Preparation of Bupleurum Nasal Spray and Evaluation on Its Safety and Efficacy

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Radix Bupleuri is widely used in traditional medicine for the treatment of fever, pain, and inflammation associated with influenza or the common cold. A number of in vivo studies have confirmed the antipyretic capacity of Radix Bupleuri in fevered animals. Oral administration of a Bupleurum decoction to rabbits with a heat-induced fever decreases body temperature to normal levels within 1.5 h. Furthermore, the antipyretic activity has also been investigated in patients with fevers caused by the common cold, influenza, malaria and pneumonia. In one clinical study of 143 patients treated with the herb, fevers subsided within 24 h in 98.1% of all cases of influenza, and in 87.9% of all cases of the common cold. The essential oil extracted from the herb is generally claimed to play the major role in the efficacious treatment of fever. The purpose of the present study was to formulate an intranasal delivery system for the essential oil in an aqueous solution used in the form of nasal spray. From 450 g Radix Bupleuri, the essential oil was extracted and formulated into a variety of dosage forms such as the favorable solubilizing agents, whose amount was respectively determined by L_{0}(4^{5}) orthogonal design. An aqueous solution with clarity and no ciliotoxicity was prepared when Tween-80 10% (w/v) solution, propylene glycol (PG) and diethylene glycol monooethyl ether (TC) were selected as the favorable solubilizing agents, whose amount was respectively determined by L_{0}(4^{5}) orthogonal design. An aqueous solution with clarity and no ciliotoxicity was prepared when Tween-80 10% (w/v), PG 14.4% (v/v) and TC 14.4% (v/v) were added. Employed to evaluate the acute toxicity, the rats grew well and were kept active and healthy within 4 d after an intranasal administration of this preparation at the dose of oil from 10 g Bupleuri/kg (50-fold higher than the clinical dose), indicating that there would be no serious toxicity at the normal dose. Intranasal administration of this preparation to 2 kg rabbits with fever induced by subcutaneous injection of turpentine decreased body temperature markedly (0.5, 0.8 and 1.0 °C respectively at the dose of oil from 1, 2 and 4 g Bupleuri/body). In addition, the administration significantly reduced fever in 200 g rats induced by intramuscular injection of colicine suspension (0.6 °C at the dose of oil from 0.8 g Bupleuri/body). The results suggest that the formulation of nasal spray for the essential oil from Radix Bupleuri can be potentially effective in the treatment of fever.

Key words Radix Bupleuri; nasal spray; antipyretic effect; nasal ciliotoxicity; essential oil

Radix Bupleuri is widely used in traditional medicine for the treatment of fever, pain, and inflammation associated with influenza or the common cold. A number of in vivo studies have confirmed the antipyretic capacity of Radix Bupleuri in fevered animals. Oral administration of a Bupleurum decoction to rabbits with a heat-induced fever decreases body temperature to normal levels within 1.5 h. Furthermore, the antipyretic activity has also been investigated in patients with fevers caused by the common cold, influenza, malaria and pneumonia. In one clinical study of 143 patients treated with the herb, fevers subsided within 24 h in 98.1% of all cases of influenza, and in 87.9% of all cases of the common cold. The essential oil extracted from the herb is generally claimed to play the major role in the efficacious treatment of fever. The purpose of the present study was to formulate an intranasal delivery system, in which the concentration of the essential oil (oil from 15 g Bupleuri/ml) was much higher than that in the injection.

Nasal sprays are of practical use for their high-bioavailability, with easy-to-use and rapid-to-act features. Recently, dihydroergotamine, sumatriptan and zolmitriptan have been prepared and marketed in the form of nasal spray to treat migraine,7-9 so have lyspressin, oxytocin and salcatonin for systemic use. Similarly, nimodipine, methotrexate and mepetazinol have been attempted in our department, their results displaying a promising use in the treatment of diseases affecting the central nervous system (CNS).10-12 Therefore, nasal spray was selected as the dosage form for the essential oil in our approach.

The essential oil extracted from Radix Bupleuri is slightly water-soluble and viscous with low-fluidity, so it can not be sprayed directly. Therefore, the crucial problem in our study was to find some favorable solubilizing agents to overcome the slight water solubility of the essential oil so as to optimize the preparation of Bupleurum nasal spray and investigate its nasal ciliotoxicity, acute toxicity and antipyretic capacity.

Experimental

Materials Both Radix Bupleuri and Bupleurum injection were supplied by Henan Nanyang Lixin Pharmaceutical Co., Ltd., China. As solubilizing agents, Tween-80 (TW-80, Shanghai Dazhong Pharmaceutical Co., Ltd., China), propylene glycol (PG, Shanghai Ciji Pharmaceutical Co., Ltd., China) and diethylene glycol monooethyl ether (TC, Transcutol P®, Colorcon Corp., America) were used. Pineapple oil (Shanghai Peacock Flavor & Fragrance Co., Ltd., China) was used as the aromatizer. The strain of Esherichia
For the intranasal administration, 60 rinsed quickly with physiological saline. Finally, the palate was dissected under a strong air flow, thus gaining about 0.2 ml essential oil. After collection, the second-distilled solution was directly distilled again, with 1350 ml distilled solution collected and saturated with NaCl. Subsequently, this solution was directly distilled again to collect 200 ml second-distilled solution, to which NaCl was added until it was saturated. At the end of the distillation step, 100 ml, 100 ml, 50 ml ethyl ether were applied in a sequential order to extract the essential oil from the second-distilled solution, and the collections were mixed with their remaining water eliminated with anhydrous Na$_2$SO$_4$. Finally, ethyl ether was removed under a strong air flow, thus gaining about 0.2 ml essential oil.

**Screening of Solubilizing Agents** The screening of solubilizing agents was performed by $L_{18}(4^{5})$ orthogonal design. As solubilizing agents, TW-80 (in 10% (w/v) solution), PG and TC were all examined at 4 levels. The essential oil, extracted from 4.5 kg Radix Bupleuri, was divided into 16 parts, and according to the $L_{18}(4^{5})$ orthogonal table (Table 1), TW-80, PG and/or TC were added to each tube before water was filled until the final volume was 18.75 ml (1 ml solution containing oil from 15 g Bupleurum), followed by stirring for 5 min. Each experiment was done in triplicate.

The clarity (CL) and cost of each solution were chosen as the indexes to evaluate its quality. The solution’s CL was estimated in 5 grades; the higher the grade, the clearer the solution. In addition, the parameter “cost integral” (CI) employed to express their cost, was calculated as follows:

$$CI = \frac{V_{TW-80}\times 0.8 + V_{PG}\times 1 + V_{TC}\times 5}{V_{TW-80} + V_{PG} + V_{TC}}$$

$V_{TW-80}$, $V_{PG}$ and $V_{TC}$ stood for the volume of TW-80, PG and TC in each test, and their relative market prices were 0.8, 1 and 5, respectively, compared with that of PG. We intended to prepare a solution with high clarity and low cost, and as the former was considered as a major evaluating mark, the weighting indexes of CL and CI were respectively fixed on 0.7 and 0.3. The integrated mark (IM) of each solution was calculated as follows:

$$IM = \left(\frac{CL}{CL_{max}}\right) \times 0.7 \times 100 + \left(\frac{CI_{max}}{CI_{max} + CI}\right) \times 0.3 \times 100$$

$CL_{max}$ and $CI_{max}$ respectively referred to the maximum value of CL and CI. The values of IM were analyzed by analysis of variance (ANOVA) or by analysis of variance (ANOVA).

**Preparation of Bupleurum Nasal Spray** In the study the solubilizing agents were screened for the best formulation selected to prepare Bupleurum nasal spray. The mixture of TW-80, PG and TC was mingled with the essential oil extracted from 450 g Radix Bupleuri, followed by stirring for 5 min. Afterwards, 0.15% (v/v), Na$_2$SO$_4$, 0.1% (w/v), pineapple oil 1% (v/v) were added in a sequential order to act as preservative, antioxidant and aromatizer, which was stirred again for 2 min before water was added until the final volume reached 30 ml. Finally, 10% (w/v) NaOH was employed to adjust pH to 4.5.

**Nasal Ciliotoxicity** Nasal ciliotoxicity studies were carried out using both in situ toad palate model and in vivo rat nasal mucosa model. In the toad experiment, the upper palate of toad (30—40 g, female) Experimental Animal Center of Fudan University, China) was exposed and treated with 0.5 ml Bupleurum nasal spray (oil from 15 g Bupleuri/ml) once a day, and then rinsed quickly with physiological saline. The palate was dissected and the mucocilia was examined under a scanning electronic microscope (S-520, Hitachi, Japan). Physiological saline and sodium deoxycholate (one of serious nasal ciliotoxicity agents, 1% (w/v) solution) were used as the negative and positive control, respectively. In the experiments the bases (the solution containing only the excepted) was also examined.

**Acute Toxicity** The acute toxicity was evaluated in Sprague–Dawley rats (200±10 g, female, Shanghai SLAC Laboratory Animal Co. Ltd., China). Bupleurum nasal spray (oil from 15 g Bupleuri/ml) was administered to the rats at the dose of oil from 10 g Bupleuri/kg (50 μl solution given every 5 min in the way described above), and then their physiological characters such as daily diet and behavior were observed and recorded after the last administration, which was performed once a day for 14 d. If one rat had died during the experiments, it would have been dissected at once for pathologic examination to identify the cause of its death. And the bases and normal saline were used as the excipient and blank control, respectively. The data were analyzed by Student’s t-test with the Stata 7.0 program.

**Antipyretic Effect on Rabbits** The New Zealand-derived white rabbits (2.0—2.2 kg, female, Shanghai Experimental Animal Center, Chinese Academy of Sciences) were used in this study. Firstly, pyrexia was induced in rabbits with a subcutaneous injection of 0.2 ml/kg turpentine. Then their temperatures were taken 16 h after the injection to determine the rabbits’ pyretic responses. Finally, the rabbits whose temperatures had risen over 1 °C were selected and divided into six groups randomly for the antipyretic test. After that, their temperatures were taken 1 h prior to drug administration and served as pre-drug control. The solution used for intranasal administration was bottled in 5 ml nasal spray bottle (Pfiffer Corp., Germany), which consisted of 5 ml brown bottle and a quantitative spray pump (each spray is 0.1 ml). The first group (the negative control) and the second group (the excipient control) received 0.2 ml/body physiological saline and 0.2 ml/body bases intranasally, respectively. The test groups were ranked the third, fourth and fifth, which received 0.1, 0.2 and 0.4 ml/body Bupleurum nasal spray (oil from 10 g Bupleuri/ml, the original nasal solution was diluted with the bases.), respectively. The sixth group was treated as the positive control, and received intramuscularly 2 ml/body Bupleurum injection (oil from 1 g Bupleuri/ml), which has been affirmed to have obvious antipyretic effect on patients. And their temperatures were recorded at predetermined time intervals after the administration.

**Antipyretic Effect on Rats** The antipyretic effect was also evaluated in rats. The colicin produced from E. coli. prior to the antipyretic tests was used as the pyrogen. Firstly, 2 ml E. coli. suspension (1 ml solution containing 1×10$^{10}$ spores) was added to 50 ml nutrient broth (Shanghai Municipal Center for Disease & Prevention, China, 1.8% (w/v) in solution) for incubation at 30 °C with gentle to-and-fro vibrations at 90 rpm for 10 d. At the end of the incubation step, the culture medium was centrifuged 8000 rpm for 8 min at 5 °C. The precipitate was collected and dissolved in 1 ml glycerol by grinding. Then both the supernatant and precipitate-glycerol suspension were sterilized at 121 °C for 15 min. Finally, the pyrogen was obtained when the precipitate-glycerol suspension was diluted to 10 ml with the supernatant, and stored at 4 °C until used.

Sprague–Dawley rats (200±10 g, female, Shanghai SLAC Laboratory Animal Co. Ltd., China) were fevered with colicin at the dose of 2 ml/kg by an intramuscular injection. Five hours later their temperatures were taken to determine their pyretic responses. Then those whose temperatures had risen more than 1 °C were selected and divided into six groups randomly for the antipyretic test. The pre-drug control temperatures were taken 1 h prior to the drug administration. For the intranasal administration, the drug solution was given via a PE 10 tube attached to a microlitre syringe inserted 1 cm into the rats’ nostrils. The first group (the negative control) and the second group (the excipient control) received 40 μl/body physiological saline and 40 μl/body bases intranasally, respectively. The third, fourth and fifth group were given 20, 40 and 80 μl/body Bupleurum nasal spray (oil from 10 g Bupleuri/ml, the original nasal solution was diluted with the bases), respectively. The sixth group treated as the positive control was injected intramuscularly 0.4 ml/body Bupleurum (oil from 1 g Bupleuri/ml). And the temperatures were recorded at predetermined time intervals after administration. All of the antipyretic tests’ results were analyzed statistically through Student’s t-test with the Stata 7.0 Program.

**Results and Discussion**

**Preparation of Bupleurum Nasal Spray** We firstly investigated how the amount of the solubilizing agents including TW-80, PG and TC affected the clarity of the aqueous solution containing the essential oil. According to ANOVA results (Table 2), TW-80, PG and TC presented marked and dose-dependent solubilizing effects, and their respective order of capacity was TC>PG>TW-80. And INAN results ($I_{1}$, $I_{2}$, $I_{3}$, and $I_{4}$) (Table 1) indicated that if the combination of A$_{2}$B$_{4}$C$_{4}$ (1.5 ml TW-80, 2.7 ml PG and 2.7 ml TC con-
tained in 18.75 ml solution) or of A2B4C4 (2.7 ml TW-80, 14.4% (v/v) PG and 2.7 ml TC contained) was adopted, the value A2B4C4 was selected as the last combination due to its lower-obtained in 18.75 ml solution) or of A 4B4C4 (2.7 ml TW-80, 14.4% (v/v) PG and 2.7 ml TC contained) was adopted, the value A2B4C4 was selected as the last combination due to its lower-

The difference source Variable A Variable B Variable C Error
Sum of squares 543.79 1052.95 1550.75 297.32
Degrees of freedom 3 3 3 6
Mean variance 181.26 350.98 516.92 49.55

F 3.66 7.08 10.43
p 0.05< p<0.01 0.01< p<0.05 p<0.01
F0.05(3,6)=3.29, F0.01(3,6)=4.76, F0.01(3,6)=9.78.

Table 2. ANOVA Results for the Screening of Solubilizing Agents

<table>
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<th>Test No.</th>
<th>1 (A)</th>
<th>2 (B)</th>
<th>3 (C)</th>
<th>4 (D)</th>
<th>5 (E)</th>
<th>CL</th>
<th>CI</th>
<th>IM</th>
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<td>1 (0.90)</td>
<td>1 (0.00)</td>
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<td>1</td>
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<td>28.00</td>
<td>21.47</td>
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<td>3 (1.80)</td>
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<td>42.00</td>
<td>17.66</td>
<td>59.66</td>
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<td>4 (2.70)</td>
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<td>15.00</td>
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<td>15.05</td>
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<td>15.11</td>
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<td>79.31</td>
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</table>

a) There were 3 variables and 4 levels studied in the experiment. b) Variable A, B and C referred to TW-80, PG and TC, respectively, and the volume (ml) of them used in each test shown in the table. And variable D and E stood for the error during the test. c) CL, CI and IM referred to clarity, cost integral and integrated marks, respectively. d) j represented variable A, B and C. And Ij, IIj, IIIj and IVj meant the sum of IM in each level of variable j.

showed that the absorbency of the distilled solution at 278 nm was greater than that obtained by the traditional method, which was consistent with the published reports.

Bupleurum injection contains a single solubilizing agent, TW-80. However, Bupleurum nasal spray holds a variety of solubilizing agents, because the concentration of the essential oil in the former is 15-fold lower than in the later. In the previously reported studies, PG was prevalently applied for its ideal solubilizing capacity, high biocompatibility and low toxicity to solubilizing the slightly water-soluble drugs.\textsuperscript{18—20} In addition, it was testified that TC could be used as a permeation enhancer to increase the drug’s transport,\textsuperscript{21,22} or utilized as a solubilizing agent.\textsuperscript{23} Therefore, in addition to TW-80, PG and TC were employed as solubilizing agents in our study.

**Nasal Ciliotoxicity** Nasal mucociliary clearance plays a crucial role in protecting the respiratory system from damage by inhaled substances.\textsuperscript{24} Therefore, it is vital to examine the influences of drugs and drug excipients on nasal mucociliary clearance before clinical application. In our study, optical microscopic results showed that there were a great number of cilia at a fast beating rate on the edge of the mucosa treated with the bases or Bupleurum nasal spray for 1 h (Fig. 1), and the beating lasted for about 12 h after the palate was dissected, indicating that both the essential oil and excipient showed no obvious effect on the cilia movement. The scanning electron microscopic results displayed that the cilia were bushy and intact on the surface of the mucosa after the continuous administration of the bases or Bupleurum nasal spray for 7 d (Fig. 2), indicating that they also presented no marked effect on the length and density of the cilia. In addition, hyperaemia, tumefaction and ulcer were all not observed on the surface of the nasal mucosa by macrography. Based on the above results, we can conclude that both the essential oil and excipient possess no serious nasal ciliotoxicity.
and irritability.

**Acute Toxicity** Both drugs and their excipient could produce some adverse effects on patients if used at an excessive dose. Therefore, it is imperative that the effective and safe doses of the drugs be prescribed before clinical research. In our approach, the acute toxicity investigation was conducted in the rats in an attempt to determine the drug's median lethal dose (LD$_{50}$) and evaluate its toxicity, with the results indicating that all of the rats, treated with oil from 10 g Bupleuri/kg (the dose was above 50-fold higher than the clinical dose), remained active and free of cough, asthma, vomit and asphyxia during the whole experiments. The test group showed a worse appetite than the controls at the first day after drug administration, but their appetites began to restore at the second day. And there were no obvious differences in weight between the test group and either of the control groups from beginning to end (Fig. 3), indicating that the spray generated no serious effect on the growth of the rats. Consequently, it could be affirmed that Bupleurum nasal spray at the dose of oil from 10 g Bupleuri/kg showed no marked effect on the rats’ physiological characters, and we could conclude that the LD$_{50}$ of Bupleurum nasal spray was
Antipyretic Effect

In order to determine the drugs’ antipyretic effect, two kinds of fevered animals (one induced by chemical and the other by microbe) are needed, with the body temperature differences between fevered and normal animals at 1 °C or above. In this study, pyrexia was induced in rabbits by turpentine, which was easily available and capable of stabilizing the fevered model for more than 8 h. Meanwhile, pyrexia was induced by colicine, rather than yeast’s suspension, in rats, because the preliminary results displayed that the fevers induced by colicine could avoid the great individual diversity found in the yeast test. In the tests on the rabbits’ fevers (Fig. 4), the maximum of $\Delta T$ (the average body temperature differences between pre-drug administration and post-drug administration) was 0.5, 0.8 and 1.0 °C, and the antipyretic capacity lasted for 4, 6 and 6 h, respectively at the doses of oil from 1, 2 and 4 g Bupleuri/body, and in the positive control group, the data were 0.5 °C and 4 h, re-

above oil from 10 g Bupleuri/kg for rats.

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above oil from 10 g Bupleuri/kg for rats.
spectively. In the tests on the rats’ fevers (Fig. 5), Bupleurum nasal spray at the doses of oil from 0.2 and 0.4 g Bupleuri/body did not exhibit obvious antipyretic effect. However, when the dose increased to oil from 0.8 g Bupleuri/body, the maximum of ΔT was 0.6°C, and the antipyretic capacity lasted for 4 h, and in the positive control group, the data were 0.5°C and 2 h, respectively. These results showed that Bupleurum nasal spray exhibited dose-dependent and significant antipyretic capacity in rabbits at all the doses used above and in rats at the dose of oil from 0.8 g Bupleuri/body, and the effects on fevered rabbits were much greater than those produced with Bupleurum injection.

The spray produced obvious antipyretic effect on the rabbits at all the doses, but on the rats only at the high dose, the reason for which was supposed that the spray was administered to the rabbits by nasal spray pump, with the drug solution spurted in mist, and to the rats by microliter syringe, with the solution streamed in drop, so the former was more likely to be absorbed than the later. When the spray is given to the patients, the nasal spray pump is employed, thus the antipyretic effect on them being more similar to the rabbits. Therefore, we can forecast that the spray would produce obvious antipyretic effect on patients with a fever at the dose of oil from 9—12 g Bupleuri/body (0.6—0.8 ml solution a day), based on rabbit to human dosage conversion formula27) showed below:

\[
\text{human equivalent dose} = \frac{\text{rabbit dose in g/kg} \times \text{(rabbit weight in kg)}}{\text{human weight in kg}}^{0.33}
\]

**Conclusion** In amount, 30 ml Bupleurum nasal spray with clarity can be prepared by mixing the essential oil, extracted from 450 g Radix Bupleuri, with TW-80 8% (v/v), PG 14.4% (v/v) and TC 14.4% (v/v). And both essential oil and excipient produced no serious nasal ciliotoxicity, irritability and acute toxicity. Moreover, Bupleurum nasal spray exhibited dose-dependent and significant antipyretic capacity on both fevered rabbits and rats, and the effects on the fevered rabbits were much greater than those produced by Bupleurum injection. Therefore, we can conclude that Bupleurum nasal spray is safe for intranasal administration and potentially efficacious against fever clinically, especially among children.

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**References**