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Preparation and evaluation *in vitro* of doxorubicin loaded mimetic exosomes-based delivery system

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Abstract: Exosomes are focused as natural drug delivery vehicles with the advantages of biocompatible, biodegradable and non-immunogenic. However, the low yield of exosomes is one of the challenges that constrain its application. Mimetic exosomes (m-Exo) are the novel cell-derived nano-carriers with similar properties to exosomes and the substantially greater yield is attractive. Herein, in order to evaluate the feasibility of m-Exo as drug delivery vehicles, M-Exo derived from red blood cells were prepared via ultrasonic method, characterized and loaded with doxorubicin (DOX-m-Exo). The preparation methods of DOX-m-Exo were optimized, drug loading as the evaluated index. The drug release and cytotoxicity *in vitro* were studied by dialysis method and MTT method, respectively. The results demonstrated that m-Exo successfully prepared showed spherical morphology and the particle size and Zeta potential were 161nm with a narrow PDI 0.238 and -25.7mV, respectively, the mixed solution of 0.085% NaCl and 0.47% glucose as the dilution medium. The drug loading of DOX-m-Exo prepared by electroporation was up to 57μg/ml. What's more, DOX-m-Exo displayed sustained release behavior and similar cytotoxicity against MCF-7 cells to DOX solution. In conclusion, the studies laid a certain foundation for m-Exo serving as novel and promising drug delivery vehicles.

Keywords: Mimetic exosomes, drug delivery system, *in vitro* release, cytotoxicity.

INTRODUCTION

It has been proven that nanoparticulate drug delivery systems as a critical issue in improving cancer therapy efficiency (Mozhgan *et al.*, 2022; Wang *et al.*, 2022). Despite the advances in liposome, nanoparticles, microemulsion and other nanoparticulate drug delivery systems, many challenges still exist in clinic including stability, safety, biocompatibility and so on (Abbasi1 *et al.*, 2022; Shinde, Panchal, Dhondwad, 2020). Recently, endogenous vehicles, such as protein, polysaccharide nanoparticles and exosomes, have been researched extensively as drug delivery vehicles to obtain better therapeutic outcomes based on the biocompatibility and low toxicity (Peng *et al.*, 2020; Baranov *et al.*, 2021; Prasher *et al.*, 2021) .

Exosomes are tiny vesicles (30-300nm) secreted by cells, also called "extracellular vesicles" (Lunavat *et al.*, 2016). As the specific vesicles, exosomes are involved in cell-to-cell communication and studies have shown that exosomes play a role in the treatment of some diseases, such as tumors, viral infections, cardiovascular diseases and neurological diseases (Ferrantelli *et al.*, 2018; He *et al.*, 2020; Huang *et al.*, 2020, Zuo *et al.*, 2020). It's worth noting that exosomes have recently been investigated as promising delivery vehicles for drugs and genes, since they are intrinsically biocompatible, biodegradable and non-immunogenic (Zheng *et al.*, 2019; Gorshkov *et al.*, 2022). In most studies, exosomes derived from tumor cells were used as the carrier to delivery drugs and displayed superior therapeutic efficiency (Heikki *et al.*, 2015; Hadla *et al.*, 2016). However, exosomes from tumor cells could promote tumor growth and/or migration and drug resistance was also unfortunately found (Whiteside, 2016; Chen *et al.*, 2018; Rizwan *et al.*, 2022; Cui *et al.*, 2022). Therefore, exosomes from tumor sources as drug delivery carriers may have potential safety hazards and are controversial. Additionally, the quantity of exosomes secreted by cells is relatively low. The large-scale production, effective separation and purification of exosomes have not been addressed to date (Wu *et al.*, 2018). Moreover, how to endow exosomes with cancer-targeting ability remains a key technical challenge (Qi *et al.*, 2016; Wang *et al.*, 2018).

Mimetic exosomes (m-Exo) are regarded as the novel cell-derived carrier, which has similar properties to exosomes and the substantially greater yield is attractive for the drug delivery systems (Jang *et al.*, 2013; Su *et al.*, 2016). Considering the abundance and availability of red blood cells, here we developed the novel mimetic exosomes (m-Exo) derived from red blood cells to solve the limited yield of exosome and optimized the preparation process. Then m-Exo loaded DOX (DOX-m-Exo) was further prepared and the drug release and cytotoxicity *in vitro* were investigated. The study preliminarily explored the possibility of mimetic exosomes as the vehicle for delivering drugs and laid a foundation for the further study of m-Exo based drug delivery systems.

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MATERIALS AND METHODS

Material

Doxorubicin hydrochloride (DOX) was supplied by Dalian Meilun biotechnology Co., Ltd (Liaoning, China). RPIM 1640 medium and fetal bovine serum (FBS) were obtained from Beijing Solarbio Science and technology Co., Ltd. and Sijiqing Biologic Co. Ltd. (Hangzhou, China), respectively. All other reagents were analytical grade.

Preparation of m-Exo

RBC membrane were firstly prepared according to the literature with a little modify (Su *et al.*, 2016). Three New Zealand albino rabbits (2.53.0Kg) were supplied by the Experimental Animal Center of Henan University of Science and Technology. In brief, fresh blood was taken from auricular vein of the donor rabbit immediately prior to the experiments and then centrifuged for 10 min at 1,000rpm and 4°C to separate RBCs. The obtained RBCs were washed for three times with 0.9% sodium chloride injection and then suspended in a cold hypotonic lysing buffer, comprised of 10 mM Tris-HCl buffer (pH 7.4), 1mM $CaCl_2$ and 1% EDTA-free protease inhibitor for 4h. Then the supernatant liquid was removed via centrifugation for 15 min at 15,000rpm. The above process was repeated and washed with PBS buffer three times to collect the purified RBC membrane.

The m-Exo was prepared via ultrasonic method. 1ml of the RBC membrane were re-suspended in 10ml dissolution medium (0.9% NaCl normal saline, phosphate buffer saline or the solution containing 0.085% NaCl+0.47% glucose) and then sonicated (JY92-II, Xinzhi Inc., Ningbo, China) to obtain the final m-Exo. In the study, Preparation process parameters and the dilution medium were optimized.

Characterization of m-Exo

The particle size and zeta potential of the m-Exo were determined by dynamic light scattering (DLS) measurement analysis using the Malvern Zetasizer Nano ZSE instrument (Malvern Instruments Ltd., UK). And the morphology of the m-Exo was observed under the transmission electron microscope (JEM-2100, JEOL Co., Ltd., Japan) with negative staining method using (2%, *w*/*v*) phosphotungstic acid.

Preparation of DOX loaded m-Exo

Co-incubation, sonication method and electroporation method are commonly used for m-Exo loading DOX (Tao *et al*., 2020). In the co-incubation method, m-Exo and DOX were incubated for 1, 2 and 4h at 37°C with shaking, respectively. Then the un-encapsulated DOX was removed via dialysis method and the DOX loaded in m-Exo was determined by HPLC method according to the literature with a little modify (Sun *et al.*, 2010). In the sonication method, m-Exo and DOX solution were mixed thoroughly. After ultrasonic treatment for 15 min and 30 min respectively, the samples were incubated for 1h at 37°C to allow for recovery of the m-Exo membrane. And the dialysis and the determination of DOX were also conducted as above. In the electroporation method, m-Exo were electroporated with a Gene Electroporator (SCIENTZ-2C, Xinzhi Inc., Ningbo, China). The electroporation was carried out after mixing m-Exo and DOX solution and the voltage was set as 200V, 300V and 400V, respectively, capacitance 500μF, Electric Resistance 500 Ω (Kamerkar *et al.*, 2017). The repairment of the m-Exo membrane, dialysis and the determination of DOX were also conducted as above. The three methods were evaluated with drug loading as the index.

In vitro release study of DOX-m-Exo

The release behavior of DOX from DOX-m-Exo was determined in phosphate bufer (pH 7.4), DOX solution as the control. Briefly, a certain amount of DOX-m-Exo (equivalent to 5μg of DOX) was placed in dialysis bags (MW cut of 12,000-14,000 kDa, soaked for 24 h before use in double-distilled water), which were put into 100mL release medium stirred at 100rpm at 37°C. An aliquot of 1ml release medium was withdrawn at predetermined time points (0.5, 1, 2, 4, 8, 12 and 24h) and replaced by the same volume of fresh medium. After passed through a 0.45μm syringe filter, each sample was determined by HPLC method according to the literature with a little modify (Sun *et al.*, 2010).

Cytotoxicity study

The *in vitro* cytotoxicity of DOX-m-Exo was tested in breast cancer cell line MCF-7 by MTT method, DOX as the reference. MCF-7 Cells were supplied by Chinese Academy of Medical Science (Beijing, China) and cultured in RPIM 1640 media supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. In brief, cells were seeded in 96-well plates at 5000 cells/well in 0.2mL medium. After adherence, the medium was removed and replaced by the fresh medium containing serial concentrations of DOX. Cells cultured with culture medium only served as a control. After further incubation for 48h, all the cells were subjected to MTT assay and examined by a plate reader spectrophotometer (FlexStation 3, Molecular Devices, USA) at 540nm. Growth inhibition was calculated as equation (1):

$$\text{cell inhibition (\%)}=(OD_c-OD_t)/(OD_c-OD_b)\times 100\% \quad (1)$$

Here, OD_c was the mean absorption value of the controls, OD_t was the mean absorption value of the wells treated with serial concentrations of DOX and OD_b was that of the blank wells.

Finally, the half maximal inhibitory concentrations (IC50) for different treatments were calculated based on the above data via SPSS 22.0.

Ethical approval

All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Henan University of Science and Technology (Grant No. SCXK(Yu) 2020-0005).

STATISTICAL ANALYSIS

All the data in the study were expressed as the mean± standard deviation (SD). Statistical analysis was conducted by Students' *t* test (Microsoft Excel 2019). *P* values less than 0.05 and 0.01 were regarded as statistically significant difference and highly significant difference, respectively.

RESULTS

Preparation of m-Exo

In the study, ultrasonication method was used to prepare mimetic exosomes. When the parameters were set as work 5s, interval 3s and circle 10 times, the effect of the power on the particle size of the mimetic exosomes was obvious. As shown in table 1, as the power increased, the Zeta potentials were almost unaffected, while the particle size decreased. With ultrasonic power from 300w to 500w, the change of particle size is negligible. Comprehensive consideration, 300w was chose to prepare m-Exo.

Table 1: The effect of the power on the particle size and Zeta potentials (n=3).

Power (W)	Particle size (nm)	PDI	Zeta potential (mV)
100	307±18	0.719±0.091	-25.3±0.87
200	243±20	0.468±0.038	-24.8±1.05
300	162±12	0.249±0.026	-25.7±0.97
400	153±6	0.264±0.027	-25.0±1.01
500	161±12	0.296±0.022	-25.5±1.37

In addition, the dilution medium also affect the particle sizes and Zeta potentials. Hence, 0.9% NaCl, PBS (pH 7.4) and 0.085% NaCl+0.47% glucose solution were investigated as the dilution medium. The particle sizes were displayed in fig. 1, showing the similar sizes but different distribution. When 0.9% NaCl and PBS were used as dilution medium, the particle size distributions were poor. What's more, both in 0.9% NaCl and PBS, the mimetic exosomes have low zeta potentials (-7.89mV and -10.2mV, respectively). The poor size distributions and low Zeta potentials were extremely detrimental to system stability (Alzoubi *et al.*, 2021). When the dilution medium was 0.085% NaCl+0.47% glucose, the Zeta potential of the m-Exo was -26.3mV, which is conducive to maintaining the stability of the system. Therefore, the mixed solution of 0.085% NaCl+0.47% glucose was used as the dilution medium of the m-Exo in the follow-up study.

In short, on the basis of the optimization about the preparation conditions, the preparation method of m-Exo was obtained as follows: 1ml of the RBC membrane were re-suspended in 10ml medium containing 0.085% NaCl+ 0.47% glucose and then sonicated under 300W with the parameters set as work 5s, interval 3s and circle 10 times. And then the samples were filtered through 0.45μm filter to obtain m-Exo stored at 4°C for characterization and further study.

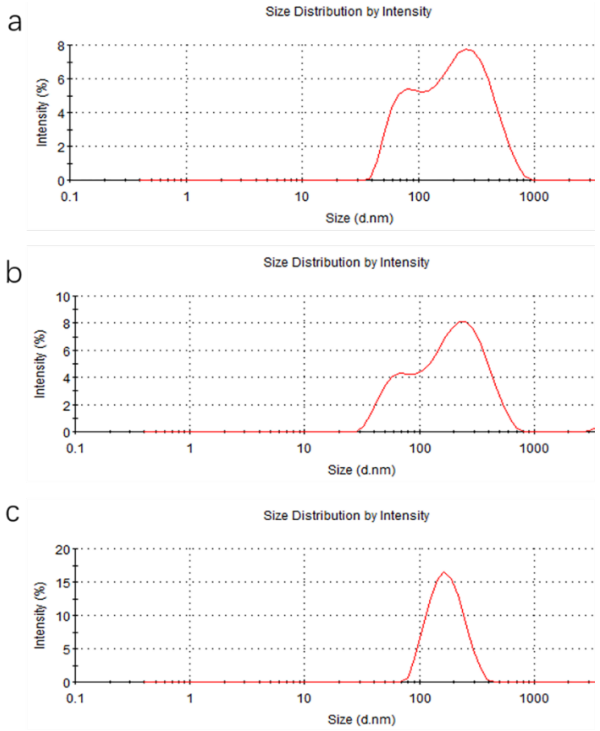

Fig. 1: The particle size distributions of m-Exo in different medium (a: 0.9% NaCl; b: PBS (pH 7.4); c: 0.085% NaCl + 0.47% glucose).

Characterization of m-Exo

The m-Exo prepared under the optimized process parameters showed spherical morphology and the size ranged from 50 to 250nm as shown in fig. 2. The particle size and Zeta potential determined by DLS were 161±10 nm with a narrow PDI 0.238±0.026 and -25.7±0.97mV, respectively. The results suggested that the particle size was uniform and the Zeta potential are appropriate, that are necessary and beneficial to maintain the stability of the system.

Preparation of DOX loaded m-Exo

DOX loaded m-Exo was prepared via co-incubation, sonication and electroporation respectively. After loading DOX, the particle sizes of DOX-m-Exo prepared via different methods had no obvious difference (data not shown). Drug loading is an important index of preparation evaluation. Meanwhile, the noteworthy difference in drug loading was displayed in table 2. We could see that the drug loading of DOX was lowest in the co-incubation

method, while the highest drug loading was obtained in the electroporation method and the drug loading tends to increase with the increase of voltage. With the voltage from 300 V to 400 V, the drug loading was basically stable. Therefore, DOX-m-Exo prepared by electroporation under the condition of power 300W, capacitance 500μF, Electric Resistance 500Ω was used for further study.

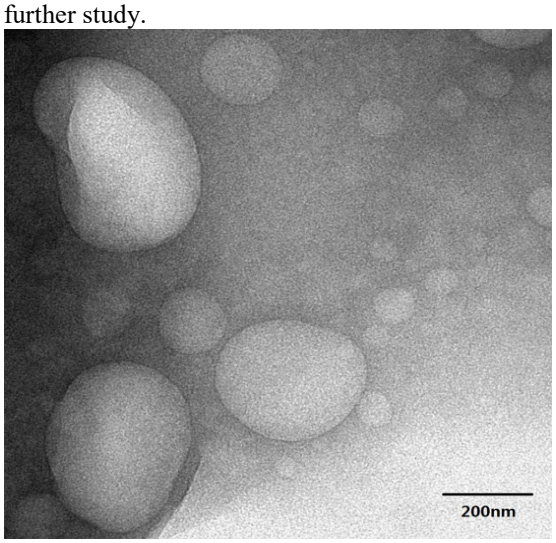

Fig. 2: The transmission electronic microscope image of m-Exo. Scale bar, 200nm.

Table 2: The drug loading of DOX in DOX-m-Exo prepared by different methods (n=3)

Methods		DOX (μg)/m-Exo(ml)
co-incubation	1h	2.24±0.41
	2h	2.85±0.32
	4h	2.79±0.37
sonication	15 min	30.1±2.41
	30 min	32.8±3.33
electroporation	200V	42.8±2.19
	300V	57.1±2.51
	400V	57.8±2.39

In vitro release study of DOX-m-Exo

The release profiles of DOX from different formulations were shown in fig. 3. It revealed that the release rate of DOX from m-Exo was considerably slower than that from DOX solution. In the solution, DOX released up to 93.4% within 4 hours, while 86.2% of DOX released from DOX-m-Exo needed 24 hours. The results suggested that DOX-m-Exo possessed sustained release effect, which was beneficial to prolong the system circulation of DOX for chemotherapy. And the phenomenon was expected.

Cytotoxicity study

The cytotoxicity of DOX-m-Exo against MCF-7 were tested and compared with that of free DOX. As shown in fig. 4, the effect of m-Exo on the cell proliferation was negligible and the cytotoxicity of DOX-m-Exo against MCF-7 was dose-dependent. There was no statistically significant difference between free DOX and DOX-m-Exo (p>0.05). However, the IC50 of DOX-m-Exo (1.04±0.055μg/ml) were higher than that of free DOX (0.83±0.045μg/ml). The cytotoxicity study showed that the cytotoxicity of DOX-m-Exo had no obvious change compared with free DOX. Therefore, m-Exo could be regarded as a superior delivery vehicle for DOX.

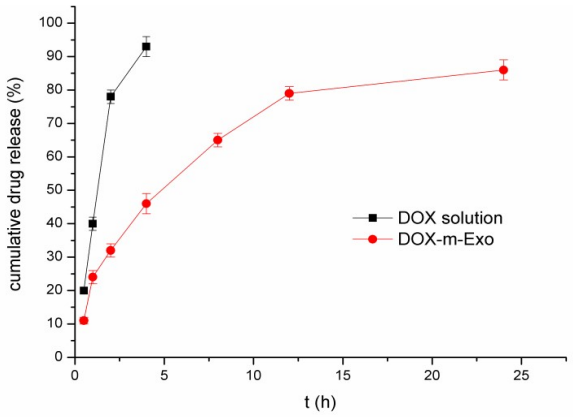

Fig. 3: *In vitro* release profiles of DOX-m-Exo and DOX in PBS (pH 7.4). (n = 3)

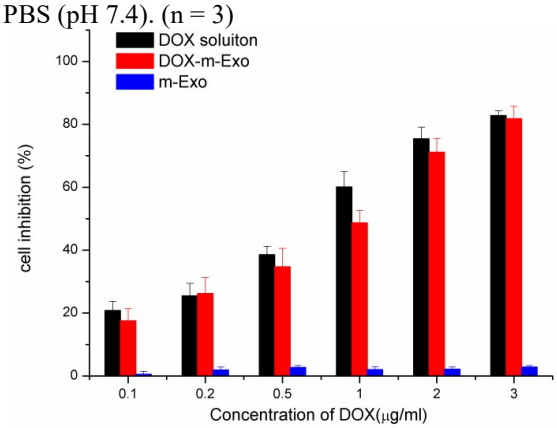

Fig. 4: Concentration-cell inhibition rate charts of blank m-Exo, DOX-m-Exo and DOX Solution against MCF-7 cells. X-axis represents a series concentration of DOX for DOX-m-Exo and DOX Solution. For m-Exo, x-axis represents a series corresponding concentration of m-Exo equal to that in DOX-m-Exo. (n=3)

DISCUSSION

It is well known that both the relatively narrow size distribution and appropriate Zeta potential are beneficial to system stability (Alzoubi *et al.*, 2021). The ionic strength is one of the main reasons that affect the Zeta potential of the particles (Chakladar *et al.*, 2022). Additionally, the shape of the particles also affected the system stability. Generally, Sphere is the most favorable shape for the stability of the system (Singh *et al.*, 2022). The surface of erythrocyte membrane is spiny both in PBS and NaCl. Fortunately, the Red blood cells can present a natural shape in the solution containing 0.085% NaCl and 0.47% glucose (Wu, Wang, Kang, 2013). What's more, 0.085% NaCl+0.47% glucose as the

dilution medium, the Zeta potential of the m-Exo was-25.7mV, which is conducive to maintaining the stability of the system. Considering comprehensively, 0.085% NaCl+0.47% glucose was selected as the dilution medium in this study to ensure the preparation of a stable m-Exo delivery system. High drug loading is also necessary for a good preparation. Therefore, in the optimization of preparation methods for DOX-m-Exo, electroporation method was selected with drug loading as the evaluated index.

The slow release rate of DOX-m-Exo could be attributed to m-Exo. Because m-Exo has a lipid double-layer membrane structure, DOX existed in m-Exo needed to pass through the double-layer membrane at first during release (Mol *et al*., 2019). And the slower release rate was beneficial to prolong the system circulation of DOX for chemotherapy.

In the cytotoxicity study, the negligible effect of m-Exo on the cell proliferation and the cytotoxicity of DOX was what we expect. The effect of m-Exo on the cytotoxicity of DOX could be explained from two aspects. On the one hand, the formulations were incubated with cells for 48 h before MTT analyzed. Therefore, a majority of DOX in DOX-m-Exo was released in the medium, which resulted in a similar cytotoxic performance with that of DOX solution. On the other hand, DOX encapsulated in the m-Exo could avoid the direct exposure to the cells (Immordino *et al.*, 2003), which decreased the cytotoxicity of DOX-m-Exo as compared with DOX solution. In order to increase the cell proliferation inhibition and even the antitumor effect *in vivo*, m-Exo could be modified with some functional groups or ligands (Feng *et al.*, 2021; Wang *et al.*, 2017).

CONCLUSION

In the study, m-Exo was designed and successfully prepared with spherical morphology. And the particle size and Zeta potential were 161nm with a narrow PDI 0.238 and -25.7mV, respectively, the mixed solution of 0.085% NaCl and 0.47 % glucose as the dilution medium. The drug loading of DOX-m-Exo prepared by electroporation method was up to 57μg/ml. What's more, DOX-m-Exo shown an expectant sustained release behavior and considerable cytotoxicity to DOX solution. We will further perform the pharmacodynamics and safety study of DOX-m-Exo *in vivo* to lay the foundation for m-Exo as an alternative and promising delivery system with the intrinsically biocompatibility and non-immunogenicity.

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