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RESEARCH ARTICLE

Exosome-loaded Paclitaxel: Preparation and toxicity evaluation on two glioblastoma cell lines

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ABSTRACT

Objective(s): Exosomes are endogenous nanovesicles act as intercellular communication tools which have been considered to utilize as drug delivery systems. As transporting therapeutic molecules into brain has obstacles, preparing exosomes which have the potential to pass through its barriers is great challenge.

Methods: Exosomes isolated from cell culture media of U87 glioblastoma cells were characterized. In the next step, paclitaxel (PTX) was loaded into them to investigate the cytotoxicity of this formulation on two cell line of glioblastoma, U87 and T98G. Pharmaceutical characterizations such as size analysis, PTX encapsulation efficiency and FESEM/TEM imaging of exosomes were also evaluated.

Results: CD9 as a biomarker of exosomes was detected in extracted samples to confirm the presence of exosomes. Size analysis and electron microscopy imaging proved nano-range of isolated and drug loaded exosomes. The cytotoxicity of empty exosomes of U87 cells was different on U87 and T98 cells. Exosomes diminished cell viability in U87 cells compared with control group while in T98 cell line they didn't have any effect on cell viability after 24 or 48 h time intervals. The cytotoxicity of drug loaded exosomes was different at two time intervals where PTX loaded exosomes had no effect or 30 % cell viability decrease on T98 cells after 24 and 48 h, respectively.

Conclusions: Increased cytotoxicity of PTX after entrapment into exosomes and BBB transport capability of exosomes promises an appropriate brain drug delivery system for in vivo characterization in GBM animal model.

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INTRODUCTION

Exosomes are cell-delivered nanovesicles to communicate between cells. They contain some materials like RNAs and proteins which modulate messages inter cellularly. Exosomes are a branch of extracellular vesicles (EVs) included apoptotic bodies (500-1000 nm), microvesicles (100-500 nm), and exosomes which have narrow and smaller size range (40-100 nm) [1]. These cell-derived vesicles have specific proteins such as tetraspanins (CD9, CD63, CD81), Alix and annexin and some nucleic

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acid compounds like tumor-sensitive gene 101 (tsg 101) on their membrane used as specific marker for exosome detection [2, 3]. As exosomes originate from cell membrane, they consist unique tissue/ cell type-specific proteins and structures that are presents on their parent cells [4]. This properties of exosomes have encouraged researchers to utilize these nanovesicles as bio-marker for diagnosis of diseases. Exosomes from urine samples were studied for diagnosis of prostate cancer [5] ovarian [6] and lung [7])and renal disease [8], and from other biologic samples for Alzheimer's disease [9], cancers (and the other diseases [10].

Because of nano-sized range and messenger role between cells for transferring of different cargos, exosomes could be used as drug delivery systems. Thus, incorporation of therapeutic agents into exosomes avoids rapid clearance of entrapped or adsorbed active pharmaceutical ingredients (APIs); on the other hand, in comparison to cell-mediated and synthetic nano drug delivery systems, toxicity of exosomes is less [11]. Meanwhile, exosomes reflect the membrane structure of their cellular source with specific cell tropism and could employ to target therapy of their parent cells or other cells.

Exosomes have been used for effective delivery of curcumine [12] as an anti-inflammatory agent and paclitaxel (PTX), a mitotic inhibitor [13, 14], as a poorly water soluble anti-neoplasm compound. Among drug delivery protocols, transferring therapeutic components to brain have specific challenges because of the blood brain barrier resistance against APIs transportation [15, 16]. In some cases, such as glioblastoma multiform (GBM), an effective cancer chemotherapeutic compounds such as PTX for GBM could be encapsulated in exosomes [17]. PTX has low water solubility (log*p* 3) and i.v. administration of this drug needs to utilize micellar (Taxol™) or nano-conjugate (Abrexane™) systems, available in drug market.

Various methods have been introduced for exosome isolation [18, 19] included differential ultracentrifugation and density gradient centrifugation [20, 21], polymer precipitation [22, 23], immuneaffinity method based on antibody (Ab) attachment [24], size exclusion chromatography [25] and microfluidic systems [26, 27]. These methods have some advantages and disadvantages through their procedure and the purity rate of obtained exosomes [28-30]. In this study we separated exosomes by Exocib® kit based on precipitation method as mentioned previously [17].

In present study, PTX was loaded in exosomes both for solubility increase and increment of its effect on two various GBM cell types (U87 MG and T98 G). Pharmaceutical characterization of PTX exosomes including size analysis, microscopic study (TEM and FESEM) and entrapment efficiency were also studied.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM) and exosome-depleted fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen Inc. Gibco BRL, USA). Penicillin plus streptomycin solution and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Exocib exosome isolation kit was provided by Cibbiotech Co. (Tehran, Iran) and CD9 TRIFicTM exosome assay (EX101) both were obtained from Cell Guidance Systems (Cambridge, UK). Bicinchoninic acid (BCA) protein assay kit was provided by Santa Cruz Biotechnology (Texas, USA). PTX was purchased from Sobhan-Oncology pharmaceutical company (Rasht, Iran). Other solvents and reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Cell culture

Human brain neuronal glioblastoma-astrocytoma U87 MG and T98 G cells (Pasture institute, Tehran, Iran) were cultured in 75 cm^2 flasks (Iwaki, Tokyo, Japan) in high glucose DMEM. The cultures were supplemented with 10% FBS, 1% penicillin and streptomycin and maintained in a humidified atmosphere of 5% CO_2 at 37 ± 0.5 °C. When 80% confluence of cell culture was achieved exosomes were extracted only from U87 cells.

Exosome isolation

Exosomes were isolated from the U87cells media by using Exocib isolation kit (contained reagent A and B). According to the manufacturer protocols, to remove cellular debris cell media were freshly collected and centrifuged 10 min at 3000 rpm by Hettich® Universal 320R centrifuge (Tuttlingen, Germany). Then the resulting supernatant was mixed with reagent A of Exocib kit at ratio 5:1, respectively, after that the mixture was mixed thoroughly by vortexing for 5 min and incubated over night at 4 °C. Next, the mixture

was centrifuged 40 min at 3000 rpm, supernatant discarded and the plate of exosomes resuspended with 100 µl of reagent B. Eventually, the product kept in -80 °C for next phases of the study.

Drug loading into exosomes

Drug incorporation into exosomes was performed by incubation method, utilized frequently for exosome loading [31, 32]. Therefore, a PTX stock solution was prepared in DMSO (50 mg/ml) and then diluted to 40 µg/ml with DMSO and PBS (pH 6.8). The isolated pellet contain exosomes obtained from the last step of previous section was resuspended with 100 µl of PTX solution in PBS and incubated at 37 ± 0.5 °C for 1 h.

Exosome characterization

Size, zeta potential, and electron microscopic images of these extracted nanoparticles were evaluated. Size analysis of exosomes was performed by photon correlation spectroscopy (PCS; Cordouan, VASCO Nano-Particle size analyzer, Pessac-Bordeaux, France). Field emission scanning electron microscopy (FESEM; Hitachi S-4160, Tokyo, Japan) and transmission electron microscopy (TEM; Philips, Germany) were used for further analysis of size and morphology. In order to prepare samples for FESEM imaging, the exosome suspension was dried at room temperature and coated with a thin layer of gold then examined by using an accelerating voltage of 20 keV. Also for TEM imaging, an accelerator voltage of 80 keV was used after exosomes samples preparation by drying on carbon coated copper grid (200 mesh). Zeta potential of exosomes that introduced surface charge of the exosomes was measured using WALLIS Zeta potential analyzer (Pessac-Bordeaux, France).

Exosome protein determination

The total protein content of exosomes should be determined to quantify amount of exosomes isolated and evaluate drug loading capacity. It was measured through BCA protein assay kit that contained standard solution, copper reagent, and BCA reagent. The standard curve was drawn for different concentration range (50-250 µg/ml) of bovine serum albumin as standard solution. The copper reagent and BCA should be mixed at 1:50 ratio, respectively. The exosome prepared samples and standards solutions mixed with combination of two reagents and incubated at 60 °C for 15 min. Then, the related absorbance at 562 nm by using NanoDrop™ spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA) was recorded.

Exosome CD9 marker detection by TRIFic™ kit

To measure the surface protein of exosomes, CD9 TRIFic™ exosome assay kit (europium time-resolved immunofluorescence based for detection of exosome antigens) was used according to manufacturing protocol. This kit consists of an streptavidin coated 96 well plate which these wells should be coated by freshly prepared dilute solution of biotinylated CD9 monoclonal Ab to capture CD9 on the surface of exosomes. After this procedure, the samples were added to wells and incubated with shake well in order to capture CD9 antigens by Ab. Afterward, europium labeled CD9 Ab dilution was added to wells to bind specifically to exosomes antigen for detecting by fluorescence reader. The excitation and emission wavelength were 340 and 615 nm, respectively and the assay was carried out by multimode microplate reader (Synergy 2, BioTek, Winooski, VT, USA). Incubation of each procedure was done in plate shaker at 750 rpm and between every step the wells were washed three times by washing buffer. The positive control was prepared by using LNCaP lyophilized exosomes through its protocol.

PTX loading determination in exosomes by RP-HPLC

Several HPLC protocols were designed and tested to find optimum resolution, retention factor (k), asymmetry factor (As), the number of theoretical plates (N) and plate heights (H) of the chromatographic peaks. The best protocol which was preferred for analysis of PTX is presented in Table 1.

Cytotoxity of exosomal formulations on U87 and T98 cell lines

Two cell lines of GBM (U87 and T98) were used to evaluate the cytotoxicity of PTX solution, empty exosomes and PTX loaded exosome formulation. In order to performing MTT test, cells were seeded in flat-bottom 96-well tissue culture microplates and incubated for 24 h (37 °C, 5 % CO_2 humidified air) to adhere. Formulations were added to the desired well in triplicate, then the survival of cells were determined at 24 and 48 h. Then over time incubation 100 µl of MTT solution (1 mg/ml

Table 1: Optimized conditions of the RP-HPLC method for PTX determination

Column: Nucleodur C18ec column (4.6 mm × 150 mm, 3.0 µm)		
Column oven temperature: 35 °C		
Mobile phase: A = H_2O, B = MeCN		
Flow rate: 1.2 ml/min		
Elution program:		
	0 -3 min	30 % B
	3 – 8 min	30 % → 80 % B
	8 - 9 min	80 % → 30 % B
	9 – 12 ,min	30 % B
Detection: UV at 227 nm		
Injection volume: 30 µm		

Table 2: Mean hydrodynamic diameter and Zeta potential of naïve and PTX loaded exosomes

Sample	Size(nm)	Zeta potential (mV)
Naïve exosome	70.69 ± 0.99	-22.18 ± 8.73
PTX loaded exosomes	89.04 ± 0.70	-18.22 ± 1.23

in culture medium) was added to each well and incubated for 2 h. The formed formazan crystals should be dissolved in 100 µl of DMSO to measure its absorbance by an ELISA plate reader (Synergy 2, Biotek, Winooski, Vermont, USA) at 570 nm. For each group 3 plates were considered as control group to determine the percentage of cell viability in compare with them.

Statistical analysis

All data were expressed in the form of the mean ± standard deviation (SD). Data were compared by one-way analysis of variance (ANOVA) with Post Hoc test, using SPSS 21 (IBM); Statistic difference significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Pharmaceutical characterization of exosomes

Exosomes have been studied wildly in diagnostic and treatment purposes due to their specific conditions such as nano size and containing parent cell-derived markers on their surface. Whereas exosomes are endogenous carriers between cells they have been find in every body fluids such as blood [33], urine [34], milk [32], saliva [35], and other physiologic fluids [36], also they could be isolated from cell culture medium.

In this study, exosomes was extracted from U87cell line and was loaded with PTX. Although exosome size might be different base on the source and method of preparation or storage condition [29, 37, 38], but they should be in size range about 40-100 nm as depicted in lipid vesicles from various neuroblastoma cells [39] or other cell lines [40]. The mean hydrodynamic diameters of naïve and drug loaded exosomes were 70.69 and 89.04 nm, respectively, as shown in Table 2. Therefore, a significant increase of exosome size was observed following PTX incorporation ($p < 0.05$). Loading of therapeutic ingredients could affect particle size of bilayer lipid vesicles such as niosomes [41] and exosomes [11] because of surface adsorption of API or rearrangement of structural components. Single or co-loading of oxaliplatin and irinotecan in nano-liposomes also resulted in different size ranges [42]. On the other hand, TEM and FESEM images showed exosomes extracted from cell culture media of U87 were in the range size of 50-150 nm (Fig. 1 and 2). The difference in mean diameters of exosomes obtained by image techniques (TEM/FESEM) and PCS or dynamic laser scattering (DLS) method is arisen from particles diffuse double layer calculation in PCS and insufficient accuracy for

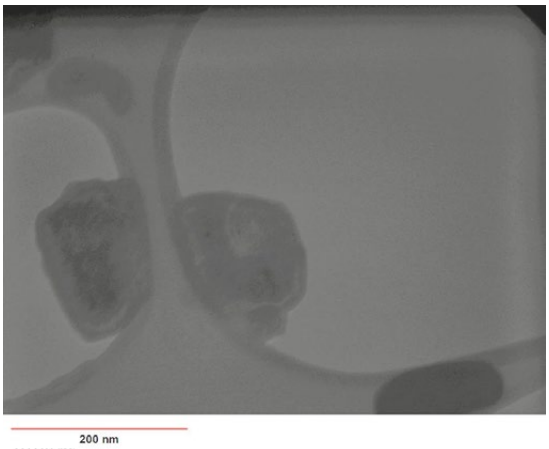

Fig. 1: TEM image of exeosomes extracted from U87 cell line

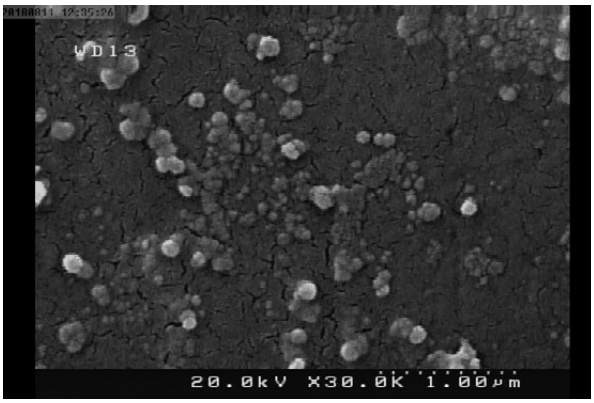

Fig. 2: FESEM image of exosomes isolated from U87 cell line

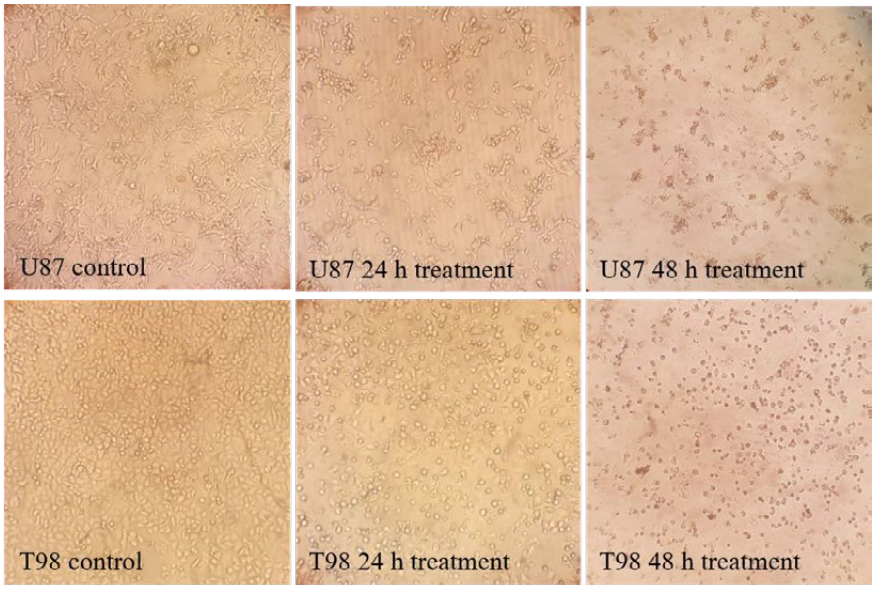

Fig. 3: Effect of PTX cytotoxicity on U87 and T98 cell lines

the DLS technique in the measurements of non-isotropic particles [43].

Zeta potential of nanoparticles could be analyzed to evaluate aggregation properties of them and the correlation between zeta potential of exosomes and their cells sources was found by Kato et al. [44]. Whereas large differences of zeta potential were reported for exosomes isolated from various cell lines [45] it was seen (Table 2) the zeta potential of these extracted exosomes were close to critical zeta potential need for high colloidal dispersion thermodynamic stability [46, 47].

Hereby, the PTX loading in exosomes is reported as loading capacity. In order to calculate loading capacity, the amount of PTX loaded on exosomes was determined through HPLC analysis and the total protein content of exosomes was evaluated through BCA method. The result of dividing PTX loaded in U87 derived exosomes to total protein was 0.74 ng/mg. Very low loading capacity in extracted exosomes in comparison to the other lipid vesicles such as invasin-functionalized nano-liposomes [48] may be consequent of protein-, not lipid content, based calculation of loading capacity in our study.

Biological characterization of exosomes

It was suggested among several exosome extraction protocols, precipitation methods had better yield and recovery compared to ultracentrifugation and density gradient procedures [49]. Furthermore, in precipitation technique by assistance of used polymer, shorter time through slower speed of centrifugation in comparison to

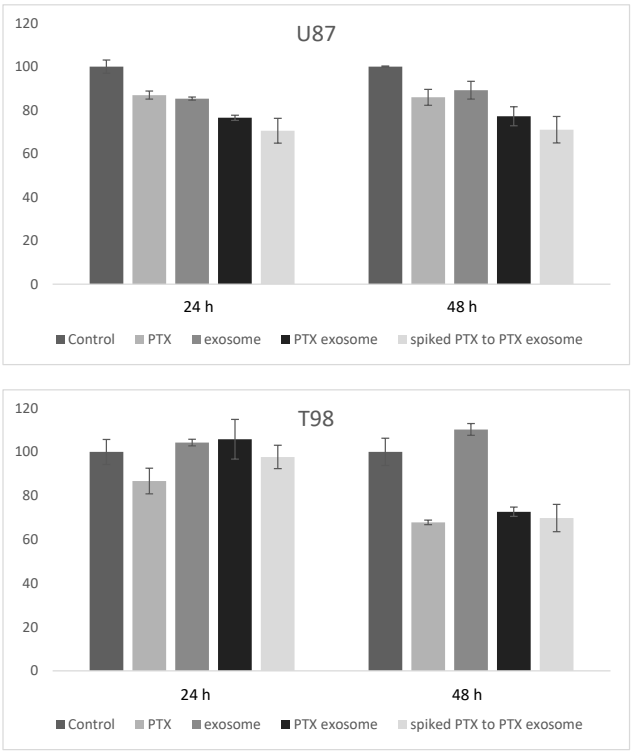

Fig. 4: Cytotoxicity plot of formulations on U87 and T98 cells

ultracentrifugation method is needed. We also used the same method as shown in our recent study [17].

In order to confirm the excreted nanoparticles which shown exosome size range, are actually exosomes, the specific biomarkers like CD9, CD63, CD81 should be found on their surface or biologic samples. In this way, CD9 TRIFic™ exosome assay kit based on monoclonal Ab- CD9 antigen affinity was used similar to Kamińska et al. [50]. The presence of CD9 on these nanoparticles was detected in both naïve and drug loaded exosomes due to similar used cell line protocol of isolation. Tetraspanin protein CD9 interacted with metalloprotease CD10 and increased its release via exosomes as depicted in Mazurov et al. study [51]; they assumed that redistribution of CD10 activity from the plasma membrane to exosomes had an important role in the regulation of extracellular microenviroments and the maturation of B lymphocytes.

Cytotoxicity of formulations

The cytotoxicity of PTX loaded and naïve exosomes and also PTX solution (with similar concentration in drug loaded exosomes) were evaluated in two different cell lines of GBM, U87 and T98, at 24 and 48 h. In similar research on PLGA nanoparticles formulations the U87 cell line was selected to investigate cytotoxicity effects. [52]. The results in Fig. 3 and 4 were suggested PTX had more cytotoxicity effect on T98 cells compare with U87 cells which this effect became greater after 48 h on T98 cells, but PTX hadn't significant effect on U87 cells after 24 h in comparison with 48h duration ($p > 0.05$). This could be related to 48 h replication time for U87 cells [53]. Exosomes extracted from U87 cells had toxicity effect on U87 cells that were their parents cells however these formulations hadn't any toxicity effect on T98 cell line. In a research that was done by Yang T. [31] it was shown exosomes isolated from U87 cell line reduced the viability of U87 cells. Cytotoxicity of PTX on U87 cells at both 24 and 48 h was increased during exosome formulation while on T98 cells exosomal formulation of PTX could not increase PTX toxicity.

CONCLUSIONS

In brief, as exosomes are endogenous nano vesicles, they were considered to using in therapeutic aspects. Here, exosomes were extracted from U87 cell line and PTX was loaded in them. The cytotoxicity of exosomes on two cell lines

were different that might be cause of the origin of exosomes they were isolated. However, more studies are needed to discuss definitely about the exosome cytotoxicity effect.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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