Cold Atmospheric Plasma Boosts Virus Multiplication via EGFR(Tyr1068) Phosphorylation-Mediated Control on Cell Mitophagy

Peiyu Han1#, Li Shen1, Nan Nan1, Renwu Zhou2, Tianci Li1, Xiaofeng Dai1,3,5#

1. Wuxi School of Medicine, Jiangnan University, Wuxi 214122, China.
2. School of Chemical and Biomolecular Engineering, University of Sydney, Sydney, 2008, Australia.
3. CAPsoul Medical Biotechnology Company, Ltd, Beijing, 100000, China.
#These authors contributed equally to this work.
Corresponding author: Xiaofeng Dai, E-mail: xiaofeng.dai@jiangnan.edu.cn. Wuxi School of Medicine, Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu 214122, China.
© The author(s). This is an open access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).
See http://ivyspring.com/terms for full terms and conditions.
Received: 2022.02.15; Accepted: 2022.04.17; Published: 2022.05.09

Abstract

Objectives: Vaccination still remains as the most effective approach for preventing infectious diseases such as those caused by virus infection, with cell-based vaccine manufacturing being one flexible solution regarding the spectrum of infectious disorders it can prevent. Rapid cell-based virus propagation can enable high yield of vaccines against viral diseases that may offer critical values in the industry when handling emergent situations such as the ongoing viral disease pandemic.

Methods: Through investigating the phenomenon and biological mechanism underlying redox-triggered cell survival towards enhanced viral particle production, this study explores novel strategies for improved yield of viral particles at a reduced cost to meet the increasing demand on cell-based vaccine manufacturing against viral diseases.

Results: We found in this study that cold atmospheric plasma (CAP), composed of multiple reactive oxygen and nitrogen species including H2O2, could effectively enhance virus replication via triggering cell mitophagy that was dynamically modulated by the p-EGFR(Tyr1068)/p-Drp1(Ser616) axis using IBRV and MDBK as the virus and cell models, respectively; and removing H2O2 can further enhance virus yield via releasing cells from excessive G0/G1 cell cycle arrest. The observed efficacy of CAP was extended to other viruses such as CDV and CPV.

Conclusion: This study provides experimental evidences supporting the use of CAP as a modulator of cell survival including mitophagy and mitochondria dynamics, and makes CAP an interesting and promising tool for enhancing the yield of viral vaccines if translated into the industry.

Key words: Cold atmospheric plasma; EGFR; Mitophagy; Cell cycle; Vaccine; Virus

Introduction

Vaccination is an effective approach for infectious disease prevention and possibly the only solution for eradicating viral diseases that have low virulence but rapid transmission and with the potential of eventually evolving into symbiosis with human. Cell-based vaccine manufacturing that utilizes cultured mammalian cells as the virus production factory represents a flexible approach for viral vaccine production regarding the yield and spectrum of viral diseases it can control [1]. Rapid vaccine production can deliver critical benefits especially under urgent situations such as the outbreak of Coronavirus Disease 2019 (COVID-19) epidemic [2]. Enhancing virus titer is a practical strategy to meet the large and increasing demand on cell-based vaccine manufacturing against viral diseases.

Cold atmospheric plasma (CAP), belonging to the fourth state of matter, has been shown capable of preventing COVID-19 [3], deactivating viruses, removing pathogens such as bacteria and fungi [4], and selectively killing cancer cells via inducing, e.g.,
cell cycle arrest [5], senescence [6], autophagy [7], apoptosis [8], ferroptosis [9, 10], ICD [11], and necrosis [12] in a dose-dependent fashion [13]. It is composed of long-lived and short-lived reactive oxygen and nitrogen species, the chemical properties of which have been intensively studied [14-19]. We had previously shown that CAP could hijack host signaling towards enhanced virus production under controlled dosing and following specified protocol [7, 20]. It is thus possible that CAP can function as a tool to boost virus multiplication that ultimately leads to rapid vaccine production for viral disease control.

Motivated as such, we explored the efficacy of CAP in triggering increased virus titration and the underlying biological mechanism with the focus laid on cell survival including selective autophagy and cell cycle arrest. Using infectious bovine rhinotrachieitis virus (IBRV) and Madin-Darby Bovine Kidney (MDBK) cells as the virus and cell models, respectively, we found that CAP could boost virus propagation through enhancing cell mitophagy that was dynamically modulated by the activity and location of phosphorylated EGFR (Tyr1068). Interestingly, removing H$_2$O$_2$ from CAP-activated medium (PAM) further enhanced virus titer via releasing cells from H$_2$O$_2$-triggered halt at the G$_0$/G$_1$ stage. Similar effect of CAP on boosting virus titer was observed for other viruses such as canine parvovirus (CPV) and canine distemper virus (CDV). Our study makes CAP an effective approach enabling rapid viral vaccine manufacturing and, also a potential dose-dependent tool for modulating cell mitophagy and mitochondria dynamics towards desirable cell outcomes for other research and clinical purposes.

Materials and methods

Home-made CAP ejection device and parameter configuration

The home-made CAP ejection device is composed of a power controller, a helium (He) gas cylinder, a rotor flow meter, and a CAP jet (Supplementary Figure 1). The peak-to-peak voltage applied to the electrode, the sinusoidal wave frequency, the He flow rate, and the distance from the CAP jet to the medium surface were set to 0.96–1.24 kV, 10 kHz, 1 L/min, and 13 mm, respectively. Plasma activated medium (PAM) was prepared by exposing 1 mL medium to CAP for 2 or 4 min for each well in a 24-well plate.

Cells and viruses

IBRV (NCBI TaxId: 79889) was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China and propagated using MDBK cells. The MDBK cell line was purchased from Cell Bank of the Chinese Academy of Sciences Cell Bank and cultured in RPMI medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Virus infection and CAP treatment

Cells were grown to 80%-90% confluence prior to incubation with different doses of PAM for 1 h. Cells were infected with viruses for 1 h. The medium was refreshed, and cells were cocultured with viruses for additional 46 h.

Tissue culture infective dose

Tissue culture infective dose (TCID$_{50}$) was measured as the virus dilution fold when 50% cells were infected. Cells were plated in a 96-well plate. Inoculating cells with a 10-gradient sequentially diluted solutions of viral fluid at a step-size of 10 folds, and monitoring cells for 5 to 7 days until the occurrence of cytopathic effect (CPE) in 50% cells. TCID$_{50}$ was calculated following previously recommended protocol [21].

qRT-PCR

Basic qRT-PCR protocol: 4 μL cDNA samples, 10 μL 2×UltraSYBR MixTure, 1 μL forward and 1 μL backward primers, 4 μL ddH$_2$O were mixed and centrifuged before running the qRT-PCR program (pre-denature at 95 °C for 10 min; 95 °C for 10 s, 60 °C for 1 min, 72 °C for 20 s, for 40 cycles) in Roche LightCycler 480 RT-PCR. Each sample had 3 replicates.

For virus detection, supernatants were collected and boiled following the protocol of UltraSYBR MixTure Kit (Catalog Number: CW0957M, Cwbio Co. Ltd., China) prior to the aforementioned basic q-PCR protocol. For EGFR mRNA quantification, intracellular mRNA was extracted followed by reverse transcription into cDNA prior to the aforementioned basic q-PCR protocol.

Primers for IBRV detection and EGFR quantification were listed in Supplementary Table 1.

Western blot

Cells were grown in 6-well plates, washed twice with PBS, lysed on ice for 20 min using RIPA lysis buffer containing protease inhibitor, and centrifuged at 12,000 g for 20 min before collecting the supernatants. Protein concentration was estimated using BCA analysis kit (Catalog Number: P0012 Beyotime Biotechnology, China). The protein per lane (20 μg) was resolved by SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skim milk powder in TBS and Tween-20 buffer, the membrane was incubated overnight at 4 °C with a suitable
primary antibody followed by incubation with the secondary antibody for 1 h at the room temperature. After TBST cleaning, the membrane was detected by Bio-Rad Gel Doc XR. Details on antibodies used in this study were summarized in Supplementary Table 2.

Immunofluorescence

Cells were fixed with cell fixature and permeabilized with 1% Triton X-100. Cells were blocked in 1% bovine serum albumin (BSA) followed by incubation with the primary antibodies for 8 h. Cells were incubated with the secondary antibody and imaged using laser-scanning confocal microscope Zeiss Axio Imager Z2 (Zeiss, Germany).

For mitochondria morphology imaging, cells were incubated with 100 nM MitoTracker (Catalog Number: M7512 Thermo Scientific, USA) for 30 min at 37 °C prior to the performance of the aforementioned protocol.

Mutation of EGFR(Tyr1068) to EGFR(Phe1068)

The pRP[CRISPR]-EGFP/Puro-hCas9-U6-[EGFR[gRNA]] and Single-stranded Oligo Donor (ssODN) (CAGGGATCCTGCATGGGATGGTGCTTTCGCTGGCTCTGTGCAGAATCCTGTCTATCACAAAGAGTTCATTAATCAGTCCGTTCCCAAAAGGCCGCTGATTACTTCACCTCTGATTTCTTTCCACTTTC) were purchased from Vector Builder. ssODN-EGFR was ethanol precipitated and re-suspended (100 μM) in 10 mM Tris-HCl (pH=7.5). Oligos of EGFR(Tyr1068) (not including the signal peptide of 24 Aa at N-terminal) were diluted to 1 μM prior to transfection. When MDBK cells grew to 60%–70% confluence in 6-well plates, 1 μM ssODN, 1 μg pRP[CRISPR]-EGFP/Puro-hCas9-U6 and 6 μL lipofectamine 3000 were added followed by incubation for 8 h.

Cell cycle assay

Cells were grown in 6-well plates, washed using PBS and digested with EDTA-free trypsin. Cells were centrifuged at 1000 rpm for 5 min to retain the pellets. Cell pellets were washed with 500 μL PBS, centrifuged at 1000 rpm/min for 5 min, and the supernatant was removed to retain the pellet. Cell pellets were re-suspended in 70% ethanol and placed in a refrigerator at 4 °C overnight for fixation. Fixed cells were centrifuged at 1000 rpm for 5 min to remove the supernatant, and cell pellets were suspended in 500 μL PBS. 5 μL propidium iodide (Catalog Number: ST511, Beyotime Biotechnology, China) was added and mixed with cells on ice for 30 min. Cell cycle detection was performed using a BD Accuri C6 flow cytometer, and data analysis was performed using Flowjo software.

ROS detection assay

DCFH-DA (Catalog Number: S0033S, Beyotime Biotechnology, China) was diluted with serum-free culture medium at a ratio of 1:1000 to make the final concentration 10 mM/L. Cell culture medium was removed followed by addition of DCFH-DA in an appropriate volume. Cells were incubated in a 37 °C cell incubator for 20 min. Cells were washed 3 times using serum-free cell culture medium to fully remove the rest of DCFH-DA. Cells were collected by trypsin without adding EDTA. Real-time fluorescence intensity detection was performed using Zeiss Axio Imager Z2 (Zeiss, Germany) before and after the signal stimulation using 488 nm excitation wavelength and 525 nm emission wavelength.

Scavenger assay

Mannitol (200 mM, Catalog Number: M8140, Solarbio, China), sodium pyruvate (10 mM, Catalog Number: SP0100, Solarbio, China), uric acid (100 μM, Catalog Number: IU0150, Solarbio, China), tiron (20 mM, Catalog Number: T104954, Aladdin, China), hemoglobin (20 μM, Catalog Number: H8020, Solarbio, China), and monopotassium phosphate (1 mM, Catalog Number: P7392, Solarbio, China) were used to quench hydroxyl radical (OH ·), hydrogen peroxide (H₂O₂), ozone (O₃), superoxide anion (O₂⁻), nitric oxide (NO ·), and electron (e⁻), respectively.

After treating cells with CAP for 2 or 4 min followed by incubation for 1 h, the quencher of each CAP component was added, separately. Viruses were added to cells and incubated for 1 h. The medium was refreshed, and cells were cocultured with viruses for additional 46 h prior to subsequent analyses.

Optical emission spectroscopy (OES)

The optical emission spectrum, in the gas phase, of CAP ejected from our home-made He-based device (Supplementary Figure 1) was detected using a spectrometer (Andor Shamrock SR-500i-A-R, England), where the optical probe was placed at a distance of approximately 10 mm from the center of the ejection device.

Gene expression determination

Gene expression, represented as FPKM (Fragments Per Kilo Per Million reads) [22], was computed using Htseq software (V 0.6.1) following Equation 1.

\[
\text{FPKM} = \frac{\text{Total exon fragments}}{\text{mapped reads} \times \text{exon length (kb)}}
\] (1)

The mapped reads represent how much percent of all reads are mapped to a gene divided by the length of the gene. FPKM normalization normalizes both the sequencing depth and the gene length to
make the expression of genes at different lengths and different sequencing depths comparable. In general, FPKM value 0.1 or 1 is considered as the threshold to determine whether a gene is expressed.

**RNA-Sequencing correlation analysis**

The correlation of gene expression levels between samples is an important index to test the reliability of the experiments. Pearson correlation was conducted among samples in a pair-wise fashion, with $R^2 > 0.8$ being considered highly correlated.

**Principle component analysis**

Principle component analysis (PCA) was conducted using R packages ‘gmodels’, ‘ggplot2’, ‘ggrepel’.

**Clustering analysis**

A hierarchical clustering analysis was performed based on the FPKM values of all genes in different samples, where ‘euclidean’ was used as the distance and ‘complete linkage’ was set as the linkage approach.

**Identification of differentially expressed genes**

Differentially expressed genes were identified using the ‘DESeq2’ bioconductor package, where genes differentially expressed over 2 folds between the compared groups were considered as differentially expressed, and those with adjusted p value ≤ 0.05 and FDR ≤ 0.05 were selected.

**Gene ontology analysis**

Gene ontology (GO) analysis was conducted using GOseq [23] that is based on Wallenius non-central hyper-geometric distribution. Threshold used for enrichment filtering was set as $p$ value ≤ 0.05 for over represented genes.

**KEGG pathway analysis**

KEGG (Kyoto Encyclopedia of Genes and Genomes) [24] analysis was conducted using the ‘clusterProfiler’ package [25] from R. The analysis was conducted using hypergeometric test where KEGG pathway was considered as the unit following Equation 2.

$$ p = 1 - \sum_{i=0}^{m-1} \binom{M}{n-i} \binom{N-M}{n-i} / \binom{N}{n} \quad (2) $$

In Equation 2, ‘N’ is the number of genes annotated in the pathways among all genes, ‘n’ is the number of differentially expressed genes among ‘N’, ‘M’ is the number of genes annotated to the specific pathway among all genes, and ‘m’ is the number genes annotated to the specific pathway among differentially expressed genes. Threshold for enrichment significance was set as $p$ value ≤ 0.05.

**Results**

**CAP enhances IBRV titration through ROS-triggered selective autophagy**

Through activating the cell culture medium (Supplementary Figure 1), CAP could substantially enhance the number of cells undergoing CPE after IBRV infection (Figure 1A) and effectively increase IBRV titer from $10^{-2.57}$/mL to $10^{-3}$/mL on 2 min CAP exposure and to $10^{-4.5}$/mL on 4 min exposure (Figure 1B). The qRT-PCR also revealed reduced PCR cycles required for IBRV detection on 2 min and 4 min CAP exposure (Figure 1C).

CAP is featured by dose-dependent cellular redox level modulation [8, 13, 26, 27]. Substantially enhanced level of ROS was observed on 4-min CAP exposure as compared with 2-min CAP exposure in IBRV-infected MDBK cells (Figure 1D).

Selective autophagy is one important process governing virus replication [20] and constitutes to an important part of cell stress responses including redox stress in mammalian cells [28]. P62 is the best characterized selective autophagy substrate in mammals that localizes at the autophagosome formation site [29], and LC3B is responsible for selective degradation of p62 through selective autophagy [30], both of which are typical markers of selective autophagy [31]. We observed considerably elevated LC3B and substantially reduced p62 expression on joint CAP exposure and IBRV infection (Figure 1E). The fluorescence imaging signal of LC3B in cells receiving both CAP exposure and IBRV infection was considerably higher than that in cells treated with CAP or IBRV alone (Figure 1F). These results collectively suggested the accompany of selective autophagy in CAP-triggered increase on virus titer.

**EGFR(Tyr1068) phosphorylation plays a central role in increasing IBRV titer on CAP exposure**

Various evidences have supported the roles of EGFR in ROS-induced cellular response [32-37]. EGFR level was considerably reduced when cells were exposed to IBRV infection and 4-min CAP treatment followed by 46 h culturing ($p<0.001$, Figure 2A). EGFR phosphorylation sites (Tyr1068, Tyr1148, Tyr1086, Tyr845) are highly conserved between human and bovine protein sequences (Figure 2B), enabling the use of human rather than bovine antibodies in the following experiments here.

For all the four phosphorylation sites examined, p-EGFR (Tyr1068) was synergistically enhanced by
IBRV infection and CAP exposure (Figure 2C). With the increase of CAP dose (from 2 min to 4 min) that did not trigger cell death (Figure 2D), p-EGFR(Tyr1068) started to accumulate in cell nucleus (Figure 2E). Joint exposure of cells to CAP and IBRV infection substantially enhanced p-EGFR(Tyr1068) level that was co-localized with mitochondria (Figure 2F), associating observed selective autophagy to mitophagy.

**EGFR(Tyr1068) phosphorylation enhances mitophagy on CAP exposure and IBRV infection through interacting with phosphorylated Drp1(Ser616)**

We next examined the role of p-EGFR(Tyr1068) in CAP-triggered mitophagy after IBRV infection. Mitochondria accumulated to the nucleus surrounding area, and the signal was intensified with the increase of CAP treatment duration (Figure 3A), implicating the occurrence of mitophagy. Mitofusin 1 (Mfn1) and dynamin-related protein 1 (Drp1) coordinate the processes of mitochondrial fusion and fission, which are canonical markers of mitophagy [38, 39]. On joint exposure to IBRV infection and CAP treatment, p-EGFR(Tyr1068) and p-Drp1(Ser616) expression were substantially increased, whereas Mfn1 level was reduced (Figure 3B), suggesting the occurrence of cell mitophagy and involvement of p-EGFR(Tyr1068) in cells’ response to both CAP exposure and IBRV infection. LC3B was co-localized with mitochondria and the signal was intensified on joint exposure to CAP and IBRV infection (Figure 3C), confirming the presence of mitophagy. Co-immunoprecipitation suggested physical interactions of p-EGFR(Tyr1068) with p-Drp1(Ser616) but not with Mfn1 on joint exposure to IBRV infection and CAP

---

**Figure 1. IBRV titer and autophagy on IBRV infection and/or CAP exposure at different doses in MDBK cells.** (A) Proportion of MDBK cells undergoing CPE after joint CAP exposure and IBRV infection in MDBK cells. IBRV titer in response to CAP 2 min and 4 min exposure as measured using (B) TCID50 and (C) qRT-PCR in MDBK cells. (D) Cellular ROS level after IBRV infection or CAP exposure in MDBK cells. (E) Western blots and their quantifications on key genes involved in autophagy (LC3B, p62) in MDBK cells. (F) Immunofluorescence imaging showing synergies between CAP exposure and IBRV infection on increasing LC3B cellular level in MDBK cells.
treatment (Figure 3D), indicating that the enhanced cell fission was mediated via the p-EGFR(Tyr1068)/p-Drp1(Ser616) axis.

Figure 2. Expression and activity of EGFR and different EGFR phosphorylation sites on IBRV infection and/or CAP exposure at different doses. (A) EGFR mRNA level on 4-min CAP exposure, IBRV infection and their combinatorial treatment. (B) Conservativity of EGFR phosphorylation sites Tyr1068, Tyr1148, Tyr1086, Tyr845 between human and bovine cells. (C) EGFR protein expression and its phosphorylation levels at Tyr1068, Tyr1148, Tyr1086, Tyr845, as well as their quantifications. (D) Imaging of cells in response to CAP exposure at different doses. (E) Immunofluorescence imaging of EGFR (Tyr1068) expression on CAP exposure at different doses. (F) Immunofluorescence imaging showing colocalization of mitochondria and EGFR (Tyr1068) on combined treatment of IBRV infection and CAP exposure at different doses.
Figure 3. CAP triggers mitophagy via direct interactions between EGFR(Tyr1068) and Drp1(Ser616). (A) Morphological alteration of mitochondria on exposure to different doses of CAP. (B) Mitochondrial dynamic-related protein expression on IBRV infection, CAP exposure, their joint treatment, and their quantifications. (C) Mitochondria and LC3B colocalization on IBRV infection, CAP exposure and their joint treatment. (D) Immunoprecipitation of Drp1(Ser616) and EGFR(Tyr1068).
EGFR(Tyr1068) mutation reduces virus titer and mitophagy on CAP exposure after IBRV infection

To consolidate the role of p-EGFR(Tyr1068) in CAP-triggered increase on virus titer and mitophagy after IBRV infection, we generated an EGFR(Tyr1068) mutant plasmid where tyrosine (Tyr) at the 1068 site was mutated to phenylalanine (Phe) (Figure 4A). The p-EGFR(Tyr1068) level was successfully reduced in cells transfected with the mutant plasmid (Figure 4E, 4F). Cells transfected with the mutant plasmid produced considerably less CPE after IBRV infection with and without CAP exposure (Figure 4B). Accordingly, virus titer reduced substantially (Figure 4C) and the Ct values from the qRT-PCR assay were significantly enhanced in EGFR(Tyr1068)-mutated MDBK cells (Figure 4D). Each primary marker used for selective
autophagy and mitophagy characterization (i.e., LC3B, p-Drp1(Ser616), Mfn1) exhibited a relapsed pattern in EGFR(Tyr1068) mutant cells as compared with the wild type under the same treatment (Figure 4E, 4G, 4H).

**The effects of CAP components on virus titer**

CAP ejected from our home-made He-based device (Supplementary Figure 1) was primarily composed of OH, N₂, N₂⁺ and O in the gas phase (Figure 5A), which became H₂O₂, O₃, O₂⁻, •OH, •NO, e⁻ in the liquid form (40) (Figure 5B). By removing/quenching H₂O₂ from PAM, cell amount (Figure 5C) and virus yield (Figure 5D) were substantially increased after PAM treatment. We next confirmed the roles of H₂O₂ in decreasing virus titer using TCID₅₀, i.e., it dropped from 10⁻⁵ to 10⁻⁵.₃₃ in IBRV-infected cells and from 10⁻⁶.₆₂₅ to <10⁻¹₀ in CPV-infected cells on CAP exposure (Figure 5E, 5F).

The p-EGFR(Tyr1068) mediated virus titer increase was primarily triggered via •NO and e⁻ given the dramatic decrease on p-EGFR(Tyr1068) expression after removing each of these species (Figure 5G, 5H). The effect of H₂O₂ on virus titer was in-dependent of p-EGFR(Tyr1068) signaling as no visible alteration was observed regarding p-EGFR(Tyr1068) expression when H₂O₂ was visible alteration was observed regarding control. A utophagy as assessed via p62 and LC3B (Figure 5G, 5K, 5L) quenchers (dramatic by adding any of these reactive species these components as the fluctuations were not combinatorial role of these species in triggering cell (Figure 5A) since the extracellular region of EGFR has been previously proposed as a potential target for anti-EGFR drug discovery (41). The top GO term and KEGG pathway identified from the 'IBRV+CAP vs IBRV+CAP-H₂O₂' comparison but not from the other two group-wise comparisons was ‘lysosome’ (Figure 6G, 6H, Supplementary Figure 3B, 3C), which is known playing essential roles in autophagy (42). Given the differential regulation of autophagy during cell cycle progression (43) and our knowledge on the roles of H₂O₂ in triggering cellular damage and consequently cell cycle arrest under certain

**Transcriptome sequencing predicts the role of cell cycle arrest in H₂O₂-triggered virus titer decrease and consolidates EGFR-mediated virus titer increase on CAP exposure**

We next conducted transcriptome sequencing to explore the mechanism of H₂O₂ in decreasing virus titer and gain a broad view on CAP-triggered cell response. Gene expression did not considerably vary among sample groups (Supplementary Figure 2A), and those of samples within the same group were highly correlated (Supplementary Figure 2B). Samples of the same group were clustered together from both PCA and clustering analyses (Figures 6A, 6B). CAP treatment caused rightward movement of samples along the first principal component (PC1) that explained 27.4% of the total variance; and removing H₂O₂ from CAP leftward shifted samples along PC1 and downward along PC2 that explained 15.8% of the total variance (Figure 6A). If PC1 reflected the effect of mitophagy, PC2 might represent another cell behavior (namely ‘behavior X’) that was affected by CAP and, in particular, H₂O₂. Additionally, removing H₂O₂ decreased the expression of under-expressed (0-0.1) genes (p=0.0129 for IBRV+CAP-H₂O₂ vs. IBRV; p=0.0019 for IBRV+CAP-H₂O₂ vs. IBRV+CAP) and caused relapsed expression of 1-3 level genes (p=0.0046 for IBRV+CAP-H₂O₂ vs. IBRV+CAP) with statistical significance that was oppositely regulated by CAP (Figure 6C). These are suggestive of the controversial roles of H₂O₂ and CAP in modulating virus titer, as well as the association of these altered genes with the ‘behavior X’. Specifically, 420, 168, 78 genes were differentially expressed in the pairwise comparisons ‘IBRV vs IBRV+CAP’, ‘IBRV vs IBRV+CAP-H₂O₂’ and ‘IBRV+CAP vs IBRV+CAP-H₂O₂’ and genes simultaneously modulated in all of these three group-wise comparisons showed a consistent regulatory direction in ‘IBRV vs IBRV+CAP’, ‘IBRV vs IBRV+CAP-H₂O₂’ but an opposite direction in ‘IBRV+CAP vs IBRV+CAP-H₂O₂’ (Figure 6D, Supplementary Table 3, Supplementary Figure 3A). These results suggested that CAP was the primary contributor to the altered transcriptome profile, and H₂O₂ played an opposite role.

Gene ontology (GO) and KEGG pathway analyses revealed that CAP-triggered cell behavior was associated with ‘extracellular space’ and ‘EGFR tyrosine kinase inhibitor resistance’, which were the top GO term and pathway, respectively, from both ‘IBRV vs IBRV+CAP’ and ‘IBRV vs IBRV+CAP-H₂O₂’ group-wise comparisons, but not from the ‘IBRV+CAP vs IBRV+CAP-H₂O₂’ comparison (Figure 6E, 6F, Supplementary Figure 3B, 3C). These results are supportive to the mediating role of p-EGFR(Tyr1068) in CAP-triggered mitophagy (Figure 4) since the extracellular region of EGFR has been previously proposed as a potential target for anti-EGFR drug discovery [41]. The top GO term and KEGG pathway identified from the ‘IBRV+CAP vs IBRV+CAP-H₂O₂’ comparison but not from the other two group-wise comparisons was ‘lysosome’ (Figure 6G, 6H, Supplementary Figure 3B, 3C), which is known playing essential roles in autophagy [42]. Given the differential regulation of autophagy during cell cycle progression [43] and our knowledge on the roles of H₂O₂ in triggering cellular damage and consequently cell cycle arrest under certain
Int. J. Biol. Sci. 2022, Vol. 18

Concentrations [44], we hypothesized H₂O₂-induced cellular ‘behavior X’ to be ‘cell cycle alteration’.

**H₂O₂ decreases virus titer through triggering G₀/G₁ cell cycle arrest**

We firstly explored CAP-imposed H₂O₂ concentration in the medium (or precisely, quenched H₂O₂ amount in the scavenger assay) via removing H₂O₂ first using 10 mM sodium pyruvate followed by H₂O₂ titration, the lowest Ct value of IBRV infected cells was achieved when 5 mM and 1 mM H₂O₂ were supplemented, respectively, with and without adding sodium pyruvate (Figure 7A, 7B); thus, H₂O₂ quenched by 10 mM sodium pyruvate in our

---

**Figure 5. Effect of each CAP primary component on virus infected MDBK cells.** (A) Optical emission spectroscopy of CAP ejected from the home-made device (Supplementary Figure 1). (B) Schematic diagram on CAP-activated medium preparation and its primary components. (C) Morphology, (D) q-RT-PCR cycle thresholds (Ct) of IBRV infected MDBK cells with and without CAP exposure after removing each primary component. (E) Plate configuration and (F) statistics showing TCID₅₀ of IBRV or CPV infected MDBK cells on CAP exposure with and without removing H₂O₂. (G) Western blots and quantification of (H) EGFR(Tyr1068), (I) p62, (J) LC3B, (K) Drp1(Ser616), (L) Mfn1 of IBRV infected MDBK cells with and without CAP exposure after removing each primary component.
experimental setting was approximately 4 mM (5 mM - 1 mM) (Figure 7C). On the other hand, virus titer decreased when cellular H$_2$O$_2$ exceeded 5 mM (Figure 7B), suggesting that the intracellular H$_2$O$_2$ raised in response to virus infection was over 1 mM (5 mM - 4 mM). Either virus-triggered H$_2$O$_2$ (over 1 mM) or CAP-imposed H$_2$O$_2$ (approximately 4 mM) led to moderate cell cycle arrest (around 40% G$_0$/G$_1$ phase) that corresponded to slightly increased virus titer. Both sources of H$_2$O$_2$ once added together, dramatically decreased virus yield as a result of substantially increased number of cells arrested at the G$_0$/G$_1$ stage (Figure 7D). Cellular ROS increased with H$_2$O$_2$ dose, and the intensity dramatically dropped at 10 mM (Figure 7E), indicative of cell death due to exceeded ROS level. Given these results, together with the known functionality of ROS in triggering cell cycle arrest [45], we assumed that the observed inhibition on virus titer was due to exceeded level of H$_2$O$_2$.

The levels of p-EGFR(Tyr1068) and p-Drp1(Ser616) were both slightly up-regulated when H$_2$O$_2$ was low (< 1 mM) and dramatically dropped under high (1-10 mM) level of H$_2$O$_2$ (Figure 7F, Supplementary Figure 4), which were supportive of the physical interactions of both phosphorylated proteins (Figure 3D) and the opposite role of high dose (1-10 mM) of H$_2$O$_2$ and CAP in triggering p-EGFR(Tyr1068)-mediated cell response. Autophagy markers p62 and LC3B showed a similar pattern with that of p-EGFR and p-Drp1 (Figure 7F). Cyclin B1 is primarily distributed in the M phase and serves as the master regulator of the G$_2$/M transition [46]. CDK1 interacts with 9 different cyclins including cyclin B1 that becomes inactivated at the end of the M phase [47]. Both cyclin B1 and CDK1 expression declined with the increased dose of H$_2$O$_2$ (Figure 7G, Supplementary Figure 4), suggestive of a suppressive role of high H$_2$O$_2$ dose on the M stage. Cyclin D1 is rapidly synthesized in the G1 phase and degraded as cells enter the S phase [48], and CDK4/6 creates complexes with cyclin D1 to mediate cell progression through the G1 phase [49]. Cyclin D1 expression showed a positive correlation with H$_2$O$_2$ dose; CDK4 expression increased with H$_2$O$_2$ concentration and declined when H$_2$O$_2$ exceeded 1 mM (Figure 7G, Supplementary Figure 4), implicating that cells were accumulated and arrested at the G$_0$/G$_1$ stage when H$_2$O$_2$ exceeded 1 mM.

**Discussion**

As integrin represents a common receptor mediating virus entry [50] and is linked to intracellular signaling via EGFR tyrosine activation, we focused on the four integrin-dependent EGFR tyrosine phosphorylation residues, i.e., Tyr1068, Tyr1086, Try845, Tyr1173 [51] in this study. We excluded Tyr1173 given its primary role in the MAPK cascade and cell proliferation [52] to narrow our focus down to cell selective autophagy/mitophagy. Among the three EGFR tyrosine phosphorylation sites, EGFR(Tyr1068) showed the highest phosphorylation level in response to joint IBRV infection and CAP treatment (Figure 2C). The expression profile of p-EGFR(Tyr1068) under varied CAP doses matched well with that of selective autophagy biomarkers (LC3B, p62, Figure 1E), in particular mitophagy markers (p-Drp1(Ser616), Mfn1, Figure 3B), and virus titer (Figure 1A-1D). In addition, p-EGFR(Tyr1068) co-localized with mitochondria (Figure 2F). These were, collectively, implicative of the regulatory role of p-EGFR(Tyr1068) in mitophagy. We, therefore, considered p-EGFR(Tyr1068) as the key EGFR phosphorylation site that drove the observed efficacy of CAP in boosting virus titer.

Selective autophagy degrades certain proteins, organelles to maintain cellular homeostasis and, on virus invasion, can be hijacked to recycle cell nutritional resources for virus propagation [20]. Elevated LC3B expression and decreased p62 level on CAP exposure after virus infection (Figure 1E, 1F) attributed the promotive role of CAP on virus multiplication to cell selective autophagy. Mitochondria accumulation at the nucleus surrounding region (Figure 3A), enhanced Drp1(Ser616) phosphorylation and reduced Mfn1 level (Figure 3B, 3C), providing additional evidence associating CAP-triggered selective autophagy to mitophagy. Mitochondrial fusion, fission, biogenesis and mitophagy constitute mitochondrial dynamics that determines mitochondrial morphology, quality and abundance [53, 54]. Activated mitochondrial fusion as represented by elevated Mfn1 [38] is protective of mitochondria on pressure. Mitochondrial fission machinery as featured by Drp1 [38] actively participates in apoptosis [55] and promotes or inhibits cell death depending on the initial lethal stimulus [56]. We observed physical interactions between phosphorylated p-EGFR(Tyr1086) and p-Drp1 (Ser616) on IBRV infection and/or CAP exposure (Figure 3D), suggesting that mitochondria fission was enhanced due to elevated level of Drp1 that was possibly increased by activated EGFR(Tyr1068). Through introducing the EGFR(Tyr1068) point mutation to native EGFR genes using the CRISPR/Cas9 system (Figure 4), we consolidated the role of p-EGFR(Tyr1068) in mediating CAP-induced virus titer increase. To this end, we could preliminarily conclude that CAP promoted IBRV titration via, at least partially, the trigger of cell mitophagy, and this process was driven by
p-EGFR(Tyr1068)/p-Drp1(Ser616)-mediated mitochondria fission. We also observed elevated expression of the pro-apoptotic protein caspase 7 (Supplementary Figure 5) that was supportive to the pro-apoptotic role of mitochondria fission.
Figure 7. Effect of H2O2 concentration on virus multiplication and cells. (A) The qRT-PCR cycle thresholds (Ct) in IBRV-infected MDBK cells supplemented with 10 mM sodium pyruvate (H2O2 scavenger) and treated with different concentrations of H2O2. The 1st control (white bar) was not supplemented with sodium pyruvate nor H2O2. (B) Ct values in IBRV-infected MDBK cells treated with different concentrations of H2O2. (C) Schematic illustration on calculating the amount of quenched H2O2 by 10 mM sodium pyruvate. (D) Cell cycle profiles, and (E) cellular ROS level of IBRV-infected MDBK cells under treatment of different concentrations of H2O2. (F) Western blots and quantification of p-EGFR(Tyr1068), p-Drp1(Ser616), Mfn1, p62, LC3B in IBRV-infected MDBK cells under different H2O2 concentrations. Simplified illustration on the relationship between EGFR, autophagy markers (Drp1, Mfn1), and mitophagy markers (p62, LC3B) is also provided. (G) Western blots and quantification of proteins critical to the G2/M, G0/G1 cell cycle stages (cyclin B1, CDK1, cyclin D1, CDK4) in IBRV-infected MDBK cells under different H2O2 concentrations. Simplified illustration on primary cyclins and CDKs controlling the four cell cycle stages is also provided.
Quenching each component of PAM using chemical scavengers, though being a qualitative approach, has been accepted as a standard measure to examine CAP-liquid interactions and the effect of CAP ingredients on cell behavior, based on the fact that each RONS scavenger has a higher reaction rate (often in an order of magnitude) with its targeted species than with other species [57, 58]. Here, we used tiron (20 mM), D-mannitol (200 mM), hemoglobin (20 µM), sodium pyruvate (10 mM), uric acid (100 µM) and monopotassium phosphate (1 mM) to trap ·O2-, ·OH, NO, H2O2, O3 and e-, respectively, with the reaction rates being 2.9×108 mol/Ls, 1×109 mol/Ls, 1×108 mol/Ls, 2.4 mol/Ls, 1.4×108 mol/Ls [58, 59]. Except for H2O2 whose removal led to a substantial increase on virus titer, eliminating each of the other ingredients unanimously decreased virus multiplication to some extent (Figure 5D), suggestive of their indispensable and collective role in promoting virus multiplication.

Exceeded level of H2O2 (1-10 mM) triggered G0/G1 cell cycle arrest that was associated with recessed cell activities and reduced virus production (Figure 7). Thus, cells underwent mitophagy in acute response to CAP-triggered redox stress, leading to the creation of a favorable environment for virus multiplication as a result of shrunken cell biomass usage. However, high H2O2 dose (1-10 mM) may result in cell cycle G0/G1 arrest due to the trigger of moderate level of DNA damage signaling (not reaching the threshold of cell apoptosis) and thus counteract virus production. Therefore, a favorable environment for virus multiplication requires enhanced available cellular biomass materials and normal/accelerated cell cycle. Importantly, these results suggested the irreplaceable role of CAP in boosting virus titer that could not be surrogated by H2O2, an easily produced CAP component, and H2O2 might function as an opposite regulator of CAP that made its effect on virus titer control reversible.

CAP is a known cocktail of ROS and non-ROS [40]. Both CAP exposure and virus infection enhanced cellular redox level [26, 60] (Figure 1D), suggesting that the promotive role of CAP in IBRV titer is due to its synergy with virus infection in enhancing cellular ROS level. We observed similar expression profiles between p-EGFR(Tyr1068) and FOXO1 (Supplementary Figure 5) on CAP exposure, where FOXO1 is a key measure of cells’ anti-oxidative ability [61]. This implicated the mediating role of p-EGFR(Tyr1068) in enhancing cellular anti-oxidant ability in response to CAP treatment and/or virus infection.

We additionally found that blocking EGF(Tyr1068) phosphorylation reduced caspase 3 expression that was elevated on CAP exposure or its joint CAP and IBRV treatments (Supplementary Figure 6). This suggested the essential role of p-EGFR(Tyr1068) in regulating cell apoptosis, and that elevated cellular ROS, on CAP exposure, triggered mitophagy and concomitantly tilted cells towards the pro-apoptotic state. This warranted us to keep appropriate CAP dosing to achieve mild yet effective boost of virus titer as over-dosing might kill the host that ultimately led to reduced virus production. We also observed rescued p65 expression in p-EGFR(Tyr1068) mutant cells on CAP or CAP+IBRV treatments (Supplementary Figure 6). This implicated that the cellular system was hijacked by viruses for maximum virus production and other cellular behaviors including cell migration (p65 is a key protein controlling cell migration [62]) were reduced, whereas p-EGFR(Tyr1068) was the hub controlling these processes.

Though we claimed on the driving role of p-EGFR(Tyr1068) and cell mitophagy in CAP-mediated boost of IBRV propagation, we could not exclude the possible involvement of p-EGFR(Try1086) and p-EGFR(Tyr845) in this process. However, these functionalities were more likely to be associated with cells’ natural response to IBRV infection rather than CAP exposure given our results.

Though different viruses may attach and/or trigger cellular signaling using different cell surface receptors beyond EGFR that may limit the generality of the uncovered driving mechanism, the facts that virus infection imposes cells with redox pressure and CAP relies on ROS to deliver its efficacy do not vary. In consistent with this, we observed enhanced titer on CAP exposure when CPV and CDV were used (Supplementary Figure 7). Therefore, the efficacy of CAP in boosting virus production may be generalizable to a broad spectrum of, if not all, viruses and hosts that deserves intensive experimental explorations and additional consolidations.

Conclusion

We report in this study that CAP can enhance IBRV propagation towards increased yield of vaccines against viral diseases through triggering cell selective autophagy and, in particular, mitophagy; this process is driven by the p-EGFR (Tyr1068)/p-Drp1(Ser616) signaling through physical interactions in response to CAP-induced cellular ROS level in a dose-dependent manner (Supplementary Figure 7A). High level of H2O2 (1-10 mM) may decrease virus titer due to enhanced G0/G1 cell cycle arrest. Thus, controlling H2O2 below 1 mM through the use of H2O2 quencher (such as sodium pyruvate) together with CAP may represent a good strategy towards maximal virus production (Supplementary Figure 7B). Our results
contribute in advancing our understandings on CAP-triggered cellular responses that make CAP a potential modulator on cell survival such as mitophagy and cell cycle arrest. Importantly, this study provides a novel strategy for boosting IBRV multiplication that can be potentially generalized to a broad spectrum of viruses towards enhanced cell-based viral vaccine production in the industry. The generality has been preliminarily examined using CDV and CPV, which needs to be validated using more virus and cell models.

Supplementary Material


Acknowledgements

Funding

This study was funded by the National Natural Science Foundation of China (Grant No. 81972789), Fundamental Research Funds for the Central Universities (Grant No. JUSRP22011), Technology Development Funding of Wuxi (Grant No. WX181VJN017). These funding sources have no role in the writing of the manuscript or the decision to submit it for publication.

Author contributions

X.F. Dai conceptualized the idea, designed and supervised this study, as well as drafted the manuscript. P.Y. Han and L. Shen conducted the experiments. P.Y. Han and N. Nan prepared figures and tables. R.W. Zhou measured the chemical spectrum of the plasma ejection device. T.C. Li assisted in the experiments and figure preparation. X.F. Dai financially supported this study. All authors have read and approved the manuscript.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Competing Interests

The authors have declared that no competing interest exists.

References

32. Zeng SY, Yan QJ, Yang L, Mei QH, Lu HQ. Inhibition of the ROS-EGFR Pathway Mediates the Protective Action of Nox1/4 Inhibitor GKT137831 against Hypertensive Cardiac Hypertrophy via Suppressing Cardiac

https://www.ijbs.com