RTCA Monitors the Inhibitory Effect of SWCNTs on the Proliferation of human liver cancer HepG2 cells

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Abstract. To investigate the inhibition of single-walled carbon nanotube (SWCNTs) on the proliferation of human hepatoma HepG2 cells by real-time label-free dynamic cell analysis (RTCA). METHODS: The RTCA system was used to dynamically monitor the effects of different doses (0, 0.5, 1 mg/mL) of SWCNTs on the growth of human hepatoma cells HepG2. RESULTS: SWCNTs at a concentration of 1 mg/mL significantly inhibited cell growth after 48 h of incubation, which was the optimal dose and duration for inhibition of HepG2 cell growth. Conclusion: RTCA system can accurately monitor the growth of HepG2 cells treated with SWCNTs.

1. Introduction
The traditional method to study the effect of nanomaterials on cells is mainly to use the endpoint labeling method such as MTT. The method is cumbersome, the repeatability and sensitivity are poor, and the modifier of the nanomaterial itself may affect the determination of the results. Real-time label-free cell analysis (RTCA) solves a series of problems in the traditional method. The basic principle is based on the detection method of electronic impedance technology [1]. As shown in Figure 1, the microgold electrode is integrated at the bottom of the E-Plate detection plate. Being attached to the surface of the micro gold electrode will cause changes in the electrical impedance of the micro gold electrode. The electrical impedance of the microgold electrode is determined by the surface area of the cell membrane in contact with the electrode and the amount of adhesion. Studies have shown that cell adhesion, morphological changes, proliferation, death and migration affect the electrical impedance of gold electrodes [2-5]. Because there is no need to label cells during the whole RTCA test, the technology can detect the change of cell state in real time, fully automatic, and continuously without damage to cells. In the process of detecting the side effects of nanomaterials on cells, the real-time unlabeled RTCA technology can truly record the effect of nanomaterials on cells, even can easily capture weak effects, making the experimental results more realistic and reliable, which is
impossible to achieve by traditional methods. This series of advantages of RTCA real-time label-free cell analysis technology has broad application prospects in the research of nanomaterials.

![Schematic diagram of the detection principle of RTCA](image)

Figure 1. Schematic diagram of the detection principle of RTCA

The single-walled carbon nanotubes (SWCNTs) have the ability to carry a variety of molecules through biological barriers and cell membranes, and their portable molecules include proteins, polypeptides, nucleic acids and drug molecules [6-9]. As a therapeutic vector, it has shown remarkable application prospects in the fields of cancer treatment, bioengineering and gene therapy.

It has been reported that SWCNTs can be toxic to HepG2 cells [10]. The apoptosis rate also increased gradually with the increase of concentration [10]. Therefore, it has a very good clinical application prospects, but the best test dose and the length of action is not clearly reported. Therefore, in this study, we explore the inhibitory effect of SWCNTs on liver cancer cells HepG2 using RTCA technology.

2. Material and Methods

2.1. Material
DMEM, GIBCO Company; fetal bovine serum, Hangzhou Four Seasons Green bioengineering company; single-walled carbon nanotubes, Sigma Odridge (Shanghai) Trading Co., Ltd.; RTCA, provided by Eisen Biology (Hangzhou) Co., Ltd.

2.2. Cell culture
HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12) supplemented with 10% FBS, streptomycin (100 mg/mL), and penicillin (100 mg/mL) at 37°C under 5% CO2. The medium changed every 2-3 days.

2.3. Determination of growth curve of HepG2 cells
HepG2 cells in good growth state were prepared to suspensions at a concentration of 1×10⁶/mL by trypsinization. 50 μL of DMEM medium was added to a 16-well plate, and then the baseline was measured by RTCA. 100 μL of cell suspensions were added to the assay plate and these cells were
cultured for 24 h. The cells were treated with SWCNTs of 0, 0.5 and 1 mg/mL with 3 replicate wells per concentration. The cell index (CI) value was tested once every 15 min and continuously measured for 144 h. CI value is the cell index unit for RTCA detection of cell activity based on microelectronic impedance detection technology, which is a dimensionless unit. After continuous measurement of cells of different densities, the growth curve was plotted with the normalized cell index (NCi) value as the Y-axis and the time as the X-axis.

The calculation formula of NCi was shown in equations (1).

\[
NCi = \frac{CI(Ti)}{CI(Tk)} \quad [i = 1, 2, 3, ..., N] \quad (1)
\]

NCi is the normalized cell index; CI (Tk) is the cell index at the last time point before the exposure; CI (Ti) is the cell index at the time point to be measured; i is 1, 2, 3, ..., N; N is the time point of all measurements.

The inhibition rate of SWCNTs is shown in equations (2).

\[
\text{Inhibition rate} = 1 - \left( \frac{CI_{(treated\ group)}}{CI_{(control\ group)}} \right) \times 100\% \quad (2)
\]

3. Results

3.1. SWCNTs can inhibit the proliferation of HepG2 cells

![Figure 2](image_url)

Figure 2. Effects of different doses of SWCNTs on proliferation of HepG2 cells.

To measure the effect of SWCNTs on the proliferation of HepG2 cells by RTCA, the cells were treated with 0, 0.5 and 1 mg/mL SWCNTs and were monitored by RTCA for 144 h. The results was shown in Figure 2. The blue line is the growth curve of HepG2 cells untreated. The red line is the growth curve of cells treated with 0.5 mg/mL SWCNTs and the green line is the growth curve of cells treated with 1 mg/mL SWCNTs. The time is taken as the transverse axis, and the CI value is taken as ordinate axis. CI values are proportional to the number of cells. From the diagram, it can be seen that the growth curve of the cells after SWCNTs treatment was lower than that of the control group. The curve of cells treated with 1 mg/mL SWCNTs was lower than that of the cells treated with 0.5 mg/mL.

The results showed that there is a good concentration dependence effect.

3.2. The inhibitory effect of SWCNTs on the proliferation of HepG2 cells was time-dependent

Table 1. Inhibitory rate of HepG2 cells after treatment of SWCNTs.

| Inhibition rate | 0.5mg/mL | 1mg/mL |
|----------------|----------|--------|
| 24h            | 38.47%   | 65.34% |
| 48h            | 53.60%   | 81.22% |
| 72h            | 63.10%   | 88.85% |
Inhibitory rates of HepG2 cells treated with SWCNTs were calculated according to formula. After the cells were treated with 0.5 mg/mL SWCNTs for 24 h, the inhibition rate was 38.47%. After SWCNT treatment for 48 h, the inhibition rate was over half, which was 53.60%. The inhibition rate could reach 63.10%, when cells were treated with 0.5 mg/mL SWCNTs for 72 h. After 24 hours of treatment with 1 mg/mL SWCNTs, the inhibition rate had reached more than half, which was 65.34%. And the inhibition rate at 48 h was 81.22%, while the inhibition rate at 72 h was 88.85%. In summary, the inhibition rate increases with the increase of SWCNT treatment time and concentration.

4. Discussion
In vitro, cell survival and growth have been basically determined by conventional methods such as tetramethylthiazole blue (MTT). These traditional methods can only be detected at artificially set time points, and the results obtained are data of one or several fixed points. Different groups of cells are used for the determination at different time points. Because of the differences between groups, the experimental results are not very accurate. RTCA, using the same batch of cells for long-term monitoring at multiple consecutive time points, which can avoid the difference between groups produced by different batches of cells. RTCA system can be used for real-time monitoring of cell proliferation.

Due to its special physical and chemical properties, such as its size, surface structure, water solubility and agglomeration, SWCNTs may have different toxic effects on cells than traditional chemicals. It has been confirmed that the adsorption of dyes by CNTs may interfere with the results obtained by using dyes. Although CNTs do not infect the formation of methylthione in cells, CNTs can adsorb methylthione and cause the adsorbed methylthione to be insoluble by DMSO, which ultimately results in low experimental results and false positivity [11-13]. RTCA obtains biological information related to cell physiological function by monitoring the change of interface impedance of adherent electrode caused by cells growing on the surface of microelectrode. Therefore, the results will not be interfered by carbon nanomaterials. The RTCA system utilizes an electronic sensor chip to continuously monitor the entire process of biological reactions, and its monitoring is unmarked and non-invasive. This high-accuracy, high-accuracy method for quantitatively measuring cell growth maximizes matches with the growth and development of cells in the natural state, which is very suitable for tumor susceptibility testing [14-15].

In this experiment, the effect of SWCNTs on the growth state of HepG2 cell line was observed in real time by RTCA system. It has been reported that SWCNTs can impair cell membrane potential, increase ROS content, and induce apoptosis [16], which has potential clinical application prospects. Our results showed that 0.5 mg/mL SWCNTs acts quickly and had obvious inhibitory effect at 24 hours. The inhibitory rate was over 50% at 48 h. Compared to that of 0.5 mg/mL SWCNTs, the inhibition of 1 mg/mL SWCNTs was more obvious and the inhibitory rate reached 65.34% at 24 h and increased significantly with time. Therefore, the dose and duration of cell growth inhibition can be visually screened through the RTCA system.

5. Conclusions
In this study, RTCA was used to observe the effects of SWCNTs on human hepatoma cell HepG2. Both 0.5 and 1 mg/mL SWCNTs significantly inhibited the proliferation of cells in time-dependent manner. Being compared to traditional proliferation experiments, RTCA has an incomparable technical advantage.

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References
[1] Zhu J, Wang XB, Xu X, et al. (2006) Dynamic and label-free monitoring of natural killer cell cytotoxic activity using electronic cell sensor arrays. J Immunol Methods, 309:25–33.
[2] Wegener J, Keese CR, Giaever I, et al. (2000) Electric Cell–Substrate Impedance Sensing (ECIS) as a Noninvasive Means to Monitor the Kinetics of Cell Spreading to Artificial Surfaces. J Exp Cell Res, 259:158–166.
[3] Abassi YA, Jackson JA, Zhu J, et al. (2004) Label-free, real-time monitoring of IgE-mediated mast cell activation on microelectronic cell sensor arrays. J Immunol Methods, 292:195–205.
[4] Solly K, Wang X, Xu X, et al. (2004) Application of Real-Time Cell Electronic Sensing (RT-CES) Technology to Cell-Based Assays. J. Assay Drug Dev Technol, 2:363–372.
[5] Xing JZ, Zhu LJ, Jackson JA, et al. (2005) Dynamic Monitoring of Cytotoxicity on Microelectronic Sensors. J. Chem Res Toxicol, 18:154–161.
[6] Zhou H, Zhou WQ. (2017) Comparison of real-time unmarked cell analysis system and traditional MTT method in the determination of breast cancer cell proliferation. J. Journal of Shenyang Medical College, 19 (03): 198–200.
[7] Zhao Z, Liu M, Jia XC, et al. (2014) Toxicity Effect of Carbon Nanotubes. J. World Scientific, 1441009(3):1-6.
[8] Zhao Z, Wang LB, Zhang Y, et al. (2008) Advances in biological safety of nanomaterials. J. Nanotechnology, 4 (02): 61-65.
[9] Zhang X, Meng L, Lu QH, et al. (2009) Targeted delivery and controlled release of doxorubicin to cancer cells using modified single wall carbon nanotubes. J. Biomaterials, 30 (30): 6041-6047.
[10] Xu Y, Xiao L. (2015) Study on in vitro toxicity of single-walled carbon nanotubes to human hepatocellular carcinoma HepG2 cells. J. Jiangxi Medicine, 50 (10): 1006-1008.
[11] LIU Yuan-fang, LIU Jia-hui, WANG Hai-fang. (2010) Cytotoxicity of pristine carbon nanotubes: mechanism and influencing factors. J. Journal of Shanghai University (Natural Science), 16(5): 447-458.
[12] Belyanskaia L, Manser P, Spohn P, et al. (2007) The reliability and limits of the MTT reduction assay for carbon nanotubes–cell interaction. J. Carbon, 45 (13):2643-2648.
[13] Simon-Deckers A, Gouget B, Mayne-L’hermite M, et al. (2008) In vitro investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes. J. Toxicology, 253(1-3):137-46.
[14] Tang C, Li C, Zhang S, et al. (2015) Novel bioactive hybrid compound dual targeting estrogen receptor and histone deacetylase for the treatment of breast cancer. J. J Med Chem, 58 (11): 4550-4572.
[15] Huang L, Pardee AB. (2000) Suberoylanilide hydroxamic acid as a potential therapeutic agent for human breast cancer treatment. J. Mol Med, 6 (10): 849-866.
[16] Chun SG, Zhou W, Yee NS. (2009) Combined targeting of histone deacetylases and hedgehog signaling enhances cytotoxicity in pancreatic cancer. J. Cancer Biol Ther, 8 (14): 1328-1339.