Bovine coronavirus nucleocapsid suppresses IFN-β production by inhibiting RIG-I-like receptors pathway in host cells

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Abstract
The present study aimed to explore if bovine coronavirus nucleocapsid (BCoV N) impacts IFN-β production in the host cells and to reveal further molecular mechanism of BCoV pathogenesis. Human embryonic kidney (HEK) 293 T cells were transiently transfected with pMyc-BCoV-N recombinant plasmids, then infected with the vesicular stomatitis virus (VSV). Expression levels of beta interferon (IFN-β) mRNA were detected using RT-qPCR. The results showed that BCoV N gene was 1347 bp that was consistent with the expected size. pMyc-BCoV-N recombinant protein was 1347 bp which was successfully transcribed and overexpressed in HEK 293 T cells. BCoV-N recombinant protein inhibited dose-dependently VSV-induced IFN-β production (p < 0.01). MDA5, MAVS, TBK1 and IRF3 could promote transcription levels of IFN-β mRNA. But, BCoV-N protein demoted IFN-β transcription levels induced by MDA5, MAVS, TBK1 and IRF3. Furthermore, expression levels of MDA5, MAVS, TBK1 and IRF3 mRNAs were reduced in RIG-I-like receptor (RLR) pathway. In conclusion, BCoV-N reduced IFN-β levels in RIG-I-like receptor (RLR) pathway in HEK 293 T cells which were induced by MDA5, MAVS, TBK1 and IRF3(5D). BCoV-N protein inhibited IFN-β production and activation of RIG-I-like receptors (RLRs) signal pathway. Our findings demonstrated BCoV N protein is an IFN-β antagonist through inhibition of MDA5, MAVS, TBK1 and IRF3(5D) in RLRs pathway, also revealed a new mechanism of BCoV N protein to evade host innate immune response by inhibiting type I IFN production, which is beneficial to developing novel prevention strategy for BCoV disease in the animals and humans.

Keywords Bovine coronavirus · Nucleocapsid protein · IFN-β · RIG-I-like receptor

Introduction
Coronavirus belongs to the order of the Nidovirales of the Coronaviridae family in the genus Betacoronavirus and the species beta-coronavirus (Lotfollahzadeh et al. 2020). Coronaviruses consists of five major structural proteins: such as the nucleocapsid (N), spike (S), membrane (M) and envelope (E) proteins. Nucleocapsid (N) protein is the most abundant viral structural protein of SARS-CoV. The S protein is cleaved into S1 and S2 sub-units (Khan et al. 2021; Rohaim et al. 2020). Coronaviruses have been organized into at least four genus: α-CoV, β-CoV, γ-CoV and δ-CoV (Dewald and Burtram 2019; Yoshizawa et al. 2020).

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Bovine coronavirus (BCoV), an important pathogen of neonatal calf diarrhea worldwide, can cause acute diarrhea in newborn calves and adult cattle (Hasoksuz et al. 2002). BCoV was detected in 18.95% (36/190) of the samples by reverse transcriptase polymerase chain reaction in China, which lead to reduction of milk yield and weight gain (Beaudeau et al. 2010; JinJing Geng et al., 2019).
Earlier research indicated that nucleocapsid (N) protein inhibited production of beta interferon (IFN-β) by targeting PKR-activating protein (PACT), or RIG-I signaling (Chen et al. 2020; Meriadeg et al. 2018). Additionally, nucleocapsid (N) protein of porcine deltacoronavirus (PDCoV) (Chen et al. 2019) and porcine epidemic diarrhea virus (PEDV) (Liu et al. 2021; Zheng et al. 2021) can inhibit production of beta interferon (IFN-β) via RIG-I signaling. N protein of peste des petits ruminants virus (PPRV) suppressed host immune response by blocking RIG-I-like receptor (RLR) pathway activation (Zhu et al. 2019). However, there are few reports on the molecular characterization of bovine coronavirus (Gomez et al. 2017; Tseng et al. 2021). Currently, scarce researches were performed on the pathogenic mechanism of BCoV (Gomez and Weese 2017; Workman et al. 2019). To better understand the role of BCoV N protein in the suppression of the RLR pathway-mediated antiviral response, we investigated the effect of BCoV N protein on type I IFN production in HEK 293 T cells so as to reveal further molecular mechanism of BCoV pathogenesis and provide experimental bases for developing novel prevention strategy of BCoV.

Materials and methods

Primers design and synthesis

Quantitative real-time PCR (RT-qPCR) was performed to determine the expression levels of IFN-β mRNA induced by vesicular stomatitis virus (VSV) infection. Specific primers of BCoV nucleocapsid (BCoV-N) with product length of 1347 bp were designed and synthesized referring to BCOV-China/SWUN/LN1/2018 strains (Genbank No.: MK095167, DQ33524) using DNAStar Primer 5.0 software (Table 1). The specificity of the primers was verified and blasted in the NCBI. The bold underlined part, respectively, indicated the enzyme digestion site. The concentrations of the primers (100 nM, 200 nM, 300 nM and 500 nM) were evaluated, and formation of primer–dimers was evaluated using the melting curve analysis. Thus, only those concentrations of primers which showed dimmer-free reactions were used for the further analysis (Wei et al. 2017).

Total RNAs were extracted from bovine coronavirus (BCoV) using the TIANamp Virus DNA/RNA Kit in accordance with the manufacturer’s standard protocol and reversely transcribed into cDNA using the M-MLV reverse transcriptase (Invitrogen) system according to the manufacture’s protocols (Wei et al. 2014, 2017). Real-time polymerase chain reaction (PCR) was conducted with SYBR Green PCR Master Mix (Bio-Rad). The full-length BCoV N gene was generated by PCR in a 25 μL reaction system, including 2.5 μL 10 × Ex Taq Buffer, 0.5 μL dNTPs (10 mM), 0.5 μL Ex Taq, 0.5 μL forward primer, 0.5 μL reverse primer, 18.5 μL diethylpyrocarbonate (DEPC) water, 2 μL cDNA, at the PCR reaction conditions 94 °C 5 min, 94 °C 30 s, 64 °C 45 s, 72 °C 1 min (35 cycles) and 72 °C 10 min.

Both BCoV N gene and pCMV-Myc vector were restricted in double enzyme systems, respectively. The reaction was carried out in 50 μL systems, including 5 μL 10× M, 2.5 μL EcoR I, 2.5 μL Kpn I, 4 μL pMyc-BCoV-N, 36 μL DEPC water at 37 °C for 4 h.

The plasmid was extracted using Transgen Biotech Plasmid Minipreparation Kit (Beijing, China) according to the manufacturer’s instruction. Extracted recombinant plasmids were verified with both double enzyme digestion and sequence.

Table 1 Primer sequences information of relative genes

| Primer name | Primer sequences (5′–3′) |
|-------------|-------------------------|
| BCoV-N F    | CGGAATTCGGATGCTTTACTCTGGAAGC -EcoRI |
| BCoV-N R    | GGATTGCCTATTTTCTGAGGTATCTTCAGT -KpnI |
| IFN-β F     | TTGTAGACACCTCCTGGCT |
| IFN-β R     | TGACTATGGTCAGCAGCAG |
| β-actin F   | TGGGACCCCAACAAATAGGA |
| β-actin R   | CTAGTCTAGATGGCCTAGAAGCA |
| ISG15 F     | CACCGTGTTCTAGAATCTGC |
| ISG15 R     | CTTATTTCGGCCCTTGGAT |
| ISG56 F     | GCTTCTGAAAGTGTGGAGGAA |
| ISG56 R     | ATCCAGGGCGATTAGGCAAGATC |

Construction of eukaryotic expression vector of BCoV N gene

Total RNAs were extracted from bovine coronavirus (BCoV) using the TIANamp Virus DNA/RNA Kit in accordance with the manufacturer’s standard protocol and reversely transcribed into cDNA using the M-MLV reverse transcriptase (Invitrogen) system according to the manufacture’s protocols (Wei et al. 2014, 2017). Real-time polymerase chain reaction (PCR) was conducted with SYBR Green PCR Master Mix (Bio-Rad). The full-length BCoV N gene was generated by PCR in a 25 μL reaction system, including 2.5 μL 10× Ex Taq Buffer, 0.5 μL dNTPs (10 mM), 0.5 μL Ex Taq, 0.5 μL forward primer, 0.5 μL reverse primer, 18.5 μL diethylpyrocarbonate (DEPC) water, 2 μL cDNA, at the PCR reaction conditions 94 °C 5 min, 94 °C 30 s, 64 °C 45 s, 72 °C 1 min (35 cycles) and 72 °C 10 min.

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Transfection of HEK 293 T cells with the recombinant plasmid and cell viability

The human embryonic kidney (HEK) 293 T cell line purchased from American Type Culture Collection was cultured in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Shanghai, China) supplemented with 10% FBS (Gibco) in 5% CO₂ at 37 °C for 24 h. HEK 293 T cells were transfected using fluid A (250 μL Opti-MEM media and pMyc-BCoV-N recombinant plasmid, pCMV-Myc and blank control) and fluid B (250 μL Opti-MEM media) and Liposome 2000 (Invitrogen; Thermo Fisher Scientific, Inc) for 6 h at 37 °C according to the manufacturer’s protocol when the cells density was 80%, or index of fusion was over 95%. After 48 h of transfection, 293 T cells were collected and measured using Cell Counting Kit-8 (CCK-8). Blank 293 T cells were used as the control group. Before co-transfection, 293 T cells were digested by trypsin, accessed to 4-well plates, 5% CO₂, and then cultured in opti-MEM media (Thermo Fisher Scientific, Shanghai, China) with 10% FBS, 1% penicillin–streptomycin and 1% glutamine in a humidified atmosphere containing 5% CO₂ at 37.0 °C for 24 h.

Cell Counting Kit-8 (CCK8, MedChemExpress LLC, Shanghai, China) was performed strictly referring to the manufacturer’s instruction, and cell viability was calculated. Additionally, HEK 293 T cells were transfected with 2 μg pMyc-BCoV-N plasmid, pCMV-Myc and blank control, respectively, so as to verify whether pMyc-BCoV-N overexpressed successfully in these cells. The tests were conducted in triplicate.

Expression levels of pMyc-BCoV-N detection with Western blotting

To determine the expression levels of pMyc-BCoV-N recombinant protein, Western blots of HEK 293 T cells were harvested, lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 0.1% SDS, 2 mM EDTA, 1 mM PMSF, 1% NP40, 5 μg/mL aprotinin, and 1 μg/mL leupeptin) on ice, and then centrifuged at 12,000 × g for 10 min.

Total protein was extracted from 293 T cells using a total protein extraction Kit (Applygen Technologies, Beijing, China) according to the manufacturer’s instructions. Protein concentrations were determined with a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haumen, China). Proteins were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% non-fat milk in 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% (w/v) Tween 20 for 2 h. The polyclonal antibody to pMyc-BCoV-N (Boster Biological technology, Pleasanton, USA; 1: 2000) was diluted and incubated at 4 °C overnight, followed by 1 h of incubation with the appropriate secondary antibody (1:500, Goat Anti-Mouse IgG). The photo was scanned with a gel image analyzer. The absorbance (A value) of each strip was analyzed using Quantity One software. The ratio of the absorbance of the proteins strip to the absorbance of the β-actin strip indicated the relative amount of proteins in the FGCs. All experiments were performed in triplicate.

BCoV N effects on expression levels of IFN-β mRNA induced by vesicular stomatitis virus infection

To investigate the interaction between BCoV N protein and host IFN-β production, HEK 293 T cells were infected with the vesicular stomatitis virus (VSV), then transfected with 2 μg pMyc-BCoV-N plasmid at four different doses and empty vector for 24 h. Expression levels of IFN-β mRNA were detected with PCR as described above.

BCoV N effects on levels of IFN-β induced by MDA5, MAVS, TBK1 and IRF3 (5D)

HEK 293 T cells were transfected with the recombinant plasmid of BCoV N gene, empty plasmid or melanoma differentiation associated protein 5 (MDA5), mitochondrial antiviral signaling (MAVS), TANK binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3), respectively. IFN-β mRNA levels of HEK 293 T cells were detected using real-time quantitative RT-PCR (RT-qPCR). Concurrently, the expression levels of MDA5, MAVS, TBK1 and IRF3(5D) were detected, respectively, in RIG-I-like receptor (RLR) pathway.

BCoV N effects on expression levels of ISG15 and ISG56-induced by VSV

As described above, HEK 293 T cells were transfected with recombinant pMyc-BCoV-N protein. Then the cells were infected with 1.0 × 10⁻⁵ VSV. After 6–8 h, total RNAs were extracted from the cells which displayed green fluorescence under the fluorescence microscope. RT-qPCR was utilized to measure expression levels of ISG15 and ISG56 genes in RLRs signal pathway.

Specific primers of ISG15 and ISG56 were designed and synthesized (Table 1).

Data statistics

Statistical analysis was performed using IBM SPSS Statistics Version 21.0 (SPSS Inc., USA) and GraphPad Prism Version 7.0 (GraphPad Software, Inc.; La Jolla, CA, USA). Data was presented as the means ± SE. Student’s t-test was used to analyze two-group differences. Inter-group differences were
analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. P values less than 0.05 were considered to indicate a statistically significant difference.

Results

Construction and verification of eukaryotic expression vector for BCoV N protein

BCoV N gene was amplified with PCR based on specific primers listed above. The product length was 1347 bp (Fig. 1a). The recombinant plasmid of pMyc-BCoV-N was successfully constructed. The recombinant plasmid was digested with EcoRI/KpnI double enzymes (Fig. 1b). The outcome demonstrated that BCoV N gene was 1347 bp, which was in agreement with the expected size.

Expression levels of pMyc-BCoV-N recombinant plasmid protein

Human embryonic kidney (HEK) 293 T cells were retrieved at 24 h and transfected with pMyc-BCoV-N recombinant plasmid, pCMV-Myc and blank control, respectively. Western blotting assay showed a clear band for pMyc-BCoV-N with 50 kD that was consistent with the predicted sizes. The outcome indicated recombinant plasmid of BCoV N protein had been successfully transcribed and overexpressed in HEK 293 T cells.

pMyc-BCoV-N suppressed VSV-induced IFN-β production

To investigate whether overexpression of BCoV N protein has an inhibitory effect on IFN-β production and the interaction between BCoV N protein and host IFN-β production, HEK 293 T cells were infected with vesicular stomatitis virus (VSV), then HEK 293 T cells were transiently cotransfected with 2 μg pMyc-BCoV-N at four different doses. The results showed that VSV could promote HEK 293 T cells to produce IFN-β production (Fig. 2). However, BCoV N proteins inhibited dose-dependently (p < 0.01) IFN-β levels in HEK 293 T cells induced by VSV. The findings indicated that overexpression of BCoV N protein inhibited production of type 1 IFN (IFN-β) that was induced by VSV.

IFN-β mRNA expression levels were detected using RT-qPCR in HEK 293 T cells. The results showed that VSV could promote HEK 293 T cells to produce IFN-β production (Fig. 2). However, BCoV N protein suppressed dose-dependently (p < 0.01) IFN-β production in HEK 293 T cells mediated by VSV.

Fig. 1 BCoV-N PCR product and double enzyme digestion of pMyc-BCoV-N recombinant protein. a PCR product of BCoV-N, M: 1000 bp DNA Marker; 1: BCoV-N with 1347 bp. b Double enzyme digestion of pMyc-BCoV-N recombinant protein; M: 1000 bp DNA Marker; 1: pMyc-BCoV-N recombinant protein; 2: pMyc-BCoV-N recombinant protein product of double enzyme digestion; 3: pCMV-Myc protein; 4: pCMV-Myc plasmid product of double enzyme digestion. NC: negative control

Fig. 2 BCoV N protein dose-dependently inhibited IFN-β production induced by VSV. HEK 293 T cells were cotransfected with vesicular stomatitis virus (VSV) and pMyc-BCoV-N. The outcomes indicated that BCoV N proteins depressed dose-dependently (500, 1000, 1500 or 2000 ng) levels of IFN-β mRNA. ***P < 0.001 as compared to VSV treatment
Transcriptional levels of ISG15 and ISG56 genes in RLRs signal

Expression levels of ISG15 and ISG56 in 293 T cells following pMyc-BCoV-N treatment were less than those of vector group ($P < 0.01$ or $P < 0.001$) (Fig. 3). These findings demonstrated that pMyc-BCoV-N could significantly decrease transcriptional levels of ISG15 and ISG56 VSV in RLRs signal pathway.

pMyc-BCoV-N protein inhibited on IFN-β expression levels induced by MDA5, MAVS, TBK1 and IRF3

HEK 293 T cells were transfected with single factors (MAD5 or MAVS or TBK1 or ORF3, respectively) as described above. The transcription levels of IFN-β mRNA in HEK 293 T cells were detected using quantitative RT-PCR. The results showed four factors of MDA5, MAVS, TBK1 and IRF3 increased the IFN-β transcription expression levels in HEK 293 T cells (Fig. 4). But, BCoV N depressed these effects. Therefore, BCoV N protein could inhibit IFN-β production that was induced by MDA5, MAVS, TBK1 and IRF3.

HEK 293 T cells were cotransfected with empty vector (pCMV-Myc), pMyc-BCoV-N protein or Flag-N-key factors including MDA5, MAVS, TBK1 and IRF3. Expression levels of IFN-β mRNA were detected using PCR.

To verify these effects, western blotting was performed for MDA5, MAVS, TBK1 and IRF3 (5D). The results showed that MDA5, MAVS, TBK1 and IRF3(5D) proteins were expressed in HEK 293 T cells (Fig. 4e).

BCoV N protein inhibited transcription levels of MDA5, MAVS, TBK1 and IRF3 (5D) in RLR pathway

Human embryonic kidney (HEK) 293 T cells were cotransfected with empty vector (pCMV-Myc), pMyc-BCoV-N protein or Flag-N-key factors. Target gene levels of MDA5, MAVS, TBK1 and IRF3 mRNA were detected in the collected cells using PCR, respectively. The pCMV-Myc vector did not increase transcription levels of MDA5, MAVS, TBK1 and IRF3(5D).

Each of MDA5, MAVS, TBK1 and IRF3(5D) accelerated greatly its self-expression level. However, pMyc-BCoV-N recombinant plasmid significantly decreased transcription levels of MDA5, MAVS, TBK1 and IRF3 by 88%, 94%, 81% and 89%, respectively (Fig. 5). The results indicated pCMV-Myc-N depressed expression levels of MDA5, MAVS, TBK1 and IRF3 (5D) mRNAs in RIG-I like receptor (RLR) pathway in HEK 293 T cells by inhibiting pCMV-Myc efficacy.

Discussion

Coronavirus (CoV) is a positive-sense RNA virus that generates dsRNA intermediates during replication, which can trigger the host innate immune defense (Chen et al. 2019; Sola et al. 2015). Bovine coronavirus (BCoV) causes calves diarrhea in the worldwide (Lotfollahzadeh et al. 2020a, b). BCoV nucleocapsid (N) protein, as a main structural protein of 50–60KD phosphoprotein, is bound to viral genomic RNA to form the helical nucleocapsid. Nucleocapsid (N) protein participates the virus replication and immune regulation (Chen et al. 2020; Geng et al. 2019).

An earlier report indicated that N protein of the identical genus virus may escape antiviral mechanism in the host cells by inhibiting production of interferon beta (IFN-β) (Liu et al. 2021). The IFN-β production is an important defense factor for host cells against infection of many pathogenic organisms. Type I IFN may locate all types of cells within bio-organisms. However, the different stimulants activate expression of type I IFN through varying ways (Daniela Klotz and Gerhauser, 2019). The type I IFN may influence
the expression and distribution of interferon stimulated genes (ISGs) (Chen et al. 2020). Some factors of ISGs are probably direct virus restriction factor which exerts their antiviral roles by blocking virus invasion, replication and release in the body (Daniela Klotz and Gerhauser 2019).

Up to date, no reports have been documented on the replication mechanism of BCoV and the escaping antiviral defense mechanism of the hosts (Lotfollahzadeh et al. 2020; Yesilbag et al. 2021). In the present study, the recombinant plasmid protein of pMyc-BCoV-N was also acquired with fragment of 1347 bp which was consistent with the expected size. Western blotting assay revealed that the recombinant pMyc-BCoV-N protein was about 50 kD that was in line with the predicted sizes. The outcome indicated this recombinant plasmid protein had been successfully constructed and transcribed in HEK 293 T cells.

To explore whether BCoV N protein assists BCoV to escape antiviral and defense mechanism by inhibiting IFN-β production, the IFN-β levels were detected in HEK 293 T cells transfected with 2 μg recombinant plasmid of pMyc-BCoV-N and also infected by vesicular stomatitis virus (VSV). VSV infection promoted IFN-β mRNA expression levels in HEK 293 T cells. But, BCoV N protein inhibited IFN-β production in HEK 293 T cells mediated by VSV.

MDA5, MAVS, TBK1 and IRF3 are important RNA sensors and can be activated by their respective RNA ligands to induce the downstream cascade pathway (Chen et al. 2019; Wu and Hur 2015).
Interferon stimulated genes (ISGs) mark an elegant mechanism of antiviral host defense that warrants renewed research focus in our global efforts to treat existing and emerging viruses (Raftery N and NJ, 2017). IFN-β can induce expression of ISGs. A few ISGs are direct inhibitor of viruses by blocking invasion, replication and release of virus into the host cells (Carty et al. 2021; Crosse et al. 2018). ISGs mark an elegant mechanism of antiviral host defense that warrants renewed research focus in our global efforts to treat existing and emerging viruses (Raftery 2017). Our study indicated pMyc-BCoV-N could significantly decrease transcriptional levels of ISG15 and ISG56 mediated by VSV, which was consistent with the previous report (Crosse et al. 2018).

In the present study, MDA5, MAVS, TBK1 and IRF3 increased the IFN-β expression levels in HEK 293 T cells. However, BCoV N protein could depress efficacy of MDA5, MAVS, TBK1 and IRF3 on enhancing IFN-β production in HEK 293 T cells. BCoV-N reduced expression levels of MDA5, MAVS, TBK1 and IRF3 mRNAs in RIG-I-like receptor (RLR) pathway which is the key innate immune receptor (González-Navajas et al. 2012; Qingshi Liu et al., 2016). Our findings demonstrated that MDA5, MAVS, TBK1 and IRF3 are key target proteins of BCoV-N protein in RIG-I-like receptor (RLR) pathway.

**Conclusions**

BCoV N protein dose-dependently depressed IFN-β production mediated by VSV, and reduced IFN-β expression levels in RLR pathway in HEK 293 T cells. MDA5, MAVS, TBK1 and IRF3 are the crucial factors of specific immune escape of BCoV. Additionally, BCoV N decreased transcriptional levels of these key proteins. Here, for the first time, our study identified inhibitory effect of BCoV N protein on IFN-β production and, thus, demonstrated the suppressive role of N protein on the host innate immune system. Meanwhile, we revealed BCoV N protein played an important role in suppression of interferon regulatory factor 3 (IRF3) function and blocking IFN-β production of RIG-I like receptors (RLRs) signal pathway. Taken together, our findings demonstrated BCoV N protein acted as an IFN-β antagonist through suppression of MDA5, MAVS, TBK1 and IRF3 in RLRs pathway, revealed a new mechanism evolved by BCoV N protein to evade host innate immune response by
inhibiting type I IFN production, which is beneficial for developing novel prevention strategy for BCoV disease in the animals and humans.

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Authorship contributions GJ optimized TaqMan probe RT-PCR. GZ Authorship made the paper design and did data analyses. WL took and detected the samples. LQ did the data statistics analyses. NY assessed the specificity and sensitivity. WS was responsible for the experimental designs and writing the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved for the final version.

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Declarations

Conflict of interest All authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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