Milk Small Extracellular Vesicles-derived miRNAs Restrict Porcine Epidemic Diarrhea Virus Infectivity

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Research

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Abstract

Background

Virus-caused diseases are a huge challenge to both animals and human beings, especially coronaviruses. Porcine epidemic diarrhea virus (PEDV), a coronavirus, causes acute diarrhea and up to 100% mortality in piglets less than three weeks of age. Maternal immunity provides protection for piglets in resisting PEDV infection. Small extracellular vesicles (sEV) contain bioactive molecules such as miRNAs to exchange genetic and epigenetic information between cells. Our previous study suggested that milk sEV facilitated intestinal tract development and prevented LPS-induced intestine damage. However, the effects of milk sEV on the inhibition of viral infections remain unclear.

Results

In this study, through in vivo experiments, we found that porcine milk sEV protected piglets from PEDV-induced diarrhea and death. In vitro, we clarified that this protective effect was partly generated through the inhibition of the PEDV-N protein and HMGB1 by sEV miR-let-7e and miR-27b, respectively.

Conclusions

In conclusion, we report that porcine milk sEVs protected piglets from PEDV-induced diarrhea and death by inhibiting virus replication, and this protective effect was partly generated through the inhibition of the PEDV-N and HMGB1 pathways by exosomal miR-let-7e and miR-27b. This study reveals a new antiviral function of milk sEVs, and the results suggest that milk sEVs may act as a mother-offspring transmission pathway for protecting newborns against PEDV infection.

Introduction

Virus-caused diseases are a huge challenge to both animals and human beings, especially coronaviruses. Porcine epidemic diarrhea virus (PEDV), a coronavirus, is an enveloped, positive-stranded RNA virus [1]. PEDV is a spherical virus 80–220 nm in diameter. The approximately 28 kb PEDV genome is an infectious single-stranded RNA [2]. PEDV encodes two polyproteins (pp1a and pp1a/b) that are processed into 16 nonstructural proteins, including an accessory protein (ORF3) and four structural proteins, namely, spike (S), membrane (M), envelope (E), and nucleocapsid (N). The transmembrane spike plays important roles in virus binding, fusing, and neutralizing host antibodies [3, 4]. PEDV causes acute diarrhea and high mortality rates in piglets less than three weeks of age [5]. Symptoms of PEDV in infected piglets include severe enteritis, watery diarrhea, vomiting, and a high mortality rate [6]. More importantly, the variant strains of PEDV severely threaten the pig industry worldwide, as the virus can cause up to 100% mortality in piglets [7]. Since the first case of PEDV was reported in England in the 1970s, PEDV has been reported in an increasing number of countries [8]. In the 1980s, PEDV was first identified in China, and the virus has caused huge economic losses [9]. Although a PEDV vaccine has been used to immunize pigs during intensive farming, piglet epidemic diarrhea still occurs occasionally [10]. Maternal immunity can provide

protection for piglets against PEDV infection [5]. Elucidating the mechanism of mother-offspring integration in resisting PEDV infection has great significance for defense against PEDV as well as other coronaviruses.

Various studies have demonstrated that breast milk not only supplies neonates with required nutrients and growth factors, but it also contains extracellular vesicles (EVs) that mediate signal transduction between mother and child [11, 12]. EVs are small lipid membrane vesicles that carry bioactive proteins, cytokines, and RNAs, and they are involved in cell-to-cell communications and are associated with attenuating intestinal inflammation. Milk is a complex fluid containing various EV subsets, the most studied of which are small extracellular vesicles(sEVs). These sEVs with ~ 100 nm in diameter are released when multivesicular bodies fuse with the cell membrane, and they pellet at centrifugation speeds above 100,000 g [13, 14] and they were previously called exosomes. sEVs donate large amounts of RNAs, such as mRNAs, miRNAs, and noncoding RNAs, to recipient cells by membrane fusion, and these sEV RNAs subsequently alter the gene expression of the recipient cells [15]. sEVs derived from milk can pass through the gastrointestinal (GI) tract and enter into the circulation and into various organs of mice [16]. Previous study has revealed that human milk sEVs can influence the infant immune system [17]. Milk sEVs also participate in metabolism regulation [18]. Milk sEVs mediate absorptive epithelium renewal, cell viability, and proliferation in the neonatal intestine after birth [19]. Our laboratory has established a method for the isolation and identification of porcine milk-derived sEVs, and we have identified their miRNAs as novel regulatory molecules transmitted mother-to-offspring [10]. Our previous studies have shown that porcine milk sEVs facilitated intestinal tract development [20] and prevented LPS-induced intestinal damage [21]. However, the effects of milk sEVs in resisting virus infection remain unclear.

Micro RNAs (miRNAs) are small noncoding RNAs that regulate gene expression by degrading mRNAs or by inhibiting translation [22]. Mammalian miRNAs target mRNA primarily by pairing with the 3’ untranslated regions (UTRs) to direct their posttranscriptional repression [23]. During host and virus interactions, miRNAs also play important regulatory roles by inhibiting viral replication through the direct targeting of viral genomic RNA [24, 25]. Increasing lines of evidence suggest that miRNAs regulate innate immune responses to viral infection. The miRNA-221 inhibited PEDV replication through targeting viral genomic RNA and activating the NF-κB Pathway [26]. Our group has completed the sequencing of porcine milk sEV miRNAs [27], and bioinformatics analysis indicates that many miRNAs potentially target the PEDV genes. In this study, we demonstrate the biological function of milk sEV in resisting PEDV infection, and our results should open an avenue for exploring the physiological functions of milk sEVs in anti-virus responses in both humans and animals.

Materials And Methods

Chemicals

The following reference compounds were commercially obtained: EGF (PeproTech, ≥ 95%), Triton X-100 (Solarbio, ≥ 98%), phenylmethanesulfonyl fluoride (PMSF) (Thermo Fisher, ≥ 99%), Formaldehyde (Tianjing DaMao, 37–40%), ethanol (Tianjing YongDa, ≥ 99.7%), hematoxylin (Acmec, ≥ 95%), eosin (Acmec, ≥ 95%).

Milk samples

Porcine milk samples were collected from day 1 to day 5 after parturition from six healthy lactating third-parity Landrace sows bred in the Shuitai pig farm, a PEDV-negative breeding farm (Yunfu, China). All of the sows had been vaccinated against PEDV during pregnancy. All of the milk samples were mixed and frozen immediately after collection and were kept at -80°C until use.

Preparation of milk sEVs

Porcine colostrum sEVs were separated and identified according to previously described methods [27], which was also recommended by MISEV(2018). Briefly, about 80 to 100 mL colostrum was centrifuged at 2000 × g and 4°C for 30 min to remove milk fat globules and mammary gland-derived cells. Defatted samples were then centrifuged at 12,000 × g and 4°C for 30 min to remove residual milk fat globules, casein, and other debris. From the supernatant, the sEVs were prepared by ultracentrifugation three times at 110,000 × g for 2 h using an SW41T rotor (Beckman Coulter Instruments, Fullerton, CA, USA). The supernatant was collected for use as a control for in vitro and in vivo experiments. The sediment was filtered by a 0.22-μm filter to prepare the sEV solution. The sEV concentration was quantified and expressed as mg total protein/μL. Milk sEVs were dissolved in Dulbecco's modified Eagle medium (DMEM/F12) (in vitro trial) or saline (in vivo trial).

sEV identification

The ultrastructure of sEV was analyzed using transmission electron microscopy as described previously [28]. Briefly, the re-suspended sEV pellet (5 μL) was fixed with 2.5% glutaraldehyde, post-fixed in buffered 1% OsO_4 with 1.5% $K_4Fe(CN)_6$, embedded in 1% agar, and processed according to the standard Epon812 embedding procedure. The sEVs were visualized on thin sections (60 nm) using a Morgagni 268 transmission electron microscope (FEI Company, The Netherlands) at 80 kV.

CD63 and CD81 protein levels in sEVs were determined by Western blotting. Protein lysate was obtained by re-suspending the sEV pellet in 100 μL of RIPA buffer (Beyotime, Jiangsu, China). Antibody information is listed in Table 3.

Nanoparticle tracking analysis (NTA) is based on the principle that the rate of nanoparticles' Brownian motion in solution is related to their size. In this method, a 405 nm laser light was directed at a fixed angle to the vesicle suspension, and the scattered light was captured using a microscope and high-sensitivity camera. By tracking the movement of individual nanoparticles, the software calculates their diameters. sEV preparations were examined using a Nanosight LM10-HS (Nanosight Ltd.) as described previously

[29] with constant flow injection. Five recordings of 30 seconds each were captured, and at least 5,000 individual particle tracks were analyzed per sample.

Animal tests

Twenty-four male Landrace piglets just after birth without sucking colostrum or vaccinations were purchased from Shuitai pig farm (Yunfu, Guangdong). All of the piglets with a birth weight of 1.3–1.5 kg were randomly divided into four groups: a control group (fed milk powder, Pigipro Milk, Schils Group; the nutritional composition of the milk powder is shown in Table 1), a sEV treatment group (fed milk powder containing porcine milk sEVs), a PEDV group (fed milk powder and with PEDV challenge), and a PEDV + sEV group (fed milk powder containing porcine milk sEVs and with PEDV challenge). The highly virulent PEDV strain GDgh (GenBank accession no. MG983755) was generously provided by Professor Changxu Song of South China Agricultural University. All of the piglets were kept in a lab without specific pathogens; the ambient temperature was controlled at 38°C, and the relative humidity was 70%. The milk powder was fed every three hours. The sEV group and PEDV + sEV group were supplied with porcine milk sEVs (containing 500 mg protein) per day. After 48 hours, the PEDV group and the PEDV + sEV group were orally challenged with 1 ml 1×10^5 $TCID_{50}$ PEDV virus per piglet once according to a previously described method [30]. The mental state and diarrhea of the piglets were observed and recorded every hour. If a piglet died, the body was dissected for further analysis. Samples of small intestine were collected, rinsed gently with PBS, and fixed in 4% paraformaldehyde for observation and analysis of virus infection. The small intestinal mucosa of piglets was collected for molecular detection. At 72 hours post challenge, the surviving piglets were euthanized, and the small intestines were dissected to observe the intestine morphology and for use in further analysis.

Table 1
the nutritional composition of milk powder

Average Analysis	
Protein	20.00%
Crude Fat	20.00%
Ash	6.50%
Moisture	4.00%
Crude fibre	0.10%
Added Vitamins per kg	
Vitamin A	50000 i.u.
Vitamine B1	5 mg
Vitamine B2	4 mg
Vitamine B3	15 mg
Vitamin B6	2 mg
Vitamine B12	0.05 mg
Vitamin C	100 mg
Vitamine D3	5000 i.u
Vitamin E	300 mg
Vitamin K	33 mg

Hematoxylin-eosin staining (H&E)

The piglet jejunum tissues (3–5 cm) were fixed in 4% formaldehyde (DaMao, Tianjing) at room temperature for 24 h and stained with hematoxylin (H69841, Acmec) and eosin (SA-15288, Acmec) according to a previous study [31]. The villus morphology was observed under the light microscope (Nikon, Tokyo, Japan) at a magnification of 40×.

Milk sEVs inactivation and sEV miRNAs extraction

We dissolved the porcine milk sEVs in a solution with a concentration of 0.018 mg protein/μL. A previous study had shown that compared with unprocessed sEVs, pasteurization led to a 302-fold decrease in sEVs ($p = 0.0021$), leaving insufficient reads for further analysis [32]. The inactivated milk sEVs were

prepared by holder pasteurization at a temperature of 62.5°C in a water bath, and the temperature was held for 30 min [32].

As for sEV miRNAs extraction, total RNAs were extracted from sEVsusing TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. We separated RNAs smaller than 50 nt by agarose gel electrophoresis. The miRNAs were recycled from the gels using a gel extraction Kit (OMEGA, Norcross, GA, USA), and the concentration of miRNAs was measured by a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Then, the miRNAs were transfected into IPEC-J2 cells (30 nM) by using Lipofectamine 3000 according to the manufacturer's instructions to elucidate the function of miRNAs in inhibiting viral replication.

Virus preparation

The PEDV strain (the avirulent PEDV strain CV777, GenBank accession no. AF353511) was generously provided by Professor Changxu Song of South China Agricultural University. Vero E6 cells (ATCC CCL-81) were cultured in DMEM (Invitrogen, Life Technologies, Carlsbad, CA, USA) containing 10% inactivated fetal bovine serum (GIBCO) and 1% penicillin-streptomycin (Sigma, USA). The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. PEDV was used to infect the cells to produce a viral stock preparation: we added 0.1 MOI PEDV to cells for 2 h at 37°C to detect the milk sEV effect on viral replication. The virus was removed, and the cells were washed twice with PBS. After that, the cell monolayers were covered by DMEM/F-12 or DMEM containing 10% inactivated fetal bovine serum and 0.3% tryptose phosphate broth (TPB, Sigma). The virus stock (1×10^5 $TCID_{50}$/ml) was used undiluted for mixed infections.

Inhibition of PEDV by porcine milk sEVs in vitro

IPEC-J2 cells were a kind gift from Professor Chen Daiwen at Sichuan Agricultural University. Cells were cultured in DMEM/F-12 (1:1) (Invitrogen, Life Technologies, Carlsbad, CA, USA), supplemented with 5 ng/mL EGF (PeproTech, Rocky Hill, NJ, USA), 10% inactivated fetal bovine serum (GIBCO), and 1% penicillin-streptomycin (Sigma, USA). IPEC-J2 and Vero E6 cells were seeded in 24-well plates at a density of $3 \times 10^4/cm^2$ and $4 \times 10^4/cm^2$, respectively. We added milk sEVs 12 h later when cell confluence reached 60%; milk sEVs, inactivated milk sEVs, or isolated sEV miRNAs were added to the cells at a concentration of 0.54 mg/cm^2 for milk sEVs and inactivated milk sEVs, or 30 nM for miRNAs, followed by cultivating for another 24 h. The medium was removed, and cells were washed twice with PBS to avoid residual sEVs or miRNAs directly binding to the virus. Then, 0.1 MOI PEDV was added to the cells and incubated for 2 h to detect the effects of milk sEVs or miRNAs on viral replication.

Crystal violet staining

After treatment, the cells were washed twice with PBS. Then, 500 μL of 10% methanol was added to each well to fix for 2 minutes, and the methanol solution was discarded. Then, 200 μL of crystal violet staining solution was added to each well and kept for 10 min. The staining solution was gently removed followed

by washing twice with PBS. The crystal violet staining results were observed under the light microscope (Nikon, Tokyo, Japan) at a magnification of 40×.

Immunofluorescence

IPEC-J2 cells were seeded in 6-well cell culture plates at a density of $2.4 \times 10^4/cm^2$. After finishing the treatment, the cells were incubated in freshly prepared 4% paraformaldehyde-neutral PBS at room temperature for 10 min. The cells were incubated with 0.5% Triton X-100 (9002-93-1, Solarbio, Beijing, China) for 5 min and then in 5% fetal serum for 2 h. The cells were incubated with rabbit anti-PEDV/NP antibody overnight at 4°C. Then, the cells were washed with cold PBS three times and incubated with FITC secondary antibody for 1 h. The cell nuclei were stained with DAPI (Beyotime, Jiangsu, China). Fluorescence was observed using a Nikon Eclipse Ti-s microscope with Nis-Elements BR software (Nikon Instruments, Tokyo, Japan) at a magnification of 200×. Five fields of view from each well were captured; each group contained six wells, and the average value was calculated.

RNA extraction, reverse transcription, and polymerase chain reaction (PCR)

Tissue and cell RNA were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (pig intestine RNA and PEDV RNA) was digested with DNase I (Promega, Madison, WI, USA), and 2 μg of total RNA was reverse transcribed with random primers. The quantitative real-time PCR was performed on a Bio-Rad system (Hercules, CA, USA). The miRNAs were quantified according to the protocol of the Mir-X miRNA First Strand Synthesis Kit (Takara Bio Company, Dalian, China). The relative expression levels of mRNAs and miRNAs were normalized to β-actin or U6 levels using the $2^{-\Delta\Delta Ct}$ method [33]. The measure using $2^{-\Delta\Delta Ct}$ was defined as the ratio of the relative mRNA or miRNA levels between the experimental group and the control group. Primers used in this study were designed using Primer Premier 5 according to the pig gene sequences obtained from NCBI. The primers used for PCR are shown in Table 2.

Table 2
The primer sequences used in this study

Gene name	Forward primer sequence (5'-3')	Reversed primer sequence (5'-3')
β-actin	GCGGGACATCAAGGAGAAGC	TGTCGGCGATGCCTGGGTA
ORF3	CGGGCTTCGTTTAGTCTGCT	GATGTAATGGTCGCCACCTTCT
S protein	GCAGTAATTCCTCAGATCCTC	GTAGTGTCAGATGCAATGAGG
N protein	AAAACGGGTGCCATTATCTCT	CCATTTGCTGGTCCTTATTCC
HMGB1	CATGGGCAAAGGAGATCCTA	TTCATCATCATCATCTTCTTCT
miR-let-7e	ACACTCCAGCTGGGTAGGAGGTTGTATAGTT	TGGTGTCGTGGAGTCG
miR-27b	ACACTCCAGCTGGGAGTGGCTAAGTTCTGC	TGGTGTCGTGGAGTCG

Bioinformatics analysis and dual-luciferase reporter assay

The target gene prediction was conducted through the software mirTargets 1.2 in conjunction with the TargetScan, MicroCosm, Pictar, and miRDB databases. The 3’ UTR sequences of HMGB1 and PEDV N were synthesized and inserted into a pmirGLO vector (Ambion, Carlsbad, CA, USA) to construct the pmirGLO-HMGB1-3'UTR and pmirGLO-PEDV N vectors, respectively. For the dual-luciferase reporter assay, HEK-293T cells (2×10^4 cells per well) were seeded in 48-well culture plates and co-transfected with the pmirGLO dual-luciferase reporter (containing the indicated miRNA-binding sites, 200 ng) and miRNA NC or mimic duplexes (30 nM) for 48 h. The activities of the Renilla and firefly luciferases were determined by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The activity of firefly luciferase was normalized to the activity of Renilla luciferase.

miRNA and plasmid transfection

IPEC-J2 cells were seeded in 6-well plates at a density of 2.4×10^4 / cm^2. Cells were transfected with pCDNA3.1-PEDV N and 30 nM miR-let-7e mimics or control NC by Lipofectamine 3000 when the cells reached approximately 80% confluency according to the manufacturer's instructions. The medium was replaced 6 h later, and the cells were maintained in the new growth medium for an additional 24 h before testing. The transfection protocols for pCDNA3.1-HMGB1 and miR-27b were similar to those described above.

To test inhibition by miRNAs on PEDV replication, 15 nM of miR-let-7e, 15 nM of miR-27b mimics, and a combination of the two miRNAs in the same amounts and scrambled NC were transfected by Lipofectamine 3000 when the cells reached approximately 80% confluence. The medium was replaced 6 h later, and the cells were maintained in the new growth medium for an additional 24 h before testing. Then, 0.1 MOI PEDV was added to cells and kept for 2 h at 37°C. The virus inocula were removed, and cell monolayers were washed twice with PBS. After that, the cell monolayers were covered by DMEM/F-12 or DMEM containing 10% inactivated fetal bovine serum and 0.3% tryptose phosphate broth. The above miRNA mimics were purchased from GENEWIZ (Suzhou, China), and the control was the scrambled NC sequence provided by GENEWIZ.

Western blotting (WB) assay

RIPA lysis buffer was used to extract proteins from the IPEC-J2 cells, milk sEVs, and intestine tissue according to the assay kit protocol (Bioteke, Beijing, China). Protein concentration was measured using the Pierce BCA Protein Assay Kit (23225, Thermo Fisher, Waltham, MA, USA) according to the manufacturer’s protocol. The method used for the Western blotting assay has been described previously [20]. The details of antibodies used in this study are listed in Table 3. ImageJ software was used for gray scan analysis.

Table 3
the details of antibodies used in this study

Primary antibody	Clone	Company	Catalog No.	Dilution
PEDV/NP	Polyclonal	Zoonogen	M100048	1:500
HMGB1	Polyclonal	Bioss	bs-20633R	1:1000
CD63	Polyclonal	CST	# 55051S	1:1000
CD81	Monoclonal	CST	# 10037S	1:1000
Clnexin	Monoclonal	CST	# 2679S	1:1000
β-actin	Monoclonal	Bioworld	BS6007M	1:5000
Secondary antibody	Conjugate Used	Company	Catalog No.	Dilution
Goat Anti-rabbit IgG	HRP	Bioss	bs-0295G	1:5000
Goat Anti-rabbit IgG	FITC	Bioss	bs-0295G-AF647	1:200

Statistical analysis

All of the data are expressed as the mean ± standard error of the mean (S.E.M.). The in vivo experiment was one independent test (N = 1), and each group contained 6 (n = 6) biological replicates. The cell experiment was designed to analyze the results of three independent experiments (N = 3), and each group contained 6 (n = 6) biological replicates. Our data were normally distributed, and the homogeneity of variance between treatment groups was confirmed by the SPSS analysis. In Fig. 1F, Fig. 2D, Fig. 4, and Fig. 5, the unpaired Student's t-test was used for p-value calculations, where * denotes $p < 0.05$; and ** denotes $p < 0.01$. For the remaining results, significant differences among groups (≥ 3) were determined by one-way ANOVA (SPSS v18.0, IBM Knowledge Center, Chicago, IL, USA). Multiple comparisons between the groups were performed using the S-N-K method. Bars with different letters indicate they are statistically significantly different ($p < 0.05$).

Results

Porcine milk sEVs inhibited PEDV replication in IPEC-J2 and Vero E6 cells

Porcine milk sEVs were isolated by ultracentrifugation. Isolated milk sEVs were identified by transmission electron microscopy, Western blotting, and nanoparticle tracking analysis. The particle size and positive detection of CD9 and CD63 (Fig. 1A, Fig. 1B, and Fig. 1C) indicated that we successfully obtained milk sEVs. To investigate whether sEVs could inhibit PEDV propagation in vitro, we tested the effect of milk sEVs on viral infection in IPEC-J2 and Vero E6 cells. The PCR results indicated that the PEDV virus was not included in the porcine milk, sEVs, or supernatant in the sEV isolation (Fig. 1D). The crystal violet staining results showed that milk sEVs treatment protected IPEC-J2 and Vero E6 cells from PEDV attack

(Fig. 1E). The results of virus copy number determined by quantitative PCR showed that milk sEVs inhibited the replication of PEDV virus in IPEC-J2 and Vero E6 cells (Fig. 1F and 1G). Western blotting revealed that the expression of the PEDV N protein was reduced after milk sEV treatment (Fig. 1H and 1I). These results indicate that milk sEVs were capable of inhibiting PEDV replication in IPEC-J2 and Vero E6 cells.

Milk sEVs reduced piglet intestinal villus damage, diarrhea, and mortality caused by PEDV

An in vivo trial was conducted to evaluate the protection by milk sEVs to piglets after artificial PEDV infection via oral administration of 1 ml 1×10^5 $TCID_{50}$ PEDV virus per piglet [34]. All of the piglets developed diarrhea within 48 hours after PEVD challenge. The total number of diarrhea events in this group was about 65 times (Fig. 2A). Interestingly, only 50% of piglets pre-fed with milk sEVs developed diarrhea within 72 hours after virus challenge, and the total number of diarrhea events in this group was about 28 times (Fig. 2A), which was much lower than for piglets without pre-feeding of milk sEVs. More importantly, without receiving milk sEVs, all of the piglets had died by 48 hours after PEDV challenge, while only 33.3% of the piglets receiving milk sEVs had died 48 hours after PEDV challenge (Fig. 2B), and piglets in the latter group were still alive up to 72 hours after challenge. H & E staining of piglet small intestines showed that compared with the control group, milk sEVs not only promoted the intestinal villi development, but they also protected the intestinal villi from PEDV-caused damage (Fig. 2C). Quantitative PCR results showed that no PEDV virus was detected in intestinal mucosal tissue of the control or milk sEV-treated groups. The number of virus copies in the PEDV infection group reached 2.2×10^9 per gram of tissue, while the number decreased to 7.5×10^6 per gram of tissue in the group with milk sEVs pre-feeding (Fig. 2D). These results were further confirmed by WB (Fig. 2E) and immunofluorescence (Fig. 2E). Immunofluorescence indicated that the PEDV virus was mainly distributed in the intestinal villi, and milk sEVs significantly reduced PEDV virus in the intestinal mucosal layer (Fig. 2F). The results strongly suggest that milk sEVs inhibited PEDV propagation in vivo and relieved the intestine villus damage, diarrhea, and mortality caused by PEDV.

Milk sEV small RNAs inhibited virus replication in IPEC-J2 cells

Since milk sEVs contain a large number of miRNAs, we further investigated whether these miRNAs played a role in resisting PEDV infection. Crystal violet staining results showed that milk sEVs promoted the cell viability after PEDV seeding, and this effect was lost after inactivation of milk sEVs by boiling for five minutes. Intriguingly, isolated sEV small RNAs (< 50 nt) still retained the protective effect toward cell viability after PEDV treatment (Fig. 3A). We then detected the virus copy number by quantitative PCR. When inactivated, milk sEVs lost the inhibitory effect on virus replication. Moreover, isolated sEV small RNAs (< 50 nt) still showed significant inhibition of virus replication (Fig. 3B). These results were also confirmed by Western blotting (Fig. 3C and 3D) and immunofluorescence detection of the N protein (Fig. 3E). Compared to controls, milk sEVs reduced the positive rate of IPEC-J2 cells infected with PEDV from 92–29%, and this reduction was diminished by inactivation of sEVs, yielding an infection rate of 78% (Fig. 3F). More importantly, isolated milk sEV small RNAs (< 50 nt) reduced the positive rate of IPEC-J2

cells (52%) infected with PEDV compared to control (Fig. 3F). These results indicate that the inactivated milk sEVs lost the protective effect, possibly due to decreasing numbers of sEV miRNAs [32]. The results suggest that small RNAs of milk sEVs are capable of inhibiting PEDV replication in IPEC-J2 cells, although the inhibitory effect is not as robust as with milk sEVs.

MiR-let-7e inhibited viral replication by targeting PEDV N protein

Since the N protein plays an important regulatory role in both virus infection and host immunity, we analyzed miRNAs targeting the PEDV N protein. The target relationship between the 50 most abundant sEV miRNAs and PEDV N was conducted using the software mirTargets 1.2 in conjunction with TargetScan, MicroCosm, Pictar, and miRDB databases. We found that miR-let-7e potentially targeted the PEDV N protein (Fig. 4A). Furthermore, a dual-luciferase reporter assay showed that miR-let-7e mimics significantly decreased the relative luciferase activity of wild-type pmirGLO-N protein-3'UTR but did not change the luciferase activity of mutated pmirGLO-N protein-3'UTR or pmirGLO (Fig. 4B). We then further evaluated the miR-let-7e function in IPEC-J2 cells. MiR-let-7e mimics enhanced the cell viability after PEDV infection, as illustrated by crystal violet staining (Fig. 4C). Moreover, miR-let-7e inhibited virus replication, as shown by quantitative PCR (Fig. 4D), and Western blotting also showed that miR-let-7e significantly decreased the N protein level in IPEC-J2 cells (Fig. 4E and 4F). The in situ immunofluorescence results illustrated that miR-let-7e significantly reduced the positive rate of IPEC-J2 cells, from 85–42% (Fig. 4G and 4H). The above results clearly indicate that miR-let-7e inhibits viral replication by targeting the N protein.

MiR-27b inhibited viral replication by targeting HMGB1

Previous studies have shown that PEDV infection led to the elevated expression and acetylation of HMGB1. It was reported that neutralizing HMGB1 inhibited PEDV replication and reduced the production of proinflammatory cytokines [35]. Bioinformatics analysis using the software mirTargets 1.2 in conjunction with TargetScan, MicroCosm, Pictar and miRDB databases revealed that there was a potential target relationship between miR-27b and HMGB1 (Fig. 5A). The results from the dual-luciferase reporter assay system indicated that miR-27b mimics significantly decreased the relative luciferase activity of wild-type pmirGLO- HMGB1-3'UTR, while the luciferase activity of mutated pmirGLO- HMGB1-3'UTR or pmirGLO was unchanged (Fig. 5B). We further evaluated the miR-27b function in IPEC-J2 cells. The miR-27b enhanced cell viability after PEDV infection as illustrated by crystal violet staining (Fig. 5C), and miR-27b inhibited virus replication as shown by the quantitative PCR detection of virus copy (Fig. 5D). Western blotting showed that miR-27b significantly decreased N protein levels in IPEC-J2 cells (Fig. 5E and 5F). The in situ immunofluorescence also showed that miR-27b reduced the positive rate of IPEC-J2 cells infected with PEDV, from 84–57% (Fig. 5G and 5H). These results suggest that miR-27b reduced HMGB1 expression, thereby inhibiting viral replication.

Synergistic function of miR-let-7e and miR-27b in inhibiting PEDV replication

To better understand the cooperative effects of milk sEV miRNAs, we co-transfected miR-let-7e and miR-27b into IPEC-J2 cells followed by PEDV infection. Crystal violet staining showed that compared with

transfection of let-7e or miR-27b alone, co-transfection of two miRNAs had a greater protective effect on cell viability after PEDV infection (Fig. 6A). Co-transfection of two miRNAs also resulted in smaller PEDV copy numbers, as revealed by qRT-PCR (Fig. 6B). N protein in situ immunofluorescence detection suggested that co-transfection with two miRNAs resulted in a smaller positive cell rate (37%) than transfection with let-7e (62%) or miR-27b (55%) alone (Fig. 6B and 6D). These results suggest that miR-let-7e and miR-27b function synergistically in inhibiting PEDV replication.

Milk sEVs inhibited expression of viral N protein and host HMGB1

Finally, we verified whether milk sEVs altered the expression of the viral N protein or host HMGB1in the intestinal mucosa of piglets after PEDV challenge. The qRT-PCR results showed that PEDV challenge did not change the expression of miR-let-7e or miR-27b, and milk sEVs significantly up-regulated miR-let-7e and miR-27b levels with or without PEDV challenge (Fig. 7A), indicating that milk sEVs transported their cargo miRNAs into recipient intestinal mucosa cells. Both qRT-PCR and Western blotting showed that after PEDV challenge, milk sEVs significantly decreased the expression of N protein and HMGB1, the respective targets of miR-let-7e and miR-27b (Fig. 7B, 7C, and 7D). These results provide direct evidence for inhibition of targeted genes by milk sEV miRNAs.

Discussion

Coronaviruses are a huge challenge to human and animal health. PEDV is a highly virulent re-emerging enteric coronavirus that causes nearly 100% mortality in neonatal suckling piglets [36]. At present, lactogenic immunity induced through the gut-mammary gland-secretory IgA axis remains the most promising and effective way to protect piglets from PEDV [37]. However, milk composition is more complex than previously thought. Recently, some novel components of milk have been identified, including EVs and oligosaccharides. These components possibly act as biological signals to modulate the intestinal environment and immune status in newborns and later in life [38]. sEVs are potent mediators of cell-cell communication. Milk sEV miRNAs modulated biological processes in the recipient cells [39, 40]. Our previous studies have shown that porcine milk sEVs facilitated intestinal tract development [20] and prevented LPS-induced intestine damage [21]. However, the effects of milk sEVs in resisting virus infection remained unclear. In this study, we demonstrated that sEVs of porcine milk significantly inhibited PEDV both in vivo and in vitro, and the antiviral function of sEVs was at least partly achieved by miRNAs.

In the present study, we tested the anti-PEDV function of milk sEVs in vitro. The IPEC-J2 cell line derived from the natural piglet small intestine [41] was used in our tests. After PEDV infection, the adhesion ability of IPEC-J2 cells became weaker, resulting in fewer cells. Interestingly, after PEDV infection, milk sEVs improved cell viability and decreased both N protein expression and virus copy number in cells. Similar results were also obtained in Vero E6 cells. These results clearly demonstrated the inhibition by milk sEVs on PEDV replication in vitro. More intriguingly, in vivo tests showed that only 50% of the piglets that received pre-feeding of milk sEVs developed diarrhea within 72 hours after virus challenge, while all

piglets developed diarrhea within 48 hours in the control group without milk sEVs. More importantly, all of the piglets died 48 hours after PEDV challenge without receiving milk sEVs, while 66.7% of the piglets receiving sEVs pre-feeding were still alive. Moreover, PEDV copy number and N protein expression were significantly reduced in intestine tissues of piglets receiving milk sEVs, as confirmed by qRT-PCR, immunofluorescence, and Western blotting, indicating the inhibition of PEDV. These results strongly suggest that maternal milk sEVs have the capability to protect piglets from PEDV infection. Furthermore, milk sEVs also protected the intestinal villi from PEDV-caused damage, similar to a previous report that porcine milk sEVs promoted intestinal villi development [42]. Our in vitro and in vivo data strongly suggest that milk sEVs have inhibitory effects toward PEDV and that this results in protection of piglets during PEDV infection. To our knowledge, this is the first report of an anti-virus effect of milk sEVs.

Our results suggest that miRNAs are possibly new active components in milk. The miRNAs are small noncoding RNAs that hybridized with complementary sequences in the mRNA 3'-untranslated regions and then silence gene expression by inducing mRNA degradation or translation inhibition [15]. Milk sEVs are bioavailable and deliver their cargo to newborn tissues [43]. Moreover, sEVs protect the miRNAs against harsh conditions such as low pH and enzymatic degradation in the gastrointestinal tract [44]. These miRNAs regulate cell proliferation, growth, differentiation, immunity, and apoptosis in newborns [45]. A previous study in our laboratory found that miR-130 was detected in different tissues and serum of miR-$130^{-/-}$ mice treated by gavage with porcine milk sEVs (unpublished data), indicating that milk sEV miRNAs could be absorbed by the intestine.

Our group has sequenced the miRNAs in porcine milk sEVs, the analysis revealed a total of 491 miRNAs, and the top 10 miRNAs were predicted to target 2333 genes enriched in metabolism, immunity, and transcript regulation [46]. In this study, to investigate whether miRNAs in porcine milk sEVs could protect piglets against PEDV infection, total RNA was extracted from porcine milk sEVs, and small RNAs < 50 nt were isolated by PAGE electrophoresis. Surprisingly, transfection with isolated small RNAs showed significant inhibition of PEDV propagation in vitro, and this was confirmed by detection of virus copy number, N protein expression, and cell viability, yielding direct evidence that small RNA components in milk sEVs may possess inhibitory effects toward PEDV. A previous study found that compared with unprocessed sEVs, pasteurization led to a 302-fold decrease in sEVs ($p = 0.0021$), insufficient reads for further analysis [32]. The inactivated sEVs were obtained by subjecting the milk sEVs to holder pasteurization at a temperature of 62.5°C in a water bath for 30 minutes. In our test, inactivation by pasteurization led to elimination of the inhibitory effect of milk sEVs toward PEDV. It should be noted that isolated small RNAs exhibited less robust inhibition of PEDV relative to milk sEVs, indicating that milk sEVs possibly contain anti-PEDV components other than small RNAs.

PEDV encodes several structural proteins, including the spike (S), membrane (M), envelope (E), and nucleoprotein (N) [47]. Previous study showed that the N-protein of coronaviruses is highly phosphorylated and forms a helical ribonucleic protein that establishes the viral core intertwining with the viral genome RNA [48]. The N-protein of coronavirus is highly conserved and is the predominant antigen produced in coronavirus-infected cells [49]. The N protein has been used as the target for accurate and

early diagnosis of PEDV infection [50]. In this study, we used the N protein as the marker in the quantitative PEDV copy number analysis. Bioinformatics prediction indicated that miR-let-7e, an RNA found in porcine milk sEVs, has a potential target relationship with the PEDV N protein. This prediction was further confirmed by the dual luciferase reporter system, since miR-let-7e mimics significantly decreased luciferase activity, and this decrease diminished when the seed sequence of miR-let-7e was mutated. Moreover, tests with IPEC-J2 demonstrated that miR-let-7e inhibited PEDV propagation by targeting the N protein. To our knowledge, this is the first miRNA confirmed to directly target the PEDV N protein.

HMGB1, previously known as HMG-1, is a structural protein of chromatin [51]. As a damage-associated molecular pattern, extracellular HMGB1 activates proinflammatory signaling pathways via TLR4 and RAGE [52]. Therefore, HMGB1 is considered as a mediator in inflammation and innate immunity [53]. PEDV infection led to the expression and acetylation of HMGB1. Neutralizing HMGB1 inhibited PEDV replication and reduced the production of proinflammatory cytokines [54]. Glycyrrhizin, the major component of licorice root extracts, inhibited the entry and replication of PEDV in Vero E6 cells through its function as a competitive inhibitor of HMGB1 [55]. In this study, we found that miR-27b inhibited PEDV replication in IPEC-J2 cells by targeting HMGB1, and this was confirmed by all tests designed as described for the target exploration of miR-let-7e. These results suggest a new pathway for exosomal miRNAs to inhibit PEDV, i.e., by regulating host genes, and this is possibly a common event in other coronaviruses.

Since both miR-let-7e and miR27b are capable of inhibiting PEDV replication, it was interesting to evaluate whether they acted synergistically. It has been reported that miR-320a, miR-34a, and miR-542 had synergistic functions in regulating MCF-7 cell fate [56]. Our previous study also found that miRNAs in porcine milk sEVs attenuated lipopolysaccharide-induced apoptosis by inhibiting NF-κB and p53 pathways in IPEC-J2 cells, and there was a synergistic mechanism among exosomal miR-4334, miR-219, and miR-338 in protecting cells against lipopolysaccharide-induced apoptosis [57]. Interestingly, co-transfection of miR-let 7e and miR-27b had a stronger inhibitory effect on virus replication than transfection of miR-let-7e or miR-27b alone.

Conclusion

In conclusion, we report that porcine milk sEVs protected piglets from PEDV-induced diarrhea and death by inhibiting virus replication, and this protective effect was partly generated through the inhibition of the PEDV-N and HMGB1 pathways by exosomal miR-let-7e and miR-27b. This study reveals a new antiviral function of milk sEVs, and the results suggest that milk sEVs may act as a mother-offspring transmission pathway for protecting newborns against PEDV infection.

List Of Abbreviations

PEDV, porcine epidemic diarrhea virus

sEV, small extracellular vesicles

GI, gastrointestinal ()

UTRs, untranslated regions

miRNAs, microRNAs ()

NTA, nanoparticle tracking analysis

WB, western blotting

Declarations

Ethics approval and consent to participate

All animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Consent for publication

All authors have read and approved for publication.

Availability of data and material

We confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Competing interests

The authors report no conflict of interest.

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Authors' contributions

M. X., Y. Z., and T. C. designed the research and wrote the manuscript; L. H., and C. S. performed the experiments, analyzed the data and wrote the manuscript; J. S., and Q. X., analyzed the data and

performed the experiments; L. Y., and Q. J. contributed new reagents or analytic tools. All authors have read and approved the final manuscript.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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Figures

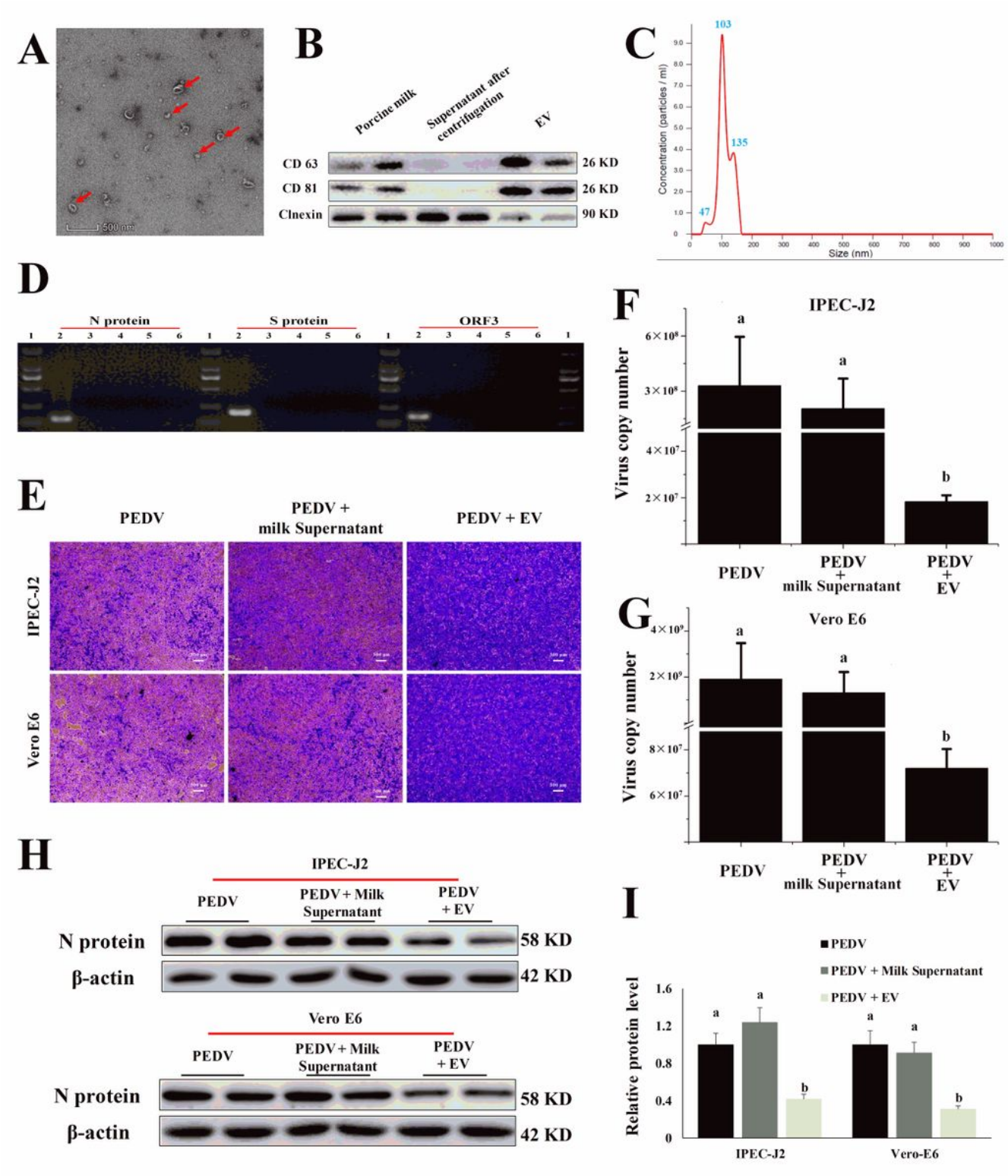

Figure 1

Milk sEVs inhibited PEDV replication in IPEC-J2 and Vero E6 cells A. Porcine milk sEVs were identified by transmission electron microscopy. (B). Porcine milk sEVs were identified by Western blotting, with positive detection of CD9 and CD63. C. Porcine milk sEVs were identified by nanoparticle tracking analysis, and the main size was about 103 nm. D. PEDV virus was not included in the milk sEVs, as indicated by PCR analysis. Lines 1-6 represent DNA marker 2000, positive control, porcine milk, milk fat from

ultracentrifugation, supernatant from ultracentrifugation, and milk sEVs, respectively. E. Milk sEVs inhibited PEDV replication, as shown by crystal violet staining analysis. Living cells are stained purple. Magnification = 200×. The scale bar on the photomicrographs represents 100 μm. F, G. Milk sEVs significantly reduced PEDV copy number as determined by quantitative PCR both in IPEC-J2 and Vero E6 cells compared to control and the supernatant. H, I. sEVs significantly reduced expression of N protein of PEDV replication as revealed by Western blotting analysis compared to control and the supernatant (n=6). The results are presented as the mean ± S.E.M. The data were normally distributed, and homogeneity of variance between treatment groups was confirmed by SPSS analysis. Significant differences among groups were determined by one-way ANOVA. Bars with different letters indicate statistically significantly differences ($p < 0.05$).

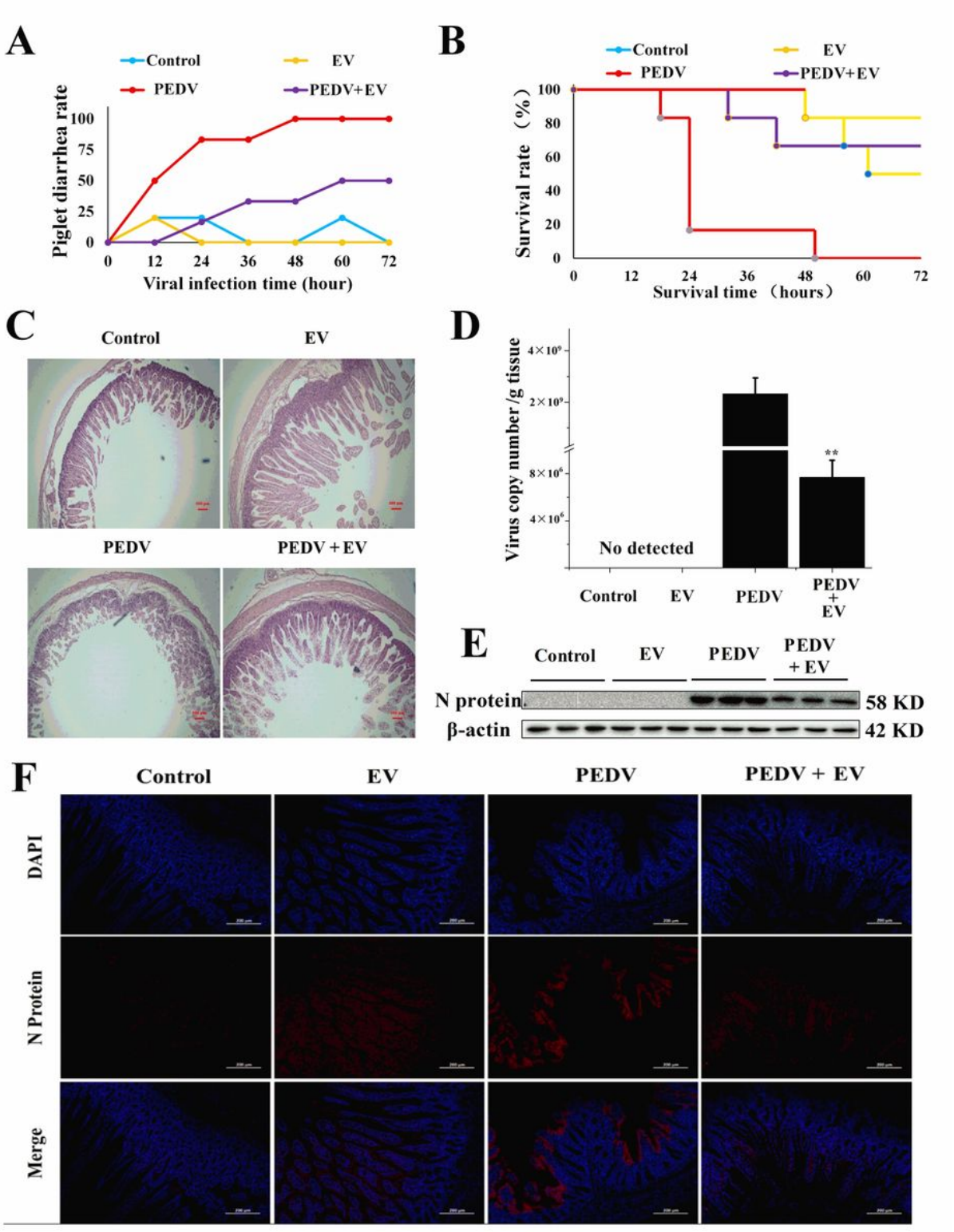

Figure 2

Milk sEVs inhibited piglet intestinal villus damage and diarrhea mortality caused by PEDV A. Piglet (N=6) diarrhea occurrence with different treatments. In piglets that received PEDV challenge, diarrhea events in the milk sEVs prefeeding group were clearly lower than in the PEDV group. B. The piglet (N=6) survival curves under different treatments. At 48 hours after PEDV challenge, all piglets had died in the PEDV group, while 66.6% of the piglets that received milk sEVs prefeeding survived up to 72 hours, when the

trial ended. C. H & E staining of piglet intestine tissue. Milk sEVs promoted development of intestinal villi and attenuated damage caused by PEDV. D. Quantitative PCR results showing that PEDV copy number in the piglet intestinal mucosa dramatically decreased, from 2.2 × 109 per gram of tissue to 7.5 × 106 when receiving milk sEVs. E. Western blotting analysis of the N protein of PEDV. No N protein was detected in piglets without PEDV challenge. N protein was significantly reduced in the milk sEVs pre-feeding group. F. In situ immunofluorescence detection of the PEDV virus and its distribution in intestine tissue. Blue: DAPI, red: N protein (n=6). The results are presented as the mean ± S.E.M. Unpaired Student's t-tests were used for p-value calculations, ** $p < 0.01$. In Figure 2C, magnification = 40×; the scale bar on the photomicrographs represents 500 μm. In Figure 2F, magnification = 100×; the scale bar on the photomicrographs represents 200 μm.

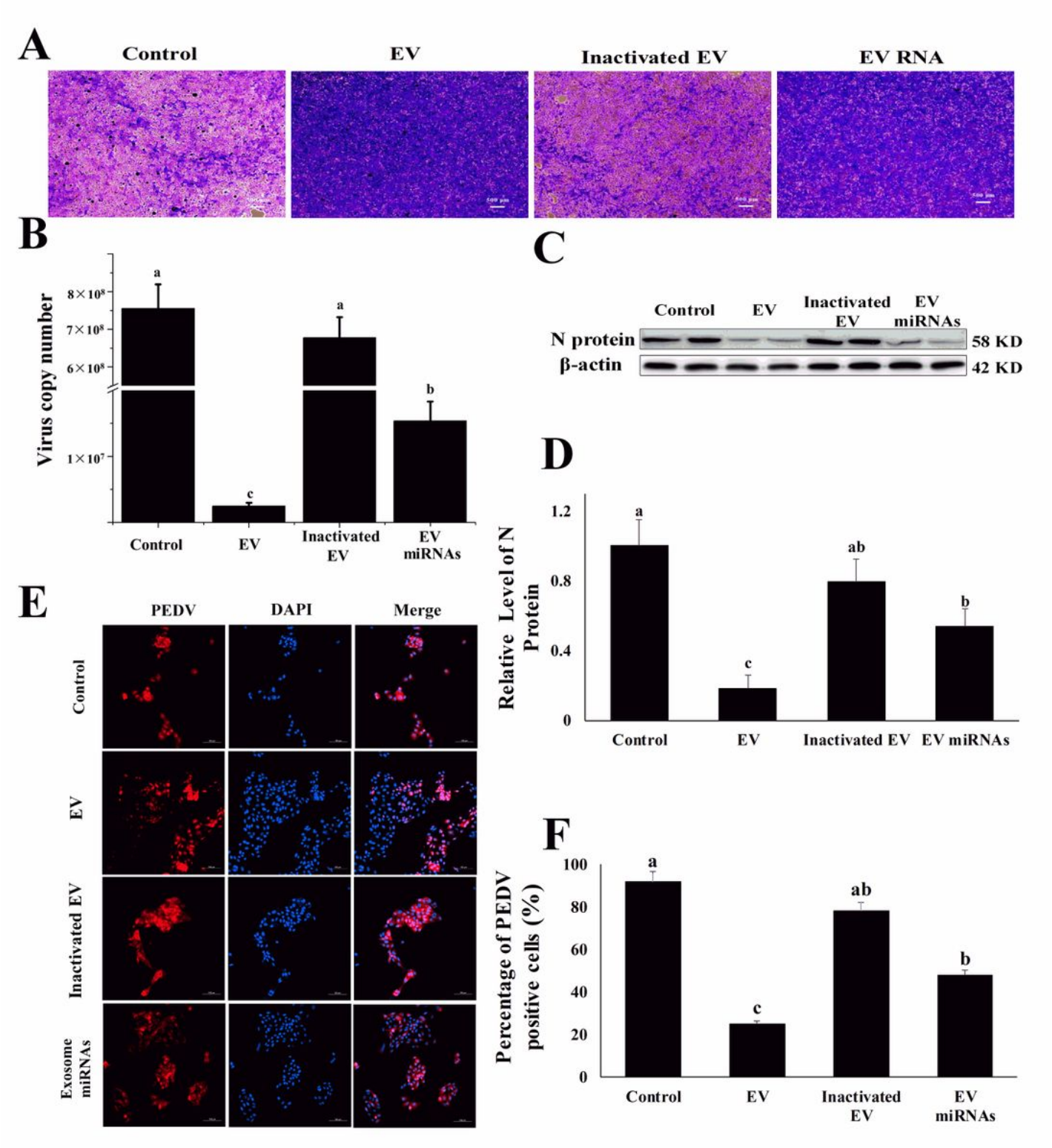

Figure 3

Milk sEV miRNAs inhibited virus replication in IPEC-J2 cells A. Crystal violet staining analysis of IPEC-J2 cells. Both milk sEVs and their isolated small RNAs were associated with higher cell viability. Living cells are stained purple. B. PEDV copy number determined by quantitative PCR. Milk sEVs robustly decreased virus copy number among groups. Isolated milk sEV small RNAs also significantly reduced PEDV copy number. Inactivated milk sEVs did not significantly affect PEDV copy number. C, D. N protein expression

detected by Western blotting in IPEC-J2 cells. E, F. in situ immunofluorescence detection of the viral N protein. Both analyses show similar results for the change profile of PEDV copy number. Blue: DAPI, red: N protein (n=6). The results are presented as the mean ± S.E.M. The data were normally distributed, and homogeneity of variance between treatment groups was confirmed by SPSS analysis. Significant differences between treatment groups were determined by one-way ANOVA. Bars with different letters indicate statistically significantly differences ($p < 0.05$). Magnification = 200×. The scale bar on the photomicrographs represents 100 μm.

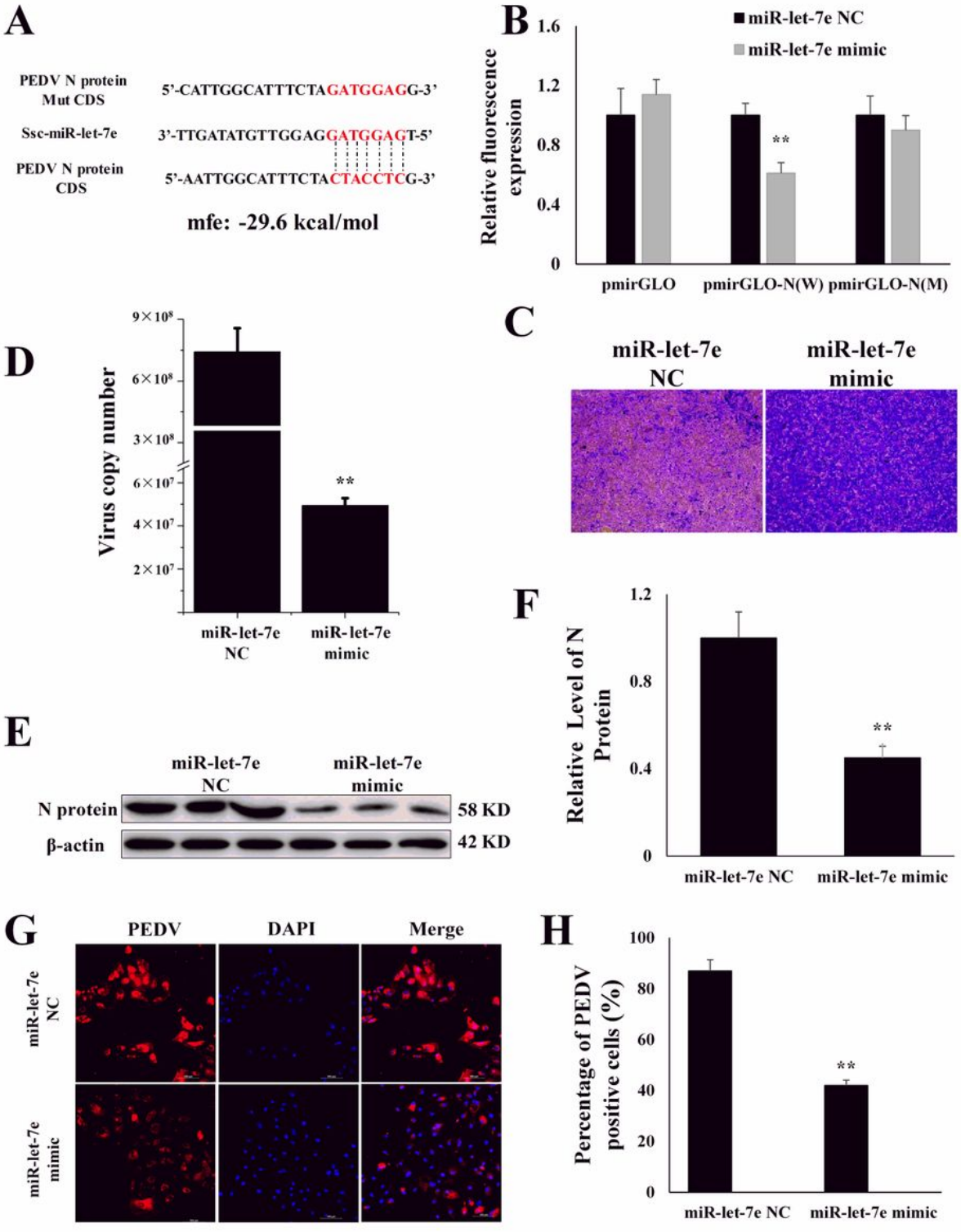

Figure 4

Milk sEV abundant miR-let-7e inhibited viral replication by targeting the PEDV N protein A. Bioinformatics predicted target relationship between miR-let-7e and N protein. B. Dual luciferase assay for detecting the target relationship between miR-let-7e and N protein. miR-let-7e mimics significantly reduced luciferase activity, while mutation of the seed sequence diminished this reduction. The mutated 5' UTR sequence is marked in red. C. Crystal violet staining analysis. Living cells are stained purple. The miR-let-7e improved cell viability under PEDV infection. D. PEDV copy number in cells as determined by quantitative PCR. The miR-let-7e significantly decreased PEDV copy number in the cells. E, F. Western blotting analysis of the N protein. G, H. in situ immunofluorescence analysis of the N protein. Both analyses showed that miR-let-7e significantly reduced N protein expression. Blue: DAPI, red: N protein (n=6). The results are presented as the mean ± S.E.M. Unpaired Student's t-tests were used for p-value calculations. ** $p < 0.01$. Magnification = 200×. The scale bar on the photomicrographs represents 100 μm.

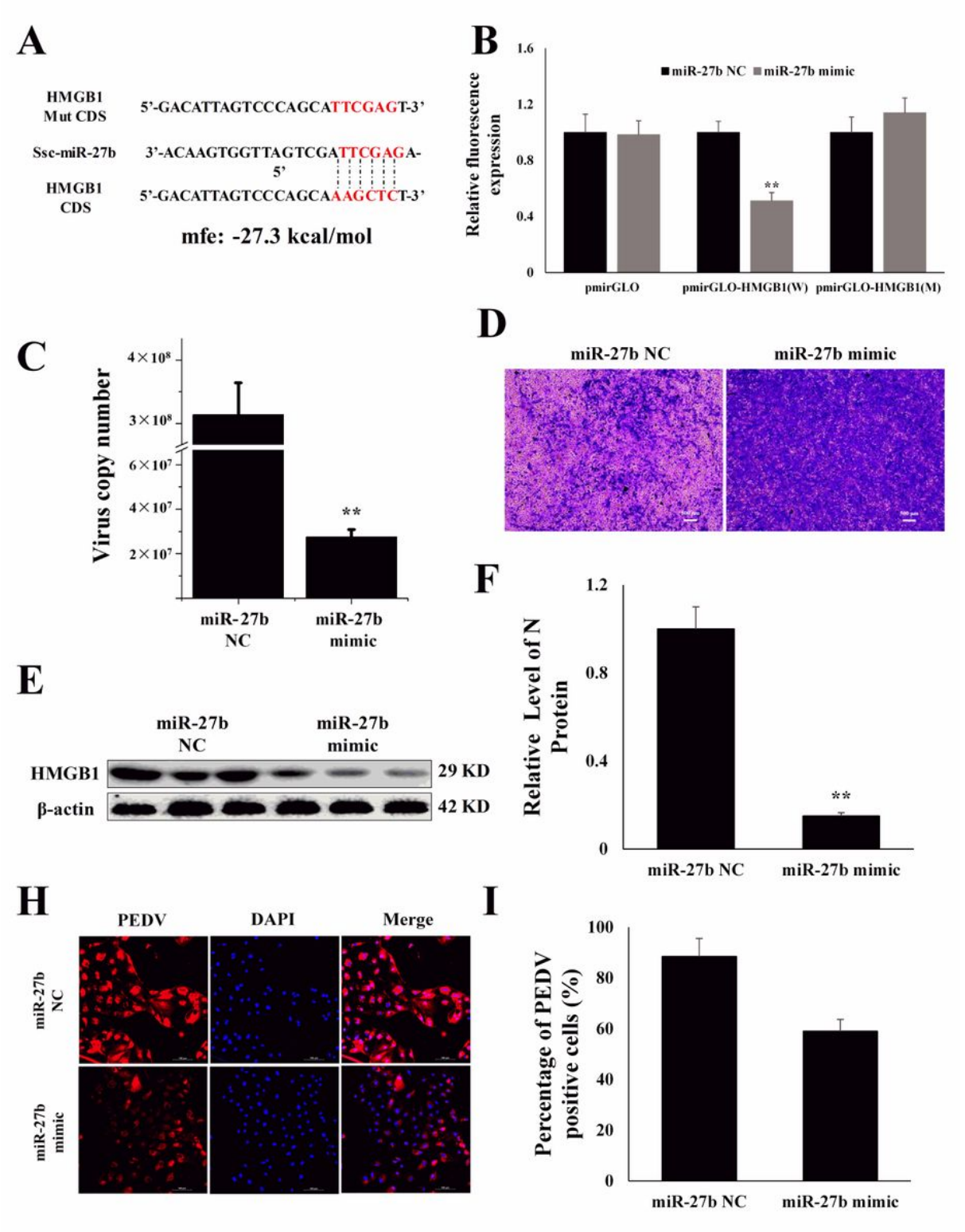

Figure 5

Milk sEV abundant miR-27b inhibited viral replication by targeting HMGB1 A. Bioinformatics predicted the target relationship between miR-27b and HMGB1. B. Dual luciferase assay for detecting the target relationship between miR-27b and HMGB1 protein. The miR-27b mimics significantly reduced luciferase activity, while mutation of the seed sequence diminished this reduction. The mutated 5' UTR sequence is marked in red. C. Crystal violet staining analysis. Living cells are stained purple. Treatment with miR-27b

improved cell viability under PEDV infection D. PEDV copy number in cells determined by quantitative PCR. Treatment with miR-27b significantly decreased PEDV copy number in cells. E, F. Western blotting analysis of HMGB1. The miR-27b significantly decreased HMGB1 expression relative to control. G, H. In situ immunofluorescence analysis of PEDV N protein. Blue: DAPI, red: N protein (n=6). The results are presented as the mean ± S.E.M. Unpaired Student's t-tests were used for p-value calculations. ** $p < 0.01$. Magnification = 200×. The scale bar on the photomicrographs represents 100 μm.

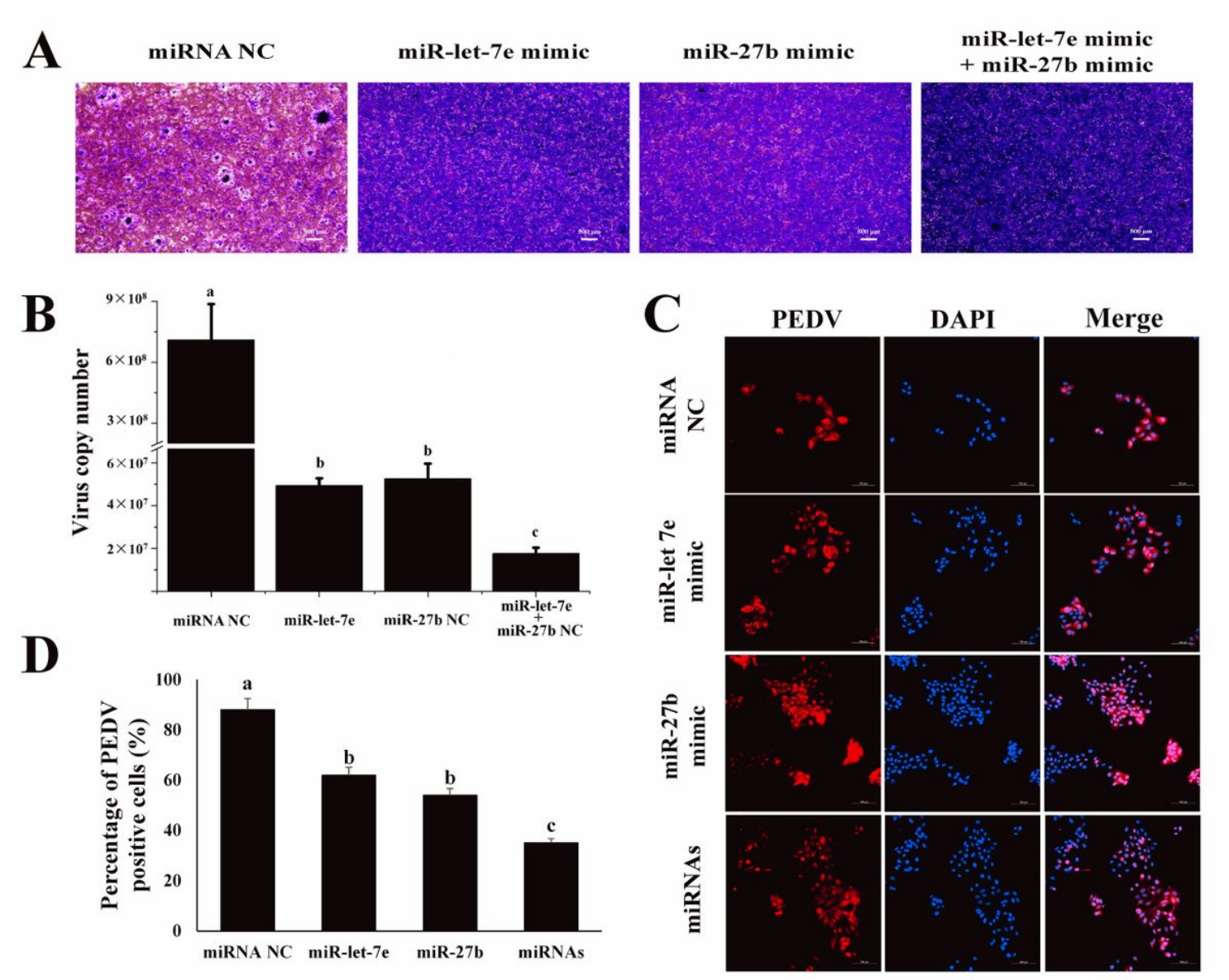

Figure 6

Synergistic function of miR-let-7e and miR-27b in inhibiting PEDV replication A. Crystal violet staining analysis of IPEC-J2 cells after PEDV challenge. Living cells are stained purple. Treatment with miR-let-7e and miR-27b yielded better protection of IPEC-J2 cells from PEDV damage. B. PEDV copy number determined by quantitative PCR. The combination of miR-let-7e and miR-27b showed the most robust decrease in PEDV copy number among groups, although miR-let-7e and miR-27b significantly decreased PEDV copy number alone. C, D. In situ immunofluorescence analysis of N protein in cells showed similar

results as for PEDV copy number. Blue: DAPI, red: N protein (n=6). The results are presented as the mean ± S.E.M. The data were normally distributed, and homogeneity of variance between treatment groups was confirmed by SPSS analysis. Significant differences between treatment groups were determined by one-way ANOVA. Bars with different letters indicate statistically significantly differences ($p < 0.05$). Magnification = 200×. The scale bar on the photomicrographs represents 100 μm.

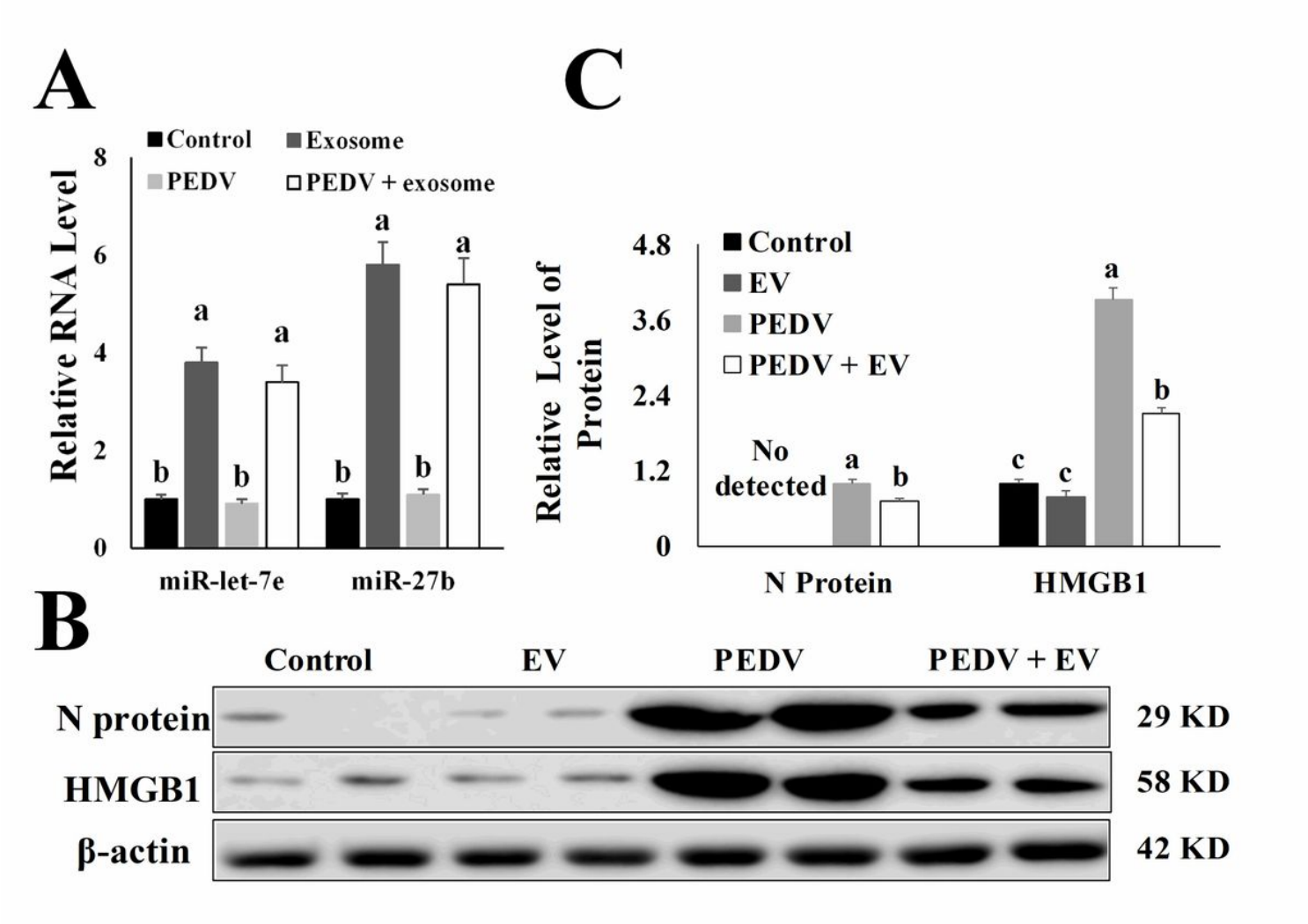

Figure 7

Milk sEVs inhibited PEDV N protein and host HMGB1 in piglet intestines A. The miR-let-7e and miR-27b levels in piglet intestine tissue deterimined by qRT-PCR. Piglets receiving milk sEVs showed significantly higher levels of both miR-let-7e and miR-27b. B, C. Western blotting analysis of N and HMGB1 protein. Milk sEVs significantly reduced expression of N and HMGB1 proteins under PEDV challenge (n=6). The data were normally distributed, and homogeneity of variance between treatment groups was confirmed by SPSS analysis. Significant differences between treatment groups were determined by one-way ANOVA. Bars with different letters indicate statistically significant differences ($p < 0.05$).