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Lamotrigine loaded nano-liposomes enhance brain selectivity *in vivo*

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Abstract: For many patients with refractory epilepsy, antiepileptic drugs (AEDs) cannot reach effective therapeutic concentration in brain due to drugtolerance. In order to increase the selectivity of lamotrigine in brain, lamotrigine loaded nano-liposomes (LTG-NL) were designed, prepared, and the physio-chemical characterizations were observed. The distribution of LTG-NL in mice was studied by detecting the concentration of LTG extracted from animal organs, then targeting efficiency (TE) and targeting index (TI) were calculated to evaluate the brain targeting effect of LTG-NL. The mechanism of LTG-NL entry into cell was determined by A549 cell internalization experiments. The results showed that LTG-NL were small and uniform spherical particles with high entrapment efficiency and release. *In vivo* distribution study showed brain selectivity of LTG-NL, and TE and TI values further demonstrated the targeting capacity of LTG-NL. The cell internalization of LTG-NL was mainly by the pathway of clathrin-mediated endocytosis and macropinocytosis. These findings suggested this lipid formulation would be a drug delivery system for insoluble drugs to promote drug release and enhance brain selectivity.

Keywords: Epilepsy, drug delivery system, lamotrigine, nano-liposomes, brain targeting.

INTRODUCTION

Epilepsy is a group of chronic brain diseases caused by abnormal discharge of neurons in brain and characterized by sudden, transient and repetitive seizures (Manford, 2017). Manyantiepileptic drugs (AEDs) are available to reduce the frequency and severity of epileptic seizures. However, because of the limitation of blood brain barrier (BBB) and some biological factors, AEDs are blocked from entering the brain and cannot maintain effective therapeutic concentration in brain (Grabrucker *et al*., 2016). In addition, some drugs are pumped back into the blood vessels by the molecular efflux pump on BBB, reducing the accumulation of AEDs in the brain (Potschka, 2012; Li *et al*., 2017). Therefore, 20-40% of patients with refractory epilepsy still cannot be treated effectively with AEDs because of drug resistance. However, several new approaches had been probed to solve these problems, including drug delivery systems, prodrugs, efflux pump inhibition and hyperosmolar BBB opening (Wang *et al*.,2015; Pardridge, 2016; Hajal *et al*., 2018). Among these methods, drug delivery systems were the main strategies to study, and nano-sized drug carriers had been already developed, such as liposome, nano-particle, nano-emulsion, etc. It is reported that Carbamazepine (CMP)-loadedmicroemulsions (CMPME) were developed for intranasal delivery in the treatment of epilepsy. The results showed higherintranasal uptake of the CMP into the rat brain. (Patel *et al*., 2016). Meenu evaluated the efficacy of sodium valproate encapsulated in nanoparticles in pentylenetetrazole (PTZ) induced acute and kindling models of seizures in male Wistar rats (Meenu *et al*., 2019).

Liposome is a kind of bilayer structure formed by phospholipids dispersed in aqueous phase which can be used as a carrier of hydrophobic, hydrophilic and amphiphilic drugs. Therefore, due to its biodegradability, nontoxicity and immunogenicity, liposome has been widely used for drug delivery. Moreover, liposome can be transported to brain via passive transport because of high lipophilia, and the surface of liposome can be further modified by different molecules to target specific organ (Khan *et al*., 2018).In this study, lamotrigine (LTG) is a new broad-spectrum antiepileptic drug, but its use was limited due to absorption affected by food (Burgoa *et al.*, 2007), low aqueous solubility and sub-therapeutic plasma drug levels (Mohan and Gundamaraju,2015),and less probability to cross the blood-brain barrier because of tight junction of p-glycoprotein (Potschka *et al.*, 2002). Furthermore, drug tolerance had been developed (Srivastava and White, 2013).Thus LTG-NL were prepared in order to enhance the selectivity of drugs in brain and reduce its side effects. The physio-chemical characterizations and distribution in mice of LTG-NL were studied to evaluate the brain targeting effect.

MATERIALS AND METHODS

Lamotrigine was purchased from Key Organics Ltd. (KS-1074, UK). Lecithin was from Acros Organics. (A0346624, USA). Cholesterol was purchased from bjke bio Ltd. (Beijing, China). Tween 80 was obtained from MP Biomedicals Co., Ltd. (103170, USA). Sephadex G-50 was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Chloroform was obtained from Modern Oriental Fine Chemicals Co., Ltd. (Beijing, China). Methanol was purchased from Fisher Scientific Inc. (USA). All the reagents were of analytical or HPLC grade.

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Male ICR mice (18-22g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in laboratory animal barrier system. Animal experiments followed the Principle of Laboratory Animal Care, and were in accordance with the protocol approved by the Experimental Animal Care Committee of Capital Medical University.

Preparation of LTG-NL

The thin-film hydration method was used to prepare LTG-NL (Hao *et al*. 2013). Firstly, moderate amount of lecithin and cholesterol were dissolved in chloroform solution containing 5% methanol. LTG was added into the above solution according to certain ratio of lipid to drug which was 1:10 (w:w) determined by orthogonal test. The solvent in solanum bottle were removed by rotary evaporator, thus the film was formed and dried under vacuum overnight. Then the phosphate buffer (pH7.45) containing 2% tween 80 was hydrated for 30 minutes at 37^{o}C. After that, the probe ultrasonic method was used to further disperse the hydrated liquid for 5 minutes under ice baths. At last, a milky light suspension was obtained and stored at 4^{o}C.

Physio-chemical characterizations of LTG-NL

LTG-NL samples were diluted with phosphate buffer saline (PBS,1:9, v/v, pH 7.45) and distilled water respectively, then the particle size and zeta potential were measured by zeta potential nanoparticle size analyzer (ZetaPlus, Brookhaven Instruments Corp, USA). Meanwhile, the samples were dispersed in distilled water and dripped onto the surface of cover slides and copper mesh and dried at 37^{o}C. Then the morphology was observed by scanning electron microscopy (SEM) (JSM-6360LV, JEOL, JAPAN) and transmission electron microscope (TEM) (JEM-1230, JEOL, JAPAN). The enthalpy values of LTG, blank liposome, physical mixtures of LTG and blank liposomes, and LTG-NL powder which was lyophilized at -80^{o}C were determined by Differential Scanning Calorimeter (DSC) (DSC 204F1 Phoenix, NETZSCH, Germany) (Lopes *et al*.2014). The scanning temperature ranged from 20 to 300^{o}C and the DSC curves were obtained.

0.5ml of LTG-NL samples were added to dextran gel column (Zhou*et al*. 2006) and eluted with distilled water at a flow rate of 1.5ml·min^{-1}. The eluent was received with EP tubes and demulsified with methanol (1:9, v/v), then the fluid was filtered with microporous membrane (0.45μm) and the drug concentration was determined by High Performance Liquid Chromatography (HPLC) (Waters e2695, Waters, USA). Meanwhile, 0.5ml of LTG-NL were directly demulsified to determine the total content of LTG in the liposomes. The encapsulation efficiency was calculated as follows (Ling *et al*., 2017):

$$\text{Encapsulation } \textit{Efficiency} = \frac{\text{The amount of drug loaded in the liposomes}}{\text{The total amount of drug}} \times 100\%$$

In vitro release

Considering the pH value of cerebral cortical fluid is weak alkaline (pH7.45), Phosphate Buffered Saline (PBS, pH7.45) was selected as *in vitro* release medium to simulate the drug release environment in brain tissue. LTG-NL were dispersed in 1ml of PBS solution and added into a dialysis bag (MW=4000), then tightly bundled dialysis bags were immersed into 30ml of release medium in EP tube. Meanwhile, LTG suspension containing the same amount of LTG was prepared as control. The EP tubes were placed into a shaking water bath with a shaking speed of 120 rpm at 37^{o}C. 1 ml of medium solution was collected at different time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24 h) and fresh PBS was supplemented in time (Mishra *et al*. 2011). The content of LTG in solution at each time point was measured by HPLC.

Biodistribution of LTG-NL in vivo

After a week of adaptive feeding, eighty-four ICR mice were randomly divided into two groups (LTG group and LTG-NL group, n=6). The mice were deprived of food, but not of water before the experiment. Then the mice were injected intraperitoneally with LTG suspension and LTG-NL (containing the same amount of LTG) respectively at a dose of 8 mg kg^{-1}. Blood was drawn from retro-orbital sinus at various time intervals (0.25, 0.5, 1, 1.5, 2, 3, 4 h) after the injection, and was centrifuged at 3000 rpm for 10min. The plasma of 200ml was transferred to another tube and added 400ml of methanol, mixed with vortex and centrifuged at 10000 rpm for 10min (Centrifuge 5810 R，eppendorf，Germany) (Lalic *et al.*, 2011; He *et al*., 2012; Russo *et al*., 2013). Following the extraction, the supernatant of 500μl was taken, concentrated and dried. At last, 200μl of methanol was added to dissolve the residue and filtered by microporous membrane (0.45μm). The blood samples were obtained and the content of LTG was detected by HPLC. Immediately after taking blood, the animals were sacrificed. The brain, liver, spleen and kidney tissues were collected, weighed and homogenized with PBS solution (pH7.45, tissue weight: PBS volume = 1:4). The tissue homogenate was centrifuged at 6000rpm for 10min and 400ml methanol was added to the centrifugal solution of 200ml and mixed with vortex. After centrifuging at 10000rpm for10min, the supernatant of 500ml was concentrated and dried under vacuum. The residue was re-dissolved by methanol and filtered by microporous membrane (0.45μm) (Walker *et al*., 2000; Gaillard *et al*., 2012). The content of LTG was analyzed by HPLC (Serralheiro *et al*., 2013) on a C18 column (250 mm×4.6mm, 5μm). The mobile phase was methanol: 5 mmol L-1 sodium dihydrogen phosphate (40:60, v: v; pH6.25) with a flow rate of 1.0ml min-1. The column temperature was 30^{o}C and the detection wavelength was 308nm.

Brain-targeting capacity evaluation

The targeting ability of LTG-NL was evaluated by the following parameters. The area under concentration-time curve (AUC) was calculated according to the logarithmic trapezoidal rule (Li *et al*., 2009), and the formula used for targeting efficiency (TE) and targeting index (TI) were as follows:

$$TE = \frac{(AUC_{0\sim\infty})brain}{(AUC_{0\sim\infty})plasma} \quad TI = \frac{(AUC_{0\sim\infty})^{i}_{LTG-NL}}{(AUC_{0\sim\infty})^{i}_{LTG}}$$

In formula i represent different tissues, including plasma, brain, liver, spleen and kidney. The higher the values for these parameters, the stronger the organ targeting ability of the formulation.

A549 cell internalization study of LTG-NL

The human non-small cell lung cancer A549 cells were inoculated with 6-well plate with 2.5×10^4 cells per hole. After 36 hours of inoculation, the medium was replaced by a cell pathway inhibitor solution diluted with serum-free 1640 medium. The four inhibitors were chlorpromazine hydrochloride (10μg ml^{-1}), M beta CD (5 mM), genistein (20μM) and wortmannin (0. 8μM) respectively. After inhibition for 1h, the medium was replaced by a co-solution of LTG-NL labeled with rhodamine (lipid concentration 20μg ml^{-1}) and the above inhibitors. The cells were collected after 3 hours of continued action of inhibitors at 37°C, then the fluorescence intensity of rhodamine was measured by flow cytometry(BD LSRF ortessa，BD, USA), and the inhibition rate of endocytosis was calculated.

STATISTICAL ANALYSIS

Data were reported as means ± standard deviation (X±SD) and analyzed by SPSS 22.0 for Windows software package. T test was used to compare differences between two groups and $p<0.05$ or $p<0.01$ was used as level of statistical significance.

RESULTS

Characterizations of LTG-NL

The average diameter of LTG-NL was (67.7±0.6) nm and zeta potential was (-11.96±0.91) mV. The morphology of LTG-NL observed by SEM and TEM was small and uniform spherical particles (fig. 1), which was consistent with the results of particle size measurement.

The entrapment efficiency of LTG-NL was determined by sephadexG-50column elution method which liposomes in solution could be separated from free drug and the content of LTG in elution fluid was determined by HPLC quantitative analysis. According to the formula, the loading efficiency was (90.54±0.31) % (n = 3).

DSC results (fig. 2) showed that the endothermic peaks of LTG and blank liposomes (BL) were 219°C (H = 145 J g^{-1}) and 141°C (H = 20 J g^{-1}) respectively. But the main phase transition temperature of physical mixture of LTG and blank liposomes (LTG + BL) were 216°C (H = 0.3 J g^{-1}) ， and a broad and flat BL curve with peak at 140.8°C (H = 5 J g^{-1}). The lower enthalpy suggested that LTG is partly covered by the physical mixture, but the blank liposomes cannot encapsulate LTG. However, the DSC curve of LTG-NL only showed an endothermic peak (132°C, H = 28 J g^{-1}) which was liposomes with increased enthalpy, but LTG peak disappeared, indicating that the prepared nano-liposomes successfully encapsulate LTG.

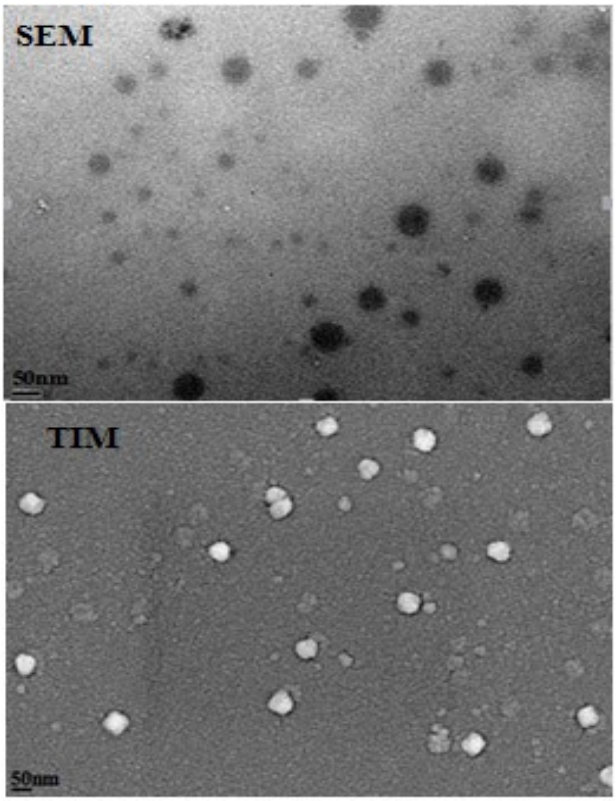

Fig. 1: The morphology of LTG-NL under SEM and TEM. (Scale bar 50nm)

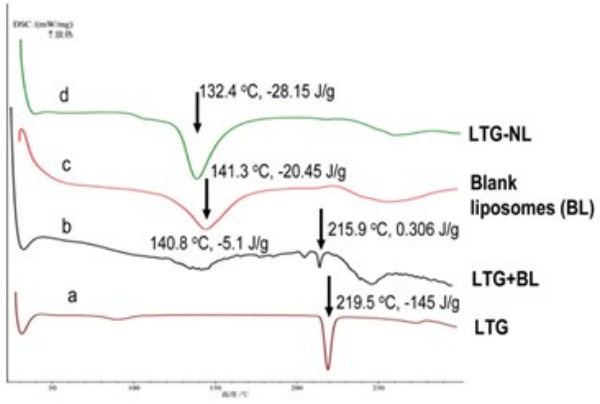

Fig. 2: DSC thermograms of free LTG (a), blank liposomes (BL, c), the physical mixture of LTG and BL (b), LTG-NL (d)

In vitro release

The molecular weight of the dialysis bag in the release medium was 4000, which was much higher than the molecular weight of the drug 256.09, so the release of LTG was the dissolution rate limit, not the diffusion controlled. The cumulative release curves of LTG suspension and LTG-NL *in vitro* were shown in fig. 3. The results showed that the drug release from LTG suspension was about 33% at 0.5 h, while was a little higher than LTG-NL (20%). However, in the following time intervals the drug release from LTG-NL was increased quickly, which was 62% and 75% at 2h and 4h respectively, but the release rate of LTG suspension was only 46% and 55% ($p<0.05$). At 8 h the drug release percentage of LTG-NL was almost complete (90%), but that of LTG suspension was only 65% ($p<0.05$) and without drug release until 24h due to its low aqueous solubility in medium. The increased drug release rate of LTG-NL compared to that of free drug suggested that nano-liposome as a drug carrier could promote drug release in brain mimic fluid.

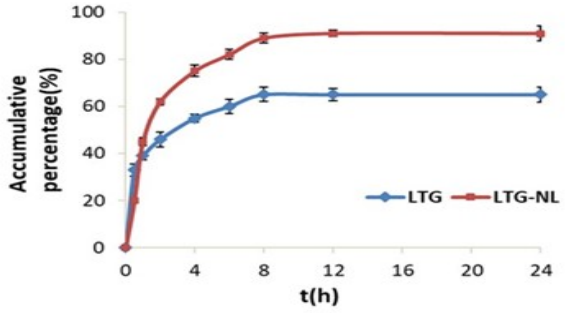

Fig. 3: *In vitro* LTG release profiles from LTG suspension (diamond) and LTG-NL (square). n=5

Biodistribution of LTG-NL in vivo

The concentration of LTG in plasma, brain, liver, spleen and kidney at different time intervals were displayed in fig. 4. At first time point (5min), although plasma concentration of LTG group was a little higher than that of LTG-NL group, in the following time intervals much higher concentration of LTG in plasma was detected in LTG-NL group than LTG suspension group ($p<0.05$/ $p<0.01$). The results were consistent with that of *in vitro* release experiment. Moreover, at each time point the level of LTG in brain of LTG-NL group were significantly higher than that of LTG group ($p<0.05$/ $p<0.01$). Meanwhile, the distribution of LTG-NL in liver and spleen was a little higher than that in LTG group ($p<0.05$), and may be due to the passive targeting effects. But in kidney the level of LTG-NL was significantly lower than that in LTG group ($p<0.05$/$p<0.01$).The outcome confirmed the amount of LTG-NL in brain were significantly higher than that of LTG group, furthermore, the higher plasma concentration and lower kidney clearance of LTG-NL prolong its duration time in brain tissue compared with LTG, suggesting LTG-NL improve the selectivity of LTG and increase the distribution of LTG into mice brain.

Brain-targeting capacity evaluation

Table 1 listed the AUC values of different organs of two groups and the corresponding targeting parameters TE and TI were calculated according to the formula. The AUC values in brain, liver and spleen of LTG-NL group were 1.47, 1.16 and 1.18 times higher than those of LTG group respectively, indicating that nano-liposomes distribute more into brain, liver and spleen compared with free drug. While, AUC value of kidney in LTG group was 1.45 times higher than that of LTG-NL group, indicating that the free drug was cleared more quickly by kidney, but the drug loaded in liposome was metabolized slowly and extend duration *in vivo*. TE evaluates the organ selectivity of free drug and formulation. If the TE values larger than 1, this means the formulation is more selective for a target organ than plasma; the greater the TE value, the stronger the selectivity. The calculated results showed TE of suspension was only 0.9, but TE of formulation was greater than 1(1.125), which revealed that LTG-NL increased the brain selectivity of LTG. TI was used to further compare the organ selectivity between free drug and formulation. TI value for brain was the highest during these organs, which demonstrated LTG-NL significantly increased the concentration of LTG in brain tissue and had good brain targeting capacity.

A549 cell internalization study of LTG-NL

The results of pharmacokinetics studies *in vivo* showed that LTG-NL had a good effect targeting the brain. In order to understand how the nano-liposomes enter into the cell, human non-small cell lung cancer A549 cell line was used as cell model to further study the endocytosis mechanism of LTG-NL at the cellular level.

Fig. 5 showed the change in endocytosis of nano-liposomes in the presence of four inhibitors. The control group was set as normal endocytosis (100%) without inhibitors. The effects of four inhibitors were as follows: chlorpromazine hydrochloride blocked reticulin-mediated endocytosis; M beta CD was a cholesterol depleting agent and blocked lipid rafts-mediated endocytosis; genistein inhibited caveolin-mediated endocytosis; and wortmannin blocked phagocytosis of macrophages pathway (Koivusalo *et al*., 2010; Rejman *et al*., 2005; Xiao *et al*., 2011). It was obvious that the cellular internalization of nano-liposomes inhibited by chlorpromazine hydrochloride and wortmannin was significantly lower than that of the control group ($p<0.01$/ $p<0.05$), while the uptake of nano-liposomes through the other two channels inhibited by M beta CD and genistein almost were not influenced. These results indicated that the cellular uptake of LTG-NL mainly through reticulin-mediated endocytosis and phagocytosis of macrophages.

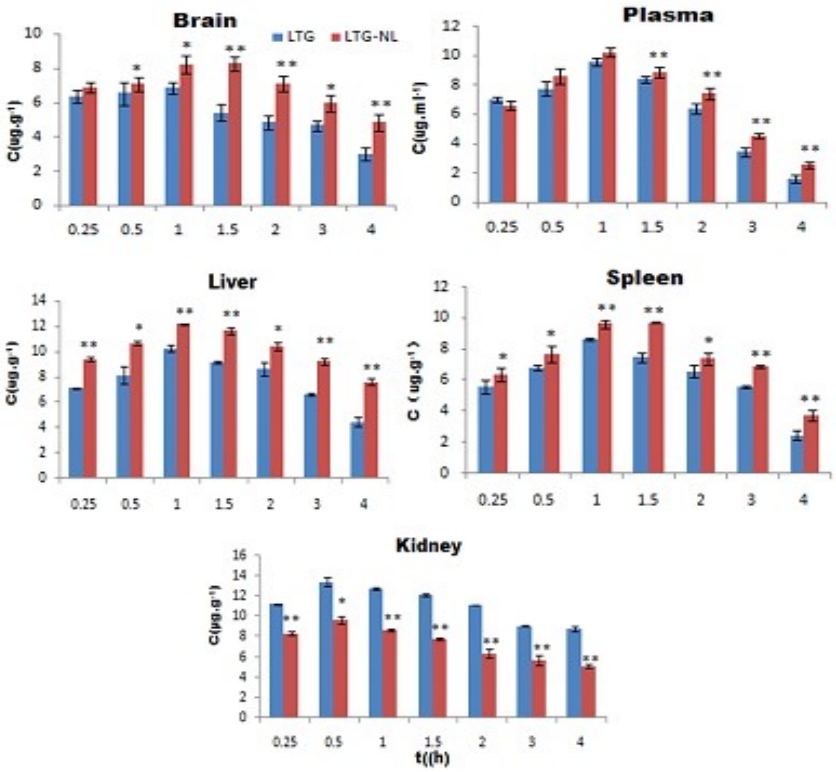

Fig. 4: Comparison of LTG concentration between LTG suspension and LTG-NL group in plasma and tissues of mice at different time point. (n=6)(*p<0.05/**p<0.01, significant difference compared with LTG group)

Table1: Comparison of AUC values between LTG suspension and LTG-NL

AUC	LTG suspension	LTG-NL
Plasma (μg.h·ml $^{-1}$)	21.196±1.52	25.223±1.642
Brain (μg.h·g $^{-1}$)	19.534±1.036	28.790±1.78
Liver (μg.h·g $^{-1}$)	29.717±2.141	34.451±2.518
Spleen (μg.h·g $^{-1}$)	23.442±1.273	27.648±2.014
Kidney (μg.h·g $^{-1}$)	39.098±2.946	26.927±1.835

AUC= area under concentration-time curve; LTG suspension= LTG suspension group; LTG-NL= lamotrigine loaded nano-liposomes group.

But the phagocytosis of macrophages is generally suitable for larger particles, so it is speculated that physical aggregation of nano-liposomes may occur *in vitro*.

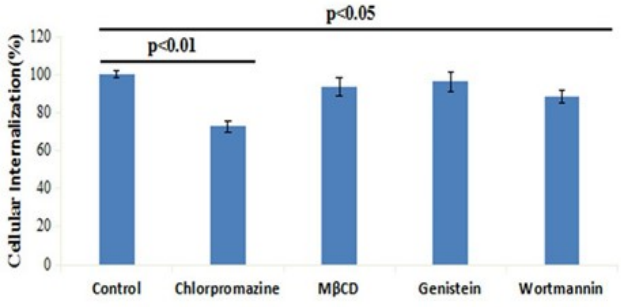

Fig. 5: A549 cell internalization study of LTG-NL with inhibitors

DICUSSION

In our study, the objectives were to develop LTG-loaded nano-liposomes to improve brain selectivity. Many experiments had been done to determine whether the formulation had the brain targeting effect. The morphology pictures showed LTG-NL were uniform little particles with average size about (67.7±0.6)nm and zeta potential was (-11.96±0.91) mV, which enhance the drug entrance into brain because the findings of MacKay et al suggested the ideal nanoparticle penetrated in brain would be less than 100nm in diameter and have neutral or negative surface charge (MacKay *et al*., 2005) The prescription of LTG-NL contained tween 80 due to tween 80 is a kind of nonsionic hydrophilic surfactant, which contains both hydrophilic and hydrophobic groups. It is reported tween 80 could improve the compatibility of

insoluble drugs with lipid components, reduce the precipitation of liposomes and improve the entrapment efficiency (Woodle and Lasic, 1992). Moreover, liposomes and tween 80 mixed together could build a "stereo" barrier layer by physical adsorption on the surface of liposomes, so as to improve the stability of liposomes (Yuan *et al*., 2017). According to DSC pattern of LTG-NL, disappeared endothermic peak of LTG proved that LTG was successfully loaded in liposomes and high entrapment efficiency was obtained for (90.54±0.31) %. Furthermore, liposomes modified with tween 80 cannot be easily recognized and ingested by plasma proteins, thus effectively increase the amount of drug reaching the target tissue (Sun *et al*., 2004; Kolter *et al*., 2015). On the other hand, the reasons for the tolerance of antiepileptic drugs mainly were the activity of efflux pump on BBB. Since tween 80 is also a P-glycoprotein (P-gp) modulator, it had been reported that nanoparticle coated with tween 80 may escape the efflux by the P-gp transporter, and then improve brain capillary endothelial cell uptake of nanoparticles (Van *et al*., 2013; Lin *et al*., 2016). These expected results were observed in the following experiments. The cumulative release curve *in vitro* showed the higher release of LTG in liposomes compared with free drug. Biodistribution *in vivo* further demonstrated the superiority of LTG-NL. At each time interval the concentration of LTG-NL in brain were significantly increased than LTG group. Although the observed times *in vivo* were only 4hours, the trend of LTG-NL distribution in brain was clearly. Moreover, the targeting efficiency TE and TI value also showed the advantages of LTG-NL compared to LTG (Pinzon *et al*., 2013). All these results indicated LTG-NL significantly enhance the concentration of LTG and prolonged its action time in brain, suggesting the targeting brain effect of LTG-NL.

Further studies at the cellular level found that LTG-NL entered into the cells mainly through the pathway of reticulin-mediated endocytosis and phagocytosis of macrophages. It is known reticulin-mediated endocytosis pathway is the main passage for nano particles to enter into cell, and the endocytosis mechanism of prepared nano-leveled LTG-NL conform to the findings. Moreover, coating with tween 80 may enhance the nanoparticles internalization via receptor mediated endocytosis by brain endothelial cells (Jose *et al*., 2014). But the phagocytosis of macrophages is generally the pathway for larger particles, the preparation process of LTG-NL should be improved to avoid physical aggregation.

CONCLUSION

In present study, developed LTG-NL showed appropriate size, high entrapment efficiency, and increased release percentage *in vitro*. Higher concentration of LTG in mice brain of LTG-NL demonstrated more LTG distributed into brain compared with LTG suspension, indicating LTG-NL could improve the entrance of LTG into brain. Moreover, TE and TI value further demonstrated the brain targeting capacity of LTG-NL. These findings suggested this lipid formulation would be a drug delivery system for insoluble drugs to promote drug release and enhance brain selectivity.

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