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CELLULAR AND MOLECULAR BIOLOGY

The effect of chitosan addition on cellular uptake and cytotoxicity of ursolic acid niosomes

ANDANG MIATMOKO, BERLIAN S. HARIAWAN, DEVY M. CAHYANI, RETNO SARI, ARISTIKA DINARYANTI & ERYK HENDRIANTO

Abstract: This study evaluated the cellular uptake and cytotoxicity of low permeable Ursolic acid (UA) on cancer cells using niosomes composed of span 60 and cholesterol. The results showed that the addition of chitosan increased particle sizes and ζ-potentials. The UA niosomes with chitosan layers had higher cytotoxicity in HeLa cells than without chitosan, however, there was no improvement observed for Huh7it cells. Moreover, chitosan layers improved the cellular uptake, which clathrin-mediated endocytosis may determine the cellular transport of UA niosomes. In conclusion, the addition of chitosan improved cellular uptake and cytotoxicity of UA niosomes in the HeLa cells.

Key words: Chitosan, niosomes, ursolic acid, cytotoxicity, cell uptake, cancer.

INTRODUCTION

Ursolic acid (UA) is a pentacyclic triterpene which promotes anti-cancer activity in humans (Ali et al. 2019). They revealed the role of UA in accelerating liver proliferation, restoring biochemical and histological functions in liver cells damaged by hepatocarcinoma, and protecting the integrity of hepatocytes against liver damage.

The use of UA in anti-cancer therapy has drawbacks related to its poor permeability and water solubility. Lawsone, an anti-cancer drug that demonstrates poor solubility and permeability, is reported to induce high cytotoxicity when in the form of niosomes (Barani et al. 2018). The increased cytotoxic effect was due to the internalization effect of the niosomes in MCF-7 breast cancer cells which was superior to that of the free form and the resulting sustained-release effect.

Research conducted by Song et al. (2014) shows that the mechanism transporting niosomes into cells is active and linked to the endocytosis pathway. Chitosan, a natural polysaccharide (Szymańska & Winnicka 2015) and a cationic polymer, has been shown to increase cell uptake (Li et al. 2013, Zhang et al. 2013). The cationic polymer charge significantly affects cellular absorption *in vitro* (Li & Ju 2017) due to the electrostatic interaction between cells and positively charged cationic polymers.

Against this background, research was carried out on cell uptake and *in vitro* cytotoxicity of UA niosomes with chitosan coating on HeLa and Huh7it cells (Guo et al. 2019, Purnamasari et al. 2019).

MATERIALS AND METHODS

In this study, UA niosomes (Nio-UA) was composed of Span 60, Cholesterol, UA at a molar ratio of 6:4:1, respectively. A solution of UA, Span 60, and Cholesterol was mixed in a round bottom flask, then the organic solvents

was evaporated until a thin lipid film was formed. This film was hydrated using Phosphate buffered saline solution pH 7.4. Furthermore, a solution of 0.005% w/v chitosan was added for producing Nio-UA-CS.

The particle size and ζ-potential were measured by means of dynamic and electrophoresis light scattering method, respectively, by the use of a Delsa™ Nano C Particle Analyzer at 25°C.

The cytotoxicity test was performed using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on HeLa and Huh7it cells after incubating Nio-UA and Nio-UA-CS for 48 hours. The absorbance of MTT was measured at λ: 560 and 750 nm using a GloMax-Multi Microplate Multimode Reader (Promega).

Furthermore, the cell uptake test was carried out on HeLa cells after incubating the UA niosomes labeled Coumarin-6 (Cou-6= 10 µg/mL). In order to determine the mechanism of cell uptake, pre-incubation was carried out with endocytosis specific inhibitors, namely; 200 µM of genistein and 450 mM of sucrose. Pre-incubation was performed on sucrose for one hour and on genistein for 30 minutes at 37°C. After pre-incubation, the media was replaced with 10 µg/mL of coumarin 6-labeled UA niosomes in the medium and incubated for two hours at 37° C. The the fluorescence intensity of Coumarin-6 in the cells was measured at λ_{ex}: 475 nm and λ_{em}: 500-550 nm using the Glomax Microplate Reader. The cell uptake was also evaluated by using a fluorescence microscope.

For statistical analysis, significance was indicated by $P<0.05$ by using one-way analysis of variance followed by a least significant difference test.

RESULTS

The data shown in Table I indicates that the particle size of Nio-UA-CS was larger than that of Nio-UA. Similarly, for niosomes labeled with coumarin, the size of Nio-UA-CS-Cou6 was greater than that of Nio-UA-Cou6. The cytotoxicity results show that the IC_{50} Nio-UA was higher than that of IC_{50} Nio-UA-CS in HeLa cells (Figure 1a). Thus, chitosan coating can increase the cytotoxicity of UA niosomes with regard to HeLa cells; however, the Huh7it cells were less sensitive to UA than HeLa cells as shown in Figure 1b.

Observations were further conducted using a fluorosense microscope after HeLa cells had been treated with UA niosomes for two hours. It can be seen in Figure 2 that the Coumarin-6 intensity of the Nio-UA-CS was higher than that of Nio-UA. Moreover, the appearance of cells in the treatment of Nio-UA indicates that Nio-UA slightly entered the cell and still mainly remained in the membrane, while Nio-UA-CS appear evenly across the cell membrane and were accumulated highly inside the cells. The Figure 2 also show that after genistein

Table I. Physical characteristics of Nio-UA, Nio-UA-CS, Nio-UA-Cou6, and Nio-UA-CS-Cou6.

Parameter	Nio-UA	Nio-UA-CS	Nio-UA-Cou6	Nio-UA-CS-Cou6
Particle Size (nm)	198.7±13.8	237.7±6.2	163.0±5.3	203.6±6.7
ζ-Potential (mV)	-57.5±11.9	3.88±1.5	-64.8±0.2	-45.7±7.6
Polydispersity index	0.29±0.02	0.33±0.03	0.16±0.03	0.22±0.09

pretreatment as transport inhibitor of Nio-UA, the cells had less intense fluorescence than that of sucrose pretreatment. On the contrary, the cells treated Nio-UA-CS had a higher intensity than that without the addition of the inhibitor, and with the addition of sucrose, these cells had relatively lower intensities than that of genistein addition.

To determine the cellular uptake mechanism of the niosome, a test was carried out involving the addition of specific endocytosis inhibitors i.e. sucrose and genistein. As in Figure 3, with the addition of genistein or sucrose as an endocytosis inhibitor of the caveolae or clathrin pathway, respectively, the Nio-UA demonstrated relatively similar levels than the Nio-UA without inhibitor. The contrast results were produced by the Nio-UA-CS, which without inhibitor, it had significant lower levels than Nio-UA-CS with the genistein.

DISCUSSION

Following the research conducted into the increase in particle size and changes in the ζ-potential value, it can be argued that chitosan layers on niosomes induce this change (Aquila 2018). The chitosan layer may be formed by electrostatic interactions increasing in particle size (Guo et al. 2003). In the niosome, ionic attraction occurs between the ammonium group of chitosan and the phosphate group of phospholipids or other negatively charged groups in lipids (Frank et al. 2020). This interaction also causes changes in the ζ-potential of the niosome as shown in Table I where the ζ-potential value becomes positive after niosome coating.

In this study, HeLa and Huh7it cells had different sensitivity for cytotoxic study of UA and niosomes since each cell type has specific biological characteristics devoted for certain functions (von Gersdorff et al. 2006). The decrease in the IC_{50} value of UA niosomes with

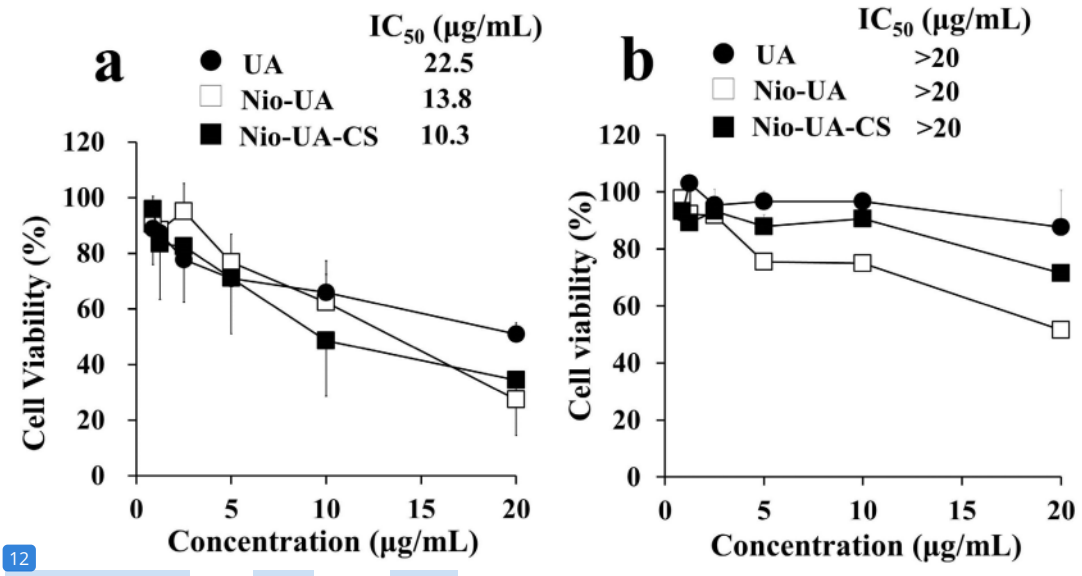

Figure 1. Viability of HeLa (a) and Huh7it (b) cells in the presence of UA, Nio-UA and Nio UA-CS treatment after incubation for 48 hours at various concentrations.

chitosan coating compared to UA niosomes is in accordance with the research findings of Ahmed et al. (2017) regarding the higher absorption of poly L-lysine silver nanoparticles than silver nanoparticles due to the interaction between positively charged poly L-lysine on the surface of the nanoparticles and the negatively charged HepG2 hepatoblastoma cell surface. In chitosan, the positive surface charge can increase the bio-nano interaction with negative surface charge of the cytoplasmic membrane resulting in an increase in cell uptake and cytotoxicity (Frank et al. 2020).

The photomicrographs of cellular uptake confirms that the niosome as a cell retrieval carrier accumulates on the surface during the cell uptake process (Kaksonen & Roux 2018). Since the addition of genistein produces higher yields, it clearly affects cell uptake from Nio-UA-CS (Quagliariello et al. 2019). Hence, the entry of Nio-UA-CS was improved with the presence of genistein as the inhibitor of the caveolae-mediated endocystosis, but no significant difference was observed after addition of sucrose. From these results, it can be concluded that Nio-UA-CS undergoes cell uptake through the clathrin-mediated endocytosis pathway, while the mechanism of Nio-UA uptake may involve both mechanisms. This finding is in accordance with that of Quagliariello's research from 2019 that cell uptake from Butyric acid in liposomes occurs through clathrin-mediated endocytosis whose mechanism is initiated when the endocytic protein layer of the cytosol clusters on the plasma membrane. After the coating is formed, the uptake molecules are concentrated in the layer area and a bond occurs which results in the flat plasma membrane becoming a 'clathrin-layered hole' containing the uptake molecules. Furthermore, the process of constriction and cutting of the neck membrane occurs with the result that the vesicles enter the cell (Kaksonen & Roux 2018).

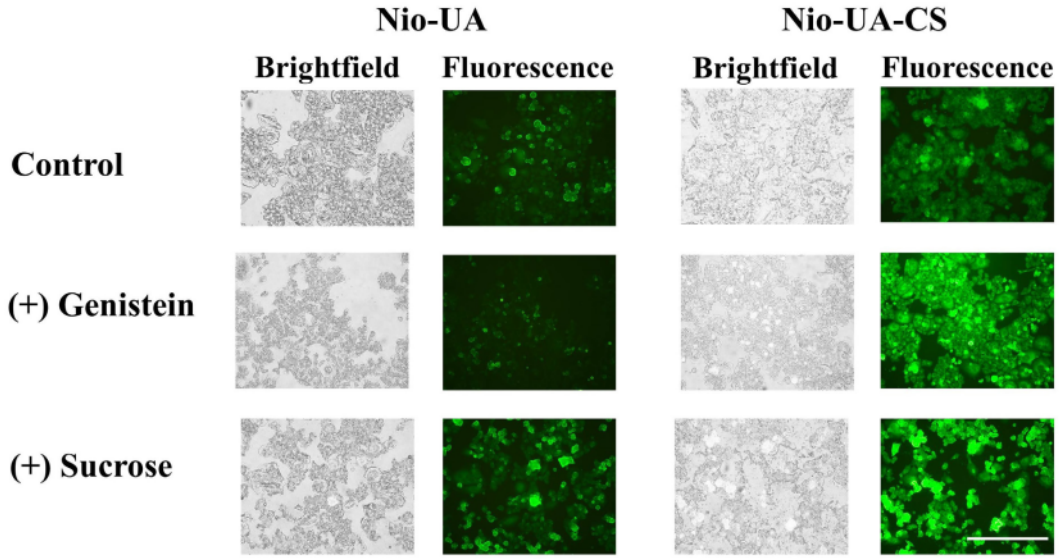

Figure 2. Photomicrographs of cellular uptake of HeLa cells after incubated with Nio-UA and Nio-UA-CS containing Coumarin-6 for two hours without and with addition of genistein and sucrose pre-treatment. Scale bar= 100 µm.

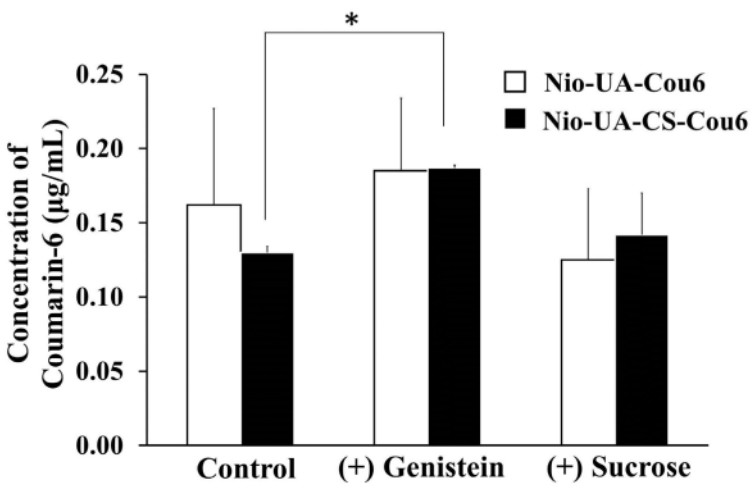

Figure 3. Coumarin-6 levels in HeLa cells after incubation with Nio-UA and Nio-UA-CS for two hours without and with addition of genistein and sucrose pre-treatment (*$P<0.05$).

CONCLUSION

It can be seen from this study that the UA niosome with chitosan layers increased cytotoxicity in HeLa cells but it was less sensitive for Huh7it cells. The difference in cell uptake between Nio-UA and Nio-UA-CS should also be investigated at greater length in order to identify the effect of chitosan addition on cellular uptake pathways.

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