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Research Article 研究报告

硒代蛋氨酸对猪 δ 冠状病毒感染小鼠肠道损伤的保护作用及机制

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摘　要：【目的】探讨硒代蛋氨酸(Se-Met)对猪 δ 冠状病毒(porcine deltacoronavirus, PDCoV)感染小鼠肠道的氧化应激、肠道屏障损伤的保护作用及其潜在调节机制。【方法】40 只雌性 C57 小鼠随机分为对照组、Se-Met 组(0.3 mg/kg Se)、PDCoV 组和 Se-Met+PDCoV 组(0.3 mg/kg Se)。饲养 23 d 后，PDCoV 组和 Se-Met+PDCoV 组小鼠灌胃 PDCoV HNZK-02-P5 株 300 μL (1×10^6 $TCID_{50}$)，其余 2 组小鼠灌胃等量的 Dulbecco's 改良细胞培养基。观察所有小鼠的临床症状、食物摄取量和体重，直至 28 d。接种病毒后 5 d 时，收集肠道组织，测定 PDCoV 滴度。采用苏木精-伊红染色监测肠道病理变化。检测氧化应激相关指标：丙二醛(malondialdehyde, MDA)、超氧化物歧化酶(superoxide dismutase, SOD)和谷胱甘肽过氧化物酶(glutathione peroxidase, GSH-PX)。采用 2′,7′-双乙酸二氯荧光素(2′,7′-dichlorofluorescein diacetate, DCFH-DA)探针测定空肠组织中活性氧(reactive oxygen species, ROS)水平。免疫荧光法分析小肠紧密连接蛋白(ZO-1 和 Occludin)的变化。采用 RT-qPCR 法分析炎症因子(TNF-α、IL-1β、IL-6 和 IL-10)、肠道紧密连接蛋白(ZO-1 和 Occludin)及 Nrf2 信号通路(Nrf2、HO-1 和 NQO1)相关基因的表达。Western blotting 分析 Nrf2 信号通路相关基因的蛋白表达。【结果】体重、摄食量、病理检查及不同肠道组织病毒 RNA 滴度的结果证实 Se-Met 可以增加 PDCoV 感染小鼠的体重，降低肠道组织病毒滴度，并减轻 PDCoV 诱导的肠绒毛结构损伤。Se-Met 通过降低 IL-1β、IL-6 和 TNF-α 的基因表达来减轻 PDCoV 诱导的肠道炎症。Se-

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Met 通过增加 ZO-1 和 Occludin 的基因表达抑制 PDCoV 诱导的肠黏膜屏障损伤。Se-Met 通过降低 ROS 和 MDA 水平，增加 GSH-PX 和 SOD 水平，改善了 PDCoV 诱导的肠道氧化应激。Se-Met 通过激活 Nrf2 信号通路抑制 PDCoV 诱导的氧化应激。【结论】Se-Met 可能通过激活 Nrf2 信号通路减轻 PDCoV 感染小鼠肠道损伤，为预防和治疗 PDCoV 感染提供了理论依据。

关键词：PDCoV；Se-Met；小鼠；肠道损伤；Nrf2 信号通路

Protective effect and mechanism of selenomethionine on intestinal injury in mice infected with porcine deltacoronavirus

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Abstract: [Objective] To explore the protective effect of selenomethionine (Se-Met) on oxidative stress and intestinal barrier damage in mice infected with porcine deltacoronavirus (PDCoV) and the potential regulatory mechanism. **[Methods]** Forty female C57 mice were randomly grouped as follows: control, Se-Met (0.3 mg/kg Se), PDCoV, and Se-Met+PDCoV (0.3 mg/kg Se). After being fed with or without Se-Met for 23 days, the mice in the PDCoV group and the Se-Met+PDCoV group were administrated with 300 μL suspension of PDCoV HNZK-02-P5 strain (1×10^6 $TCID_{50}$) by gavage, while those in the other two groups were administered with the same volume of Dulbecco's Modified Eagle Medium (DMEM). All the mice were observed daily for clinical signs, food intake, and body weight changes until day 28. At five days post-inoculation (dpi), intestinal tissues were collected and PDCoV titers were determined. Hematoxylin staining and eosin staining were used to monitor pathological changes in intestinal tissues. Oxidative stress-related indicators such as malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) were investigated. The level of ROS in the jejunum tissue was measured *via* a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) probe. Immunofluorescence was used to analyze the changes of small intestinal tight junction proteins (ZO-1 and Occludin). The mRNA levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-10), intestinal tight junction proteins (ZO-1 and Occludin), and the Nrf2 signaling pathway-associated factors (Nrf2, HO-1, and NQO1) were determined by RT-qPCR. Western blotting was employed to assess the protein levels of factors related to the Nrf2 signaling pathway. **[Results]** The results of body weight, food intake, pathological examination, and viral RNA titers in different intestinal tissues revealed that Se-Met might increase the body weight, decrease viral titers in intestinal tissues, and attenuate PDCoV-induced structural damage of intestinal villi in PDCoV-infected mice. Se-Met attenuated PDCoV-induced inflammation by lowering the mRNA levels of major inflammatory cytokines, such as IL-1β, IL-6, and TNFα in

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the jejunum. Se-Met ameliorated PDCoV-induced intestinal mucosal barrier damage by up-regulating the mRNA levels of ZO-1 and Occludin in the jejunum. Se-Met ameliorated PDCoV-induced oxidative stress by decreasing the levels of ROS and MDA and increasing the levels of GSH-PX and SOD in the jejunum. Se-Met inhibited PDCoV-induced oxidative stress by activating the Nrf2 signaling pathway. **[Conclusion]** Se-Met may attenuate the intestinal injury in mice infected with PDCoV by activating the Nrf2 signaling pathway, which provides a theoretical basis for the prevention and treatment of PDCoV infection.
Keywords: PDCoV; Se-Met; mice; intestinal injury; Nrf2 signaling pathway

Coronaviruses (CoVs) are a family of enveloped, single-stranded, positive-strand RNA viruses classified within the Nidovirales order, which has become one of the most important pathogens threatening human and animal health. Studies have shown that CoVs can jump from animal hosts to humans, and cause zoonotic diseases. Swine enteric coronaviruses (SECoVs), including transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV), the recently emerged swine acute diarrhea syndrome coronavirus (SADS-CoV), can infect pigs of different ages, and cause enterocyte loss and atrophy of villi, which leads to watery diarrhea and vomiting with high morbidity and mortality in piglets[1-3], and pose a heavy disease burden on livestock. Notably, PDCoV has been proven to have the ability of cross-species transmission. Recent studies have confirmed that PDCoV can infect SPF chickens and mice and induce intestine injury[4-6]. Furthermore, PDCoV was detected and isolated from the plasma samples of three Haitian children with acute febrile illness[7], which suggests that PDCoV has posed a threat to public health security. Unfortunately, there is no effective drug or vaccine against PDCoV.

Reactive oxygen species (ROS) production, as a consequence of microbial intrusion, has long been acknowledged for its role in the antimicrobial defense of phagocytes[8]. Activation of the antiviral and inflammatory signaling pathways has also been associated with the production of ROS[9-10], which involves oxygen ions, peroxides, and oxygen free radicals, which are byproducts of aerobic metabolism. Because of the high chemical reactivity of ROS, cells possess antioxidant defense systems that maintain redox homeostasis. ROS-mediated oxidative stress activates the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2), and Nrf2 binds to downstream antioxidant response elements (AREs), which promotes the transcriptional activation of antioxidant genes and reduces oxidative stress by bursting ROS[11-12].

In many viral infections, the increase in ROS generation and alterations in redox homeostasis contribute to viral pathogenesis by modifying biological structures and inducing extensive cell death[13]. In the SECoVs family, TGEV and PEDV have been shown to promote oxidative stress and manipulate antioxidant system, leading to cell injury[14-16]. Additionally, PDCoV infection can significantly reduce the activity of GSH-PX, increase the amount of ROS, and further cause oxidative stress, thereby promoting viral replication *in vitro*[17]. Furthermore, PEDV infection triggers aberrant regulation of hepatic antioxidant genes through epigenetic inhibition of ROR/NRF2-

mediated transcription[18].

Selenium (Se) is an essential trace element for living organisms and has been implicated in antioxidant mechanisms, regulation of immune function, and thyroid hormone metabolism[19]. Se is involved in the regulation of defense mechanisms of various viral diseases. Studies have shown that Se reduces oxidative stress induced by viral infections, protects cells from oxidative damage, and maintains the integrity of the cell[20]. Se also can promote the number of $CD4^+$ T cells, which plays a role in enhancing the immune response to viral infections[21]. Se's antioxidant bioactivity and immunomodulatory functions are of paramount importance, which are mainly derived from the insertion of selenoproteins, such as glutathione peroxidase (GSH-PX) and thioredoxin reductase (TrxR)[22-23]. Other studies have shown that dietary selenium deficiency can alter the viral genome, causing the virus to become hypervirulent in response to oxidative stress[24-26]. Se deficiency weakens defense against infectious diseases by reducing the expression of selenoproteins. Thus, it is now recognized that the nutritional status of the host plays a dominant role in the prevention of viral diseases[27-31]. Multiple pieces of evidence have shown a close interdependence between Nrf2 regulation and the antioxidant system of Se[32-33].

Because of its high bioavailability and greater safety, organic Se is more easily absorbed by the body and is commonly used as an alternative for nutritional treatment[34]. Selenomethionine (Se-Met), as the main chemical form of dietary se in our daily supplement, has been widely used a variety of health food products[35]. It has been reported to inhibit PDCoV replication *in vitro* due to the fact that Se-Met36 enhances the antioxidant capacity and activates the cellular immune system[36]. However, the specific mechanism of PDCoV-induced intestinal damage in mice and the effectiveness of Se-Met as a potent antiviral therapy are unknown.

1 Materials and Methods

1.1 Chemicals and materials

Se-Met (98%) was obtained from J&K Scientific Ltd. (Beijing, China), T-SOD (hydroxylamine approach), MDA (TBA approach) and GSH-PX (colorimetry) detection kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

1.2 Virus and animals

The PDCoV HNZK-02 passage 5 (P5) (GenBank accession number MT260149) was provided by Professor Zhanyong Wei at Henan Agricultural University.

Four-week-old female C57 mice with a specific pathogen-free health status were provided by the Experimental Animal Center of Zhengzhou University. All experimental procedures were approved by the Medical Ethics Committee of Yan'an University Affiliated Hospital and was approved (YAU-G20230081).

Forty mice were randomly assigned with similar body weight ((16.05±0.40) g) to 4 groups (10 mice per group and 2 replicates per group), namely Control group, Se-Met group, PDCoV group and Se-Met+PDCoV group. After acclimatization for 1 week, the mice were provided with the standard diet, Se-Met-treated diet (0.3 mg/kg Se), standard diet and the Se-Met-treated diet (0.3 mg/kg Se), respectively. The trial lasted for 28 d. On the day 23 of the experiment, the mice in the PDCoV group and Se-Met+PDCoV group were intragastrically inoculated with 300 μL

of PDCoV HNZK-02-P5 strain (1×10^6 $TCID_{50}$), whereas the mice in the other two groups were orally administered the same volume of Dulbecco's Modified Eagle Medium (DMEM). All mice were observed daily for clinical signs and body weight evaluation. The intestinal tissues were quickly removed. The intestinal length was measured using a measuring scale. After being rinsed with ice-cold sterile deionized water, the partial intestinal tissues were ground into homogenates, and the other part of the intestinal tissue was fixed in 4% formaldehyde solution. All remaining intestinal tissues were stored at −80 ℃ until needed.

1.3 Se concentration detection

Inductively coupled plasma mass spectrometry (ThermoFisher Scientific) was used to estimate Se concentrations in the feed. Detailed experimental procedures referred to the previous publication[37]. Briefly, the feed sample (0.33 g) and the water for mice to drink (0.2 mL) were digested in a microwave digestion system with 6 mL of HNO_3 (65%) and 2 mL of H_2O_2 (30%) and diluted to 10 mL with deionized water. The blank group digest was performed by the same method. The digested samples were filled with ultrapure water and then analyzed by inductively coupled plasma mass spectrometry.

1.4 Histological analysis of the jejunum

The jejunum tissues taken from a 4% formaldehyde solution were embedded in paraffin. 5 μm-thick sections were stained with hematoxylin and eosin (H&E) and examined following previously described methods[38]. The ratio of villus height to crypt depth (VH/CD), intestinal wall thickness, and number of internal villi per 1 000 μm were measured and calculated by computer-assisted microscopy (NIS-Elements).

1.5 Total RNA extraction and RT-qPCR

Total RNA was extracted from intestine tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and then RNA was reverse-transcribed (RT) into cDNA using a reverse transcription kit (Vazyme Biotech). Later, RT-qPCR was conducted using SYBR premix *Ex Taq* (Vazyme Biotech). RT-qPCR was used to quantify the titer of PDCoV in mouse intestinal tissues as previously reported[4]. The relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized against β-actin gene expression. The primer sequences were shown in Table 1 (accession numbers NMDCX0002104).

1.6 ROS detection

ROS levels in jejunum tissues were measured using a 2′,7′-dichlorofluorescein diacetate (DCFH-

Table 1 The primers used in the present study

Primers name	Primer sequence (5′→3′)
PDCoV	CAACCGTCTTGAGGAAGTAGAG
	TCAACGGTGAGGTTGAGAATAG
ZO-1	ACCCGAAACTGATGCTGTGGATAG
	AAATGGCCGGGCAGAACTTGTGTA
Occludin	GGACCCTGACCACTATGAAACAGACTAC
	ATAGGTGGATATTCCCTGACCCAGTC
IL-10	GGTTGCCAAGCCTTATCGGAAATG
	GCCGCATCCTGAGGGTCTTC
IL-1β	CTCGCAGCAGCACATCAACAAG
	CCACGGGAAAGACACAGGTAGC
IL-6	TTCTTGGGACTGATGCTGGTGAC
	CTGTTGGGAGTGGTATCCTCTGTG
TNF-α	CACCACGCTCTTCTGTCTACTGAAC
	AGATGATCTGAGTGTGAGGGTCTGG
Nrf2	AAGCACAGCCAGCACATTCTCC
	TGACCAGGACTCACGGGAACTTC
HO-1	TCCTTGTACCATATCTACACGG
	GAGACGCTTTACATAGTGCTGT
NQO1	GAAGACATCATTCAACTACGCC
	GAGATGACTCGGAAGGATACTG
β-actin	GATGGTGGGAATGGGTCAGAAGG
	TTGTAGAAGGTGTGGTGCCAGATC

DA) probe according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). Briefly, frozen jejunum slides were co-incubated with a DCFH-DA (10 μmol/L) probe at 37 ℃ for 30 min. After washing with PBS 3 times, the sections were stained with DAPI for 10 min. The fluorescence images were captured using a Nikon microscope (TE2000 U).

1.7 Detection of redox state in jejunum tissues

Freshly collected jejunum tissues were ground into homogenates, and the levels of total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) were assayed using commercially available kits according to the instructions of the reagent company (Nanjing Jiancheng Bioengineering Institute). A 10% tissue homogenate was prepared by grinding approximately 1 g of jejunal tissue using a tissue homogenizer. The supernatant was extracted and the protein concentration in the supernatant was detected. The SOD activity, GSH-PX activity, and MDA content of the samples were determined according to the operating instructions, respectively.

1.8 Western blotting (WB) assay

Total proteins were obtained from jejunum tissues using the RIPA containing 1 mmol/L PMSF (Beyotime). The BCA protein assay reagent was used to determine the protein concentrations (Beyotime). Next, the proteins were separated using 12% SDS-PAGE, transferred onto PVDF membranes, blocked with 5% skimmed milk (Servicebio) for 1.5 h, and incubated overnight with diluted primary antibody against nuclear factor-E2-related factor 2 (Nrf2) (1∶2 500 dilution, Proteintech), heme oxygenase (HO-1) (1∶2 500 dilution, Proteintech), and quinone oxidoreductase 1 (NQO-1) (1∶2 500 dilution, Servicebio) at 4 ℃, washed five times with PBST. Then it was incubated with HRP-conjugated goat anti-rabbit secondary antibody (1∶3 000 dilution, Servicebio) at room temperature for 60 min and washed again. Immunoreactive bands were visualized using ECL reagent and images were captured using a Fluor Chem system (Alpha Innotech). The protein levels were quantified using ImageJ software.

1.9 Immunofluorescence staining of jejunum

Immunofluorescence was performed as previously described[39]. Briefly, dewaxed jejunum sections were repaired with 0.01 mol/L sodium citrate buffer (pH 6.0). After blocking with 5% BSA for 30 min, the sections were incubated overnight at 4 ℃ with rabbit anti-ZO-1 and rabbit anti-Occludin in a dilution of 1∶100. Subsequently, they were incubated with biotin-conjugated secondary antibody that was added dropwise for 2 h at room temperature in the dark, followed by incubation for 30 min at 37 ℃ and staining with Antifade Mounting Medium with DAPI. The fluorescence intensity was observed under a confocal microscope. The immunoreactivity density was analyzed using ImageJ.

1.10 Statistical analysis

One-way analysis of variance (ANOVA) was performed to analyze the significance between groups, and Tukey post hoc test was used for multiple comparisons. GraphPad Prism 6.0 was used for statistical tests. All experiments were at least technical triplicates for each experimental group and multiple independent experiments were performed. Results were presented as mean and standard deviation (SD). Statistical details can be found in figure legends. $P<0.05$ was considered

statistically significant. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

2 Results

2.1 Se-Met ameliorated the manifestations in PDCoV-infected mice model

To observe the beneficial effects of Se-Met in mice, we treated mice with Se-Met (Figure 1A). Food intake and body weight were used to monitor the general state of the mice. There was no significant difference ($P<0.05$) in the amount of food intake and body weight among the groups for 0−23 days of the test (Figure 1B, 1D). To observe the beneficial effects of Se-Met on PDCoV-infected mice, mice treated with or without Se-Met were infected with PDCoV (Figure 1A). At day 1 post-inoculation (dpi) (at the 24 day of the experiment), PDCoV-infected mice showed a significant reduction in the amount of food intake compared with the Control group ($P<0.05$ or $P<0.01$). Rapid recovery of feed intake was observed in mice of the Se-Met+PDCoV group, which was significantly higher than that of mice in the PDCoV group at 2 − 5 dpi ($P<0.001$ or $P<0.01$) (Figure 1C). Compared to the Control group, the body weight of mice in the PDCoV group began to decline sharply from 3 dpi (decrease of 7.9%, $P<0.001$), followed by a slow increase, but the weights were still lower than those of the Control group ($P<0.001$). However, Se-Met treatment significantly reversed this change ($P<0.001$ or $P<0.01$) (Figure 1E). In addition, the feed intake and body weight of mice in the Se-Met group were significantly increased compared to the Control group (Figure 1C, 1E). Taken together, the results showed that Se-Met treatment could increase the food intake and body weight of mice and ameliorate the manifestations in mice infected with PDCoV.

According to macroscopic analysis, the intestinal walls of mice infected with PDCoV became translucent and thinned at 5 dpi, and some yellow liquids accumulated in the jejunum of mice, while Se-Met treatment attenuated intestinal pathologic changes in mice, which suggested that Se-Met alleviated intestinal pathologic damage in PDCoV-infected mice (Figure 1F). Further, in PDCoV-infected mice, the length of the mice intestine was found to be significantly shorter ($P<0.001$), whereas Se-Met treatment significantly improved the change in mice infected with PDCoV ($P<0.01$) (Figure 1G, 1H).

2.2 Se-Met reduced virus load in intestinal tissues and attenuated intestine injury in mice

To determine the distribution in different intestinal tissues of PDCoV, qRT-PCR was used to detect viral RNA from different intestinal segments collected at 5 dpi (Figure 2A). In the PDCoV group, viral RNA was detected in different intestinal segments, with the highest viral load in the jejunum (6.33 $\log_{10}$ GE/mL), followed by the ileum (5.61 $\log_{10}$ GE/mL), the duodenum (5.21 $\log_{10}$ GE/mL), the rectum (5.27 $\log_{10}$ GE/mL), colon (5.23 $\log_{10}$ GE/mL), and the cecum had the lowest load (4.94 $\log_{10}$ GE/mL). All of them were higher than the Se-Met+PDCoV group. The results indicated that Se-Met reduced the PDCoV load. No viral RNA was detected in tissue samples from uninfected mice.

In addition, we observed the destructive effects of PDCoV in the jejunum and the therapeutic effects of Se-Met, as shown in Figure 2B. Compared with the Control group, the villi of

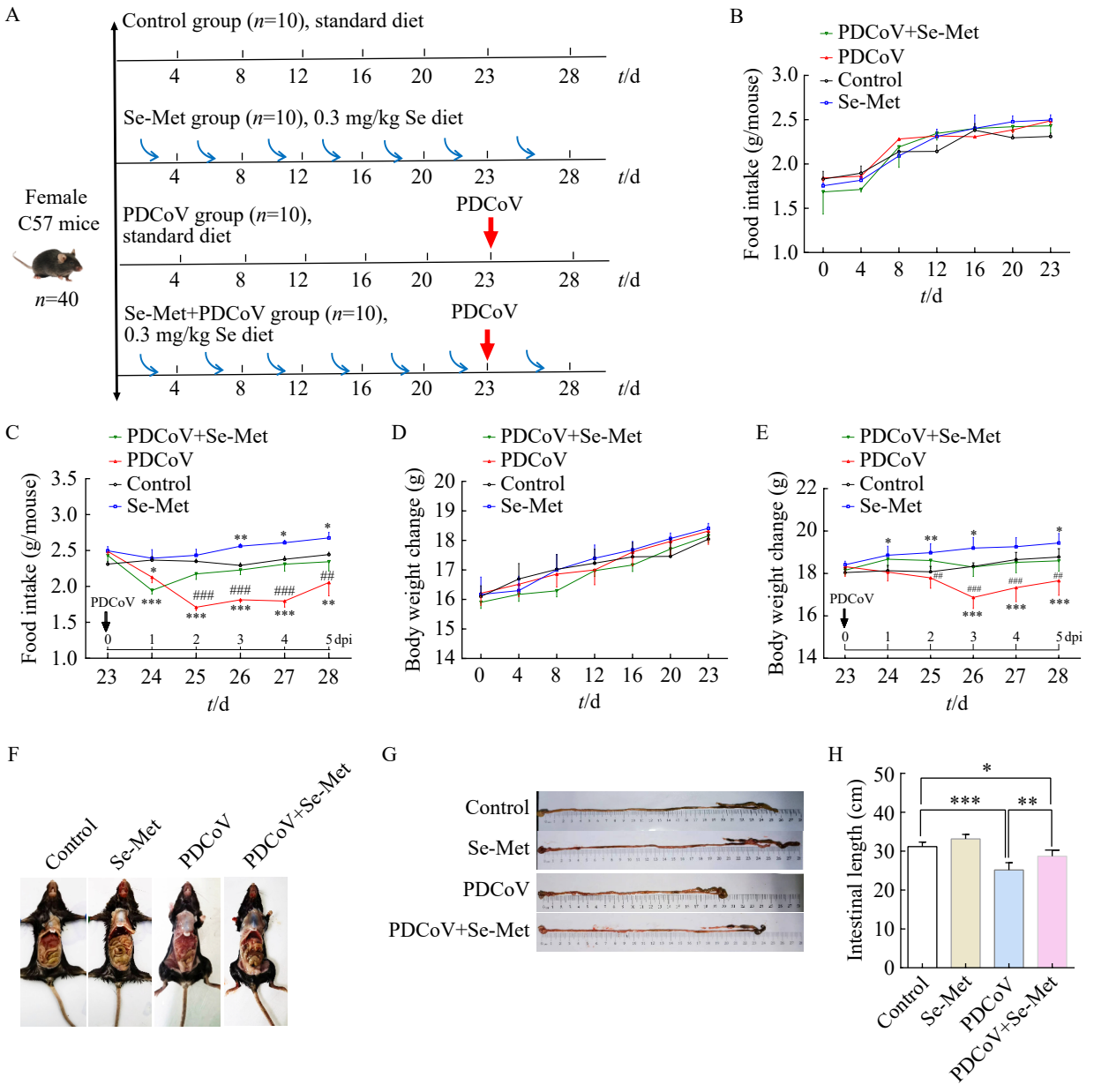

Figure 1 Se-Met alleviates the manifestations in PDCoV-infected mice models. A: Timeline of the animal experiment; B, C: Food intake change; D, E: Body weight change; F: Macroscopic lesions of the small intestines; G, H: Small intestine length. Data are presented as mean±SD (n=6). *: P<0.05, **: P<0.01 *vs.* Control group; $^{\#\#}$: P<0.01 *vs.* PDCoV+Se-Met group; ***: P<0.01 *vs.* Control group; $^{\#\#\#}$: P<0.01 *vs.* PDCoV+Se-Met group; dpi: Days post-inoculation.

the jejunum in the PDCoV group were atrophied and sparsely distributed, and even necrotic and detached, with a significant decrease in the villus height/crypt depth ratio (*V*/*C*) (P<0.001) (Figure 2C), a significant decrease in the number of villi (P<0.01) (Figure 2D), and a significant thinning of the entire intestinal wall (P<0.001) (Figure 2E). Se-Met treatment significantly improved jejunal

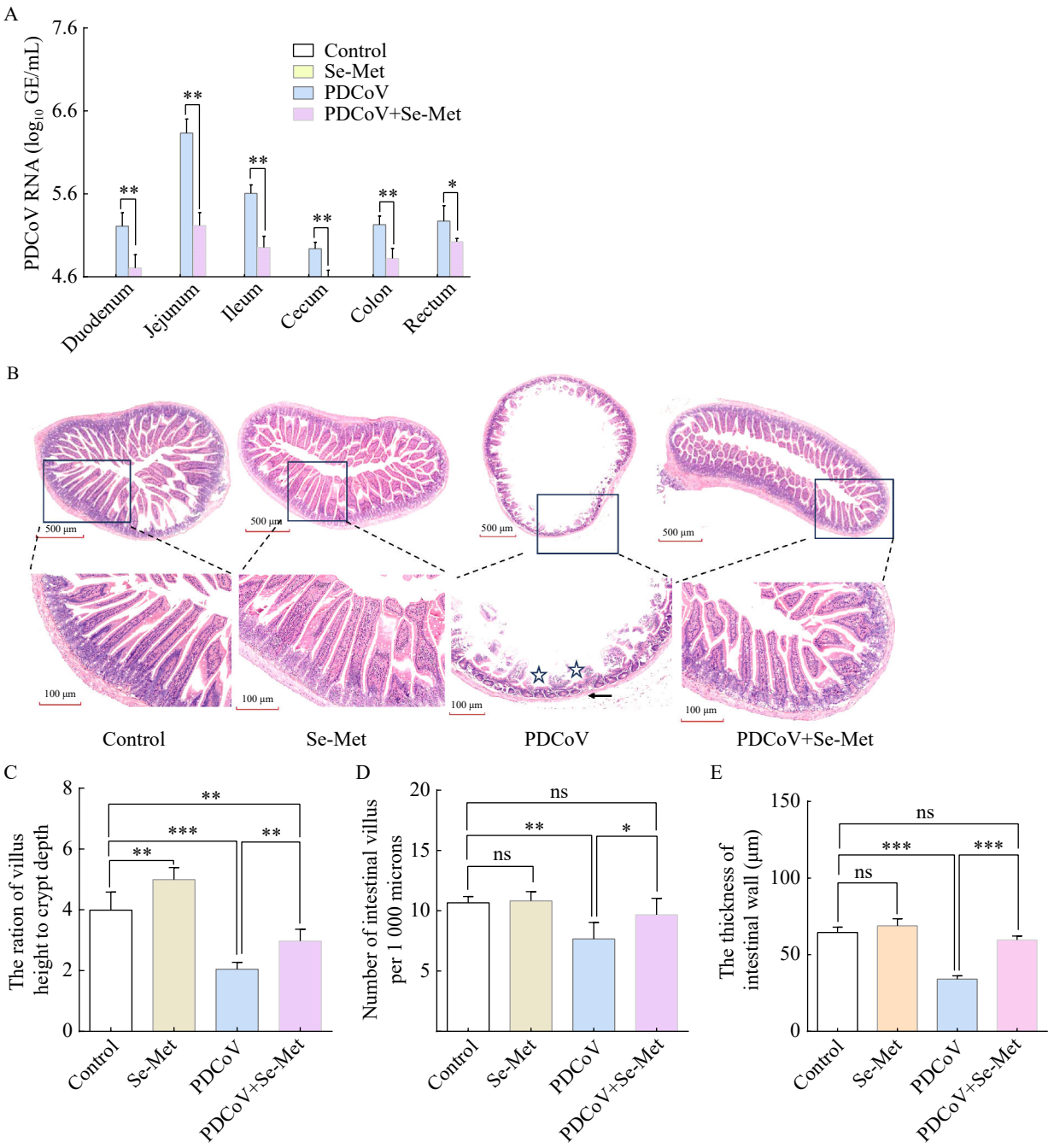

Figure 2 The effects of Se-Met on viral titers in the intestinal tissues and jejunum injury in mice induced by PDCoV. A: Inoculated with PDCoV *via* RT-qPCR assay; B: Paraffin sections of jejununal tissues from each group of mice (n=6) stained with H&E (Scale bars were different and labeled at the bottom left corner of each photo, and the boxed region in the image is enlarged below, each image is representative of six mice. ←: Intestinal wall thinning, ☆: Villous atrophy and sparse); C–E: Data analysis of the reparative effects of Se-Met on PDCoV-induced decreases in *V*/*C* ratio (Villus number, and intestinal wall thickness in mice jejuna, data are presented as mean±SD (n=6). ns: No significance; *: P<0.05; **: P<0.01; ***: P<0.001).

injury, with significant recovery in *V*/*C* (P<0.01), villous number (P<0.05), and intestinal wall thickness (P<0.001) (Figure 2B−2E).

2.3 Se-Met inhibited PDCoV-caused intestine inflammation and protected against PDCoV-induced intestinal barrier dysfunction in mice

Many studies have reported that Se-Met has an inhibitory effect on inflammation. Similarly, our study revealed the anti-inflammatory impact of Se-Met on PDCoV-induced intestine damage (Figure 3A−3C). The mRNA levels of TNF-α, IL-1β, and IL-6 were significantly increased after PDCoV treatment compared to the Control group (P<0.001). Furthermore, the Se-Met treatment attenuated the inflammatory response to PDCoV in the jejunum. In the PDCoV group, the anti-inflammatory cytokine IL-10 showed a significant reduction compared to the Control group (P<0.001) (Figure 3D). In addition, tight junction-associated proteins (ZO-1, Occludin), which maintain intestinal barrier function[40], were decreased in the PDCoV group compared with the Control group and the PDCoV+Se-Met group (Figure 3E − 3F). Furthermore, we detected their fluorescence intensity by immunofluorescence staining. The relative fluorescence intensities of ZO-1 (P<0.001) and Occludin (P<0.01) in jejunal tissues were significantly higher in the PDCoV+Se-Met group compared with the PDCoV group compared with the PDCoV group (Figure 3G − 3I). To summarize, these findings indicated that Se-Met had the ability to fight the development of inflammation in the mice infected with PDCoV.

2.4 Se-Met alleviated PDCoV-caused intestinal oxidative stress in mice

Due to the close relationship between oxidative stress and inflammation, we further examined oxidative stress-related marker changes in jejunal tissues. ROS levels in mouse jejunal tissues were detected *via* frozen section ROS staining (Figure 4A, 4B). Activities of T-SOD, GSH-PX, and contents of MDA were also determined (Figure 4C − 4E). The results showed that PDCoV significantly increased ROS accumulation and MDA levels (P<0.001) and significantly decreased SOD and GSH-PX levels in mouse jejunal tissue (P<0.001) compared with the Control group. Furthermore, the Se-Met treatment significantly reduced ROS accumulation (P<0.001) and MDA levels (P<0.001), while increasing SOD and GSH-PX levels induced by PDCoV (Figure 4A − 4E). In addition, we found Se-Met supplementation significantly increased GSH-PX levels in the Se-Met group compared with the Control group (P<0.01). Therefore, our results found that Se-Met could increase antioxidant capacity and alleviate PDCoV-caused intestinal oxidative stress in mice.

2.5 Se-Met inhibited PDCoV-induced oxidative stress by the Nrf2 signaling pathway

Considering the strong correlation between the Nrf2 pathway and oxidative stress, we investigated the role of PDCoV in the Nrf2 pathway (Figure 5). RT-qPCR analysis revealed that the mRNA levels of Nrf2 signaling pathway-related genes (Nrf2, HO-1, and NQO1) were significantly reduced in the PDCoV group compared to the control group (P< 0.001) (Figure 5A−5C). Similarly, Western blotting showed the PDCoV-induced downregulation of Nrf2, HO-1, and NQO1 expression in mice (P< 0.001). After being treated with Se-Met, these results were reversed in the mice infected with PDCoV. Moreover, we noted that the gene and

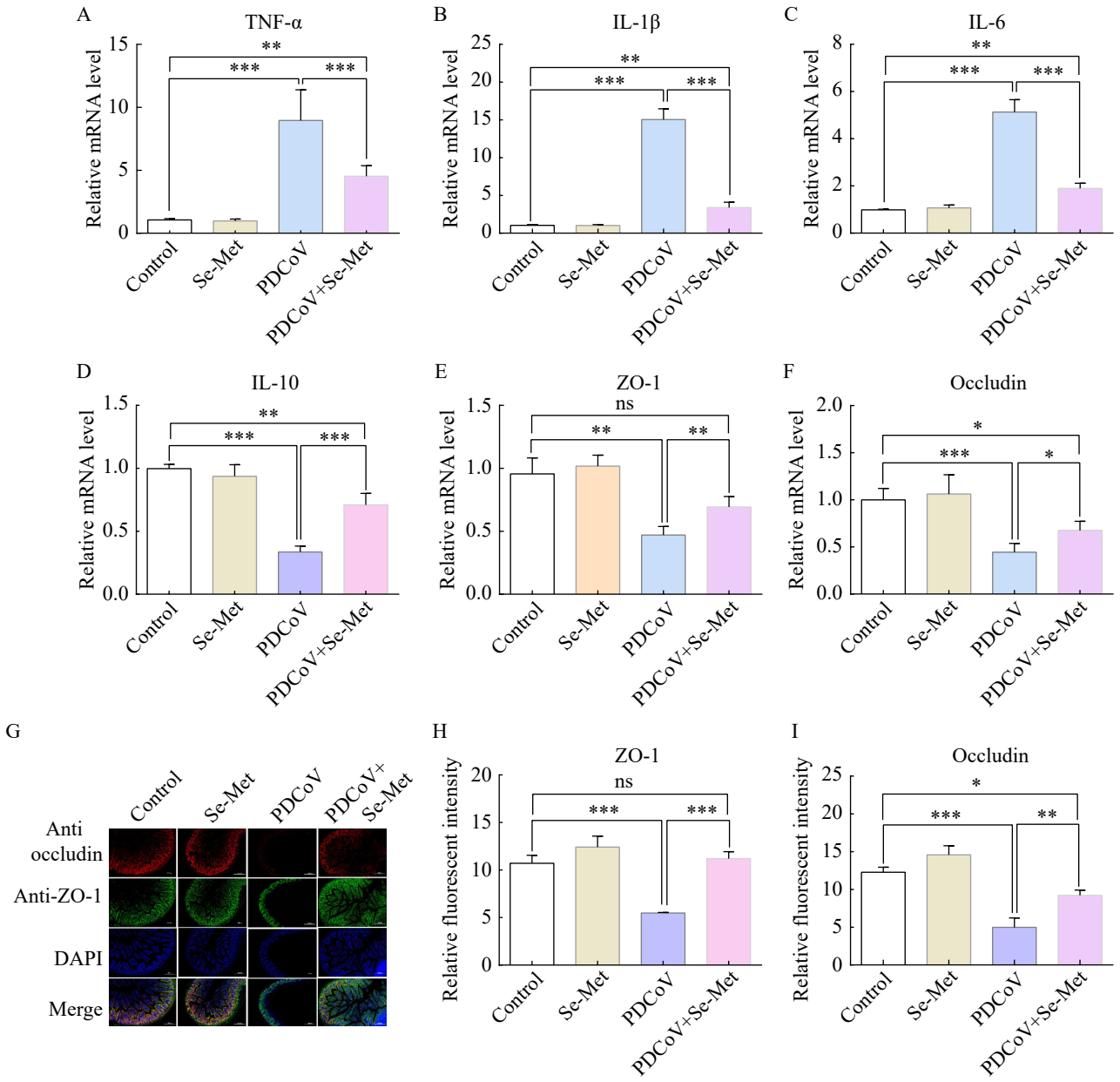

Figure 3 Reparative effects of Se-Met on mice jejunal inflammation and mucosal damage induced by PDCoV. A – D: mRNA expression of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-10) in jejuna; E, F: mRNA expression of ZO-1 and Occludin in jejuna, G: Immunofluorescence images showing changes of ZO-1 and Occludin in jejuna of mice from different groups (Scale bar=200 μm, each image is representative of six mice); H, I: The quantification of G, data were presented as mean±SD (n=6). ns: No significance; *: P<0.05, **: P<0.01; ***: P<0.001.

protein expression of Nrf2, HO-1, and NQO1 were notably higher in the Se-Met group than in the Control group (Figure 5D – 5E). Overall, Se-Met acted on the Nrf2 pathway by increasing the expression of HO-1 and NQO1 in mice, which protected against oxidative stress and inflammation

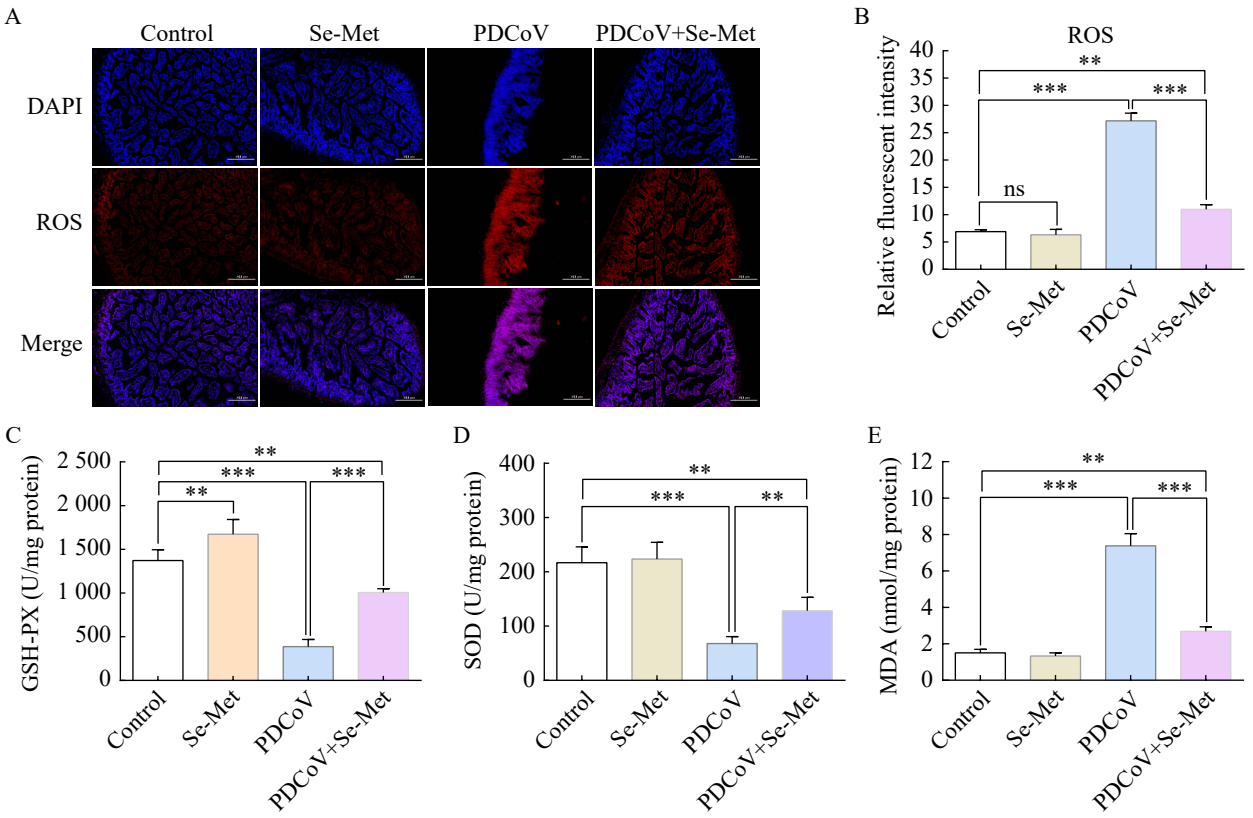

Figure 4 Effects of Se-Met on PDCoV-induced oxidative stress in the jejunum of mice. A: ROS fluorescence images showing changes of ROS in jejunum of mice from different groups (Scale bar=200 μm, each image is representative of six mice); B: The quantification of A; C: The contents of GSH-PX in mouse jejunum and the concentrations of GSH-PX were expressed as U/mg protein; D: The contents of total SOD were measured in mouse jejunum and the concentrations of SOD were expressed as U/mg protein; E: The contents of MDA in mouse jejunum and the concentrations of MDA were expressed as nmol/mg protein. Data are expressed as mean± SD (n=6). ns: No significance; **: P<0.01; ***: P<0.001.

injury in PDCoV-infected mice.

3 Discussion

As a destructive enteropathogenic animal coronavirus, PDCoV can cause intestinal damage in pigs, chickens, and mice, which seriously affects their survival prognosis[4-6,41]. Most alarmingly, the cross-species transmissibility of PDCoV has posed a potential threat to humans. Se-Met is an essential micronutrient with important roles such as antioxidant, antiviral, and anti-inflammatory, and in order to better understand the role of Se-Met in the cross-species transmission of PDCoV, we used mice as a model of PDCoV infection. The main purpose of this study was to explore the protective and reparative effects of Se-Met on PDCoV-induced mucosal barrier disruption in the intestinal epithelium, as well as its potential mechanisms, which will provide an essential theoretical basis for preventing or treating new cross-species transmission in the future.

In the present study, after inoculating with

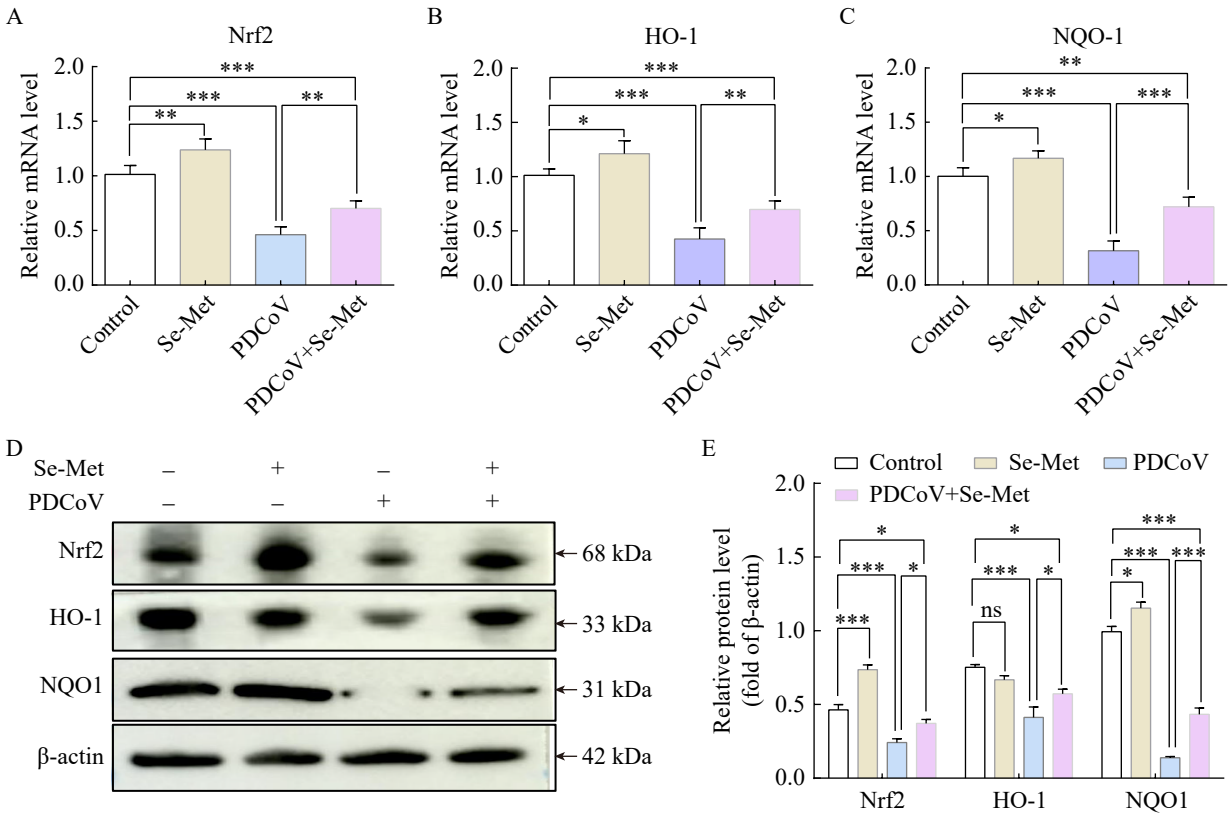

Figure 5 Effects of Se-Met on genes and proteins associated with the Nrf2 pathway in the jejunum of PDCoV-infected mice. A−C: mRNA expression of Nrf2, HO-1 and NQO1 in the jejuna; D: Protein expression of Nrf2, HO-1 and NQO1 in the jejuna; E: The quantification of D. Data are presented as mean±SD (n=6). ns: No significance, *: P<0.05; **: P<0.01; ***: P<0.001; +: Contains the substance; −: Does not contain the substance.

PDCoV, there were significant body weight losses from 2 to 5 dpi in C57 mice, which was consistent with the PDCoV-induced weight loss in BALB/c mice[6]. The intestinal walls were found to be translucent and thinned and some yellow liquids accumulated in the jejunum of the PDCoV-inoculated mice. In addition, jejunal villi were found to be atrophied and sparsely distributed, and even necrotic shedding. The length of the intestinal tract of PDCoV-infected mice was significantly shorter relative to that of uninfected mice, and viral RNAs were detected in the different intestinal tissues. These findings match those observed in earlier studies[6,42]. It indicated that the PDCoV-infected mouse model was successfully constructed. Therefore, the tissues treated with PDCoV were used for subsequent analysis.

PDCoV has become a potential threat to humans due to its ability to spread across species. Effective antiviral drugs are urgently needed to treat the pathogenic potential of PDCoV. Several have sequentially explored the potential of broad-spectrum antiviral drugs for the treatment of PDCoV. For example, lithium chloride and diammonium glycyrrhizinate and Se-Met could inhibit PDCoV replication in LLC-PK cells in a dose-dependent manner[36,43]. In addition, remdesivir showed high efficacy against PDCoV *in*

vitro[44]. The role of Se in inhibiting viral replication in many animal models has been extensively studied[24-26], as well as moderate amounts of Se can promote animal growth performance and health. Studies have shown that the supplementation of Se (0.3 mg/kg) can increase average daily weight gain and decrease the feed-to-gain ratio of piglets under stress[45-46]. In this study, Se concentration in the standard diet, Se-Met-treated diet, and drinking water were about 139.38, 424.27, and 31.39 μg/kg, respectively (Table 2, accession number NMDCX0002104). These data showed that the dose of Se used in test mice was less than 0.5 mg/kg, which is in line with the levels of Se in the range of normal supplementation[47-48].

Previously, it was found that Se-Met exhibited antiviral effects in LLC-PK cell experiments[36]. Therefore, in this article, we constructed a PDCoV-infected mouse model and continued to explore whether Se-Met could play antiviral effects in PDCoV-infected mice. The result showed that Se-Met significantly reduced virus load in intestinal tissues and attenuated intestinal injury, thus maintaining the body weight of PDCoV-infected mice. We performed intestinal tissue measurements and H&E staining of mouse jejunal tissues, and the results showed that Se-Met significantly improved intestine length, significantly increased the *V*/*C* ratio and villi count, and thickened the intestinal wall in PDCoV-infected mice, indicating that Se-Met significantly reduced the pathological features of jejunum tissue.

Table 2 Selenium concentrations in the feed and water

Items	Selenium (μg/kg)
Feed (standard diet)	139.38±6.66
Feed (Se-Met-treated)	424.27±10.32
Drinking water	31.39±3.23

Date are expressed as means±SD (*n*=3).

Cytokines play a crucial role in the regulation of inflammatory response in the gastrointestinal tract. During the inflammatory response, up-regulation of IL-1β, IL-6, and TNF-α inflammatory mediator expression results in further intestinal damage[49], while anti-inflammatory cytokines, including IL-10, may also attenuate or protect against intestinal inflammation[50]. Therefore, decreasing the concentration of proinflammatory cytokines may be potentially beneficial in improving intestinal injury. PDCoV showed toxic effects in the intestine and induced inflammation[5,51]. In this experiment, Se-Met downregulated TNF-α, IL-6, and IL-1β levels and elevated IL-10 levels. Thus, Se-Met is important in the attenuation or containment of inflammatory processes.

In addition, the cytokines have important pathological and physiological effects on the intestinal tight junction (TJ) barrier. It was reported that all of these pro-inflammatory cytokines could play a key role in the increase of paracellular permeability of intestinal epithelial cells through the redistribution and the expression of TJ proteins[50]. These alterations trigger a disturbance in the intestinal TJ barrier and raise its permeability[52-53]. A previous study showed that PDCoV infection could impair the TJs in the intestinal epithelium of piglets and cause intestinal barrier dysfunction, resulting in severe chronic diarrhea[54]. In our study, we found that PDCoV down-regulated the mRNA expression of ZO-1 and Occludin, whereas Se-Met improved PDCoV-decreased mRNA expression of jejunal ZO-1 and Occludin, implying that Se-Met attenuated intestinal barrier damage and improved intestinal health.

Viral infections are often accompanied by oxidative stress, which triggers an inflammatory

response. Se was previously reported to attenuate virus-induced oxidative stress. For example, in a PDCoV-infected LLC-PK cell model, the addition of Se-Met inhibited the increase in PDCoV replication by enhancing GSH-PX activity and suppressing the content of H_2O_2[36]. SOD is an important antioxidant enzyme for scavenging superoxide radicals in the body. In this experiment, PDCoV decreased the activity of GSH-PX and SOD, increasing the content of ROS and MDA in the jejunum of mice. After using Se-Met, the ROS and MDA expression levels exhibited a reduction, and GSH-PX and SOD levels were also reversed in the jejunum of PDCoV-infected mice. The results were consistent with previous results *in vitro* experiments on PDCoV[36]. From this, we can infer that Se-Met can alleviate PDCoV-induced oxidative stress by improving the intestinal antioxidant capacity, thus achieving the effect of mitigating intestinal barrier damage.

Among the signaling pathways that regulate oxidative stress responses, the Nrf2 signaling system is perhaps the most important cellular defense against oxidative stress and toxicants[55]. As a crucial endogenous antioxidant factor, Nrf2 binds to ARE in the promoter regions of many cell defense genes and activates their transcription (NQO-1, HO-1, TrxR-1, and Trx) in response to cellular stress and to maintain cellular homeostasis[11-12,56]. Nrf2 has shown potential natural or pharmacological protective functions in a variety of diseases[57], However, the protective capacity of Nrf2 could be hijacked under many pathological conditions, including virus infection[58]. In this study, we observed that Nrf2 protein levels and target genes such as HO-1 and NQO1 were downregulated in the PDCoV group. At the same time, Se-Met ameliorated the PDCoV-induced decrease in Nrf2, HO-1, and NQO1 protein and gene expressions in the jejunum of mice. Therefore, we hypothesized that Se-Met inhibited ROS production by activating Nrf2/HO-1, thereby reducing intestinal tissue inflammation and increasing barrier functions. Our study was the first to report that Se-Met protected the intestine from injury by inhibiting ROS. Our data demonstrated that Se-Met may improve the intestinal antioxidant capacity by scavenging ROS, which is associated with its potent antioxidant capacity.

4 Conclusion

In conclusion, Se-Met could alleviate PDCoV-induced intestinal injury. The underlying mechanism may be that Se-Met activates the Nrf2 signaling pathway, which improves the antioxidant capacity of the intestines of PDCoV-infected mice and attenuates intestinal barrier damage. This study may reveal a novel mechanism by which Se-Met alleviates oxidative stress in PDCoV infection and contribute to its application in the cross-species transmission of PDCoV.

Credit authorship contribution statement

LI Haiyan: Writing-review & editing, Writing-original draft, Visualization, Methodology, Investigation, Formal analysis, and Conceptualization; ZHANG Tongjun: Visualization, Methodology, Investigation, Resources, and Conceptualization; GUO Xin: Formal analysis; GUO Yongpeng: Writing-review & editing, Visualization, Investigation, and Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests.

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