell surface and bind to serosa proteinogen, through which it contributes to the mucosal surface localization of bacterial pathogens and assists their invasion of host cells [32].

Eno can binds to 40S ribosomal protein SA (RPSA) on the surface of porcine brain microvascular endothelial cells leading to activation of intracellular p38/ERK-eIF4E signalling, which promotes intracellular expression of HSPD1 (heat-shock protein family D member 1), and initiation of host-cell apoptosis, and increased BBB permeability facilitating bacterial invasion [33]. Sun et al. found that Eno promoted the release of IL-8 and other cytokines by increasing BBB permeability during the onset of meningitis [34]. Lu et al. also found that *S. suis 2* enolase is an important protective antigen that protects mice from the deadly S.suis 2 infection [32].

Surface antigen I (SAO)

Li Y et al. [35] found Sao protein by using *S. suis 2* gene expression library and recovered serum antibody library of pigs after infection. Western blot proved that the protein existed in most serotypes of Streptococcus suis, and immunoelectron microscopy technology proved that the protein existed on the surface of *S. suis 2*. Sao occurs as one of three alleles and contains the Leu- Pro- V- Thr- Gly (LPVTG) motif at the C-terminal [36]. Sao is present in most *S. suis 2* and has classic characteristics of Gram-positive membrane anchoring proteins, for instance MRP and LPXTG membrane anchoring structure. Although the Sao protein of different *S. suis 2* strains has polymorphism, animal protective tests have proved that Sao protein has good protective effect on different *S. suis 2* strains [37]. It can be seen that Sao protein is an excellent candidate for protein subunit vaccine, and its role in *S. suis 2* infection and pathogenesis need to be further studied.

H factor

The H factor plays a crucial role in negative regulatory complement pathways. Many exposed pathogens can recruit the factor H to their cell surfaces, reducing phagocytosis and facilitating bacterial adhesion and invasion of host cells [38]. The first discovered H factor binding protein was named Fhb. Fhb mutants with defects were found to be completely non-toxic in swine infection models, and reduced survival rates of whole-person blood or human neutrophils [39]. Later, the second H factor binding protein, named Fhbp, was reported. Pre-incubation of eight *S. suis 2* Fhbp proteins with FhbP monoclonal antibody significantly decreased *S. suis 2* binding to H factor . Studies have shown that the binding of H factor to cells enhances *S. suis 2* adhesion and invasion of human throat cancer epithelial cells (HEp-2).

HP0197

A surface protein known as HP0197 is present on almost all virulent *S. suis 2* strains. Except for a 14% homologous G5 superfamily domain, HP0197 shares no similarity with any other known proteins. Recombinant HP0197 protein-immunized mice and piglets can withstand the test with a fatal dose of *S. suis 2*. Additionally, high titer anti-HP0197 serum can effectively boost passive immunity. This protein can therefore be employed as a potential *S. suis 2* subunit vaccination [40]. An 18 kDa domain in the N-terminal of HP0197 serves as the binding site for glucosaminoglycans (GAGs) on the host cell surface [41]. Additionally, HP0197 participates in HP0197-heparin-mediated HEp-2 cell adhesion. Ser-46's level of phosphorylation in the *S. suis 2* Hpr protein is controlled by HP0197 [42]. Since hpr is a cofactor for CcpA, phosphorylated hpr can make it easier for CcpA to bind to metabolite-responsive

elements (cre), which controls downstream gene expression and controls bacterial pathogenicity. Ser-46's level of phosphorylation in the *S. suis 2* Hpr protein is controlled by HP0197 . Since hpr is a cofactor for CcpA, phosphorylated hpr can make it easier for CcpA to bind to metabolite-responsive elements (cre), which controls downstream gene expression and controls bacterial pathogenicity [43].

Sortase

Sortase (Srt) is an enzyme expressed in Gram-positive bacteria that covalently immobilizes bacterial surface proteins on peptidoglycan. These surface proteins have a conserved LPXTG-motif and are linked to the C-terminus cell wall sorting signal [44]. Many diseases express the five types of sortases: A, B, C, D, and E. Gram-positive bacteria frequently contain the conservative protein SrtA . In Staphylococcus aureus, deletion of srtA severely impairs the anchoring of bacterial surface proteins, which greatly lowers the bacteria's adherence to and invasion of host epithelial cells and, ultimately, reduces their pathogenicity [45]. In *S. suis 2* isolate 05ZYH33, six sortase-encoding genes have been found. MRP and Sao proteins were improperly attached to the surface of S. SUIS 2 cells in the srtA-deficient mutant. The mutation impaired the pathogenicity in piglets and reduced their ability to adhere to ECM [46]. SrtB, SrtC, and SrtD deletions greatly reduced the capacity of the virus to adhere to HEp-2 cells, but they had no effect on the virus' pathogenicity in mice when compared to the wild strains. According to [47], srtF has no impact on the strain's pathogenicity or ability to adhere.

Elongation factor thermo unstable (EF-Tu)

A conserved multifunctional protein called EF-Tu plays roles in a number of

cellular functions and disorders, including apoptosis, cytoskeletal structure, signal transmission, and translation regulation [48, 49]. Amino acid tRNAs can enter particular regions of the ribosome thanks to EF-Tu. Barbier demonstrated that EF-Tu strongly influences the promotion of early Pseudomonas aeruginosa colonization and may play a role in bacterial pathogenicity [50]. The main metabolism of the recipient cells may be destroyed by Tse6 toxicity, according to Cabeen and Losick, who also described a sudden requirement for EF-Tu [51]. EF-Tu from S. suis may selectively combine with human fibronectin (FN) and laminin (LN), and EF-Tu-FN or EF-Tu-LN interactions play crucial roles in bacterial adherence and colonization, according to an immunoproteomics investigation [52]. In our earlier research, we discovered that S. suis antisera could be recognized using recombinant EF-Tu. EF-Tu can be employed as a successful subunit vaccine candidate antigen for *S. suis 2* because it stimulated strong immune responses and shielded New Zealand rabbits against lethal *S. suis 2* infective doses.

1.3 Infection mechanism of *S.suis*

Studies on the pathogenesis of *S.suis* strain are mainly limited to how *S.suis* causes meningitis. It is generally believed that *S.suis* first colonizes on the mucosal surface of tonsil, then breaks through the barrier of tonsil and upper respiratory tract, reaches the central nervous system through blood circulation, locally stimulates and produces a large number of cytokines, and the inflammatory exudate accumulates in the central nervous system, leading to increased intracranial pressure and typical meningitis symptoms [53].

1.3.1 Study on adhesion mechanism

CPS on the surface of *S.suis* made *S. suis 2* strains have strong phagocytosis resistance. Compared with the deficient strains, *S.suis* with capsular can resist the phagocytosis of mononuclear macrophages. CPS is a negative regulator of *S.suis* phagocytosis, inhibiting its adhesion and invasion of epithelial cells. Vanier G [54] studied the interaction of *S. suis 2* strain with porcine brain microvascular endothelial cells and found that CPS interfered with the adhesion and invasion ability of Streptococcus suis to varying degrees. Benga L [55] reported that the *S. suis 2* strain could not penetrate pig endothelial cells but adhered to them. The pretreatment of *S. suis 2* strain with healthy pig serum can enhance the adhesion of *S. suis 2* strain to endothelial cells. It is speculated that *Streptococcus suis* may interact with the serum components through the binding site on the surface of the bacteria, so as to enhance the adhesion to endothelial cells. Haataja S [56] demonstrated that the thickness of the capsule affects the agglutination effect of *S. suis 2* on erythrocytes. Many studies have also confirmed that CPS contributes to adhesion, especially its sialic acid component. It demonstrated that *S. suis 2* strain could adhere to and invade porcine brain microvascular endothelial cells [57].

The adhesion of *S. suis 2* may be closely related to the protein molecules on its surface. MRP is a kind of galactose adhesion, with blood clotting activity , in bacteria addicted to its sexual organs of engraftment and further infection plays an important role, its expression and virulence of *S. suis*. Fibulin is a substrate for bacterial adhesion, and it has been proved that fibulin is one of the receptors for *S.suis* adhesion to host cells, and FBPS have an effect on *S. suis 2* pathogenicity . Brassard J [58] confirmed

that glyceraldehyde 3-phosphate dehydrogenase with a relative molecular mass of 39ku was associated with bacterial adhesion as a surface protein of *S.suis*. *S.suis* specifically adheres to P group oligosaccharide receptors in blood, which makes it easier to colonize on epithelial cells and mediates *S.suis* invasion into epithelial cells [59]. The oligosaccharide chains of glycoproteins and glycolipids are ideal receptors for bacterial adhesion. The polyphasic oligosaccharide structure and the agglutinin specific adhesion of *S. suis 2* strains give *S.suis* the ability to adhere to different cell types. Vanier G demonstrated that *S. suis 2* can adhere to and invade the microvascular endothelial cells of pig brain and survive in the cell, but can only adhere to human microvascular endothelial cells SS, which is speculated to be because pigs are the natural host of *S.suis*, and the surface of human microvascular endothelial cells lacks the specific receptor of S *S. suis 2* strain.

In conclusion, *S. suis 2* adhesion is mediated by something on the cell wall, and the presence of capsular polysaccharides may reduce this effect. It is generally believed that *S. suis 2* strains first adhered to and colonized porcine nasal and pharyngeal mucosal epithelial cells, and then spread to respiratory tract or enter blood to cause disease. However, the capsular polysaccharide could not inhibit the adhesion of *S. suis 2* to cerebral microvascular endothelial cells.

1.3.2 Study on invasion mechanism

Studies on the interaction between host cells and *S.suis* mainly focus on *S. suis 2*. Due to the large number of serotypes of *S.suis* and the presence of some uncategorized strains of *S.suis*. *S.suis* has a variety of virulence characteristics, and whether *S.suis* invade host cells is still controversial. Benga L [60] subjected HEp-2 epithelial cells to

10 *S. suis 2* strains and 4 uncategorized *S.suis* strains to evaluate their ability to adhere to and invade cells. Only the untyped strain and the uncapsulated mutant showed strong adhesion and invasion [61]. There may be a unique molecular mechanism for the invasion of uncategorized strains by electron microscopy. Uncategorized *S.suis* strains have the ability to invade and survive in epithelial cells, and this characteristic of *S.suis* regulated by environmental signals and other factors has a certain relationship with the diversity of virulence of the strains. Norton P [62] reported that the *S. suis 2* strain was able to invade human epithelial cells to some extent. Sly positive strains are toxic to epithelial cells, and anti-Sly monoclonal antibodies inhibit this effect. Swildens B [63] demonstrated that the intestine is the entry portal of EF positive *S. suis 2*. Since sly has a strong cytoidal effect, it is speculated that *S.suis* enters the central nervous system mainly through brain microvascular endothelial cells under the combined action of adhesion and sly.

1.3.3 Study on blood transmission mechanism

After breaking through the mucosal barrier, *S. suis 2* enters the bloodstream and spreads, which may have the following mechanisms. (1) phagocytosis by mononuclear phagocytes. In the blood, *S. suis 2* is phagocytosed by macrophages, resulting in two outcomes: ① The strain is killed intracellular. ② It survives by some mechanism, travels with macrophages to other tissues and even the brain, and then invades the central nervous system. *S. suis 2* can be phagocytic by pig or human monocytes. Quessy S [64] demonstrated that *S. suis 2* was phagocytic by pig or human mononuclear cells using flow cytometry. (2) Free *S. suis 2* diffuses with the blood circulation. Although phagocytosis has been observed at some level, many Streptococcus suis still survive

outside the cell. In the peripheral blood of bacteremia pigs, the proportion of monocytes that phagocytic *S. suis 2* is less than 2% [65]. Moreover, the capsule has anti-phagocytic effect, so most *S. suis* is free in the blood and diffuses with the blood circulation. *S. suis 2* can adhere to macrophages at a high level without being phagocytic, so it is inferred that a large number of extracellular bacteria in the bloodstream also spread by adhering to the cell surface. (3) Change the permeability of infected cells and help *S.suis* invade cells to pass through the cell layer. During meningitis caused by *S.suis*, the interregulation of various cytokines causes many inflammatory responses, including the fixation of white blood cells at the site of infection. In the study of Vadeboncoeur N [66], *S. suis 2* induced human microvascular endothelial cells to produce cytokines such as IL-6, IL-8 and Monocyte Chemoattractant Protein-1 (MCP-1), which increased the permeability of cerebral microvascular endothelial cells in important parts of the blood-brain barrier and even caused cell rupture, and then passed through the cell layer, resulting in brain edema, increased intracranial pressure and blood flow obstruction and other typical meningitis lesions.

1.4 Interaction of *S. suis 2* with host cells

Neutrophils

Neutrophils are the white blood cells with the largest number and the strongest swallowing ability in peripheral blood. The host's main first defense against *S. suis 2* is the phagocytosis of neutrophils. When pathogenic *S. suis 2* infects pigs, neutrophils are significantly increased in tonsils, and neutrophils are often infiltrated in the injured sites caused by infection [67]. In the absence of specific antibodies, *S. suis 2* is not effectively killed by porcine neutrophils. CPS can protect *S. suis 2* from phagocytosis

by neutrophils [68].

Macrophage

Tonsil, the gateway for *S. suis 2* colonization and invasion, significantly increased macrophages in the crypt epithelium at the initial stage of swine *S. suis 2* infection [69]. *S. suis 2* not only attaches to macrophages. Also resist the swallowing of macrophages. As the concentration of bacteria increases during incubation, it becomes toxic to macrophages.

The interaction between *S. suis 2* and macrophages can induce host cells to release proinflammatory cytokines and chemokines. Like tumor necrosis factor-α (TNF-α), IL-l, 1L-6, 1L-8. *S. suis 2* with capsule can resist phagocytosis of macrophages. The capsule is a virulence factor for *S. suis 2*. The capsulated *S. suis 2* defies macrophage phagocytosis by inhibiting the activation of PI3K/A kT/PKCa signaling pathways used for phagocytosis [70].

Epithelial cell

The adhesion, invasion and toxicity of *S. suis 2* on different epithelial cell lines A549, Hela, PK15, LLC-PK1 and MDCK were studied [71]. The results showed that *S. suis 2* had no ability to invade epithelial cells. Adherent epithelial cells were time-dependent and concentration-dependent. CPS seems to have no effect on adhesion. But lack of CPS. The adhesion rate increased significantly. Other studies have also revealed that *S. suis 2* may adhere to epithelial tissue and then act on epithelial tissue through Sly «Perforation», resulting in infection of epithelial tissue.

Endothelial cell

In vitro experiments showed that *S. suis 2* had adhesion and invasion effect on BMEC. The surface proteins and cell wall components (mainly teichoic acid) of bacteria are involved in the interaction between *S. suis 2* and BMEC [72].

Before entering BBB, *S. suis 2* can secrete bacterial capsular polysaccharide down-regulation, reduce capsular synthesis and thickness, and expose invasive proteins such as bacterial adhesion virulence factor, lipoteichoic acid, sorting enzyme and enolase as much as possible, and promote interaction between *S. suis 2* and BMEC through Sly protein toxicity. It can combine with extracellular matrix protein, which is good for the colonization of fine bacteria [73, 74].

Adhesion of *S. suis 2* to B, E and C results in the production of cytokines and chemokines, increased leukocyte attraction and BBB permeability. However, the proinflammatory factors in cerebrospinal fluid during meningitis may be derived from microglia, endothelial cells and migrating white cells. Other cytokines such as TNF-α or IL-l and *S. suis 2* can stimulate the production of proinflammatory cytokines.

Porcine choroid plexus epithelial cells

Chorioidea epithelial cells are the structural basis of the blood-cerebrospinal fluid barrier and play an active role in host defense against bacterial meningitis. Mar et al. [75] confirmed that inflammatory response plays an important role in the pathogenesis of *S. suis 2* infection by using a mouse model of *S. suis 2* infection. The choroid plexus and lining brain endothelial cells have early transcriptional activation of TLR2, CDl4 and inflammatory cytokines.

Adam RA et al. confirmed in vitro that Interferon-7 (IFN-7) and TNF-a activated

indoleamino-2 in porcine choroid plexus epithelial cells. 3 A pair of oxygenase inhibits *S. suis 2* growth [76]. *S. suis 2* has a destructive effect on the blood-cerebrospinal fluid barrier, affecting its function and integrity [77]. Different strains of *S. suis 2* had different effects on the destruction of blood-cerebrospinal fluid barrier. The main mechanism of this destructive effect is that *S. suis 2* leads to cell necrosis of chorioidal epithelial cells [78].

1.5 Clinical signs and symptoms

Septicemia

Septicemic streptococcus is mainly caused by group C streptococcus, group D streptococcus, and group E streptococcus. The most acute cases often result in sudden death without any symptoms. The symptoms of the disease are more gradual and include a rise in body temperature above 41℃, muscle tremors, loss of appetite or paralysis, constipation, visual cyanosis of mucous membranes, flushing of the conjunctival membranes, and tears. The skin color in the ear root, neck, abdomen and other places is purple, and breathing difficulties appear in the later stage of the disease [79]. Death occurs within 3 days after the disease, and dark red blood flows from natural pores after death. Autopsy showed serous cavity effusion, cellulose attachment, nasal mucosa congestion and bleeding, larynx and trachea congestion, a large number of bubbles in the trachea, spleen enlargement of 1 ~ 3 times of normal, kidney swelling and bleeding, digestive tract mucosa with varying degrees of congestion and edema [80].

Meningitis

This type of suis streptococcus disease is caused by group R streptococcus and

Group C streptococcus, with a small number of cases caused by L or S streptococcus infections. Diseased pigs present with elevated body temperature, constipation, refusal to feed, and serous or mucous rhinorrhea. Soon after the disease appeared neurological symptoms, ataxia, empty chewing, spinning, hind limb paralysis, limbs were swimming. Post examination of diseased pigs showed meningeal congestion and bleeding, some of the diseased pigs had symptoms of submeningeal effusion, and white matter and gray matter were scattered in hemorrhagic spots under the section of the brain.

Arthritis

This type of pig is mainly converted from the first two types of disease, the course of disease is slightly longer than the last two types, the symptoms are relatively mild, the body temperature sometimes increased and sometimes normal, mental and appetite instability, one or more joint enlargement, lameness, difficulty standing, and emaciation [81]. There are yellow jelly-like liquids in the joint capsule, and some are cellulose purulent substances.

Lymph node abscess

The main manifestations of infected pigs are suppurative lymphadenitis, the main lesions are mandibular lymph nodes, and sometimes the lymph nodes in the pharynx, ear and neck will also be damaged [82]. The lesions of lymph nodes are inflamed, swollen, palpated hard and hot pain, which will affect feeding, chewing and swallowing, and seriously affect breathing. When the purulent site ruptured, the systemic symptoms were relieved, the course of the disease was 3–5 weeks, and the mortality was low.

1.6 The overview and mechanism pyroptosis and the diseases related to pyroptosis

1.6.1 Overview of Pyroptosis

Cells are the basic unit of life, and the metabolism of the body is often accompanied by the occurrence of cell death. Cell death plays a key role in the development of the body, the maintenance of homeostasis and the occurrence and development of diseases. The modes of cell death are mainly divided into passive cell death and active cell death. Passive cell death is a self-protection mechanism produced by cells when they are stressed, injured or infected by pathogenic microorganisms. In this process, inflammation is produced, so it is also called inflammatory death. Typical passive cell death has cell coke. apoptosis and cell necrosis [83]. Active cell death mainly refers to the cell self-regulation process produced by organisms in order to regulate the number of cells, promote morphogenesis, and remove harmful or abnormal cells, mainly including apoptosis and autophagic death, both of which are cell behavior regulated by genes [84, 85]. Pyroptosis is a newly discovered way of cellular program death in recent years. It is the body's primary non-specific defense mechanism. It has an irreplaceable role in preventing external pathogen invasion and sensing endogenous danger signals [86]. This article reviews the discovery and nomenclature, morphological and molecular features, molecular mechanisms and pyroptosis-related diseases of pyroptosis in recent years.

The discovery and naming of pyroptosis

Pyroptosis was initially proposed in 2001 by Cookson and Brennan to describe pro-inflammatory programmed necrosis that occurs in *Salmonella* - infected

macrophages in a caspase‐1‐dependent manner [87]. The term «pyropto-sis» comes from the Greek roots pyro, which means "fire" or "fever," and ptosis to denote a falling. The combination of the two words reflects the inflammatory nature of this method of cell death [88]. However, how the activation of inflammatory caspase causes cell pyrolysis has not been answered. It was not until the publication of two independent research results in 2015 that this question was initially answered [89, 90]. They all found that gasdermin D (GSDMD) is a substrate of inflammatory caspase, which causes pyrolysis by forming small holes in the cell membrane after lysis [91]. Therefore, pyroptosis is defined as gasdermin family-mediated programmed cell necrosis.Shao, et al found pyrolysis can also be caused by the activation of caspase-4/5/11 by Lipopolysaccharide (LPS) in the cytoplasm. The activated caspase-4/5/11 will eventually induce pyrolysis through the cleavage of gasdermin family proteins [92]. Therefore they defined pyroptosis as Gasdermin family-mediated programmed cell necrosis .

Morphological and molecular features of pyroptosis

Morphological features of pyroptosis.

Pyroptosis is morphologically characterized by both cell necrosis and apoptosis. When cells undergo pyroptosis, the nucleus is condensed, chromatin DNA is randomly fragmented and degraded, the cells are swollen in a circular shape, and multiple vesicular protrusions are formed. Numerous pores appear on the surface, causing the cell membrane to lose its integrity [93]. Blister-like protrusions are similar in size to apoptotic bodies and are called pyroptotic bodies. The formation of pores in the cell membrane is mainly a non-ion-selective channelformed by GSDMD [94].

The cell membrane loses the ability to regulate the entry and exit of substances, the cell loses the balance of internal and external ions, osmotic swelling occurs and the membrane ruptures, releasing active substances such as cell contents, stimulating the body's immune response, recruiting more inflammatory cells, and expanding the inflammatory response [95, 96].

Molecular features of pyroptosis Caspase family

Caspase family is a homologous and structurally similar proteolytic enzyme in cytoplasm, which selectively recognizes and cleaves peptide bonds behind downstream target aspartic acid residues. In normal cells, caspase protein usually exists in the inactive pro-caspase state, and only after hydrolysis of amino acid sequence into active caspase can play its role. So far, 15 caspases have been identified in mammals, 13 caspases in humans and 11 caspases in mice [97]. According to the differences in structure and function,caspase can be divided into apoptotic and inflammatory types. Among them, Apoptosis caspase includes caspase-2/3/6/7/8/9/10, represented by caspase3, which is related to apoptosis. But it was found that caspase-3 also can induce pyroptosis by cleaving gasdermin E(GSDME) [98]. Moreover, caspase-8 which is related to apoptosis can also straightly cleave GSDMD to induce pyroptosis [99]. Inflammatory caspases include caspase-1/4/5/11/1213/14, which mediate inflammatory responses [100, 101]. Activation of inflammatory caspase-1 and caspase-4/5/11 ultimately leads to cell apoptosis.

Gasdermin family

Gasdermins(GSDMs) are a family of functionally diverse proteins expressed in a variety of cell types and tissues [102, 103]. The earlier identified GSDMs in the

gastrointestinal tract and dermis were named «gas-dermin» [104]. 6 GSDMs were found in humans and 10 GSDMz were found in mice. GSDMs consists of Gasdermin A (GSDMA), Gasdermin B (GSDMB), Gasdermin C (GSDMC), Gasdermin D (GSDMD), Gasdermin E (GSDME) and Pejvakin (PJVK). GSDMA and GSDMB are mainly expressed in esophagus and intestinal cells, and are associated with hair loss, asthma and inflammatory diseases [105, 106]. Human GSDMC protein is expressed in epithelial cells of stomach, esophagus and spleen, and is inhibited in cancer cells such as gastric cancer, and its biological function is still under study [107]. GSDMD and GSDME are widely expressed in different cell tissues. GSDMD is the executioner of pyroptosis due to its ability to form membrane pores [108]. GSDMD can be specifically activated by inflammatory Caspase-1, 4, 5, 11, and cleaved into GSDMD-N (p30 fragment) and GSDMD-C (p20 fragment). GSDMD-C exists in the cytoplasm, and GSDMD-N has lipophilic and can binds specifically to phosphatidylinositol on the inside of the cell membrane and cardiolipin on the outside of the bacterial plasma membrane, oligomerizes in the membrane and forms a pore with a diameter of 10–16 nm [109]. The pore secretes a substrate of smaller diameter, eventually causing the membrane to rupture and releasing the entire cell contents [110]. When stimulated by chemotherapy drugs, tumor necrosis factor and virus infection, GSDME can be activated by caspase-3 of apoptotic signaling pathway to punch holes in cell membranes and transform the cells that should undergo apoptosis into pyroptosis [111, 112]. Usually GSDME is expressed at a high level in normal cells, while cancer cells undergo epigenetic modifications such as DNA methylation and histones, and most of them are in the state of GSDME inhibited expression or low-level expression [113]. Pyroptosis of

normal cells expressing GSDME may be one of the reasons for the toxic side effects of conventional chemotherapy drugs.

1.6.2 The mechanism of pyroptosis

The occurrence of pyroptosis can be divided into two ways: caspase-1-dependent and non-caspase-1-dependent [114]. The way of cell death that depends on caspase-1 is called classical pathway pyrolysis, while the way of cell death that is not dependent on caspase-1 is caused by human caspase-4 and -5 or Caspase-11 induction in mice is called non-classical pathway pyrolysis. The morphological characteristics of pyrolysis in the classical pathway and the non-classical pathway are similar.

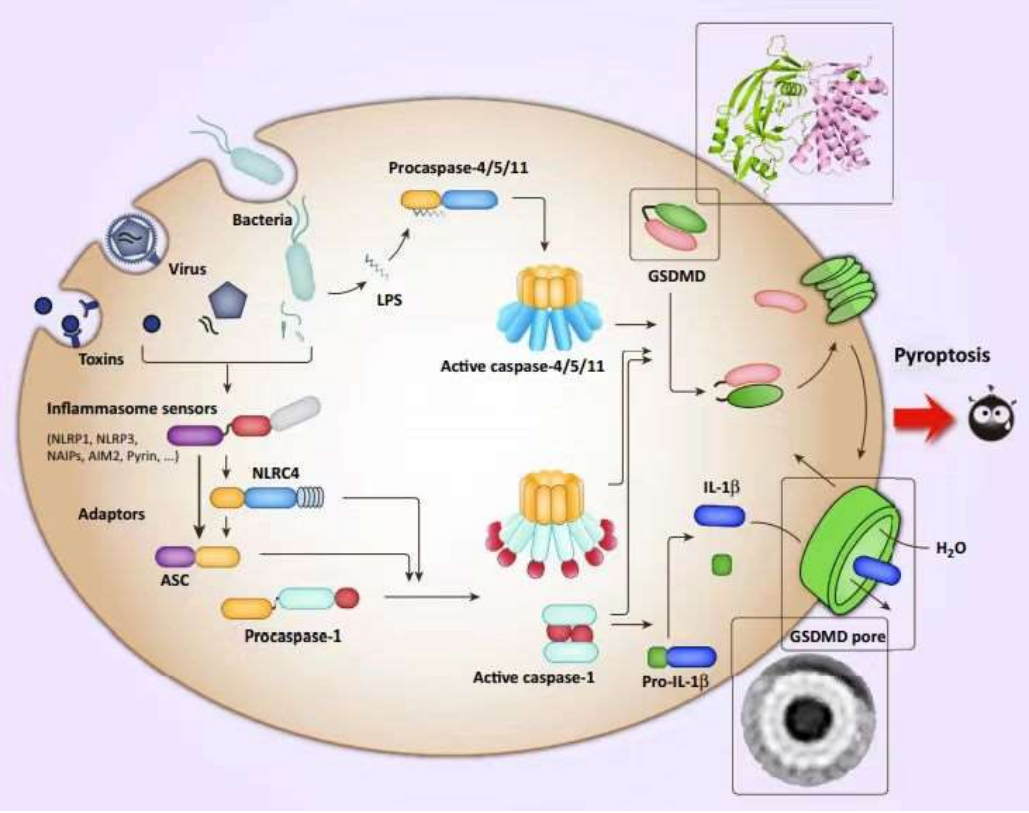

Fig 1.2 The mechanism of pyroptosis [91]

Both pathways cause the release of IL-1beta and IL-18, which are involved in inflammasome activation. IL-1beta induces tissue inflammation, vasodilation, and

extravasation of immune cells, and also plays a role in adaptive immune responses [115]. IL-18 can promote the production of interferon-γ by Th1 cells, Natural killer(NK) cells and cytotoxic T cells, promote the development and maturation of Th2 cells, and enhance local inflammatory response [116].

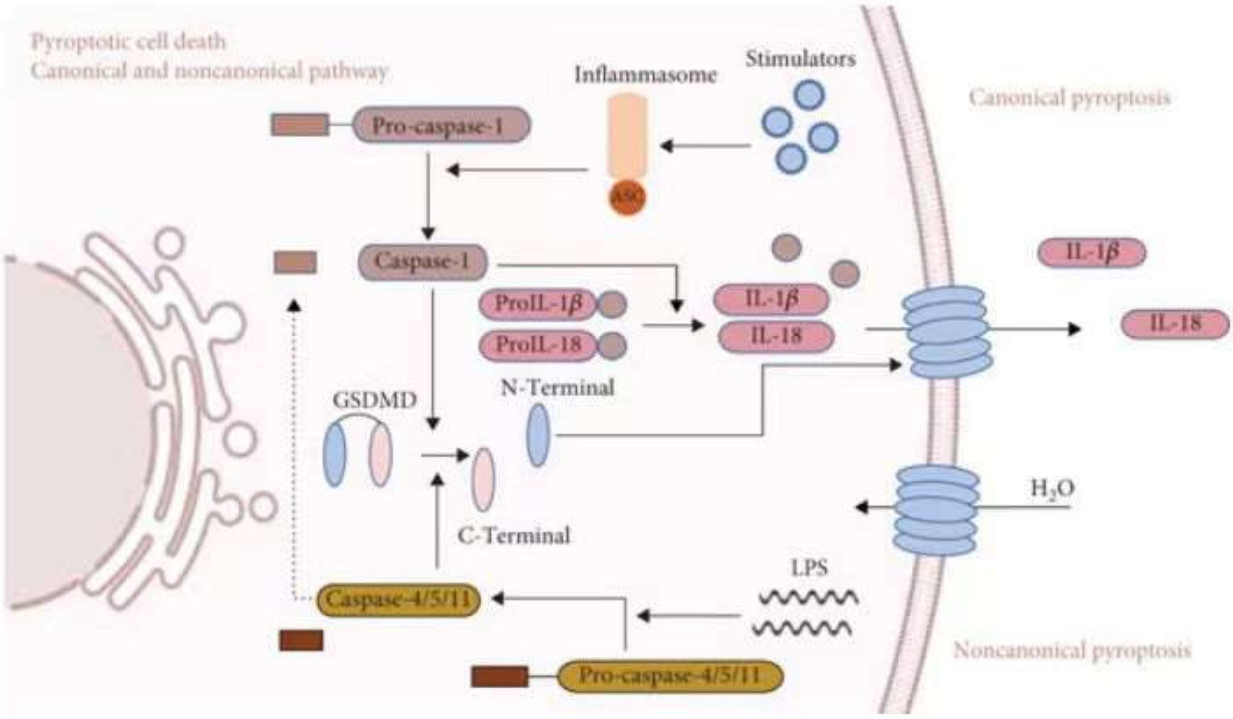

Fig 1.3 The pathway of pyroptosis

Canonical pathway [117]

Canonical pyroptosis is mediated by inflammasome assembly with GSDMD cleavage and IL-1β and IL-18 release [118, 119]. Inflammasomes are multimolecular complexes that are activated when the host becomes resistant to microbial infection.

When pathogens invade host cells, specific pattern recognition receptors (pattern recognition receptors, PRRs) on the cell surface or inside recognize pathogen-related molecular patterns (PAMPs) structure and endogenous risk-associated molecular patterns (DAMPs) [120]. Pattern recognition receptors bind to specific ligands, and then combine with other proteins to form inflammasomes. If the Nod-liker Recptor Protein

3(NLRP3) inflammasome is activated, its ligands can stimulate eukaryotic cells to generate reactive oxygen species (ROS) and damage lysosomes to release lysosomal proteases to mediate NLRP3 activation [121]. Activated NLRP3 converts biologically inactive pro-caspase-1 into active caspase-1. The caustic executive protein GSDMD is cleaved by activated caspase-1 at the Asp275 site, forming a 31 kDa N-terminus (N-GSDMD) and. a 22 kDa C-terminus (C-GSDMD) . N-GSDMD penetrates the cell membrane to form non-selective pores, resulting in cell swelling and pyroptosis [122, 123]. At the same time, caspase-1 also cleaves the precursors of IL-1β and IL-18 into mature IL1β and IL-18, which are released through the pores formed by GSDMD, leading to pyroptosis [124, 125].

Non-canonical pathway

Non-classical pyroptosis is activated by the activation of Caspase-4/5/11 as the premise pathway, mainly through the direct binding of the inflammatory Caspase-4/5/11 protein precursor to the LPS in the cytoplasm to assemble and trigger cell pyroptosis [126]. When pathogenic microorganisms infect host cells, PRRs located in the cytoplasm are recognized and bound to corresponding ligands, assembled to form multi-protein complexes in the cytoplasm, and activate inflammatory Caspase-4/5/11 to further cleave GSDMD protein to the cell membrane Punch holes to promote the occurrence of pyroptosis. At the same time, the inflammasome acts on downstream molecules to promote the release of mature and ruptured cell membranes such as inflammatory cytokines (such as IL-1β, IL-18, etc.), chemokines, and adhesion molecules to the outside of the cell, recruiting and activating more inflammatory cells. trigger an inflammatory response [127].

In addition, Pannexin-1 is found to be another key protein in mediating pyroptosis in the non-classical pathway induced by caspase-11 [128]. Upon stimulation with LPS, activated caspase-11 can specifically cleave and modify Pannexin-1, elicited intracellular ATP release and thereby induce pyroptosis mediated by the ion channel P2X7 receptor.

Caspase-3/8-mediated pathway

Members of the gasdermins protein family are highly conserved in structure. With the exception of DFNB59, all gasdermins contain C-terminal and N-terminal domains, the N-terminal being the executor of pyroptosis [129]. Caspase-3 has long been considered as an important marker of apoptosis. Recently, Wang et al. Found that caspase-3 can affect and activate gsdme and promote the occurrence of focal death. In tumor cell lines with high expression of gsdme, chemotherapeutic drugs can induce the activation of Caspase-3 and cleave gsdme. The generated gsdme-n can punch holes in the cell membrane and cause the scorch death of tumor cells [130]. Sarhan et al. Reported that caspase-8 can cleave gsdmd and mediate cell death during the inhibition of TGF-β-activated kinase 1(TAK1) by pathogenic Yersinia through effector YopJ [131, 132]. TNF-mediated apoptosis is converted to pyroptosis by programmed death-ligand 1(PD-L1) in breast cancer cells.Under hypoxic conditions, the nuclear translocation of PD-L1 is promoted by p-Stat3, which together enhance GSDMC transcription. Under the stimulation of tumor necrosis factor-α(TNF-α), Caspase-8 specifically cleaved GSDMC to generate N-GSDMC, and formed pores in the cell membrane to induce pyroptosis .

Granzyme-mediated pathway

Recently, Shao,etc, found for the first time that gasdermin can perform the perforation function through serine protease gzma hydrolysis at non ASP sites, and proved the cell death induced by cytotoxic lymphocytes as pyroptosis [133]. This discovery rewrites the conclusion that focal death can only be activated by caspase. The serine protease granzyme A in cytotoxic lymphocytes (such as CTLs, NK cells, etc.) can enter the target cells through perforin, and the target cells can be induced to scorch by hydrolyzing lys229 / lys244 sites of gasderminb (gsdmb) molecules [134]. GSDMB has tissue-specific expression and is highly expressed in digestive system epithelial cell-derived tumor cells. Induction of focal death by gsdmb will enhance antitumor immunity and will become a potential target for the treatment of these tumors .

1.6.3 Diseases associated with pyroptosis

Inflammatory bodies formed during pyroptosis can stimulate tumor cell pyroptosis and decrease tumor cell growth.. The accumulation of inflammatory bodies, on the other hand, can create a favorable milieu for tumor cell growth [135]. GSDMD activation causes the release of inflammatory cytokines such IL-1 and IL-18, which activate immune cells, chemokines, cytokines, and adhesion molecules, so amplifying the inflammatory response [136]. IL-1 β is an endogenous heat source that promotes fever, vasodilation, chemotactic migration of leukocytes, cytokine increase and hyperalgesia. Its unregulated discharge causes autoimmune disorders to develop(periodic syndrome, Mediterranean fever) . By boosting the production of IFN-γ , Il-18 causes inflammation. It is a well-known antibacterial inflammatory cytokine that causes T cells and macrophages to become activated. At the same time, excessive

pyrotopia activation can result in a significant number of cell death, tissue damage, organ failure, and even autoimmune inflammation, septic shock, or tumor, resulting in irreversible body harm [137].

Pyroptosis and Infectious diseases

Pyroptosis is closely associated with multiple bacterial, fungal and viral infections In the pyroptotic pathway, PAMPs and LPS can be recognized by the corresponding inflammasomes and caspases, respectively, and activate the downstream pyroptotic pathway. Therefore, pathogen infection is the main way to induce pyroptosis. Pyroptosis has been found in Shigella, anthrax, tuberculosis, Brucella infection and bacillary dysentery [138, 139]. Pyroptosis functions as a host defense mechanism when a pathogen infects the body, activating the innate immune system to fight infections . When cells are infected by *Salmonella*, the activation of caspase-1 will lead to the production of inflammatory factors, cell membrane damage, and even cell rupture, which is beneficial to the removal of intracellular bacteria [140]. When Shigella infectes cells, it rapidly invades the intestinal mucosa quickly, cause inflammatory reaction, and eventually result in bacterial dysentery [141]. In Lei et al.'s studyin the pathogenesis of enterovirus 71, the enterovirus protease 3C was found to cleave gasdermin D [142]. The cleavage site is distinct from the caspase-induced cleavage site and physiologically inactivates the N-terminal fragment, thereby disabling the downstream pyroptosis pathway.

Enterovirus 71 escapes the resistance mechanism of the host cellular immune system by directly disrupting key factors in the pyroptosis pathway, providing a new perspective for reassessing pathogen resistance to host pyroptosis

Pyroptosis and Cardiovascular diseases

Atherosclerosis(As) is a chronic progressive disease characterized by lipid accumulation and inflammatory cell infiltration [143]. Many factors such as hyperlipidemia, hyperglycemia and smoking can promote the progression of As [144].

High fat environment can induce the increase of reactive oxygen species (ROS), trigger endothelial cell scorch death and downstream inflammatory waterfall, and aggravate the development of as. It can also promote the expression of AIM2, GSDMD-N and other genes in smooth muscle cells, increase the area of plaque and the number of dead cells in mice by inducing the scorch death of smooth muscle cells, and increase the instability of plaque [145].

Oxidized low density lipoprotein (ox-LDL) has a strong as promoting effect. It can induce endothelial cell death through ERS/ASKI axis or miR-125a-5p expression [146, 147]. While ox-LDL induces macrophage focal death, it promotes the occurrence of cell focal death by limiting autophagy, and promotes the formation of necrotic nuclei and plaque instability [148].

High density lipoprotein (HDL) can play an important role, but when combined with chronic inflammatory diseases, it can be oxidized and modified to promote oxidation and inflammation. Oxidized HDL can induce NLRP3 mediated cell scorch death in macrophages, thereby promoting the progression of as plaque .

Pyroptosis and Central nervous system disease

Studies have shown that cell death is involved in the pathogenesis of central nervous system degenerative diseases progress, such as Alzheimer's disease (AD), Parkinson's disease(PD) and stroke . The pathological features of AD are synaptic loss,

neuronal death and extracellular neuroinflammatory plaques β－ Amyloid- β， Aβ）, which can interfere with the function of membrane and cause the outflow of K + from neurons. Low K concentration can activate nlrp1 and cause cell pyroptosis [149].

Pyroptosis activated by the PD-causing protein α-synuclein is closely related to the development of PD-induced neuroinflammation [150]. Normally, aggregated α-synuclein can be released from impaired neurons and recognized by Toll-like receptors on microglia to activate the NF-B pathway and the NLRP3 inflammasome, thereby inducing microglia Pyroptosis and neuroinflammation [151].

Inflammation activated by inflammasome and pyroptosis is closely related to stroke pathology [152]. Increased expression of NLRP3, NLRP1, caspase-1, IL-1β and IL-18 was observed in brain samples from stroke patients [153]. Activation of the NLRP3/caspase-1/GSDMD pathway induces microglia and astrocyte pyroptosis in a mouse model of middle cerebral artery occlusion (MCAO) [154, 155]. In addition, absent in melanoma 2 (AIM2) and NOD-like receptor containing 4(NLRC4) inflammasomes in microglia and NLRP6 and NLRP2 inflammasomes in astrocytes have been shown to activate GSDMD-mediated pyroptosis and inflammation, leading to models of ischemic brain injury damaged neuronal cells [156, 157].

The role of Pyroptosis in Tumors

Pyroptosis can affect the occurrence and progression of tumor, which regulates the proliferation, invasion and metastasis of tumor cells through some non-coding RNA and other molecules.

Studies have found that inflammatory bodies can also exist in tumor cells, and these bodies can promote and inhibit tumor growth [158, 159]. Because inflammatory

corpuscles are the key molecules that guide caspase-1 in cell focal death, it may be an important node between tumor cells and pyroptosis. Different tumors involve different inflammatory bodies. For example, NLRP3 widely exists in tumor cells [160], and related tumors include nasopharyngeal carcinoma, colorectal cancer , and lung adenocarcinoma [161, 162]. In addition, liver cancer is also associated with aim2 inflammatory bodies [163]. Although it can be inferred that cell death is related to tumor, the relationship between them is relatively complex. Studies have shown that cell death can inhibit the occurrence and development of tumor, but on the other hand, cell death can promote inflammatory death and form a microenvironment suitable for the growth of tumor cells, so as to promote the growth of tumor [164].

Pyroptosis and lung cancer

Lung cancer is the most common cancer in the world and one of the leading causes of death [165, 166]. In non-small cell lung cancer (NSCLC), GSDMD was found to be elevated [167]. Furthermore, a high level of GSDMD aided tumor spread and predicted a poor outcome in lung adenocarcinoma (LUAD) patients. Activation of the pyroptotic signaling pathway (NLRP3/caspase1) promoted apoptosis but not pyroptosis in GSDMD-deficient tumor cells. Furthermore, inhibiting tumor proliferation by inhibiting the epidermal growth factor receptor / Protein Kinase B(EGFR/Akt) signaling pathway in NSCLC was achieved by silencing GSDMD [168]. Xi et al. reported in 2019 that GSDMD colocalized with GzmB near immunological synapses, and that a deficiency in GSDMD reduced CD8+ T cell cytolytic capabilities, suggesting that GSDMD is required for tumor cell immune response [169]. GSDME is found in a variety of molecular subtypes of lung cancer. In A549, PC9, or NCI-H3122 cells,

GSDME or caspase-3 reduction drastically decreased GSDME-dependent pyroptosis [170]. Both paclitaxel and cisplatin were shown to trigger apoptosis in A549 cells by Zhang et al., however some of the dying cells had a morphology that was very similar to pyroptosis [171].

Pyroptosis and gastric cancer

Gastric cancer is a cancer that starts in the cells of the stomach and has a poor prognosis and a high mortality rate [172, 173]. GSDMA was found to be a tumor suppressor gene in gastric cancer , but it was also found to be overexpressed in some gastric cancer cells, suggesting that it could operate as an oncogene. GSDMB was found to be strongly expressed in the majority of malignant tissue samples but not in the majority of normal gastric tissues, suggesting that it may be linked to invasion [174]. On the other hand, GSDMC was shown to be downregulated in gastric cancer, suggesting that it may act as a tumor suppressor. Wang et al. found that GSDMD can inhibit extracellular-signal-regulated kinase1/2(ERK1/2), Signal transducer and activator of transcription 3(STAT3) and phosphatidylinositol-3-kinase/Protein Kinase B(PI3K/AKT) in gastric cancer (GC) cells, thereby reducing the expression of Cyclin A2 and Cyclin DependentKinase (CDK2). Therefore, the reduction of GSDMD expression in GC cells increases the expression of Cyclin/CDK complex as a substance that regulates cell cycle, promotes the transition from S phase to G2 phase, and accelerates GC cell proliferation [175]. Chemotherapeutic medicines were discovered to cause pyroptosis rather than apoptosis in gastric cancer cells with high GSDME expression. The stomach cancer cell lines that had been treated with 5-fluorouracil (5-FU) looked to go into pyroptosis .

Pyroptosis and breast cancer

GSDMB overexpression was linked to tumor growth in breast malignancies, and overexpression predicted a poor response to HER-2 targeted treatment [176]. This suggests that GSDMB could be a new tumor prognostic marker. Furthermore, high GSDMC levels have been linked to a poor prognosis in breast cancer patients . Antibiotics such as doxorubicin, daunorubicin, actinomycin D, and epirubicin have been shown to increase the expression of nuclear PD-L1 and GSDMC and facilitate caspase-8 activation, resulting in pyroptotic death in breast cancer cells . Pizato et al. found that compared with untreated breast cancer cells, caspase-1 was activated,gasdermin D was cleaved,IL-1β secretion was enhanced, and high mobility group protein B1(HMGB1) was secreted in breast cancer cells treated with docosahexaenoic acid (DHA). It is proved that DHA can induce pyroptosis in breast cancer cells [177]. GSDME expression was shown to be low in various malignancies, and low levels of GSDME were also linked to poor breast cancer patient survival [178]. The P2X7 signaling pathway has been linked to cancer [179, 180]. Ivermectin regulates the sensitivity of extracellular ATP and HMGB1 by mediating P2X4/P2X7-gated Pannexin-1 channel, and activates caspase-1 to induce apoptosis and pyroptosis [181].

Pyoptosis has been clarified as an inflammatory and planned mode of cell death, but there are still some questions to be answered, such as what function other members of the gasdermin family play in pyroptosis. Pyroptosis plays an important role in the maintenance of normal physiological function and morphology of tissues. At the same time, it is also involved in the occurrence of severe pathological damage and the development of clinical diseases, especially in tumor.

More and more researches focus on the phenomenon of pyroptosis in tumors, and the current research mainly focuses on the compounds or molecules activating inflammasomes such as NLRP1/3, NLRC4, and AIM2 and promoting cell pyroptosis. They have the potential to become new drugs for treating tumors. However, we do not fully understand the mechanism that these molecules affect tumor cell pyroptosis. Future research towards elucidating the mechanism of pyroptosis will help us improve our understanding of tumor cell pyroptosis and help develop anti-tumor drugs based on pyroptosis.

CHAPTER 2

OBJECTS AND METHODS

2.1 Research material

Object of the study- Through the detection of secreted cytokines, to determine whether there is the occurrence of pyroptosis during infection.

The subject of the study- cytokines, the mechanism of pyroptosis, pathogenesis of *S. suis* infection**.**

2.2 Research methods

Consumables gloves, syringes and needles, petri dishes, cell culture plates, methyl alcohol, 1.5ml Centrifugal tubes, 15ml Centrifugal tubes, Cell freezing tubes, Ethanol, PrimeScript™ RT Master Mix(Takara), cDNA synthesis kit, mouse IL-6 ELISA kit, mouse IL-18 ELISA kit, mouse IL-1β ELISA kit, mouse IL-6 ELISA kit, mouse caspase-1 ELISA kit, BCA Protein Colorimetric Assay Kit, Antibody(IL-18, IL-6, IL-1β and caspase-1).

Equipment 1. Biochemical incubators (Thermo, USA); 2. Q5 PCR meter(ABI, USA); 3. Biosafety cabinets (Thermo, USA); 4. Carbon dioxide incubators(Thermo, USA); 5. Autoclave sterilizers (Hirayama, Japan); 6. Micropipettes (Eppendorf, Germany); 7. Shaker (Zhicheng science and technology, China); 8. Fully automated enzyme markers(Thermo, USA); 9. Ultra-micro spectrophotometer(Biodrop, UK); 10. Fluorescent microscope (Nikon, Japan); 11. Electronic scales(Sartorius, Germany); 12. Electrophoresis instrument (Liuyi , China); 13. Constant temperature incubators(Yiheng, China); 14. Ice machines (Sanyo, Japan); 15. High-speed frozen centrifuges(Thermo,

USA); 16. Ultra-low temperature refrigerators (Thermo, USA); 17. Ultra Pure Water Machine (Thermo, USA);

Chemicals and solvents 1. DMEM medium (Hyclone); 2. Dimethyl Sulfoxide (Merck); 3. 2.5%Typsin-EDTA (Hyclone); 4. Fetal bovine serum (Hyclone); 5. Trizol(Invitrogen); 6. Paraformaldehyde (Merck); 7. Triton X-100 (Sigma); 8. PBS (Hyclone); 9. Primary antibody (Abcam); 10. Fluorescent secondary antibody (Abcam); 11. DAPI (Beyotime); 12. RIPA Lysate(Beyotime); 13. Chloroform (Merck); 14. Isopropyl alcohol(Merck); 15.Tris (Merck); 16. Carbinol(Merck); 17. SDS (Sigma); 18. Ammonium persulfate (Beyotime) ; 19. Acrylamide (Sigma).

2.2.1 The activation, culture and counting of *S. suis 2*

S.suis serotype 2 was originally isolated from Jilin University in China and is currently stored at the Laboratory of Animal Science and Technology College, Henan Institute of Science and Technology.

Any tube of frozen bacteria was taken and melted on ice. 5 mL of bacteria solution was taken and added into 5 ml THB liquid medium. The bacteria suspension was shaken overnight at 37 degrees with 180rpm/min. The plate was lined with a inoculating ring and overnight cultured at 37 ℃ upside down.

Single colonies were placed in liquid THB medium and shaken overnight at 37℃ with 180rpm. 100 μL bacterial solution was added to 5 mL liquid THB medium and shaken for 4 hours for frozen storage. 100 μL bacterial solution was taken from one of the frozen storage tubes and added into 5 mL THB liquid medium, shaken at 37 ℃ and 180rpm/min for 12 hours. Then the bacterial solution is coated and counted.

2.2.2 Bacterial count

Bacterial solution preparation

Streptococcus was removed from the refrigerator at -80℃, the bacterial solution was inserted into 5mLTHB medium with inoculation ring, and the culture was oscillated for 12h. After two times of activation, a single colony was obtained by a continuous streak method. The purified single colony was selected and inoculated in 10mLTHB medium for 12h to obtain streptococcal suspension.

Take 5mL bacterial solution and inject it into 5mL normal saline to make 1:2 diluent. Then draw 5mL diluent and inject 5mL normal saline to make 1:4 diluent. Repeat the above operation until the bacterial suspension is close to clear water, and dilute 8 times to obtain a series of bacterial suspensions with 9 concentration gradients.

Plate count

The bacterial suspension with a series of concentration gradients is compared with the McTurbidimetric tube, and the bacterial suspension with a turbidimetric ratio close to 0.5 McTurbidimetric is counted, that is, the tubulin solution is diluted with equal concentration gradient (0.2mL/1.8mL). Then, 1mL of diluted bacterial suspensions of 10-5, 10-6, 10-7 and 10-8 were absorbed and added into the sterile plate respectively. At the same time, the AGAR medium cooled to 45℃ -48 ℃ was poured into the plate and cultured at 37℃for 48h. The number of colonies was counted with a plate counter. The number of colonies on each plate was recorded, and plates with a colony number between 30 and 300 were selected as the standard for measuring the total number of colonies, and the average number of colonies growing on plates with the same dilution was calculated. For each dilution, three plates were used to calculate the

average.

Determination of absorbance value

A series of bacterial suspensions with different concentration gradients were obtained from the above-mentioned dilution, and OD values at different wavelengths (450, 570, 600, 650nm) were determined by 721 visible spectrophotometer, and the measurements were repeated for 3 times.

Establishment of standard curve

The OD values measured at different wavelengths were taken as the horizontal coordinate, and the number of bacteria obtained by plate count (×106CFU/mL) was taken as the vertical coordinate, and the standard curve of the correlation linear relationship between the number of standard streptococcus bacteria and OD values was established.

Application of standard curves

Two tubes of streptococcal bacterial fluid (bacterial fluid 1 and bacterial fluid 2) with different concentrations were taken for plate counting and OD values were determined. For specific operation methods, refer to «1.2.2» and «1.2.3». OD values obtained from measurement were added into the established standard curve regression equation, and the calculated results were compared with the actual plate counting results for verification to calculate the degree of fit.

2.2.3 The preparation, identification and culture of brain microvascular endothelial cells

The preparation of brain microvascular endothelial cells

The mice were sacrificed by neck removal, and then sterilized in 75 % alcohol for

5 minutes. The cranial cavity was opened with ophthalmic forceps, and the brain was aseptically removed. The brain was placed in a glass Petri dish filled with cold D-Hank solution to remove the cerebellar tissue. After that, the diencephalon, hippocampus and brain white matter were removed, and the cerebral cortex was retained. Then, the brain was placed in a new glass culture dish with cold D-Hank solution. After rinsed with D-Hank solution for 3 times, an appropriate amount of Dulbecco's modified eagle medium (DMEM) was added, and it was cut into meat slime with ophthalmic scissors. 4mL of 20 % BSA was added into the meat slime and homogenize it 4 times. The homogenate was centrifuged with 1000r/min for 10 minutes, and the supernatant was collected and centrifuged again. The precipitate after two centrifugations was mixed, and 8 mL of 0.1 % type II collagenase was added and digested at 37°Cfor 30 min, centrifuged (1000 r/min,10 minutes), and the supernatant was discarded to obtain the microvascular segment precipitate.

Primary culture of brain microvascular endothelial cells

Add 4mL of 0.1% gelatin into the culture flask, place it at room temperature for 1~2h, then suck out the gelatin, and add the isolated microvascular segment into the culture flask. At the same time, DMEM complete medium (containing 20 % FBS, ECGF15μg/mL, Penicillium 100 U /mL, streptomycin 100 ug/L, L-glutamine 2mmol/L) was added, mixed, and placed in a 37 °C, 5 % CO2 incubator for static culture. After 24 hours, puromycin was added to the final concentration of 2 mg/L, retained for 60 h, and then replaced with conventional DMEM medium. Every three days after that. When the cell density reaches 90%, it can be passed.

Subculture of brain microvascular endothelial cells

The medium was sucked out and the cells were washed twice with PBS preheated at 37°C, and 1ml trypsin-EDTA solution was added to digest for 2~5 minutes. When most of the cells contracted and turned into a garden, and the intercellular connection relaxed, sufficient complete medium was added immediately to terminate digestion, and the cells were gently blown off with a pipette, mixed, and centrifuged to collect cell precipitation. The cells were suspended by adding an appropriate amount of complete medium, mixed, and seeded into cell culture flasks at a passage ratio of 1:2. The cells were incubated in a 5%CO2 incubator at 37°C.

The identification of brain microvascular endothelial cells

The cells were sub-cultured on confocal cell culture plates. When the cells grew to 80%~ 90%, the medium was sucked out and the plates were rinsed three times gently with 37°C preheated PBS. Then pre-cooled 95%ethanol was added and the samples were fixed at 20°Cfor 20 minutes. Ethanol was sucked out, and the plates were rinsed with PBS for 3 times with 3 minutes each time. 1 mL factor VIII antibody for mouse was added into the culture wells, and the samples were incubated at 37°C for 4 hours. The plates were rinsed with PBS three times with 3 minutes each time. 1 mL rabbit anti-mouse antibody with FITC label were added and incubated for 2 hrs. The samples were rinsed three times with 3 minutes each time. The plates were rinsed with deionized water and observed and photographed under a confocal laser microscope.

Determination of brain microvascular endothelial cells growth curve

Microvascular endothelial cells growing in monolayer were selected to make cell suspension, and 1×103 cells per well were inoculated in 96 well plates with 100 μL per

well. There were 7 groups from day 1 to day 7 with 3 replicates in each group. Blank withered group only add culture medium without inoculation of cells. The cells were cultured at 37°C and 5 % CO2.The original medium was sucked out on day1, day 2, day 3, day 4, day 5, day 6 and day 7 and 180 μL of DMEM and 20 μL of MTT were added to each well at the same time. The culture was continued for 4 hours. Supernatant was sucked out and 150μL Dimethyl Sulphoxide (DMSO) was added to each well to dissolve the crystals. Cells were shaken on room temperature for 10 minutes and the absorbance value (A value) of each well was measured at 492 nm with a microplate reader. The absorbance value can reflect the number of live cells per day.

2.2.4 Screening of optimal multiplicity of infection and duration

Preparation of bacterial solution

S. suis 2 was taken out from -80 refrigerator and 100 microliters was inoculated in THB liquid medium and shook in a shaker at 37 degrees overnight. Bacteria was counted by plate count method. Then 10 ml of bacterial liquid were added into 10 centrifuge tubes separately and tubes were centrifuged at 3000 rpm for 5 minutes. The supernatants were discarded and 10 ml sterile PBS were added into tubes separately and suspend bacteria. The bacteria liquid were centrifuged at 3000 rpm for 5 minutes. The supernatants were discarded and 10 ml DMEM culture media without fetal bovine serum (FBS) were added into tubes and suspend them.

Cell culture

When brain microvascular endothelial cells are in logarithmic growth phase, cells were plated in a 6-well plate at a density of 1.0×106 cells/well and cultured at 37°C, 5% CO2 for 12 hours. When the cell density reaches 80%~90%, *S.suis2* were added into

well respectively with 1.4μL, 14μL, 140μL and 280μL, the corresponding multiplicity of infection (MOI) are 1, 10, 100 and 200 respectively. The plates were cultured continuously in the incubator.

Detection of qPCR

According to the different culture time, the medium were discarded from 6-well plate respectively after 6hs, 9hs, 12hs, 18hs and 24hrs. The plates were washed twice with PBS and 1 mL of Trizol was added into each well to extract total cell RNA according to the instructions. Nanodrop 2000 was used to detect the concentration and purity of RNA to make sure the values of OD260/OD280 are between 1.8~2.4 and OD260/OD230 are 1.5~2.4 and the concentration is greater than 100ng/μL.

The total RNA was reverse transcribed to obtain cDNA using a reverse transcription kit, and the one-step method was used to detect IL-18 mRNA, IL-1beta mRNA, IL-6 mRNA, and IL-10 mRNA(table x).

The upstream primer sequence of IL-18 is GACTCTTGCGTCAACTTCAAGG and the downstream primer sequence of IL-18 is CAGGCTGTCTTTTGTCAACGA. The length of the target fragment is 169bp. The upstream primer sequence of IL-1beta is TTCAGGCAGGCAGTATCACTC and the downstream primer sequence of IL-1beta is GAAGGTCCACGGGAAAGACAC. The length of the target fragment is 75bp.

The upstream primer sequence of IL-6 is AGTTGCCTTCTTGGGACTGA and the downstream primer sequence of IL-6 is TCCACGATTTCCCAGAGAAC. The target fragment length is 159bp. The upstream primer sequence of IL-10 is CTGCTATGCTGCCTGCTCTTACTG and the downstream primer sequence of IL-10 is ATGTGGCTCTGGCCGACTGG. The target fragment length is 104bp. The upstream

primer sequence of GAPDH is AGGTCGGTGTGAACGGATTTG and the downstream primer sequence is TGTAGACCATGTAGTTGAGGTCA. The length of the target fragment is 123bp.

qPCR was performed using SYBR@ Premix Ex Taq™ Ⅱ（Takara Bio Inc. Dalian, RR820A）by Q5 detection system(ABI). All reaction was carried out in a 20μL reaction volume (Table 2.1). Amplification conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C (Table 2.2). Each sample was set with 3 parallel replicate wells. Using GAPDH as the internal reference gene, the relative expression of the target gene was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Table 2.1

Reaction System of qPCR

Reagents	**Volume(20 μ L)**
TB Green Premix	10 μ L
Forward Primer	1 μ L
Rerverse Primer	1μL
cdna	1μL
ddH_2O	7μL

Table 2.2

The Steps of qPCR

Steps	Temperature	Time
predenaturation	95℃	30 min
Denaturation	95℃	30s } 40 cycles
annealing	60℃	34s
Solution curve analysis	95℃	15s
	60℃	1min
	95℃	15s

2.2.5 Bacterial infected cell

Brain microvascular endothelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO2 incubator. When the cells in the culture bottle occupied 75% of the bottom of the bottle, the cells were digested with pancreatic enzyme and spread on the 6-well plate. When the cells in the 6-well plate reached 80% of the bottom of the hole, brain microvascular endothelial cells were infected with *S. suis 2*.

Brain microvascular endothelial cells were divided into 4 groups: control, *S. suis 2* infection, LPS+ATP infection, *S. suis 2*+Ac-YVAD-CMK infection.

Cells were pre-incubated with Ac-YVAD-CMK for 1h. Then *S. suis 2* group, CMK group were treated with *S. suis 2* at a multiplicity of infection (MOI) of 100 for 12 h. Control group was treated with PBS. Brain microvascular endothelial cells 3 were pretreated with LPS for 4 h and then ATP was added for 8 h.

2.2.6 Total RNA extration and reverse transcription

S. suis 2 was taken out from -80 refrigerator and 100 microliters was inoculated in THB liquid medium and shook in a shaker at 37 degrees overnight. Bacteria was counted by plate count method. Then 10 ml of bacterial liquid were added into 10 centrifuge tubes separately and tubes were centrifuged at 3000 rpm for 5 minutes. The supernatants were discarded and 10 ml sterile PBS were added into tubes separately and suspend bacteria. The bacteria liquid were centrifuged at 3000 rpm for 5 minutes. The supernatants were discarded and 10 ml DMEM culture media without fetal bovine serum (FBS) were added into tubes and suspend them. When brain microvascular endothelial cells are in logarithmic growth phase, cells were plated in a 6-well plate at a density of 1.0×106 cells/well and cultured at 37°C, 5% CO2 for 12 hours. When the cell density reaches 80%~90%, *S.suis2* were added into well respectively with 1.4μL, 14μL, 140μL and 280μL, the corresponding multiplicity of infection (MOI) are 1, 10, 100 and 200 respectively. The plates were cultured continuously in the incubator.

2.2.7 QPCR detection

According to the different culture time, the medium were discarded from 6-well plate respectively after 6hs, 9hs, 12hs, 18hs and 24hrs. The plates were washed twice with PBS and 1 mL of Trizol was added into each well to extract total cell RNA according to the instructions. Nanodrop 2000 was used to detect the concentration and purity of RNA to make sure the values of OD260/OD280 are between 1.8~2.4 and OD260/OD230 are 1.5~2.4 and the concentration is greater than 100ng/μL.

The total RNA was reverse transcribed to obtain cDNA using a reverse transcription kit, and the one-step method was used to detect IL-18 mRNA, IL-1beta

mRNA, IL-6 mRNA, and IL-10 mRNA(table x).

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The upstream primer sequence of IL-6 is AGTTGCCTTCTTGGGACTGA and the downstream primer sequence of IL-6 is TCCACGATTTCCCAGAGAAC. The target fragment length is 159bp. The upstream primer sequence of IL-10 is CTGCTATGCTGCCTGCTCTTACTG and the downstream primer sequence of IL-10 is ATGTGGCTCTGGCCGACTGG. The target fragment length is 104bp. The upstream primer sequence of GAPDH is AGGTCGGTGTGAACGGATTTG and the downstream primer sequence is TGTAGACCATGTAGTTGAGGTCA. The length of the target fragment is 123bp.

qPCR was performed using SYBR@ Premix Ex Taq™ Ⅱ（Takara Bio Inc. Dalian, RR820A）by Q5 detection system(ABI). All reaction was carried out in a 20μL reaction volume (Table 1). Amplification conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C (Table 2). Each sample was set with 3 parallel replicate wells. Using GAPDH as the internal reference gene, the relative expression of the target gene was calculated according to the $2^{-\Delta\Delta Ct}$ method.

2.2.8 Western blot detection

Extraction and concentration determination of cellular proteins

The cells in the 6-well plate were collected and centrifuged at 2000rpm, 4℃ for

5min. The cell precipitate was collected and 250 μL of RIPA lysis solution was added to each centrifuge tube, placed on ice for 30min, then centrifuged at 12000 rpm, 4℃ for 10min. The supernatant was collected, which was the total protein solution. The protein concentration was determined according to the method in the BCA kit instructions.

SDS-PAGE electrophoresis

Protein was uploaded in a volume of 20 μg and 12% of the separation gel was electrophoresed with 5% of the concentrate. SDS-PAGE gel was transferred onto PVDF membranes by a semi-dry transfer machine. The PVDF membrane was placed in 5% skimmed milk powder prepared in TBST at 4 degrees overnight at 50 rpm for blocking. 1:1000 primary antibody was applied for 2h at 37 degrees and washed 3 times in TBST. 1:5000 secondary antibody with enzyme label was added at 37 degrees for 1h and washed 3 times in TBST. Colour development with ECL luminescent solution. Protein bands were analysed by Image J software for grey scale values

2.2.9 ELISA detection

ELISA is a common enzyme immunoassay technique, which is based on the adsorption of a known antigen or antibody to the surface of the solid phase carrier, the antigen-antibody reaction is carried out on the solid phase surface. The method has the advantages of simple operation, strong specificity, high sensitivity and low price. IL-6, IL-10, IL-18, IL-1beta, caspase-1 were detected by corresponding ELISA kits.

After cultured with a 6-well plate, the cell supernatant was absorbed into a sterile centrifuge tube. Centrifuge tube was centrifuged with at 2000 rpm for 5 min. Standard wells, sample wells and blank wells were set up. Standard wells are filled with 50 μL standard solution of different concentrations. 50 μL sample solution were added into

sample wells. 100 μL of enzyme-labeled reagent was transferred into each well, except the blank well. After the plate was sealed with the sealing plate film, it was placed in a temperature chamber at 37℃ for 1h. The liquid was discarded, and the wells were washed 5 times. Substrate A and B were mixed in a ratio of 1:1 and all wells were filled with 100ul of substrate solution and incubated at 37 °C for 15 minutes. 50μL termination solution were added into all wells and the absorbance value were read on spectrophotometer.

2.2.10 Lactate dehydrogenase (LDH) detection

LDH release detection was performed according to lactate dehydrogenase cytotoxicity detection kit. The procedures were as follows: (1) Total LDH release of brain microvascular endothelial cells: add LDH release agent, blow and mix repeatedly for several times, incubate in a cell incubator for 1h, and absorb 120 μL of cell superserum of each group; (2) Preparation of working liquid for LDH determination: Add 20 μL lactic acid solution to each hole of 96-well plate, dilute 20 μL to 1X 1NT solution and 20 μL enzyme solution, the total volume is 60 μL; (3) Incubation: LDH assay working solution was added into the supernatant of each group of cells absorbed, and incubated for 30min in the dark; (4) Calculation: the enzymoleter is set at 490nm wavelength to measure the absorbance, and calculated according to the absorbance value, LDH/% = (absorbance value of the treated sample - absorbance value of the blank control hole)/(absorbance value of the LDH full release hole - absorbance value of the blank control hole).

2.2.11 Electron microscopy observation

Transmission electron microscopy (TEM) projects an accelerated and

concentrated beam of electrons onto a very thin sample. The electrons collide with the atoms in the sample and change direction, resulting in solid Angle scattering. The size of the scattering Angle is related to the density and thickness of the sample, so different images can be formed. Usually, the resolution of the transmission electron microscope is 0.1~ 0.2nm, and the magnification is tens of thousands to millions of times, which is used to observe the ultrastructure, that is, the structure that is less than 0.2 microns and can not be seen under the optical microscope, also known as "submicroscopic structure". For example, transmission electron microscopy was used to observe the three-layer structure of the biofilm and the morphological structure of various organelles in the cell.

Six cells in 6-well plates were collected in a centrifuge tube at 2000 rpm for 10 min. The supernatant was discarded, and the cell precipitation was fixed with 2.5% glutaraldehyde. The sample was rinsed with 0.1mol/L phosphate buffer for 3 times, and then fixed with l % osmic acid. The samples were dehydrated with 50%, 70%, 80%, 90% (twice) and 100% (twice) acetone, respectively, for 10 min/time. The samples were impregnated with acetone and embedding agent at the ratio of 1:1, 1:2 and 1:3, respectively, for 60min/ time. After the embedding agent was soaked for 3h, the embedded sample was polymerized in a 37℃ oven for 12h and 60℃ for 48h respectively. Then the ultra-thin slices with a thickness of 60nm were prepared. After staining with uranyl acetate and lead citrate, the samples were observed by electron microscope.

CHAPTER 3

RESULTS OF OWN RESEARCH

3.1 Bacterial count

The optical density values of streptococcal bacterial solution with different dilution at 4 wavelengths and the plate count results are shown in Table 3.1

Table 3.1

The number of *S. suis 2* in cells

Dilution gradien	Absorbance values at different wavelengths				Plate counting results (× 10^6 CFU/mL)
	450nm	570nm	600nm	650nm	
1	1.154	1.138	1.147	1.324	4736.32
1:2	1.128	1.109	0.964	1.055	2386.16
1:4	0.954	0.886	0.768	0.796	1184.08
1:8	0.628	0.513	0.492	0.413	592.04
1:16	0.376	0.225	0.241	0.209	296.02
1:32	0.198	0.168	0.137	0.143	148.01
1:64	0.116	0.082	0.072	0.059	74.01
1:128	0.041	0.046	0.041	0.032	37.05
1:256	0.023	0.021	0.02	0.019	17.02

At the wavelength of 450nm, when the absorbance value is between 0.023 and 0.628, there is a good linear relationship between the bacterial number of streptococcus suis and the absorbance value, and the regression equation established is y= 1461.7x-19.135 (R2=0.9913) (Fig. 3.1).

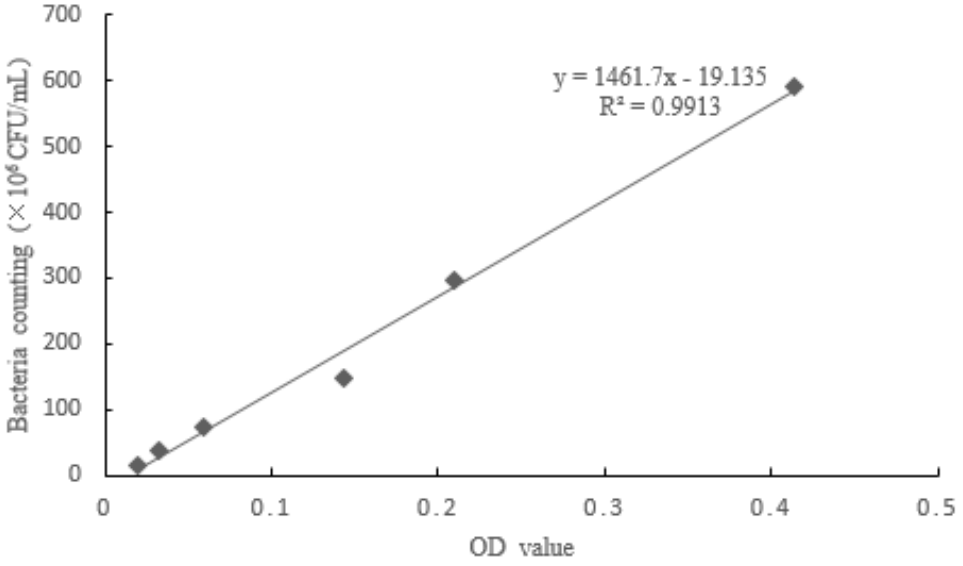

Fig. 3.1 Standard curve of the absorbance value of *Streptococcus suis* and the number of bacteria at the wavelength of 450 nm

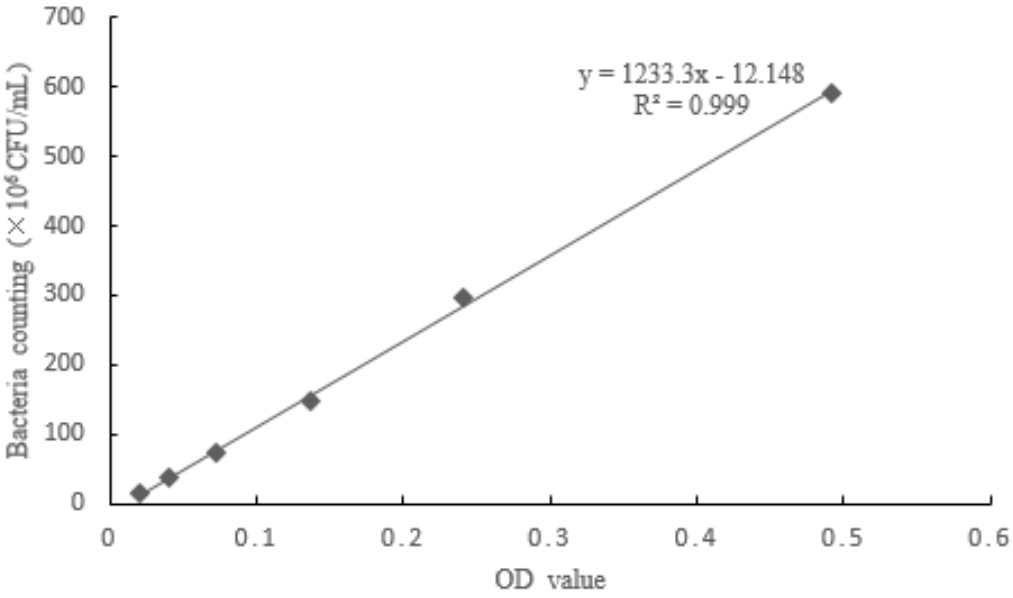

Fig. 3.2 Standard curve of the absorbance value of *Streptococcus suis* and the number of bacteria at the wavelength of 570 nm

At the wavelength of 570nm, when the absorbance value is between 0.021 and 0.513, there is a good linear relationship between the bacterial number of streptococcus

suis and the absorbance value, and the regression equation established is y= 1233.3x-12.148 (R^2=0.999) (Fig. 3.2).

At the wavelength of 600nm, when the absorbance value is between 0.02 and 0.492, there is a good linear relationship between the bacterial number of streptococcus suis and the absorbance value, and the regression equation established is y= 1233.3x-12.148 (R^2=0.999) (Fig. 3.3).

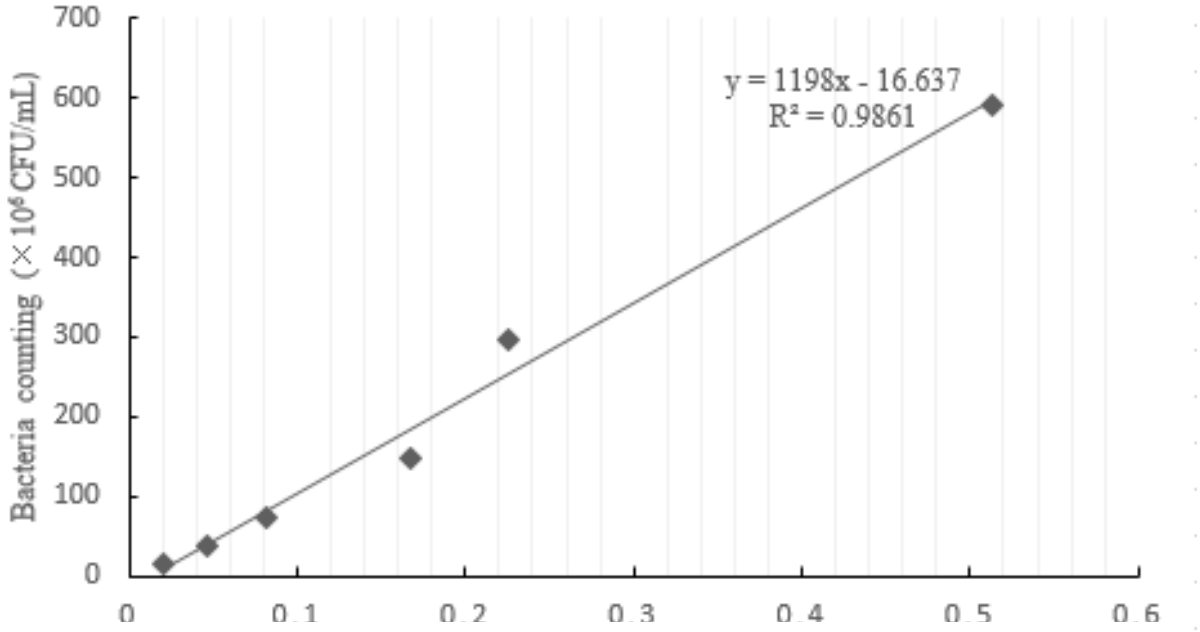

Fig. 3.3 Standard curve of the absorbance value of *Streptococcus suis* and the number of bacteria at the wavelength of 600 nm

At the wavelength of 650nm, when the absorbance value is between 0.019 and 0.413, there is a good linear relationship between the bacterial number of streptococcus suis and the absorbance value, and the regression equation established is y= 935.6x-21.484 (R^2=0.9877) (Fig. 3.4).

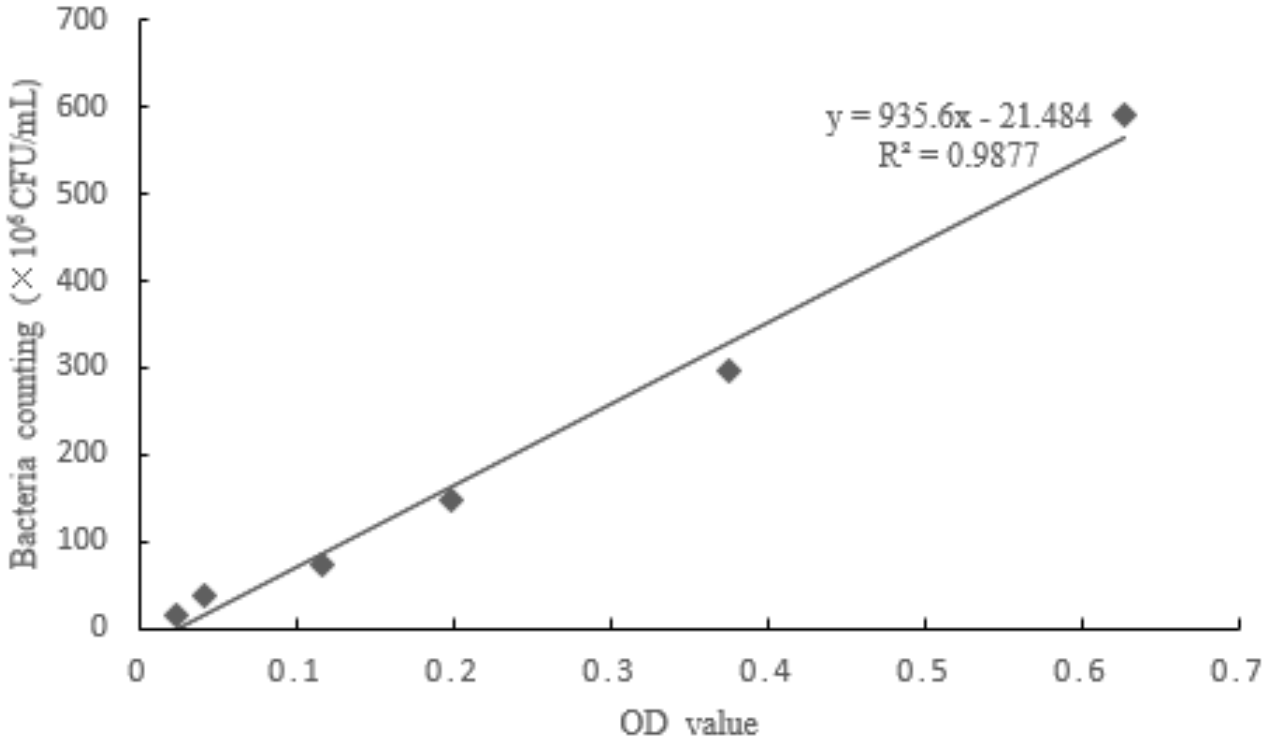

Fig. 3.4 Standard curve of the absorbance value of *Streptococcus suis* and the number of bacteria at the wavelength of 650 nm

At 570nm wavelength, plate counting and optical density value measurement were carried out on two tubes of streptococcal bacteria solution (bacteria solution 1 and 2) with different concentrations respectively, and optical density values measured were added into the established standard curve regression equation.

The calculated results were compared with the actual plate counting results, and the degree of fitting was calculated, as shown in Table 3.2. At 570 nm wavelength, the calculated values of the bacterial solution and the calculated values of the curve fit 96 %.

Table 3.2

Application results of standard curve

Bacteria solution	OD value	Counting results（× 10^6 CFU/mL）	
		Plate counting results	312.28
		Curve caculation results	323.31
		Coincidence	96.5%
Bacteria solution 1	0.272		
		Plate counting results	604.23
		Curve caculation results	621.77
		Coincidence	97.2%
Bacteria solution 2	0.514		

3.2 The preparation, identification and culture of brain microvascular endothelial cells

Under the inverted microscope, it can be observed that the newly seeded micro-vessels are bead-like, with different lengths, smooth walls and strong reflections. After 12 hours of culture, most of the microvascular segments were completely adherent, and individual cells grew out of the microvascular segments. After 24 hours of culture, a large number of cells grew out of the microvascular segment, in the shape of a short spindle or polygon. The cells began to spread and grew around 6 to 7 days, forming a dense monolayer of cells and showing a typical endothelial cell shape (Fig. 3.5).

Cells are mostly short fusiform or polygonal.

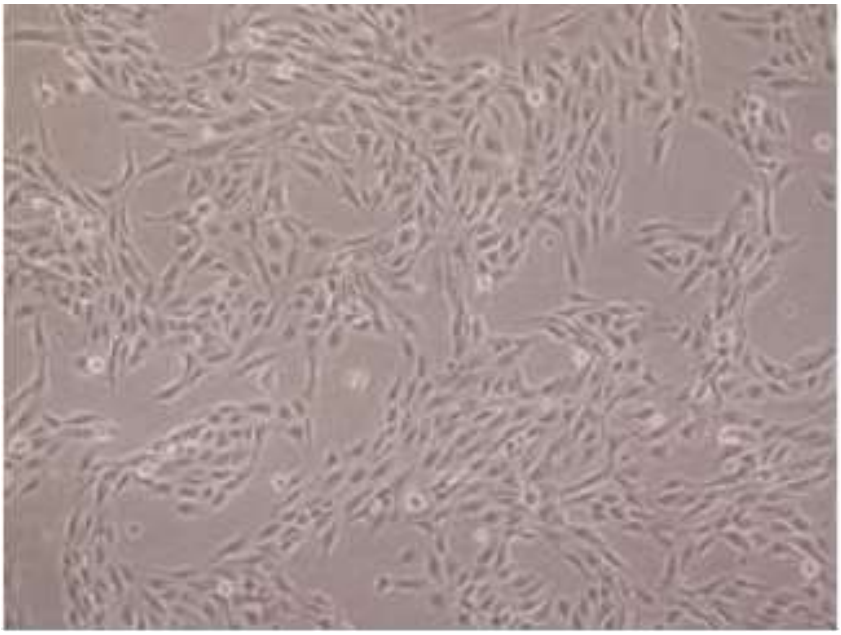

Fig. 3.5 Morphology of BMECs on day 7 (×100)

Immunofluorescence identification of factor VII-related antigens in brain microvascular endothelial cells. After 6~7 days of culture, brain microvascular endothelial cells were stained with factor VIII-related antigen immunofluorescence, and observed under an inverted fluorescence microscope. The positive expression of factor VIII-related antigen (green fluorescence) in brain microvascular endothelial cells was observed (Fig. 3.6). DAPI-stained nuclei showed blue fluorescence. It can be seen that the experimental method adopted in this study successfully cultivated brain microvascular endothelial cells.

The growth curve of brain microvascular endothelial cells was determined by thiazolyl blue (MTT) colorimetric method. It can be seen that on the 1st to 2nd day, the cell growth was in the incubation period, and it entered the logarithmic growth phase from the 3rd day (figure 3.7). Compared with the A value of the first day, there was a significant difference between the day's A value and the first day (all $P<0.05$), and the growth peak was on the 6th to 8th day (compared with the 5th day, all $P<0.05$).

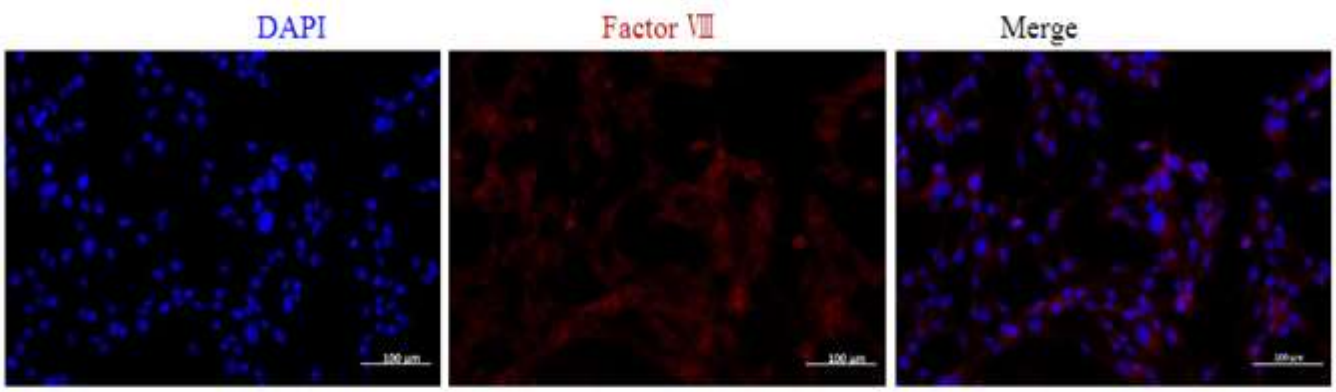

Fig. 3.6 BMECs stained positive for Factor VIII-related antigen（×100）

The growth curve of brain microvascular endothelial cells was determined by thiazolyl blue (MTT) colorimetric method. It can be seen that on the 1st to 2nd day, the cell growth was in the incubation period, and it entered the logarithmic growth phase from the 3rd day (Fig. 3.7). Compared with the A value of the first day, there was a significant difference between the day's A value and the first day (all $P<0.05$), and the growth peak was on the 6th to 8th day (compared with the 5th day, all $P<0.05$).

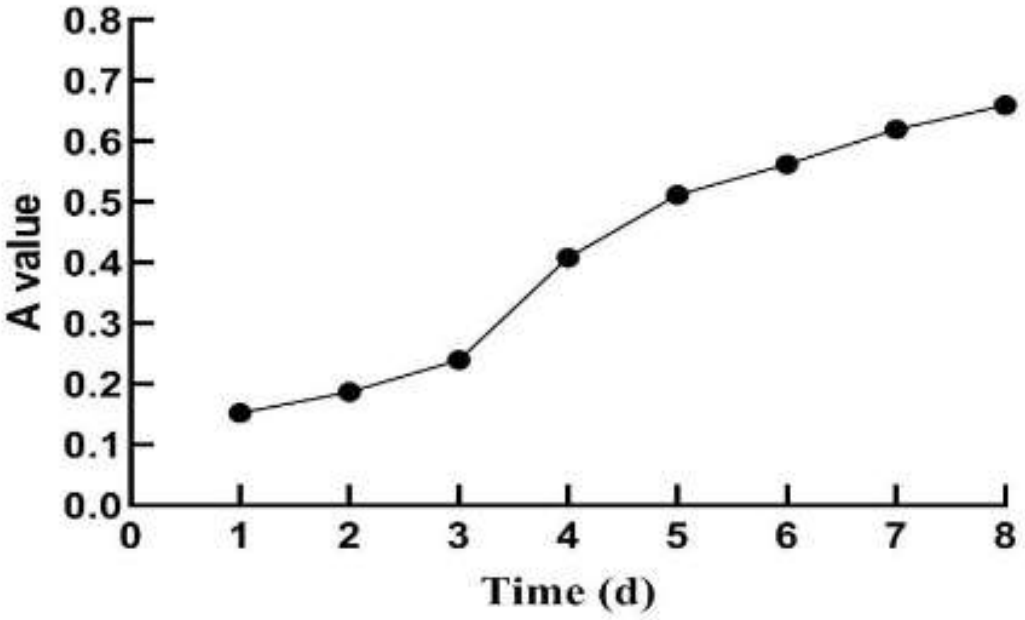

Fig. 3.7 The growth curve of BMECs

3.3 Screening of optimal multiplicity of infection

The relative expression of cytokines in each MOI group are all higher than control group with different infection time.When MOI is at 1, the relative expression of cytokines in each group reach a peak at 18hrs after infection (Fig. 3.8). When MOI is at 10, the relative expression of cytokines in each group reach a peak at 12hrs after infection (Fig. 3.9). When MOI is at 100, the relative expression of cytokines reach a peak at 12hrs after infection (Fig. 3.10). When MOI is at 200, the relative expressions of each cytokine reach a peak at 6hrs after infection (Fig. 3.11).

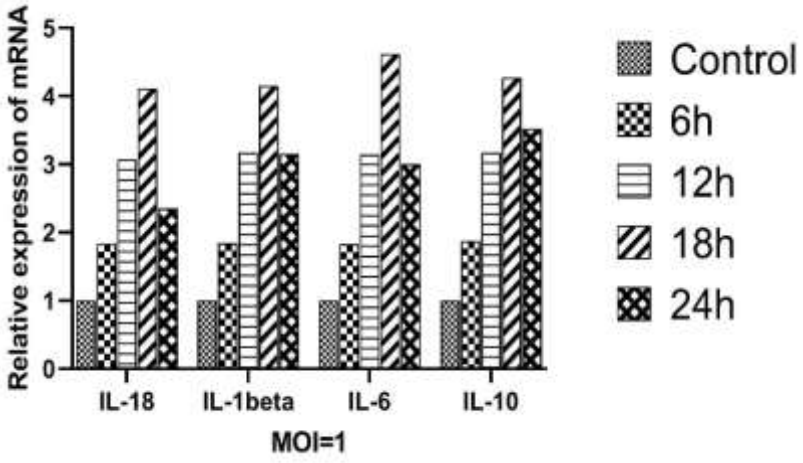

Fig. 3.8 Relative expression of mRNA when MOI=1

When MOI=1, the mRNA expression of IL-18, IL-1beta, IL-6 and IL-10 increased with the extension of bacterial infection time. Compared with the control group, the mRNA expression of cytokines increased significantly at 6 hours after infection. Compared with 6 hours after infection, the expression of cytokine mRNA increased significantly at 12 hours after infection. Similarly, cytokine mRNA expression increased significantly at 18 hours after infection compared with 12 hours after infection. But as the infection lasted longer, the cells began to die in large numbers.

Therefore, after 24 hours of infection, the expression of several cytokines is significantly reduced. The lowest mRNA expression of each cytokine was 6 hours after infection, and the highest mrna expression was 18 hours after infection.

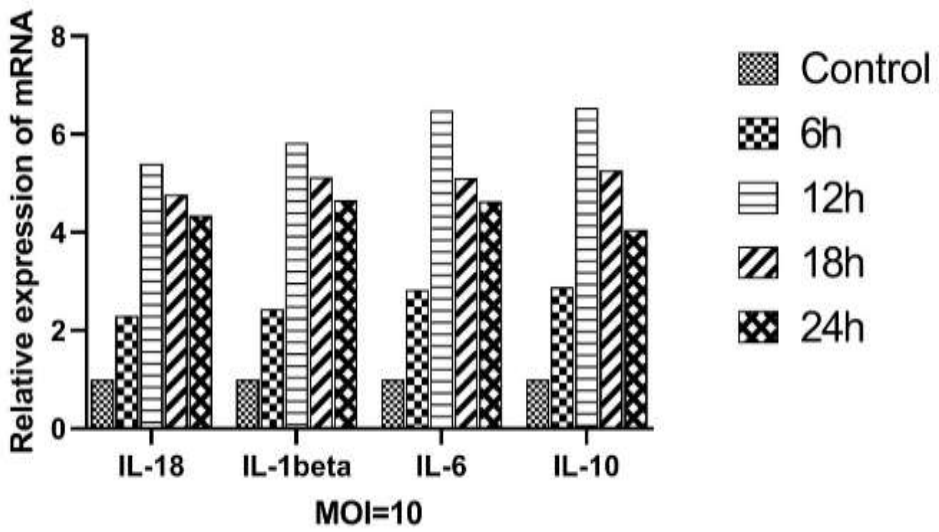

Fig. 3.9 Relative expression of mRNA when MOI=10

When MOI=10, the mRNA expression of IL-18, IL-1beta, IL-6 and IL-10 increased with the extension of bacterial infection time. Compared with the control group, the mRNA expression of cytokines increased significantly at 6 hours after infection. Compared with 6 hours after infection, the expression of cytokine mRNA increased significantly at 12 hours after infection. Compared with 12 hours after infection, cytokine mRNA expression began to decrease at 18 hours after infection, and the difference was significant. By 24 hours after infection, a large number of cells had died, so the expression of several cytokines continued to decrease after 24 hours of infection, and the difference was significant. The lowest mRNA expression of each cytokine was 6 hours after infection, and the highest mRNA expression was 12 hours after infection.

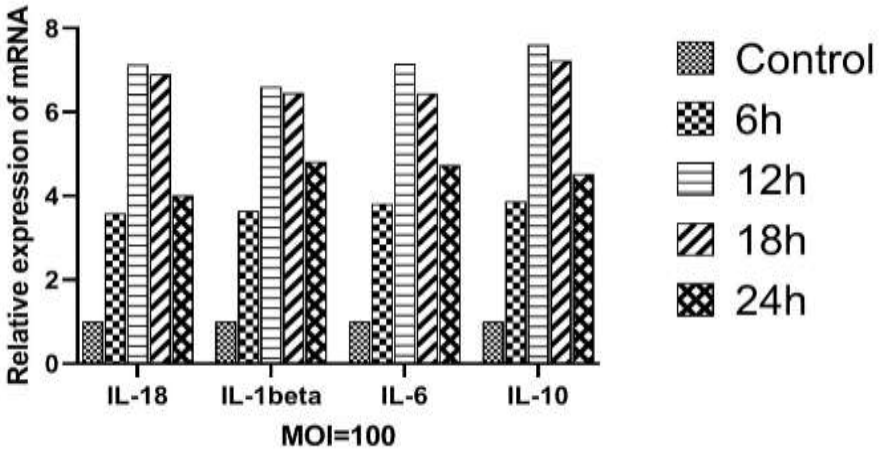

Fig. 3.10 Relative expression of mRNA when MOI=100

When MOI=100, the mRNA expression of IL-18, IL-1beta, IL-6 and IL-10 increased with the extension of bacterial infection time. Compared with the control group, the mRNA expression of cytokines increased significantly at 6 hours after infection. Compared with 6 hours after infection, the mRNA expression of cytokines increased significantly at 12 hours after infection. Compared with 12 hours after infection, cytokine mRNA expression began to decrease slightly at 18 hours after infection, and the difference was significant. By 24 hours after infection, a large number of cells had died, so after 24 hours of infection, the expression of several cytokines was significantly reduced, and the difference was very significant. The lowest mRNA expression of each cytokine was 6 hours after infection, and the highest mrna expression was 12 hours after infection.

When MOI=200, due to the large number of infected bacteria, the mRNA expressions of several cytokines IL-18, IL-1beta, IL-6 and IL-10 increased sharply 6 hours after infection compared with the control group, and the differences were extremely significant.

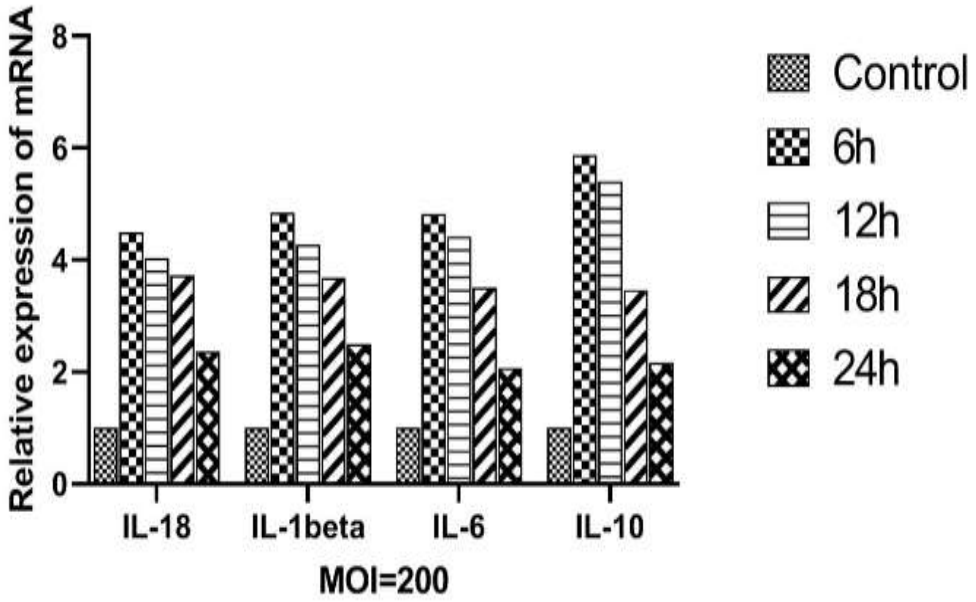

Fig. 3.11 30Relative expression of mRNA when MOI=200

Compared with 6 hours after infection, the cells began to die at 12 hours after infection, and the mRNA expression of several cytokines decreased slightly, the difference was significant. Compared with 12 hours after infection, a large number of cells began to die at 18 hours after infection, and the expression of cytokine mRNA began to decrease significantly, and the difference was extremely significant. At 24 hours after infection, the cells continued to die in large numbers, and the number of surviving cells was very small, so the expression of several cytokines continued to decrease after 24 hours of infection, and the difference was significant. The lowest mRNA expression of each cytokine was 24 hours after infection, and the highest mrna expression was 6 hours after infection.

In addition to the detection of cytokine mrna expression, lactate dehydrogenase tests are used to screen for optimal infection conditions. The percentage of lactate dehydrogenase varies with different multiplicity of infection values. Compared with

multiplicity of infection (1), the lactate dehydrogenase release of multiplicity of infection (10) is greatly increased, similarly, the lactate dehydrogenase release of multiplicity of infection (100) is much higher than that of multiplicity of infection (10), while the lactate dehydrogenase release of multiplicity of infection (200) is not much higher than that of multiplicity of infection (100) (Fig. 3.12).

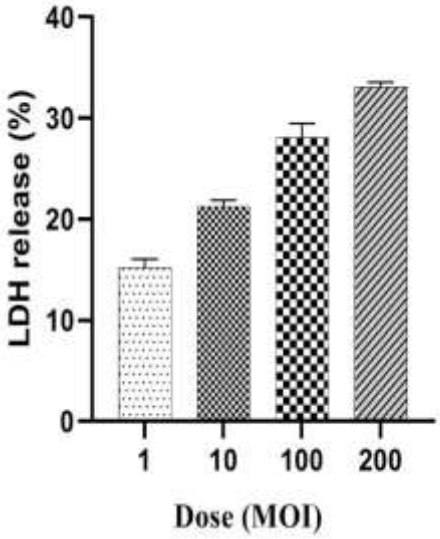

Fig. 3.12 LDH detection with different MOI

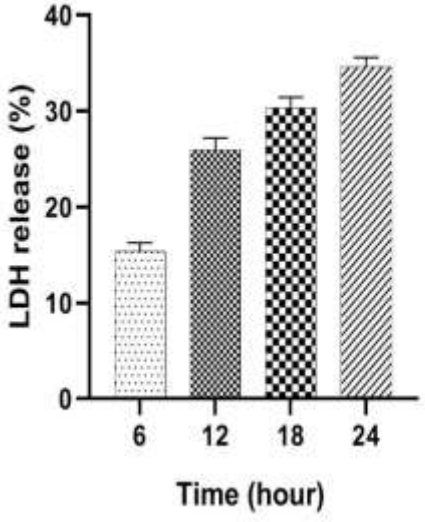

Fig. 3.13 LDH detection with different time

The percentage of lactate dehydrogenase was also different at different time of

infection. With the extension of infection time, lactate dehydrogenase release also gradually increased, but the two are not proportional. Compared with 6 hours of infection, lactate dehydrogenase release increased greatly at 12 hours of infection. Lactate dehydrogenase release increased at 18 h compared with 12 h, but at a slower rate, and LDH release at 24 h did not increase much compared with 18 h (Fig. 3.13).

Based on the mRNA relative expression of each cytokine under different conditions and lactate dehydrogenase detection with different MOI and infection time, when the MOI=100 and the infection time was 12h, the mRNA expression of each cytokine was the highest.

3.4 Cellular total RNA extration and reverse transcriptionp

Extracted total RNA were detected by Nanodrop instrument and the concentration and purity were obtained (Tabl. 3.3).

Table. 3.3

Concentration and purity of RNA

Sample	Repeats	Average	OD260/280	OD260/230
Control	3	895	1.81	2.06
S. suis 2	3	1183	1.82	2.18
LPS+ATP	3	1023.9	1.95	2.09
S. suis 2+CMK	3	1113.3	1.86	2.12

There are several values used to evaluate the quality of nucleic acids. The purine and pyrimidine molecules contained in nucleic acids have conjugated double bonds and

have maximum absorption peaks at 260nm wavelength. Proteins have maximum absorption peaks at 280nm. OD260 reflects the concentration of nucleic acids in the solution, and OD280 reflects the concentration of proteins or amino acids in the solution. The ratio of OD260/280 can reflect the purity of nucleic acid. In theory, the value of OD260/OD280 in the case of pure RNA is 2, and the value of OD260/OD280 in the case of pure DNA is 1.8. OD260/OD280=1.9~2.0 RNA, the purity is already very high.

OD230 was used to evaluate the presence of some contaminants in the sample, such as carbohydrates, peptides, phenols, etc., and the OD260 /OD 230 ratio of purest nucleic acids was greater than 2.0. The OD260/OD280 of RNA extracted from several groups of cells was between 1.8-2.0, indicating high purity. The values of OD260/OD230 are both greater than 2, indicating that the content of pollutants in the extracted RNA is very small.

3.5 QPCR detection

Fluorescence quantitative PCR was first called TaqMan PCR, later also called

Real-Time PCR, is the United States Perkin Elmer company in 1995 developed a new nucleic acid quantitative technology. The quantitative function of this technique is realized by adding fluorescent labeled probe or corresponding fluorescent dye on the basis of conventional PCR. The principle is that with the PCR reaction, the PCR reaction products continue to accumulate, and the fluorescence signal intensity also increases proportionally. After each cycle, a fluorescence intensity signal is collected, so that we can monitor the change of product quantity through the fluorescence intensity change, thus obtaining a fluorescence amplification diagram.

SYBR Green I is the most commonly used DNA-binding dye for fluorescence quantitative PCR, binding non-specifically to double-stranded DNA. In the free state, SYBR Green I fluoresces weakly, but once bound to double-stranded DNA, its fluorescence increases by a factor of 1,000. Therefore, the total fluorescence signal from a reaction is proportional to the amount of double-stranded DNA in the outgoing line, and will increase with the increase of the amplified product.

Fluorescent quantitative PCR is mainly used in the following aspects: nucleic acid quantitative analysis, gene expression differential analysis, SNP detection and methylation detection. In order to confirm the occurrence of pyroptosis, we detect the mRNA levels of pyroptosis-related genes, such as Caspase-1, IL-1β, IL-18, IL-6, IL-10 and INF-γ. The results show that, compared to control group, the mRNA levels of pyroptosis-related genes significantly increase in LPS+ATP group and *S. suis 2* group, respectively ($P < 0.01$). Compared with SS2 group, the mRNA levels of pyroptosis-related genes significantly decrease in SS2+CMK group ($P < 0.01$) (Fig. 3.14, 3.15).

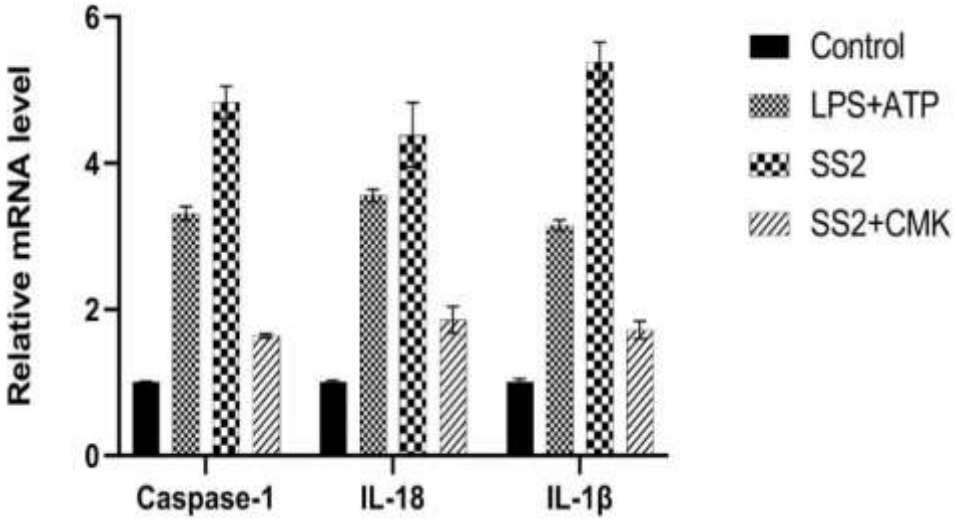

Fig. 3.14 The mRNA levels of caspase-1,IL-18 and IL-1β in different groups

When MOI=100 and streptococcus suis infected brain microvascular endothelial cells 12 hours later, cells were collected and RNA was extracted for QPCR detection of mRNA expression levels of caspase-1, IL-18 and IL-1β. For caspase-1, compared to control group, the mRNA levels of caspase-1 significantly increase in LPS+ATP group and *S. suis 2* group, respectively ($P<0.01$), and the difference was extremely significant. Compared with *S. suis 2* group, the mRNA levels of caspase-1 genes significantly decrease in *S. suis 2*+CMK group ($P<0.01$) , and the difference was extremely significant. For IL-18, compared to control group, the mRNA levels of IL-18 significantly increase in LPS+ATP group and *S. suis 2* group, respectively ($P<0.01$) and the difference was extremely significant. Compared with *S. suis 2* group, the mRNA levels of caspase-1 genes significantly decrease in *S. suis 2*+CMK group, and the difference was extremely significant ($P<0.01$). For IL-1β, compared to control group, the mRNA levels of caspase-1 significantly increase in LPS+ATP group and *S. suis 2* group, respectively, and the difference was extremely significant ($P<0.01$). Compared with *S. suis 2* group, the mRNA levels of IL-1β genes significantly decrease in *S. suis 2*+CMK group, and the difference was extremely significant ($P<0.01$).

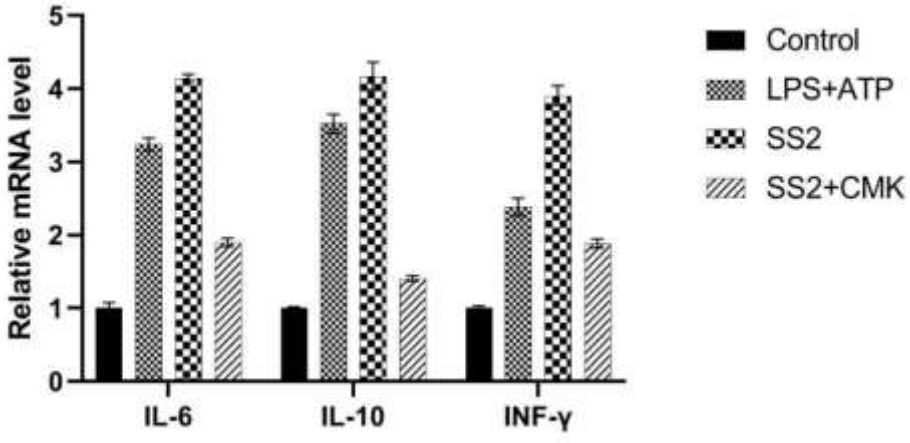

Fig. 3.15 The mRNA levels of IL-10,IL-6 and INF-γ in different groups

When MOI=100 and streptococcus infected rat brain microvascular endothelial cells 12 hours later, cells were collected and RNA was extracted for QPCR detection of mRNA expression levels of IL-6, IL-10 and INF-γ. For IL-6, compared to control group, the mRNA levels of IL-6 significantly increase in LPS+ATP group and *S. suis 2* group respectively, and the difference was extremely significant ($P<0.01$). Compared with *S. suis 2* group, the mRNA levels of IL-6 genes significantly decrease in *S. suis 2* +CMK group, and the difference was extremely significant ($P<0.01$). For IL-10, compared to control group, the mRNA levels of IL-10 significantly increase in LPS+ATP group and *S. suis 2* group respectively, and the difference was extremely significant ($P<0.01$). Compared with *S. suis 2* group, the mRNA levels of IL-10 genes significantly decrease in *S. suis 2* +CMK group, and the difference was extremely significant ($P<0.01$). For INF-γ, compared to control group, the mRNA levels of caspase-1 significantly increase in LPS+ATP group and SS2 group respectively, and the difference was extremely significant ($P<0.01$). Compared with *S. suis 2* group, the mRNA levels of INF-γ genes significantly decrease in *S. suis 2*+CMK group, and the difference was extremely significant ($P<0.01$).

3.6 Western blot detection

Western Blot (WB) is an experimental method commonly used in molecular biology, biochemistry and immunogenetics. It is a protein detection technology that transfers proteins from cells or tissues separated by electrophoresis from the gel to the solid phase support NC membrane or PVDF membrane, and then uses specific antibodies to detect a specific antigen. It has been widely used in the study of gene expression at protein level, antibody activity detection and early diagnosis of diseases.

In the study, WB was used to detect the protein expression of cytokines which are related to pyroptosis, such as IL-18, IL-1β, caspase-1, GSDMD and GSDME. The Image J software was used for quantitative analysis of WB strips. Compared with the control group, the protein expressions of IL-18, IL-1β, caspase-1, GSDMD and GSDME in LPS+ATP group were significantly upregulated and increased by 0.477, 0.088, 0.378, 1.118 and 3.05 times, respectively ($P<0.05$). The protein expressions of IL-18, IL-1β,caspase-1,GSDMD and GSDME in *S. suis 2* group were significantly upregulated and increased by 1.024, 0.066, 0.376, 0.453 and 1.654 times, respectively ($P<0.05$). Compared with *S. suis 2* group, the protein expression in *S. suis 2* + CMK group was significantly decreased by 36.7%, 60.45%，70.25%, 25.74% and 41.33% , respectively ($P < 0.05$). The results showed that *S. suis 2* infection of brain microvascular endothelial cells caused strong inflammatory reaction and even pyroptosis of cells. At the same time, the inhibitor CMK played a good inhibitory effect (Fig. 3.16).

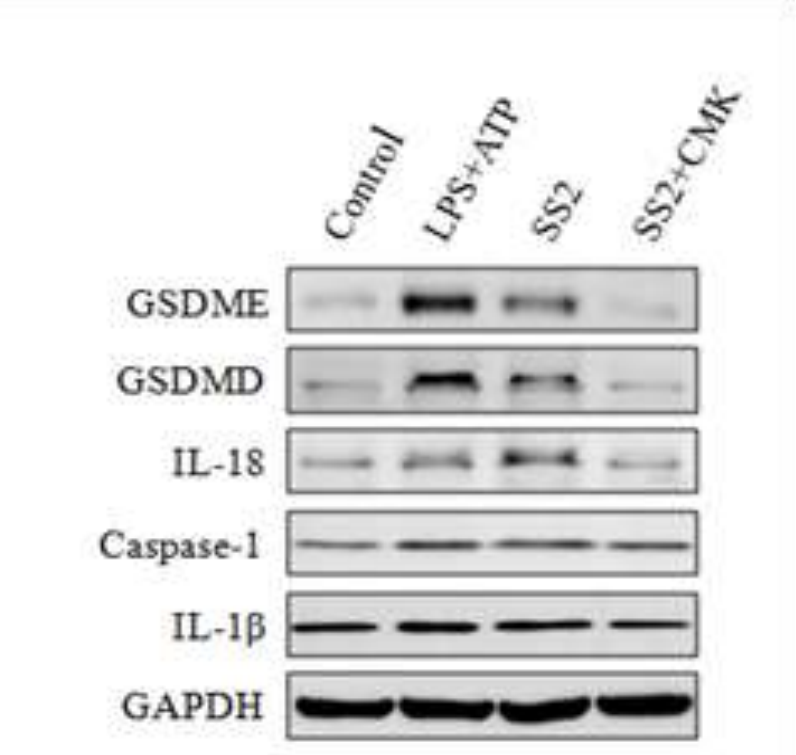

Fig. 3.16 Different protein expression levels of pyroptosis-related genes

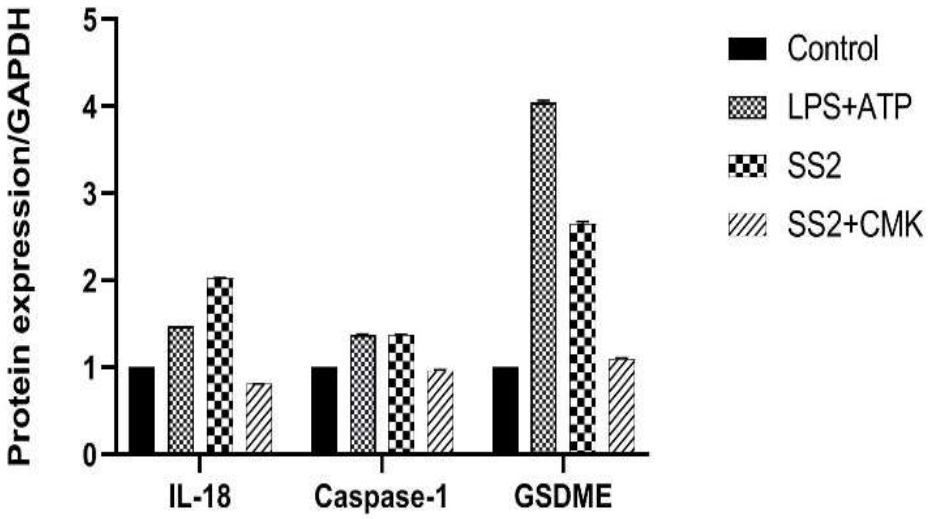

Fig. 3.17 The relative proteins levels of Caspase-1, GSDME, and IL-18 in defferent groups

For IL-18, under the action of LPS+ATP, the protein expression level of IL-18 increased significantly compared with the control group, and the difference was significant. Meanwhile, under the action of *S. suis 2*, the protein expression level of IL-18 increased significantly compared with the control group, and the difference was extremely significant. Under the action of *S. suis 2* +CMK, the expression of IL-18 protein was inhibited, and the expression level was significantly decreased, even lower than that of control group (Fig. 3.17).

For caspase-1, under the action of LPS+ATP, the protein expression of caspase-1 was significantly increased compared with the control group, and the difference was significant. Meanwhile, under the action of *S. suis 2*, the protein expression of caspase-1 was significantly increased compared with the control group, and the difference was extremely significant. However, compared with the LPS+ATP group, the change of protein expression of caspase-1 in *S. suis 2* group was small and the difference was not significant. Under the action of *S. suis 2* +CMK, the protein expression of Caspase-1

was inhibited, and the expression level was significantly decreased, which was lower than that of the control group.

For GSDME, under the action of LPS+ATP, compared with the control group, the protein expression of GSDME increased sharply, and the difference was extremely significant; meanwhile, under the action of *S. suis 2*, compared with the control group, the protein expression of GSDME increased significantly, and the difference was extremely significant, but compared with the LPS+ATP group, the protein expression of GSDME decreased significantly. The difference is very significant; Under the action of *S. suis 2* + CMK, the protein expression of GSDME was significantly inhibited, and the expression level was sharply decreased, even lower than that of the control group.

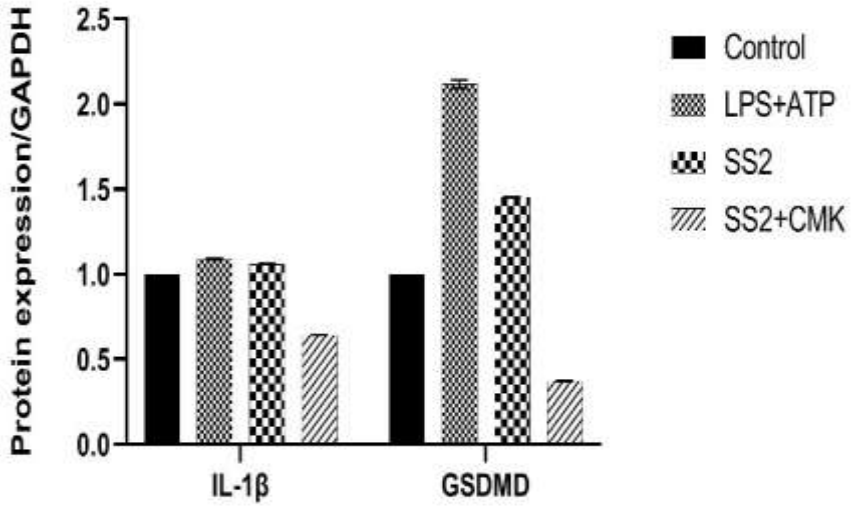

Fig. 3.18 The relative proteins levels of IL-1β and GSDMD in different groups

For IL-1β, under the action of LPS+ATP, the protein expression level of IL-1β was slightly increased compared with the control group, and the difference was significant. Meanwhile, under the action of *S. suis 2*, the protein expression level of IL-1β was slightly increased compared with the control group, and the difference was significant.

Compared with the LPS+ATP group, the protein expression level of IL-1β was slightly decreased. The difference is significant; Under the action of *S. suis 2* +CMK, the protein expression of IL-1β was inhibited, and the expression level was significantly lower than that of the control group (Fig. 3.18).

For GSDME, under the action of LPS+ATP, compared with the control group, the protein expression of GSDME increased sharply and the difference was extremely significant. Meanwhile, under the action of *S. suis 2*, compared with the control group, the protein expression of GSDME increased sharply and the difference was extremely significant. Compared with the LPS+ATP group, the protein expression of GSDME decreased significantly. The difference is very significant; Under the action of *S. suis 2* +CMK, the protein expression of GSDME was inhibited, and the expression level decreased sharply, which was much lower than that of the control group.

3.7 ELISA detection

The experiment was repeated three times, and the results were shown in Fig.13. Compared with control group, the relative expression of TNF-α, IL-6, IL-10, IL-18, IL-1β in cell supernatant of *S. suis 2* group and LPS+ATP group was significantly increased, and the difference was statistically significant ($P<0.01$). Compared with *S. suis 2* group and LPS+ATP group, the relative expression level of TNF-α, IL-6, IL-10, IL-18, IL-1β in *S. suis 2* + CMK group was significantly decreased, and the difference was statistically significant ($P<0.01$), indicating that cell death caused by *S. suis 2* and LPS+ATP could be inhibited by CMK (Fig. 3.19, 3.20, 3.21, 3.22, 3.23).

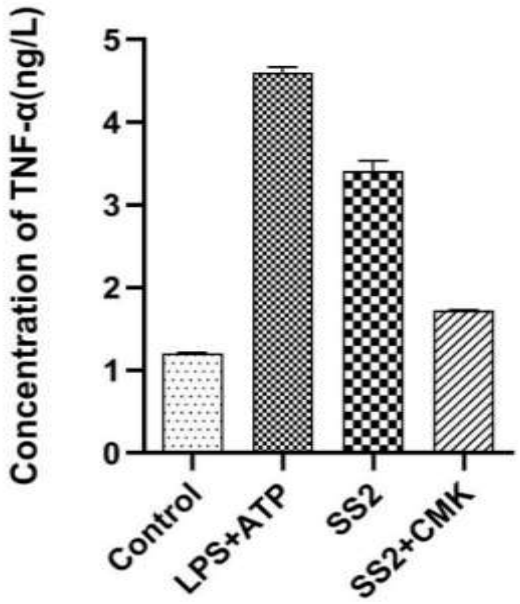

Fig. 3.19 The concentration of TNF- α in supernatant

For TNF-α, the content of TNF-α in cell supernatant of LPS+ATP group was significantly higher than that of control group, and the difference was extremely significant. The content of TNF-α in cell supernatant of *S. suis 2* group was also significantly higher than that of control group, but lower than that of LPS+ATP group. Under the action of CMK, the secretion of cytokines was inhibited, and the content of TNF-α in the cell supernatant of *S. suis 2*+CMK group was much lower than that of *S. suis 2* group, and the difference was extremely significant.

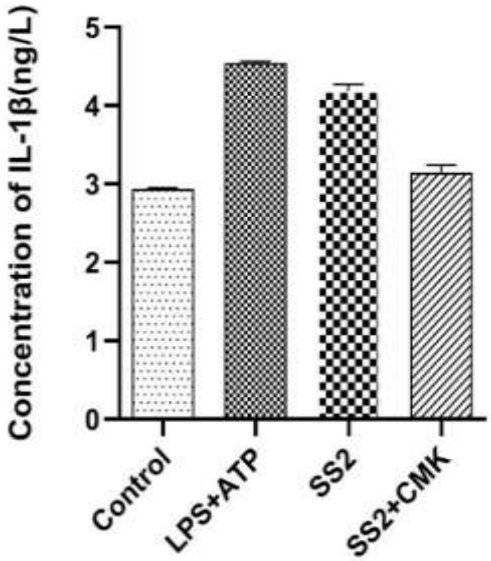

Fig. 3.20 The concentration of IL-1 β in supernatant

For IL-1β, the content of IL-1β in cell supernatant of LPS+ATP group was significantly higher than that of control group, and the difference was extremely significant. The content of IL-1β in cell supernatant of *S. suis 2* group was significantly higher than that of control group, but lower than that of LPS+ATP group. Under the action of CMK, the secretion of cytokines was inhibited, and the content of IL-1β in the cell supernatant of *S. suis 2* + CMK group was lower than that of *S. suis 2* group, and the difference was very significant.

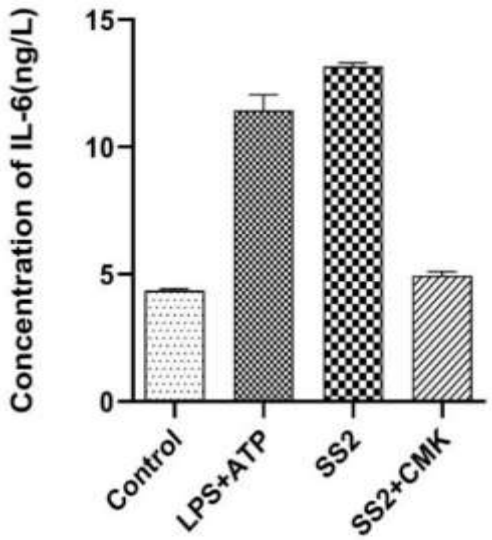

Fig. 3.21 The concentration of IL-6 in supernatant

For IL-6, the content of IL-6 in cell supernatant of LPS+ATP group was significantly higher than that of control group, and the difference was extremely significant. The content of IL-6 in cell supernatant of *S. suis 2* group was also significantly higher than that of control group, and the difference was extremely significant, and higher than that of LPS+ATP group. Under the action of CMK, the secretion of cytokines was inhibited, and the content of IL-6 in the cell supernatant of *S. suis 2*+CMK group was sharply decreased, which was lower than that of *S. suis 2* group, and the difference was extremely significant.

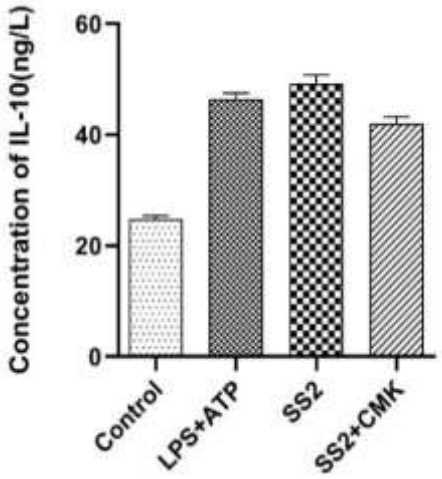

Fig. 3.22 The concentration of IL-10 in supernatant

For IL-10, the content of IL-10 in cell supernatant of LPS+ATP group was significantly higher than that of control group, and the difference was extremely significant. The content of IL-10 in cell supernatant of *S. suis 2* group was also significantly higher than that of control group, and the difference was extremely significant, and slightly higher than that of LPS+ATP group. Under the action of CMK, the secretion of cytokines was inhibited, and the content of IL-10 in cell supernatant of *S. suis 2*+CMK group was decreased, which was lower than that of *S. suis 2* group, and the difference was significant.

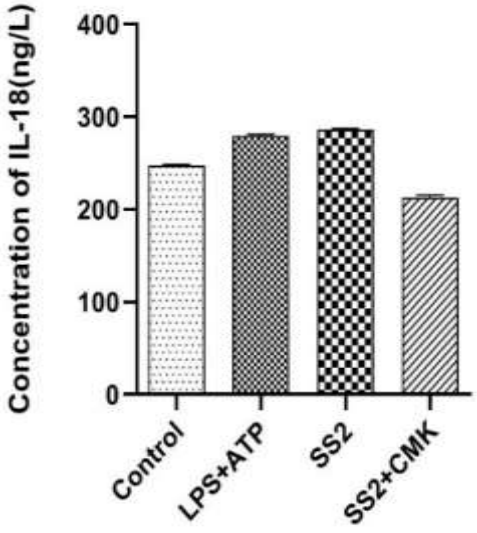

Fig. 3.23 The concentration of IL-18 in supernatant

For IL-18, the content of IL-18 in cell supernatant of LPS+ATP group was higher than that of control group, and the difference was significant. The content of IL-18 in cell supernatant of *S. suis 2* group was significantly higher than that of control group, and slightly higher than that of LPS+ATP group. Under the action of CMK, the secretion of cytokines was inhibited, and the content of IL-18 in the cell supernatant of *S. suis 2* + CMK group was decreased, which was lower than that of *S. suis 2* group, and the difference was significant.

3.8 Lactate dehydrogenase detection

Lactate dehydrogenase (LDH) is a common measure of cytotoxicity. Under normal conditions, lactate dehydrogenase exists stably in the cytoplasm. When the cell pyrodeath occurs, enzymes in the cytoplasm are released due to the destruction of the cell membrane structure, including lactate dehydrogenase with relatively stable enzyme activity. Therefore, lactate dehydrogenase release can be used as an important index to judge cell membrane integrity, and the loss of cell membrane integrity is a sign of cell pyroptosis, so we can detect the situation of cell pyroptosis by detecting lactate dehydrogenase release in the experiment (Fig. 3.24).

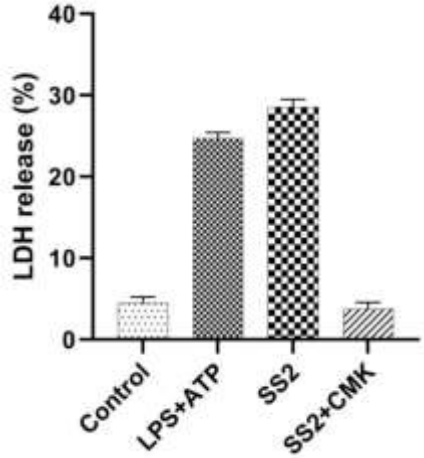

Fig. 3.24 The LDH release

The lactate dehydrogenase release rate of brain microvascular endothelial cells in the LPS+ATP group was significantly higher than that in the control group, and the lactate dehydrogenase release rate of brain microvascular endothelial cells in the *S. suis 2* group was also higher than that in the control group, the difference was extremely significant, and the lactate dehydrogenase release rate was also higher than that in the LPS+ATP group. The lactate dehydrogenase release rate of cerebral microvascular endothelial cells in *S. suis 2* +CMK group was significantly inhibited, much lower than that in *S. suis 2* group, and the difference was extremely significant.

3.9 Electron microscopy observation

In addition to the study of pyroptosis from the perspective of genes and proteins, the morphological observation of cells under electron microscopy is also an important step. In this study, the morphology of treated brain microvascular endothelial cells was observed by electron microscopy, and the results were shown in the figure below (Fig. 3.25).

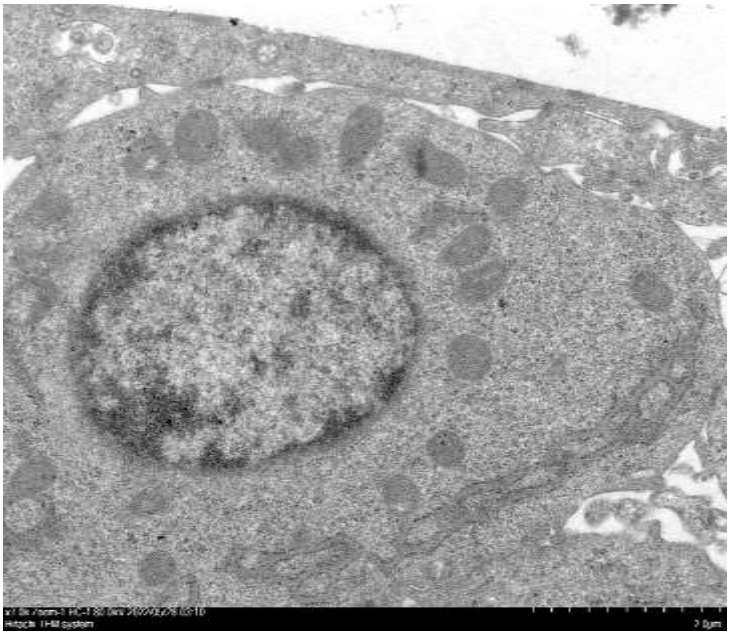

Fig. 3.25 The cell morphology of control group under electron microscope (× 20000 times)

In the control group, the cell morphology and size of brain microvascular endothelial cells were normal, the cell membrane was intact, and the organelles such as endoplasmic reticulum and mitochondria were normal under electron microscope (Fig. 3.25).

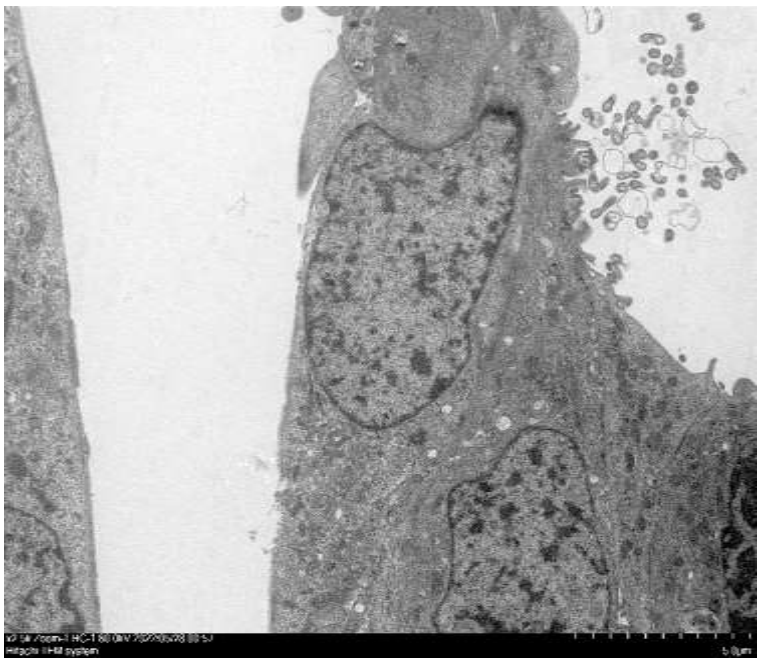

Fig. 3.26 The cell morphology of LPS+ATP group under electron microscope (× 20000 times)

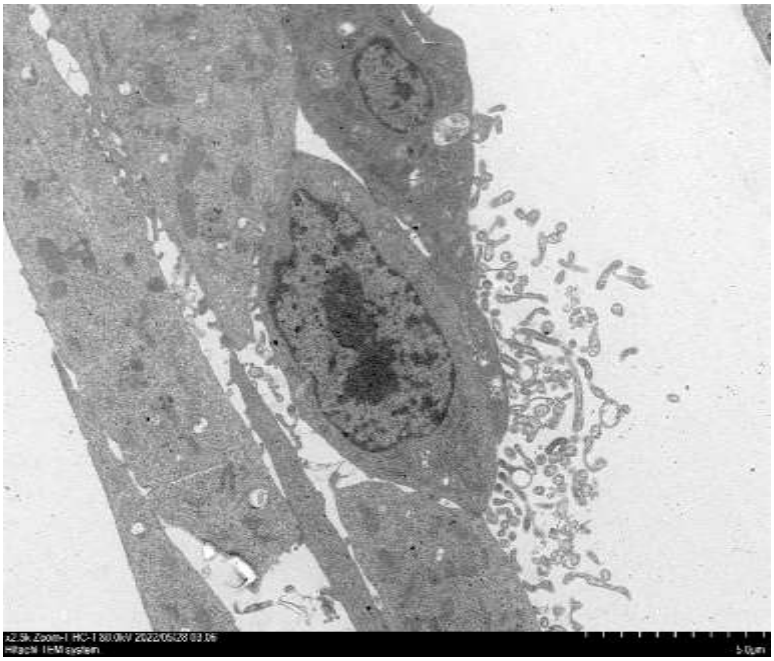

Fig. 3.27 The cell morphology of *S. suis 2* group under electron microscope (× 20000 times)

In the *S. suis 2* group, cerebral microvascular endothelial cells in expanded, deformed, cell membrane ruptured, cell contents flowed out, and organelles deformed under electron microscope (Fig. 3.27).

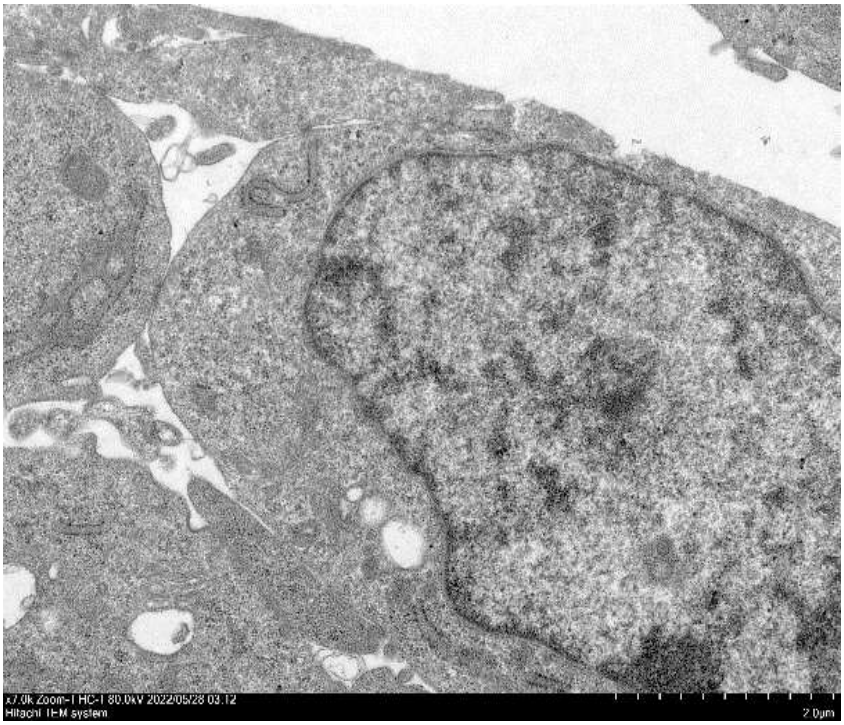

Fig. 3.28 The cell morphology of *S. suis 2* +CMK group under electron microscope (× 20000 times)

In *S. suis 2* + CMK group，the effect of *S. suis 2* was inhibited by CMK, and the shape and size of cerebral microvascular endothelial cells tended to be normal, the cell membrane was relatively complete, and the organelles such as endoplasmic reticulum and mitochondria were normal under electron microscope (Fig. 3. 28).

3. 10 Conclusions to chapter 3

According to the research results, it was theoretically and experimentally established for the first time that pyroptosis takes part in the pathogenetic process of developing meningitis due to streptococcal infection of pigs. The purpose of this study was to

explore the mRAN expression of genes related to pyroptosis, the protein expression and the changes of cell morphology of infected cells when *S. suis 2* infected brain microvascular endothelial cell (b.End3), so as to infer whether pyroptosis occurred during infection. Brain microvascular endothelial cells were identified as infection objects of *Streptococcus suis,* these cells were identified as infection objects of *Streptococcus suis*. The conditions for *S. suis 2* to infect brain microvascular endothelial cells were explored, and the optimal number of infection was established as 100:1, the best duration of infection was 10 hours. The mRNA expression levels of various cytokines in *S. suis 2* group and LPS+ATP group were significantly higher than those in control group, while the mRNA expression levels of various cytokines in *S. suis 2* + inhibitor group were significantly lower than those in *S. suis 2* group. Protein levels of pyroptosis-related genes in *S. suis 2* group and LPS+ATP group were significantly higher than those in control group, while the protein levels of pyrogen-related genes in *S. suis 2* + CMK group were significantly lower than those in *S. suis 2* group. ELISA test showed that protein content in cell supernatant in *S. suis 2* group was significantly higher than that in control group, while the protein level in *S. suis 2* +CMK group was significantly lower than that in *S. suis 2* group. Under electron microscope, the cell membrane of *S. suis 2* -infected cells was broken, the cell shrank, and the cell contents were leaked out. Combined with the above results, it can be concluded that pyroptosis occurred during *S. suis 2* infection with brain microvascular endothelial cells. The results of the study confirmed that during the bacterial infection, the cells were scorched. It was established for the first time that cell pyroptosis is involved in the occurrence of meningitis.

CHAPTER 4

SUMMARY AND ANALYSIS OF RESULTS

In vitro isolation and culture of primary mouse BMECs, the main difficulty lies in the difficulty of obtaining vascular segments, poor activity and easy contamination by pericytes, glial cells and fibroblasts [182, 183]. Therefore, the isolation and culture of primary cerebrovascular endothelial cells has always been a key factor restricting in vitro experiments. In order to successfully isolate mouse brain microvascular segments, we took the following measures: (1) Mice aged 6–8 weeks were selected. We have tried to select older mice and found that their cell proliferation activity is relatively decreased, and the cells grow and proliferate slowly in vitro. Newborn mice were also tried, and their proliferative activity was similar to that of 6–8 weeks, but because of their relatively small brain size, the number of cells obtained was relatively small.（2） isolation operation is performed on ice. The pia mater and large blood vessels are carefully dissected. Cerebellum and hippocampus are removed and smooth muscle cell, fibroblast and neuron growth are inhibited, and endothelial cell purity is improved . (3) Through homogenate grinding, more fine tissue can be obtained, which is beneficial to the digestion of endothelial cells [184]. (4) Enzyme digestion was used twice. Type II collagenase can loosen the connection between endothelial cells and basement membrane, destroy the tight junctions between endothelial cells, and completely separate brain microvascular segments from brain tissue [185]. Collagenase/dispersion is used for the second digestion. Enzyme removes the miscellaneous cells on the microvascular segment [186]. Digestion time is the key to preserving cell viability.

Excessive digestion time will lead to decreased cell viability [187]. If digestion time is too short, pericytes, glial cells and fibroblasts will be mixed. The digestion time of the experiment is 1.5h, which not only obtains enough microvascular segments, but also removes the foreign cells. After two consecutive centrifugations, the obtained microvessels were combined, which greatly reduced the loss of microvessels caused by centrifugation, and more microvessels were obtained. (5) In order to ensure the nutritional requirements of primary endothelial cell growth, the concentration of foetal bovine serum, in DMEM complete culture medium can be appropriately increased, we used 20–30% foetal bovine serum. At the same time, there is no screen filtration in the process of separating microvessels, which not only reduces the possibility of contamination, but also avoids the loss of cells. （6）The concentration of purinomycin is 2mg/L. Purinomycin can inhibit protein synthesis, and endothelial cells can effectively pump purinomycin out of the cell due to the expression of multidrug resistance protein (MDR1), so as to survive and grow. Other cells gradually die because purinomycin inhibits protein synthesis, so adding purinomycin to cell culture serves to purify endothelial cells. In previous literature, the concentration of 4 mg/L was mostly used [188]. In this study, it was observed that purinomycin at this concentration could purify endothelial cells, but it would affect the proliferation activity of endothelial cells, resulting in slow cell proliferation and decreased production. The purification ability of 2 mg/L purinomycin on endothelial cells was similar to that of 4 mg/L purinomycin, but it had little effect on cell proliferation.

In the culture of microvascular segment after separation, the cell culture flask should be coated with 0.1% gelatin firstly, which can promote the adhesion of

microvascular endothelial segment and facilitate the growth of cells [189]. The complete medium contained 30% FBS to ensure the nutritional requirements of endothelial cell growth. At the same time, endothelial cell growth factor was added to promote the proliferation of endothelial cells. At the same time, heparin sodium was added to inhibit the growth of smooth muscle cells.

Plate counting method is often used to calculate bacteria. The method includes plate mixed culture and spread plate method. Mixed plate culture needs mix bacteria liquid and agar medium together when the temperature of agar medium is about 40 degree [190]. It is easy to affect the activity of bacteria when the temperature is not controlled well and then the density of bacteria is affected subsequently [191]. Spread plate method put bacteria liquid after the agar medium is cold and solidified. It avoids the shortage of mixed plate culture and was used in this study [192].

The plate counting method is the most commonly used method for the determination of the total number of bacteria in the laboratory. However, in actual work, it is found that there are often sediment, particles and other substances that are not easy to be emulsified in the sample, which is easy to be confused with small colonies in the bacterial count, affecting the accuracy of the bacterial count. In addition, the plate counting method is complex and time-consuming, requiring the experimenteer to use the naked eye to count, which is time-consuming and laborious, increasing the difficulty of bacterial counting.

In addition to using the plate counting method, this study also established the bacterial curve for counting. Studies and analyses have shown that there is a positive correlation between the absorbance of bacterial suspension and the number of bacteria,

and bacteria can be counted by measuring the absorbance value of bacterial solution. Therefore, the bacteria count of *Streptococcus suis* was carried out by spectrophotometry in this experiment. At the wavelengths of 450nm, 570nm, 600nm and 650nm, the bacterial suspension showed a good linear relationship with the number of bacteria. But at 570nm wavelength, the obtained goodness of fit R2 (R2=0.999) of the standard curve regression equation is the largest.

Therefore, after analysis and comparison, the best wavelength for measuring the concentration of *Streptococcus suis* was 570 nm. At 570 nm wavelength, when the absorbance value ranges from 0.021 to 0.513, the standard curved line equation for establishing a linear correlation between the absorbance value of *Streptococcus suis* and the number of bacteria is y=1233.3x-12.148. The curve was verified by the isolated *Streptococcus suis*, and it was found that the degree of fitting of the curve was 96%, which had high reliability.

However, it should be noted that when using spectrophotometry to count Escherichia coli bacteria, in addition to considering the optimal wavelength for measuring streptococcus concentration, attention should also be paid to the measurement range of bacterial absorbance value. Only when the absorbance value of bacterial liquid is within the range of 0.021-0.513. Only the total number of bacteria and absorbance A value can show a good linear relationship. Compared with the traditional counting method, using bacterial curve to count bacteria saves time and effort, is convenient to operate and has good repeatability. At the same time, it provides technical support for the rapid counting of bacteria in laboratory tests such as the determination of minimum inhibitory concentration of drugs and drug sensitivity test.

In addition to the plate count used in this study, there are several other methods. 1. Bacteria counting plate method. The principle and components of the bacteria counting plate and the blood cell counting plate are the same, except that the bacteria counting plate is thinner and can be observed with an oil mirror; However, the blood cell counting plate is thick, and the oil mirror cannot be used, and the bacteria under the counting plate is not easy to see.

A certain volume of sample bacterial suspension was placed in the counting chamber of the bacterial counting plate, observed and counted with a microscope. Since the volume of the counting tank is certain, the number of bacteria in the sample can be calculated according to the number of bacteria in the counter scale. This method is simple and practicable, and results can be obtained immediately. Larger yeast or mold spores can also be counted using blood cell counting plates. This method is suitable for simple conditions and can only yield a rough value. 2. McGregor Nephelometry. McFarland invented a standard turbidity tube of different turbidity for microbial turbidity. The prepared bacterial suspension was compared with a standard McTurbidimetric tube of 0.5 McTurbidimetric, and the bacterial solution with the same turbidity was 0.5 McTurbidimetric. MCF is the MCF turbidity unit, and 0.5 MCF concentration bacterial solution is the common concentration of microbial drug sensitivity experiments. It is generally believed that when the concentration of bacterial solution is mixed into 0.5 MCF turbidimetric tube, it is equivalent to 1.5×108 bacterial number / mL. 3. Spectrophotometer method. In general, the laboratory has a spectrophotometer that can also measure the turbidity of bacterial suspension, and adjusting the bacterial solution to the OD (625) value between 0.08-0.13 is equivalent to

0.5 McGlob turbidity. However, because different bacteria refractive index is different, so this is only a rough conversion relationship, only has a certain reference value. 4. CCU method. CCU is a unit of color change, which takes the metabolic activity of microorganisms in the medium as an indicator and counts the number of microorganisms according to the change in the color of the medium. The bacterial counting method using CCU/mL as the bacterial counting unit is the CCU method.

The distribution of swine streptococcal disease is worldwide, for infected pigs, mainly living in the tonsils and nasal cavity and other locations, if people are in direct contact, it will make people suffer from sepsis, meningitis and other diseases, serious threats to human life safety. Swine streptococcus disease is widely endemic.

Once the infectious disease breaks out, pigs will die and even infect the breeders for unknown reasons, which not only threatens human health, but also causes huge economic losses, which is not conducive to the sustainable development of pig breeding industry. After infection in pigs, it will enter the blood circulation system, which needs to break through the tonsils and upper respiratory tract, and adhere to the surface of macrophages, causing obvious symptoms of disease. For sick pigs, some will gradually develop sepsis, and eventually form arthritis, meningitis and so on. Streptococcus suis disease has no obvious seasonality, mainly through respiratory tract, broken skin and digestive tract transmission, and there are many causes of the disease, such as poor sanitation, poor ventilation and so on.

Pigs with streptococcal disease can be divided into four clinical symptoms according to the location of the disease, which are arthritis, lymph node swelling, septicemia and meningitis. (1) Arthritis type: A variety of disease transformation is the

main cause of the formation of arthritis type of streptococcal disease, during the disease, although many joints will appear swelling phenomenon, but the overall disease is relatively mild, while the pig's fur is rough, messy, accompanied by weakness of the limbs, so the pig is often unable to walk, or even unable to stand. (2) Lymph node swelling type. Lymph node swelling type of streptococcal disease generally occurs in pigs at the fattening stage or weaning stage. After the disease, the lymph nodes have obvious heat pain, especially the swelling degree of the lymph nodes under the jaw of the sick pigs is more serious, and at the same time, it causes mild or severe respiratory diseases. (3) Septicemia type. Septicemia type is associated with higher morbidity and mortality than other types of streptococcal disease.

The body temperature of the sick pigs increased, loss of appetite, poor mental state and other conditions, and the respiratory rate changed, the overall short and rapid, if not timely relief and treatment, within a few hours after the onset of the disease, the death rate increased, extremely easy to die. (4) Meningitis type. In general, meningococcal suis disease is mainly concentrated in newborn piglets, and the specific symptoms are similar to septicemia.

Preliminary diagnosis can be made based on clinical symptoms and autopsy characteristics, and laboratory tests should be performed if a definitive diagnosis is needed. The liver and other disease materials were collected by aseptic method and inoculated into Martin broth medium or blood AGAR medium. After one day of culture, the broth was uniformly and slightly turbidized, and there were round, smooth translucent colonies on the blood AGAR medium with β hemolytic ring. The obtained colonies were examined by microscopy after smear, and a single spherical bacteria with

short chain arrangement could be seen. It was identified as Gram-positive by Gram staining. The obtained colonies were separated and purified. During biochemical experiments, if the colonies could decompose starch, glucose, sucrose, sorbitol and fructose, but could not decompose mannitol well and did not produce hydrogen sulfide, it could be considered that the infected pig was infected with Streptococcus suis.

The main measure to prevent streptococcal disease is vaccination. Currently, the vaccines available for immunization can be divided into three categories, which are whole bacteria inactivated vaccines, live vaccines and genetically engineered vaccines. Whole bacteria inactivated vaccine mainly refers to the own vaccine, but because the immune effect is uncertain and easy to cause disease transmission due to incomplete vaccine inactivation, it is not recommended to use.

The live vaccine is made of ST171 strain of Group C streptococcus as immunogen. Sucrose gelatin is used as adjuvant to keep the vaccine stable, and the protection rate of the vaccine for live pigs can reach 80%-100%. The genetically engineered vaccine is only in the experimental research stage, and there is no stable finished vaccine to choose from. Genetic engineering vaccine is mainly for the research of certain virulence factors are true gene deletion vaccine, as well as the research of gene recombinant subunit vaccine. At present, the analysis, recombination and expression of immunogenic protein fragments of *Streptococcus suis type 2* have been achieved, and it is expected that a good immune protection streptococcus suis vaccine will be developed in the near future.

There are many drugs to treat swine streptococcal disease, mainly the application of streptococcal sensitive antibiotics for treatment, and symptomatic treatment for pigs

with relatively serious symptoms. Conditional pig farms should conduct drug sensitivity tests, select the most sensitive drugs isolated from the infected pigs of the pig farm for clinical treatment, such drug use is more targeted, the course of disease will be greatly shortened. However, because the drug sensitivity test needs to separate and purify the bacteria first, the requirements for the operator are relatively high

Therefore, in practice, broad-spectrum antibiotics are often chosen for treatment. Antibiotics that have a good inhibitory effect on streptococcus include penicillin, ampicillin, gentamicin, sulfadiazine, ceftiofuran sodium, etc. These drugs can be used for the treatment of streptococcal disease. When piglets are sick, often accompanied by meningitis, at this time should be used with antibiotics with dexamethasone, which can alleviate the symptoms of meningitis, improve the survival rate of piglets. For pigs with lymph node abscess type, the abscess should be symptomatic treatment. When the abscess is mature, the skin of the affected area should be disinfected with iodophor first, and the abscess should be performed under sterile conditions to help the affected pig discharge pus as soon as possible, and then the wound should be treated with erythromycin ointment to reduce inflammation. Usually after 1 week of treatment, the affected pig can recover.

Meningitis is one of the common diseases that restrict the development of pig industry. S.suis2 infection is the main pathogenic factor of meningitis [193]. Exploring the relationship between streptococcus and meningitis is an important basis for the prevention and treatment of the disease.

Although the research attention on *Streptococcus suis* has increased significantly in recent years, the mechanism of *streptococcus suis* causing meningitis has not yet

been fully clarified. Most studies focus on the screening and functional exploration of virulence factors affecting the blood-brain barrier, while there are few studies on how Streptococcus suis crosses the blood-brain barrier to the central nervous system, destroys brain tissue and leads to meningitis. The blood-brain barrier is an important barrier in the body. Its main function is to prevent pathogens and other macromolecules from entering the brain tissue through the blood circulation, and it is a barrier with selective permeability [194, 195]. The key to the journey from infection with Streptococcus suis to bacterial meningitis is the way it crosses the blood-brain barrier and reaches the central nervous system. As cerebral microvascular endothelial cells are the most critical component of the blood-brain barrier, they play an important role in the occurrence of meningitis. Therefore, the study of the interaction between *Streptococcus suis* and cerebral microvascular endothelial cells and the changes of cerebral microvascular endothelial cell membrane during this process will help us to discover the mechanism of bacterial meningitis.

Numerous studies have shown a link between streptococcal infection and apoptosis [196, 197]. *Streptococcus suis* hemolysin is a cholesterol-dependent cell pore-forming toxin, an important virulence factor secreted by Gram-positive bacteria when infecting the host [198]. It can oligomerize on the surface of cholesterol-rich cell membranes, and then punch holes on the surface of the cell membrane to destroy the cell by changing the permeability of the cell membrane [199]. Apoptosis of cerebral microvascular endothelial cells is induced, which crosses the blood-brain barrier and causes meningitis.

Muramidase-released protein has the function of inducing epithelial cell fusion

and apoptosis, which can destroy the originally dense cell structure and thus make the epithelial cell structure lose its barrier function. Muramidase-released protein is one of the key mechanisms of breaking through the tonsil and brain blood-brain barrier during SS2 infection. After apoptosis induced by muramidase-released protein, a large number of nuclei and cell fragments are released into the surrounding tissue environment, causing a large number of phagocytes to accumulate, and the clearance process leads to serious inflammatory reactions in parenchymal organs, and then produces symptoms and sequelae.

S. suis 2 enolase (Eno) is an important virulence factor. It was found that Eno plays a role in the destruction of blood-brain barrier by *S. suis 2* through the interaction of *S. suis 2* gene library with co-cultured blood-brain barrier model in vitro. The effect of Eno on the function of porcine primary cerebral microvascular endothelial cells showed that Eno can induce the apoptosis of PBMEC, and apoptosis can promote the adhesion and invasion of *S. suis 2* to primary cerebral microvascular endothelial cells and the destruction of blood-brain barrier. To explore the mechanism, it was found that p38 MAPK could mediate apoptosis.

As another way of cell death, Is pyroptosis involved in the occurrence of meningitis? The main features of pyroptosis are cell enlargement, cell membrane rupture and release of cell contents represented by IL-18 and IL-1β [200].

The characteristics of pyroptosis and apoptosis are similar, but the death process of pyroptosis is different from apoptosis. The specific differences are as follows: (1) The cells with pyrodeath gradually expanded, and then the nucleus gradually rounded and mildly condensed. With pyrodeath, the chromosomal DNA degraded and showed

positive results in TUNEL assay, which was also observed in apoptotic cells Appear; (2) The DNA degradation degree and TUNEL staining intensity of pyrodead cells were not as high as those of apoptotic cells, and there was a small amount of ladder DNA. The integrity of the nucleus of cells with pyrosis remains unchanged, and spherical vesicles are formed around the nucleus [201], which is different from apoptosis. The biggest difference between the two is that during pyroptosis, the cells lose the integrity of the plasma membrane, the cell membrane loses the ability to regulate the entry and exit of substances and rapidly expands, which eventually leads to the dissolution of the cell membrane and the cell to undergo osmotic disintegration, while releasing large amounts of inflammatory cytokines IL-1β and IL-18 into the extracellular environment and generating pro-inflammatory signals to neighboring cells , recruit more inflammatory cells, and then induce inflammatory response, but apoptotic cells, the integrity of the cell membrane remains unchanged and no inflammatory response occurs.

Other studies have shown that the breakdown of the plasma membrane of scorch dead cells is closely related to Gasdmin D protein. caspase-1 or caspase-11 can cut the splice structure of Gasdmin D and release the Gasdmin-N terminal domain, which then combines with the internal lobules of the plasma membrane to oligomerize and form 1-2nm pores on the membrane, thereby increasing the permeability of the cell membrane, potassium ion outflow, sodium ion and water flow in the cell, resulting in the disappearance of the ionic gradient of the cell, the increase in the osmotic pressure of the cell membrane, the loss of the ability to regulate the entry and exit of substances, rapid expansion, and finally leading to the dissolution of the cell membrane and the cell osmotic disintegration.

IL-6 is the most typical cytokine associated with inflammation. It plays an important role in host defense by regulating immune and inflammatory responses. IL-6, a member of the pro-inflammatory cytokine family, activates the signaling pathway by binding to membrane-bound IL-6R or circulating soluble IL-6R and glycoprotein gp130, thus initiating its pleiotropic effects.

IL-10 is a pleiotropic cytokine with important immunomodulatory functions. It is involved in the activity of many types of cells in the immune system. During acute infection, dendritic cells produce pro-inflammatory signals after recognizing pathogen patterns. At the same time, natural killer cells that recognize pathogen patterns and/or are stimulated by pro-inflammatory signals further enhance inflammation. In this pro-inflammatory condition, dendritic cells can promote an antiviral T cell response that clears the infection. Activation of dendritic cells, T cells, and natural killer cells also leads to the production of the immunomodulatory cytokine IL-10 to balance inflammation.

As inflammatory factors, IL-6 and IL-10 are normally present in very low levels in the body. When the body is infected by microorganisms, the immune system is activated, and the contents of IL-6 and IL-10 will increase [202]. High levels are positively correlated with inflammation. The more severe the inflammation, the higher the contents are. Especially in bacterial infections, the elevated levels of IL-6 and IL-10 were significantly higher than those in non-bacterial infections [203].

The results showed that the mRNA expression of the corresponding cytokines increased with the increase of MOI value when brain microvascular endothelial cells was infected with different MOI. When MOI is at 1, due to the small number of infected

streptococcus, the mRNA expression of cytokines in each group was not significantly increased compared with the control group, and reached a peak at 18h. When MOI is at 10, the mRNA expression of cytokines in each group increased relative to MOI is at 1, and reached a peak at 12h. With the increase of the number of *S.suis2*, the expression of cytokines in each group increased significantly when MOI is at 100, compared with other infection time and 12h and 18h were relatively more expressed. However, when the MOI is at 200, the mRNA expression of cytokines in each group decreased again, mainly because the infection time was too long, a large number of cells died, and they were lysed into cell fragments.

First identified in 1992, pyroptosis was given this name in 2001 [204]. Pyroptosis was always thought to be a mode of cell death dependent only on Caspase-1 activity until 2011, when it was found that mouse Caspase-11 could sense bacterial infection in a way independent of Caspase-1 and cause pyroptosis [205].

In 2014, Shao Feng et al. 'reported that intracellular Caspase-4/5/ll could act as a receptor to directly bind to LPS on the surface of gram-negative bacteria, mediating the non-classical pathway of cell pyrodeath. In 2015, it was found that GSDMD, a substrate of Caspase-1/11, is a key executive molecule in mediating pyrodeath. In 2017,Wang et al. [98] found that Caspase-3 could recognize and cut GSDME, another member of the Gasdermin family, and the N-terminal fragment formed could also mediate pyroptosis. In 2018, EgilLien's team reported in Science that Yersinia infection can cause pyrodeath in host cells dependent on Caspase-8 activity. In 2018, Kambara et al. [206] found that neutrophil serine protease ELANE can cut GSDMD in a Caspase-1/11 independent manner, triggering neutrophil pyrosis. Therefore, these findings suggest that pyroptosis

is not determined by Caspase, but by its recognition of hydrolyzed substrate Gasder-min family proteins.

Thus, the definition of pyroptosis was gradually refined from «pyroptosis caused by Caspase-1/11 cleavage of Gasdermin family proteins» to «a cell death dependent on Gasdermin family protein pore-forming toxicity, often but not always due to Caspase activation».

Bacterial infection is one of the main causes of pyroptosis. Bacteria activate inflammasome through different components, e.g. Salmonella activates NLRC4 inflammasome through bacteriophage protein M, Listeria monocytogenes activates AIM2 inflammasome through cytoplasmic dsDNA [207, 208].

NLRC4 Inflammatory body (NLR family, CARD domain containing) More than 4, NLRC4) is a kind of protein complexes, raise structure by urinary day N pride side containing protease domain (Caspase recruitment domain, CARD), combined with domain structure in the middle of the nucleotides (Nucleotide binding oligo merization domain, NOD) and leucine-rich C-terminal protein interaction domains. Over the past 10 years, it has been found that the activation of inflammasome activates caspase-1 of cysteine, cleaving inactive caspase-1 precursor (pro-caspase-1) to active caspase-1, thereby promoting inactive IL-1β precursor (pro-IL-1β) and IL-18 precursor (pro-IL-18) is cut into mature IL-1β and IL-18, which are released into the extracellular to participate in the process of body inflammation and injury.

The LRR domain of NLRC4 consists of 15 repeating building units with a total of 440 amino acids. It is called a leucine-rich repeat because each unit is connected to a helical structure containing 8 to 15 amino acids. This domain is used to recognize

ligands such as the pathogen associated molecular pattern. The NOD domain belongs to the superfamily of AAA+ATPase and mediates NLRC4 oligomerization itself. The CARD domain is usually composed of the first 94 amino acids of NLRC4, folded into six inverted parallel alpha-helical coils around the hydrophobic core, which can connect the adapter protein ASC and the effector caspase-1 to mediate downstream signal transduction.

The congenital immune receptor melanoma deficiency factor (absent in melanoma 2, AIM2), originally isolated from human melanoma cells, has been identified as a novel inflammator-activating protein. AIM2 directly binds to double-stranded DNA (dsDNA) and initiates the recruitment of apoptosis-associated speck-like protein (ASC) and Caspase-1 containing cysteine to assemble multiprotein inflammator-complex, which induces Interleukin 1β. Maturation and activation of IL-1β and IL-18, inducing pyrodeath [209].

AIM2 is the only member of the HIN-200 family that interacts specifically with ASC. When AIM2 inflammasome is activated, ASC acts as an important cohesion molecule, recruiting the effector protein Caspase-1 into the inflammasome complex and converting it into Caspase-1 with proteolytic activity through dimerization and self-proteolysis. Cutting Gasdermin D (GSDMD) releases GSDMD-NTER fragments, which polymerize in the plasma membrane to form cytotoxic pores, destroy the integrity of the cell membrane, induce cell pyrodeath and release of cell contents [210].

At the same time, AIM2 catalyzes IL-1β and IL-18 precursors into their activated forms IL-1β and IL-18 by activating Caspase-1, and exacerbates cell pyrodeath . Studies have shown that AIM2 inflammatosomes have a new function of inducing

Caspase-8-dependent apoptosis. In Caspase-1-deficient mice, AIM2 inflammatosomes can promote Caspase-8 activation, leading to Caspase-1-independent cell death and cell necrotic apoptosis [211]. AIM2 inflammatosomes can simultaneously cause pyroptosis and apoptosis, and the balance between the two is determined by the amount of DNA in the cell. Apoptosis requires a lower transfection DNA concentration and has a higher activation threshold than pyroptosis.

Innate immune response, as the first line of defense of the body, maintains the stability of the internal environment by resisting infection by pathogenic microorganisms [212]. When the body is subjected to various stimuli, pattern recognition receptors of immune cells recognize either the pathogen-associated molecular patterns outside the cell or the damage associated molecular molecules inside the cell patterns, triggering innate immune responses [213]. PRRs can be divided into membrane type, endosomal type, cytoplasmic type and secretory type, while NLRP3 inflammatory bodies belong to cytoplasmic PRRs.

The NLRP3 inflammasome is an important signaling molecule for tissue cells to regulate inflammatory response. It is mainly composed of NOD-likereceptor protein3(NLRP3) and apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1 composition [214]. After activation of the NLRP3 inflammatome, it can induce the cleavage and activation of Caspase-1 precursor, and the activated Caspase-1 can lyse GSDMD and release its N-terminal domain, which migrates to the cell membrane and forms pores, mediates the release of cell contents, including IL-1β and IL-18, activates the pyrodeath pathway and induces pyroptosis [215].

In addition, the action of ATP on P2X7 receptor can also induce the formation of

NLRP3 inflammasome. In the process of inducing pyrodeath, the administration of LPS and ATP alone could not cause the significant increase of caspase-1 and IL-1β, while the administration of ATP after the stimulation of cells by LPS could significantly increase caspase-1 and IL-1β [216].

NLRP3 activation requires the participation of two signals. The first signal is TLR4 / LPS, which can promote the formation of pro-IL-1β. The second signal is the stimulation of substances such as ATP. LPS can only promote the formation of pro-caspase-1 and pro-IL-1β. When stimulated by ATP, LPS can activate the NOD-likereceptor protein3 inflammasome, and then activate caspase-1, IL-1β, IL-18 in turn, causing pyroptosis.

When the intracellular ROS content is increased, it will promote the activation of NOD-likereceptor protein3 inflammasomes, and subsequently activate caspase-1, IL-1β, and IL-18 in large quantities, resulting in cell pyrodeath, aggravated cell damage, and increased lactate dehydrogenase level [217].

SS activate inflammasome NLRP3, mainly through haemolysin, which further activates caspase-1, ultimately inducing pyroptosis. Tang observed a significant onset of pyroptosis after LPS/ATP stimulation of endothelial cells, with marked increases in relative mRNA levels of caspase-1, IL-1β and IL-18 [218]. Antushevich [219] reported a significant increase in mRNA levels of genes associated with pyroptosis when SS2 acted on BMEC.

Brain microvascular endothelial cells (BMEC) form the structural basis of cell monolayer through three kinds of connections, including tight junction, adhesive junction and gap junction, and constitute the barrier basis of BBB, which is the basic

structure of blood-brain barrier [220]. Most bacterial meningitis causes the disease. Brain microvascular endothelial cells is the initial site of blood-brain barrier damage. microvascular endothelial cells can be used not only as the target cell of cytokines and inflammatory factors, but also as the secretory organ of these factors. It is involved in regulating the body's immune response and inflammation.

Current studies have demonstrated that microvascular endothelial cells are the primary target cells attacked by a variety of bacterial toxins (e.g. LPS, Shiga-like toxins) and viruses (e.g., sporospora virus, HIV). Therefore, the injury of microvascular endothelial cells has been regarded as the pathological basis of the occurrence and development of various disease syndromes such as trauma, shock, infection, cardiovascular disease, tumor and acute lung injury.

After *S. suis 2* adhesion to brain microvascular endothelial cells, streptococcal virulence factors can induce brain microvascular endothelial cells to release inflammatory mediators such as cytokines, and these inflammatory mediators not only protect the corresponding tissues, but also cause brain microvascular endothelial cells damage. Combined with the toxic damage effect of virulence factors on brain microvascular endothelial cells, the permeability of blood-brain barrier is increased. SS2 enters the central nervous system.

In the present study, intracellular mRNA levels of NOD-likereceptor protein3, caspase-1, IL-1β and il - 18 were significantly higher in *S. suis 2* infected brain microvascular endothelial cells than in the control group, and showed a trend of first increase and then decrease, which is consistent with the literature. The findings suggest that *S. suis 2* activates the pyroptosis signalling pathway. Expression of caspase-1, IL-1β

and IL-18 was significantly reduced after caspase-1 inhibitor treatment.

AC-YVAD-CMK is a specific irreversible and permeable cell inhibitor of caspase-1. AC-YVAD-CMK can directly inhibit the activity of caspase-1 and block its molecular signaling pathway mediating inflammatory response. Studies on Caspase-1 inhibitors mostly focus on brain damage of the central nervous system [221]. Studies have found [222] that caspase-1 inhibitor has significant efficacy in animal models of brain injury and brain abscess, which can reduce brain edema, protect and promote the repair of neurons after cerebral ischemia, and promote the progression of inflammatory response.

CONCLUSIONS

The dissertation presents an experimental study of the pathogenesis of meningitis due to streptococcal infection of pigs based on the study of the pathogenetic processes of pyroptosis through the expression of mRAN genes and proteins associated with pyroptosis, changes in the morphology of endothelial cells of the microvessels of the brain of white mice (b.End3) during infection with *S. suis serotype 2*.

The conclusions of this study are as follows:

1. Brain microvascular endothelial cells were identified as infection objects of *Streptococcus suis*. The self-made cells were identified by immunofluorescence as mouse brain microvascular endothelial cells.

2. The conditions for *S. suis 2* to infect brain microvascular endothelial cells were explored, and the optimal number of infection was established as 100:1, the best duration of infection was 10 hours.

3. The mRNA expression levels of various cytokines in *S. suis 2* group and LPS+ATP group were significantly higher than those in control group, while the mRNA expression levels of various cytokines in *S. suis 2* + inhibitor group were significantly lower than those in *S. suis 2* group. Protein levels of pyroptosis-related genes in *S. suis 2* group and LPS+ATP group were significantly higher than those in control group, while the protein levels of pyrogen-related genes in *S. suis 2* + CMK group were significantly lower than those in *S. suis 2* group.

4. ELISA test showed that protein content in cell supernatant in *S. suis 2* group was significantly higher than that in control group, while the protein level in *S. suis 2*

+CMK group was significantly lower than that in *S. suis 2* group.

5. Under electron microscope, the cell membrane of *S. suis 2* -infected cells was broken, the cell shrank, and the cell contents were leaked out. Combined with the above results, it can be concluded that pyroptosis occurred during *S. suis 2* infection with brain microvascular endothelial cells.

6. The results of the study confirmed that during the bacterial infection, the cells were scorched. It was established for the first time that cell pyroptosis is involved in the occurrence of meningitis.

PRODUCTION PROPOSALS

1. On the basis of our recearsh Methodological Recommendations «Streptococcus suis infection (etiology, epidemiology,laboratory diagnosis, prevention and treatment)», Protocol № 18, dated 29.05. 2023, authors: Mingcheng Liu, Kasianenko Oksana, introduced into the educational process during the teaching of the disciplines: «Epizootology and infectious diseases», «Veterinary technologies for the prevention of infectious diseases of animals», «Antiepizootic measures in animal husbandry» at the department of Epizootology and Parasitology in the training of specialists for with the degree of higher education "Master" in specialty 211 "Veterinary Medicine at the Sumy National Agrarian University and for the courses «Veterinary microbiology» and «Veterinary immunology» for masters of the Henan Institute of Science and Technology (HIST).

2. We recommend using the materials of the dissertation work when studying the course «Veterinary microbiology» of the Henan Institute of Science and Tech nology (HIST).

3. We recommend that the study of pyroptosis can provide an in-depth understanding of the pathogenesis of porcine meningitis, provide new ideas for the clinical prevention and treatment of meningitis, and provide a theoretical basis for the development of new drugs.

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APPLICATIONS

Appendix A

List of works published on the topic of the dissertation

LIST OF WORKS PUBLISHED ON THE THEME OF THE DISSERTATION

Articles in scientific professional publications of Ukraine:

1. **Liu Mingcheng,** Kasianenko Oksana (2022). Screening of infection conditions for brain microvascular endothelial cells infected by Streptococcus suis. *Ukrainian Journal of Veterinary and Agricultural Sciences,* 5, № 2, 28–31. DOI: https://doi.org/10.32718/ujvas5-2.04

(PhD participant in carrying out of experimental researches, processing of results , preparation of article for printing).

2. **Liu Mingcheng,** Xiaojing Xia, Xingyou Liu, Kasianenko Oksana (2021). Research Progress on the pathogenic mechanism of Streptococcus suis 2. *Scientific Messenger of LNU of Veterinary Medicine and Biotechnologies. Series Veterinary Science,* 23, № 104, 30–35. DOI: https://doi.org/*10.32718/nvlvet10405*

(The applicant participated in research, analysis of the results and writing the article).

3. Kasianenko O., **Mingcheng Liu** (2022). The molecular mechanism of pyroptosis and its related diseases. *Bulletin of Sumy National Agrarian University*, 2 (57), 16–26. DOI: https://doi.org/10.32845/bsnau.vet.2022.2.3

(The applicant participated in research, analysis of the results and writing the article).

4. **Liu Mingcheng** (2023). Primary culture and identification of mouse brain microvascular endothelial cells. *Scientific and Technical Bulletin of State*

Scientific Research Control Institute of Veterinary Medical Products and Fodder Additives and Institute of Animal Biology, 24, №1, 74–80. DOI: https://doi.org/10.36359/scivp.2023-24-1.11

(The applicant participated in research, analysis of the results and writing the article).

5. Kasianenko O., **Liu Mingcheng** (2023). Streptococcus suis infection (diagnosis, prevention and treatment). *Bulletin of Sumy National Agrarian University*, 3 (62), 125–130. DOI: https://doi.org/10.32782/bsnau.vet.2023.3.18

(The applicant participated in research, analysis of the results and writing the article).

Theses of scientific reports:

6. **Liu Mingcheng**; Kasianenko Oksana (2021). Extraction and reverse transcription of total RNA from mouse brain-derived endothelial cells.3 infected by *Streptococcus suis 2*. *Proceedings of the 5th Annual Conference*, Tallinn, Estonia, 39–41. DOI: https://doi.org/10.21303/2585-6634.2021.002153

PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).

7. **Liu Mingcheng,** Kasianenko Oksana (2021). Scientific research on surface proteins of Streptococcus suis type 2. *Materials of the Scientific and practical conference of teachers, graduate students and students of the Sumy National Agrarian University*. Sumy, 214.

(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).

7. **Люй Мінченг,** Касяненко О. (2022). *Екстракція загальної РНК з мікросудинних ендотеліальних клітин головного мозку білих мишей. Тези доповідей XX Всеукраїнської науково-практичної молодих вчених, присвяченої 90-річчю від дня народження доктора біологічних наук, професора, члена-кореспондента НААН, заслуженого діяча науки і техніки України Макара Івана Арсентійовича.* Львів, Біологія тварин, т 24, № 2, *52.*

PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).

8. **Liu Mingcheng,** Kasianenko Oksana (2022). *Gasdermin and its role in pyroptosis. III CISP Conference «Science of post-industrial society: globalization and transformation processes»*. Grail of science. Veterinary sciences, 17, 207–209. DOI: https://doi.org/10.36074/grail-of-science.22.07.2022.037

(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).

10. **Liu Mingcheng,** Kasianenko Oksana (202**2**). Screening of optimal multiplicity of infection and optimal infection time of mouse brain microvascular endothelial cells infected with Streptococcus suis 2. *Materials of the All-Ukrainian scientific conference of students and graduate students dedicated to the International Student Day*, Sumy, 163.

(PhD participant in carrying out of experimental researches, processin g of results, preparation of article for printing).

11. **Mingcheng Liu,** Kasianenko Oksana (2020). Epizootologycal investigation of swine epidemic diarrhea in Jiaozuo of Henan province, China *Materials of the Scientific*

and practical conference of teachers, graduate students and students of the Sumy National Agrarian University. Sumy, 231.

(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).

12. Mingcheng Liu, Kasianenko Oksana (2023). Mechanism of escape neutrophil extracellular traps from Streptococcus suis. *International scientific and practical conference of scientific and pedagogical workers and young scientists «Current aspects of the development of veterinary medicine in the conditions of european integration», dedicated to the 85th anniversary of the establishment of the Faculty of Veterinary Medicine of OSAU*, Odesa, 338. *(The applicant participated in research, analysis of the results and writing the these).*

Methodological recommendations:

13. **Liu Mingcheng,** Kasianenko Oksana (2023). Streptococcus suis infection (etiology, epidemiology, laboratory diagnosis, prevention and treatment). Scientific and practical recommendations. Sumy, 27 (Approved at the meeting of the Academic Council of the SNAU (Protocol № 18, dated 29.05. 2023).

Appendix B

Methodical Guidelines

Mingcheng Liu, Kasianenko Oksana (2023). Streptococcus suis infection (etiology, epidemiology, laboratory diagnosis, prevention and treatment). Scientific and practical recommendations. Sumy, 27 (Approved at the meeting of the Academic Council of the SNAU (Protocol № 18, dated 29.05. 2023).

Appendix C

Conclusion of the commission on bioethics in English

Laboratory Animal Welfare and Ethical review of Henan Institute of Science and Technology

Application Number: 202009-023 Application Date: September 10, 2020 Issue No.

<table>
<tr><td colspan="3">Program and No: Experimental research on pathogenesis of Streptococcus suis infection</td></tr>
<tr><td rowspan="2">Applicant</td><td>Name: Mingcheng Liu</td><td>Organization: College of animal science and veterinary medicine</td></tr>
<tr><td>E-mail: liumc80@163.com</td><td>Phone Number: 15893836036</td></tr>
<tr><td rowspan="2">Animals</td><td colspan="2">Animal Souce: Henan Animal Experiment Center</td></tr>
<tr><td colspan="2">Animal Grade(Normal、SPF or Others) : Normal</td></tr>
<tr><td colspan="3">(Experimental objective, necessity and significance and how the program has been designed to achieve the objectives of the research):
Objective: Through the detection of secreted cytokines, to determine whether there is the occurrence of pyroptosis during infection.
Necessity and significance: It is helpful to carry out accurate and targeted drug screening and vaccine development for meningitis.
Experimental steps:
1. The mouse brain microvascular endothelial cell were infected with SS2 and the cells were collected.
2. A series of tests are performed on infected cells, such as QPCR,WB, ELISA and electron microscopy.</td></tr>
<tr><td>Animal Care</td><td colspan="2">The nursing standards of ordinary mice were adopted</td></tr>
<tr><td rowspan="2">Animal Disposition</td><td colspan="2">Death conduct:
□ CO_2 suffocated ■ Exsanguinations with anesthesia
□ Cervical dislocation □ Anesthesia overdose
□ Other, detailed description</td></tr>
<tr><td colspan="2">Not for the death of the animal disposition:
□ Continue to use □ Save in the agency
□ Release to the wild
□ Others, detailed description</td></tr>
<tr><td colspan="3">Poisonous(harmful) material (infection, radiate, chemical poison and other) being used
□ Yes ■ No
Declare:</td></tr>
<tr><td colspan="3">Supplementary instruction for investigate
No</td></tr>
<tr><td colspan="3">Declaration for the information disclosure and confidentiality requirements, declaring the information need to be kept secret, the information can be disclosed
No</td></tr>
<tr><td colspan="3">Claiming jurors for being debarb
No</td></tr>
<tr><td colspan="3">Applicant's Declaration:
1. I will abide by the law and regulation stipulation, and accept the supervision and inspection by the committee and laboratory animal department.
2. The information I have given is accurate, detailed and comprehensive.</td></tr>
</table>

Applicant Signature(Seal): Xiaojing Xia

September 10, 2020

Approval opinions:

■ Approval □ Not approve

Authorized Personnel Signature (Stamp):

September 10, 2020

Appendix D

Laboratory Biosafety certificate

实验室生物安全证书

我校博士生刘明成，在开展研究：链球菌感染的发病机制的实验研究过程中，涉及病原微生物的实验。该生在实验过程中，严格遵守国家及学校生物安全相关规定，并根据《人间传染的病原微生物名录》（卫科教发：[2006]15 号）规定的生物安全防护和标准操作规程开展实验研究，并确保实验室生物安全。

Laboratory Biosafety Certificate

Mingcheng Liu, PhD student who involved in the use of pathogenic microorganisms in the research on Experimental research on pathogenesis of Streptococcus suis infection. During the experiment, He strictly abide by the relevant national and school biosafety regulations, and carry out experimental activities in accordance with the standard operating procedures in the biosafety protection level laboratory stipulated in the "List of Pathogenic Microorganisms Infected Between Heumans"（Wei Ke Jiao Fa:[2006] No.15）. And ensure laboratory biosafety.

Henan institute of Science and Technology

Date: September 6, 2021

Appendix E

ACT

on the implementation / use of the results of the dissertation work in the educational process

APPROVED BY

Vice-Rector of academic

and Educational work

_____ Ihor Kovalenko

September "22", 2023

ACT

on the implementation / use of the results of the dissertation work in the educational process

This act states that the results of the PhD Thesis (Specialty: 211 –veterinary medicine), which are presented in the scientific and practical recommendations "Streptococcus suis infection (etiology, epidemiology,laboratory diagnosis, prevention and treatment)", Protocol № 18, dated 29.05. 2023, authors: Mingcheng Liu, Kasianenko Oksana, introduced into the educational process during the teaching of the disciplines: "Epizootology and infectious diseases", "Veterinary technologies for the prevention of infectious diseases of animals", "Antiepizootic measures in animal husbandry" at the department of Epizootology and Parasitology in the training of specialists for with the degree of higher education "Master" in specialty 211 "Veterinary Medicine at the Sumy National Agrarian University.

Head of the Department of Epizootology and Parasitology
Sumy NAU,
Doctor of Veterinary Science, Professor Oksana Kasianenko