Selenium Nanoparticles Inhibited H1N1 Influenza Virus Induced Apoptosis By Improving The Level of Gpx1

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Abstract

Influenza A (H1N1) viruses are distributed around the world and pose a threat to public health. Vaccination is the main treatment strategy to prevent influenza infection, but antiviral drugs also play an important role in controlling seasonal and pandemic influenza. Currently, influenza viruses may emerge antiviral resistance, new agents with different modes of action are being investigated. Recently, selenium nanoparticles (SeNPs) which have antiviral effects attracted more and more attention in biomedical interventions. The appearance of nanotechnology attract great attention in the nanomedicine field. SeNPs constitute an attractive vector platform for delivering a variety of drugs to action targets. SeNPs is being explored for potential therapeutic efficacy in a variety of oxidative stress and inflammation-mediated diseases, such as cancer, arthritis, diabetes, and kidney disease. SeNPs could inhibit infection of Madin Darby Canine Kidney (MDCK) cells with H1N1 and prevent chromatin condensation and DNA fragmentation. ROS play a key role in physiological processes on apoptosis. SeNPs significantly inhibited the production of reactive oxygen species (ROS) in MDCK cells. Mechanistic investigation revealed that SeNPs inhibited the apoptosis induced by H1N1 virus infection in MDCK cells by improving the level of GPx1. Our results suggest that SeNPs is an effective selenium source to obtain H1N1 influenza antiviral candidate.

Introduction

Influenza A (H1N1) viruses are distributed globally and pose a threat to public health [1]. Now, H1N1 continues to spread around the world. The great pandemic occurred in 2009 have caused great social panic and economic losses. It is significant to strengthen and coordinate preventive activities at a global level to control the spread of the virus [2]. Swines play a key role in the transmission of this virus to humans as a natural host and mixing carrier of influenza A (H1N1) virus. Furthermore, swine influenza A (H1N1) viruses have provided all eight genes or some genes to the genomes of influenza strains that have historically caused human pandemics. Therefore, continuous surveillance of influenza A (H1N1) virus in swine herds contributes to help prevent and control this virus [3]. Influenza A virus infections occur in different species, including pigs, causing mild to severe respiratory symptoms that lead to a significant burden of disease. Hence, pigs are thought to be a genetic mixing vessel for humans and avian influenza viruses. Classical H1N1 swine influenza viruses (SIVs) cause sporadic zoonotic infections, while recombinant swine-origin influenza A H1N1 virus (A(H1N1)pdm09) caused human influenza pandemic in 2009 [4].

So far, vaccination is still considered the best way to control infection. Fortunately, in August and September 2009, a vaccine against H1N1 was reported. However, it is important to note that, this vaccine is not suitable for children and people who are allergic to eggs [2]. In addition, between the rapid evolution of the virus and vaccine development restrain the spread of influenza infections is the long period [5]. These synthetic drugs have led to the emergence of drug-resistant strains, which cause trouble to humans [6]. The research and development of antiviral drugs is very important. Selenium (Se) is an essential trace element in human body [7-9]. It is very important physiological process and involving
many physiological functions and its biological functions are mainly manifested in low molecular Se compounds and Se-containing proteins [10, 11]. It is incorporated into selenoproteins as selenocysteine (Sec) representing the most important part of the active center of their enzymatic activities [12]. Se controls several key biological processes, such as elimination of reactive oxygen species (ROS) and the regulation of specific enzyme [13-15]. Selenium deficiency can lead to susceptibility to infections, including respiratory viruses [16].

Nanotechnology has been used to study the mechanisms of disease and treatment options in the past three decades [17, 18]. Many kinds of nanostructures, including polymers, dendrimers, liposomes, metal nanoparticles (Ag, Au, Ce, Cu, Eu, Fe, Se, Ti, Y, etc.), silicon and carbon based nanomaterials have been used as successful therapeutic agents and drug delivery carriers [18]. Nanoparticles have unique features such as small size, large surface area, surface charge, surface chemistry, solubility and versatility [19]. Among these nanoparticles, selenium nanoparticles (SeNPs) are one of the most extensively studied. Because Se is toxic and has a narrow margin of safety. The use of Se in the form of nanoparticles has basically solved the toxicological concerns associated with Se. SeNPs has attractive anticancer activity and reduced toxicity [18]. As demonstrated by mechanisms investigation, SeNPs inhibited H1N1 influenza virus from infecting MDCK cells through inducing apoptosis via suppression AKT and p53 signaling pathways [20]. This study was to verify selenium nanoparticles inhibited H1N1 influenza virus induced apoptosis through improving the level of GPx1.

**Materials And Methods**

**Materials**

Madin Darby Canine Kidney cells (MDCK) were used in the current study. All cells were purchased from ATCC and cultured according to the ATCC recommendations. They were cultured with serum-free minimum essential medium (MEM; Gibcol, USA) at 37°C incubator [21]. Na₂SeO₃, vitamin C, MTT, DAPI, DCF-DA were purchased from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM) and Fetal bovine serum (FBS) were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). H1N1 influenza virus was provided by Guangzhou Women and Children's Medical Center of Guangzhou Medical University.

**Preparation and Characterization of SeNPs**

Selenium nanoparticles (SeNPs), a new form of selenium, have attracted worldwide attention because of their biological activity and low toxicity. The main factors determining its efficiency are the size, stability and surface properties of the nanomaterial. SeNPs was prepared according to the method reported in the literature [22]. Briefly, the 0.1 M Na₂SeO₃ was freshly prepared with ultrapure water. 0.25 ml stock solution of Na₂SeO₃ (0.1 M) was gradually added into 2 ml stock solution (50 mM) of Vitamin C. Finally, the solution was dialyzed in the dark at for overnight. Its purpose is to remove superfluous Vitamin and Na₂SeO₃. SeNPs was sonicated and then filtered through 0.2 µm pore size. 5 µL of samples were dripped
onto a copper grid which is coated with a holey carbon film to dry and observed by transmission electron microscopy. The TEM images were taken on H-7650 (Hitachi, Tokyo, Japan) performed at 80 kV. Use Nova Nano scanning electron microscope 430 (FEI, Hillsboro, OR, USA) equipped with EDX. SEM micrographs and elemental composition of SeNPs can be obtained by using the Oxford system [23]. The particle size distribution, mean diameter and Zeta potential of SeNPs were measured by using Zetasizer Nano ZS90 particle analyzer (Malvern, Worcestershire, UK).

Determination of cell viability

As described in the references, the cytotoxicity of SeNPs was assessed by cell viability. The anti-proliferative activity of SeNPs in vitro was explored by the MTT reduction method [24]. MDCK cells were inoculated on a 96-well plate for 24 h in an incubator at 37°C, 5% CO₂. MDCK cells were added with H1N1 influenza virus for 2 h, and then the indicated concentrations of SeNPs were added to MDCK cells for 24 h. 20 µL/well MTT in serum-free medium was added to each well and let stand for 5 h. Dissolve Formazan crystals by adding 150 µL/well dimethyl sulfoxide. Absorbance values were recorded at 570 nm using a VersaMax ELISA Microplate Reader (Molecular Devices, San Jose, CA, USA) [25]. Cell viability was conducted using MTT reduction method, detect the cell proliferation and investigate the antivirus activity of SeNPs.

Detection of mitochondrial membrane potential (ΔΨm)

Jc-1 is a perfect fluorescent probe to detect mitochondrial membrane potential ΔΨm. The fluorescence intensity from JC-1 monomers was used to estimate the status of ΔΨm in MDCK cells exposed to SeNPs [26]. As mentioned above, Plasma membrane alterations was detected in MDCK cells treated with SeNPs. Cells were trypsinized and resuspended in PBS buffer with 10 µg/mL of JC-1. Then incubated at 37°C for 30 min. The MDCK cells were then collected by centrifugation, resuspended in PBS and analyzed by flow cytometry [27]. JC-1 fluorescence was measured with excitation (485 nm) and dual emission (shift from green at 530 nm to red at 590 nm) [28].

TUNEL-DAPI co-staining assay

The effect of SeNPs on DNA fragmentation was detected with fluorescence staining by the TUNEL apoptosis kit as described above. MDCK cells were confirmed with TUNEL for 1 h and incubated with DAPI for 15 min, and nuclear staining was performed at 37°C.

Caspase-3 activity

Colorimetric assay kit was used to conduct the enzymatic activity of caspase-3. In brief, MDCK cells were treated with SeNPs for 48 h, and then lysed in cell lysis buffer for 1 h. After centrifugation, 50 µL of cell lysate was mixed with 50 µL of reaction buffer, and then added to the 96-well plate. Then, 5 µL of the caspase substrate was added into each well and the plate was incubated at 37°C in the dark for 1 h. The absorbance values were measured at 380 nm (excitation) and 460 nm (emission) and caspase activity
was expressed as fold change compared to the control group [29]. Then we will use TUNEL apoptosis detection kit was used to detect DNA fragmentation with fluorescence staining. MDCK cells were confirmed with TUNEL for 1 h and incubated of DAPI for 15 min at 37°C for nuclear staining.

Determination of reactive oxygen species

Mitochondrion is a significant part of intracellular ROS production. The overexpression of ROS causes damage to mitochondrial ATP synthesis and leads to mitochondrial dysfunction which further induces cell apoptosis [30]. ROS play a key role in physiological processes on apoptosis [31-34]. Fluorescence intensity analysis of DCF with 500 nm excitation and 529 nm emission to mitochondria ROS levels [35].

Statistical analysis

All the data are presented as mean ± SD. One-way analysis of variance (ANOVA) was used in multiple group comparisons. Difference with $P<0.05$ (*) or $P<0.01$ (**) was considered statistically significant.

Results And Discussion

Preparation and Characterization of SeNPs The optical images and Tyndall effect of SeNPs were as shown in Figure 1A and 1B, the results indicate that SeNPs nanoparticles were composited. The morphology of SeNPs presented spherical particles in Figure 1C. As shown in Figure 1D, energy-dispersive X-ray spectroscopy indicated the signal of C (18%), the percentage of Se atoms was 82%. As shown in Figure 1E and 1F, SeNPs was 200 nm and the zeta potential of SeNPs (-16.9 mv). Antiviral of SeNPs MTT assay was used to detect the effects of virus and SeNPs on MDCK cells. Figure 2A shows the morphology of the cells in the corresponding dose-adding group, the figure that the morphology of the cells infected with the virus alone significantly changed. When cells treated with H1N1, they showed cytoplasmic shrinkage, loss of cell contract, and reduction in cell numbers. With the increase of the concentration of SeNPs, the virus-infected cells were effectively inhibited, and appeared healthy, with regularity in shape. Figure 3B shows that after virus infection, the cell survival rate was 34.5%, which decreased significantly compared with the control group. After adding different concentrations of nano-selenium, the survival rate of cells infected with virus was 40.2% and 65.4%, respectively increased compared with the control group. When the SeNPs concentration reached 0.25 μM, the survival rate increased to 82.5%. The results showed SeNPs effectively inhibited H1N1 influenza virus replication. Depletion of mitochondrial membrane potential ($\Delta \Psi_m$) induced by SeNPs Increase in intracellular and mitochondrial ROS can cause oxidative stress and lead to the loss of mitochondrial membrane potential. As shown in Figure 3, MDCK cells treated with H1N1 virus showed increased mitochondrial depolarization and dysfunction. Compared to virus-infected group, when MDCK cells were exposed to SeNPs, the percentage of mitochondrial membrane potential was increased significantly. The mitochondrial membrane potential of virus group is 27%, and the SeNPs treated group markedly dropped to 53% (0.0625 μM), 69% (0.125 μM) and 89% (0.25 μM), compared with the virus group. It can be concluded that SeNPs inhibited H1N1 influenza virus through apoptotic by inducting mitochondrial dysfunction in MDCK cells. Inhibition of H1N1 infection of MDCK cells by SeNPs TUNEL staining is the terminal transferase
marker, which can accurately reflect the biochemical and morphological characteristics of apoptotic cells. As shown in Figure 4, MDCK cells exhibited apoptotic characteristics, DNA fragmentation and nuclear condensation under the action of H1N1 influenza virus. However, treatment with SeNPs significantly prevented the H1N1-induced nuclear morphology changes. It can be concluded from the figure, SeNPs rescues the apoptosis of MDCK induced cells by H1N1. Detection of Caspase-3 activity Apoptosis induced during H1N1 virus infection. Apoptosis was detected by inhibiting caspase-3 activation. In the research of this paper, MDCK cells were treated with different concentrations of SeNPs after H1N1 virus infection. Then activity of caspase-3 was detected by Enzyme standard instrument. As was reflected in Figure 5, caspase-3 activity of the control group in which cells were uninfected was 100%, however, caspase-3 activity in untreated H1N1 virus-infected cells was 501%. MDCK cells activity remarkably increased after treatment with H1N1 influenza virus. While the SeNPs treated group markedly dropped to 382% (0.0625 μM), 260% (0.125 μM) and 160% (0.25 μM), compared with the virus group. These results suggested that H1N1 virus may induce apoptosis through caspase-3 and be effectively suppressed by SeNPs. Inhibition of apoptosis signaling pathways by SeNPs As shown in Figure 6, the MDCK cells were treated with SeNPs, and Gpx1 activity was detected. The concentrations of SeNPs were 0.0625 μM, 0.125 μM and 0.25 μM. In the virus group alone, the activity of Gpx1 is 24.5%. With the addition of SeNPs, the activity of Gpx1 gradually increases to 35.6%, 56.7% and 78.5%, respectively. Compared with the virus-infected group, the activity of Gpx1 in MDCK cells treated with SeNPs was increased and correlated with the concentration of SeNPs. It can be seen from the figure that H1N1 influenza virus can significantly reduce the activity of Gpx1, while SeNPs can increase the activity of Gpx1. It can be concluded from the figure, SeNPs may inhibit the proliferation of H1N1 influenza virus by increasing the activity of Gpx1. Ultimately leads to DNA damage by regulating apoptosis signaling pathway. The ROS production was detected by DCF-DA to indicate the action mechanisms of SeNPs. After treatment with H1N1, intracellular ROS production increased to 470%, as shown in Figure 7A. However, MDCK cells infected with H1N1 were preincubated with 10 μM DCF for 30 min and then treated with SeNPs. The concentrations of SeNPs were 0.0625 μM, 0.125 μM and 0.25 μM. The intracellular ROS generation were 362% (0.0625 μM), 280% (0.125 μM) and 160% (0.25 μM). The higher the concentration of SeNPs, the more significant the reduction rate of ROS genes. As shown in Figure 7B, the fluorescent intensity of DCF was found by H1N1 influenza virus. The fluorescent intensity of the treated with H1N1 influenza virus group was much stronger than SeNPs. These results show that SeNPs can effectively inhibit the production of ROS of H1N1 virus. In general, as shown in Figure 8, H1N1 infection induced intracellular apoptosis in MDCK cells and SeNPs inhibited the generation of apoptosis by upregulation of Gpx1 level in this study.

Conclusions

In conclusion, in this study, SeNPs have lower toxicity and exhibit superior antiviral abilities to restrain H1N1 influenza virus infection. The underlying mechanisms of antiviral indicated that SeNPs inhibited caspase-3 mediated MDCK cells apoptosis through improving regulate the level of Gpx1. In conclusion, this study elaborates that SeNPs has been widely used for functional research of antivirals and the
strategy to use SeNPs could be a highly efficient method to control H1N1 transmission, which is critical for the development of new antiviral drugs.

**Abbreviations**

DMEM: Dulbecco’s modified Eagle’s medium; FBS: fetal bovine serum; MTT: thiazolyl blue tetrazolium bromide; PBS: phosphate-buffered saline; SeNPs: selenium nanoparticles; TEM: transmission electron microscopy;

**Declarations**

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**Authors’ Contributions**

Xia Liu, Danyang Chen and Jingyao Su designed the study, analyzed the experimental data and drafted the manuscript. Ruilin Zheng and Zhihui Ning carried out the experiments. Mingqi Zhao analyzed the data. Bing Zhu and Yinghua Li refined the manuscript and coordination. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data are available on request.

**Competing interests**

The authors declare that they have no competing interest.

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**Figures**
Figure 1

Characterization of SeNPs. (A) and (B) Tyndall effect in SeNPs. (C) TEM images of SeNPs. (D) EDX analysis of SeNPs. (E) Size distribution of SeNPs. (F) Zeta potential of SeNPs.
Figure 2

Effects of SeNPs on the growth of H1N1 infection of MDCK cells by MTT assay. (A) Morphological changes in H1N1-infected MDCK cells observed by phase-contrast microscopy. (B) Antiviral activity of SeNPs. The concentrations of SeNPs were 0.0625 μM, 0.125 μM and 0.25 μM. Bars with different characters are statistically different at *p<0.05 or **p<0.01
Figure 3

The fluorescence intensity from JC-1 monomers was used to estimate the status of ΔΨm in MDCK cells exposed to SeNPs, then it analysed by flow cytometry. JC-1 fluorescence was measured with excitation (485 nm) and dual emission (shift from green at 530 nm to red at 590 nm). The concentrations of SeNPs were 0.0625 μM, 0.125 μM and 0.25 μM. Bars with different characters are statistically different at *p<0.05 or **p<0.01.
SeNPs induced apoptosis in H1N1 infection of MDCK cells. (A) The cell cycle distribution after different treatments was analyzed by quantifying DNA content using flow cytometric analysis. (B) DNA fragmentation and nuclear condensation as detected by TUNEL-DAPI costaining assay. All results were representative of three independent experiments.
Figure 5

Inhibition of caspase-3 activity by SeNPs. MDCK Cells were treated with SeNPs and caspase-3 activity was detected by synthetic fluorogenic substrate. Concentration of SeNPs was 0.0625 µM, 0.125 µM and 0.25 µM. Bars with different characters are statistically different at *p<0.05 or **p<0.01 level.
Figure 6

GPx1 activity of SeNPs. The MDCK cells were treated with SeNPs, and Gpx1 activity was detected. The concentrations of SeNPs were 0.0625 μM, 0.125 μM and 0.25 μM. Bars with different characters are statistically different at *p<0.05 or **p<0.01
Figure 7

ROS overproduction was inhibited by SeNPs. (A) ROS levels were detected by the DCF fluorescence intensity. (B) MDCK cells infected with H1N1 were preincubated with 10 μM DCF for 30 min and then treated with SeNPs. The concentrations of SeNPs were 0.0625 μM, 0.125 μM and 0.25 μM. Bars with different characters are statistically different at *p<0.05 or **p<0.01
Figure 8

Intracellular apoptotic by SeNPs in H1N1 infection of MDCK cells.