Studies of Mucosal Irritation and Cellular Uptake Mechanisms of Xingnaojing Nanoemulsion

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Xingnaojing (XNJ) injection was used to treat pneumonia and stroke in clinic in China, but with poor patient compliance. Xingnaojing nanoemulsion for intranasal delivery was developed to improve it. This article tried to evaluate the mucosal irritation of Xingnaojing nanoemulsion and investigate cellular uptake mechanism of its encapsulated lipophilic drugs. The toad palate model and rat nasal mucosa model were used to study the nasal ciliotoxicity and nasal mucosal irritation of nanoemulsion to evaluate its safety intranasally. The cellular uptake mechanism was studied by Calu-3 cell model. Coumarin 6 was encapsulated in nanoemulsion and the endocytic pathways were studied by cellular uptake experiments after being treated with different inhibitors. In toad palate model, the cilia movement of Xingnaojing nanoemulsion group last for 467.40 ± 39.02 min, which was obviously longer than deoxycholate group (90.60 ± 15.40 min). Studies on rats showed that the damage caused by nanemulsion is capable of being recovered. Nanoemulsion uptake was reduced obviously when cells were treated with wortmannin, and it also decreased about 13% when the temperature reduced from 37°C to 4°C. Mucosal irritation caused by nanoemulsion is low and the damage is recoverable. The cellular uptake of Xingnaojing nanoemulsion is energy-dependent, and macropinocytosis was the most important pathway for cellular uptake.

Keywords: Nasal ciliotoxicity. Endocytosis. Energy-dependent. Micropinocytosis.

INTRODUCTION

XNJ is a compound preparation composed of both lipophilic compounds (borneolum, muscone, curcumae volatile oil) and hydrophilic compound (gardenia extract). Stroke is characterized by high incidence, high disability rate, high mortality rate and high recurrence rate. It is one of the main diseases that threaten the aging society, and it is considered as the top four major incurable diseases in traditional chinese medicine. XNJ injection is a well-known patent medicine in China, which has the functions of clearing heat, transforming phlegm, and opening the orifices, and has been widely used in the treatment of pneumonia, hyperpyrexia, and stroke in clinic (Wang et al., 2011).

Nanoemulsion is a drug delivery system with low-viscosity and thermodynamic stability, formed by oil phase, water phase, emulsifier and co-emulsifier in an appropriate ratio. As a new drug carrier, it can improve the dispersion of drugs, pass through the biological barriers easily, and significantly increase the rate of cell uptake of drugs, thereby it enhances the brain-targeting of drugs (Kumar et al., 2008a, 2008b; Pangeni et al., 2014). Compared with semi-solid and solid preparations (such as nasal dry powder preparations), liquid preparations have good fluidity, which are more convenient for

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administration, complicated delivery device is not required. For aqueous solutions, nanoemulsion has a certain viscosity, which can prolong the contact time of the drug with the nasal mucosa and decrease the clearance of nose. Compared with nanoparticles, nanoemulsions have a higher drug loading (Luo et al., 2014). In addition, nanoemulsion contains a certain proportion of emulsifier, which is also helpful for drug absorption (Comfort et al., 2015). Compared to liposomes, nanoemulsions are more stable. So, the use of nanoemulsion in nasal delivery system may increase the amount of drugs in brain and improve the bioavailability.

A study showed that the nasal routes can be exploited for the delivery of drugs (Ruigrok, De Lange, 2015). Nasal administration is a high-potential way with good patient compliance (Kameswara et al., 2015). Nasal epithelial membrane is of high permeability, and it can provide a pathway from the nose to the brain directly, avoiding the first-pass metabolism, which is of great advantage for brain diseases (such as stroke) treatment (Graff, Pollack, 2005; Illum, 2010). The nasal preparation of nanoemulsion can not only take advantage of the characteristics of rapid absorption, convenient use, and high-efficiency of brain targeting, but also has “super” dissolving ability, which is especially suitable for large-dose traditional Chinese medicine with ingredients of different polarity. Wen (2014) Pharmacokinetic studies of borneolum, a liposoluble component, after administration of XNJ nanoemulsion via different routes (intravenous injection and nasal administration), showed that it could be rapidly absorbed into the blood \( T_{\text{max}} < 2 \text{ min} \) after nasal administration, and the AUC was higher than that of intravenous administration, and the relative bioavailability was 125.59%; and the mean retention time (MRT) after the nasal administration was significantly longer than that of intravenous administration. In addition, intravenous injection operation is inconvenient for most patients to deal with when there is a medical emergency. To some extent, this inconvenience may delay the rescue time and increase the risk of physical disability or death, especially in the areas with insufficient emergency medical resources. Therefore, nasal administration is more appropriate in this case. However, the nasal mucosa is relatively vulnerable, the pharmaceutical excipients in the preparation of XNJ nanoemulsion may cause some irritation. In some severe cases, they may affect the normal physiological function of the nasal cavity (Chu et al., 2013). It is necessary to evaluate the nasal mucosa irritation of the XNJ nanoemulsion. In this study, assessments were carried out with the toad palate model and rat nasal mucosa model. At the same time, Calu-3 cells were cultured for the uptake study of XNJ nanoemulsion to clarify some transport mechanisms in the process of transnasal mucosal administration. Coumarin compounds have the properties of reducing the background fluorescence interference from cell components and physiological fluids, and are widely used in the study of biological pathways. Among them, Coumarin-6, a lipophilic fluorescent substance, is stable and has a sufficiently large Stokes shift to avoid the overlap of excitation spectrum and emission spectrum (Zhang et al., 2013). With a small amount of Coumarin 6 encapsulated in the drug delivery system, it can show strong fluorescence (Eley, Pujari, McLane, 2004; Li et al., 2017). Since the surfactant formed an emulsified film, lipophilic drugs were encapsulated in the nanoemulsion. Both Borneolum and Coumarin 6 are lipophilic molecules, so Coumarin 6 can be used as a model drug to visualize the transport and uptake of nanoemulsion. In this study, Coumarin 6 was encapsulated in nanoemulsion to display the position of nanoemulsion particles in the cell under confocal laser scanning microscope.

**MATERIAL AND METHODS**

**Reagents**

Borneol was purchased from Guizhou Golden Pharmaceutical Co., Ltd. Gardeniae fructus \((Gardenia jasminoides\) Ellis) extract (aqueous extract), Curcumae rhizoma volatile oil \((Curcuma wenyujin\) Y.H. Chen et C. Ling) and Muschus (Drug Standard of Ministry of Public Health of the People’s Republic of China, WS-210(Z-32)-93) volatile oil (steam distilled) were self-made in laboratory according to the drug standard of Ministry of Health (WS3-B-3353-98). Isopropyl myristate (IPM) was purchased from Alfa Aesar Chemical Co.,

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Experimental animals

Mucosal irritation of XNJ nanoemulsion

Preparation of XNJ nanoemulsion

Toads (Bufo bufo gargarizans) male and female were obtained from Beijing Jinmuyang Experimental Animal Breeding Co., Ltd. Sprague Dawley (SD) rats male, 200-220 g, No.: SCXK 2010-D002 were provided by Beijing Weitonglihua Experimental Animal Technology Co., Ltd. In this research, all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Beijing University of Chinese Medicine (The approval number of the animal ethics committee is BUCM2010102003).

Mucosal irritation of XNJ nanoemulsion

Preparation of XNJ nanoemulsion

The borneol, Muschus volatile oil, Curcumae rhizoma volatile oil, IPM, Cremophor EL-35, and ethanol were accurately weighted according to the prescription dose and added in a 10 mL volumetric flask, and mixed with a magnetic stirrer (300 rpm) at 25°C until they were fully mixed. Gardenia extract (0.336 mg) was dissolved in 5 mL purified water then the solution was added into the mixture solution above slowly with continuous stirring. Finally pure water was added to volume and mixed well. Tyndall effect can be observed when the nanoemulsion was prepared.

Preparation of blank nanoemulsion

IPM, ethanol, Cremophor EL-35 were accurately weighted according to the proportion of prescription and mixed, stirring with water bath at 25°C, 300 rpm. Then 5 mL pure water was added in, mixed well.

Preparation of XNJ suspension

Borneol 0.4 g, muschus volatile oil 60 μL, curcumae rhizoma volatile oil 48 μL, gardenia extract 0.336 mg were accurately weighted in a 10 mL volumetric flask, then physiological saline was added to volume, stirring with a magnetic stirrer (300 rpm) at 25°C until mixed well. It was shaken well before use.

Nasal ciliotoxicity study of toad palate model of XNJ nanoemulsion

Nasal ciliotoxicity was studied using an in situ toad palate model (Na et al., 2010). In short, the upper palate of the toad (n=5) was exposed and immersed with tested formulation (XNJ nanoemulsion 0.2 mL, and XNJ suspension 0.2 mL) for 30 min, respectively. The physiological saline (0.5 mL) and the blank nanoemulsion (0.5 mL) were used as negative controls. The sodium deoxycholate solution (Zhu et al., 2012) (0.2 mL) which has serious nasal ciliotoxicity was used as a positive control. Then the drug solutions were washed with physiological saline. A piece of palate mucosa (about 5 mm × 5 mm) was separated and observed under a 40× optical microscope and the duration of cilia movement was recorded (Cai et al., 2011).

Nasal mucosa irritation study in rats of XNJ nanoemulsion

Nine SD rats were randomly divided into 2 groups. Six rats were treated with XNJ nanoemulsion. Three rats treated with physiological saline were taken as control group. Each rat was administrated 3 times a day (50 μL for each nostril); 24 h later, 3 rats in each group were sacrificed. After a 7-day recovery, the other animals of XNJ nanoemulsion group were sacrificed. Nasal septa were separated and stained with HE for microscopic observation (Wang, Li, Wang, 2016).
The cellular uptake mechanism of nanoemulsion

Characterization of Coumarin 6 nanoemulsion

Coumarin 6 nanoemulsion was prepared as mentioned above (XNJ was replaced by Coumarin 6). Nanometer emulsions were diluted with 5, 10, 50, 100 and 500 times respectively, and particle sizes of different groups were analyzed by Malvern Zetasizer Nano (Malvern, Worcestershire, UK) to check stability, which was expressed as mean ± standard deviation. The morphology of nanoemulsion was analyzed by a transmission electron microscope (Hitachi H-800, Japan). Nanoemulsion was diluted 100 times and then placed on a carbon-coated copper grid. The sample was prepared with negative staining: it was diluted 100 times and a drop of the resulting nanoemulsions was placed gently on a carbon-coated copper grid. After 10 minutes, 2% phosphotungstic acid (pH 7.4) was added in and then air dried.

Cell culture

Calu-3 cells were commonly used to simulate the nasal mucosa in vitro experiments to study nasal drug absorption (Martens, Hellings, Steelant, 2018). Calu-3 cells (purchased from China Infrastructure of Cell Line Resources, Beijing, China) were cultured with MEM (minimum essential medium) medium containing 10% FBS, 1% penicillin, and 1% MEM NEAA in 25 cm² culture flasks and were placed in a humidified atmosphere (5% CO₂, 95% humidity, 37°C). All procedures were performed on a super-clean bench (Heal Force; Shanghai Lihua Technology Co., Ltd., Shanghai, China) with aseptic techniques.

Cytotoxicity assays

The cytotoxicity of Coumarin 6 nanoemulsion on cells was evaluated by the methyl thiazolyl tetrazolium (MTT) colorimetric assay (Kadan et al., 2013). Cells were seeded at a density of 1 × 10⁵ cells/mL in 96-well flat-bottomed microtiter plates with 100 μL of culture medium per well. Then the 96-well plates were incubated in the incubator (series ||; Thermo Fisher Scientific, Waltham, MA) at 37°C with 5% CO₂ for 48 h. Then the medium was removed and replaced with 100 μL Hank’s buffered salt solution (HBSS, pH 7.4) containing a series of different dilutions of the nanoemulsion. 100, 300, 400, and 500-fold dilutions were prepared. HBSS (Hank’s Balanced Salt Solution) was taken as control. After incubation for 4 h, 20 μL of a 5 mg/mL MTT solution in culture medium was added to each well and the mixtures were incubated at 37°C for 2 h until blue deposits were visible. Later, after the MTT solution was discarded, the colored metabolite was completely dissolved by the addition of 150 μL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 490 nm using a Multiskan GO microplate reader (Thermo Fisher Scientific, USA). The mean absorbance of six measurements for each group was expressed as the percentage of the absorbance of the untreated control (Chen et al., 2014; Zhang et al., 2016, 2017).

Cellular distribution of the nanoemulsion

Calu-3 cells were cultured as mentioned above. Prior to treatment day, cells were seeded in a glass bottom dish at a density of 1 × 10⁵ cells/mL and cultured for 48 h. After a rinse with HBSS, the cells were incubated with Coumarin 6 solution and Coumarin 6 nanoemulsion respectively for 1 h. Extracellular fluorescence was quenched by 0.4% Trypan Blue (TB). Then, cells were washed 3 times with HBSS and 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiI, Solarbio, Beijing Solarbio Science and Technology Co., Ltd, Beijing, China) was used for membrane stain. After 3 times washing with HBSS, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Solarbio, Beijing Solarbio Science and Technology Co., Ltd, Beijing, China) was used for nuclear stain. Cells were washed 3 times again and observed with a confocal laser scanning microscope (CLSM, Olympus Corporation, Tokyo, Japan) at an excitation wavelength of 488 nm and emission wavelength of 574 nm (Yang et al., 2017).

Energy dependence cellular uptaking study

In order to investigate whether nanoemulsion uptake was an active or passive process, uptaking experiments
were carried out at different temperature (37°C and 4°C) or with uptake inhibitor. Energy dependence experiments were performed by pre-incubating the cells at 37°C, 4°C or with sodium azide (NaN_3)/2-DG (2-deoxy-D-glucose) incubation, which is known as the active uptake inhibitor. After the pre-incubations for 30 min, Calu-3 cells were incubated with nanoemulsion for 1 h, either in the presence of inhibitor or at 37°C, 4°C. Fluorescence extracellular was quenched by 0.4% Trypan Blue (TB) and then cells were washed with PBS (1 mL) three times. After that, cells were digested by 0.25% trypsin. After digestion was terminated by MEM, cell suspension was centrifuged (TDL80-2B; Shanghai Anting Scientific Instrument Factory, Shanghai, China) at 1000 rpm for 3 min, and re-suspended in 0.3 mL PBS and analyzed by a flow cytometer (BD FACS Canto TM ||, BD, New Jersey, USA).

Cellular uptake pathways of nanoemulsion

In order to investigate which pathway was involved in nanoemulsion endocytosis, Calu-3 cells were treated with four inhibitors of three major pathways: chlorpromazine hydrochloride (CPZ) and high concentrations of sucrose (0.45M) for clathrin mediated endocytosis (CME) pathway, methyl-β-cyclodextrin (M-β-CD) for caveolae/lipid raft-mediated endocytosis pathway, Wortmannin for macropinocytosis pathway. Calu-3 cells were pre-incubated with different inhibitors for 30 min, then Coumarin 6 nanoemulsion was added in and the uptaking experiments were carried out for 60 min with the presence of inhibitors. After that, fluorescence extracellular was quenched by 0.4% Trypan Blue (TB) and fluorescence intracellular was measured by a flow cytometer as mentioned above (Foerg et al., 2007; Li et al., 2010; Kennedy et al., 2013; Huang et al., 2015; Batrakova et al., 2001).

Statistical analyses

The absorbance data of MTT assay were expressed as ± SD, analyzed by ANOVA. Significance was set at P < 0.05.

Cell viability (percentage) from the MTT assay was calculated using the following equation and analyzed with SAS 8.2 software:

\[
\text{Viability} \% = \frac{A_{\text{sample}}}{A_{\text{blank}}} \times 100 \%
\]

RESULTS AND DISCUSSION

Mucosal irritation of XNJ nanoemulsion

Nasal ciliotoxicity study of toad palate model of XNJ nanoemulsion

In this study, the nasal mucosal movement time was taken as an indicator to investigate the ciliotoxicity just like some bioadhesive dosage forms (like in situ gel delivery system and bioadhesive delivery system) reported. Cilia movement time of different group was recorded and compared with physiological saline group. The relative percentage P (%) of cilia movement time was obtained by the time of experimental group divided by the time of physiological saline group. The higher the value is, the lower the irritation will be. The results are shown in Table I.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>The Cilia Sustained Movement Time (min)</th>
<th>The Relative Percentage P (%) of The Cilia Sustained Movement Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>physiological saline</td>
<td>1188.20 ± 25.67</td>
<td>100</td>
</tr>
<tr>
<td>sodium deoxycholate solution</td>
<td>90.60 ± 15.40*</td>
<td>7.62</td>
</tr>
<tr>
<td>XNJ nanoemulsion</td>
<td>467.40 ± 39.02*</td>
<td>39.34</td>
</tr>
<tr>
<td>XNJ suspension</td>
<td>213 ± 27.89*</td>
<td>17.93</td>
</tr>
<tr>
<td>blank nanoemulsion</td>
<td>503.6 ± 79.91*</td>
<td>42.38</td>
</tr>
</tbody>
</table>

Values are the mean ± SD. *P < 0.05, compared with physiological saline group.
As was shown in the Table I, compared with physiological saline, significant irritations were observed in the other groups. XNJ nanoemulsion, XNJ suspension and blank nanoemulsion can reduce the cilia movement duration, but their irritations were lower than sodium deoxycholate. Compared with XNJ suspension, nanoemulsion can reduce the irritation caused by drugs. No difference was observed between XNJ nanoemulsion and blank nanoemulsion, which indicated that the volatile oil and borneol encapsulated in the nanoemulsion may have strong irritation.

Nasal mucosa irritation study in rats of XNJ nanoemulsion

The pathological sections of different groups were shown in Figure 1. For physiological saline group (Figure 1-A), epithelial cells were arranged neatly, no hyperplasia and no edema were observed. For XNJ nanoemulsion group (Figure 1-B), cilia loss was observed on epithelial cells, gland hyperplasia can be observed in mucosa lamina propria, blood vessels were widened slightly, but these damages can be repaired after 7 days recovery, as was shown in Figure 1-C. The results indicated that, XNJ nanoemulsion has slight irritation, which may cause damage to nasal mucosa, but it is not a permanent damage, which can be recovered in a week. Irritation evaluation study illustrated the potential drug ability of XNJ nanoemulsion. XNJ can be developed into intranasal nanoemulsion replacing the injection used in clinic now.

**FIGURE 1** - Rat nasal mucosa section of XNJ nanoemulsion. A: physiological saline group; B: XNJ nanoemulsion group; C: XNJ nanoemulsion group after a recovery of a week.

The **cellular uptake mechanism of nanoemulsion**

**Preparation and characterization of Coumarin 6 nanoemulsion**

Coumarin 6 nanoemulsion was successfully developed using the same formulation and technology as XNJ nanoemulsion, as shown in Figure 2. The particle size is $17.99 \pm 0.09$ nm (diameter) with a PDI of 0.058 $\pm 0.01$ (mean $\pm$ SD, $n=3$). Moreover, no difference was observed between different dilution groups, which indicated that the nanoemulsion can keep stable when it was diluted 500 times.

It is generally believed that particle size is a key factor for nanocarriers to overcome the barrier of mucus and epithelial cells. Relevant research on particle transport has proved that diffusion is dependent on particle size, and only particles $<500$ nm can diffuse
through mucus (Sanders, Smedt, Demeester, 2000). In addition, Ahmad et al., 2017) showed that nanocarriers with a particle size of about 100 nm can be transported along the olfactory or trigeminal nerve pathways. At present, no studies have shown that transnasal delivery systems have particle size requirements for nanoparticles clearly. However, our recent research shows that when the particle size of the nano-formulation is below 40 nm, its transport characteristics in the mucosa shows moderate absorption, while the transport characteristics is difficult to absorb when the particle size is greater than 60 nm. The uptake of OECs cells to nano-formulations decreases with increasing particle size. It can be seen that the control of particle size parameters is of great value in the preparation of nano transnasal drug delivery system for increasing the amount of drugs into the brain. Based on the study of the pharmacological properties of Xingnaojing nanoemulsion in the early stage of the research group (Wen, 2014), the average particle size of Xingnaojing nanoemulsion was 17.83 nm. In this paper, the research shows that the average particle size of coumarin 6 nanoemulsion is 17.99 nm. Both of them are less than 20nm, so they can improve the bioavailability, and have wide application prospects.
Confocal laser scanning microscope presented the distribution of the Coumarin 6 nanoemulsion, as shown in Figure 3. According to Figure 3-A, Calu-3 cells were incubated with Coumarin 6 solution for 1 h, after the extracellular fluorescence was quenched by TB, fluorescence emitted by Coumarin 6 can hardly be observed in cells. However, the absorption of Coumarin 6 encapsulated by nanoemulsion was highly improved, as shown in Figure 3-B, which indicated that nanoemulsion can deliver more lipophilic drugs into cells. It is an efficient drug carrier for brain-targeting drug delivery system.

**Cytotoxicity assays**

The cytotoxicity of Coumarin 6 nanoemulsion on Calu-3 cells was shown in Table III. There was no cell cytotoxicity observed in the dilution range of 300-500-fold. So, cellular distribution and cellular uptake mechanism studies can be carried out by diluting the nanoemulsion 300 times.

**TABLE II - Particle sizes of different dilution groups**

<table>
<thead>
<tr>
<th>Different dilution times</th>
<th>$\bar{x} \pm$ SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>16.67 ± 0.0141</td>
</tr>
<tr>
<td>10</td>
<td>17.99 ± 0.0990</td>
</tr>
<tr>
<td>50</td>
<td>17.47 ± 0.1202</td>
</tr>
<tr>
<td>100</td>
<td>17.64 ± 0.0919</td>
</tr>
<tr>
<td>500</td>
<td>17.60 ± 0.0848</td>
</tr>
</tbody>
</table>

**TABLE III - The cytotoxicity to Calu-3 cells induced by Coumarin 6 nanoemulsion (n = 6)**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>$\bar{x} \pm$ SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.4056</td>
<td>0.3584</td>
<td>0.3750</td>
<td>0.3725</td>
<td>0.3900</td>
<td>0.4006</td>
<td>0.3837 ± 0.0181</td>
</tr>
<tr>
<td>100</td>
<td>0.3138</td>
<td>0.3133</td>
<td>0.3178</td>
<td>0.3143</td>
<td>0.3131</td>
<td>0.3194</td>
<td>0.3153 ± 0.0027*</td>
</tr>
<tr>
<td>300</td>
<td>0.3789</td>
<td>0.3814</td>
<td>0.3876</td>
<td>0.3746</td>
<td>0.3794</td>
<td>0.3793</td>
<td>0.3802 ± 0.0043</td>
</tr>
<tr>
<td>400</td>
<td>0.3753</td>
<td>0.4021</td>
<td>0.3955</td>
<td>0.3953</td>
<td>0.3709</td>
<td>0.3817</td>
<td>0.3868 ± 0.0043</td>
</tr>
<tr>
<td>500</td>
<td>0.3857</td>
<td>0.3950</td>
<td>0.3839</td>
<td>0.3826</td>
<td>0.3625</td>
<td>0.3725</td>
<td>0.3804 ± 0.0113</td>
</tr>
</tbody>
</table>

* $P < 0.05$, compared with control group

**Cellular distribution of the Coumarin 6 nanoemulsion**

The cytotoxicity of Coumarin 6 nanoemulsion on Calu-3 cells was shown in Table III. There was no cell cytotoxicity observed in the dilution range of 300-500-fold. So, cellular distribution and cellular uptake mechanism studies can be carried out by diluting the nanoemulsion 300 times.
Energy dependence cellular uptaking study

As shown in Figure 4, compared with the fluorescence intensity of 37 °C, nanoemulsion uptake was reduced from 100% to 87.32% at 4 °C. When Calu-3 cells were incubated with (NaN$_3$)/2-DG, nanoemulsion uptake was obviously inhibited (from 100% to 44.48%). The results indicated that nanoemulsion uptake is energy dependent (Gromnicova et al., 2016; Gratton et al., 2008). Nanoemulsion intake is affected by temperature, which may influence the transport from nose to brain.
**Cellular uptake pathways of nanoemulsion**

After the extracellular fluorescence was quenched by TB, fluorescence intracellular of different inhibitor groups was measured. Calu-3 cells treated with no inhibitor were taken as control group. The result was shown in Figure 5. From the results thus obtained, all the inhibitors influence the uptake of nanoemulsion. However, the inhibition by wortmannin was higher than the other inhibitors, which indicated that macropinocytosis was the major endocytosis pathway for the nanoemulsion. The uptake of nanoemulsion may also be involved with caveolae/lipid raft-mediated pathway and clathrin-dependent pathway, for hyperosmotic sucrose (reported to impair the formation of both coated and uncoated invaginations at the plasma membrane), chlorpromazine hydrochloride (causes the assembly of adaptor proteins and clathrin on endosomal membranes thus depleting it from the PM, leading to a block in CME), and methyl-β-cyclodextrin (used to deplete cells of plasma membrane cholesterol, leading to a block in endocytosis of various toxins and GPIAPs) could also affect the endocytosis of nanoemulsion.

![Cellular uptake of Coumarin 6 nanoemulsion with different endocytosis inhibitors.](image)

*P* < 0.05, compared with control group.

The nasal cavity drug delivery system has great development potential in the treatment of brain diseases. An important part of the evaluation of the nanoparticle delivery system is to study the safety of the preparation and its absorption and transport in vivo and in vitro. The study of cellular uptake mechanism found that the absorption of nanoemulsion drops is energy dependent and it mainly endocytosed through macropinocytosis pathway. Considering that the volatile active ingredients in XNJ may cause irritation to the nasal mucosa, XNJ was made into nanoemulsion and the nasal mucosal irritation was compared in the toad palate model and the rat model. Volatile drugs may have strong irritation to the nasal mucosa, but after XNJ is made into nanoemulsion, its irritation to the nasal mucosa is reduced and the damage is reversible. Because XNJ nasal nanoemulsion is mainly used for the treatment of acute cerebral apoplexy, it is a short-term medication. Although it can produce certain irritation to the nasal mucosa, it is not permanent damage and...
can be recovered within a week, suggesting that XNJ nasal nanoemulsion has potential value.

CONCLUSIONS

Nanotechnology is essential in the field of drug delivery, not only to protect the drug from degradation, but also to improve the absorption and uptake of drugs by the olfactory mucosa and central nervous system.

The above research results indicate that this preparation has the potential to be developed as a nasal delivery system, and that further enhancement of transnasal absorption can be considered in subsequent studies by promoting the relevant absorption pathways (such as promoting the expression of genes and proteins related to transport pathways).

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DISCLOSURE

The authors declare that they have no competing interests.

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