Evaluation of Pegylated Nanoniosomal Cisplatin on Rat C6 in Vitro.

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Abstract
Cisplatin is one of the most useful drugs in chemotherapy, which have several therapeutic properties. Nevertheless, this drug carries some side effects. In order to reduce such effects, nanotechnology has been a great help. In this study pegylated nanoniosomal Cisplatin was prepared through reverse phase evaporation technique. Certain ratios of span 60, cholesterol and polyethylene glycol (3000 Da) were synthesized to prepare pegylated nanoniosomal Cisplatin. The mean diameter, size distribution and zeta potential of pegylated nanoniosomal containing drug and without drug was measured 205.5±4.60 nm, 0.253±0.0036 and -21.4 mv; 251.1±3.93 nm, 0.058±0.0075 and -22.6 mv, respectively using Zetasizer. Encapsulation and drug loading efficiency of pegylated nanoniosomal Cisplatin was determined by spectrophotometry method that were 48.2±2.05% and 4.38±0.28%, respectively. The percent of drug released from niosomes was performed by dialysis for 48 hours. The amount of released drug from niosomes indicated that 82.73±1.36% of drug released into the PBS. Also, this study investigated the cytotoxicity effect of pegylated nanoniosomal Cisplatin using MTT assay. The results showed that IC50 of the pegylated nanoniosomal Cisplatin formulation is less than free drug on C6 cell line.

Keywords: Cisplatin, Reverse phase evaporation, Nanoniosome, IC50, C6.

1. Introduction
Cancer is the second mortality factor among human societies. It takes thousands of lives every year (American Brain Tumor Association, 2014). Factors affecting fertility, environment, lifestyle and physical activity explain this trend (Pathya et al., 2011). Chemotherapy is one of the most common therapies in different kinds of cancer. Among chemotherapy agents, Cisplatin (cisdiamminedichloroplatinum) is commonly used as an anti-cancer agent. Cisplatin, which is considered in platinum group, is used to treat different types of cancers (Johnson et al., 2005). Cisplatin is also a DNA-damaging agent (Jordan & Carmona-Fonseca, 2000). The drug is used in treatment of ovarian, testicular, bladder, cervical, head and neck, pharyngeal and laryngeal and small cell lung cancers(Giordano & Hortobagyi, 2003). However, Cisplatin has some side effects such as nephrotoxicity, nervous system toxicity, auditory toxicity and nausea and vomiting(Kelland, 2000). These side effects limit consumption dosage of the drug in patients (Giaccone, 2000).

Nowadays, we use technologies to decrease side effects and increase the efficiency of drugs. Nanotechnology has created a revolution in cancer diagnosis and treatment. The development of nanotechnology makes it possible, and we can target animals and human diseases, and it yet decreases side
Effects (Mozafari, 2006). Injected carriers in nanoscale are utilized to cross biological barriers, protect drugs and release the optimize dosage of the drug (Costantino & Boraschi, 2012). Niosomes are novel drug delivery systems (Makeshwar & Wasankar, 2013). They are nonionic surfactants with multi lamellar vesicles obtained on hydration of synthetic nonionic surfactants, with or without incorporation of sterol, such as cholesterol or other lipids (Cosco et al., 2009). These carriers have potential for direct targeting by drugs (Marianeccia et al., 2014). Since niosomes are nonionic, they cause drugs to have less cytotoxicity and better therapeutic index (Pawar et al., 2012; Sankhyan et al., 2012).

In this study, in order to prepare pegylated nanniosomal Cisplatin, reverse phase evaporation technique was used (Gopalakrishnan & Chenthilnathan, 2012), and properties of nanoparticles, such as size, morphology, stability, encapsulation and loading efficiency, in vitro release and cytotoxicity are evaluated and are compared with the free drug.

2. Materials and Methods

2.1 Materials

Span 60, cholesterol, polyethylene glycol (3000 Dalton) (PEG-3000), Cisplatin and MTT reagent (0.5 mg/ml) were purchased from Sigma company (SIGMA, USA). Ethanol and RPMI 1640 culture medium were purchased from Merck (Merck, Germany) and Invitro gen Corporation, respectively. C6 cells and A172 cells were supplied from cell bank of Pasteur Institute of Iran.

2.2 Preparation of nanoparticles

To prepare pegylated naniososomal Cisplatin nonionic surfactant (span 60), cholesterol and PEG-3000 (ratio of 6:3:1 weight ratio) were dissolved in 10 ml of ethanol98% by magnetic stirrer (300 rpm, room temperature, 30 min). Afterward, 5 mg Cisplatin was added to the final solution. The solvent phase was evaporated by rotary evaporator (50°C, 90 rpm) (Heidolph Co., Germany). The reproduced gel was dissolved in 10 ml physiological phosphate buffered saline pH 7.4 (300 rpm, 40°C, 10 h). Then, the obtained formulation was sonicated for 15 min (Bandelin Sonorex Digitec, 60Hz).

2.3 Characterization of nanoparticles

The mean diameter of the nanoparticles was measured by Zetasizer (Malvern Instruments Ltd, UK) and the morphology of nanoparticles was studied by Scanning Electron Microscope (SEM) (KYKY-EM3200, China).

2.4 Entrapment efficiency

In order to determine the amount of entrapped drug, 2 ml (1 mg) of pegylated naniosomal Cisplatin was centrifuged (13000 rpm, 1 h, 4°C). Then, the absorbance of naniososomal drug supernatant was measured at 300 nm using spectrophotometer (UV 1601PC, Shimadzu Co.). Afterward, the encapsulation and drug loading efficiency was calculated using formula 1 and 2.

\[
\text{Encapsulation} = \frac{\text{Init. conc. of cis.} - \text{conc. of cis. supernatant}}{\text{Init. conc. of cis.}} \times 100 - (1)
\]

\[
\text{Loading efficiency } (\%) = \frac{\text{Amount of Cisplatin in nanoparticle (mg)}}{\text{Weight of nanoparticle (mg)}} \times 100 - (2)
\]

2.5 Drug release studies

To study the release pattern of naniososomal Cisplatin, 1 ml (0.5 mg/ml of Cisplatin) of niosomal Cisplatin and niosomal suspensions were poured in a dialysis bag (Cut off 12000Dalton, SIGMA), and submerged in 20 ml phosphate buffered saline (PBS) pH 7.4, and stirred (100 rpm, 48 h, 37°C). To evaluate the pattern of drug release at various times, 1 ml of PBS buffer was removed and replaced with a 1 ml of fresh phosphate buffer. Then, the released drug in PBS was measured at 300 nm in different time intervals within 48 hours by spectrophotometry. Afterward, the percentage of released Cisplatin of pegylated naniososomal formulation was obtained using standard curve.
2.6 Evaluation of Cellular Toxicity
The cytotoxicity of standard Cisplatin and pegylated nanoniosomal Cisplatin were determined by MTT assay on C6. The cells were seeded in a 96-well plate at a density of \(1\times10^4\) and cultivated with 5% CO\(_2\) at 37°C in RPMI-1640 culture medium containing 10% fetal bovine serum, 100 unit/ml penicillin antibiotics and 100 unit/ml streptomycin antibiotics. They were allowed to attach for 24h. After removing supernatant, cells were treated with standard Cisplatin and pegylated nanoniosomal Cisplatin in concentrations of 0, 0.25, 0.0125, 0.0625, 0.0312, 0.0156 and 0.0078 mg/ml and incubated for a further 24 hours. After 24 hours, culture supernatants were removed, and MTT solution (0.5 mg/ml) was added. After 3 hours of incubation, the amethyst crystal (formazan) was dissolved in 100 µl Isopropanol. Then, the optical absorption was measured at 540 nm by Elisa reader (BioTek Instruments, VT, USA), and IC\(_{50}\) was calculated using Pharm software.

2.7 Statistical analysis
The statistical analysis of the data was carried out using SPSS, version 13.0. The data were expressed as the means of three measurements ± SD. The \(P<0.05\) was considered statistically significant.

Results and Discussion
3.1 Characterization of Nanoparticles
The mean diameter, size distribution and zeta potential of nanoparticle of pegylated nanoniosomal containing drug and without drug were 205.5±4.60 nm, 0.253±0.0036 and -21.4 mv (Figure 1); 251.1±3.93 nm, 0.058±0.0075 and -22.6 mv (Figure 2), respectively, which was obtained by Zetasizer.

3.2 Entrapment Efficiency
Encapsulation efficiency and loading efficiency was calculated according to formula 1 and 2 using spectrophotometry method, which was obtained 48.2±2.05% and 4.38±0.28%, respectively.

3.3 Drug Release Studies
The amount of released Cisplatin from the prepared drug formulation in PBS buffer during time periods of 2, 4, 6, 8, 23, 27, 31 and 48 h was obtained using the standard curve (Figure 3). Our findings indicated that 82.73±1.36% was released into the PBS in pegylated nanoniosomal Cisplatin formulation.

3.4 Evaluation of Cellular Toxicity
Drug toxicity with different concentrations was studied through MTT assay. Figure 4 indicates the IC\(_{50}\) for different forms of Cisplatin including standard drug and pegylated nanoniosomal drug on C6 cell line.
Fig. 4. The comparison between IC50 of standard Cisplatin and pegylated niosomal Cisplatin on C6 cell line. Results are presented from three independent tests.

Discussion

In this study, Cisplatin was selected as a drug with low solubility in order to encapsulate niosomes. Moreover, nonionic surfactant was selected, because of low cytotoxicity (Makeshwar & Wasankar, 2013). The niosomes entrap drugs like liposomes, but they have more stability in vitro than liposomes. The carriers have less production cost and their storage and maintenance is easier than liposomes (Paolino, 2008; Verma, 2010; Ismail, et al., 2007). Cholesterol was added to the formulation as a membrane stabilizing agent (Tseng et al., 2007). Unlike other studies which had used surfactant and cholesterol with equal ratio (Paolino, 2008; Bayindir & Yuksel, 2010), in this study, the ratio of surfactant and cholesterol was two to one, and nanoparticles with suitable properties were produced. Some coating surfactants, such as span 60, can help nanoparticles to cross the blood-brain barriers (Ma et al., 2011). Also, using the productions method was easy, low cost and with appropriate efficiency. The size of particles is one of the most important agents in drug delivery. Regarding previous studies, particles smaller than 400 nm can pass through endothelial vessels, and they accumulate in tumor site (Duan et al., 2010). In this study, the mean diameter of synthesized nanoparticles was determined based on DLS (Dynamic Light Scattering). The result of SEM verified the mean diameter of nanoniosomal formulation to be in nano scale sizes (Mansour et al., 2009). Nanoparticles surface charge is the another important characteristic in drug delivery, because this factor affects stability of nanoparticles suspension and their reaction with cell membrane (Duan et al., 2010). By comparing the surface charge of nanoparticles, we observed that nanoparticle charge was a bit more positive, due to the positive charge of Cisplatin. Investigation of the nanoparticles size distribution showed control and loaded nanoparticles had appropriate size distribution, and with respect to related curve, these nanoparticles had uniform distributions. Another considerable point in the study was also the size of nanoparticles containing drug was smaller than nanoparticles without drug. This phenomenon can be associated with drug role in the process of producing nanoparticles. Since we observed Cisplatin causes decreasing pH of reaction medium, perhaps changing pH as a result of ionic mechanism produces smaller nanoparticles. The study of nanoniosomal drug release pattern indicated the released drug is relatively slow and as expected. Encapsulation and drug loading efficiency of nanoniosomal nanoparticles, which were determined using spectrophotometry method, were low. This result can be concerned to Cisplatin drug characteristic, because it has low hydrophilic and lipophilic and solubility (1mg/ml). Considering that the amount of drug loading has a direct proportion to the solubility in the reaction medium, and also entrapping the drug in the nanoparticles are physical, the loading was influenced by the low solubility of Cisplatin. In this study, drug loading efficiency was calculated to be 4.38%, and it was shown the method can be used for encapsulation of Cisplatin with appropriate entrapment efficiency. This is lead to increase drug bioavailability. The vesicle produced by the method preserves the properties of anticancer drug, and improves its efficiency. As these nano devices are 100 to 1000-fold smaller than cancer cells, they can be easily transferred through leaky blood vessels (Wang et al., 2008). In order to investigate and compare cytotoxicity of Cisplatin loaded nanoparticles with free Cisplatin, MTT assay is used on C6 cell line. With respect to results of MTT assay, it is identified that the IC50 amount of Cisplatin loaded nanoparticles on C6 cell line...
is less than IC50 of free drug. It is also specified that response of different cells to Cisplatin is different, further on the amount of drug resistance depends on cell type (Babaei et al., 2014).

**Conclusion**

In this study, reverse phase evaporation technique was used to prepare nanoniosomal Cisplatin as an appropriate method. The properties of nanoparticles, such as the mean of diameter, size distribution, zeta potential, encapsulation and loading efficiency, release, morphology and cytotoxicity of pegylated nanoniosomal Cisplatin were evaluated. The results showed the nanotechnology was effective in drug delivery and increase therapeutic index of drugs. This formulation is recommended to in vivo studies.

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**References**


