The Antiviral Activity of Kaempferol Against Pseudorabies Virus in Mice

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Research article

Keywords: Kaempferol, Pseudorabies virus, Antiviral activity
Abstract

Background: Pseudorabies virus (PRV), a member of the neurotrophic Alphaherpesviruses, is one of the most important pathogens that harm the global pig industry. Accumulated evidence indicated that PRV could infect humans under certain circumstances, inducing severe clinical symptoms such as acute human encephalitis. Currently, there are no antiviral drugs to treat PRV infections, and vaccines available only for swine could not provide full protections. Thus, new control measures are urgently needed.

Results: In the present study, kaempferol exhibited anti-PRV activity in mice through improving survival rate by 22.22% at 6 days post infection (dpi) which is higher than acyclovir (Positive control) with the survival rate of 16.67%, and in the infected-untreated group the survival rate was 0%. Kaempferol could inhibit the virus replication in the brain, lung, kidney, heart and spleen, especially in the brain the viral gene copies were reduced by over 700-fold, which is further confirmed by immunohistochemical examination. The tissue lesions induced by PRV infection in these organs were also alleviated. The expressions of the early genes EPO and TK in the brain were significantly inhibited by kaempferol. The expression of latency-associated transcript (LAT) were also inhibited in the brain, which suggested kaempferol could inhibit the PRV latency. Kaempferol-treatment could induce higher levels of IL-1β, IL-4, IL-6, TNF-α and IFN-γ in the serum at 3 dpi which were then declined to normal levels at 5 dpi, suggesting that kaempferol could exert anti-PRV activity through regulation of the immune function.

Conclusions: These results suggest that kaempferol is expected to be a new alternative control measure for PRV infections.

1. Background

Pseudorabies virus (PRV), a member of the neurotrophic Alphaherpesviruses, is the causative agent of pseudorabies, also called Aujeszky's disease (AD) [1]. Apart from the direct effects of the disease, the trading of PRV-infected pigs and their products are globally restricted. Most mammals are susceptible to PRV, including ruminants, carnivores and rodents, and infections often lead to death except pigs which are the only natural hosts for PRV [2]. Whether PRV is able to infect humans is still controversial. In 1914, suspected human infections with PRV were first reported, then there were 8 human cases of infection with PRV (22 humans) over the past 100 years [3]. Recently, four cases of acute human encephalitis caused by PRV variant strain were reported and a PRV strain was firstly isolated from patient [4]. Thus, there is increasing evidence that PRV could infect humans under certain circumstances. In addition to concerned about the control of PRV infection, PRV has been used as a model organism to study the molecular biology of herpesviruses [5]. The PRV-infected pigs exhibit a variety of clinical symptoms, including high mortality in piglets, growth retardation in adult pigs, and reproductive failure of sows, which causes substantial economic losses to swine industry [6]. PRV usually induces latent-infection in adult pigs, and the hosts behave normally and show no clinical symptoms [7]. However, once the host is stressed or its resistance becomes reduced, PRV can be re-activated and a large number of virions were produced to infect the central nervous system [8], causing the re-emergence of PRV infection, even the
host becomes a source of infection [9]. The PRV infections are mainly achieved by controlling the transcription levels. PRV’s gene expression can be divided into three stages: immediate early gene (IE), early gene (E) and late gene (L). In those stages, IE180 is the only immediate early gene expression product that is an effective transcriptional activator of viral genes involved in DNA replication and RNA transcription [10]. The early genes that have been reported are only EP0, TK and UL54 [11–13]. EP0 is expressed as an early protein in the PRV lifecycle which can transactivate viral promoters, such as IE180, TK and gG [14]. The viral gene is not expressed during latent infection, and only a segment of the non-coding region of PRV genome transcribes the latency-associated transcript (LAT) [15].

Although AD has been eradicated from domestic pig populations in the United States and some European countries, PRV epidemic is especially prominent in regions of South America and Asia [16]. At present, there are no effective drugs for treating PRV infections, and vaccination is still the main measure to prevent AD. Many vaccines against PRV have been successfully developed including inactivated and attenuated vaccines and genetically engineered marker vaccines [17; 18]. Although vaccines can effectively control the virus spread, it doesn't protect against infections by variant strains, PRV excretion and establishment of latency of wild-type virus [19]. Since 2011, the outbreaks of PRV variants in Bartha-K61 vaccinated pig herds have been constantly reported, and the origin of these variant viruses remains to be addressed [20]. The variability of viral strains and the insecurity of attenuated vaccines are a serious threat to the pig industry. Thus, newly control measures are urgently needed.

Kaempferol, 3, 5, 7 - trihydroxy – 2 - (4-hydroxyphenyl) – 4H – 1 - benzopyran – 4 - one, is a natural flavonol which is mainly derived from the rhizome of the ginger family and present in different plant species, such as tea, broccoli, purple cabbage, beans, chicory, leeks, tomatoes, strawberries and grapes [21; 22]. It is a tetrahydroxy flavone in which the four hydroxy groups are located at positions 3, 5, 7, and 4′ [23]. It has received widespread attention because of its anticancer, anti-inflammatory, antioxidant, antibacterial and antiviral effects as well as treatment of diabetes and osteoporosis [24; 25; 26; 3]. Previously, we found that kaempferol possesses the ability to inhibit PRV replication in a dose-dependent manner in vitro with a 50% inhibitory concentration of 25.57 µM. In the present study, PRV infected mice were established for evaluation of anti-PRV potency of kaempferol in vivo for the purpose of development of complementary control measure for PRV infections.

2. Results

2.1. Survival rate

After artificial infection of healthy mice for 2 days, symptoms similar to natural infections appeared. The mice began to show itching symptoms. The survival rates of mice in each group were shown in Fig. 1. There were no deaths in each group at 2 dpi. At 3 dpi, except for the uninfected-untreated and KM-H groups, the infected mice in other groups began to die. The survival rates of the KM-L, KM-M and infected-untreated groups were below 50% at 4 dpi; in the KM-H and acyclovir groups, the survival rates were 50% and 57.69%, respectively. The KM-H group exhibited the highest survival rate (36.36%) at 5 dpi, while the
survival rate of the acyclovir group was 31.81%. At 6 dpi, all mice in the KM-L and infected-untreated groups died. The survival rate of the KM-H group was 22.22%, in contrast, the survival rate of acyclovir group was 16.67%. These results suggested that kaempferol at the dose of 240 mg/kg had higher activity to protect mice from PRV- induced death than acyclovir did.

2.2 Organ coefficient

Organ coefficient is the ratio of the organ weight to the body weight, which is important for identification of potentially harmful effects of various pathogens. The results of organ coefficients were shown in Table 2. PRV infection significantly decreased the lung coefficient at 3 dpi, which was significantly increased by acyclovir-treatment. At 4 dpi, PRV infection significantly decreased the spleen coefficient, and the brain coefficient of the KM-H group was significantly increased. Acyclovir-treatment significantly decreased the spleen coefficient at 5dpi.

2.3. Virus load

The virus load in heart, spleen, liver, lung, kidney and brain were reflected by viral gene copies which were determined through FQ-PCR. As can be seen from Fig. 2, with the increase of infection time, the viral loads of the infected-untreated group showed an upward trend in liver, lung and brain. The viral loads reached the peak at 4 dpi in heart, spleen and kidney. After treated with acyclovir and kaempferol, the viral loads were significantly reduced in the test organs. In liver, lung and brain, the viral loads of kaempferol-treated groups were significantly lower than that of acyclovir-treated group, and kaempferol at a dose of 240 mg/kg exhibited the highest potency.

As the gene copies of PRV in the brain are the highest, immunohistochemical examination is employed for further detection of virus reproduction in the brain of PRV-infected mice. The brain tissue slides were stained by SABC method, and the SABC staining positive cells mainly showed brown and the section background mainly showed light yellow. As shown in Fig. 3, there were no obvious positive cells in the uninfected-untreated group and the number of positive cells in the infected-untreated group was higher than those of the treated groups, which showed the consistent results with the viral load study.

2.4. Histopathological examination

Viral infections usually cause damages to healthy tissues, thus the organs of PRV-infected mice including heart, liver, spleen, lung, kidney and brain in each group at 5dpi were further subjected to histopathological examination, and the following pathological changes were found.

Heart: the myocardial fibers in the infected-untreated group were disordered and a large number of myocardial fibers were broken (Fig. 4B); localized myocardial fiber arrangement was slightly disordered and broken in the acyclovir-treated group (Fig. 4C); the blood vessel wall of the heart was completely shed, and most of the myocardial fibers were broken in the KM-L group (Fig. 4D); In the KM-M (Fig. 4E) and KM-H (Fig. 4F) groups, varying degrees of disordered and broken myocardial fibers and localized vacuolar degeneration were observed; the uninfected-untreated group showed no obvious lesions (Fig. 4A).
Liver: in the infected-untreated group, blood vessel walls were severely shed, liver cells were concentrated and swollen, liver cords were disorderly arranged, and the liver sinusoids became narrowed and diffused (Fig. 4H); the structure of hepatic cord was partly disappeared in the KM-L group (Fig. 4J); the KM-M (Fig. 4K), KM-H (Fig. 4L) and acyclovir (Fig. 4I) groups showed slightly localized separation of blood vessel wall, and the liver cells were slightly swollen and diffused; the uninfected-untreated group showed normal structure of liver (Fig. 4G).

Spleen: the red pulp area of the infected-untreated group was significantly reduced with vacuolar degeneration, and the white pulp area had abnormal and unclear boundaries (Fig. 4N); the red pulp area of the acyclovir group was decreased and the structure of the white pulp became unclear (Fig. 4O); the area of the red pulp in the KM-L group (Fig. 4P) was reduced, and the boundaries of the white pulp area were unclear; the area of white pulp in the KM-M group decreased, and slight vacuolar degeneration occurred (Fig. 4Q); the structure of the red pulp and white pulp in the KM-H group was normal, and only slight vacuolar degeneration occurred (Fig. 4R); the uninfected-untreated group showed normal structure of spleen (Fig. 4M).

Lung: PRV infection induced massive bleeding, inflammatory cell infiltration, and thickened alveolar septum in the infected-untreated group (Fig. 4T); After treatment with acyclovir and kaempferol, the lesions were alleviated (Fig. 4U-4X); the uninfected-untreated group showed normal structure of lung (Fig. 4S).

Kidney: in the infected-untreated group, the cortical area is congested, and a large number of renal tubular epithelial cells are shedding and swollen (Fig. 4Z); in the acyclovir group, the cortical area is mildly congested and the glomeruli are slightly swollen (Fig. 4ZA); localized congestion and slightly shedding of renal tubular epithelial cells could be observed in the kaempferol-treated groups (Fig. 4ZB-ZD); the uninfected-untreated group showed normal structure of kidney (Fig. 4Y).

Brain: a small amount of cell edema occurred in each group (Fig. 4ZF-ZJ); the uninfected-untreated group showed normal structure of brain (Fig. 4ZE).

The pathological changes of each group were scored for tissue lesions. The results (Table.4) showed that compared with the infected-untreated group, kaempferol had different degrees of therapeutic effects on PRV-induced tissue lesions.

### 2.5. The changes of serum cytokines

Cytokines are involved in the immune response and inflammatory response process, which play a key role in protecting the body from foreign pathogens. TNF-α, IL-1β, IL-6 and IFN-γ, belonging to the pro-inflammatory cytokines, are involved in promoting acute inflammation to defense against infection [27].

PRV infection induced increased levels of TNF-α, IL-1β, IL-6 and IFN-γ. After treatment with kaempferol, the levels of IL-1β, IL-6 and IFN-γ (Fig. 5) were increased at 3 dpi in comparison with the uninfected-untreated and infected-untreated groups, and then gradually declined to normal levels at 4 and 5 dpi. The high dose of kaempferol group (240 mg/kg) still maintained higher levels of IL-1β and IFN-γ at 5 dpi. The TNF-α
concentration showed a normal level in the treated groups except the high dose of kaempferol group (240 mg/kg) at 5 dpi. As one of the important anti-inflammatory factors, IL-4 can help fight viral infections. PRV infection increased the IL-4 level (Fig. 5). The kaempferol-treated groups had a significantly higher IL-4 levels in comparison with the uninfected-untreated and infected-untreated groups at 3 dpi, and then declined to the level of the infected-untreated group at 4 and 5 dpi (Fig. 5).

2.6. Transcription levels of EPO, TK and LAT

Viral gene transcription is the key step of virus replication, thus it is an effective way to inhibit virus multiplication through blocking gene transcription. The EPO and TK are the early genes of PRV [28], which were significantly inhibited in the brain at 5dpi (Fig. 6). LAT (latent related transcript), a large amount of RNA that is present and transcribed during the incubation period of PRV, plays an important role in the establishment, maintenance, and reactivation of PRV latency [29]. Compared with the infected-untreated group, kaempferol significantly reduced the expression of LAT, but acyclovir could not inhibit the LAT transcript (Fig. 6). Kaempferol showed higher inhibitory effects than acyclovir.

3. Discussion

Since the 1980s, the spread of AD worldwide has inevitably caused serious economic losses [30]. The farms in China currently rely on vaccination to prevent and treat AD, including inactivated vaccines, attenuated vaccines and gene-deleted vaccines [17]. However, during the past decades, the PRV strain has mutated, and the existing vaccines have been unable to control all infections, which eventually led to the repeated outbreak of AD. Recent case reports of human PRV infections suggested possible transmission to the humans [4]. Therefore, development of alternative control measures is still the main task at present. Kaempferol is a very common dietary flavonoid compound that has inhibitory effects on several viruses, such as bovine herpesvirus 1 [31], HSV [32], Japanese encephalitis virus [33], and enterovirus 71 [34].

In this study, kaempferol could effectively protect mice from PRV infection through improvement of survival rate by 22.22% (Fig. 1). In addition to pigs, many mammals are susceptible to PRV, and often died after infection. Mice would die quickly after being infected with PRV, and the mortality rate is mostly 100% within 3–7 dpi [35]. The same result was observed in this study that the mice in the infected-untreated group had all died at 6 dpi. Acyclovir and its analogues are the most frequently used drugs approved for the treatment of HSV, which can inhibit the viral DNA polymerase [36]. Aciclovir also showed antiviral activity against other herpesviruses, such as varicella zoster virus, Epstein-Barr virus, cytomegalovirus and human herpesvirus 6 [37], thus it was served as positive control. In recent human cases, acyclovir were used for antiviral treatment of PRV-induced acute human encephalitis [4]. In this study, acyclovir exhibited antiviral activity against PRV in mice by improvement of 16.67% survival rate, but it is lower than kaempferol which suggested a potential application of kaempferol in control of PRV infections in animals and humans.
Viral load is a direct parameter in the evaluation of antiviral effects in vivo, which can reflect the virus replication in different organs [38; 39; 40; 41]. In this study, PRV reproduction in the test organs, including brain, heart, spleen, lung and kidney, were observed. The results (Fig. 2) showed that PRV replicated most in brain, it was about 50-fold than other test organs. The reason could be attributed to PRV belonging to a neurotropic alphaherpesvirus which can invade the CNS via the trigeminal nerve, as well as by sympathetic and parasympathetic pathways [42]. The main feature of PRV infection is encephalomyelitis, which is often accompanied by inflammation of the upper respiratory tract and lungs [43]. Kaempferol could significantly inhibit virus replication in the test organs, especially in the brain that the viral gene copies were decreased by more than 700-fold. In contrast, the acyclovir showed lower ability to inhibit virus reproduction than kaempferol, and the viral gene copies were decreased by about 10-fold in the brain. The immunohistochemical study also demonstrated that the amount of progeny virus in the brain was markedly decreased after kaempferol or acyclovir treatment (Fig. 3). In the antiviral effects of resveratrol in piglets infected with PRV, resveratrol can significantly inhibit the virus titer in the brain, and the levels of viral copies in the brain were positively linked to the clinical parameters of infected piglets [44]. Kaempferol were found effective to delay and inhibit the clinical symptoms in the PRV-infected mice which may attributed to inhibition of virus replication in the brain.

After mice were infected with PRV, histopathological analysis found that the brain, spleen, and liver tissues had different degrees of lesions [45]. Studies have shown that PRV could cause lethal respiratory disease in an animal model of PRV-infected BALB/c mice [46]. In the present study, mild tissue damages were observed in the PRV-infected mice after treated with kaempferol (Fig. 4), indicating that kaempferol can alleviate the histopathological changes.

Although the innate immune system is the first line of defense by triggering an inflammatory response to prevent the spread of viral infections, the main challenge is to ensure that inflammation is resolved [35], then the body's homeostasis can be restored to normal. When the inflammatory response is uncontrolled, it usually leads to more severe inflammation, which may cause damages to the host [47]. In this study, the levels of pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α and IFN-γ, were elevated after infection, suggesting the innate immunity was activated by PRV. After kaempferol treatment, the levels of these pro-inflammatory cytokines were higher than the infected control at 3 dpi, and then almost declined to the normal level at 5 dpi, suggesting that kaempferol could enhance the inflammatory responses at the early stage of infection to inhibit viral replication, then recovered it to normal to avoid severe inflammation. The anti-inflammatory cytokine IL-4, produced by Th2 cells, NKT cells, basophils and mast cells, has a wide range of Immunological functions, such as regulating the function of macrophage [48; 49]. Studies have shown that IL-4 could suppress PMA-induced HIV expression at the transcriptional level in monocytic U1 cell [50]. PRV infection increased the IL-4 level in mice. After kaempferol treatment, IL-4 level was significantly higher than that of the infected-untreated control at 3 dpi, indicating that kaempferol can suppress PRV by enhancing immune function.

After PRV enter into the host cells, the capsid is transported along microtubules to the cell nucleus and the viral DNA was injected into the nucleus[43]. Then, the only immediate-early gene of PRV, IE180, is
transcribed directly following infection [10]. The product of IE180 gene was the transactivator required for early genes transcription [28; 10]. In this study, the transcriptional level of early genes, EPO and TK was inhibited by kaempferol in the brain of PRV-infected mice, which may indicate that the function of IE180 protein was blocked (Fig. 6). After the body is infected with the virus for the first time, the virus can establish latent infection in the sensory ganglion and brain [51]. The latent infection of the virus can trigger a continuous inflammatory response, which can directly cause damage to the nerve tissue. LAT plays an important role in the establishment, maintenance, and reactivation of PRV latency. LAT prevents the expression of the immediate early gene IE180 during the incubation period, which could prevent the virus from entering the lytic infection period [52; 53]. The present study found that the transcriptional level of LAT in brain was inhibited, suggesting that kaempferol could inhibit PRV latency in mice.

4. Conclusions

Kaempferol exhibits effective antiviral activity against PRV infection in mice, which is better than acyclovir. Kaempferol can increase the survival rate, reduce the virus titers and gene expressions, and alleviate tissue lesions. The anti-PRV activity attributes to regulation of immune response. Kaempferol exhibits the potential to control PRV.

5. Methods

5.1 Virus and chemicals

PRV (Ra strain) were preserved in the Natural Medicine Research Center Sichuan Agricultural University (Chengdu, China) [44], and the 50% tissue culture infective dose (TCID$_{50}$) was determined as $10^{-7.43}$/mL. The virus was diluted to $1 \times 10^4$ TCID$_{50}$ with PBS before use. Kaempferol (No. MB2171) and acyclovir (No.MB1002) were purchased from Dalian Meilun biotechnology Co., Ltd. (Dalian, China), and dissolved in 0.5% carboxy methyl cellulose sodium solution.

5.2 Animals and experimental design

The experimental protocol was approved by the National Institute of Animal Health Animal Care and Use Committee at Sichuan Agricultural University (approval number 2018-012).

180 male specific pathogen-free KM mice (body weight $20 \pm 2$ g) were commercially obtained from the Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China), and kept in the BSL-2 lab at the Sichuan Agricultural University (Ya’an, China). They were housed at 20–25 °C with a relative humidity of 55 ± 5% and a 12 h light-dark cycle. After acclimating for a week, the mice were randomly divided into the following 6 groups ($n = 30$ in each): low dose of kaempferol group (KM-L, 80 mg/kg), medium dose of kaempferol group (KM-M, 160 mg/kg), high dose of kaempferol group (KM-H, 240 mg/kg), acyclovir group (ACV, 160 mg/kg), infected-untreated group and uninfected-untreated group. All mice, except those in the uninfected-untreated group (0.1 mL PBS), were intraperitoneally injected with 0.1 mL of $1 \times 10^4$
TCID\textsubscript{50} PRV. In the treated groups, the mice were orally administered with 0.2 mL kaempferol or Acyclovir once a day for 6 successive days. In the infected-untreated group and uninfected-untreated group, the mice received 0.2 mL 0.5% CMC-Na. Four mice were randomly selected in each group for sample collection at 3, 4 and 5 days post-infection (dpi), respectively. Blood sample collection was performed by retro-orbital puncture under anaesthesia by isoflurane inhalation. In order to minimize animals suffering during blood samples collection, an eye drop of tetracaine 1% was applied. Then, animals were euthanized by cervical dislocation and subjected to full dissection.

5.3 Survival rate

The number of deaths in each group was recorded daily. The survival rate was calculated as follows:

\[
\text{Survival rate} = \frac{\text{number of surviving mice}}{\text{total number of mice}}
\]

5.4 Organ coefficient

After dissection, the heart, liver, spleen, lung, kidney, thymus, brain were exercised and weighed. The relative organ weight was calculated according to the formula:

\[
\text{Organ coefficient (mg/g)} = \frac{\text{organ weight}}{\text{body weight}}.
\]

5.5 Viral load assay

The liver, heart, spleen, brain, lung and kidney were collected from each group and immediately frozen with liquid nitrogen, followed by homogenization. Total DNA of tissue sample (25 mg) was extracted by DNA Extraction Kit (Biomed DL107-01; Beijing, China) according to the manufacturer’s instructions. The viral copies were determined by the fluorescent quantitative polymerase chain reaction (FQ-PCR) method described by Zhao et al., 2017 [54]. The FQ-PCR was performed at 95 °C for 120 s, 95 °C for 5 s and 56.5 °C for 30 s (40 cycles) by using a CFX connect™ real-time PCR detection system (Bio-Rad, USA).

5.6 Serum cytokines assay

Blood samples collected from eyeball were allowed to coagulation at room temperature for 30 min. Serum was separated by centrifugation at 3000×\(g\) for 5 min. The levels of IL-1\(\beta\), IL-4, IL-6, TNF-\(\alpha\) and IFN-\(\gamma\) in serum were measured using the ELISA kits according to the manufacturer’s instructions (Beijing Gersion Bio-Technology Co., Ltd, China).

5.7 Histopathological examination

During dissection, the heart, liver, spleen, lung, kidney and brain were taken and fixed in 4.0% paraformaldehyde for 2 days and embedded in paraffin. Sections (4 \(\mu\)m) were cut and stained with hematoxylin-eosin (HE) solution. Histopathological changes were observed under a microscope (Nikon eclipse 80i, Tokyo, Japan). Three slides containing sections randomly selected from different part of each sample, with an area of 2000–2500 mm\(^2\) per slide, were analyzed to determine the lesion scores of each organ. The whole lesions for each tissue were scored by multiplying the degree of severity (0 = no lesions, 1 = mild lesions, 2 = moderate lesions, and 3 = severe lesions) with the extent of lesions (1 = low extent, 2
= intermediate extent, and 3 = large extent) [55]. For each organ, three slides from different part of each tissue were analyzed, the maximal lesional score was 9 and the minimal score was 0.

5.8 Immunohistochemistry

The tissue slides of brain from different groups were deparaffinised in xylene following dehydration with ethanol. Endogenous peroxidase activity was blocked by treatment with 0.3% H₂O₂ in methanol for 20 min at RT. After antigen retrieval, the tissue slides were blocked with 5% goat serum for 20 min at RT and then probed with anti-Pseudorabies Virus antibody (No.ab3534; Abcam, England) at 4°C overnight. After washing, the tissue slides were incubated with biotinylated goat anti-rabbit IgG (No.ab205718; Abcam, England) at RT for 1 h, stained with DAB, and counterstained with haematoxylin. Then, the slides were evaluated under a microscope (Nikon, Japan).

5.9 Gene expression assay

The expressions of PRV genes related to transcription and latent infection (EPO, IE180, LAT and TK) were detected through real-time PCR assay. Briefly, total RNA from brain was extracted using the RNAiso Plus (No.9108; TaKaRa, China) according to the manufacturer’s instructions. Equal amounts of the RNA samples were immediately reverse transcribed into cDNA using the RevertAid First Strand cDNA kit (No. K1622; Thermo Scientific™) according to the manufacturer’s instructions. The primers were listed in Table 1. The real-time PCR was performed with a CFX connect™ real-time PCR detection system (Bio-Rad, USA) using the iQ SYBR Green Supermix kit (Bio-Bad, USA) at 95 °C for 45 s, followed by 39 cycles of 95 °C for 7 s and 62 °C (60 °C for TK gene) for 34 s. A melting curve of the products (55–95 °C) was also conducted to confirm the absence of artefacts. The relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta CT}$ method using Bio-Rad CFX Manager software.

5.10 Statistical analysis

Results were expressed as means ± standard deviation (SD). Significant differences were determined using a one-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range test in SPSS 19.0 (IBM Corp., Armonk, NY, USA) at p < 0.05 for significance.

Abbreviations

PRV
Pseudorabies virus
AD
Aujeszky’s disease
LAT
Latency-associated transcript
TCID₅₀
50% tissue culture infective dose
ACV
Acyclovir
CMC-Na
Carboxymethylcellulose sodium
HE
Hematoxylin-eosin
KM-L
Low dose of kaempferol group
KM-M
Medium dose of kaempferol group
KM-H
High dose of kaempferol group
ELISA
Enzyme-linked immunosorbent assay

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the National Institute of Ethics Committee at Sichuan Agricultural University [approval number SYXK (Sichuan) 2018-012]. The humane endpoints were a weight loss above 15% of initial weight or animals in a state of prostration. Animals that reach one of these endpoints were euthanized by cervical dislocation by caretakers. All efforts were made to minimize suffering of animals.

Consent for publication

All authors consent to publish this study in BMC Veterinary Research.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was financially supported by the Program Sichuan Veterinary Medicine and Drug Innovation Group of China Agricultural Research System (SCCXTD-2020-18), the Science and Technology Project of Sichuan Province (Grant Nos. 2018HH0076, 2018NZ0043 and 2018NZ0064). The funding
body doesn't have any roles in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

RW carried out laboratory work, wrote the manuscript and performed the data analyses. RW, YH QY designed the experiment, wrote the manuscript and performed the data analyses. XC, XL, CH, LY, GY, YZ, LL, HT and GY carried out laboratory work. RJ, XS conceived and supervised this work, revised manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

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Tables

Table 1  
The primers used for real-time PCR

| Gene name | Primer sequence (5’→3’) | T_m (℃) |
|-----------|-------------------------|---------|
| β-actin   | F: GGCTGTATTCCCCTCCATCG  | 62      |
|           | R: CCAGTTGGTAACAATGCCATGT |         |
| EPO       | F: GGGTGTGAACTATATCGACACGTC | 62     |
|           | R: TCAGAGTCAGAGTGTGCCTCG  |         |
| IE180     | F: CATCGTGCTGGACACCATCGAG | 62     |
|           | R: ACGTAGACGTGGTAGTCCCCCA |         |
| LAT       | F: GGCAGCAGGACTACTGTCA   | 62      |
|           | R: GTCTTGGTGGAAGAAGTA     |         |
| TK        | F: ATGACGGTCGTTTGACCGCCAC | 60     |
|           | R: CGCTGATGTCCCCGACGATGAA |         |

F, forward primer; R, reverse primer.
Table 2
Organ coefficient of mice

| Group   | Organ coefficient /10^{-3} |        |        |        |        |        |        |
|---------|---------------------------|--------|--------|--------|--------|--------|--------|
|         | Heart                     | Liver  | Spleen | Lung   | Kidney | Thymus | Brain  |
| KM-L    | 3dpi                      |        |        |        |        |        |        |
|         | 5.28 ± 0.87               | 49.18 ± 5.80 | 3.68 ± 0.83 | 6.23 ± 0.76 | 15.55 ± 1.72 | 1.58 ± 0.40 | 13.35 ± 1.97 |
| KM-M    | 6.28 ± 1.82               | 53.08 ± 5.93 | 3.80 ± 0.37 | 6.20 ± 0.86 | 15.98 ± 1.76 | 1.73 ± 0.74 | 12.30 ± 2.58 |
| KM-H    | 5.48 ± 1.53               | 48.53 ± 1.05 | 4.00 ± 0.32 | 5.53 ± 0.83 | 13.60 ± 2.13 | 1.80 ± 0.59 | 13.35 ± 1.41 |
| ACV     | 6.38 ± 1.30               | 50.48 ± 7.86 | 3.25 ± 0.42 | 6.45 ± 0.75* | 15.78 ± 0.96 | 1.60 ± 0.68 | 14.20 ± 0.65 |
| Untreated | 5.95 ± 1.52               | 47.60 ± 6.32 | 3.68 ± 0.93 | 5.18 ± 0.35# | 14.68 ± 1.84 | 1.30 ± 0.43 | 13.35 ± 0.62 |
| Control  | 7.48 ± 1.26               | 50.68 ± 7.44 | 3.95 ± 0.31 | 5.80 ± 0.56 | 14.68 ± 1.09 | 1.13 ± 0.33 | 12.68 ± 0.72 |
| KM-L    | 4dpi                      |        |        |        |        |        |        |
|         | 7.33 ± 1.77               | 54.75 ± 2.17 | 3.93 ± 0.62 | 5.65 ± 1.16 | 15.78 ± 1.53 | 1.13 ± 0.21 | 15.05 ± 0.79 |
| KM-M    | 8.05 ± 1.56               | 51.50 ± 2.78 | 3.15 ± 1.22# | 5.60 ± 0.67 | 15.10 ± 0.22 | 0.98 ± 0.36 | 14.25 ± 1.48 |
| KM-H    | 7.25 ± 1.13               | 52.2 ± 3.89 | 3.25 ± 0.79# | 6.98 ± 0.96 | 13.80 ± 0.94 | 0.95 ± 0.31 | 16.78 ± 0.93*# |
| ACV     | 5.95 ± 1.48               | 51.23 ± 9.42 | 3.00 ± 1.11# | 6.70 ± 0.86 | 14.88 ± 2.05 | 0.75 ± 0.42 | 14.53 ± 1.45 |
| Untreated | 6.85 ± 1.66               | 48.55 ± 1.93 | 3.18 ± 0.21# | 5.55 ± 0.93 | 15.68 ± 0.63 | 1.08 ± 0.33 | 13.88 ± 1.67 |
| Control  | 7.78 ± 2.12               | 56.27 ± 12.39 | 4.80 ± 1.42* | 6.23 ± 1.56 | 16.03 ± 1.93 | 1.48 ± 1.30 | 13.90 ± 0.56 |
| KM-L    | 5dpi                      |        |        |        |        |        |        |
|         | 7.63 ± 1.46               | 58.37 ± 8.31 | 2.27 ± 0.68 | 7.20 ± 1.82 | 16.53 ± 1.06 | 1.07 ± 0.50 | 15.60 ± 2.72 |
| KM-M    | 6.23 ± 0.38#              | 57.43 ± 5.92 | 3.80 ± 0.85 | 6.18 ± 1.05 | 15.28 ± 2.10 | 1.25 ± 0.34 | 14.00 ± 1.16 |

The heart, liver, spleen, lung, kidney, thymus and brain were collected and weighed at 3, 4 and 5 dpi (n = 4). The relative organ weight was calculated according to the formula: organ coefficient (mg/g) = organ weight / body weight. “*” represents the significant differences (p < 0.05) observed between the untreated group and the treated groups, and “#” represents the significant differences (p < 0.05) observed between the normal group and infected groups.
The heart, liver, spleen, lung, kidney, thymus and brain were collected and weighed at 3, 4 and 5 dpi (n = 4). The relative organ weight was calculated according to the formula: organ coefficient (mg/g) = organ weight / body weight. **"** represents the significant differences (p < 0.05) observed between the untreated group and the treated groups, and **"#"** represents the significant differences (p < 0.05) observed between the normal group and infected groups.

Table 3 Tissue lesions score on 5dpi

| Group             | Tissue lesions score | Heart       | Liver       | Spleen      | Lung        | Kidney      | Brain       |
|-------------------|----------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Infected-untreated control |                      | 6.34 ± 2.05 | 4.67 ± 0.94 | 6.33 ± 2.05 | 7.00 ± 1.41 | 6.33 ± 2.05 | 2.00 ± 0.87 |
| KM-L              |                      | 4.67 ± 0.94 | 3.67 ± 0.47 | 3.67 ± 0.47 | 3.67 ± 1.70 | 3.00 ± 0.82 | 1.33 ± 0.47 |
| KM-M              |                      | 4.34 ± 1.25 | 4.33 ± 1.25 | 4.67 ± 0.94 | 3.00 ± 0.82 | 2.67 ± 0.94* | 0.67 ± 0.94 |
| KM-H              |                      | 3.67 ± 0.47 | 3.00 ± 0.82 | 3.00 ± 0.82 | 2.67 ± 0.94* | 2.33 ± 0.47* | 1.33 ± 1.25 |
| ACV               |                      | 5.34 ± 0.94 | 4.33 ± 1.25 | 3.33 ± 0.47 | 3.33 ± 0.47* | 3.67 ± 0.47 | 2.00 ± 1.41 |

Lesional scores of each organ (n = 4) were obtained by multiplying the degree of severity (0 = no lesions, 1 = mild lesions, 2 = moderate lesions, and 3 = severe lesions) with the extent of lesions (1 = low extent, 2 = intermediate extent, and 3 = large extent). **"** represents the significant differences (p < 0.05) observed between the infected-untreated group and the treated groups.

Figures
Figure 1

The survival rate of mice in each group. A, chemical structure of kaempferol: 3, 5, 7 - trihydroxy - 2 - (4-hydroxyphenyl) - 4H - 1 - benzopyran - 4 - one. B, The number of alive mice in each group was recorded daily, respectively. The survival rate of each group was then calculated according to the formula: survival rate = number of surviving mice / total number of mice. The KM-H, KM-M and KM-L represent the PRV-infected groups which were treated with kaempferol at doses of 240, 160 and 80 mg/kg· body weight, respectively. ACV, the acyclovir-treated group (160 mg/kg· body weight).
Figure 2

Virus load of heart, liver, spleen, lung, kidney and brain in each group (n=4). The mice were randomly dissected at 3, 4, and 5 dpi to collect these tissues. After homogenization with liquid nitrogen, the tissue samples (25mg per sample) were used for DNA extraction. The virus gene copies were detected by FQ-PCR. The KM-H, KM-M and KM-L represent the PRV-infected groups which were treated with kaempferol at doses of 240, 160 and 80 mg/kg• body weight, respectively. ACV, the acyclovir-treated group (160 mg/kg• body weight). Symbols “*, **” and “***” represent p < 0.05, p < 0.01 and p < 0.001, respectively, between the infected-untreated control and the treated groups. Symbols “ξ, ξξ” and “ξξξ” represent p < 0.05, p < 0.01 and p < 0.001, respectively, between the acyclovir group and kaempferol groups.
**Figure 3**

Immunohistochemical examination of the brain at 5 dpi. The KM-H, KM-M and KM-L represent the PRV-infected groups which were treated with kaempferol at doses of 240, 160 and 80 mg/kg• body weight, respectively. ACV, the acyclovir-treated group (160 mg/kg• body weight). (A) DAB chromogen substrates were used for antigen visualization. The SABC staining positive cells mainly showed brownish-yellow (indicated by arrows), and the section background mainly showed light yellow. Each tissue undergoes three SABC stains to check its repeatability. The uninfected–untreated control had no obvious positive cells, and the infected-untreated group had the most number of positive cells. Compared with the infected-untreated group, the number of positive cells in the treated group decreased. (B) No brownish-yellow cells detected in the uninfected-untreated control were negative; the scattered distribution of brownish-yellow cells in the tissue is less than 5% indicates mildly positive; the proportion of brownish-yellow cells in the tissue is 5% - 50% indicates positive; the proportion of brownish-yellow cells in the tissue is greater than 50% indicates strong positive.

**Figure 4**

Histopathological examination of the tissues including heart, liver and spleen on 5dpi. In the heart (A-F), PRV infection causes breakage of myocardial fibers and disordered arrangement (denoted by arrowhead “←”), the blood vessel wall of the heart appears shedding to varying degrees (denoted by arrowhead “→”), and localized vacuolar degeneration (denoted by arrowhead “↑”). In the liver (G-L), the main histopathological changes are shedding of blood vessel walls (denoted by arrowhead “←”), disordered arrangement of hepatic cords (denoted by arrowhead “→”), hepatocellular swelling (denoted by arrowhead “↓”), and foam-like changes (denoted by arrowhead “↑”). In the spleen (M-R), the lesions showed a decrease in the area of the red pulp area (denoted by arrowhead “←”) and white pulp area (denoted by arrowhead “→”), vacuolar degeneration (denoted by arrowhead “↑”). In the lung (S-X), the main lesions included bleeding (denoted by arrowhead “←”), alveolar septal rupture and thickening (denoted by arrowhead “→”), inflammatory cell infiltration (denoted by arrowhead “↓”). In the kidney (Y-ZD), the lesions mainly included hyperemia in the cortical area (denoted by arrowhead “←”) and medulla area (denoted by arrowhead “→”), shedding of renal tubular epithelial cells and glomerular swelling (denoted by arrowhead “↓”). In the brain (ZE-ZJ), the lesions mainly included cell edema (denoted by arrowhead “←”).
Figure 5

The concentrations of IL-1β, IL-6, TNF-α, IL-4 and IFN-γ in serum at 3, 4 and 5 dpi (n=4). The concentrations of these cytokines were determined through ELISA. The KM-H, KM-M and KM-L represent the PRV-infected groups which were treated with kaempferol at doses of 240, 160 and 80 mg/kg· body weight, respectively. ACV, the acyclovir-treated group (160 mg/kg· body weight). Symbols “*, ** and ***” represent p < 0.05, p < 0.01 and p < 0.001, respectively, between the infected-untreated control and the infected-treated groups. Symbols “#, ## and ###” represent p < 0.05, p < 0.01 and p < 0.001, respectively, between the uninfected-untreated control and infected groups.
Figure 6

The gene expressions of EPO, TK and LAT in brain at 5 dpi (n=4). Infected control, the infected-untreated control. The KM-H, KM-M and KM-L represent the PRV-infected groups which were treated with kaempferol at doses of 240, 160 and 80 mg/kg • body weight, respectively. ACV, the acyclovir-treated group (160 mg/kg • body weight). Symbols “**” and “***” represent p < 0.01 and p < 0.001, respectively, between the infected-untreated control and the treated groups. Symbols “ξ” and “ξξ” represent p < 0.05, p < 0.01 and p < 0.001, respectively, between the acyclovir group and kaempferol groups.

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