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Inhibition of H3N2 Influenza Virus Induced Apoptosis by Selenium Nanoparticles with Chitosan through ROS-Mediated Signaling Pathways

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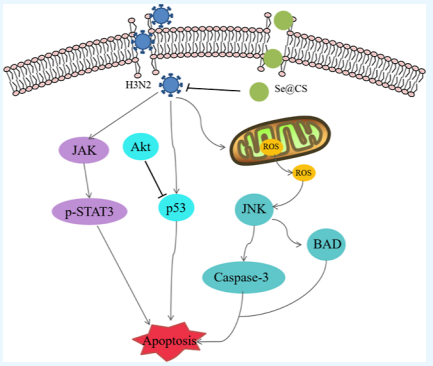
ABSTRACT: In recent years, nanotechnology has received more and more attention in the antiviral field. Among them, selenium nanoparticles (SeNPs) have received a lot of attention. Chitosan, as a substance with antiviral effect, is limited by water solubility, low bioavailability, and poor stability. In this study, the combination of SeNPs with chitosan (Se@CS) showed less toxic and good anti-H3N2 infection effect. CCK-8 and RT-PCR showed that Se@CS effectively prevented H3N2 infection of MDCK cells by inhibiting viral replication and preventing cell fragmentation and cell aggregation. In addition, Se@CS can inhibit the excessive production of ROS and the change of mitochondrial membrane potential. More importantly, Se@CS can inhibit the late apoptosis of cells caused by virus, which may be related to the inhibition of apoptotic proteins in the ROS/JNK apoptotic signaling pathway. Finally, Se@CS was also found to inhibit H3N2-induced inflammation and alleviate infection. These results prove that Se@CS is a promising inhibitor for controlling influenza H3N2 virus infection.



1. INTRODUCTION

Influenza virus is an important species of the *Orthomyxoviridae* family; it is classified by the difference of hemagglutinin (HA) and neuraminidase (NA).[1] The main influenza viruses prevalent in humans are type A and type B, of which type A influenza viruses are H1N1 and H3N2.[2] In 1986, a global pandemic caused by H3N2 killed more than one million people.[3] The death toll was not as alarming as the H1N1 influenza outbreak in 1981, but it also was significant enough.[4] Because of the seasonal nature and the frequent occurrence of antigenic drift of influenza, although there is a vaccine for influenza, influenza vaccines need to be constantly updated.[5] Because of the constant changes in HA and the surface glycoprotein of influenza, it is difficult to predict, which also makes the treatment and prevention of influenza not as effective as expected. Therefore, it is necessary to develop a drug against the influenza virus infection.

Chitosan is the main derivative of chitin, which is converted from chitin by enzymatic or chemical processes.[6] It is produced by partial deacetylation of chitin under an alkaline condition and is structured as residue 1 of β-(1,4)-2-amino-2-deoxy-D-glucopyranose and *N*-acetyl-D-glucosamine.[7] The characteristics of chitosan are biocompatibility, cytocompatibility, bioabsorbability, and biodegradability, so it has been studied in many biomedical fields, such as drug delivery,[8] gene delivery,[9] and transfection and immune adjuvant.[10] Recently, numerous studies have also demonstrated the antibacterial and antioxidant properties of chitosan and its derivatives.[11]

Nanotechnology has been developing rapidly in the past decade, involving a wide range of fields, such as cosmetics,[12] food and agriculture,[13] cell-membrane-coating nanotechnology,[14] drug delivery,[15] and so on. Selenium is a trace element required by the human body and has a positive trend in the diagnosis and treatment of many diseases.[16] In the study by Guillin et al., it was found that selenium has antioxidant effects and maintains redox homeostasis mainly through the regulation of selenoproteins.[17] Numerous studies have found that the combination of nanoparticles and selenium to form selenium nanoparticles is well applied in the biomedical field. SeNPs have good biocompatibility and can effectively target drugs to specific cells, increasing efficacy. SeNPs also has a wide range of applications.[18] It was proved to have the least toxic effect on normal cells in the application of cancer, and a significant anti-cancer effect was observed.[19] Rehman et al. demonstrated the ability of selenium nanoparticles to resist oxidative stress-mediated inflammation, thus exhibiting potent anti-rheumatic effects.[20] Cong et al. found that selenium nanoparticles are effective in alleviating neurological damage and motor dysfunction caused by Huntington's disease.[21]

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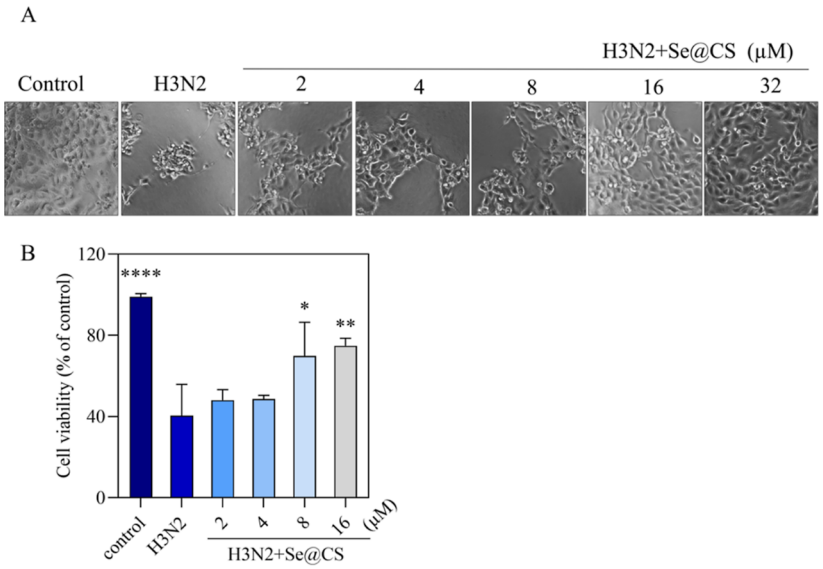

Figure 1. Antiviral effect of chitosan was detected by CCK-8 assay. (A) Morphological changes of MDCK cells infected with H3N2 and after Se@CS treatment were observed by contrast microscopy. (B) Effect of Se@CS on the growth of MDCK cells infected with H3N2 was determined by CCK-8 assay. The titer of H3N2 was 100 $TCID_{50}$/0.1 mL. MDCK cells were infected with H3N2 for 2 h and then treated with chitosan at the corresponding concentration for 48 h. Comparisons between groups were made with H3N2 groups. Bars with different characters are statistically different at the $*p < 0.05$ or $**p < 0.01$ or $****p < 0.0001$ level.

Apoptosis is a highly regulated programmed cell death. Apoptosis is divided into two main pathways, extrinsic and intrinsic pathways. The extrinsic apoptotic pathway is mainly caused by receptor mediated apoptosis, and the intrinsic apoptotic pathway is caused by non-receptor mediated and mitochondrial regulation.[22] It is well known that the main function of mitochondria is oxidative phosphorylation (OXPHOS) to provide energy to the cell and in parallel with OXPHOS, ROS is produced.[23] During influenza virus infection, ROS production always increases.[24] The effects of ROS and mitochondria are mutual. On the one hand, an excess of ROS disrupts antioxidant defenses in cellular compartments, leading to mitochondrial damage.[25,26] On the other hand, decreasing in mitochondrial membrane potential due to mitochondrial membrane damage would in turn stimulate ROS production and excessive ROS can bring about oxidative damage to important biomolecules such as proteins, lipids, and nucleic acids.[27] Excessive ROS also activates ROS-mediated JNK signaling pathway, activates caspase-3, and causes apoptosis.[28] Stopping viral infection by inhibiting ROS production may be a direction for treatment. On the other side, influenza virus infection can induce the host innate immune response and release large amounts of inflammatory factors through a variety of regulation mechanisms.[29] Therefore, in addition to inhibiting the influenza virus, the treatment of influenza virus can also start from inhibiting the virus-induced apoptosis and inhibiting the inflammatory response.

In this study, surface-modified SeNPs by chitosan are used to improve the antiviral benefits of chitosan and for better access to the cell.[30] SeNPs as a carrier can help chitosan to better resist certain viral infections by improving physical properties mentioned. The aim of this study is to investigate whether Se@CS have anti-H3N2 virus activity and the underlying mechanism.

2. MATERIALS AND METHODS

2.1. Materials. Na_2SeO_3, vitamin C, chitosan, propidium iodide, and 6-coumarin were purchased from Sigma. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were gained from Gibco. Thiazolyl blue tetrazolium bromide (MTT) and lyso tracker were used from Sigma in the study. JNK, p53, caspase-3, BAD, JAK, p-STAT3, Akt, and β-actin monoclonal antibodies were obtained from Cell Signaling Technology (CST). Madin-Darby canine kidney cells (MDCK) were obtained from American Type Culture Collection (ATCC CCL-34). H3N2 influenza virus was provided by Virus laboratory, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University.

2.2. Determination of Cell Viability. MDCK cells were grown in 96-well plates for 24 h, incubated with H3N2 for 2 h, and grouped with 2, 4, 8, and 16 μM Se@CS for 48 h. Then, the CCK-8 reagent was added for 1 h and observed at 540 nm absorbance with an enzyme-labeled instrument. The cytotoxicity and antiviral effects of Se@CS were detected by reflecting the cell survival rate.

2.3. Fluorescence Real-Time PCR. Viral replication was detected by RT-PCR. H3N2 was added to MDCK cells grown for 24 h in 6-well plates and incubated for 2 h, followed by the addition of 8 and 16 μM Se@CS for 48 h.

2.4. Detection of Intracellular Cytokines. Centrifuge the cells (3000 rpm, 10 min) and then collect the supernatant to remove cell debris. Add a mixture containing 20 μL of the bead mixture, 20 μL of the sample solution, and 20 μL of the assay antibody mixture incubated for a 96-well filter plate and for 2 h at 500 rpm in the dark environment. Then, 20 μL of PE-labeled *Streptomyces*-plant cyanate (SA-PE) solution is put in the wells for 30 min (500 rpm) at room temperature. Lastly, the corresponding fluorescence is measured by flow cytometry and the level of intracellular cytokines in the sample is obtained by analyzing the fluorescence intensity of the immune complex; fluorescence data were collected using BD FACS

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Diva software and intracellular cytokine levels were evaluated using FCAP Array 3.0 analysis software.[31]

2.5. Annexin-V Assay. The apoptosis of MDCK cells was detected by fluorescein isothiocyanate (FITC)-conjugated annexin-V. After treating H3N2-infected MDCK cells with 16 μM Se@CS for 48 h, the cells were fixed with 70% ethanol at 4 °C for 6 h, and then, apoptotic cells were detected by the Annexin-V kit. The apoptosis rate was detected by flow cytometry.

2.6. Measurement of Mitochondrial Membrane Potential by JC-1 Fluorescence. Mix MDCK cells in a 6-well plate with 1 mL of JC-1 solution and incubate in an incubator at 37 °C for 20 min; then capture the fluorescence image under a fluorescence microscope.[32]

2.7. Determination of ROS Generation. Se@CS inhibits ROS production in MDCK cells infected with H3N2. Collect MDCK cells by centrifugation and then add them to PBS containing 10 mM DCFH-DA. ROS levels were measured at wavelengths of 500 nm (excitation wavelength) and 529 nm (radiation wavelengths) for 20 min at 37 °C with a fluorescence microscope.[33]

2.8. Western Blot. MDCK cells were grown to 80% on 10 cm dishes, incubated with H3N2 for 2 h, and then treated with 16 μM Se@CS for 48 h. Total protein was extracted, and the protein concentration was determined using the BCA kit. After denaturation at 95 °C for 10 min, the protein samples were separated by 10% SDS-PAGE gel and transferred to a difluoride film in polyvinylidene. Polyvinylidene difluoride membranes were sealed with 10% BSA for 2 h and incubated overnight with primary antibodies including JNK, p53, caspase-3, BAD, JAK, p-STAT3, Akt, and β-actin antibodies. Then, the membrane was washed with TBST and incubated with the secondary antibody for 2 h. After the membrane was fully mixed with luminescent solution, the protein band images were exposed and preserved with a gel imager, and the exposure process should be carried out without light.

2.9. Statistical Analysis. All data are expressed as mean ± standard deviation. Multiple groups were compared using one-way analysis of variance (ANOVA). $P < 0.05$ (*) or $P < 0.01$ (**) were considered statistically significant different.

3. RESULTS AND DISCUSSION

3.1. Se@CS's Results of CCK-8. The toxicity and antiviral effects of the drug were detected by CCK-8 assay. According to the results of drug toxicity results, Se@CS was not toxic at 2, 4, 8, 16, and 32 μM, compared with controls. As shown in Figure 1B, the cell viability of MDCK infected with H3N2 decreased to 40%. After treatment with Se@CS, there was a significant raise in the two concentration groups of 8, 16 μM, compared with the H3N2 group. Figure 1A shows that the MDCK infected with H3N2 under the phase contrast microscope could not see the cell morphology, shriveled cytoplasm, clustered cells, and reduced cell numbers. After different concentrations of Se@CS treatment, the cell morphology gradually returned to normal. These results showed that Se@CS had some anti-H3N2 effect and could inhibit the morphological changes of cells induced by H3N2.

3.2. Inhibition of Viral Replication by Se@CS. The RT-PCR experiment was used to test whether Se@CS inhibited the replication of H3N2. Figure 2 shows that the RNA content of H3N2 was clearly higher than that of the H3N2 + Se@CS group, and the RNA content of 16 μM H3N2+Se@CS group was further reduced than that of 8 μM H3N2 + Se@CS. Thus, the results showed that Se@CS can effectively inhibit H3N2 replication.

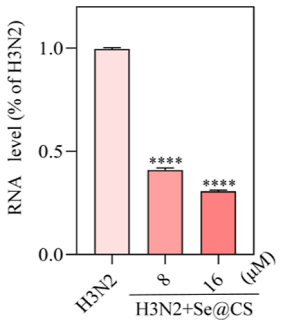

Figure 2. Se@CS inhibited the replication of H3N2. The inhibitory effect was detected by RT-PCR. Comparisons between groups were made with H3N2 groups. Bars with different characters are statistically different at the ****$p < 0.0001$ level.

3.3. Cytokine Release. The effect of Se@CS on H3N2-mediated inflammation was investigated by flow cytometry assay, which showed that H3N2 infection has led to an increase in TNF-β, IL-6, IL-8, IL-4, and IL-5(Figure 3A–E). As shown in Figure 3A–E, compared with the control group, the expression levels of TNF-β, IL-6, IL-8, IL-4, and IL-5 in the H3N2 group were increased, indicating that these cytokines were significantly activated as inflammatory mediators during H3N2 infection. After treatment with Se@CS, it can be seen that there is some decrease in the expression of these cytokines. Overall, these results suggest that Se@CS inhibits the inflammatory reaction caused by H3N2 infection.

3.4. Effect of Se@CS in Virus-Induced Apoptosis. Annexin V-FITC was used to study the effect of H3N2 on apoptosis and Se@CS. The experiment was divided into four groups: control group, H3N2 group, Se@CS group, and H3N2 + Se@CS group. In Figure 4, compared with the control group, the percentages of late apoptotic cells in the H3N2 group increased to 7.8%, while the percentages of viable cells decreased to 77.6%. However, after Se@CS intervention, the percentages of late apoptotic cells increased to 0.3%, and the percentages of viable cells increased to 94.8%. This experiment indicated that Se@CS inhibited H3N2-induced late apoptosis of MDCK cells.

3.5. Changes of Mitochondrial Membrane Potential by Se@CS. The mechanisms of apoptosis are associated with the decrease of mitochondrial membrane potential. The JC-1 fluorescent probe experiment was conducted to detect the change of membrane potential of MDCK under different treatments. The experiment was divided into four groups: control group, H3N2 group, Se@CS group, and H3N2 + Se@CS group. As shown in Figure 5, the amount of fluorescence of depolarized monomers in the H3N2-infected group was clearly increased compared with the control group. After addition of Se@CS, depolarizing monomers were significantly reduced and red fluorescence increased. These results mean that Se@CS could inhibit cell apoptosis by inhibiting H3N2-induced changes in mitochondrial membrane potential. The decrease in mitochondrial membrane potential leads to the production and accumulation of ROS, which is associated with an imbalance in REDOX.[34] Excessive ROS not only can cause damage to nucleic acids and proteins in cells[35] but also can cause damage to the JNK pathway and activate caspase-3, leading to apoptosis.[36]

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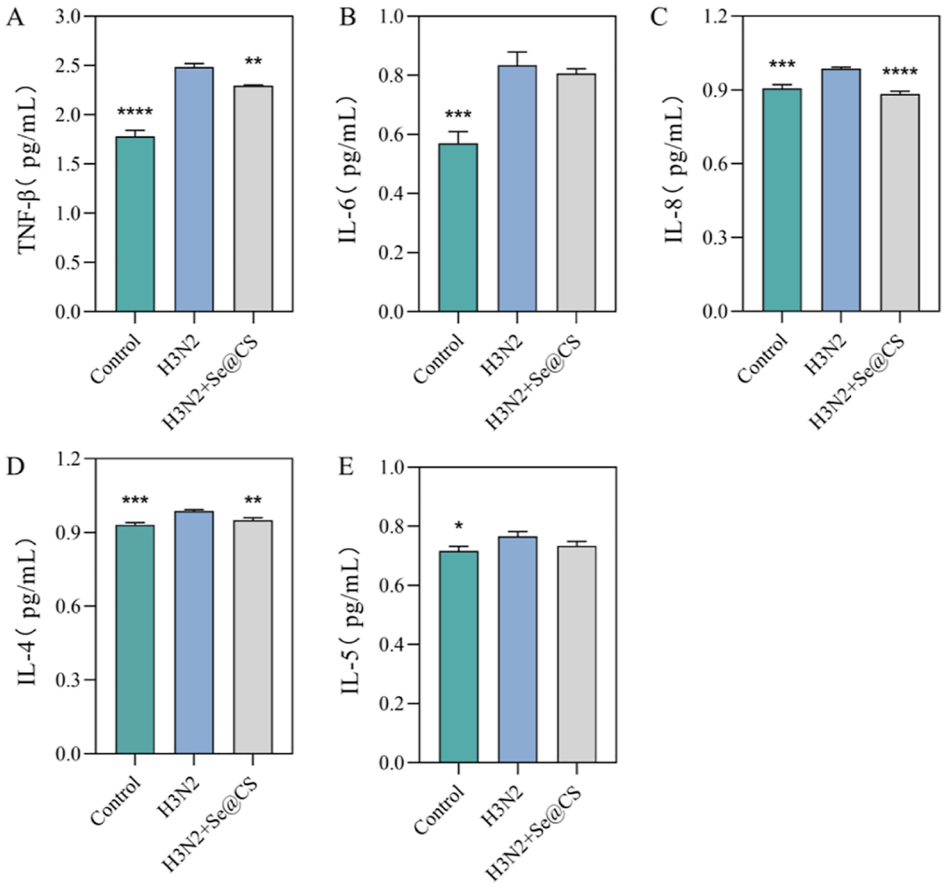

Figure 3. Se@CS inhibited H3N2-mediated release of inflammatory cytokines. (A) TNF-β of MDCK cells. (B) IL-6 of MDCK cells. (C) IL-8 of MDCK cells. (D) IL-4 of MDCK cells. (E) IL-5 of MDCK cells. All MDCK cells after 16 μM Se@CS treatment were detected by flow cytometry. The titer of H3N2 was 100 $TCID_{50}$/0.1 mL. Comparisons between groups were made with H3N2 groups. Bars with different characters are statistically different at the *p < 0.05 or **p < 0.01 or ****p < 0.0001 level.

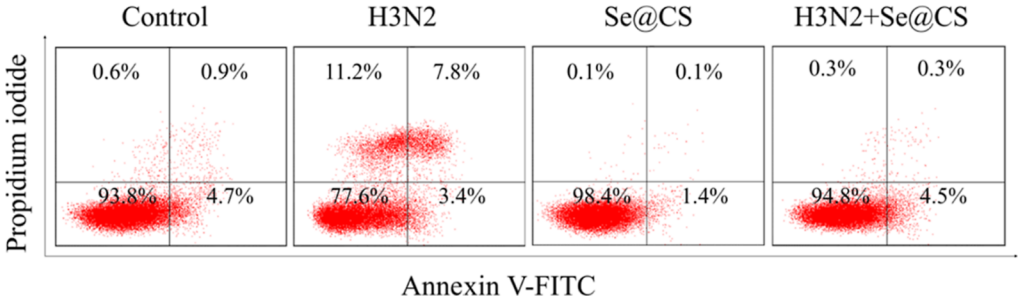

Figure 4. Effect of different treatments on H3N2-mediated apoptosis by annexin V-FITC staining. MDCK cells were infected with H3N2 for 2 h and then treated with 16 μM Se@CS for 48 h.

3.6. Inhibition of ROS Production Mediated by H3N2 by Se@CS. The decrease in mitochondrial membrane potential caused by influenza virus leads to the accumulation of ROS and further fragmentation of nucleic acids. The effect of H3N2 on ROS was assessed by ROS kits. As shown in Figure 6, under the fluorescence microscope, the green fluorescence of the H3N2 infection group was very distinct compared with the control group. However, the addition of Se@CS treatment significantly weakened the green fluorescence. These results displayed that Se@CS inhibited ROS production induced by H3N2.

3.7. Se@CS Mediated Signaling Pathways. The mechanism of nano-chitosan on apoptosis was studied. As shown in Figure 7, H3N2 viral infection increased the expression of JNK, P53, caspase-3, and BAD and decreased the expression of JAK, p-STAT3, and Akt. After Se@CS treatment, in contrast to the virus group, the expressions of JNK and P53 were decreased, and the expressions of p-STAT3 and Akt were increased. It is worth noting that the expressions of caspase-3 and BAD were similar to those of the normal group, thus revealing that Se@CS inhibited H3N2-induced cell apoptosis by down-regulating the ROS-mediated JNK signal pathway. β-Actin was an internal reference for protein expression. The study of influenza virus-induced apoptosis by western blotting assay revealed that influenza virus induces apoptosis mainly by inducing the accumulation of ROS and activating the ROS-mediated ROS/JNK signaling cascade. This experiment illustrates that nanosized chitosan blocks

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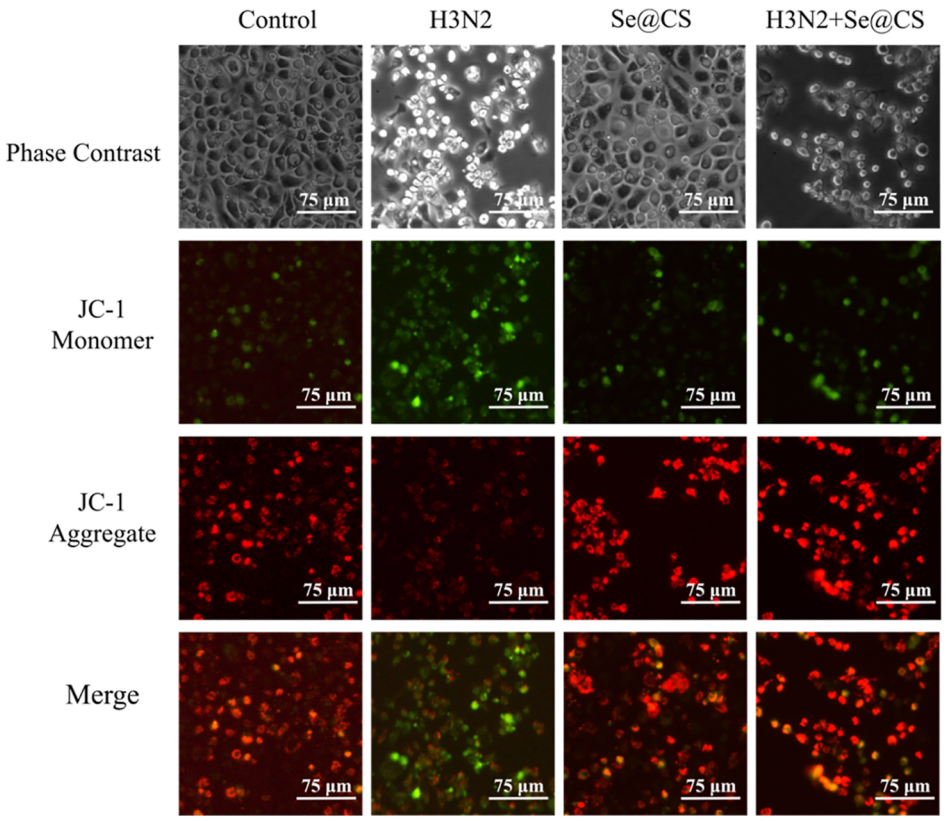

Figure 5. Changes of mitochondria membrane potential under different treatments were detected by the JC-1 kit. The titer of H3N2 was 100 $TCID_{50}$/0.1 mL. MDCK cells were infected with H3N2 for 2 h and then treated with 16 μM Se@CS for 48 h.

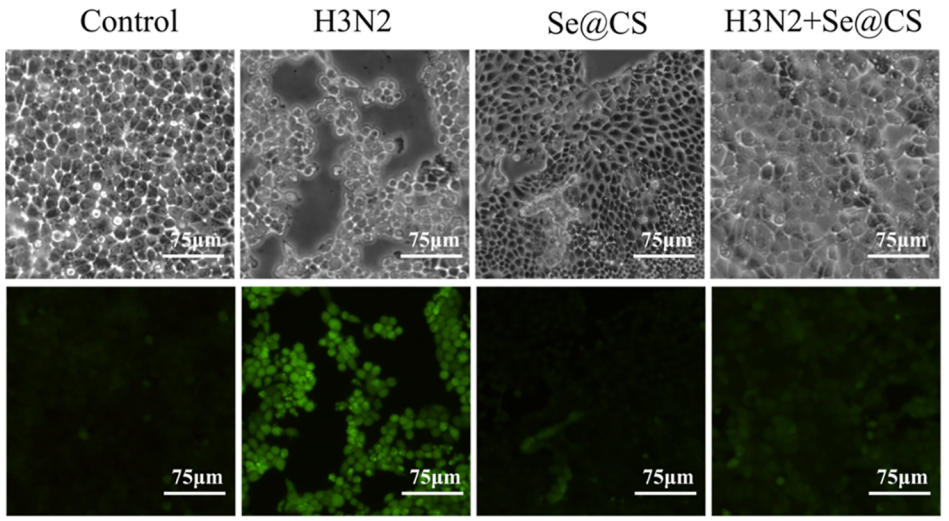

Figure 6. Se@CS inhibited ROS production in H3N2 infection of MDCK cells. MDCK cells were infected with H3N2 for 2 h, preincubated with 10 μM DCF for 30 min, and then treated with 16 μM Se@CS.

apoptosis mainly by inhibiting ROS production. p53 is a positively related protein that regulates apoptosis.[37] The JAK/STAT signaling pathway is a widely expressed intracellular signal transduction pathway associated with cell growth, apoptosis, and immune regulation.[38] We also found that the H3N2 virus could decrease the JAK and P-STAT3 proteins, while the expression of JAK and P-START was significantly increased in the Se@CS group. Therefore, we suspected that the influenza virus might also go through the JAK-START pathway to control cells.

4. CONCLUSIONS

In this study, we demonstrated the effectiveness and mechanism of nanoselenium-loaded chitosan against H3N2 virus. It can inhibit the replication of H3N2 influenza. The mechanism is that by inhibiting ROS production, regulating apoptotic proteins affects the ROS/JNK pathway, alleviating the decrease in mitochondrial membrane potential and reducing mitochondrial damage. In addition, Se@CS can inhibit the overproduction of inflammatory cytokines in infected virus cells and reduce the inflammatory response

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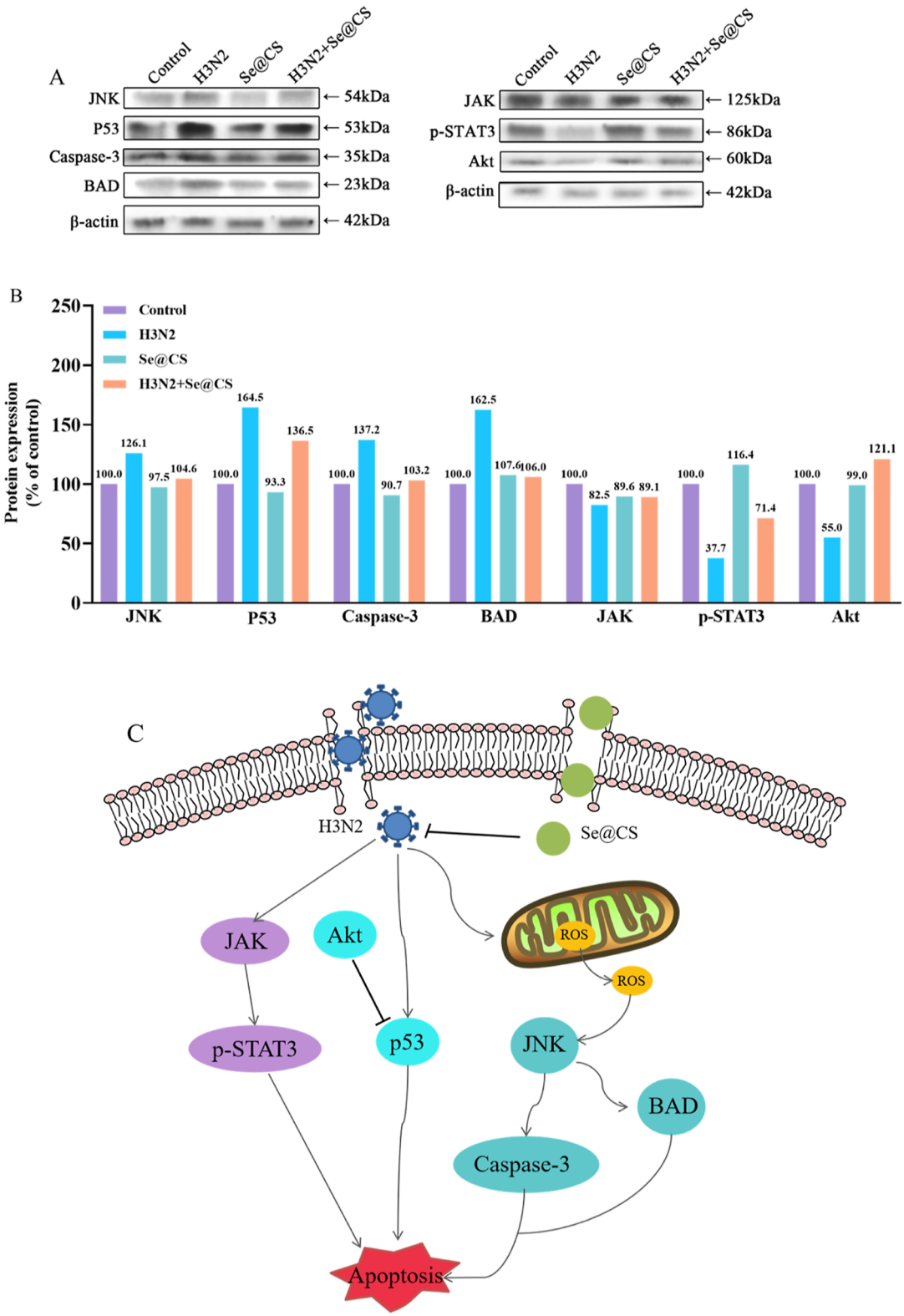

Figure 7. Signaling pathway participated in the inhibition of H3N2 by Se@CS. (A) Regulation of apoptotic proteins. (B) Content of each apoptotic protein. (C) Scheme of apoptosis signaling pathways. The titer of H3N2 was 100 $TCID_{50}$/0.1 mL. MDCK cells were infected with H3N2 for 2 h and then treated with 16 μM Se@CS for 48 h.

caused by H3N2 infection. However, the anti H3N2 influenza mechanism of Se@CS still needs to be investigated, as well as its influence on inflammatory response and flu-mediated signal pathway of apoptosis. In conclusion, Se@CS is a promising antiviral drug.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07575.

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Original western blots of JNK, caspase-3, BAD, β-actin, JAK, p-STAT3, Akt, and β-actin (PDF)

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Author Contributions

†T.X. and J.L. contributed equally to the work. T.X. and J.L. designed the study, analyzed the experimental data, and drafted the manuscript. J.S. and D.C. carried out the experiments. M.Z. analyzed the data and drafted the manuscript. Y.L. and B.Z. refined the manuscript and coordination. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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