Protective effect of chrysophanol on canine renal cell injury induced by canine parvovirus

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ABSTRACT

Canine parvovirus (CPV) causes apoptosis of canine kidney cells. As a polyphenol compound, chrysophanol (CHR) has antioxidant effects. However, it is not yet clear whether CHR has a protective effect on CPV-induced renal cell damage. In order to clarify the role of CHR in the lesion caused by CPV, this study constructed a model of Madin Darby Canine Kidney (MDCK) cell line injured by CPV *in vitro* and evaluated the effect of chrysophanol on the oxidative stress, expression of inflammatory cytokines and apoptosis of renal cell line induced by CPV through biochemical analysis, flow cytometry and real-time fluorescence quantitative polymerase chain reaction (RT-PCR). In this study, it has found that Pre-treatment with 40 μM CHR inhibited cell viability and the release of lactate dehydrogenase (LDH) of MDCK cells, and induced a decrease in malondialdehyde (MDA) content, reactive oxygen species (ROS) level, and expression of pro-inflammatory factors IL-6 and TNF-α. Besides, A reduction in mitochondrial membrane potential (ΔΨ), activities of Caspase-9 and Caspase-3 and apoptosis rate of CPV-infected MDCK cells which were pre-treated with CHR was observed. Therefore, CHR has a protective effect on renal cells caused by CPV and is closely related to depressing oxidative stress, inflammatory responses, and apoptosis, revealing that CHR is a potential cellular protective compound to combat viral infection.

Introduction

Canine parvovirus (CPV), an important pathogen, affects canine animals worldwide. Since the late 1970s, CPV has been reported worldwide(1). CPV belongs to a single-stranded DNA virus, and its epidemic is seasonally dependent. So far, CPV infection is still a common animal infectious disease. After the onset of the disease, the affected animals exhibit severe symptoms including systemic symptoms, especially in young dogs(2). The main therapy methods nowadays are mainly based on symptom therapy, such as addressing gastrointestinal symptoms and treating and preventing potential sepsis. For example, antibiotics, antiemetics and painkillers are often used(3), but there are still many cases of ineffective treatment in clinical practice. Continuous evidence showed that CPV causes cellular lesions. CPV-induced cell cycle arrest and DNA damage, leading to cell death in a Caspase-dependent manner in Madin Darby Canine Kidney (MDCK) cells which is commonly used as model cells for CPV research(4). After CPV infection, the transient depolarization of cell mitochondria across membrane potential and the increase of reactive oxygen species (ROS) level were observed, and the activation of cell survival signal through ERK1/2 cascade was also found in the early stage of CPV-infected cells. Whatmore, at 12 h after infection, the mitochondrial structure was damaged(5).

Chrysophanol (CHR), also known as rhein, is an anthraquinone compound extracted from various medicinal plants such as rhubarb. Previous studies have revealed that CHR has a wide range of benefits, including tumor inhibition, virus killing, and anti-inflammatory(6). In addition, CHR has a protective effect on various normal cells, plays a vital role in antioxidant effects and modulates apoptosis(7). However, it is not yet known whether CHR exerts a protective effect on cellular lesion induced by CPV infection.

In this study, it is demonstrated that CPV induces oxidative stress, apoptosis and inflammatory responses in MDCK cells, while CHR is able to dose-dependently alleviate CPV-induced damage by inhibiting oxidative stress, endogenous pathways of apoptosis and inflammatory responses. Therefore, CHR is a promising natural compound for the prevention and therapy of CPV infectious diseases.

Materials and Methods

Cell culture

MDCK cell line was purchased from Procell Co., Ltd. (Wuhan, China), and cultured in Dulbecco's Modified Eagle Medium (DMEM, Procell, Wuhan, China) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, USA), 1% of penicillin as well as streptomycin (Procell). MDCK cell line was placed in a cell incubator at 37 °C with 5% carbon dioxide and 95% air.

MTT analysis

MDCK cell line (1×10^4) was seeded into 96-well plates and cultured with culture medium overnight. CHR (Yuanye, Shanghai, China) at the final concentration of 0, 10, 20, 40, 80 and 160 μM in DMEM was used to incubate with MDCK cells for 28 h. In the experiment for exploring the

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effect of CPV on MDCK cells, CPV (0, 0.05, 0.1, 0.2, 0.4 and 0.8 MOI) in DMEM was added to infect MDCK cells for 24 h. In the experiment for exploring the effect of CHR on CPV-induced cellular injury on MDCK cells, MDCK cells were incubated with CHR at the concentration of 10, 20 and 40 μM for 4 h and then treated with a mixture of various concentrations of CHR and 0.2 MOI CPV for additional 24 h. The culture supernatant of MDCK cells was collected for subsequent LDH and MDA detection. Cell viability was measured via 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A volume of 20 μL MTT reagent (Solarbio, Beijing, China) and 100 μL fresh DMEM were added to each well. The cells were cultured in the incubator for 4 h at 37 °C, then the culture medium was discarded and 150 μL DMSO was placed onto each well. The reaction system was lightly shaken for 10 min, then the absorbance was measured by a microplate reader at 490 nm. Cell viability was converted based on the relative percentage of absorbance in the control group.

LDH detection

LDH content in the culture supernatant of MDCK cells was detected by a commercialized kit (Solarbio). After centrifugation, reaction reagents were sequentially added to the supernatant of the cells, and a standard curve was constructed at the same time. The absorbance was measured at 450 nm, and LDH concentration was calculated based on the standard curve. The percentage of LDH concentration was standardized with the concentration of the control.

MDA determination

MDA content in the culture supernatant of MDCK cells was determined by a colorimetry method via a commercialized kit (Solarbio), according to the indicated protocols.

ROS analysis

MDCK cells (1×10^5 cells per well) were plated in 6-well plates. In the next day, The cells were incubated with 10, 20 and 40 μM CHR for 4 h, then treated with a mixture of various concentrations of CHR and 0.2 MOI CPV for 24 h. After the indicated incubation, the ROS level was tested by flow cytometer with a ROS assay kit (Solarbio) with reference to the kit's instructions. In brief, MDCK cells were incubated with DCFH-DA, a fluorescent probe, and then the cells were collected for analysis through flow cytometry.

RT-PCR detection

To demonstrate the effects of CHR on the pro-inflammatory cytokines IL-6 as well as TNF-α expression levels, MDCK cells were seeded in 6-well plates at a density of 1 $\times 10^5$ cells/well then cultured overnight. Then cells were incubated with 10, 20 and 40 μM CHR for 4 h, then treated with a mixture of various concentrations of CHR and 0.2 MOI CPV for an additional 24 h. Total RNA was extracted by Trizol reagent with reference to the manufacturer's instructions. Subsequently, the total RNA from MDCK cells was reverse transcribed via SuperMix (TransGen, Beijing, China) into cDNA. Then RT-PCR Super Mix (TransGen) was used to assay expression levels of IL-6 as well as TNF-α, with reference to the kit's instructions. The primers of the target gene, IL-6 as well as TNF-α, and internal reference gene β-actin are listed in Table 1. The relative expression levels of the IL-6 as well as TNF-α were calculated based on $2^{-\Delta\Delta Ct}$.

Mitochondrial membrane potential (ΔΨ) assay

MDCK cells were seeded in 6-well plates and cultured overnight. Then cells were cultured with 10, 20 and 40 μM CHR for 4 h, and ultimately treated with a mixture of CHR at the concentration of 10, 20 and 40 μM and 0.2 MOI CPV for 24 h. MDCK cells were collected and dyed with JC-1, then Δψm of MDCK cells was estimated using a Δψm detection kit (Solarbio) by a flow cytometer according to the instructions.

Measurement of activity of Caspase-9 and Caspase-3

Caspase-9/3 activity assay kits (Beyotime, Shanghai, China) were used to analyze the Caspase-9 and Caspase-3 in MDCK cells. 100 μL lysis solution was added for obtaining cell lysates. Caspase-9 catalyzed the substrate Ac-LEHD-pNA (acetyl-Leu-Glu-Hes-Asp p-nitroanilide) to produce yellow p-nitroanilide (pNA), thus detecting the activity of Caspase-9 by measuring absorbance at 405 nm. However, Caspase-3 catalyzed the substrate Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide) to produce yellow pNA, thereby detecting the activity of caspase-3 by measuring absorbance at 405 nm.

Apoptosis determination

MDCK cells in 6-well plates were incubated with 10, 20 and 40 μM CHR for 4 h, then treated with a mixture of CHR and CPV for 24 h. The cells were harvested by trypsin and washed with an ice-cold phosphate buffer solution. Next, MDCK cells were incubated with Annexin V- fluorescein isothiocyanate (FITC) and propidine iodide for 30 min, and the apoptosis rate of MDCK cells was evaluated by a flow cytometer according to a detection kit protocol (Solarbio).

Statistical analysis

GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, USA) was applied to data processing. All data are shown as mean ± standard deviation. One-way ANOVA

Table 1. Primer sequences for RT-PCR.

No.	Gene	Primer sequences	Notes
1	IL-6	TCTTGCAGTCGCAGGATGAG TGCCCAGGCTACATTATCCG	
2	TNF-α	CCCCAAGTGACAAGCCAGTA TCAGCTGGTTGTCTGTCAGC	(8)
3	β-actin	GAACGGAGAAGGTGACAGCA GGCTTTTGGGAAGGCAAAGG	

was used to analyze the statistical differences in the mean between groups. *P < 0.05* were regarded as a significant difference.

Results

Effect of CHR and CPV on the injury of MDCK cells

Firstly the safe concentration of the test drug CHR needs to be identified. As shown in Figure 1, 80 and 160 μM CHR treated with MDCK cells for 28 h significantly inhibited cell viability (Figure 1A) and promoted LDH release to the culture medium (Figure 1B), while 10 to 40 μM CHR has no obvious influence on the viability and LDH release of MDCK cells (Figure 1A and B). Thus, these results showed that 10, 20 and 40 μM CHR within 28 h is non-toxic to MDCK cells. Based these findings, 10, 20 and 40 μM CHR were selected for the next intervention experiment.

In addition, MTT analysis showed that 0.1 MOI CPV infection for 24 h dramatically depressed cell viability of MDCK cells and 0.2 MOI CPV resulted in a decrease of approximately 50% in the cell viability (Figure 2A). Moreover, 0.1 MOI of CPV significantly increased the release of LDH of MDCK cells (Figure 2B). Considering the moderate degree of cellular damage caused by the CPV, 0.2 MOI CPV was adopted for subsequent damage model research *in vitro*.

Effect of CHR on MDCK cell injury induced by CPV

As shown in Figure 3A, 0.2 MOI CPV reduced MDCK cell viability, while incubation with 40 μM CHR for 4 h in advance and subsequent co-incubation of CHR and CPV for 24 h raised the cell viability of MDCK cells. In Figure 3B, a significant enhancement in LDH release in CPV-in-
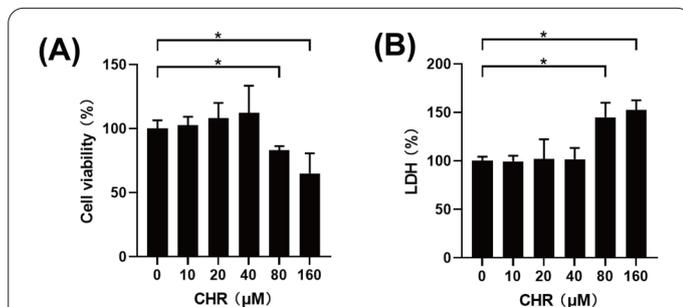

Figure 3. The effect of CHR on the survival of MDCK cells damaged by CPV. (A) Cell viability of MDCK cells pre-treated with CHR for 4 h and then incubated with CHR and CPV for 24 h; (B) Concentration of LDH in the culture medium of MDCK cells pre-treated with CHR for 4 h and then incubated with CHR and CPV for 24 h. * *P < 0.05.*

fected cells was inhibited by incubation with 40 μM CHR. Additionally, 10 and 20 μM CHR pre-treatment had no apparent effects on MDCK cell injury induced by CPV (Figures 3A and B).

Effect of CHR on oxidative stress of MDCK cells induced by CPV

0.2 MOI CPV infection for 24 h caused an apparent increment in MDA production, while 40 μM CHR pre-incubation suppressed the overproduction of MDA in MDCK cells triggered by CPV infection (Figure 4A). At the same time, similar results were found in the ROS level alteration of MDCK cells (Figure 4B).

Effect of CHR on inflammatory responses of MDCK cells induced by CPV

To examine the effect of CHR on inflammatory responses of MDCK cells induced by CPV, RT-PCR was applied to determine the expression level of pro-inflam-

Figure 1. The effect of different concentrations of CHR on the survival of MDCK cells. (A) Cell viability of MDCK cells treated with CHR for 28 h; (B) Concentration of LDH in the culture medium of MDCK cells treated with CHR for 28 h. * *P < 0.05.*
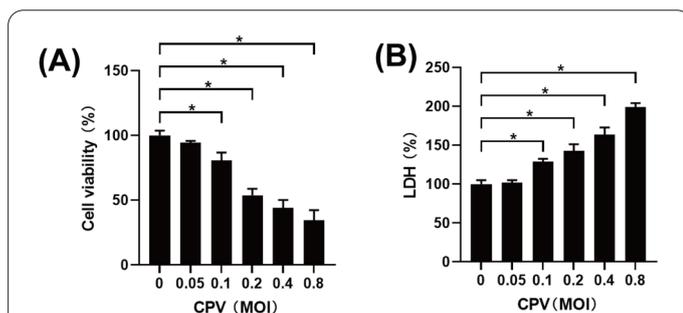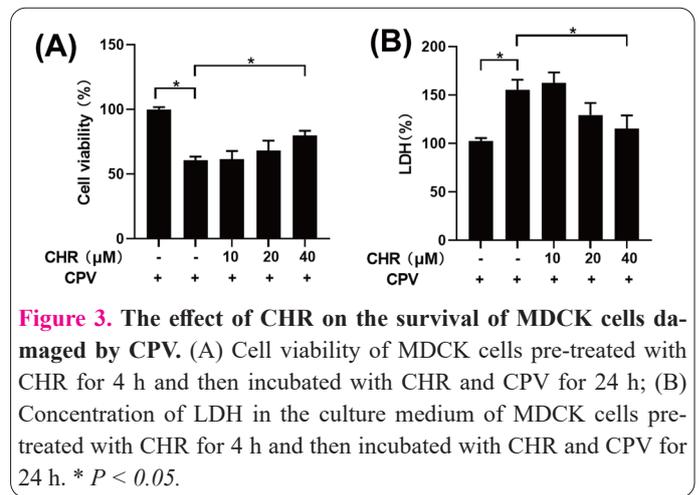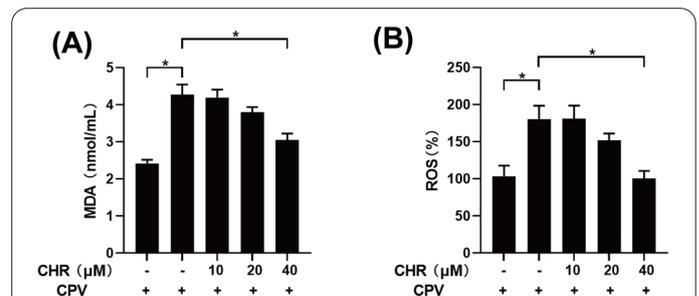

Figure 4. The effect of CHR on the oxidative stress of MDCK cells induced by CPV. (A) MDA concentration in the culture medium of MDCK cells; (B) ROS level of MDCK cells. * *P < 0.05.*

Figure 2. The effect of different MOI of CPV on the survival of MDCK cells. (A) Cell viability of MDCK cells treated with CPV for 24 h; (B) Concentration of LDH in the culture medium of MDCK cells treated with CPV for 24 h. * *P < 0.05.*
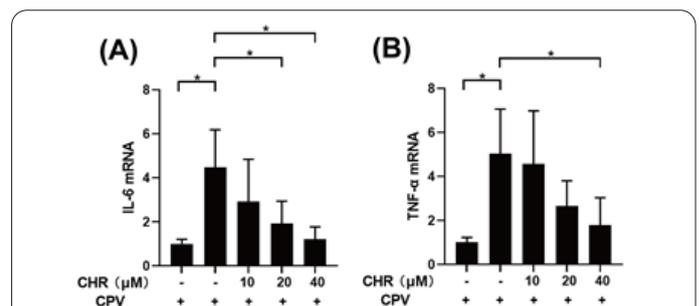

Figure 5. The effect of CHR on the inflammatory reactions of MDCK cells induced by CPV. (A) The expression level of IL-6 mRNA of MDCK cells; (B) Expression level of TNF-α mRNA of MDCK cells. * *P < 0.05.*

matory cytokines IL-6 and TNF-α. The results showed that both IL-6 and TNF-α were over-expressed in mRNA level after CPV infection and 40 μM CHR pre-incubation reversed the outcome of the expression of IL-6 and TNF-α in MDCK cells (Figures 5A and B).

Effect of CHR on apoptosis of MDCK cells induced by CPV

To validate the effect of CHR on apoptosis of MDCK cells infected by CPV, the ΔΨm in MDCK cells with 0.2 MOI CPV infection or CHR pre-treatment was detected. The results showed that The low ΔΨm was induced by CPV and CHR upregulated ΔΨm reduced by CPV in a concentration-dependent manner in MDCK cells (Figure 6A). Besides, CHR pre-treatment reversed the increment in the activity of Caspase-9 and Caspase-3 triggered by CPV in MDCK cells (Figures 6B and C). Importantly, 0.2 MOI CPV elevated the apoptosis rate of MDCK cells, while CHR caused a sharp decline in the apoptosis rate (Figure 6D).

Discussion

Although vaccines have played some roles in preventing CPV infectious diseases, in reality, CPV infectious diseases often occur due to the neglect of vaccination, virus variation and limited protection rate of vaccines. So developing drugs to prevent and treat this infectious disease through other means has important practical values. CPV has been revealed to induce apoptosis of infected cells, which has a negative effect on the normal physiological function of animals[4]. Natural products are an important treasure trove for drug development. CHR has been proven to be a natural compound with multiple biological activities, particularly playing a vital function on cellular protection[9]. Canine kidney cells MDCK can be infected by CPV, causing DNA damage and apoptosis[4]. It can be seen that the MDCK cell line could be used as model cells to evaluate the protection of candidate compounds for CPV-damaged cells.

The succinate dehydrogenase in the mitochondria of living cells reduces exogenous MTT to water-soluble blue-purple crystalline formaldehyde and deposits it in the cells. DMSO further dissolves the crystals, while dead cells have no the same function. In addition, LDH exists within cells under normal circumstances. When cells are stimulated by damage sources, LDH seeps out of the cells as the cell membrane ruptures[10]. Therefore, in the MTT experiment, the darker the color of the blue-purple solution in the detection system, the higher the cell activity; Outside the cell, an increase in the concentration of LDH indicates more severe damage to the cells. In this study, the high concentration of CHR caused damage to canine kidney cells, leading to increased LDH release, while 10 to 40 μM CHR is non-toxic to canine kidney cells *in vitro*. In addition, CPV induced the release of a large amount of LDH from cells, indicating a cytotoxic effect, which is consistent with existing literature results[11]. Interestingly, in the CHR intervention experiment, it was found that CHR pre-treatment with MDCK cells had an inhibitory effect on CPV-induced LDH release, indicating that CHR has a positive protective effect on CPV-triggered damage. This is similar to the discovery that CHR produces cytoprotection in other virus-induced damage[12].
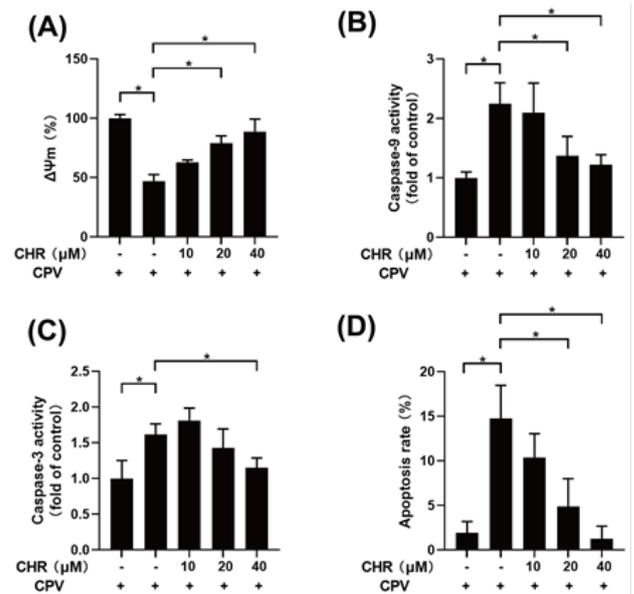

Figure 6. The effect of CHR on the apoptosis of MDCK cells induced by CPV. (A) ΔΨ of MDCK cells; (B) Caspase-9 activity of MDCK cells; (C) Caspase-3 activity of MDCK cells; (D) Apoptosis rate of MDCK cells. * *P < 0.05.*

The theory of oxidative stress is considered a major mechanism of cell damage[13]. Damage factors break the inherent redox system and promote the production of a large amount of oxidizing substances and products. ROS is the most representative oxidative factor that causes cell membrane oxidation, such as lipid peroxidation of biomembrane, which generates MDA[14, 15]. Previous studies have shown that blood cell lipid oxidation levels in dogs infected with CPV are at a high level[16]. What's more, the symptoms of dogs with CPV disease were significantly improved after treatment with the antioxidant N-acetylcysteine[17]. This study found that CPV triggered oxidative stress, and CHR possessing antioxidant effects also reduced oxidative stress in canine kidney cells triggered by CPV. These evidences reveal that targeting oxidative stress is an important way to prevent and treat CPV infectious diseases.

Inflammatory reactions can be mediated by ROS[18]. The release of a large number of pro-inflammatory cytokines is an important manifestation of inflammatory responses, including IL-6 and TNF-α, which activate the surrounding immune cells and generate more inflammatory factors, causing further cellular damage. In this exploration, CPV intensified the expression of pro-inflammatory factors in canine kidney cells, while CHR reversed this pathological change. This is similar to the effect of CHR[19].

Excessive ROS aggravates mitochondrial destruction, provokes changes in mitochondrial membrane permeability, reduces membrane potential, and activates apoptosis pathway-associated proteins such as Caspase-9 and Caspase-3[20]. This is the classic that mediates apoptosis[21]. Among them, Caspase-9 is the initiator of apoptosis, and Caspase-3 is the executor of apoptosis[22, 23]. Apoptosis is exacerbated after the caspase cascade reaction is triggered. This study elucidated that CPV increased the apoptosis rate in canine kidney cells. This is consistent with existing research findings and also mediates cell apoptosis through endogenous pathways[4]. Importantly, CHR pre-treatment inhibited endogenous apoptotic signaling and significantly

improved CPV-induced apoptosis in canine kidney cells. These evidences indicate that CHR has a protective effect on CPV-induced damage to canine kidney cells, and it can also be inferred that this compound may have a similar relieving effect on damage to other cells.

However, there are still some shortcomings in this study, such as specific signal transduction mechanisms, *in-vivo* application effects of CHR in animals, and whether CHR can directly kill or inhibit CPV. Further research is needed to solve these issues in the future.

In conclusion, CPV induces canine renal cell apoptosis and inflammatory responses through the oxidative stress pathway, while CHR can dose-dependently improve CPV-induced damage by inhibiting endogenous pathways of apoptosis and inflammatory responses. In addition, CHR is a low-cost natural compound with the economic cost advantage of promoting its use. Thus, it can be seen that CHR could be used as a candidate natural compound to prevent and treat CPV infectious diseases, which is of great significance to maintaining animal health and welfare.

Funding

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