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Histopathological and cytopathological findings in minipigs infected with *Streptococcus suis* serotype 2

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Abstract

Five pathogen-free miniature pigs (minipigs) were infected with the virulent strain SH08 of *Streptococcus suis* 2 (SS2) by intramuscular injection. The pigs died consecutively within 72 h after the challenge. An additional five non-infected pigs were euthanised and used as controls. Microstructural observations showed that degeneration, bleeding, congestion, cellular necrosis, and an increase in inflammatory cells were present in all organs and tissues except the brain. Ultrastructural observations revealed mitochondrial vacuolation and malformed or missing cristae, indicating that infection of minipigs with strain SH08 of SS2 can lead to extensive lesions in major internal organs and tissues. The findings also demonstrated that the minipig is a useful model for the study of SS2 infection.

Key words: miniature pig, *Streptococcus suis* 2, experimental infection, histopathology, cytopathology.

Introduction

*Streptococcus sui*s infection is a zoonosis leading to meningitis, arthritis, endocarditis, septicaemia, and pneumonia in pigs. Although 35 serotypes have been described so far, serotype 2 (SS2) is the most prevalent type isolated from diseased pigs. Two outbreaks of SS2 infection have been recorded in China and it has resulted in human death (37). There have also been a few reports of SS2 infection in other countries in recent years (31, 16). It mainly affects people who come into close contact with infected pigs or raw pork products. SS2 has been recognised as an emerging zoonotic agent, which seriously threatens public health and food safety.

In previous studies, the virulence and pathology of the bacterium (2), airborne transmission (3), and virulence factors of infection were evaluated (9, 30, 34, 39). However, elucidation of the pathogenesis of SS2 remains a challenge. SS2-infected animal models are useful tools for investigating this virulent pathogen (11, 15, 18, 22, 33). However, in these models, the histopathological and cytopathological changes in major organs following SS2 infection have not been thoroughly and systematically reported. Understanding these changes is very important for further investigations of the pathogenic mechanisms. Therefore, in the study, different organs and tissues of miniature pigs infected experimentally with SS2 were investigated histopathologically and cytopathologically, and by electron microscopical examination.

Material and Methods

Animals. Ten clinically healthy, 40-day-old, female Chinese miniature pigs (Bama minipigs) were provided by the Experimental Animal Centre, Chinese Academic of Sciences (Beijing, China). The Bama minipig strain is characterised by a high inbreeding coefficient (32). All studies were performed with the approval of the Experimental Animal Committee at the Shanghai Jiao Tong University.

Bacterial strain. The SH08 virulent strain of SS2 was isolated in the Zoonosis and Comparative Medicine Laboratory, Shanghai Jiao Tong University,

China, as described previously (36). The presence of muramidase-released protein (MRP), extracellular factor (EF), suilysin, adhesin, glutamate dehydrogenase (GDH), and fibronectin-binding protein was confirmed by PCR assay (14, 21, 28).

Challenge. The animals were randomly divided into two equal groups. A 2 mL volume of the bacterial suspension (5×10^7 CFU/mL) (infected group) or 0.9% normal saline (control group) was administered by intramuscular injection into the neck. The reason for choosing intramuscular injection was the prolongation of the disease process in order for typical pathological changes to develop instead of the causing sudden death, which would have resulted from intravenous injection. The infected animals were housed separately and observed for clinical signs of the disease. The pigs died consecutively within 72 h after challenge. The control animals were euthanised by exsanguination.

Preparation of microscope sections. Samples of the heart, liver, spleen, lung, kidneys, tonsils, mesenteric lymph nodes, and brain of the minipigs were fixed in 4% neutral buffered formaldehyde for 24 h, dehydrated in alcohol, cleared in xylen, and embedded in paraffin. Paraffin blocks were cut into 6 μm sections, which were stained with haematoxylin and eosin.

Electron microscopy. For ultrastructural examination, 1.0 mm^3 tissue blocks were fixed in 2.5% glutaraldehyde (pH 7.2) for 24 h, followed by pre-cooled 2% osmium acid for 1 h, and then rinsed three times (10 min each) with phosphate-buffered saline (PBS). The samples were then dehydrated in a gradient series of ethanol solutions and embedded in Epon 812 using standard procedures. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined using a Hitachi 600H transmission electron microscope (Hitachi, Japan).

Ethics statement. This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. The protocol was approved by the Shanghai Animal Management Committee.

Results

Clinical signs were observed only in the infected group, with an onset 24 h after challenge. The infected pigs lost appetite, became apathetic, and their body temperatures rose to 38.8°C during 24 h and reached 40.1°C 36 h after challenge. Congestion and haemorrhages were found on the abdominal skin, and the joints of the posterior limbs were slightly swollen. The animals died within 36-72 h after challenge.

Gross lesions. The gross findings were characteristic of septicaemia. Subcutaneous haemorrhages were observed on the abdomen (Fig. 1a). During the autopsy, petechial haemorrhages were found to be widespread in all tissues (Fig. 1b). Pinpoint haemorrhages were seen on the endocardial surfaces (Fig. 1c). The liver was enlarged and its texture had become fragile (Fig. 1d). The spleen was enlarged, haemorrhagic, and dark red (Fig. 1e). Pulmonary oedema and pinpoint haemorrhages were seen on the surface of the lungs (Fig. 1f). Pinpoint haemorrhages were also seen on the surface of the kidneys (Fig. 1g). Scattered petechial haemorrhages were also present in the cortex of the kidneys (Fig. 1h). Swelling of the tonsils and bleeding points on their surface were found (Fig. 1i). Systemic lymphadenectasis and bleeding, particularly in the submandibular lymph nodes, inguinal lymph nodes, and mesenteric lymph nodes were seen (Fig. 1j). Bleeding points were found on the meninges of one animal (Fig. 1k).

Histopathological findings. Under a light microscope, the following lesions were observed in challenged animals: fibrinopurulent peritonitis; subcapsular hepatic necrosis and fibrinopurulent perihepatitis in the liver; necrosis and bleeding in the spleen and lymph nodes; fibrinopurulent pleuritis and embolic pneumonia; fibrinopurulent nephritis; heterophils in the crypt epithelium and lumen of tonsils; and diffuse fibrinopurulent meningitis, in one animal also with focal submeningeal encephalitis (Fig. 2).

Ultrastructural findings. The ultrastructure of organs in challenged animals showed cell edema, heterochromatinisation, dilation of the endoplasmic reticulum, chondriosomal swelling, and ridge disappearance. These lesions were not found in the control animals (Figs 3-10).

Discussion

In order to investigate the pathological effect of *Streptococcus suis* strain 2 (SS2) on the Bama minipig, some of these animals were infected with a Shanghai strain of SS2 (SH08), which was isolated in the Zoonosis and Comparative Medicine Lab., Shanghai Jiao Tong University, China. Clinical symptoms, necropsy, histopathology, and cytopathology were carried out to evaluate the utilisation of the pigs as a standard experimental animal model for studying SS2.

There are many reports on the mechanism of the pathogenic action of SS2 (7, 23, 34, 35, 38). However, this mechanism *in vivo* is still unknown, and one of the major reasons is that a standard animal model is still unavailable. Therefore, it is of great importance to establish proper animal models to understand the pathogenicity mechanism of this bacterium. Most of the investigations carried out to date on SS2 have utilised mice, guinea pigs, piglets, and other animals to establish models. Although attempts have been made to find other animal models, the recent studies demonstrate that SS2 exhibits species-specificity. Mice are not suitable for investigations the virulence and

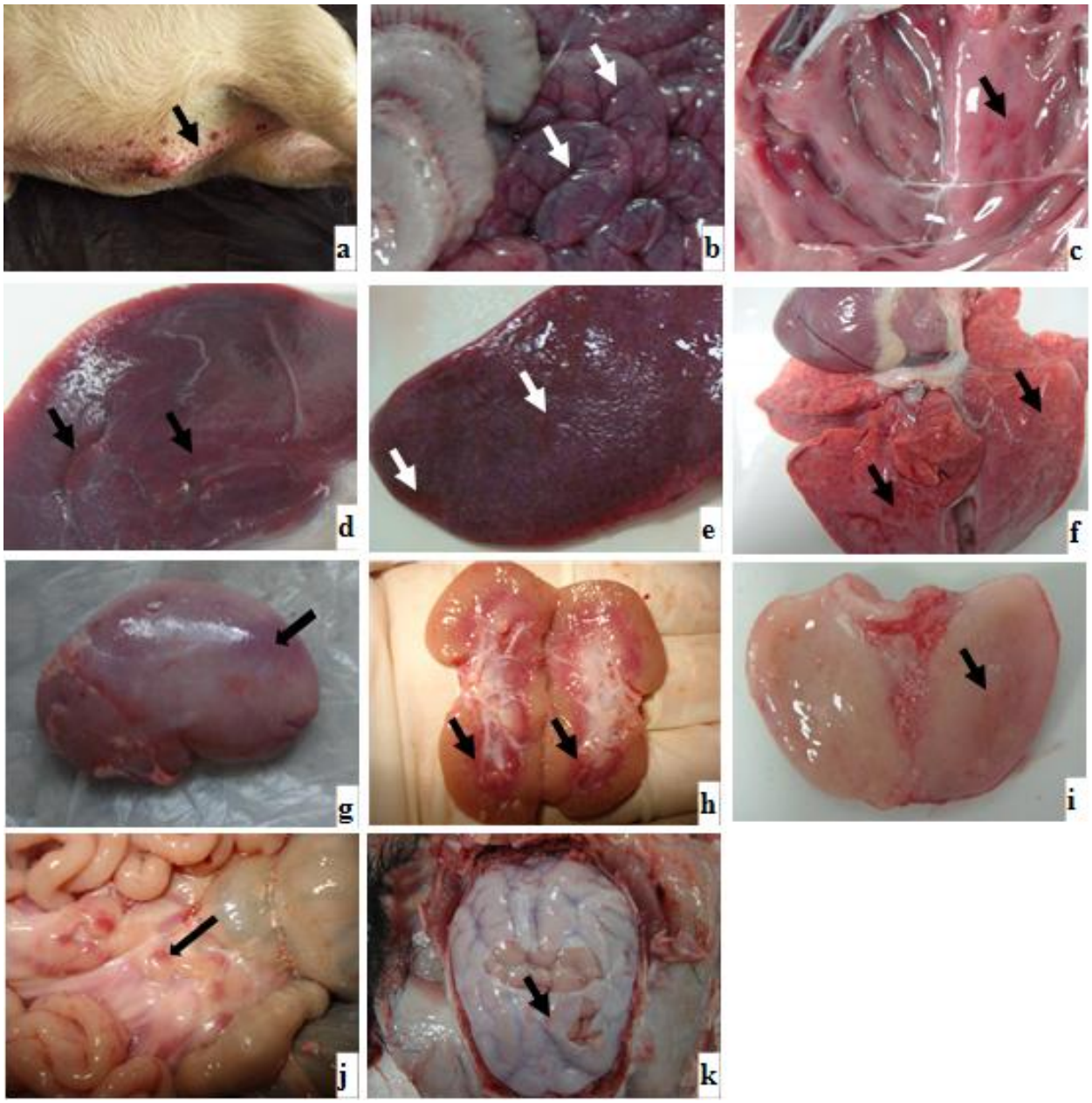


Fig. 1. Gross lesions of organs 72 h after challenge. a. Subcutaneous haemorrhages in the abdomen; b. Petechial haemorrhages, characteristic of septicaemia, widespread in all abdominal tissues; c. Pinpoint haemorrhages on the endocardial surfaces; d. Enlarged liver; e. Enlarged, haemorrhagic, and dark red spleen; f. Pulmonary oedema and pinpoint haemorrhages on the surface of the lung; g. Pinpoint haemorrhages on the surface of the kidneys; h. Scattered petechial haemorrhages in the cortex of the kidney; i. Swelling of tonsils and bleeding points on their surface; j. Lymphadenectasis and bleeding in the lymph nodes; k. Bleeding points on the meninges
Note: arrows indicate haemorrhagic lesions

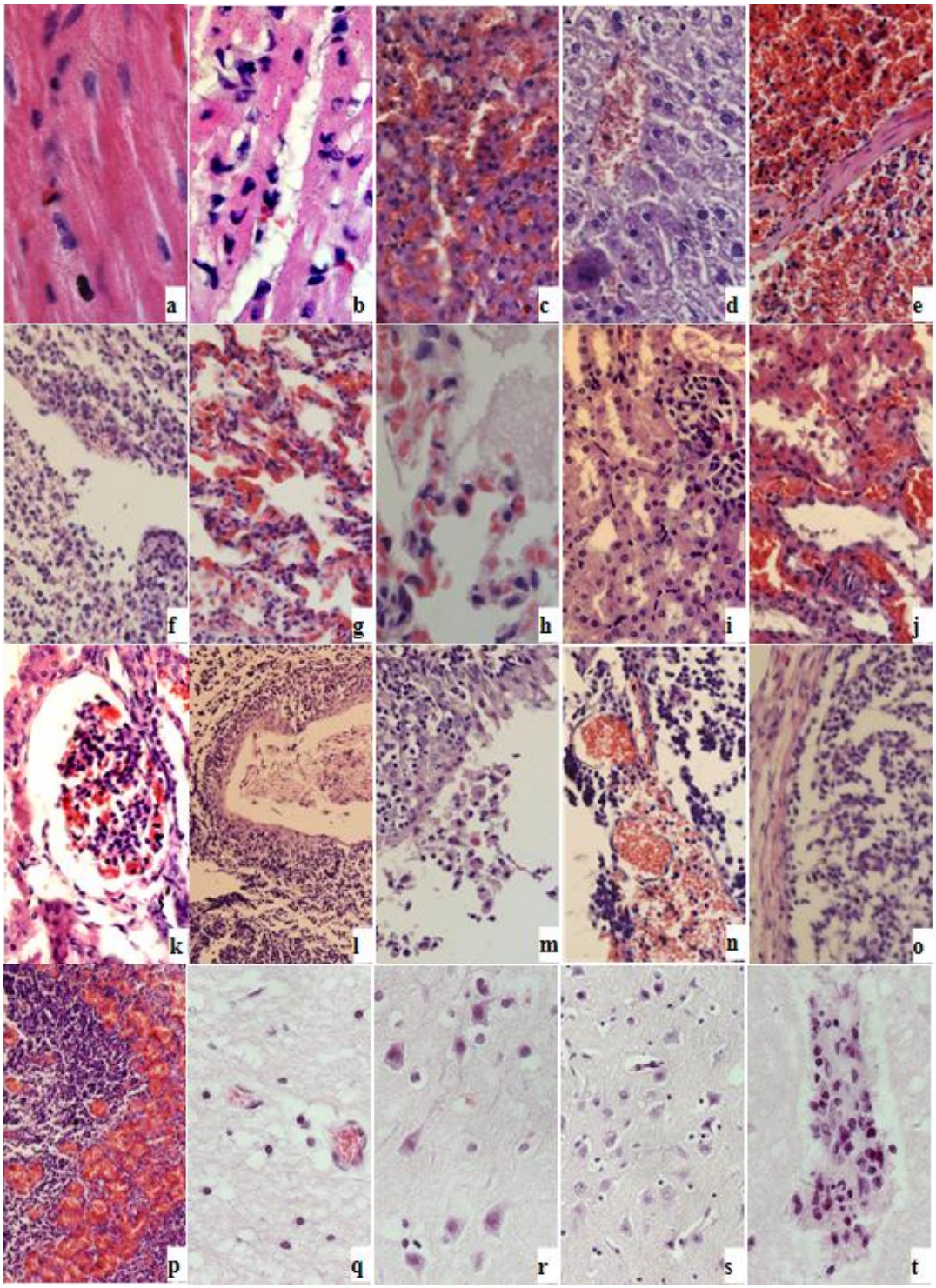


Fig. 2. Histopathological findings in different organs 72 h after challenge**.** a (heart). Damaged structures of myocardial tissues, no remaining intercalated discs, denatured granules of myocardial cells, swollen myocardial cells, large amount of red protein particles is in the cytosol. 1000×. b (heart). Vacuolated degeneration of myocardial cells, swollen and lightly stained myocardial cells. 1000×. c (liver). Damaged structure of hepatic lobules, no remaining cord-like structures, hepatic sinusoids stretched and filled with erythrocytes and other blood cells. 400×. d (liver). Solidification and karyolysis of nuclei, swollen, vacuolated, and denatured hepatic cells, congestion of central veins. 400×. e (spleen). Large areas of haemorrhages with mixed red and white pulp, lymphatic nodules not obvious, neutrophil infiltration and lympho-cytopenia. 400×. f (spleen). Necrotic lesions. 400×. g (lung). Congestion of interalveolar septa, oedema, inflammatory cell infiltration, decrease in pulmonary alveoli space. 400×. h (lung). Large amounts of fibrinous effusion, scarce amounts of neutral inflammatory granulocytes, and exudative alveolar epithelial cells in the alveolar interstitium. 400×. i (kidney). Swollen glomeruli and completely filled Bowman's space. 400×. j (kidney). Haemorrhagic renal tubule. 400×. k (kidney). Swollen tubular epithelial cells, thickened tube wall, and some detatched endothelial cells in some areas of the renal tubules. 400×. l (tonsil). Large amounts of fibrinous effusion in tonsillar crypts. 200×. m (tonsil). Detached cryptic epithelium dropped into tonsillar crypts. 400×. n (tonsil). Large number of necrotic erythrocytes and lymphocytes in blood vessels. 400×. o (mesenteric lymph node). Bleeding and congestion. 400×. p (lymph node). Necrosis of cortical and medullar lymphocytes, nuclear disruption and karyolysis. 400×. q (cerebrum). Congestion of small vessels. 400×. r (brain). Slight degeneration of neurons. ×400. s (brain). Increased number of colloid cells. 200×. t (brain). Necrotic lesions in some nervous tissues. 400×

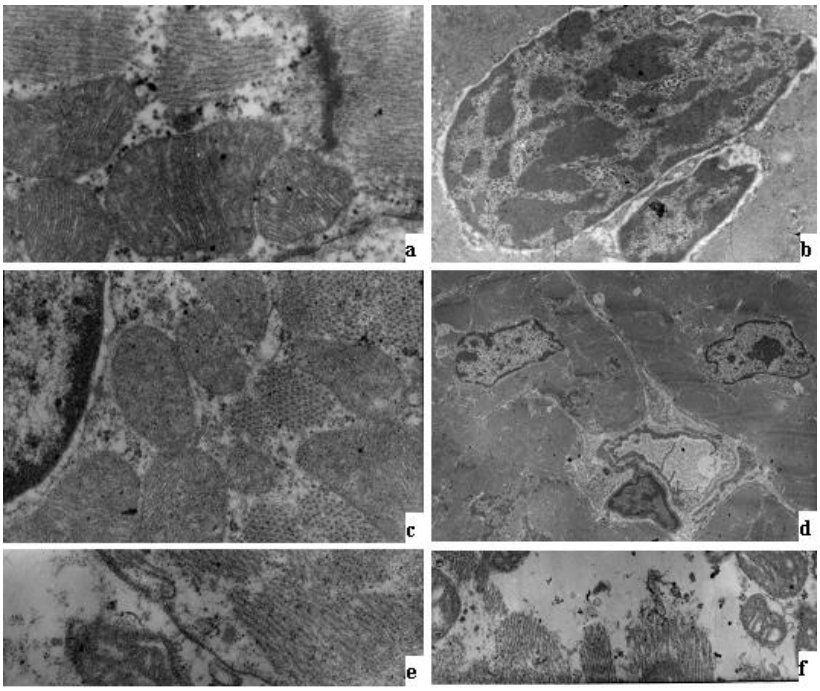


Fig. 3. Ultrastructural findings in the heart 72 h after challenge. a. Regularly arranged muscle fiber bundles and electronically dense intercalary discs in normal hearts. 30 000×. b. Oval-shaped nuclei of myocardial cells, uniform chromatin, and intact double membrane structure. 10 000×. c. Unclear cristae in most mitochondria. 10 000×. d. Deformed and heterochromatinised nuclei in some myocardial cells. 30 000×. e. irregularly arranged some muscle fiber bundles. 30 000×. f. disrupted some muscle fiber bundles. 20 000×

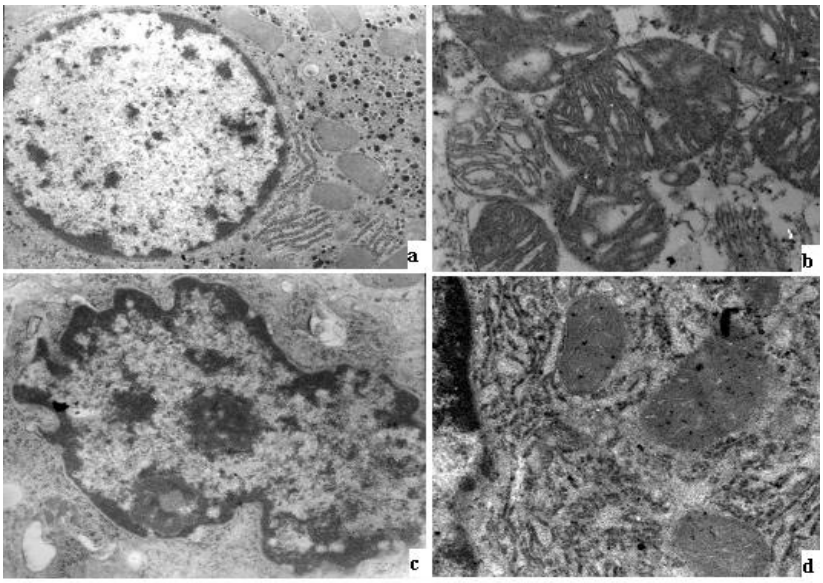


Fig. 4. Ultrastructural findings in the liver 72 h after challenge. a. Uniformly distributed nuclear chromatin of hepatic epithelial cells in normal liver and regular shape of these cells. 10 000×. b. Distinct mitochondrial cristae and double membrane structure. 40 000×. c. Markedly deformed some hepatic epithelial cells with heterochromatinised nuclei. 11 000×. d. Swollen mitochondria, disappeared cristae, and unclear structures. 40 000×

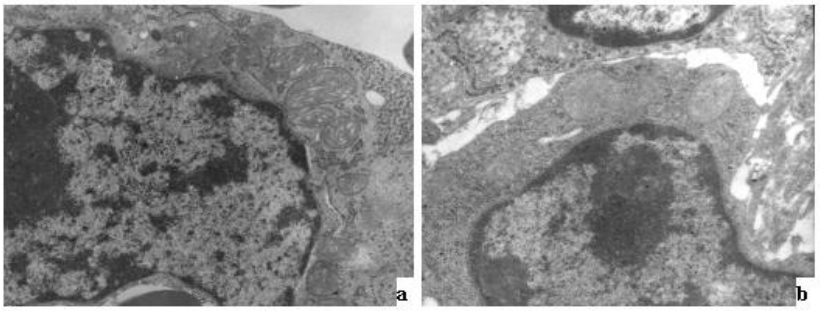


Fig. 5. Ultrastructural findings in spleen lymphocytes 72 h after challenge. a. Double membrane structure in the mitochondria of normal spleen lymphocytes. 20 000×. b. Swollen mitochondria, disappeared cristae, and only a blurred outline could be identified. 20 000×

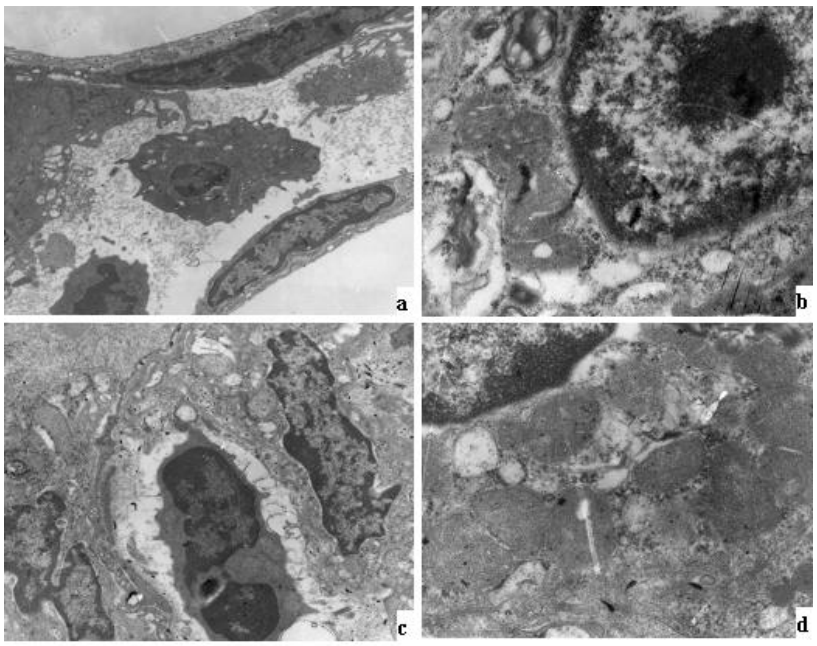


Fig. 6. Ultrastructural findings in the lung 72 h after challenge**.** a. Flat and thin nuclei of normal alveolar epithelial cells, distinct nucleoli, and uniform chromatin in the normal lung. 6000×. b. Relatively distinct structures of mitochondria and visible double membrane structure. 20,000×. c. Significantly deformed alveolar epithelial cells with heterochromatinised nuclei 8000×. d. Absent mitochondrial cristae and only a blurred identifiable outline 30,000×

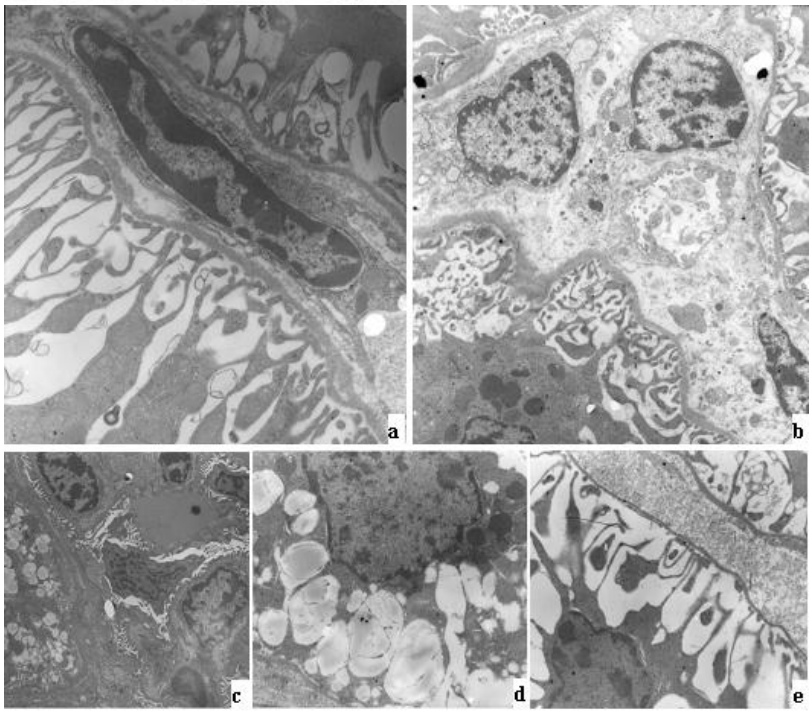


Fig. 7. Ultrastructural findings in the kidneys 72 h after challenge. a. Flat and thin nuclei of renal tubular epithelial cells in normal kidneys, chromatin distributed in the peripheral areas of nuclei, glomerular basement membrane relatively thin, and foot processes obvious and regular. 10,000×. b. Round endothelial cells and karyoplasm uniformly distributed. 6000×. c. Deformed renal tubular endothelial cells with heterochromatinised nuclei. 4000×. d. A large number of vacuoles in the cytosol. 8000×. e. Fused foot processes and thickened basal membrane. 10,000×

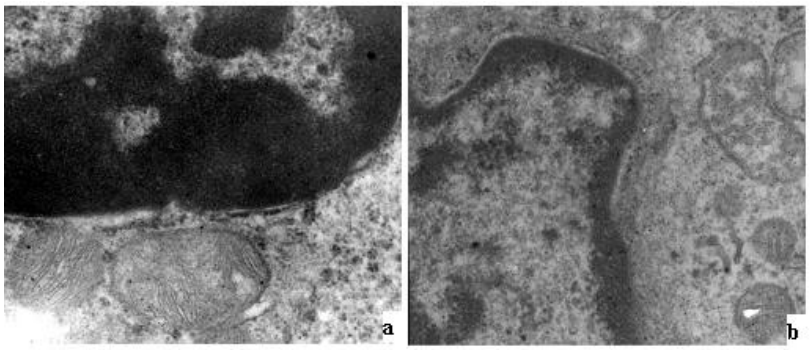


Fig. 8. Ultrastructural findings in tonsil lymphocytes 72 h after challenge. a. Double membrane structure in the mitochondria of normal tonsil lymphocytes. 30,000×. b. Swollen mitochondria and absent cristae, only a blurred outline. 10,000

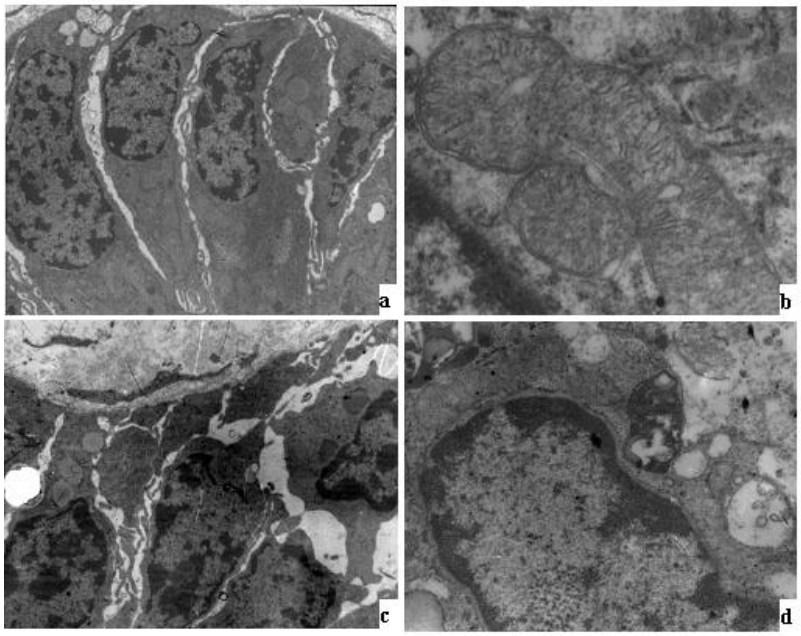


Fig. 9. Ultrastructural findings in mesenteric lymph nodes 72 h after challenge. a. Oval and regularly arranged endothelial cells of normal mesenteric lymph nodes with chromatin uniformly distributed. 6000×. b. Distinct mitochondrial cristae and double membrane structure. 40,000×. c. Deformed endothelial cells with heterochromatinised nuclei and chromatin accumulated in peripheral areas of the cells. 6000×. d. Swollen, vacuolated, or absent mitochondria. 30,000×

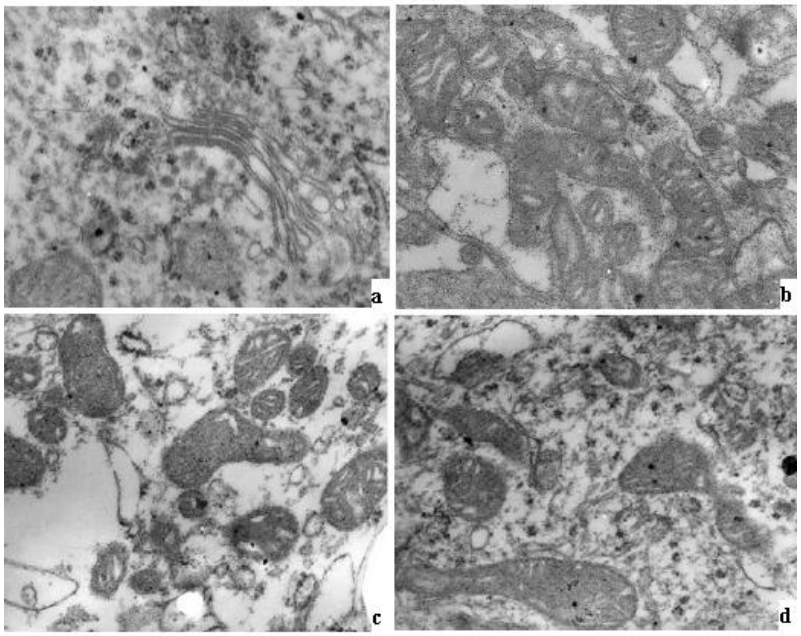


Fig. 10. Ultrastructural findings in brain colloid cells 72 h after challenge**.** a. Abundant organelles in the cytosol of normal brain colloid cells: smooth endoplasmic reticulum, lysosomes, and mitochondria. 30,000×. b. Mitochondrial cristae and a double membrane structure. 30,000×. c. Only mitochondria are visible, and the smooth endoplasmic reticulum is seldom seen. 30,000×. d. Some swollen and deformed mitochondria, absent cristae, and lack of double membrane structure. 30,000×

pathogenic mechanisms of SS2 (4). Although rabbits can be infected with SS2, their morbidity and mortality are extremely irregular, and thus they are not suitable for use as models (27). Wu *et al.* (33) infected guinea pigs with the SS2 strain isolated in Jiangsu and established an animal model for study of encephalitis and septicaemia. They found that the incubation period in guinea pigs was delayed as compared with that of piglets, which may suggest that guinea pigs are less susceptible than piglets.

Piglets are the best models for investigations of SS2. However, a requirement is that recessive germ-carrying animals must be excluded, as it has been reported by many previous investigators (2, 3, 5, 10, 11, 15, 18, 22). However, the Bama minipigs used in our studies came from a closed population, with a well-established background. Tests for streptococcal antigen and antibody detection were negative. Previous investigations have shown that infection of Bama minipigs with SS2 can well represent natural infections. Furthermore, minipigs are small and convenient for manipulation, thus making them suitable for investigations of the virulence and pathogenic mechanisms of SS2 (29).

Isolates of SS strains differ significantly in their pathogenicity. The strain that was used in the study was isolated from infected pigs in Shanghai. The PCR was able to detect lysozyme-releasing protein, extrinsic factors, erythrocytolysin, adhesin, glutamate dehydrogenase, and fibronectin-binding protein. The animal model of SS2 was successfully reproduced in Bama minipigs by artificial infection. Relatively more

cases have been reported regarding the symptoms of SS-induced meningitis (2, 8, 19, 26); however, the SH08 strain used in the study led mainly to septicaemia and arthritis in pigs, with only one animal showing symptoms of meningitis, which was a different outcome to that associated with the ZY05719 and HA9801 strains in China. These two velogenic strains mainly induced symptoms of meningitis and toxic shock syndrome, which can lead to the death of great numbers of infected pigs, as well as human infection and death (12, 13, 25).

The pigs died consecutively within 72 h after challenge, indicating that the virulence of the SH08 strain was very high. We carried out other experiments to infect Bama minipigs with identical infectious doses of the SH08 and ZY05719 strains. The animals that were infected with the SH08 strain died, whereas the animals infected with ZY05719 strain survived, demonstrating only slight clinical symptoms (data not shown). We have speculated that virulence factors of SH08 strain must obviously be different than those of ZY05719 strain.

Lesions in the lungs, in the form of suppurative, fibrinous, and interstitial pneumonia, are frequently seen in animals infected by *Streptococcus suis* (7, 16, 20). Fibrinous pneumonia and interstitial pneumonia were detected as pathological impairments in the lungs in the present study, which also explains respiratory distress in some infected pigs in clinical investigations. However, granular and vacuolated degenerations of myocardial cells have seldom been seen in previously reported cases.

Among all minipigs that were subjected to experiments in the present study, slight meningitis lesions were only found in one animal. It is generally considered that the SS2 strain initially attaches to the surface of the mucosal membranes of the tonsils and then penetrates the tonsils and upper respiratory tract barriers, finally reaching the central nervous system *via* blood circulation (1). This causes the production of copious amounts of cytokines under local stimulation, with a large amount of inflammatory effusion accumulating in the central nervous system and leading to an increase in intracranial pressure and typical symptoms of meningitis (1). However, the ability of pathogenic strains to penetrate the blood-brain barrier is related to many factors, such as virulence factors, cytokines, and others. Vadeboncoeur *et al.* (24) reported that SS2 strain could induce endothelial cells in the human brain microvasculature to produce IL26, IL28, MCP21, and other cytokines. Furthermore, infection with SS2 increases the permeability of endothelial cells in the human brain microvasculature at important positions of the blood-brain barrier, or even leads to cell disruption. Thus, SS2 could penetrate the intercellular layer, finally causing typical meningitis lesions, such as cerebral oedema, increased intracranial pressure, blood flow obstruction, and other symptoms (24).

In ultrastructural observations, we found that SS2 mainly induced lesions that were related to nuclei and mitochondria. The major functions of mitochondria are oxygenolysis by utilising their nutrients and synthesis of large amounts of adenosine triphosphate (ATP) by utilising the released energy to satisfy the requirements for organisms' metabolic activities (9). It can be assumed that when the structure of mitochondrial membranes is damaged, mitochondria become swollen or disappear, which may disrupt the normal progression of the tricarboxylic acid cycle, lead to functional disturbance in metabolism, and thus inevitably affect the physiological functions of the organism. Therefore, cells undergo degeneration, cellular necrosis, and other pathological changes, and the normal physiological functions of organs and tissues, particularly those requiring more energy, are affected. Thus, myocardial cell degeneration, cellular necrosis, or even myocarditis may be induced.

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