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Comparison of antiviral activity of realgar and nano-realgar against herpes simplex virus type II (HSV-2) *in vitro*

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In traditional folk prescriptions, realgar, as a traditional Chinese medicine, has been widely used to treat herpes caused by virus. To compare the antiviral activity of realgar and nano-realgar against herpes simplex virus type Ⅱ(HSV-2) *in vitro*, the Vero cells model of HSV-2 infection was established, and cytotoxicity of realgar and nano-realgar on Vero cells were determined by the cell counting kit-8 (CCK8) in this study. Subsequently, the antiviral effects of realgar and nano-realgar on infected cells were also evaluated by CCK8 method under the three modes including prevention, treatment and direct inactivation. The results showed that the 50% cytotoxic concentration (CC_{50}) of realgar and nano-realgar on Vero were 18.75 μg/mL and 150 μg/mL, respectively. In the preventive mode, 50% effective concentration (EC_{50}) of realgar on HSV-2 infected cells was 10.19 μg/mL, while nano-realgar had an EC_{50} value of 2.26 μg/mL for infected cells. Under treatment mode, the EC_{50} value of realgar and nano-realgar on infected cells were 3.94 μg/mL and 1.13 μg/mL, respectively. In the direct inactivation mode, the EC_{50} of realgar on infected cells was 10.23 μg/mL, while the EC_{50} value of nano-realgar on infected cells was 2.92 μg/mL. In conclusion , the cytotoxicity of nano-realgar was lower than realgar's. Both realgar and nano-realgar can play a good antiviral activity on HSV-2 infected cells in the three ways of prevention, treatment and direct inactivation modes. Meanwhile, in prevention, treatment and direct inactivation modes, nano-realgar has an EC_{50} value lower than that realgar on HSV-2 infected cells, so the anti-HSV-2 efficacy of nano-realgar on infected cells is better than realgar's antiviral efficacy in three modes.

Keywords: Realgar, nano-realgar, herpes simplex virus type II (HSV-2), antiviral

INTRODUCTION

There is increasing awareness of the importance of the skin infection disease caused by herpes simplex virus type II (HSV-2). Furthermore, several of emerging rare cases [1, 2] were found continuously in recent years, which indicated an upward trend in their incidence. HSV-2 can typically cause serious afflictions in a significant proportion of individuals, mainly because of the generation of genital lesions and severe infections like life-threatening encephalitis and disseminated infections in neonates [3-5]. Additionally, HSV-2 infection also significantly increased the risk of the host to acquire HIV [6]. Clinically, the most common drugs used for treating viral infection are acyclovir (ACV) and its derivatives. With the wide use of them, the disadvantages of narrow antiviral spectrum, drug resistance and high costs were more concerned, because of its huge burden on people's life [7-9]. Beyond that, the reserves shortage of ACV had been also extremely concerned in America [10]. Besides, no effective HSV-2 vaccine is available until now in the world. For these reasons, there is a need for the development of novel antiherpes drugs which are safe and preferably inexpensive with limiting the primary infection and supporting further treatment.

Realgar, as a traditional Chinese medicine, has been used for treating diseases more than many years owning a wide range of sources and has good antitumor, antibacterial, antiviral effects, and so on [11-14]. The main component of realgar is As_4S_4, with disadvantages of large particles, low bioavailability, and insolubility in water [15]. In order to increase the solubility of realgar, enhance antiviral efficacy and reduce toxicity, nano-realgar whose average particle size was about 72.79 nm was therefore gifted by Li huijie, a teacher from shandong traditional Chinese medicine university [16, 17]. The existing clinical data shown that, as the main drug in the prescription, realgar had a significant effect on herpetic lesions appearing on the human surface [18]. But until now, there is no

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relevant experimental study on separately realgar anti-HSV-2 activity. Therefore, the anti-HSV-2 activities of realgar and nano-realgar were compared to elucidate their activity more clearly in this study. Cell culture technology was used to investigate the antiviral activities of realgar and nano-realgar against HSV-2 *in vitro*, which can provide a theoretical basis for clinical herpes treatment and the invention of an antiviral drug that is widely available and cheap enough for Chinese people.

EXPERIMENTAL

Cell lines, virus strain, traditional chinese medicine and reagents

African green monkey kidney cells (Vero) and HSV-2 virus G strain, kindly donated by prof.X.A WU from department of pathogenic microorganism of airforce medical university. Monkey kidney cells Vero were incubated under dulbecco-modified eagle's medium (DMEM, high glucose) with 10% fetal bovine serum (FBS) at 37℃ in atmosphere containing 5% CO_2 in CO_2 incubator (KZX00016904, obtained from Thermo). HSV-2 virus G strain were grown for 3 ~ 4 days on Vero cells in an atmosphere of 5% CO_2 at 37℃, and the virus stock solution was stored at -80℃ until use.

Realgar was purchased from shaanxi Conway pharmaceutical co.LTD, and was milled to nanometer level, also known as nano-realgar, by longmai fine grinding machine (Model: LVM-80WE) of shandong longmai technology development co.LTD. And the particles size and size distribution of nano-realgar were determined by the photon-related nano-laser particle size analyzer (Model: Winner801) of jinan micro-nano particle instrument co.LTD. The results showed that the average particle size of nano-realgar was 72.79 nm, which met the requirements of nano-drug preparation [16,17]. Nano-realgar was donated by Li huijie, a teacher from shandong traditional Chinese medicine university.

Crystal violet is purchased from xi'an yongyi biotechnology co.LTD. Sodium carboxymethyl cellulose was purchased from Sigma-Aldrich. All of the high glucose DMEM medium, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) and cells counting kit-8 (CCK8) were obtained from shanghai sangon bioengineering co.LTD. Ultra-clean bench (SW-CJ-2F) was purchased from Shanghai xinmiao medical equipment manufacturing co.LTD. Other instruments and equipment are as follows: LABGARD series biosafety cabinet (Nuair, America), Inverted microscope (EKY0014477; OLYMPUS-CKX31), Full wavelength microenzymelabeling apparatus (BioTek).

Cytotoxicity determination of realgar and nano-realgar on Vero cells

The cytotoxicity of both realgar and nano-realgar were determined by cell counting CCK-8 kit-8 [19]. Vero cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured in DMEM with 10% FBS overnight. The medium was then removed and the cells were washed twice with phosphate buffered saline (PBS). Realgar and nano-realgar at various concentrations (200, 150, 100, 50, 25, 12.5, 6.25, 3.13, 1.57, 0.78, 0.39, 0 μg/mL) were added to individual well of Vero cells severally in plates and the plates were incubated 24 h under 37℃ and 5% CO_2. Cells treated without the realgar and nano-realgar were used as control. After incubation for 24 hours at 37℃, the supernatant medium of each well was replaced with 100 μL DMEM, then 10 μL CCK-8 solution was added to the cells, and cells were cultured for 4 h avoiding light. The absorbance (A) of each well was measured at 450 nm using BioTek synergy 2 microplate reader. The cell viability was calculated using following formula. Cell viability (%) = (A_s – A_b) / (A_c – A_b) × 100%, where A_s and A_c refer to the absorbance in the presence and absence of realgar or nano-realgar, respectively, and A_b stands for blank control. GraphPad Prism 7.0 software was used to calculate the 50 % cytotoxic concentration (CC_{50}) of realgar and nano-realgar on Vero according to bliss principle.

Virus infection and titer determination

The HSV-2 virus stocks were generated by infecting Vero cells monolayer for 2 ~ 3 days in the culture flask, then the infected cells were not lysed by three freeze-thaw cycles between -80℃ and room temperature until the cytopathic effect (CPE) up to 90%. The viral lysates, also was called the first generation viral supernatant, were collected and stored at -80℃. A total of five generations viral supernatant were obtained through the above method. The titer of the primary purified HSV-2 virus and the fifth generation viral supernatant on Vero cells were determined by plaque assay [20]. Vero cell monolayer in 6-wells plates was infected with HSV-2 virus at multiple dilutions (10^{-1}, 10^{-2},

10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) and incubated at 37℃with 5% CO_2 for 2 h. The infected cell monolayer was then overlaid with overlapping solution containing 2% carboxymethyl-cellulose sodium salt. After 4 days, cell monolayer was washed three times with PBS and strained with 1% crystal violet solution. Plaques were counted and plaque formation units (PFUs/mL) were calculated as $\bar{x}$ / (n × v) × d, where $\bar{x}$, n, v and d refer to the average numbers of plaques, repetitive holes numbers, viral load and dilution factor, respectively.

Antiviral activity of realgar and nano-realgar against HSV-2 under prevention, treatment and direct inactivation modes

In prevention, treatment and direct inactivation assays, the antiviral activities of realgar and nano-realgar on HSV-2 infected cells were assessed by the CCK-8 assay combined with cytopathic observation. For treatment assay, Vero cells cultured in 96-well plates were first infected with HSV-2 at 100 multiple tissue culture infective dose (100 $TCID_{50}$) for 2 h to allow viral attachment. Following 2 hours' incubation, the realgar or nano-realgar with indicated concentrations (20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0 μg/mL) were added to the infected Vero cells, of which cells without treatment of realgar or nano-realgar was used as a virus control group. Following incubation, the supernatant medium of each well was not replaced with 100 μL DMEM until the virus control group showed 80 % ~ 90 % cytopathic effects, then 10 μL CCK-8 solution was also added to the cells, and cells were cultured for 4 h avoiding light. The absorbance (A) of each well was measured at 450 nm using BioTek synergy 2 microplate reader. The antiviral activity of realgar or nano-realgar on HSV-2 infected cells was calculated using following formula. Antiviral activity (%) = (A_s - A_v) / (A_c - A_v) × 100%, where A_s and A_c refer to the absorbance in the presence and absence of realgar or nano-realgar, and A_v stands for the absorbance of virus control group, respectively. GraphPad Prism 7.0 software was used to calculate the 50 % effective concentration (EC_{50}) of realgar and nano-realgar on HSV-2 infected Vero cells. For cellular prevention assay, Vero cells monolayer was first treated with realgar or nano-realgar at different concentrations for 2 h at 37 ℃ before HSV-2 infection at 100$TCID_{50}$ for CCK-8 assay combined with cytopathic observation. For viral direct inactivation assay, the HSV-2 virus suspernatant and realgar/ nano-realgar at indicated concentrations were added to the Vero cell monolayer, simultaneously. The infected cell monolayer was co-cultured with HSV-2 and realgar/ nano-realgar for CCK-8 assay combined with cytopathic observation.

Cell morphology changes

When Vero cells were cultured to a nearly monolayer state at 37℃ in atmosphere containing 5 % CO_2, the original culture medium was discarded and the HSV-2 virus solution was added to cells for 2 h to allow viral attachment. After 2 hours' incubation, the HSV-2 virus solution was replaced with the maintenance media (DMEM supplemented with 2 % FBS). The state of the Vero cells were observed and photographed under optical microscope 20× magnification using Inverted microscope (EKY0014477; OLYMPUS-CKX31) under a visible light.

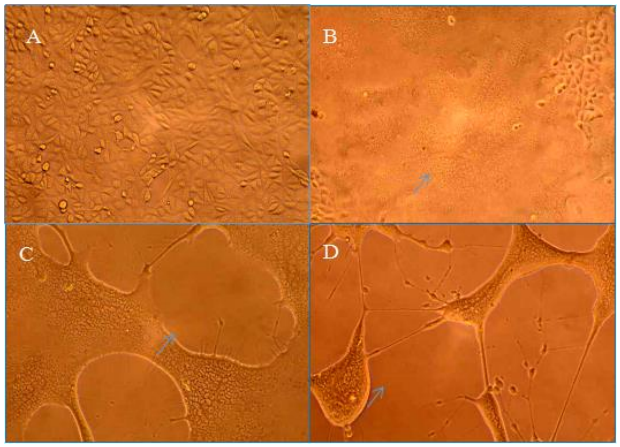

Fig.1. The morphological changes of normal Vero cells and Vero cells infected with HSV-2 (× 20). A. The normal Vero cells, cells were closely arranged with fusiform morphology and intact cytomembrane, and the cell membranes were clearly visible with a strong refractive index; B. Vero cells infected with HSV-2 for 12 h, the cytomembranes of cells were blurred ; C. Vero cells infected with HSV-2 for 24 h, multiple cells were gradually merged to form a multinucleated giant cell; D. Vero cells infected with HSV-2 for 30 h, multiple cells were gradually merged to form a multinucleated giant cell.

RESULTS AND DISCUSSIONS

Morphological changes of Vero cells after HSV-2 infection

Morphological changes of the cells can be seen in Fig.1. Normal Vero cells were closely arranged with fusiform morphology and intact

cytomembrane, and the cell membranes were clearly visible with a strong refractive index (Fig.1-A). However, the cytomembranes of Vero cells infected with HSV-2 for 12 h were blurred (Fig.1-B), and multiple cells infected with HSV-2 for 24 h were gradually merged to form a multinucleated giant cell (Fig.1-C), which multiple cells were gradually merged to form a multinucleated giant cell in 30 h (Fig.1-D).

Suspension degree of Nano-realgar is superior to realgar in DMSO solution

Realgar can be suspended in DMSO, and the suspension is dark brown. Realgar tended to settle rapidly to the bottom of the eppendorf (EP) tube, resulting in solution delamination in Fig.2A. However, nano-realgar can be completely suspended in DMSO solution. The solution of nano-realgar in DMSO solution is pale yellow and its sedimentation rate was extremely slow in Fig.2B. These phenomena illustrated that suspension degree of nano-realgar is superior to realgar in DMSO solution.

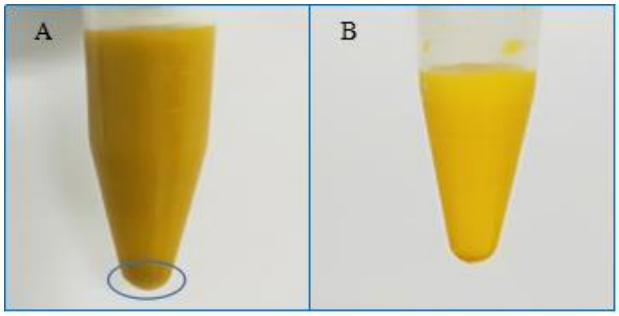

Fig.2. The appearance of realgar and nano-realgar suspensions. A. Realgar suspension; B. Nano-realgar suspensión

The cytotoxicity of nano-realgar on Vero was lower than realgar's

In order to evaluate the usability of realgar and nano-realgar on the Vero cells, their cytotoxicity on cells were determined by CCK-8 assay.

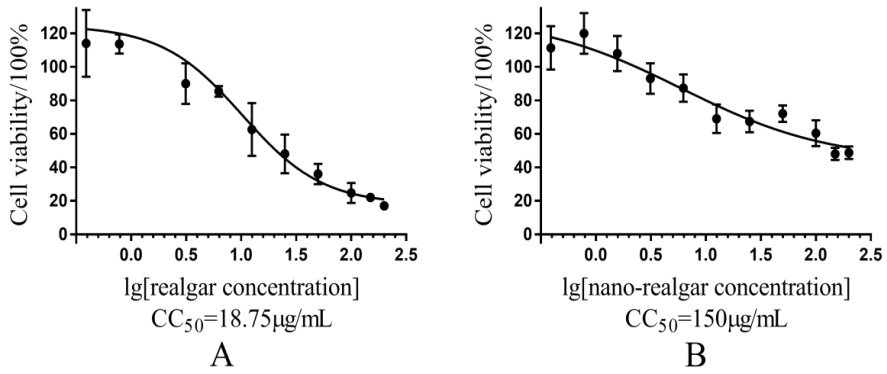

Fig.3. The cytotoxic effects of realgar and nano-realgar on Vero cells. A. realgar; B. nano-realgar

As shown in Fig.3, the cell viabilities of Vero cells treated with realgar at different concentrations were gradually decreased with the dose rising. In other words, the cytotoxicity of realgar on Vero cells was increased in a dose-dependent manner. Similarly, the cell viabilities of Vero cells treated with nano-realgar were also weakened. By calculation, the 50 % cytotoxic concentration (CC_{50}) of realgar and nano-realgar on Vero were 18.75 μg/mL (Fig.3A) and 150 μg/mL (Fig.3B), respectively. There was a significant difference between the cytotoxicity of realgar and nano-realgar on Vero cells ($p < 0.001$). Therefore, the cytotoxicity of nano-realgar on Vero was lower than realgar's. In order to reduce their toxic effects on normal cells, 20 μg/mL was selected as the maximum initial concentration for subsequent experiments.

The Vero cells model establish of HSV-2 infection for determining the antiviral activity of realgar and nano-realgar against HSV-2 through viral titer by plaque assay

In order to determine the antiviral activity of realgar and nano-realgar against HSV-2, the Vero cells model of HSV-2 infection was established by infecting cells with the fifith gengertion HSV-2 virus supernatant. The titer of the primary purified HSV-2 virus and the fifth generation viral supernatant on Vero cells were determined by plaque assay.

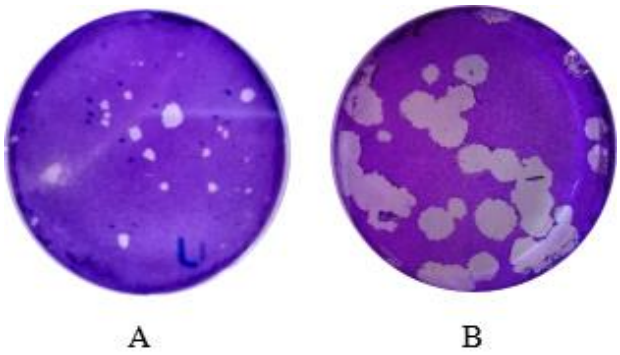

Fig.4. The plaque results of the HSV-2 virus on Vero cells. A. the primary purified HSV-2 virus; B. the fifth generation HSV-2 viral supernatant.

As shown in Fig.4, the plaque result of the Vero cells infected primary purified HSV-2 virus at 10^{-4} dilution showed a total of 17 plaques with small spots, and the titer of primary purified HSV-2 virus on Vero cells was 5.28 logPFUs/mL by calculation. However, the plaque number of the Vero cells infected the fifith gengertion HSV-2 virus supernatant was eighteen at viral 10^{-6} dilution, and the spots were large. The titer of the fifth generation HSV-2 virus supernatant on Vero cells was 7.30 logPFUs/mL. The results showed that the virulence of HSV-2 virus was enhanced through generations of HSV-2 infection on Vero cells. Therefore, the Vero cells model of HSV-2 infection was established by infecting cells with the fifith gengertion HSV-2 virus supernatant at $100TCID_{50}$, whose $100TCID_{50}$ value was 10^{-5}.

Antiviral activity of realgar and nano-realgar against HSV-2 under prevention, treatment and direct inactivation modes

Antiviral activity of realgar and nano-realgar against HSV-2 under prevention, treatment and direct inactivation modes were shown in Fig.5.

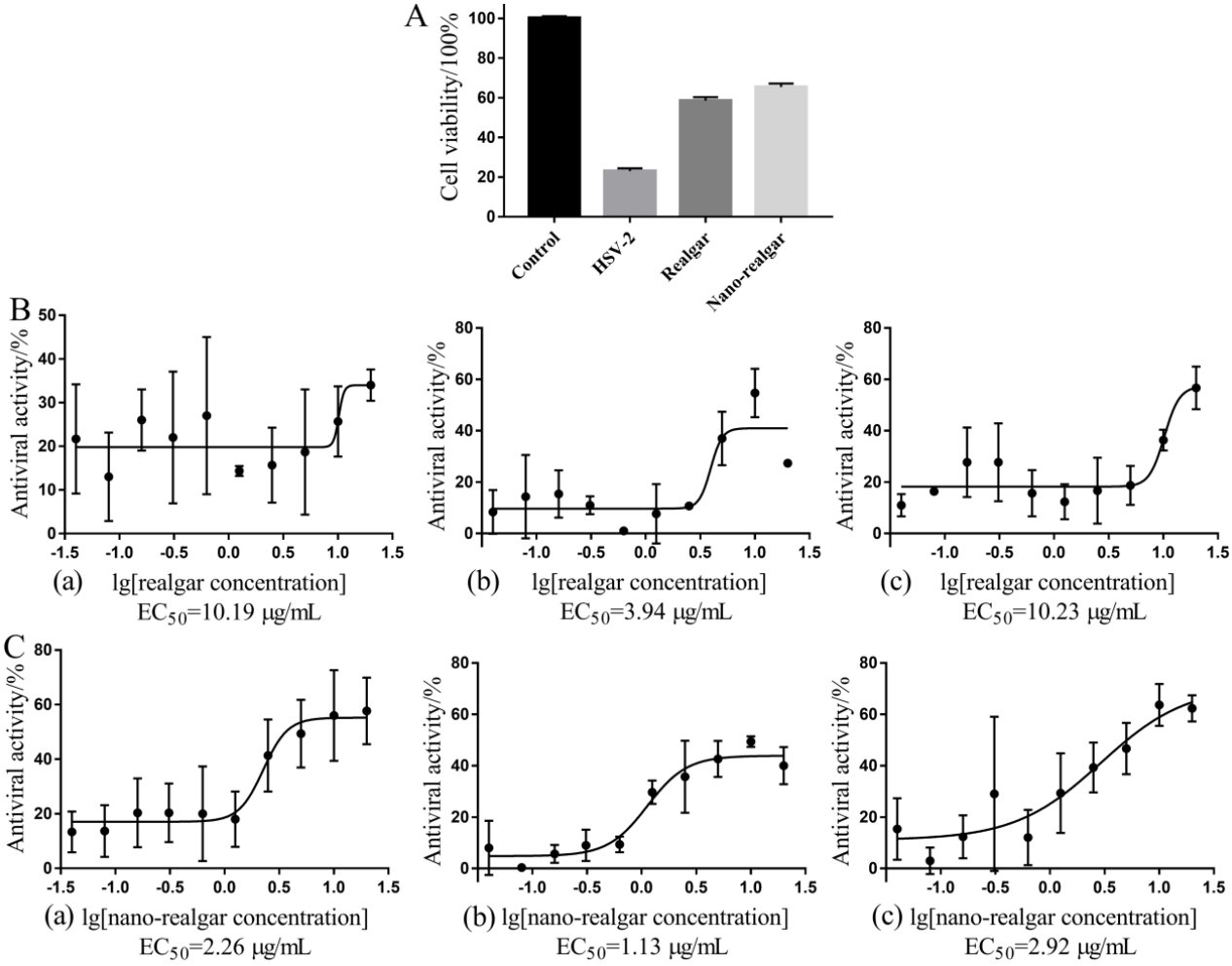

Fig.5. The dose-response curves of realgar and nano-realgar against HSV-2 in prevention, treatment and direct inactivation modes. A. the cellular activities of Vero cell treated with realgar and nano-realgar; B. the dose-response curves of realgar; C. the dose-response curves of nano-realgar; (a) prevention; (b) treatment; (c) direct inactivation

The cell viability of Vero cells infected with HSV-2 was reduced to 23 % compared with that of the normal cells group. However, the cell viability of Vero cells treated with realgar and nano-realgar at 20 μg/mL concentration was decreased to 58 % and 65 %, respectively (Fig.5-A). Therefore, the cytotoxicity of nano-realgar is lower than that of realgar at the same concentration. In the prevention mode, 50 % effective concentration (EC_{50}) of realgar on Vero cells infected with HSV-2 was 10.19 μg/mL, while nano-realgar had an EC_{50} value of 2.26 μg/mL for infected cells. Under treatment mode, the EC_{50} value of realgar and nano-realgar on infected cells were 3.94 μg/mL and 1.13 μg/mL, respectively. In the direct inactivation mode, the EC_{50} of realgar on infected cells was 10.23 μg/mL, while the EC_{50} value of nano-realgar on infected cells was 2.92 μg/mL. These results showed that both realgar and nano-realgar can exert variously anti-HSV-2 effects in three action modes. And nano-realgar has an lower EC_{50} value than realgar on HSV-2 infected cells in prevention, treatment and direct inactivation modes, which indicated that the anti-HSV-2 efficacy of nano-realgar on infected cells is better than realgar's antiviral efficacy in three modes.

CONCLUSIONS

In this paper, the antiviral activities of both realgar and nano-realgar on HSV-2 infected Vero cells were investigated and compared *in vitro*. Firstly, the cytotoxicity of nano-realgar on normal Vero cells was lower than that of realgar. Then, the anti-HSV-2 activitiy experiments *in vitro* indicated that both realgar and nano-realgar can play a good antiviral activity on Vero cells infected HSV-2 in prevention, treatment and direct inactivation modes. And the anti-HSV-2 efficacy of nano-realgar on infected cells is better than realgar's antiviral efficacy in all three modes. This study provides a certain theoretical basis on comparison of antiviral activity of realgar and nano-realgar against herpes simplex virus type II (HSV-2) *in vitro* and demonstrates the potential of nano-realgar as a mineral medicine to control human skin disease caused by HSV-2.

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