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20 (s)-ginsenoside Rg3-loaded magnetic human serum albumin nanospheres applied to HeLa cervical cancer cells in vitro[1]

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Abstract.20(s)-ginsenoside Rg3 is extracted from traditional Chinese medicine, red ginseng. However, due to its poor aqueous solubility and low oral bioavailability, the use of 20(s)-Rg3 is limited. This study aimed to explore a method of preparing nano-sized 20(s)-ginsenoside Rg3 particle named 20(s)-ginsenoside Rg3-loaded magnetic human serum albumin nanospheres (20(s)-Rg3/HSAMNP) to change dosage form to improve its aqueous solubility and bioavailability. 20(s)-Rg3/HSAMNP were prepared by the desolvation-crosslinking method. The character of 20(s)-Rg3/HSAMNP was detected. An antiproliferative effect and cell apoptosis rates of 20(s)-Rg3/HSAMNP on human cervical cancer cells were determined by the MTT assay and flow cytometry, respectively. TEM analysis showed that 20(s)-Rg3/HSAMNP were approximately spherical and uniform in size. Thermodynamic testing showed that the corresponding magnetic fluid of a specific concentration rosed to a steady temperature of 42–65°C. Iron content was approximately 3 mg/mL. Drug encapsulation efficiency was approximately 70%. The potential of 20(s)-Rg3/HSAMNP combined with magnetic hyperthermia therapy to inhibit cell growth and induce apoptosis was much more prominent than that of the other groups. A new dosage form of 20(s)-Rg3 was prepared, which effectively induced apoptosis in HeLa cervical cancer cells in vitro when combined with hyperthermia.

Keywords: Magnetic nanoparticles, human serum albumin nanospheres, 20(s)-ginsenoside Rg3, cervical cancer cells

1. Introduction

Ginseng is a valuable traditional Chinese medicine and famous for its effect on enriching consumptive disease. Bioactive compounds in ginseng possess a series of beneficial effects in the prevention of cancer [1], diabetes [2] and cardiovascular disease [3]. 20(s)-ginsenoside Rg3 (20(s)-Rg3) is extracted from red ginseng with a molecular formula of $C_{42}H_{72}O_{13}$ and can suppress tumor cell proliferation and induces tumor cell apoptosis [4]. However, due to its poor aqueous solubility and low oral bioavailability, the use of 20(s)-Rg3 is limited. A safer and more efficient agent is needed to improve 20(s)-Rg3 bioavailability. Biocompatible and biodegradable albumin microspheres are made with human or animal albumin and have the size of a micron grade ball. They have the advantage of being non-toxic and

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nonimmunogenic [5]. Human serum albumin (HAS) is a versatile drug carrier because of its small size and abundance in blood plasma. The accumulation in malignant and inflamed tissue of HSA agents can confer good biodistribution and deliver a significantly increased dose in tumors.

Magnetic fluid hyperthermia(MFH) involves injecting a nanoscale magnetic liquid into the target area of the tumor to attain a temperature effective for killing cancer cells upon exposure to an alternating magnetic field, resulting in a therapeutic temperature around the tumor but without damage to normal tissue. Tumor cells have a stronger ability to absorb nanoscale magnetic nanoparticles and a higher sensitivity to hyperthermia, which also provides broad prospects for tumor hyperthermia [6,7]. Fe_3O_4 has tremendous properties of magnetism, catalysis, and wave absorption, which makes it the most commonly used magnetic fluid for tumor hyperthermia [8]. However, Fe_3O_4 has a short half-life and no specific tumor-targeting effects, because it is easily taken up by reticuloendothelial cells and removed by macrophages. Consequently, it is important to identify a good carrier to overcome these disadvantages.

In this study, HAS nanospheres was firstly designed to encapsulate Chinese herbs extracts, 20(s)-Rg3 to improve its solubility and bioavailability. Then Fe_3O_4 was contained into to improve the therapeutic efficacy by combining chemotherapy with Hyperthermia.

2. Materials and methods

2.1. Materials

HeLa cells were obtained from Shanghai Institute of Cell Biology (Shanghai, China) and cultured in RPMI 1640 medium (folic acid-free, FA-free) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Shanghai Institute), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% CO_2.

HSA and 20(s)-Rg3 were purchased from Pureone Biotechnology (Shanghai, China). The Annexin V-FITC Apoptosis Detection Kit was supplied by Beyotime Institute of Biotechnology (Shanghai, China). Fe_3O_4 was prepared as described by Li [9]. All reagents were of analytical grade.

2.2. Synthesis of 20(s)-Rg3/HSAMNP

20(s)-Rg3/HSAMNP were prepared by a desolvation-crosslinking technique [10]. Briefly, HSA (250 mg), Fe_3O_4 (50 mg), and 20(s)-Rg3 (5mg) were dissolved in deionized water (25 mL). The pH was adjusted to 8.0 after mixing. Absolute alcohol (150 mL) was added to the solution and stirred at room temperature until a precipitate appeared. Subsequently, 2.5% glutaraldehyde (50 μL) solution was added to the solution and stirred for 12 h. Finally, the mixture was centrifuged at 1500 rpm for 10 min to remove unencapsulated Fe_3O_4 and at 21,000 rpm for 30 min to remove unencapsulated 20(s)-Rg3. Magnetic human serum albumin nanospheres(HSAMNP) and 20(s)-Rg3 human serum albumin nanospheres (20(s)-Rg3/HSANP) were prepared using the same method.

2.3. Characterization of 20(s)-Rg3/HSAMNP

2.3.1. Morphology assessed by TEM

The morphology of 20(s)-Rg3/HSAMNP was assessed by TEM.

2.3.2. Dynamic light-scattering analysis

The particle size and size distribution of the 20(s)-Rg3/HSAMNP were assessed by dynamic light-scattering analysis (DLS; Brookhaven Instruments Co., Holtsville, NY, USA).

2.3.3. Iron content and thermodynamic test

Iron content was measured using 1,10-phenanthroline spectrophotometry as described by Wu [11]. The sample in the tube was placed on SPG-06A high-frequency induction heating equipment (Shenzhen, China) (f = 200 kHz, I = 20 A) for 60 min for the thermodynamic test with different iron concentrations. The distance between the bottom of the tube and the center of the alternating magnetic field (AMF) heating coil was 0.5 cm. The temperature was tested using a digital thermometer every 5 minutes.

2.3.4. Drug encapsulation, loading, and drug release

Vanillin-sulfuric acid spectrophotometry was used to detect 20(s)-Rg 3 content. A 1-mL aliquot of 20(s)-Rg3/HSAMNP was dispersed into 9 mL 0.5% pepsin aqueous solution and digested for 3 h at 37°C. After centrifuging for 30 min at 21,000 rpm, the sediment was added into 5% vanillin-acetic acid and perchloric acid and dissolved in an 80°C water bath for 10 min. Finally, 4.0 ml glacial acetic acid was added after allowing the solution to stand for 15 min. The solution was analysed by using UV spectrophotometry (DU 800, Beckman Coulter, Brea, CA, USA) for the drug content at 588 nm. The absorbance of the diluted sample obtained from the 20(s)-Rg3 standard solution was used for standard curve preparation. Drug encapsulation efficiency (DEE) was calculated by the formula: DEE (%) = actual amount of drug encapsulated in MNP/initial amount of drug used in the fabrication of MNP×100%. Drug loading efficiency was calculated by the formula: Drug loading efficiency (%) = actual amount of drug encapsulated in MNP/actual amount of MNP×100%.

Free 20(s)-Rg 3 and 20(s)-Rg 3 nanoparticle release characteristics were tested in vitro. A 20(s)-Rg 3 solution (15 mL) and sample (15 mL) were placed in a dialysis bag, which was immersed in a 50-mL centrifuge tube containing 30 mL PBS (pH 6.0). The tube was placed in a shaking incubator at 37°C and 110 rpm. A 5-mL sample was removed from the centrifuge tube and replaced with 5 mL fresh PBS at regular time intervals. The 20(s)-Rg 3 concentration in each sample was quantified using a standard curve, and the cumulative release rate Q (%) was calculated by Eq. (1):

$$Q(\%) = \sum_{i=1}^{n=j} CiVi + CnV / W \times D \times 100\% \quad (1)$$

where Ci and Cn are the drug concentration in released mediators, Vi is the volume of mediator removed, V is the total amount of leaching mediator, and W and D are the weight of the microspheres and drug concentration in the microspheres, respectively.

2.4. MTT assay

The MTT method was used to observe inhibition of HeLa cell proliferation. HeLa cells were seeded in 96-well plates at a density of 4×10^3 cells/well and cultured for 24 h. The cells were divided into five groups of six wells each: (a) a negative control group (RPMI 1640 medium containing 10% fetal calf serum); (b) the hyperthermia only group (HSAMNP); (c) the chemotherapy only group (20(s)-Rg3/HSANP); (d) the chemotherapy group using 20(s)-Rg3/HSAMNP; and (e) the hyperthermia

combined with chemotherapy group (20(s)-Rg3/HSAMNP). Treatments (b) and (e) were placed on a heating coil under SPG-06A (f = 200 kHz, I = 20 A) for 60 min. Then, all groups were incubated for 48 h. MTT solution (20 μL) was added to each well for 4 h. DMSO (200 μL) was added and mixed until the precipitate disappeared. The plates were read at 590 nm on a spectrophotometer (Bio-Tek, Winooski, VT, USA). The cell growth inhibition rate (IR) was calculated by the formula: IR(%)=1-the experimental group OD/the negative control group OD×100%.

2.5. Apoptosis assay

HeLa cells were divided into four groups: (a) the negative control group (RPMI 1640 medium containing 10% fetal calf serum); (b) the chemotherapy only group (20(s)-Rg3/HSANP); (c) the hyperthermia only group (HSAMNP), and (d) the hyperthermia combined with chemotherapy group (20(s)-Rg3/HSAMNP). (c) and (d) groups were placed on SPG-06A (f = 200 kHz, I = 20 A) for 60 min. After a 48-h incubation, annexin V-FITC (5 μL) and propidium iodide (PI, 5 μL) were added to the tubes in the dark. The effect of nanospheres on HeLa cells was detected by flow cytometry (FACSCalibur; Becton Dickinson).

2.6. Statistical analysis

Values were shown as means ± standard deviation (SD). The data were analyzed with the SPSS 16.0 program. A p value of <0.05 was considered significant.

3. Results and discussion

3.1. Characterization of 20(s)-Rg3/HSAMNP

The TEM sample (Figure 1) was round and uniform in size. 20(s)-Rg3/HSAMNP had a magnetic core and led to no aggregation with each other. The nanoparticle size and size distribution measured by DLS are shown in Figure 2. The diameter of 20(s)-Rg3/HSAMNP was 179.8±1.2 nm, which is a size that can be easily taken up by tumor cells. 20(s)-Rg3 is given priority in preparing oral medications because of its poor solubility in water. The structure of it was shown in Figure 3. It is easily metabolized by intestinal flora and enzymes, coupled with the first-pass effect, which lead to low plasma concentrations. However, it showed good water solubility when coated with HSA. HSA had stable performance, no toxic side effects, no immunogenicity, and good biocompatibility, which make it an ideal drug carrier, particularly for antitumor drugs.

The iron content of 20(s)-Rg3/HSAMNP was 3 mg/mL. When placed on SPG-06A, 20(s)-Rg3/HSAMNP showed good heating ability, which was stabilized after 35 minutes (Figure 4). The iron concentration was correlated with temperature: the greater the iron concentration, the stronger the heating and the higher the rise in temperature.

The regression equation of the drug was $C = 0.01648A + 0.05757$, $r^2 = 0.9998$. A and C are the A values and the 20(s)-Rg3 concentration, respectively. It was confirmed that the DEE was approximately 70%, and drug loading efficiency was approximately 1.3%. The free 20(s)-Rg 3 and 20(s)-Rg 3HSAMNP release characteristics were displayed in Figure 5. The results showed that free 20(s)-Rg3 was completely released within 8h. The 20(s)-Rg3/HSAMNP have good sustained drug release effect, and the cumulative release percentage was 80% after 190 h. The release pattern could not only make

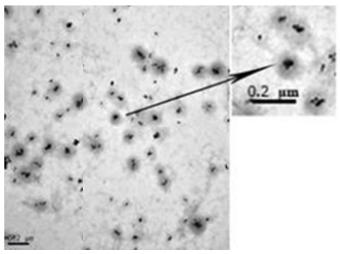

Fig.1 TEM morphology

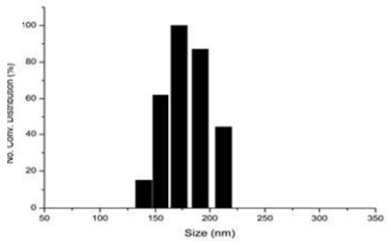

Fig.2 DLS drawing of 20(s)-Rg3/HSAMNP

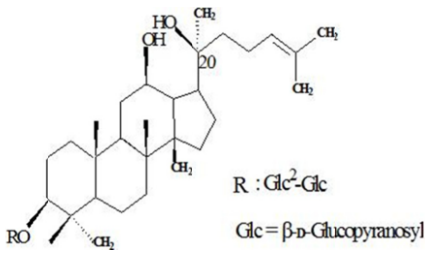

Fig.3 20(s)-Rg3 structure

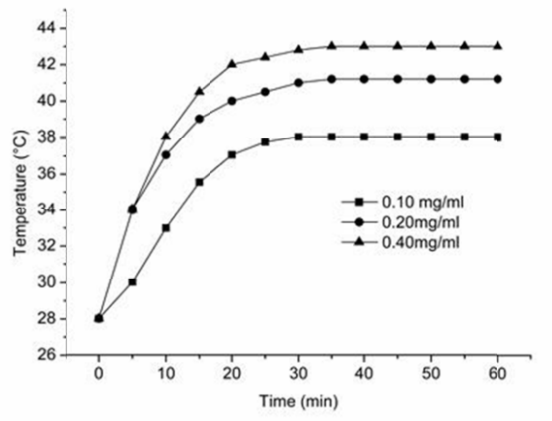

Fig. 4 Temperature increase of FA-DOX-HSAMNP

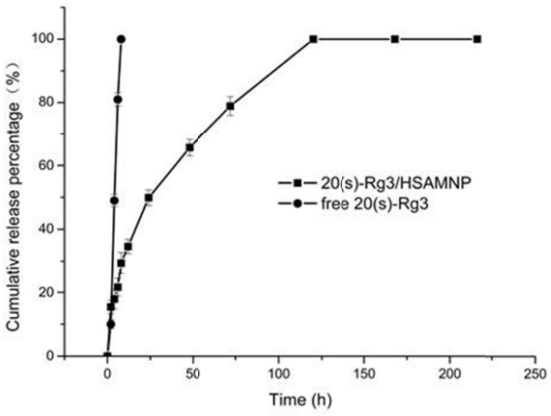

Fig.5 Free 20(s)-Rg3 and 20(s)-Rg3/HSAMNP in vitro release drug characteristics

the harmful side effects of antitumor drugs minimize but also reduce dosing frequency and improve patient compliance when used in clinical.

3.2. MTT assay

Results of the MTT assay (Table 1) showed that the 20(s)-Rg3/HSANP group and the 20(s)-Rg3/HSAMNP with hyperthermia group significantly stimulated the growth of HeLa cells (24.78% vs. 89.44%, $p<0.05$). The HeLa cells were sensitive to heat, and the inhibition rate of hyperthermia using HSAMNP was 56.4%. The inhibition rate of 20(s)-Rg3/HSAMNP without hyperthermia was not significantly different ($p<0.05$) from that of the 20(s)-Rg3/HSANP group. The morphological changes of HeLa cells after treatment were observed by inverted microscopy (Figure 6). The results were coincident with the MTT's. The density of cells in each treat group decreased. The density and morphology of HeLa cells changed obviously in 20(s)-Rg3/HSAMNP with hyperthermia. According to Table 1 and Figure 6, hyperthermia combined with chemotherapy was more effective at inhibiting cell proliferation than that of the other treatments.

Innovation of Chinese traditional drug agents is an important part in improving the quality of the medicine and accelerating the development of the new medicine. One of the most important dosage form of Chinese traditional medicine is decoction, the process is too cumbersome for nowadays. 20(s)-Rg3, has good antineoplastic activity, but its clinical application is limited due to poor water solubility. Above all, using HSA to encapsulate 20(s)-Rg3 is feasible in practice.

Table 1

Inhibition rate of nanoparticles against HeLa cells

Groups	OD values ($\bar{x} \pm S$)	IR (%)
a Negative control group	$1.007 \pm 0.019^{*\#}$	0
b Chemotherapy only group	$0.757 \pm 0.015^{*}$	24.78
c Hyperthermia only group	$0.439 \pm 0.022^{*\#}$	56.4
d Chemotherapy group using 20(s)-Rg3/HSAMNP	$0.751 \pm 0.013^{*}$	25.41
e Hyperthermia combined with chemotherapy group	$0.106 \pm 0.016^{*\#}$	89.44

Notes: Data are presented as means ± standard deviation. Each experiment was repeated at least three times. *Comparisons between the experimental groups and the negative control group, P<0.05, #comparison with the other groups, P<0.05.

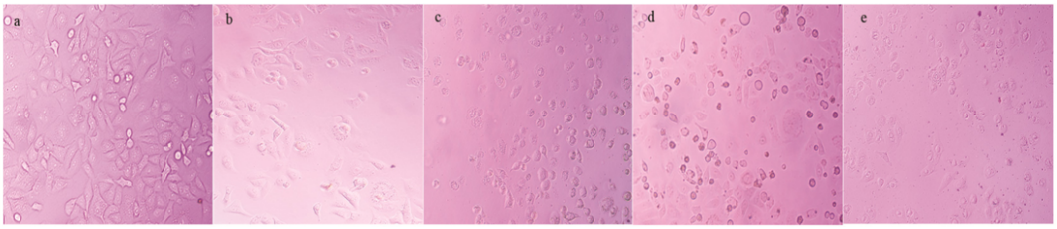

Fig. 6. Morphology analysis of HeLa cells after treated with different methods (a) Negative control group (b) Chemotherapy only group (c) Hyperthermia only group (d) Chemotherapy group using 20(s)-Rg3/HSAMNP (e) Hyperthermia combined with chemotherapy group.

3.3. Apoptosis assay

According to Table 1, no difference in the MTT assay results was observed between the 20(s)-Rg3/HSANP group and the 20(s)-Rg3/HSAMNP without hyperthermia group. The apoptotic cells were not detected in the negative control group without hyperthermia group (Figure 7). The rate of apoptosis in the hyperthermia only group was between that of the chemotherapy only group and that of the hyperthermia combined with chemotherapy group. The rate of apoptosis in the hyperthermia combined with chemotherapy group was the highest ($p<0.05$) (Table 2). The flow cytometry analysis showed similar results to those of the MTT assay.

MFH is a new breakthrough in thermal therapy because of its unique properties in targeting heating temperatures. Magnetic fluid injected directly into tumor tissues produces a high-temperature area to promote apoptosis and simultaneously activate the body's immune system to attack the tumor [12]. Magnetic Fe_3O_4 nanoparticles prepared by Meng showed nearly no toxicity to normal cells and could been quickly distributed to various organs [13]. The mechanisms of hyperthermia and chemotherapy in the treatment of tumor cells have been explored in many experiments. The results showed that hyperthermia has synergistic effects with chemotherapy and played vital roles in combined tumor therapy [13]. Heating can increase the cellular toxicity of some drugs, including platinum and anthracycline-based drugs, and increase tumor local blood flow to promote absorption of medications. Moreover, tumor cell membrane lipid fluidity increases with heating, making it is easy for drugs to enter cells. Above all, magnetic medicine-carried nanoparticles can be served as a center for hyperthermia, chemotherapy as well as targeted therapy, which will have a bright future in the treatment of tumor.

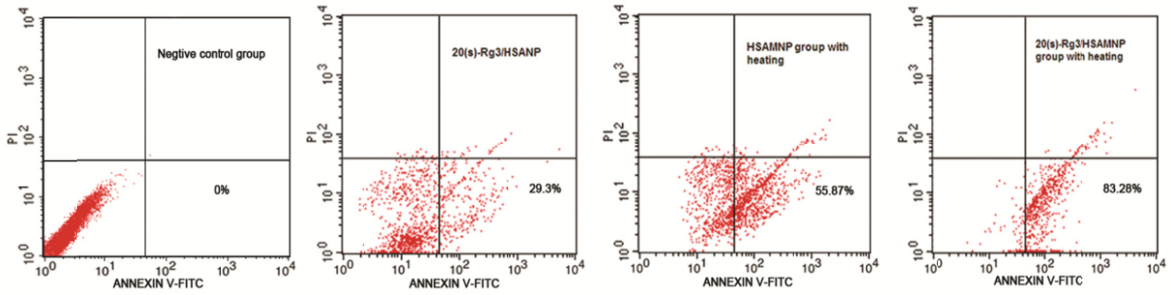

Fig. 7. The apoptotic rates of HeLa cells showed by flow cytometry 20(s)-Rg3/HSANP was chemotherapy only group. HSAMNP group with heating standed the hyperthermia only group. 20(s)-Rg3/HSAMNP group with heating was the hyperthermia combined with chemotherapy group.

Table 2

Nanoparticle-induced apoptosis of HeLa cells

Groups	Apoptotic cells (%)
Negative control group	0*
Chemotherapy only group	23.68 ± 3.23*#
Hyperthermia only group (HSAMNP)	58.24 ± 2.77*#
Hyperthermia combined with chemotherapy group	90.24 ± 6.67*#

Notes: Data are presented as means ± standard deviation. Each experiment was repeated at least three times. *Comparison between the experimental groups and the negative control group, $P<0.05$, #Cmparison of the hyperthermia combined with chemotherapy group with the cemotherapy only group and hyperthermia only group, $P<0.05$.

4. Conclusion

A more effective nanocarrier of Chinese medicine for treating human cervical cancer has been created. They showed good slow-release properties, inhibited HeLa cell proliferation, and induced apoptosis. But more work is needed to optimize this therapy and determine whether it will be effective in vivo.

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