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Study of Virus Infection Procedure using Atomic Force Microscope

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***Abstract*—This work mainly uses AFM to study the procedure of virus infecting cells and the mechanism of action of antiviral drugs. Firstly, the change trend of mechanical properties during cell apoptosis was studied, including normal cells and infected one by virus. The procedure of adhesion change was from soft to hard and then soft, in the same experiment. The Young's modulus was also measured under the same condition. Then the effect of virus dose on the process of infecting cells was studied. By quantitative analysis of the trend of mechanical properties, it was found that the change rate of the mechanical properties would speed up with the number of viruses infecting cells increasing. The quantity increase of the virus will accelerate the process of cell apoptosis. Finally, the anti-viral drug Ribavirin inhibiting the process of virus infecting cells was studied by comparing the two designed experiments. The Ribavirin was found to modify the surface structure of the virus for inhibiting the virus infection.**

I. INTRODUCTION

The virus is a simple structure of microorganisms at nanoscale, which exist in human living environment [1] and can lead to global large-scale infectious diseases. Meanwhile, however, viruses can also be used as a very important carrier for treatment of diseases, such as adenoviral vectors carrying therapeutic genes into tumor cells, cleaving viral capsids, and releasing therapeutic genes to achieve therapeutic goals[2]. We recognize that viral membrane proteins are invaded by viruses such as HIV, adenoviruses, and herpes. In the process of virus infecting cells, these membrane proteins play a crucial role in mediating [3-6]. From the perspective of how to modify membrane protein structure of virus, the researchers studied how to effectively control the cell infection at the beginning. Therefore, detection of procedure of virus infecting cells has become one of the hotspots of scientific research today.

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As the smallest unit of living things, a single cell has the potential to reveal the mystery of life. The study of single cell can greatly supplement the shortcomings of traditional macro-cluster detection methods and generate new insights into cell biology [7]. The life system consists of molecules, cells, tissues and organs. The basic units of any scale have their unique heterogeneous structural, physical and chemical properties. These characteristics are constantly changing dynamically during life activities and are closely related to various physiological functions [8]. Among these characteristics, the mechanical properties of cells have proved to be a new biomarker. However, due to the lack of systematic understanding of the mechanical properties of cells after viral infection [9], and the lack of mechanical properties characterization of virus-infected cells, the further study on the mechanism of action of viruses and cells is hindered. Also further research on the mechanism of interaction the development of antiviral drugs and gene therapy methods has limited. By understanding the changes of mechanical properties of individual cells before and after infection, people can be more deeply aware of the key factors that affects the virus invasion of cells.

At present, the main methods for studying virus infection of cells include Optical Tweezer (OT), Dielectrophoresis (DEP) and AFM robotic nano-manipulation. The OT method is used to fix the host cells and study the changes of the physiological characteristics of the cells after infection by a single virus [10,11]. The DEP method is to concentrate multiple viruses into one area using an applied electric field, and then the OT method is used to transfer a single virus to the cell surface for studying the infection process [12]. In the above procedure of manipulating viruses and infecting cells, the experimental environment is subject to more factors of random disturbance, and the uncertainty in measurement and manipulation significantly increases. At the same time, the virus needs to be driven and controlled in the micrometer-scale aperture, and the control precision is not accurate. Compared with OT, DEP and other methods, AFM Micro/Nano-manipulation has a unique mechanical force mechanism and can be used to study the structure and properties of biological samples under physiological conditions at Micro/Nano scale.

In the past few decades, AFM has obtained a huge success in single-cell observation and manipulation for biomedical applications, which illustrates that AFM has excellent capabilities in solving biological problems. AFM has unprecedented spatial and temporal resolution

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in single-cell imaging [13], while AFM manipulation has multiple imaging modes compared to OT, DEP, etc. [14], particularly when imaging under Peak Force mode. This work mode can make the probe tip have tiny force on the sample, do not damage the surface structure of the sample, and obtain information such as the shape profile, elastic modulus and adhesion of the sample.

In this work, the advantages of AFM imaging and manipulation are mainly adopted to study the process of virus infecting cells and the action mechanism of antiviral drugs. Firstly, we studied the general trend of change in mechanical properties during cell apoptosis, which includes two parts. One part is that the process of adhesion changing during normal cell apoptosis is from hard to soft, and the other part is that cell adhesion after infection gradually softens when it go through a process from soft to hard. Then, in order to study the effect of virus dose on the process of infecting cells and the degree of mechanical properties changing, the change trend of mechanical properties during virus infection of cells were quantitatively analyzed. According to the experimental results, it is found that the increase in the number of viruses infecting cells raises the rate of change in mechanical properties, and the increase in the dose of the virus accelerates the process of cell apoptosis.

Finally, the two experiments are contrastively performed to studying inhibition process of the anti-viral drug Ribavirin, and found that the surface structure of the virus is modified by the Ribavirin, and furtherly inhibit the virus infection

II. Materials And Methods

A. Cell Culture

293 cells (human normal renal epithelial cells), purchased from the Chinese Academy of Sciences Cell Bank (Shanghai), cell culture medium was purchased from Hyclone Corporation of the United States. 293 cells were cultured at 37 ° C (5% CO 2) containing 90% high sugar medium, 10% fetal bovine serum and 1% double antibody. The cells were cultured in a petri dish. When the cells were spread to about 80% of the bottom of the dish, they were passaged and cultured for 2 to 3 days before the experiment.

B. AFM Imaging

The experiment was performed on a Bioscope Catalyst AFM (Bruker, USA). AFM was used in conjunction with an inverted microscope (Ti, Nikon, Japan). The tip material was silicon nitride, cantilever beam elasticity. The coefficient is 0.08N/m (Bruker). The imaging environment is medium, and the imaging mode chooses Peak Force Tipping mode. The scanning frequency is 0.5 Hz. The scanning line is 256, and the sampling point of each scanning line is 256.

C. AFM Cell Mechanical Properties Measurement

The mechanical properties of normal 293 cells and 293 cells infected with virus were measured in the medium by AFM indentation test. The thermal coefficient method was used to accurately correct the elastic coefficient of the cantilever beam [15]. Under the guidance of the optical microscope, the tip movement was controlled. Go to the center of the cell (Fig. 1). Then control the tip to measure the elasticity and adhesion of the cells in the approximation-retraction mode [16]. The force curve is recorded by the AFM control software, and the adhesion and Young's modulus between the cells and the tip is extracted from the force curve. Cells are randomly selected during force curve measurement and measured near the center of the cell. Each cell is measured continuously for about 10 times.

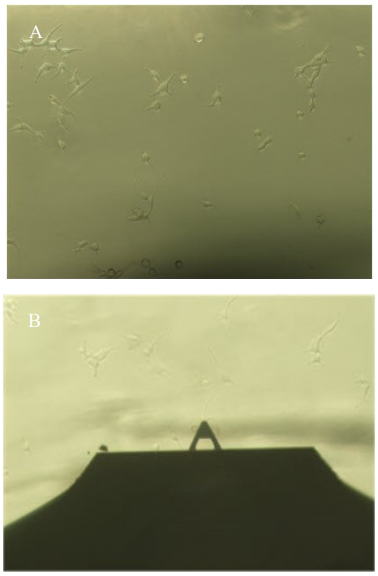

Fig.1. A is a normal adherent cell, and B is an approximation-retraction operation of the cell using the tip.

III. Data Analysis

The data analysis consists of two parts, one of which is to obtain the adhesion between the cell and the substrate, and another one is the Young's modulus of the cell. The force curve contains the approximation curve and the regression curve. The approximation curve is used to calculate the cell Young's modulus ,and the regression curve is used to analyze the adhesion between the tip and the sample [17], as shown in fig2:

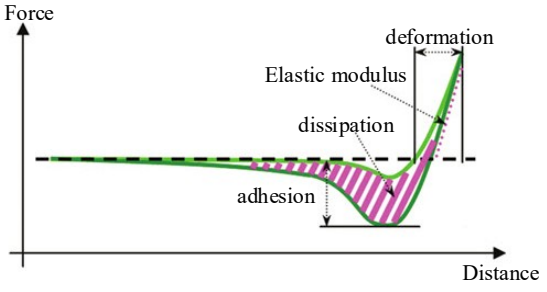

Fig.2. Force-distance curve

A single cell cannot be considered as a thick and elastic half space because its internal structure is composed of materials with different mechanical

properties. The cell cytoskeleton consists of elastic rigid fibers with hundreds of megapascals or even thousands of gigapascals surrounded by viscous cytoplasm. Although single-cell mechanical property modeling is evolving, Hertzian contact theory still dominates in single-cell elastic analysis. It has been developed by Sneddon[18]

The approximating curve is extracted from the force curve by the Hertz-Sneddon model [18]. The Hertz model is suitable for spherical tip, and the Sneddon model is suitable for conical tip. This work uses scan-fluid tip for image scanning and force. Curve measurement

$$F(\delta) = \frac{2\tan\alpha \cdot E'}{\pi}\delta^2 \tag{1}$$

Where δ is indentation depth and α is half open angle of tapered tip, E' is the falling value of Young's modulus and F is the loading force.

$$E' = \frac{E_c}{1-\nu_c^2} \tag{2}$$

Where ν_c is the cell Poisson's ratio(Cells are usually considered as incompressible materials [19,20]),therefore ν_c=0.5 and E_c is Young's modulus Calculated according to Hooke's law.

$$F = k \cdot x \tag{3}$$

Where k is the Cantilever beam spring constant and x is the Cantilever beam deflection(it can be obtained directly from the original force curve) The approximation curve is converted to an indentation curve based on the contact point on the approximation curve (indicated by the black arrow in Fig 2A) (the indentation depth is equal to the tip vertical position change minus the tip cantilever deflection)

The Hertz-Sneddon model is used to fit the indentation curve to obtain the Young's modulus of the cell. The Fig. 2 is the force curve obtained by the cone tip, the Fig 3 is the Sneddon fitting result of the indentation curve of Fig. 2., so it can be utilized that the Sneddon model is fitted. It can be seen that the theoretical curve fit the experimental indentation curve.

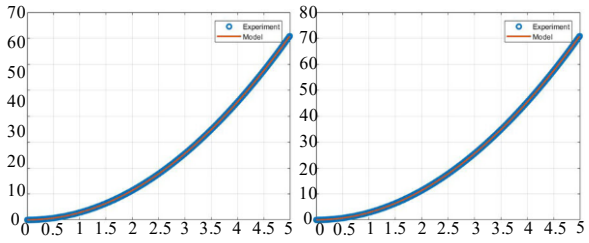

Fig.3. It shows the Young's modulus results fitted by the Sneddon model.

IV. Experiment Procedure And Result Analysis:

It mainly consists of three experiments. The first one is to measure the force curve of normal 293 cells and image the cells as shown in Fig 4. The second one is to infect the normal 293 cells by adding different doses of virus, and image the surface of the cell surface. The size of scanning is 1μml, the scanning frequency is 0.5Hz, and the force curve is obtained from the time of the cells are infected to apoptosis. And the last one, the normal 293 cells are added with virus and antiviral drugs (Ribavirin spray)by two ways, the first way is to add the virus directly into the cell culture dish, add 30mg Ribavirin after put it into the incubator 2h; and re-culture in the incubator again, the second way is to mix the virus with 30mg Ribavirin for 10 minutes, add it in the cell culture dish, place it in the incubator to culture, and observe the both of them by fluorescence microscope. The main steps are as follows:

A. Preparation:

The 293 cells with good culture were taken out from the CO2 incubator and divided into three groups (1), (2) and (3), of which group (3) served as the control group; Take out three well-cultured 293 cells from the CO2 incubator, place them in the clean bench after spraying the alcohol;

Absorb the original culture solution, add it in 2ml PBS, add 4.5ml complete medium, add cells according to different MOI (add 2μml virus in (1), add 5μml virus in (2), mix and put 37 ° C incubator infecting for 2h;

After 2 hours, the original culture solution containing the virus was aspirated and observed with a fluorescence microscope. The photograph was taken as Fig 5. In the Fig 5, The image on the right side of the figure showed no fluorescence reaction and did not successfully infect the cells within two hours after the virus was added.

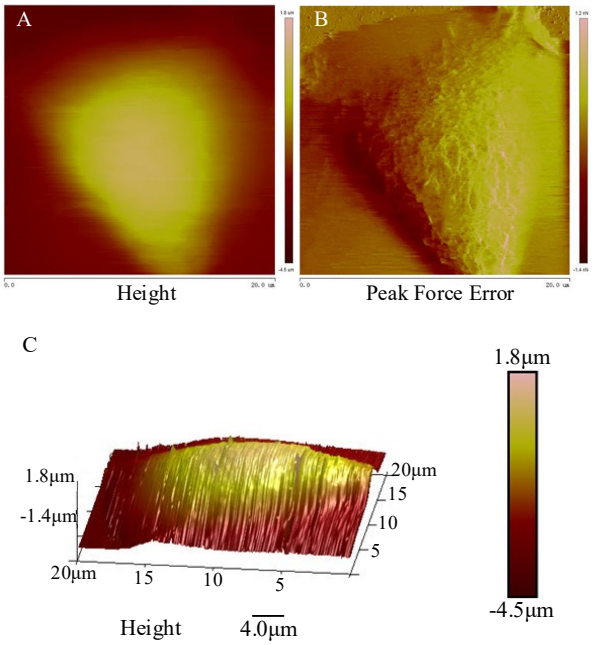

Fig.4. A shows normal cell height information, B shows peak force error, C is a three-dimensional map

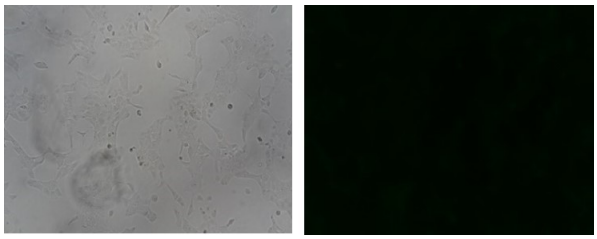

Fig.5. The left picture shows the bright field only after 2 hours of infection, and the right picture shows the closed field only under fluorescent illumination.

B. Virus imaging

As can be seen from the figure below, after two hours of virus addition, the cells were not infected. After the fresh culture medium was changed, the virus adhered to the cell surface, and the virus image was scanned by catlast as shown in the Fig 6.

Adenovirus is a non-encapsulated spherical structure, and its virions are often arranged in a lattice in the infected nucleus. The scanning position selects the cell near the edge, and the scanning range is 1 μm, as shown in the Fig 6, the virus diameter is about 50-60 nm.

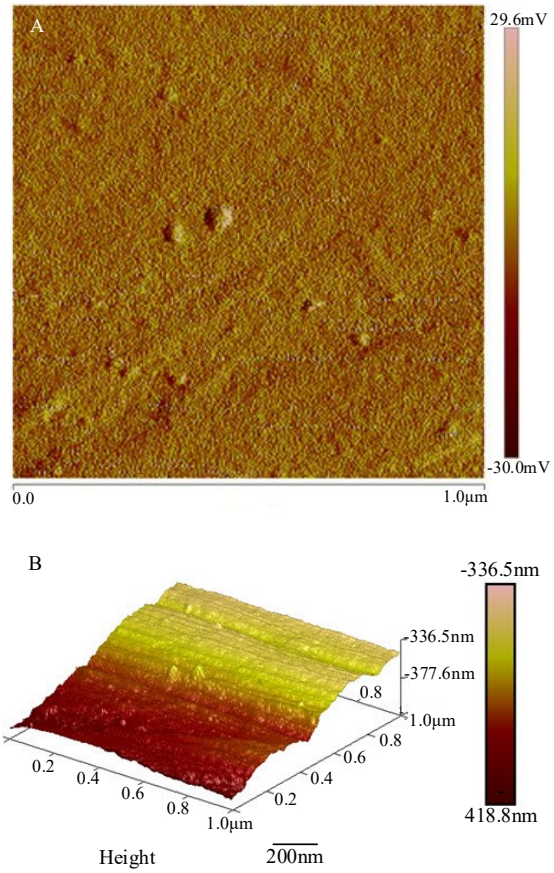

Fig.6. The above picture shows a partial scan of the cell. The scan range is 1 square micron. The cell surface virus can be observed in the figure, and its diameter is 50-60 nm. A shows the peak force error and the B shows the three-dimensional map.

C. The cells are completely infected

After the experimental sample was incubated in the incubator for 2 hours, the fresh medium was replaced and re-cultured in a 37° C incubator for 16 hours. Cells are taken by fluorescence microscope and the results is shown in the Fig 7.

After being infected for 16 hours, each group of 293 cells was infected completely that they were added with 2μml and 5μml virus (upper and lower respectively 2μml and 5μml part).

Passing the infested sample for subculture and providing experimental samples for subsequent observation of the infection process;

Take out the sample to be tested, the force curve is measured with an atomic force microscope, and the Young's modulus and adhesion of the cells are analyzed according to the approximate curve and the contraction curve, An eight-hour measurement is taken, with each measurement being approximately two hours apart.

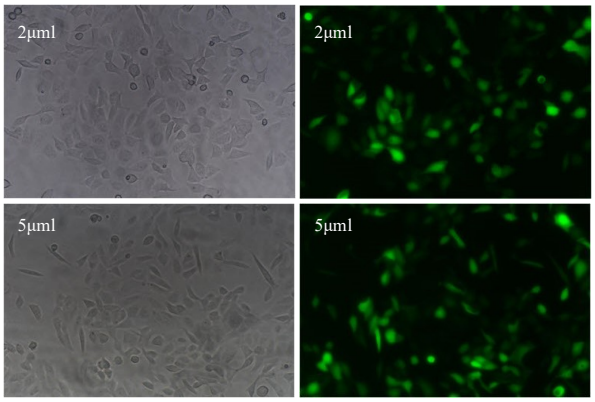

Fig.7. Different amounts of virus were added to the two groups of cells, and the results were taken with a fluorescence microscope after 16 hours.

1) *Adhesion calculation*

Randomly select 5 different cells every 2 hours to measure the force curve. Each cell receives 10-12 force curves and continuously measures for 8 hours. Where the abscissa represents the measured force, the ordinate represents the number of occurrences, and the image is Gaussian fitted to obtain the mean.

As shown in Fig. 8, the result is 2μml, which approximates the Gaussian distribution, and the mean value of Gaussian fitting for 2μml and 5μml respectively is shown in Fig. 9.

From the distribution of mean force in the figure, after 293 cells were infected by adenovirus, the change of adhesion within 8 hours reflected the changing trend of cells from soft to hard and turn back soft, and the number of virus infected also affects the speed of the change process, that is, the more the number of virus doses, the faster the changing process.

Since the experimental sample is still cultured in the incubator without measuring force curve and it has been infected. It will undergo a process from normal cells to apoptosis, and the apoptosis time in the incubator can be used as a reference time, using this method can eliminate the influence of some external factors.

In the next 8 hours, the cells gradually become apoptotic. The infected cells are marked by the cells not

attached to the surface of the medium, eventually, the cells are completely floating.

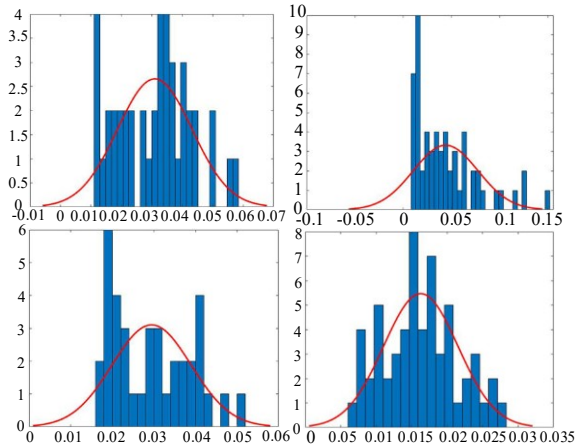

Fig.8. Gaussian fitting results of four different time periods of 2μml experimental samples

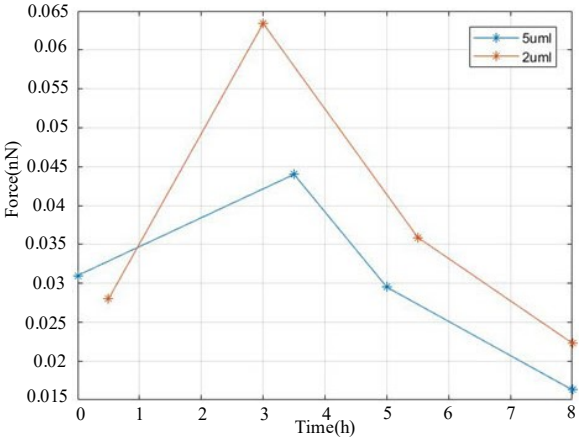

Fig.9. Mean distribution map of 2μml and 5μml fitting results

2) *Young's modulus calculation*

In order to verify that the Young's modulus is consistent with the changing trend of adhesion, a similar experiment is added with the former, that is, 5 μml of virus is added to the same batch of normal 293 cells for infecting, and after 16 hours, it had completely infected, continuously measured force curve for 9.5 hours, each interval is about 2 hours, and at the corresponding time point, each cell selects five consecutive force curves, and the Hertz-Sneddon model is used to fit the indentation curve, each cell has 5 Young's modulus, and randomly select 5 different cells at each time point, that is, 25 results at a single time point. Since the timing is continuous, the same cell fitting results are similar, the results of the 25 experiments were averaged, it is shown in TABLE I, and the trend of the results is also consistent with the trend of adhesion.

TABLE I. YOUNG'S MODULUS FITTING RESULT

Young's modulus	2.50	1.86	3.45	2.10	1.49	0.98
Time(h)	0	1.5	3.5	5.5	7.5	9.5

3) *Drug inhibition*

The mediated process of virus-infecting cells mainly has two stages. The virus is added during the process of virus infection, and the detection of changes in mechanical properties cannot directly reflect which of the mediated processes of virus-infecting cells. The stage plays a role in inhibition (reducing the binding force or inhibiting the cleavage of the capsid). Therefore, two infected modes were designed to infect the cells. The first one was to add 5μml virus to the cell culture dish and put it into the incubator for two hours. Add 30mg of the drug, mix it and put it into the incubator again. The second way is to mix 30mg of the drug with 5μml virus for 10 minutes, then add it to the cell culture dish for infection, and add another cell culture with only 5μml virus. The dish was tested as a control and the results are shown in Fig 11.

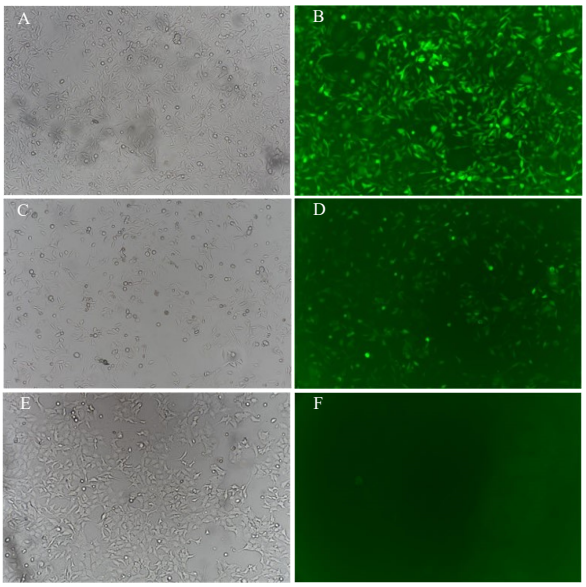

Fig.11. A and B are the results of the experiment after adding only the virus. C and D are the results of adding the virus to the culture dish for 2 hours, and the results of E and F are mixed the virus with the drug, and then added to the culture dish.

After 16 hours, the results of cells showed that in the sample with only virus added, the cells were completely infected. Only the second method in the two ways can inhibit the virus, that is, the surface structure of the virus is modified by the Ribavirin, and furtherly inhibit the virus infection.

V. CONCLUSION:

Single cell research has been hindered due to the uncertainties in the AFM system, the influence of the external environment such as room temperature, and the unstable state of different batches of cells. Therefore, this work mainly adopts the advantages of AFM imaging and manipulation for studying the whole cell apoptosis process of the same batch under the same experimental environment. And the force curve was measured in the approximation-retraction mode. It was found that the

cell adhesion of infected cells by viruses changes from small to large, and then gradually reduces. Similarly, Young's modulus of the infected cell also indicates a this process, which represents softening process of the cells at initial stage, and then changing from soft to hard. Then quantitative experiments are on virus infecting cells, the analysis is performed to obtain the result that increasing the dose of the virus in an appropriate amount can accelerate the progress of cell apoptosis.

Finally, the antiviral drug Ribavirin is exerted on the cells at different stages of virus infecting cells. The conclusion is obtained that Ribavirin can modify the surface structure of the virus for inhibiting the infection of the virus.

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