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ORIGINAL ARTICLE

Investigation the Characteristics of Carboplatin loaded onto Pegylated Liposomal Nanoparticles on the Rat Glioma Cell line C6

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ABSTRACT
Brain cancer is a severely life-threatening problem worldwide. The brain cancer chemotherapy has pursued two purposes: prevention of irregular cell duplication and induction of apoptosis in tumor cells. Carboplatin is a selective chemotherapeutic agent for brain cancer. The study aimed to encapsulate carboplatin into pegylated liposomal nanoparticles and evaluate its efficacy against brain cancer in vitro environment. Nanoparticles were prepared using reverse phase evaporation technique and characterized by dynamic light scattering (DLS), light microscopy, spectrophotometry, dialysis bag and MTT assay. Unilamellar vesicles (ULVs) were formed with the size and zeta potential of 497.3±20.5 nm and -26.4±1.4 mV, respectively. Drug loading and encapsulation efficiency were found to be 3.2% ± 0.15 and 74.07%±2.5 respectively. The drug release study confirmed the power of nanoparticles to drug retention by 71.8%±4.3 release in a period of 48 h. In addition, nanoparticles were increased the cytotoxicity of carboplatin by 64% against brain cancer cell line C6. Findings of study suggested liposome nanoparticles can be used as proper carrier for carboplatin delivery into rat glioma cell line C6.
***Keywords**: Brain cancer, Carboplatin, Liposomes nanoparticle, DLS, Dialysis membrane.*
***MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium*

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INTRODUCTION

Brain cancer with the spread ratio of 3.5 per 100000 people in the world have seriously threaten human health because of rapid growth and poor prognosis 1. glioma the most common primary brain tumor has constituted 29% of all primary brain and Central nervous system (CNS) tumors and 80% of malignant brain tumors 2. The median survival of glioblastoma bearing patients is 14.6 months considering various treatment options including surgery, radiotherapy and chemotherapy 3. Blood brain barrier is the main obstacle for brain tumor drug delivery 4.

However a range of strategies have been used to overcome this barrier including nanoparticles containing drugs 5. Many researchers have frequently applied liposome nanoparticles in this regards 6-8. They have increased the efficacy of drugs and concurrently reduce the side effects 9. in addition the adaptability of liposomes, their capability to proficiently protect the encapsulated agents in blood

circulation and simplicity of surface modification have made liposome nanoparticles distinctive compared to other nanoparticle drug delivery systems [10].
Carboplatin is a chemotherapeutic agent used for treatment of brain cancer [11, 12]. the cytotoxicity effects of drug has exerted by binding to DNA molecule and generating the inter strand cross link 13. However, various side effects of drug such as myelo-suppression restricted the recommended dose 14.
We here encapsulated the carboplatin into pegylated liposomal nanoparticles by reverse phase evaporation technique. polyethylene glycol (PEG) was used because of its effect on the improving of stability 15. Nanoparticles were characterized in terms of size, zeta potential, drug loading and encapsulation efficiency and drug retention capability. In addition, carboplatin loaded liposomal nanoparticles were observed by light microscopy. After that the cytotoxicity effects of nanoparticles were evaluated on the brain cancer cell line C6 in vitro environment.

MATERIALS AND METHODS

Materials

Carboplatin was purchased from Shanghai Luke Chemical Co., Ltd. (China). Cholesterol and ethanol were prepared from Merck (Germany). Kimyagaran Emrooz Chemical Ind. (Iran) provided the polyethylene glycol 6000 (PEG_{6000}). Sigma Company (USA) supplied lecithin and MTT. The rat glioma cell line C6 was provided from the nation cell bank of Iran (Pasteur institute of Iran). All materials were analytical grade. Distilled water was used throughout the study.

Preparation of nanoparticles containing drug

Nanoparticles were prepared by reverse phase evaporation technique. Lecithin, cholesterol, carboplatin and polyethylene glycol were dissolved in ethanol 96% in the molar ratio of 10, 7, 1 and 1. The solvent was then being removed using rotary evaporation instrument (Heidolph, Germany) in 37˚C and 130 rpm. After that Phosphate Buffer Saline (PBS, pH7.4) was added to the thin film formed at the bottom of round bottom flask. The final concentration of lecithin, cholesterol, PEG and carboplatin were 13, 9, 1 and 1 mM, respectively. Nanoparticles were sonicated with a probe sonicator (50. W, Bandelin Sonopuls HD 2070, Bandelin Elec., Germany) for 3 min. Blank nanoparticles were prepared with the same technique without adding of the drug.

Characterization of nanoparticles

The size and zeta potential of nanoparticles were determined by Zetasizer instrument (Nano ZS3600, Malvern Instruments, UK). For this purpose, the suspension of nanoparticles was diluted in PBS and its absorbance was estimated in 630 nm followed by examination in Zetasizer instrument.

Drug loading and encapsulation efficiency

Drug loading and encapsulation efficiency were determined by spectrophotometry method. Suspension of nanoparticles was centrifuged (21000 rpm, 4˚C and 30 min) and the amount of drug in supernatant was estimated according to the standard curve at 220 nm. Drug loading and encapsulation efficiency were calculated according to the below formulae:

$$\text{Drug loading efficiency (\%)} = \frac{\text{The amount of drug into nanoparticle}\left(\frac{\text{mg}}{\text{ml}}\right)}{\text{Weight of nanoparticle}\left(\frac{\text{mg}}{\text{ml}}\right)} \times 100$$

$$\text{Drug encapsulation efficiency (\%)} = \frac{\text{Initial drug concentration}\left(\frac{\text{mg}}{\text{ml}}\right) - \text{supernatant drug concentration}\left(\frac{\text{mg}}{\text{ml}}\right)}{\text{Initial drug concentration}\left(\frac{\text{mg}}{\text{ml}}\right)} \times 100$$

Evaluation of nanoparticles by light microscopy

Liposomal nanoparticles containing carboplatin were evaluated for size, shape, and probable crystallization using light microscopy (Nikon, Tokyo, Japan).

Drug release study

The experiment was performed using dialysis membrane technique. Briefly, sediment of drug loaded nanoparticles was prepared by centrifugation process as mentioned previously. The sediment was resuspended into 5 ml fresh PBS and with standard drug were poured in two separate dialysis tubes (Sigma, cut off 10000 Da) and immerse into PBS and stirred (50 ml, 150 rpm). 2 ml of buffer was

withdrawn on the predetermined time intervals and replaced with 2 ml fresh PBS. The amount of released drug in the PBS was calculated using spectrophotometry technique.

Investigation of cytotoxicity

C6 cell line was cultured under humidified atmosphere containing 5% CO2 in RPMI-1640 cell culture supplemented by 10% Fetal Bovine Serum (FBS), penicillin/streptomycin antibiotics (0.1 and 0.06 mg/ml respectively) at the density of 10^4 per each well of 96 well plates. After 24 h, cell culture was removed and carboplatin in the form of standard or encapsulated into nanoparticles at the same concentrations was added into the wells. After 24 h of incubation, the medium was removed and MTT solution (0.5 mg/ml PBS) was added into each well and incubated for 3 h. The formazan crystals formed were dissolved in isopropanol 100% and the absorbance was read at 540 nm using a microplate scanning spectrophotometer (ELISA reader, Organon Teknika, Netherlands). Cell viability was evaluated by following formula

$$\%\ \text{Cell Viability} = \frac{\text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

In addition, the half maximal inhibitory concentration (IC_{50}) was calculated using Pharm program.

Statistical analysis

For statistical analysis, SPSS software version 18 was used and P values 0.05 was considered as significance.

RESULTS

Characterization of nanoparticles

The size and zeta potential of drug loaded nanoparticles were calculated 497.3±20.5 nm and -26.4±1.4 mV respectively.

Drug loading and encapsulation efficiency

The results of encapsulation and loading efficiency were estimated 74.04±2.5 and 3.2±0.15, respectively. In other words, 74% of used drug become associated with nanoparticles and carboplatin accounts for 3.2% of nanodrug weight.

Evaluation of nanoparticles by light microscopy

Evaluation of formulation by light microscopy confirmed preparation of nanoparticles. They were unilamellar vesicles (ULVs) with spherical to ellipsoid hallow forms dispersed throughout the matrix (Fig.1). Also, nanoparticles were found without crystallization.

Drug release

The results of drug release demonstrated a sustained release pattern. Regarding the nanodrug, release curve initiated with a burst release (30% of encapsulated drug) followed by mild ascending slope with maximal release of 16.6% after 48 h. In contrast, standard drug was shown faster release pattern in which 51% of standard drug was found into PBS (Fig.2)

Cytotoxicity effects of drug and nanodrug

Regardless of standard or encapsulated form, cytotoxicity effects were consistent with carboplatin concentration. These effects however become intensified for encapsulated drug (Fig.3).

Also, IC_{50} of nanodrug and drug was found to be 102 and 150 µM respectively. In other words, liposomal nanoparticles enhance the efficacy of carboplatin by 64%.

DISCUSSION

Nanoparticle drug delivery systems have received considerable attention because of two features including size and biodegradability. Small size of nanoparticles make them to penetrate into target cells through tiny capillaries and nanoparticles construction by biodegradable materials provide the possibility of drug release over a period of several days or even weeks [16]. Appropriate properties of nanoparticles confirmed reverse phase evaporation technique is a proper method for preparation of carboplatin loaded liposomal nanoparticles. PEG was used in the study because of proper stability in the blood circulation, water solubility, low immunogenicity and antigenisity and is able to extend the period of drug release [15]. Nanoparticles were found ULVs that may results from sonication effects. Zeta potential of nanoparticles is correlated with suspension stability [17]. Zeta potential of - 26 mV confirmed the proper stability of particles. Various liposomal formulations of carboplatin were constructed [18, 19]. Zhang et al prepared carboplatin loaded liposomal nanoparticles using thin film hydration method [18]. The prepared particles had the size of 82 nm. They were used diverse materials compared to our study. Lecithin was used here whereas they developed particles using phosphatidyl ethanolamine. Furthermore, the PEG

molecular weight was diverse in two studies. Drug encapsulation efficiency equal to 74% confirmed the appropriate efficiency of technique. in the Liu et al study this value was reported 49% [19].

Drug release is a determinant factor in drug delivery systems [20]. A sustained release of carboplatin from nanoparticles was here discerned. The release was initiated with a burst release indicating the release of adsorbed drug from nanoparticles. While 16.6% of drug was released after 48 h, this value was found to be 51% for standard drug. PEG in the nanoparticle structure could enhance the stability and augment the chance of drug delivery to tumor [21]. Therefore, the power of retention capability may be partially come from the presence of PEG in the formulation. In addition, augment the stability of particles improve the efficacy of drug by enhance the drug delivery to tumors.

Various nanoparticles containing carboplatin were constructed by researchers [22, 23]. Arshad et al used of Poly (lactic-co-glycolic acid) (PLGA) as carboplatin carrier and studied the efficacy of nanoparticles containing drug in vitro and in vivo environment [24]. They demonstrated nanodrug significantly improved the cytotoxicity effects of carboplatin. Hamelers et al, also encapsulated carboplatin into a lipid formulation [25]. Formulation robustly intensified the cytotoxicity effects of drug by three orders of magnitude compared to the standard drug. In the present study, liposome nanoparticles improve the anticancer activity of carboplatin against rat glioma cell line C6. Cytotoxicity effects were directly associated with drug concentrations. Nanodrug with the IC_{50} of 102 µM showed the superior cytotoxicity compared to the standard carboplatin with IC_{50} of 150 µM. This phenomenon was due to the sustained release of drug from the nanocarrier. As far as we know, this is the first study that has evaluated the efficacy of carboplatin loaded liposomal nanoparticles on the rat glioma cell line C6. In conclusion, liposomal nanoparticles are proper carrier for carboplatin delivery to rat glioma cell line C6.

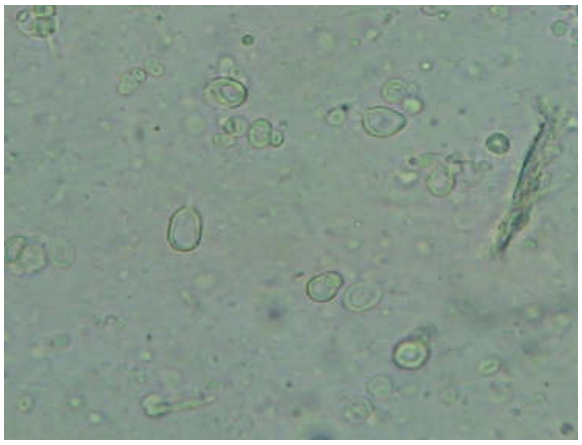

Fig. 1 Light microscopy of carboplatin loaded pegylated liposomal nanoparticles.

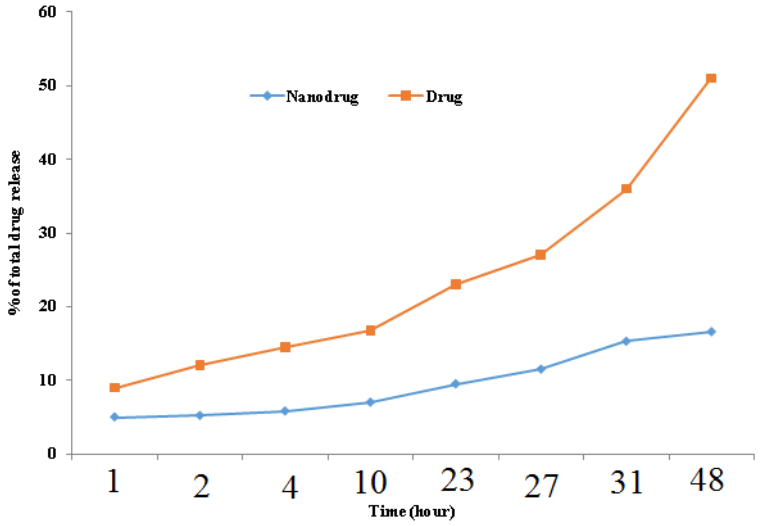

Fig. 2 carboplatin release pattern in the standard and encapsulated form. Results were expressed as mean ± 5% values

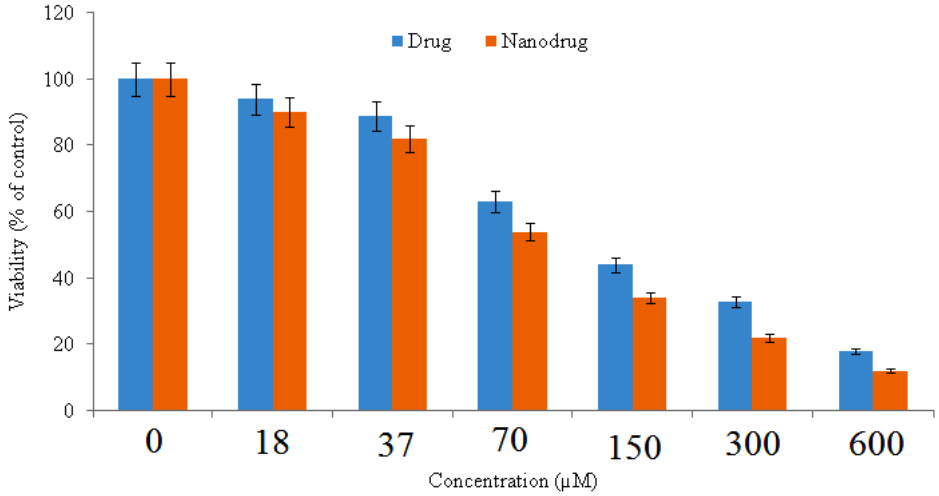

Fig. 3 the cytotoxicity effects of Carboplatin in the standard form or encapsulated into liposome nanoparticles on the viability of rat glioma cell line C6. Results were expressed as mean ± 5% values of three independent experiments.

CONCLUSION

Reverse phase evaporation technique was approved as proper technique for preparation of carboplatin loaded liposomal nanoparticles. The size, zeta potential, drug loading and encapsulation efficiency and drug retention capability of nanoparticles containing drug were evaluated and was found proper. The particles were ULVs. The study was followed by evaluation the efficacy of nanodrug on the rat glioma cell line C6 which demonstrated superior cytotoxicity of nanodrug compared to standard drug. The results of study demonstrated pegylated liposomal nanoparticles are proper carrier for carboplatin delivery to rat glioma cell line C6.

CO-AUTHOR CONTRIBUTIONS

Mehdi Izadi and Reihaneh Asachi contributed to develop and characterize the nanoparticles. Meysam Ebrahimifar and Azim Akbarzadeh designed the study. Hasan Ebrahimi Shahemabadi conducted the MTT assay. Hemen Moradi-Sardareh performed the statistical analysis. Faezeh Safdari and Leila Kanaani performed the release study.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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