



Antiviral Effect of Selenomethionine on Porcine Deltacoronavirus in Pig Kidney Epithelial Cells

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Porcine deltacoronavirus (PDCoV) is an emerging porcine intestinal coronavirus in recent years, which mainly causes different degrees of vomiting and diarrhea in piglets and has caused great harm to the swine husbandry worldwide since its report. Selenium is an essential trace element for organisms and has been demonstrated to have antiviral effects. In this study, pig kidney epithelial (LLC-PK) cells were used to study the antiviral activity of selenomethionine (Se-Met) (2, 4, 8, and 16 μ M) against PDCoV by detecting the replication of the virus, the expression of the mitochondrial antiviral signal protein (MAVS) protein, and the phosphorylation of interferon regulatory factor-3 (IRF-3), IFN- α , and IFN- β , and the changes in glutathione content, glutathione peroxidase, superoxide dismutase activity, and hydrogen peroxide content in the cells. The results showed that Se-Met at higher than physiological concentrations (16 μ M) could significantly inhibit the replication of PDCoV in LLC-PK cells and enhance the expression of MAVS protein and the phosphorylation of IRF-3. In addition, Se-Met also improved the intracellular production of IFN α/β and antioxidant capacity with increasing doses. These data suggest that the availability of selenium through selenomethionine supports the antiviral response in porcine kidney cells, and the specific mechanism is attributed to the improved cellular antioxidant capacity and activation of the MAVS pathway by Se-Met.

Keywords: porcine deltacoronavirus, antiviral activities, antioxidant, selenomethionine, innate immunity

INTRODUCTION

Coronaviruses are single-stranded positive capsular RNA viruses that mainly infect mammals and birds (Niederwerder and Hesse, 2018). According to the differences of genetic characteristics and serology, it can be divided into four genera: Alphacoronavirus, Betacoronavirus, Gamacoronavirus, and Deltacoronavirus (Ma et al., 2015). Among them, PDCoV belongs to the genus Deltacoronavirus in the Coronaviridae family and is a newly discovered porcine enteropathogenic coronavirus, which is mainly characterized by digestive system symptoms, such as diarrhea, dehydration, and varying degrees of vomiting in piglets (Xu et al., 2018; Yin et al., 2020). PDCoV

Abbreviations: GSH, glutathione; GSH-PX, glutathione peroxidase; HIV, human immunodeficiency virus; H₂O₂, hydrogen peroxide; IRF-3, interferon regulatory factor-3; IFN, interferon; LLC-PK, Pig Kidney Epithelial; MAVS, mitochondrial antiviral signal protein; PDCoV, Porcine deltacoronavirus; PCV, Porcine circovirus; RLR, RIG-I-like receptor; Se-Met, selenomethionine; SOD, superoxide dismutase; TrxR, thioredoxin reductase; TCID₅₀, tissue culture infectious dose.

was first reported in Hong Kong in 2012 and is now a pandemic worldwide (Zhang, 2016). The outbreak of PDCoV has caused serious economic losses to swine farming, but there is no widely used drug and vaccine in production. Therefore, we need to find a drug or nutrient with an antiviral effect commonly used in production to fight PDCoV infection.

As an essential nutrient element, selenium mainly exists in organic and inorganic selenium. Selenoprotein and selenoamino acids are the most common organic selenium, and selenomethionine (Se-Met) is the most common selenium form ingested by the organisms from food (Weekley and Harris, 2013). The antioxidant and immunomodulatory functions of selenium are most important, and these functions are mainly exerted by selenoproteins, such as glutathione peroxidase (GSH-PX) and thioredoxin reductase (TrxR). GSH-PX 1-4 is involved in hydrogen peroxide (H₂O₂) signal transduction and maintaining the cellular redox state (Brigelius-Flohe and Maiorino, 2013). In addition, GPX1, GPX4, and TrxR1 are also the most abundant selenoproteins in a variety of immune cells, and they play an important role in T cell proliferation and NK cell activation (Chu et al., 1992; Wingler and Brigelius-Flohe, 1999; Lei et al., 2007; Huang et al., 2012; Ingold et al., 2018).

Innate immunity acts as the first line of defense against pathogenic microorganisms. RIG-I-like receptor (RLR) is a member of innate immunity, which plays a significant role against RNA viruses (Ren et al., 2020). RLR recognizes the virus and can activate the mitochondrial antiviral signal protein (MAVS) and interferon regulatory factor (IRF), which in turn secrete interferon (IFN) to achieve antiviral effects (Hou et al., 2011). Currently, selenium has been found to have antiviral effects, including against coxsackie virus, influenza virus, human immunodeficiency virus (HIV), and porcine circovirus (PCV) in humans and animals (Beck et al., 1994; Schrauzer and Sacher, 1994; Nelson et al., 2001; Jaspers et al., 2007; Qian et al., 2018; Guillin et al., 2019; Bermanno et al., 2021). The antiviral effect of selenium is mainly attributed to its antioxidant and immunomodulatory effects. For example, Se-Met (2, 4 mM) can inhibit PCV2 replication by inhibiting H₂O₂-mediated oxidative stress (Chen et al., 2012). In 450 HIV-1 seropositive patients, daily supplementation with 200 µg of yeast selenium not only decreased the viral load of HIV-1 but also increased the number of CD4⁺ T cells (Hurwitz et al., 2007).

Given the current situation of PDCoV, which is seriously harmful, there is no specific drug treatment. In this assay, we first examined the replication effect of Se-Met on PDCoV *in vitro*. Then, we further explored the potential mechanism of PDCoV inhibition by Se-Met from the perspective of innate immunity and anti-oxidation. It provides some theoretical basis and guiding significance for reducing PDCoV infection from antiviral nutrition.

MATERIALS AND METHODS

Cells Culture and Reagents

Pig Kidney Epithelial (LLC-PK) cells were provided by Professor Zhanyong Wei of Henan Agricultural University. Cells were

cultured in Minimum Essential Medium (MEM, Solarbio). MEM was supplemented with 8% fetal bovine serum (FBS, Gibco), 1% HEPES (Gibco), 100 IU/mL penicillin, and 100 IU/mL streptomycin solution. Se-Met was purchased from Sigma, United States. Referring to the results of Pan's study (Pan et al., 2008), we confirmed that 16 µM Se-Met was not toxic to cells. Se-Met was diluted to 2, 4, 8, and 16 µM with MEM.

Virus Stocks and Titration

PDCoV HNJK-04 strain (provided by Professor Zhanyong Wei, Henan Agricultural University) was used in this study. PDCoV was propagated in a maintenance medium (MEM supplemented with 1% antibiotics, 1% HEPES, and 5 µg/mL trypsin) containing LLC-PK cells. The number of infectious PDCoV particles was determined based on the 50% tissue culture infectious dose (TCID₅₀) in LLC-PK cells, according to the method described by Zhai's study (Zhai et al., 2019).

Assays for Antiviral Activity of Selenomethionine

To understand the inhibitory activity of Se-Met (2, 4, 8, and 16 µM) on PDCoV, cells were added to six-well plates and allowed to grow to about 90%. The virus stock was diluted to 100 TCID₅₀ and inoculated into cells for 1 h. The virus liquid was discarded, washed twice with D-Hanks, and Se-Met solution was added and cultured at 37°C for 24 h. The virus infection control group (group V) and the blank control group (group C) were randomly within the plates. At last, the viral load of each group was measured.

Assays of Virus Titer

RNA from LLC-PK cells was extracted using TRIzol reagent (Invitrogen) and cDNA synthesis using cDNA Synthesis Super (TransGen Biotech) according to the manufacturer's instructions. Then, RT-qPCR was performed using Perfect StartTM Green qPCR Super (TransGen Biotech). The virus was determined by absolute RT qPCR, and the primers for the PDCoV M gene are shown in **Table 1**. The 2^{-ΔΔCT} method was used to differentiate between control and treated cells.

Assays of Interferon

According to the kit instructions (Jiangsu MEIMIAN Co., Ltd. China), expression of IFN-α and IFN-β was determined using ELISA and RT qPCR. Primers for RT qPCR are shown in **Table 1**,

TABLE 1 | Primer sequence.

Primer	Sequence (5'→3')	Bp
M	F: CGCGTAATCGTGTGATCTATGT R: CCGGCCTTTGAAGTGGTTAT	
IFN-α	F: CTTCTGGACCTGGTTGCCC R: GCTCCTGGCACAAATGAGGA	103
IFN-β	F: GGAGATTATGCAACCACCA R: CCAGCCAGTGCTAGAGAAA	112
β-actin	F: CTGCGGCATCCACGAACT R: AGGGCCGTGATCTCCTCTG	147

and β -actin was used as a reference gene (Chen et al., 2012). There are three independent replicates done for treatment.

Detection of Oxidative Stress and Antioxidant Indicators

The cell pellet was collected at the end of cell culture after washing and centrifugation. One milliliter of the extract was added to the cell pellet, and the cells were disrupted by sonication for subsequent testing. The contents of glutathione (GSH) and H_2O_2 , as well as the activities of GSH-PX and superoxide dismutase (SOD), were measured using kits (Nanjing Jiancheng Bioengineering Institute, China). There are three independent replicates were done for treatment.

Western Blot Analysis

The expression of MAVS protein and the phosphorylation of IRF-3 in innate immunity was examined using Western blot to determine whether Se-Met affects innate immunity. The following primary antibodies were used: Anti-Phospho-IRF-3 (Ser396), Monoclonal Antibody (MA5-14947) (Invitrogen Corporation, NY, United States), Anti-IRF-3 Polyclonal antibody (11312-1-AP), and Anti-MAVS antibody (14341-1-AP) (Proteintech Group, Wuhan, China).

Statistical Analysis

Results were expressed as the means \pm standard deviation (SD). The test data were analyzed for the significance of difference by one-way ANOVA using SPSS 26 ($P < 0.05$).

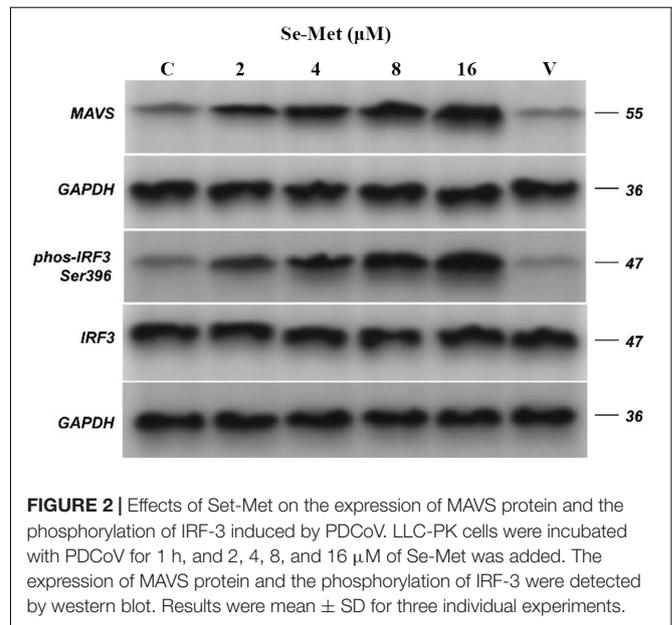
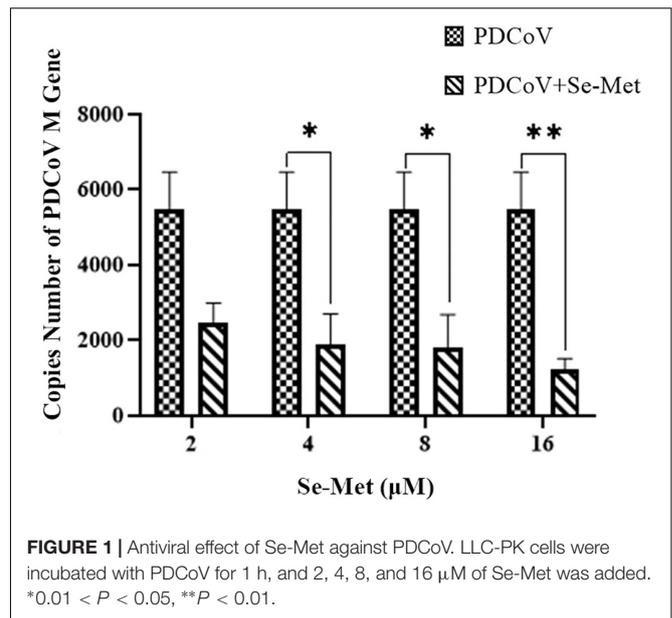
RESULTS

Selenomethionine Has Antiviral Activity on Porcine Deltacoronavirus

LLC-PK cells were treated with different concentrations of Se-Met (2, 4, 8, and 16 μ M) after inoculation with PDCoV. The copy number of the PDCoV M gene in each group was detected using RT qPCR 24 h after virus infection. The results are shown in **Figure 1**. The 4 and 8 μ M Se-Met could significantly inhibit the copy number of virus M gene ($0.01 < P < 0.05$). Moreover, after the virus was treated with 16 μ M Se-Met, the replication was extremely decreased ($P < 0.01$). In conclusion, Se-Met can inhibit the replication of PDCoV in a dose-dependent manner, with 16 μ M Se-Met having the best effect.

Selenomethionine Can Enhance Cellular Immunity After Porcine Deltacoronavirus Infection

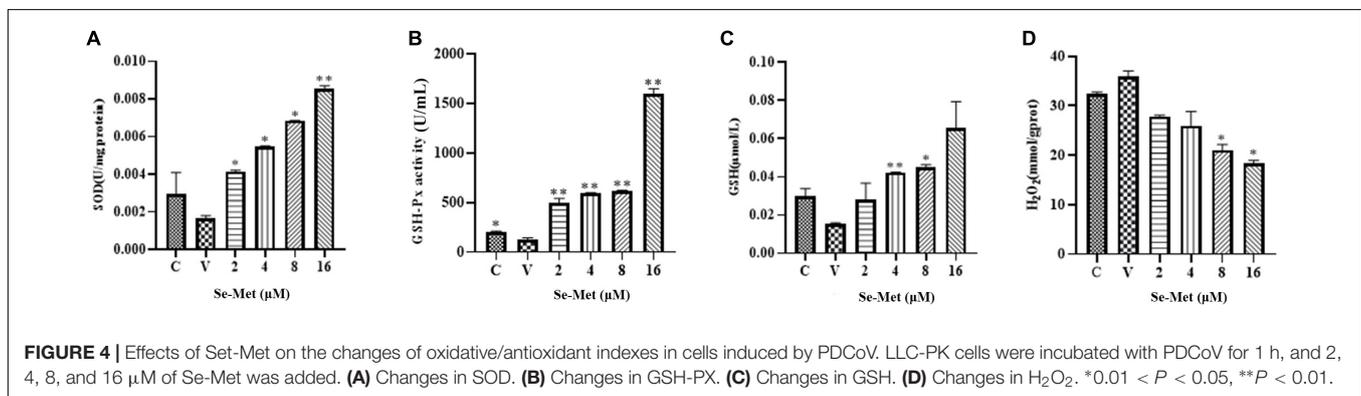
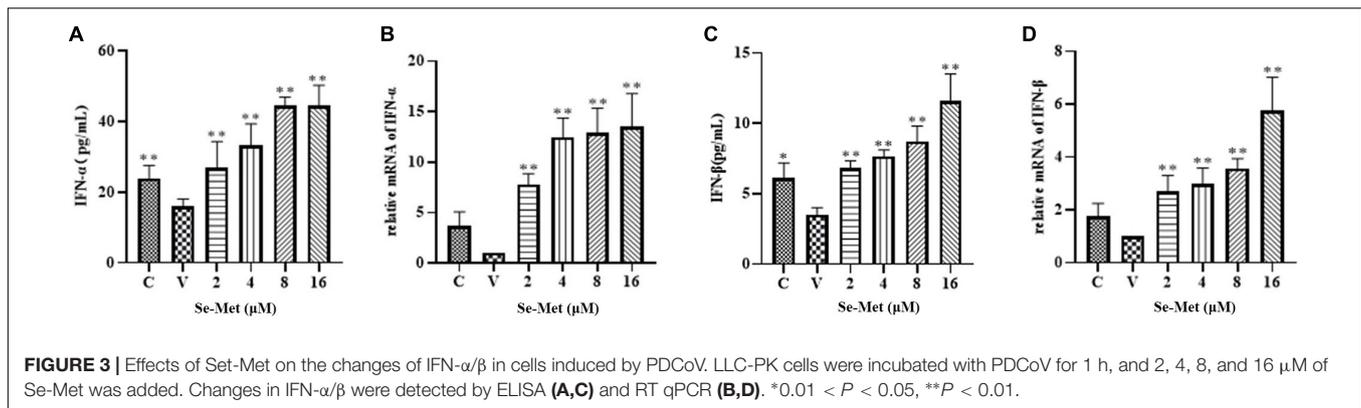
Evasion of innate immunity has emerged as a way for the virus to maintain replication. We treated LLC-PK cells with Se-Met in four concentrations to further investigate whether Se-Met inhibits viral replication by improving cellular immunity. First, we used Western blot to detect the expression of MAVS protein and phosphorylation of IRF-3 intracellularly and then used ELISA and RT-qPCR to detect the changes of IFN- α and IFN- β . Western blot results showed that PDCoV was able to significantly



reduce the protein expression of MAVS and the phosphorylation of IRF-3 ($0.01 < P < 0.05$). After Se-Met treatment, the protein expression of MAVS and the phosphorylation of IRF-3 were significantly increased in all concentration groups ($P < 0.01$, **Figure 2**). We can see from **Figure 3** that all concentrations of Se-Met significantly increased the production of IFN α / β in cells compared with group V ($P < 0.01$).

Selenomethionine Can Enhance the Antioxidant Capacity of Cells After Porcine Deltacoronavirus Infection

The effect of Set-Met on oxidative/antioxidant factors of LLC-PK cells induced by PDCoV. Se-Met and virus were applied



to LLC-PK cells using the modalities described above. After cytocentrifugation, cells were disrupted with ultrasound and used to detect GSH-Px, H₂O₂, SOD, and GSH. As shown in **Figure 4**, PDCoV was able to reduce the activity of GSH-Px in the cells significantly ($0.01 < P < 0.05$), and although the contents of SOD and GSH were also decreased, they did not change significantly ($P > 0.5$). We found that 16 μ M Se-Met was able to significantly increase the activity of GSH-Px and the content of SOD ($P < 0.01$). After PDCoV was treated with 8 and 16 μ M Se-Met, the H₂O₂ content was significantly reduced ($0.01 < P < 0.05$). In summary, PDCoV induces oxidative stress in cells; however, Se-Met can alleviate this damage.

DISCUSSION

Since PDCoV was first reported in 2012, PDCoV infections have been reported in regions every year, and new lineages have been slowly discovered. PDCoV is currently seriously damaging the development of the pig industry, but there are no commercial vaccines and antiviral drugs commonly used in the breeding industry. To develop a good drug against PDCoV infection, we found that the nutrient selenium has biological functions such as antiviral, antioxidant, and immunomodulatory (Guillin et al., 2019; Bermano et al., 2021). Whether Se-Met, as an organoselenium, has an inhibitory effect on the infection of PDCoV is unknown. In this experiment, we first determined that the TCID₅₀ of PDCoV for LLC-PK cells was $10^{-4.15}/0.1$ mL.

Subsequently, referring to the results of Pan's study (Pan et al., 2008), we confirmed that 16 μ M Se-Met did not affect the growth of cells, which was used as the maximum effective concentration of Se-Met in this experiment for the determination of subsequent antiviral and antioxidant assays.

Given the current situation that there is no specific drug treatment for PDCoV, some people have successively explored the potential of broad-spectrum antiviral drugs in treating PDCoV. For example, lithium chloride and diammonium glycyrrhizinate could inhibit PDCoV replication in LLC-PK cells in a dose-dependent manner (Zhai et al., 2019). In addition, Zhang explored the inhibitory effect of LJ001 on PDCoV in three ways (Zhang et al., 2020). He found that the antiviral effect of pretreatment was not significant, which may be because the receptor sites available for virus attachment on the cell membrane are altered after the cells are pretreated with the drug, which affects the fusion of virus and cells (Zhang et al., 2020). Studies on the inhibition of viral replication by selenium have been reported, for example, HIV, Coxsackie virus, influenza virus, and PCV (Beck et al., 1994; Schrauzer and Sacher, 1994; Nelson et al., 2001; Jaspers et al., 2007; Qian et al., 2018). In this experiment, PDCoV was treated with Se-Met in a post-treatment manner. The results showed that Se-Met significantly inhibits PDCoV replication, and 16 μ M Se-Met has the best effect; 16 μ M Se-Met showed better antiviral activity, but 16 μ M was far higher than the normal physiological concentration, and too high concentration of selenium would be counterproductive (Sivertsen et al., 2007; Zhao et al., 2016). We found that Se-Met at 8 and 16 μ M had

similar antiviral activity, and combined with the dangers of high concentrations of selenium, we believe that Se-Met at 8 μM is in line with clinical application. However, if we want to treat PDCoV, 8 μM Se-Met will not necessarily be clinically effective, so we also need to combine *in vivo* experiments.

After virus infection, the oxidative stress state of the organism can destroy the immune system in the body, which in turn facilitates virus replication (Zhang Z. et al., 2019). GSH-PX is an important enzyme in the biological function of selenium, and it achieves antioxidant effects by scavenging peroxides in the body (Tian et al., 2021). It was previously reported that selenium could alleviate virus-induced oxidative stress. For example, in the PK-15 cell model of PCV infection, 6 μM Se-Met can inhibit the increase of PCV2 replication by enhancing the activity of GSH-PX1 and inhibiting the production of H_2O_2 (Chen et al., 2012). SOD is an important antioxidant enzyme in organisms that scavenges superoxide radicals. Styblo et al. (2007) found that after mice were infected with the influenza virus, selenium supplementation significantly increased the SOD activity of the mouse liver. In addition, oseltamivir is an effective antiviral drug. Nano-selenium is surface-modified by oseltamivir, which significantly inhibits ROS generation induced by H1N1 (Li et al., 2017). This experiment verified that Se-Met could inhibit the replication of PDCoV in LLC-PK cells, and the GSH-Px, H_2O_2 , GSH, and SOD changes in the virus groups and the treatment groups were further compared. The experimental results are consistent with previous results on other viruses; that is, PDCoV inhibits the viability of cellular GSH-Px, increasing the content of H_2O_2 in the cell, which in turn causes oxidative stress. The addition of Se-Met can significantly increase the ability of GSH-PX and GSH in cells. From this, we can infer that Se-Met can alleviate the oxidative stress caused by PDCoV by improving the antioxidant capacity of cells, thereby achieving the effect of inhibiting virus replication.

During virus infection and replication, innate immunity acts as the first line of defense of the immune response, clearing the virus from the host. As a key receptor for the recognition of RNA viruses, RLR can activate the downstream MAVS after binding to the virus, which in turn stimulates the expression of IRF and nuclear factor kappa-B (Chen et al., 2018; Ren et al., 2020). Then, the release of IFN, a key cytokine for the host to see viral immunity, is stimulated by IRF. However, viruses have been able to evade or fight the host's immune system in various ways in continuous evolution (Wang et al., 2016; Kouwaki et al., 2021). It has been found that Hepatitis B can competitively bind

MAVS with the help of lactate and further interfere with the binding of RLR to MAVS to achieve the effect of evading innate immunity (Zhang W. et al., 2019). Then, At least eight proteins encoded by severe acute respiratory syndrome coronavirus have been identified as interferon antagonists (Devaraj et al., 2007; Kopecky-Bromberg et al., 2007; Wathelet et al., 2007; Siu et al., 2009). In addition, PDCoV was also found to evade host immune responses, and it not only avoided IFN- β activation but also inhibited IFN- β production induced by SeV or Poly (I: C) (Luo et al., 2016). This reason may be attributed to the degradation of interferon by the ubiquitin proteasome encoded by PDCoV (Ji et al., 2020). Based on previous studies, we hope to improve the antiviral effect of host innate immunity by adding Se-Met. As expected, Se-Met could significantly increase the expression of MAVS protein, the phosphorylation of IRF-3, and the mRNA level of IFN- α/β . Therefore, we conclude that Se-Met can activate MAVS and IRF-3 in innate immunity and secrete a series of IFN to inhibit PDCoV replication.

CONCLUSION

In summary, this study suggests that Se-Met could inhibit PDCoV replication in a dose-dependent manner. The underlying mechanism may be attributed to the activation of MAVS and IRF-3 in innate immunity by Se-Met, which in turn secretes a series of cytokines. In addition, it may also be because Se-Met can improve the antioxidant capacity of cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

ZR, ZCZ, JD, YH, ZJZ, and SY contributed to the conception and design of the study. TD, HH, and YY performed the statistical analysis. GJ wrote the first draft of the manuscript. The rest of the authors reviewed and revised the manuscript. All authors reviewed the manuscript, read, and approved the submitted version.

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