Paclitaxel-loaded hybrid exosome for targeted chemotherapy of triple-negative breast cancer

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Abstract

Background: Triple-negative breast cancer (TNBC) is a highly invasive malignant tumor with bleak prognosis. Paclitaxel (PTX) is the first-line chemotherapeutic drug for TNBC management. It is of great significance to develop carriers for targeted delivery of PTX to enhance the anti-tumor efficacy and reduce the side effects for TNBC management. Exosomes are small extracellular vesicles and emerging competent nanocarriers for targeted drug delivery. However, the application of exosomes has been challenged by the production, drug loading and quality control. The introduction of synthetic nanomaterials may help to overcome the limitations of exosomes and generate alternatives. Herein, we performed a biohybrid approach to fuse exosomes with liposomes to produce hybrid exosome (HE) with improved PTX loading capacity and enhanced tumor-targeting ability for TNBC chemotherapy.

Methods: HE and PTX-loaded HE (PTX-HE) were prepared by co-extrusion of exosomes with liposome (lipo) or PTX-loaded liposome (PTX-lipo) respectively. The size distribution of HE was measured by nanoparticle tracking analysis (NTA) and dynamic laser-light scattering (DLS). The morphology was observed by transmission electron microscopy (TEM). The protein profile of HE was determined by western blot and Coomassie Blue staining. Drug loading capacity and encapsulation efficiency were measured by high-performance liquid chromatography (HPLC). Cellular uptake, tumor cell killing effect and anti-migration ability of PTX-HE were evaluated in vitro. Biodistribution, anti-tumor therapeutic efficacy and safety of PTX-HE were evaluated in orthotopic TNBC mice models.

Results: The characterization results manifested HE was spherical structure with uniform size distribution (157.28 nm). HE had improved loading capacity (6.20 ± 0.79%) and higher encapsulation efficiency (86.79 ± 11.07%) of PTX than lipo. More importantly, PTX-HE possessed higher cellular uptake efficiency, lower IC50 (4.32 ± 0.48 μg/mL) and stronger anti-migration ability (cell mobility: 16%) as compared with PTX-lipo. In TNBC-bearing mice, PTX-HE accumulated at tumor sites and suppressed tumor growth (tumor inhibition rate: 60%) with minimal systemic toxicity.

Conclusions: HE exhibits improved drug loading capacity, targeting ability and cancer cell-killing effect. Overall, HE developed by fusing exosomes with liposomes provides a promising strategy for the large-scale generation of exosome alternatives as drug carriers for targeted chemotherapy.

1. Background

Breast cancer is the most common malignant tumor in women and the leading cause of cancer-related mortality among women worldwide[1]. Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer defined by the absence or low expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2)[2, 3]. Clinical management of TNBC is a great challenge owing to the rapid relapse, high incidence of systemic metastases, and lack of effective therapeutic targets.
Strategic TNBC therapy involves mastectomy (total excision of the breast), usually followed by breast reconstruction, or lumpectomy (breast-conserving surgery) with neoadjuvant or adjuvant therapy. Due to the absence or low expression of ER, PR and HER2, the endocrine therapies such as aromatase inhibitors and selective ER modulators are ineffective in TNBC treatment [4, 5]. For early-stage TNBC patients, neoadjuvant chemotherapy is considered as the standard approach to reduce tumor burden [6]. Also, chemotherapy is generally the only available systemic option to reduce and prevent tumor recurrence and visceral metastasis in TNBC management [7]. Currently, chemotherapy still remains the standard of care (SOC) for TNBC treatment in clinical practice [8, 9]. Paclitaxel (PTX), a tricyclic diterpene alkaloid compound, is the first-line therapeutic drug for TNBC chemotherapy [10]. PTX promotes the assembly of tubulin into microtubules and prevents the dissociation of microtubules, blocking cell cycle progression, inhibiting mitosis and exerting cytotoxic effects, thereby inducing cellular apoptosis [11, 12]. However, PTX is associated with serious adverse effects, including bone marrow suppression, neurotoxicity and metastasis since it lacks tumor targeting ability [13]. Ineffective PTX delivery often leads to undesirable side effects and long-term adverse health consequences, seriously impairing patient’s quality of life. Hence, it's of great value to develop tumor-targeted approaches for the delivery of PTX to reduce the side effect and augment the anti-tumor effect of PTX chemotherapy.

In recent years, nanoparticle drug delivery systems are emerging as promising approaches to enhance the therapeutic efficacy for the chemotherapy of TNBC [14, 15]. Over the past decades, exosomes have been increasingly considered as biological substitutes for nanoparticle drug delivery systems. Exosomes (exos) are nano-sized extracellular vesicles (EVs), ranging from 30 to 150 nm, released by the majority of cell types. Exosomes are vital endogenous "messengers", playing a significant role in intercellular communications, immune response regulation, and bioactive molecules transfer including RNA, proteins and lipid [16–18]. Compared with synthetic nanoparticles, exosomes possess distinct advantages in drug delivery, such as low immunogenicity, low toxicity, excellent biocompatibility and homologous targeting capacity [19–21]. Besides, most cancer cell-derived exosomes, such as exosomes secreted by 4T1 cells, carry CD47, a membrane protein that interacts with the signal regulatory protein alpha receptor (SIRPα) in phagocytes to help exosomes escape from the clearance by the mononuclear phagocytic system (MPS), resulting in prolonged circulation [22, 23]. However, the application of exosomes as drug delivery platforms poses numerous challenges, including low yield, tough quality control and poor drug loading capacity for lipid-soluble drugs [24].

The introduction of liposomes (lipo) may help to overcome these hurdles. Liposomes are synthetic nanoparticles that have been widely applied as drug carriers for anti-cancer therapy [25]. Liposomes, with a bilayer membrane structure, possess excellent drug loading capacity which protect drugs from degradation and thus prolong the half-time of the drugs [26]. Also, liposomes are scalable for large-scale production and facile to surface modification [27–29]. Nevertheless, the rapid capture and clearance by the reticuloendothelial system, induction of immunosuppression, and the lack of targeting ability are still great challenges for the clinical applications of liposomes in drug delivery [30]. Given the fact that the composition of the exosome phospholipid bilayer membrane resembles that of liposomes, the biohybrid approach of fusing exosomes with liposomes to obtain membrane fusion-based hybrid exosome (HE) as
nanocarriers may be a promising strategy for the targeted delivery of anti-tumor therapeutics. HE not only obtain the characteristics of high biocompatibility, homologous targeting capacity and low immunogenicity of exosomes, but are also endowed with facile large-scale production and excellent drug loading capacity of liposomes. Rayamajhi et al. hybridized macrophage J774A.1-derived exosomes with liposomes to obtain hybrid exosome and loaded doxorubicin into it, which showed great cytotoxicity against cancer cells *in vitro* [31]. Nevertheless, the tumor-targeting and endocytosis efficiency of macrophages-derived exosomes is much lower than that secreted by tumor cells, thus producing hybrid exosome by fusing liposomes with tumor cell-derived exosomes may possess superior targeting ability and anti-tumor efficacy for cancer management.

Different approaches have been developed to obtain hybrid exosomes, including freezing-thawing, incubation, extrusion and ultrasonication [32–35]. Freezing-thawing and ultrasonication method tend to disrupt exosome membrane and affect the protein integrity, and the incubation method is time-consuming and with low fusion efficiency. Compared with freeze-thawing and incubation methods, the hybrid vesicles prepared through membrane extrusion exhibit more uniform size distribution. Thus, we prepared HE through the co-extrusion method. In this study, we focus on developing PTX-encapsulated hybrid exosome (PTX-HE) by fusing triple-negative breast cancer cell-derived exosomes with PTX-loaded liposomes for the treatment of triple-negative breast cancer (Fig. 1). We demonstrate that PTX-HE showed strong cytotoxicity and anti-migration ability against 4T1 cells. Also, PTX-HE possessed superior tumor-targeting ability and anti-tumor efficacy in orthotopic TNBC models with optimized safety. These results suggest that HE may emerge as an exciting platform for drug delivery against TNBC.

## 2. Materials And Methods

### 2.1. Cell culture and animals

The murine 4T1 breast cancer cells was obtained from the Chinese Academy of Sciences Cell Bank, cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) medium containing 10% fetal bovine serum (VivaCell, Shanghai, China), 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA), 1% sodium pyruvate (Gibco, Grand Island, NY, USA) and 1% GlutaMax™-1 (100X) (Gibco, Grand Island, NY, USA) at 37°C with 5% CO2. All animal studies were approved by the Department of Laboratory Animals of Central South University and all animals were treated following the Institutional Animal Care and Use Committee (IACUC) approved procedures.

### 2.2 Preparation of exosomes, lipo and HE

4T1 cells were cultured in RPMI 1640 supplemented with 10% exosome-depleted fetal bovine serum and 1% penicillin-streptomycin, 1% sodium pyruvate and 1% GlutaMax™-1. Exosomes secreted by 4T1 cells were isolated by differential ultracentrifugation method. Briefly, the cell culture supernatant containing exosomes was centrifuged at 500 × g for 10 min to remove cells, and then centrifuged at 2000 × g for 20 min and 10,000 × g for 30 min, to eliminate dead cells and deplete residual debris respectively. Afterward,
ultracentrifugation at $150,000 \times g$ for 90 min was conducted to pelleted exosomes. The isolated exosomes were then washed with PBS at $150,000 \times g$ for 90 min and finally resuspended in PBS. All centrifugation process was performed at 4°C. The total protein level of exos was measured by BCA assay (Beyotime, China). The isolated exosomes were stored at -80°C.

Synthetic liposomes were prepared through thin film hydration technique followed by membrane extrusion. Briefly, the lipid material DMPC (Avanti Polar Lipids, USA), cholesterol (Sigma-Aldrich, USA), and DSPE-PEG 2000 (Lipoid GmbH, Germany) were dispersed in methanol in a mass ratio of 10:1:2. The mixture was sonicated for 2 mins and then evaporated to form a layer of lipid film. The dried film was hydrated by distilled water, followed by suspension extruded through 400 nm and 200 nm polycarbonate membrane filters (Whatman) using a mini-extruder (Avanti Polar Lipids, USA) for 15 times to form lipo.

HE was prepared by the co-extrusion of liposomes and exosomes. Briefly, the isolated exosomes were added to liposome suspensions (protein equivalent of exosomes to the lipid mass in ratio of 1:100). The mixture was co-extruded through the 200 nm polycarbonate membranes for 15 times to obtain nano-sized HE.

2.3 Characterizations of exosomes, lipo and HE

Size distribution and yield of 4T1 cells-derived exos was analyzed using nanoparticle tracking analysis (NTA) (Nanosight NS300, Malvern, UK). The size and surface charge of lipo and HE were measured by dynamic light scattering (DLS) (Zetasizer Nano ZS90, Malvern, UK). Surface morphology of exos, lipo and HE were observed by Tecnai G2 Spirit 120kV TWIN Electron Microscope (TEM) (FEI, USA). The presence of protein markers CD47 (ab175388, Abcam, UK), CD63 (ab216130, Abcam, UK), CD9 (ab92726, Abcam, UK) and TSG101 (sc-7964, Santa Cruz, USA) on exos, lipo and HE were detected via western blotting. Briefly, exos, lipo and HE (20 µg) were loaded into 10% SDS-polyacrylamide gel in the Bio-Rad Electrophoresis System to separate the proteins with different molecular weights. PVDF membranes were blocked with 5% skimmed milk for 1 hour and incubated with antibodies against CD47, CD63, CD9 and TSG101 at 4°C for 16 h. The blots were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Anti-rabbit IgG, ZB-5301; ZSGB-BIO, China) for signal visualization. Protein profiles of exos, lipo and HE were compared by Coomassie Blue staining. Protein signals were detected using a gel imaging system (ChemiDoc™ Touch, Bio-Rad, USA).

Storage stability of lipo and HE under different temperatures was also investigated. Briefly, the particle size of lipo and HE stored at 4°C, -20°C and −80°C were monitored for 28 days, respectively.

2.4 PTX loading and release profile

PTX-loaded lipo were prepared by the thin film hydration method. Briefly, DMPC, cholesterol, DSPE-PEG 2000 and PTX (Yuanye Bio-Technology, Shanghai, China) (mass ratio 10:1:2:1) were dissolved in methanol and the follow-up operation is consistent with 2.2, obtaining PTX-lipo. PTX-HE was prepared through the co-extrusion of exosomes and PTX-lipo. Free PTX was removed by centrifugation at $3000 \times g$ for 5 min. The size of PTX-loaded formulations were then measured and compared.
The PTX loading capacity was measured via high-performance liquid chromatography (HPLC) (LC-20AT, SHIMADZU, Kyoto, Japan). Briefly, 900 µL chromatographic methanol was added to 100 µL PTX-lipo and 100 µL PTX-HE respectively, followed by sonication for 2 mins. The mixture was then filtered through a 0.22 µm filter (Biosharp, Hefei, China) and measured by high-performance liquid chromatography. The drug loading was calculated based on the peak area of PTX concentration. The percentage of PTX loading capacity and loading efficiency were calculated according to the following equations:

\[
\% \text{ loading capacity} = \left( \frac{\text{PTX in nanoparticles}}{\text{Total lipid materials added}} \right) \times 100
\]

\[
\% \text{ loading efficiency} = \left( \frac{\text{PTX in nanoparticles}}{\text{Initial input of PTX}} \right) \times 100
\]

Drug release study was carried out in physiological (pH 7.4) and acidic (pH 5.3) conditions. PTX released from the formulations within 60 h was measured at 37°C, using an ultrafiltration tube with 10 kDa cutoff (Millipore) against PBS containing 30% ethanol. The released PTX was then quantified by HPLC.

### 2.5 Cellular uptake

Lipo and HE were labeled with Dil dye (Yeasen Biotech Corporation, Shanghai, China) as previously described[36]. To investigate the impact of the quantity of exos in HE on cellular uptake, 4T1 cells were incubated with Dil-lipo and three Dil-HE groups in different exosome-liposome hybridization ratio, respectively. Briefly, 4T1 cells were evenly planted on a 96-well cell culture plate (NEST Biotechnology) at a density of 5×10^4 cells per well. Dil-HE in three different exosome-liposome hybridization ratios (the protein equivalent of exosomes to total lipid mass in ratios of 1:100, 1:200, 1:300) were added to incubate 4T1 cells for 2 h. Meanwhile, we compared the uptake efficiency of HE at different time. 4T1 cells in a 24-well plate were treated with Dil-labeled HE (the protein equivalent of exosomes to total lipid mass in 1:100) for 1 h, 2 h, and 4 h followed by fixing with 4% paraformaldehyde (Biosharp, Hefei, China) and nuclei staining with DAPI (Yuheng Biotechnology Corporation, Suzhou, China). Cellular internalization was observed under an Olympus IX73 fluorescence microscope (Olympus, Japan). Image J was used to analyze the fluorescence intensity of cell uptake and performed statistical comparisons.

### 2.6 Cell viability and inhibition of 4T1 cell migration

Cell viability was determined by CCK-8 assay (NCM Biotech, Suzhou, China) following the manufactory’s protocol by measuring the absorbance at 450 nm using a Multiskan FC (Thermo Fisher Scientific, USA). Experiments were repeated three times and representative data were shown. The IC50 of 4T1 cells in each preparation group were calculated.

The inhibition of 4T1 cell migration by PTX-HE was investigated via the scratch assay. Briefly, 4T1 cells on a 6-well plate were evenly scratched on the bottom surface using a 200 µL pipette tip, followed by treatment with PTX, PTX-lipo and PTX-HE (4 µg/mL) for 24 h respectively. The scratch size was measured using a microscope.
2.7 Animal models

Female BALB/c mice (6 weeks) were purchased from Hunan Slake Jingda Experimental Animal Co., Ltd and maintained in specific pathogen-free (SPF) faculties. An orthotopic allograft triple-negative breast cancer (TNBC) model was generated by subcutaneously injecting $1 \times 10^6$ 4T1 cells in the right mammary gland of mice. The in situ TNBC model was successfully established in 5–7 days.

2.8 In vivo distribution of HE

Lipo and HE were labeled with DiR (Yeasen Biotech Corporation, Shanghai, China) and the unbounded DiR dye was removed by ultrafiltration. When the tumor volume approached nearly 100 mm$^3$, mice were randomly divided into five groups with the different treatments of free DiR, DiR-lipo and DiR-HE via tail vein injection, respectively. To investigate the distribution of HE and lipo, fluorescent images of each mouse were obtained at 4 h, 8 h and 24 h after injection by using the in vivo Imaging System FX Pro (Bruker, Germany). The tumor-bearing mice were then euthanized to remove the major organs and tumors to capture the fluorescent images.

2.9 In vivo anti-tumor study and safety of PTX-HE

Subcutaneous orthotopic TNBC mice models were developed for a comprehensive comparison of the anti-tumor efficacy of PTX-lipo and PTX-HE. The tumor-bearing mice were injected with PBS, HE, PTX, PTX-lipo and PTX-HE at an equivalent dose of PTX (5 mg/kg) once a day with an interval of two days for four times in total. Tumor volume and body weight of TNBC-bearing mice were measured every day.

After the intervention, mice were sacrificed, and blood samples, tumors and major organs were excised and weight. Tumors and organs were fixed in 4% paraformaldehyde (PFA) and then stained with hematoxylin and eosin (H&E). Tumors were also stained by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) to observe cell death. The collected serum was used to examine liver and kidney functions. Related factors including aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea nitrogen (BUN) and creatinine (CR) were detected to evaluate the safety of PTX-HE.

2.10 Statistical analysis

Data were presented as means ± Standard deviation (SD) for control and experimental samples. Graphpad Prism 8 was used to plot and compare the data. A two-tailed Student’s t-test was applied to test the statistical significance of the difference between the two groups. One-way analysis of variance (ANOVA) was applied to test the statistical significance of difference among three or more groups. The statistical significance was set at * $P < 0.05$, ** $P < 0.01$ and ***$P < 0.001$.

3. Results

3.1. Preparations and characterizations of exo, lipo and HE
4T1 cell-derived exosomes were prepared through classic differential centrifugation as described (See Supplementary Fig. 1A, Additional File 1). Nanoparticle tracking analysis (NTA) and dynamic laser-light scattering (DLS) demonstrated that the average particle size of 4T1 cell-derived exosomes (Fig. 2A-B) was 113 nm. Liposomes were prepared by thin film hydration technique and coupled with extrusion through 200 nm polycarbonate membrane to obtain HE (See Supplementary Fig. 1B, Additional File 1). Compared to exosome and lipo (mode size: 134.23 nm), the particle size of HE (mode size: 157.28 nm) was slightly larger but with a more homogeneous size distribution (Fig. 2B). The increase in the size of HE may due to the content of exosomes was inserted into the lipid bilayer of liposomes which increased the interaction with water, leading to a slightly increase in size. Zeta potential results (Fig. 2C) revealed that all formulations were negatively charged. The zeta potential of exos (-21.18 ± 0.63) and lipo (-31.54 ± 0.29) were both slightly lower than HE (-34.40 ± 3.01), indicating the superior stability of HE in circulation owing to the electrostatic repulsion. The morphologies of exos, lipo and HE were observed by TEM images (Fig. 2D), showing both the nano-formulations were spherical structures with bilayer membrane structures. Similar expression levels of exosomal protein markers CD9, TSG101, CD63, and CD47 on exos and HE were confirmed by western blotting (Fig. 2E). Also, Coomassie blue staining further showed similar protein compositions of exos and HE (Fig. 2F).

To assess the stability of the prepared formulations, they were respectively stored at 4°C, -20°C and -80°C for 28 days and checked for particle size (See Supplementary Fig. 2, Additional File 1). The stability results of HE and lipo stored at 4°C, -20°C and -80°C exhibited negligible changes in particle size within 30 days, revealing the potential of HE for long-term storage and transportation. Notably, the particle size of both lipo and HE changed markedly within 28 days at -80°C as compared to stored at 4°C and -20°C. HE is recommended to be stored at 4°C or -20°C instead of -80°C, which are beneficial for maintaining the basic function of HE.

### 3.2 Drug loading and in vitro release study

The size of both lipo and HE slightly increased after the encapsulation of PTX (See Supplementary Fig. 3, Additional File 1), but still in a proper range. A stable and precise HPLC method for PTX was developed and validated (See Supplementary Fig. 4–5, Additional File 1). As shown in Fig. 2G, the drug-loading capacity of PTX-HE was 6.20 ± 0.79%, higher than PTX-lipo (3.47 ± 0.26%). Also, compared to PTX-lipo (48.60 ± 3.68%), the encapsulation efficiency of PTX-HE (86.79 ± 11.07%) was prominently improved.

The cumulative release rate of PTX under the conditions of pH 5.3 (in vivo neutral physiological environment) and pH 7.4 (acidic microenvironment of tumor) was analyzed. Very little PTX was detected by HPLC under pH 7.4 condition. The cumulative release of PTX from PTX-HE was 34.6%, slightly lower than that released from PTX-lipo (45.8%) at pH 5.3 within 60 h (Fig. 2H).

### 3.3 Cellular uptake assay

To investigate the impact of the mass ratio of exosomes on the cellular uptake of HE, we first incubated 4T1 cells with HE in three different exosome-liposome hybridization ratios. A very similar cellular uptake efficiency of HE in the three groups with different hybridization ratios was observed (Fig. 3A). The uptake
efficiency of HE was higher than lipo as observed by the fluorescence microscope, indicating that HE had superior affinity to TNBC cells. Besides, a significantly higher increase of fluorescence intensity of 4T1 cells treated with Dil-HE by time was observed (Fig. 3B), showing that the cellular uptake of HE by 4T1 cells was time-dependent.

3.4 *In vitro* anti-tumor effect

The *in vitro* anti-tumor effect of free PTX, PTX-lipo and PTX-HE were evaluated and compared.

All the treatments suppressed 4T1 cell viability, and a dose-response relationship of cytotoxicity was observed (Fig. 3C). After continuous incubation for 24 h, free PTX led to the most significant decrease in cell viability while PTX-lipo and PTX-HE exhibited comparable less cytotoxicity to 4T1 cells. The IC50 of the free PTX group was the lowest (3.09 ± 0.60 µg/mL) as compared to the PTX-lipo group (6.32 ± 3.08 µg/mL) and PTX-HE group (4.32 ± 0.48 µg/mL).

The scratch assay was used to investigate the ability of PTX-lipo and PTX-HE to inhibit tumor cell migration. Free PTX showed the strongest inhibitory effect with a cell mobility of 9% (Fig. 3D). Compared to PTX-lipo (cell mobility: 34%), the anti-migration ability of PTX-HE group (cell mobility: 16%) was notably stronger.

3.5 *In vivo* distribution of HE

The tumor-targeting ability of HE was evaluated in *in situ* TNBC mice models. DiR-labeled lipo and HE reached and accumulated at tumor sites at 4 h while free DiR exhibited no fluorescence signals at the tumor site for 24 h (Fig. 4A), indicating both lipo and HE possessed tumor-targeting ability. Notably, the fluorescence intensity of the tumor site markedly decreased after the injection of lipo at 8 h, while the tumor fluorescence signal of mice exposed to HE was significantly stronger. After the injection of HE for 24 h, the tumor site of mice injected HE still exhibited strong fluorescence signal. Mice were sacrificed to remove the major organs and tumors after the injection for 24 h, and the excised tumor also confirmed a markedly stronger fluorescence intensity than that of lipo (1.9-fold) (Fig. 4B-C).

3.6 *In vivo* anti-tumor effect on the orthotopic 4T1 tumor model

The therapeutic efficacy of PTX-HE was evaluated in orthotopic TNBC models (Fig. 5A). Mice receiving the treatment of PTX-HE exhibited a decrease in tumor volume as compared with those exposed to PBS, HE, free PTX and PTX-lipo (Fig. 5B-C). PTX-HE greatly suppressed tumor growth among PTX-HE group, compared to PBS group (Fig. 5D). Size (Fig. 5E) and weight (Fig. 5F) of the excised tumors also confirmed enhanced anti-tumor efficacy of PTX-HE. Notably, the tumor inhibition rate of the PTX-HE group was 60%, and the average tumor weight in the PTX-HE group (0.37 ± 0.21 g) was greatly decreased as compared to PBS group (0.95 ± 0.24 g).

Simultaneously, H&E staining and TUNEL analysis were performed on tumor tissues (Fig. 5G), confirming significant cell death in mice receiving the treatment of PTX-HE, with both internal and external parts of
the tumor showing extensive apoptosis. These results further displayed the therapeutic benefits and application potential of PTX-HE.

3.7 Evaluation of the in vivo safety of PTX-HE

The weight of the mice was monitored for 11 days (See Supplementary Fig. 6, Additional File 1). The overall trend of body weight of the mice exposed to the five treatments showed a slightly downward trend since the feeding process was affected by the changes in the physiological process as the tumor grew. Collectively, each intervention had little impact on the body weight of the mice.

Tumor-bearing mice receiving PTX-HE treatment exhibited a decrease in serum levels of AST, ALT, BUN and CR as compared to the control group (PBS) (Fig. 6A-D). Compared with mice received the treatment of HE, free PTX and PTX-lipo, the serum levels of AST, ALT, BUN and CR of the mice exposed to HE was lower or comparable. Meanwhile, the main organs of mice were also stained with H&E to evaluate the safety of PTX-HE. No obvious damage was observed in the heart, spleen, lung and kidney of mice (Fig. 6E). The liver of the mice receiving free PTX treatment showed significant inflammatory cell infiltration while no damage was observed in mice exposed to PTX-HE, suggesting that PTX-HE has minimal toxicity in in situ TNBC mice.

4. Discussion

In this study, the aim of developing HE was to inherit the advantages of both cancer cell-derived exosomes and liposomes for drug delivery. Here, we successfully prepared HE through the co-extrusion of 4T1 cell-derived exosomes and artificial liposomes.

The particle size of nanoparticles within an appropriate range is one of the key factors that affects the clinical application in drug delivery. Particles in large sizes are arduous to be internalized by cells and tend to be easily cleared by the reticuloendothelial system in circulation, resulting in ineffective drug delivery. In our study, HE had suitable and uniform size distribution and negatively charged membrane surface. TEM image confirmed that HE was spherical structure with a lipid bilayer, indicating the successful fusion of exosome and liposome membrane. The western blotting and Coomassie brilliant blue staining results confirmed a similar protein expression on HE and exosome. However, it is reported that the shear stress generated during the extrusion process may damage the protein integrity of exosomes[37]. Here, the intensity of the protein bands of HE and exosome in both western blotting assay and Coomassie brilliant blue staining was comparable, indicating that the membrane extrusion frequency (15 times) has little impact on protein degradation. Also, HE was prepared at the protein equivalent of exosomes to the lipid mass in ratio of 1:100, which helped to overcome the hurdle of low yield of natural exosomes, making HE possible and suitable for scalable production of nanocarriers. We then loaded the lipid-soluble PTX into HE to investigate the loading capacity of HE for lipid-soluble drugs. The PTX loading study validated that HE notably improved the loading capacity for lipid-soluble drugs. The cumulative release revealed that PTX was slowly released from PTX-HE on the tumor sites within 60 h. Compared with PTX-lipo, the slower release of PTX from PTX-HE at pH 5.3 may due to that extrusion
process embedded the protein on the exosome membrane into the lipid bilayer and filled the gap region, slowing the drug leakage. These advantages suggested that HE has great potential to be further applied as a drug carrier candidate for cancer management.

The fluorescence co-localization experiment showed that HE possessed enhanced cellular uptake. The fluorescence intensity of all the three HE groups was stronger than lipo due to that the 4T1 cell-derived exosomes possessed homologous targeting capacity. However, the specific molecules related to the targeting mechanism still need further investigation. Meanwhile, no difference was observed in the cellular uptake efficiency of HE with different exosome hybridization ratios. This may due to the added exosomes were still in the same order of magnitude, which was inadequate to make a notable difference in cellular uptake. Continuing to increase the quantity of exosomes in HE may further promote the cellular internalization. The uptake of HE by TNBC cells increased with the increasing incubation time, indicating that there is a time dependence in the absorption of PTX.

The scratch assay showed that the inhibitory effect of PTX-HE was notably stronger than PTX-lipo, but slightly poorer to free PTX. Still, the CCK-8 assay revealed that the in vitro anti-tumor effect of PTX-HE on TNBC cells was markedly stronger than that of PTX-lipo but also poorer to free PTX. Free PTX may enter cells more easily than nano-formulations in in vitro cell culture and resulting in killing 4T1 cells directly, while PTX-HE needs to firstly enter TNBC cells through endocytosis and then gradually release PTX before it exerts cytotoxic effect.

As a first-line chemotherapeutic drug for TNBC treatment, PTX must first enter into cells to exert cytotoxic effects and kill tumor cells while the direct administration of PTX has serious side effects. Herein, the tumor-targeting ability of PTX-HE are crucial for alleviating the side effects and enhancing the anticancer efficacy. The in vivo tumor targeting ability and the anti-tumor effect of HE were then investigated in orthotopic TNBC mice models. The in vivo targeting results revealed that HE possesses superior tumor-targeting ability and prolonged circulation time as compared with lipo, which may because of the homologous targeting ability of exosomes and the CD47 on the exosome surface serving as a “do not eat me” signal thus evading the clearance by reticuloendothelial system. Simultaneously, the tumor volume and tumor weight of the mice exposed to PTX-HE were markedly reduced as compared with free PTX and PTX-lipo groups, demonstrating that PTX-HE significantly inhibited tumor growth. H&E staining and TUNEL analysis also confirmed the most significant cell death in mice receiving PTX-HE. Taken together, PTX-HE is of significantly improved tumor-targeting ability and enhanced antitumor efficacy. These advantages of HE allowed forceful tumor-targeted delivery of chemotherapeutic agents, ensuring therapeutic effect of TNBC chemotherapy. Meanwhile, the weight of the mice exposed to PTX-HE was comparably similar compared to the controlled group. No increase in serum levels of AST, ALT, BUN and CR in tumor-bearing mice receiving PTX-HE treatment was witnessed, and also no damage in the excised organs was observed. Compared with mice administrated free PTX, PTX-HE exhibited minimal damage to normal tissues in TNBC management. Collectively, PTX-HE was found to have superior targeting ability and enhanced therapeutic effect for the treatment of TNBC, with minimal systemic toxicity.
5. Conclusions

In this study, HE was developed via fusing 4T1 cell-derived exosomes with artificial liposomes, which inherited the advantages of both liposomes and exosomes. HE possessed improved lipid-soluble drug-loading capacity, higher cellular uptake efficiency and escalated tumor-targeting ability. PTX-HE exhibited prominent anti-tumor efficacy and improved safety in *in situ* TNBC mice models. Therefore, fusing cancer cell-derived exosomes with synthetic liposomes to develop biohybrid nanoparticles provides a promising and scalable strategy for the large-scale utilization of exosome alternatives in drug delivery for future targeted cancer therapy.

**Abbreviations**

TNBC
triple-negative breast cancer
PTX
paclitaxel
HE
hybrid exosome
PTX-HE
hybrid exosome loaded with paclitaxel
PTX-lipo
PTX-loaded liposome
NTA
nanoparticle tracking analysis
DLS
dynamic laser-light scattering
TEM
transmission electron microscopy
HPLC
high-performance liquid chromatography
ER
estrogen receptor
PR
progesterone receptor
HER2
human epidermal growth factor receptor
exos
exosomes
EVs
extracellular vesicles
Declarations

Ethics approval and consent to participate

The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC), The Second Xiangya Hospital, Central South University (Approval No.: 2021839) and all animal studies were carried out following the IACUC approved procedures.

Consent for publication

Not applicable

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

J.L and Y.L designed the study. J.L and Y.T performed the experiments. X.H and S.H collected the data. W.X, X.H and M.Z analyzed the data. Y.T wrote the manuscript. Y.L revised the manuscript. J.W and D.X supervised the study. All authors read and approved the final manuscript.

**Acknowledgements**

Fig. 1 and Fig. 5A are drawn by BioRender.

**References**


Figures
Figure 1

Preparation and application of paclitaxel (PTX)-loaded hybrid exosome (PTX-HE) for treatment of triple-negative breast cancer (TNBC) in mice.
Figure 2

Characterizations of exos, lipo and HE. (A) Size distribution of 4T1-derived exos measured by nanoparticle tracking analysis (NTA). (B) Size distribution and (C) zeta potential of lipo and HE measured by dynamic light scatting (DLS). (D) TEM images of exos, lipo and HE, scale bar = 100 nm. (E) Western blotting analysis of exosome marker proteins of exos, lipo and HE. (F) SDS-page analysis of protein composition of exos, lipo and HE. (G) The loading capacity and encapsulation efficiency of PTX-lipo and PTX-HE.

<table>
<thead>
<tr>
<th>sample</th>
<th>Loading capacity(%)</th>
<th>Encapsulation efficiency(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-lipo</td>
<td>3.47 ± 0.26</td>
<td>48.60 ± 3.68</td>
</tr>
<tr>
<td>PTX-HE</td>
<td>6.20 ± 0.79</td>
<td>86.79 ± 11.07</td>
</tr>
</tbody>
</table>
PTX-HE. (H) Accumulative PTX release profile from PTX-lipo and PTX-HE under pH 5.3 within 60 h at 37°C.

Figure 3

Cellular uptake and *in vitro* anti-tumor effect. (A) The cellular uptake of lipo and HE with different hybridization ratios, and the semi-quantitative analysis of fluorescence intensity. (B) The cellular uptake
of lipo and HE at different time, and the semi-quantitative analysis of fluorescence intensity. (C) The cell killing effect of PTX, PTX-lipo and PTX-HE measured by CCK-8 assay. (D) The inhibition of tumor cell migration evaluated by scratch assay, and the semi-quantitative analysis of cell mobility. Data are shown as mean ± SD. *P < 0.05, **P < 0.01.

Figure 4

The *in vivo* distribution of PTX-HE. (A) *In vivo* biodistribution of lipo and HE in TNBC mice. (B) *Ex vivo* biodistribution of lipo and HE in major organs and tumors from *in situ* TNBC mice. (C) The semi-quantitative analysis of fluorescence intensity of the excised organs. Data are shown as mean ± SD. *P < 0.05.
Figure 5

The *in vivo* anti-tumor effect. (A) Treatment schedule of various formulations for subcutaneous orthotopic TNBC mice models. (B) Average absolute and (C) Relative tumor growth curves of mice receiving different formulations (*n* = 6). (D) Individual tumor growth after different treatments. (E) Visual comparison of tumors excised from the mice in each group. (F) Weight of excised tumors in mice after
In vivo safety evaluation of PTX-HE. (A) AST, (B) ALT, (C) BUN and (D) CR in tumor-bearing mice two days after treatment for four times with an interval of two days. (E) H&E-stained hearts, livers, spleens, lungs and kidneys of mice after receiving the treatment of PBS, HE, PTX, PTX-lipo and PTX-HE.
Supplementary Files

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