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ORIGINAL RESEARCH

Photoresponsive nanocapsulation of cobra neurotoxin and enhancement of its central analgesic effects under red light

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Abstract: Cobra neurotoxin (CNT), a peptide isolated from snake venom of *Naja naja atra*, shows central analgesic effects in our previous research. In order to help CNT pass through blood–brain barrier (BBB) and improve its central analgesic effects, a new kind of CNT nanocapsules were prepared by double emulsification with soybean lecithin and cholesterol as the shell, and pheophorbide as the photosensitizer added to make it photoresponsive. The analgesic effects were evaluated by hot plate test and acetic acid-induced writhing in mice. The CNT nanocapsules had an average particle size of 229.55 nm, zeta potential of −53.00 mV, encapsulation efficiency of 84.81% and drug loading of 2.98%, when the pheophorbide content was 1% of lecithin weight. Pheophorbide was mainly distributed in outer layer of the CNT nanocapsules and increased the release of the CNT nanocapsules after 650 nm illumination. The central analgesic effects were improved after intraperitoneal injection of CNT at 25 and 50 $\mu g \cdot kg^{-1}$ under 650 nm irradiation for 30 min in the nasal cavity. Activation of pheophorbide by red light generated reactive oxygen species which opened the nanocapsules and BBB and helped the CNT enter the brain. This research provides a new drug delivery for treatment of central pain.

Keywords: cobra neurotoxin, nanocapsules, photoresponsive, central analgesic effects, red light, drug delivery, photosensitizer

Introduction

Pain, a stress response or symptom caused by physical damage or disease, is closely associated with signal molecules in cells, including hormones, cytokines, neurotransmitters, lymphatic factors, growth factors and chemokines, which could change ion channels, the activities of relevant enzymes and gene expression.[1,2] Nonsteriodal anti-inflammatory drugs (NSAIDs) or morphine-like painkillers are widely adopted as therapy for chronic pain in clinics, but NSAIDs can cause gastrointestinal damage such as hemorrhage and ulcer after long time use,[3] and morphine-like drugs have undesirable complications and side effects such as addiction, tolerance, etc.[4] Therefore, current analgesic research focus on natural medicines without tolerance, dependence and other side effects.[5,6]

Cobra neurotoxin (CNT), a short-chain peptide isolated from snake venom of *Naja naja atra*, has central analgesic effects and is appropriate for therapy of chronic pain, including cancer and neuropathic pain.[7,8] But it is difficult to pass through the blood–brain barrier (BBB) due to the molecular weight of 7,000 Da,[9] and it has no ability of targeting central tissues after peripheral administration, which limits its practical use in clinics. Many methods of BBB permeation have been studied to help

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the macromolecules go through, such as conjugation with avidins, transport proteins or virus,[10,11] but their stability and safety are unsolved yet.

Liposome has become a promising drug delivery system with the advantages of biocompatibility, biodegradability, ability to target specific cells or tissues, possibility of controlled release and protection against degradation of the drugs.[12] The phospholipids bilayer of liposome could flexibly pass through the membrane of the cells via endocytosis and pinocytosis, which helps the drugs penetrate the cytoplasm.[13] Furthermore, the liposome can be modified to be pH responsive, thermal responsive and enzymatic responsive in order to achieve drug release control.[14]

Liposome nanocapsules have the excellent capacity to transport hydrophilic compounds into cells in particle size of 100–300 nm due to their similarity with cell membrane, and they are usually prepared by film dispersion, membrane extrusion, emulsion evaporation and so forth.[15] Among these methods, the double-emulsification method could achieve the controllability and flexibility of encapsulation for targeted delivery and release system.[16]

On the basis of our previous finding that the photosensitizers could assist the macromolecules permeate into the BBB but cause little injury,[7] we designed a new kind of photoresponsive CNT nanocapsules by photosensitizer excitation of red light. We prepared it by the double-emulsification method and evaluated its central analgesic effects. Because red light can penetrate deeper tissues than ultraviolet and other visible light without side effects,[16] it will achieve targeting the drug to central tissues by adjustment of BBB permeation and drug release.

Materials and methods

Chemicals

Crude cobra venom was purchased from Jiangxi snake farm (Jiangxi, People's Republic of China), and its neurotoxin (96%) was isolated and purified during our previous research.[17] Pheophorbide (98%) was extracted from silkworm feces in our laboratory.[18] Soybean lecithin and cholesterol were bought from Guangzhou Qiyun Biotech Company (Guangzhou, People's Republic of China). Other chemicals were all analytical reagents without further purification and purchased from Qianhui Reagent Company (Guangzhou, People's Republic of China).

Animals

The experiments were performed on Kunming mice of weight 30±5 g, supplied by the Experimental Animal Center of South Medical University (Guangzhou, People's Republic of China). The animals were housed under conditions of 25°C±2°C, 50%±10% humidity with a 12 h light/dark cycle. Food and water were accessible ad libitum. The experiments were performed in accordance with the Chinese Guidelines for the use of laboratory animals and received approval (No 20160612) from the Animal Experimentation Ethic Committee of South China University of Technology.

Isolation of CNT

The CNT was isolated from venom of *Naja naja atra* according to Guo.[17] Briefly, 1.5 g of crude venom was dissolved in 10 mL of 0.01 mol·L^{-1} acetate ammonia buffer (pH 5.0), passed through CM-AgroseCL-6B column (10×500 mm) and then eluted by 0.1 mol·L^{-1} to 1.5 mol·L^{-1} NaCl in acetate ammonia buffer (pH 5.0) with linear gradient at 0.5 mL·min^{-1}. The third peak was collected and further purified by Sephadex G-50 column (10×1,000 mm) with elution by 0.01 mol·L^{-1} acetate ammonia buffer (pH 5.0) to 0.5 mL·min^{-1}. The peak was collected, lyophilized and stored at −20°C.

Preparation of photoresponsive CNT nanocapsules

The CNT nanocapsules were prepared by a two-step emulsification procedure according to references with some modifications.[16] First, soybean lecithin (0.5 g), cholesterol (0.05 g) and pheophorbide (0%, 0.2%, 0.5%, 1% and 2% of lecithin weight) were dissolved in 10 mL of chloroform as phase A. CNT (0.02 g) was dissolved in 5 mL of phosphate buffer solution (0.1 mol·L^{-1} phosphate-buffered saline [PBS], pH =7.4) as phase B. Phase B was added dropwise to phase A with stirring at 6,000 r·min^{-1} for 15 min to obtain the water/oil emulsion. Second, the water/oil emulsion was added dropwise to 5 mL of PBS (0.1 mol·L^{-1}, pH =7.4) and constantly stirred at 6,000 r·min^{-1} for 15 min to form the water/oil/water emulsion. Finally, it was concentrated at 50°C for 2 h to remove chloroform by RE-52AA rotary vacuum evaporator (Shanghai Yarong Instrument Factory, Shanghai, People's Republic of China) and then dispersed in 25 mL of distilled water to pass through 0.45 μm membrane. The CNT nanocapsules (0.7 g) were obtained after lyophilization. Blank nanocapsules were prepared in the same process with 1% pheophorbide but no CNT was added.

Determination of encapsulation efficiency (EE) and drug loading

The sample (10 mg) was dispersed in 10 mL of distilled water and centrifuged at 20,000 r·min^{-1} in 4°C for 20 min.

The supernatant was kept as solution A, and the sediment was dispersed in 10 mL of distilled water and treated by ultrasonic for 5 min to break the nanocapsules, then filtered using a 0.22 μm membrane to make solution B. The concentration of CNT in solutions A and B was determined by HP 1200 high-performance liquid chromatography (Agilent Technologies Co., Santa Clara, CA, USA) in the following conditions: Column: TSK-GEL G2000SWXL (7.8×300 mm, 7 μm); mobile phase: 0.1 mol·L^{-1} PBS (pH =6.7) with 0.05% NaN_3; flow rate: 0.7 mL·min^{-1}; temperature: 30°C; injection volume: 20 μL; wavelength: 280 nm.[19] EE%, was calculated as the percentage of (CNT content in solution B)/(CNT content in solution A + solution B); drug loading content (DLC%) was calculated as the percentage of (CNT content in solution B)/(weight of the sample).

Thermogravimetry and differential scanning calorimetry (DSC)

Thermogravimetric analysis (TGA) and DSC were performed on an SDT-Q6000 thermogravimetric analyzer (TA Incorporated, New Castle, DE, USA). The CNT nanocapsules (4 mg) were weighed and heated from 30°C to 180°C at a rate of 5°C·min^{-1} under 100 mL·min^{-1} nitrogen flow.

Measurement of particle size and zeta potential

Particle size and zeta potential were determined in this research using a Nano-2S MDT-2 Malvern particle size analyzer (Malvern Instruments Limited, Malvern, UK). The sample of 5 mg was dispersed in 10 mL of deionized water and then measured at 25°C to show a refractive index 1.33. Data were calculated as the average of 5 repetitions.

Morphological observation of the particles

The CNT nanocapsules were diluted to a concentration of 0.5 μg·mL^{-1} with deionized water, and then dropped on to a glass slide and observed by Y-2 Inverted Fluorescence Microscope (Shenzhen Aosvi Optical Instrument Company, Shenzhen, People's Republic of China) under white light and 415 nm excitation.

Nanocapsule release test

The release of CNT nanocapsules was measured by the dialysis diffusion method.[20] The CNT nanocapsules (5 mg) were dispersed in 10 mL of pH 7.4 PBS (0.1 mol·L^{-1}), and put in the dialysis bag (molecular weight cutoff 10,000 Da), which was placed in the container with 100 mL of pH 7.4 PBS (0.1 mol·L^{-1}), stirred at 100 r·min^{-1} and maintained at 37°C±0.5°C. After irradiation by 100 mW laser (Three Top Photoelectric Company, Dongguan, People's Republic of China) at 650 nm for 0, 15 and 30 min, 1 mL of the solution was consecutively sampled at intervals of 1 h, and the same volume of 0.1 mol·L^{-1} pH 7.4 PBS was supplemented at designated intervals. The concentration of CNT in the solution was determined by high-performance liquid chromatography for three repetitions, and the cumulative release was calculated during 12 h test.

Hot plate test in mice

The hot plate test in mice was performed in order to evaluate the response latencies according to Hayashi et al.[21] The female mice were divided into 10 groups of 8 mice each: normal saline, blank nanocapsules, CNT nanocapsules containing 0.7% pheophorbide at high dose (HD) and low dose (LD) with and without nasal cavity irradiation at 650 nm for 30 min. Based on effective and safe doses,[22] the medicated groups of mice were given intraperitoneal injection of CNT at 50 μg·kg^{-1}, blank nanocapsules at 50 μg·kg^{-1}, CNT nanocapsules at 25 μg·kg^{-1} (LD) and 50 μg·kg^{-1} (HD). The control group was injected with the same volume of normal saline instead. The latency time for paw licking or jumping was recorded as pain threshold when mice were exposed to the hot plate surface at 55±0.5°C. Basic pain threshold was determined before administration, and mice responses were recorded at 0.5, 1, 2, 4, 6, 8, 12 and 18 h after administration. Pain inhibition was evaluated by pain threshold increase, which was calculated according to the following equation: Pain threshold increase (%) = $(P_t - P_0) \times 100/P_0$ where P_0 and P_t separately represent basic pain threshold and pain threshold at "t" time interval.

Acetic acid-induced writhing test in mice

Writhing in mice was induced according to the procedures described previously.[23] The mice were divided into 10 groups with 8 mice in each. Each group had equal number of male and female mice. The groups and administration were the same as hot plate test. Afterward, the animals received nasal cavity irradiation at 650 nm for 30 min before intraperitoneal injection of acetic acid (0.6%, v/v) at 0.1 mL·10 g^{-1}. The number of abdominal contraction was counted over a period of 30 min and expressed as writhing numbers. Antinociceptive activity was expressed as percent inhibition of writhing number in control animals.

Statistical analysis

Data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and analyzed with SPSS17.0 software (IBM, Armonk, NY, USA). Significant tests among the groups were based on

Table 1 Properties of the cobra neurotoxin nanocapsules with different concentrations of pheophorbide

Pheophorbide concentration (% lecithin weight)	Encapsulation efficiency (%)	Drug loading content (%)	Average particle size (nm)	Zeta potential (mV)
1 (blank nanocapsule)	/	/	244.15±2.76	−47.00±4.20
0	65.60±3.4	2.30±0.12	204.15±2.75	−62.15±1.48
0.2	74.46±2.5	2.61±0.09	210.65±3.25	−58.55±0.07
0.5	77.52±3.0	2.72±0.11	218.60±3.74	−57.30±3.95
1	84.81±2.0	2.98±0.07	229.55±0.49	−53.00±0.56
2	68.92±3.6	2.42±0.13	234.35±2.21	−50.50±1.56

Notes: Data are presented as mean ± standard deviation (n=5). All data show significant differences between treatments ($P<0.01$).
Abbreviations: CNT, cobra neurotoxin; EE, encapsulation efficiency.

one-way analysis of variance and Student–Newman–Keuls test with 99% confidence limit and probability $P<0.01$.

Results and discussion

Characteristics of the CNT nanocapsules

The characteristics of the nanocapsules prepared by the double-emulsification method are shown in Table 1. The EE and DLC constantly increased until pheophorbide reached up to 1% content of lecithin weight, but decreased thereafter. The EE and DLC were 84.81±2.0% and 2.98±0.07%. The average nanoparticle size and zetapotential were 229.55 nm and −53.00 mV, respectively, suggesting that proper pheophorbide concentration is advantageous to compactness and stability of the nanocapsule, but excess pheophorbide was destructive. The size distribution and zeta potential are illustrated in Figure 1. The average size distribution in the range of 200–240 nm indicated that CNT was ideal for the nanocapsule preparation. The nanocapsules were homogeneous and stable because of appropriate negative charge repulsion.

Morphology of the nanocapsules was observed and photographed by fluorescence microscope. The images of CNT nanocapsules displayed good dispersity (Figure 2A) and sphericity with red fluorescence in borderline (Figure 2B). In the formulation of CNT nanocapsules, only pheophorbide had red fluorescence under the illumination at 415 nm.

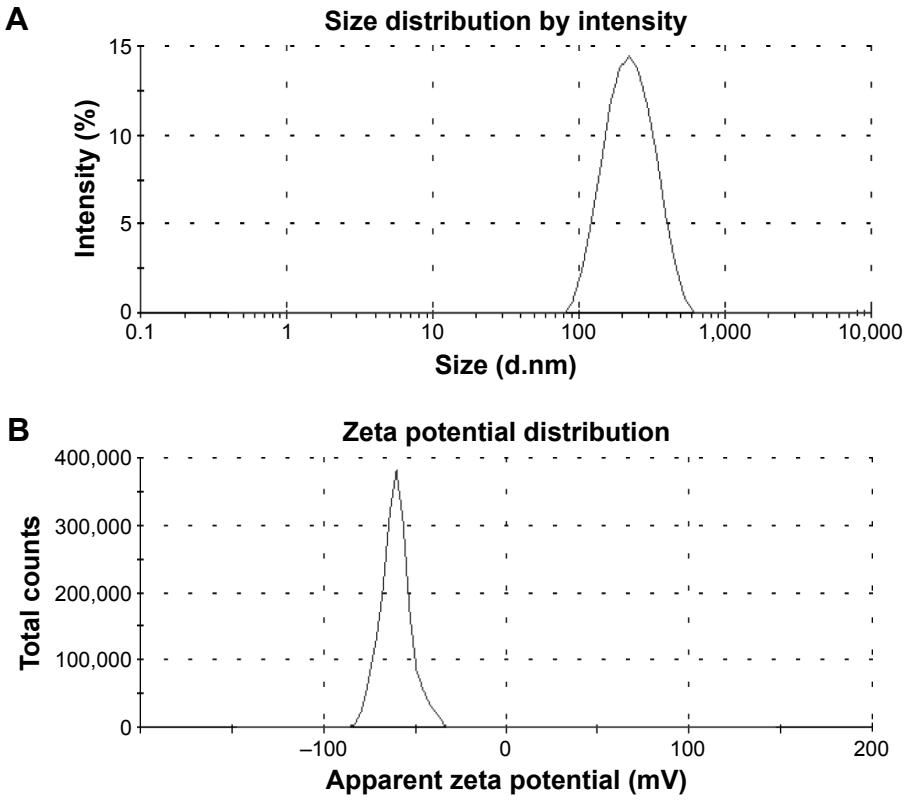

Figure 1 Size distribution (**A**) and zeta potential (**B**) of cobra neurotoxin nanocapsules.

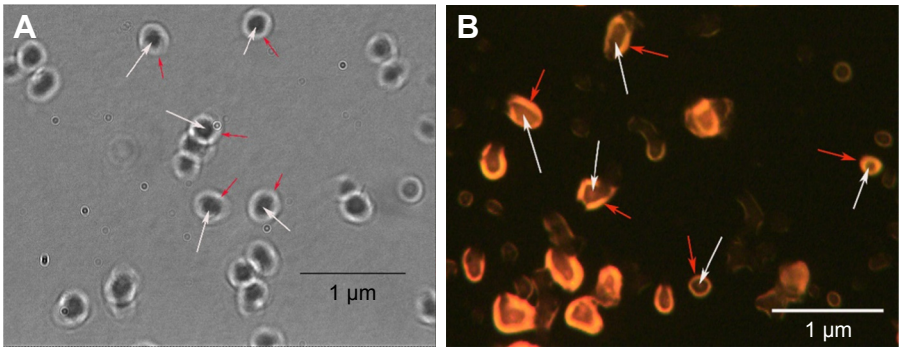

Figure 2 Photograph under white light (**A**) and fluorescence photograph under 415 nm light excitation (**B**) of the CNT nanocapsules by microscope at 1,000× amplification.
Red arrows represent pheophorbide in the outer shell and white arrows represent CNT in the inner core of the nanocapsules.
Abbreviation: CNT, cobra neurotoxin.

It indicated that pheophorbide is mainly distributed in the outer shell of the nanocapsules, and water soluble CNT is encapsulated by a bilayered structure of lecithin and cholesterol without molecular agglomeration.

TGA and DSC were applied as a simple method to determine the thermostability of the nanocapsules. As shown in Figure 3, the nanocapsules exhibited <5% weight loss at 80°C in TGA curve and a single endothermic melting peak at 78°C in the DSC curve. It confirms that CNT nanocapsules are stable for application below that temperature.

The pheophorbide, a porphyrin isolated from excrement of silkworm, is hydrophobic with a single hydrophilic carboxyl group. Hence, a certain amount of pheophorbide can combine with lecithin and enhance the stability of hydrophobic block in the nanocapsules, but excess pheophorbide vastly impedes tight junction of lecithin, leading to the decrease in DLC and EE.[24] CNT, a water-soluble peptide, can change the distribution of negative and positive charges of polarity zones via ion–dipole interaction on the surface of the nanocapsules and hinder the aggregation and formation of larger particles.[25] The average size of CNT nanocapsules is smaller than blank nanocapsules due to interfacial interaction between CNT and lecithin. The positive charge of CNT can attract the negative charge of lecithin, making the nanocapsules compact and small.

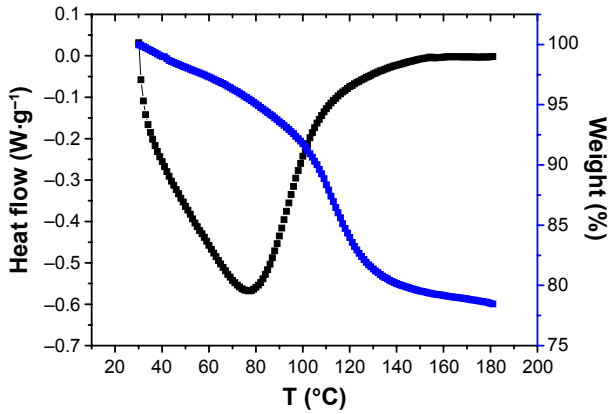

Figure 3 Differential scanning calorimetric and thermogravimetric chart of cobra neurotoxin nanocapsules.
Note: The black line represents heat flow; the blue line represents weight percentage.

Photoresponsiveness of the CNT nanocapsules

The cumulative release curves of CNT nanocapsules were significantly different as based on various illuminating time as shown in Figure 4. The release rate could reach 67.35% and 82.17% at the interval of 12 h with 15 min and 30 min irradiation, respectively, but only 30.11% in the same time interval without irradiation. The results demonstrate that CNT nanocapsules release slowly, remain stable under no illumination and have photoresponsiveness under 650 nm irradiation.

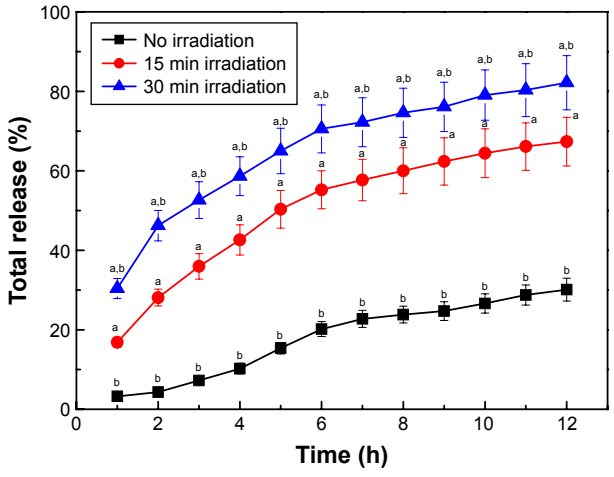

Figure 4 Total release ratio of the cobra neurotoxin nanocapsules at different times of irradiation at 650 nm.
Notes: Data were presented as mean ± standard deviation in 3 repetitions. (a) $P<0.01$, compared with no irradiation; (b) $P<0.01$, compared with 15 min irradiation.

Currently, most photoresponsive formulas utilize azobenzene as the photosensitive agent and require excitation by ultraviolet light (UV), but UV light is easily absorbed by tissues and harms the cells.[26] On the contrary, tissues absorb less red light, and it can penetrate deeper into the tissues without harming them. Pheophorbide, an effective photosensitizer in photodynamic therapy, could generate singlet oxygen excited by red light.[27] Furthermore, the cell membrane composed of phospholipids linked by phosphodiester bonds, could be broken by reactive oxygen species.[28] Therefore, the photoactivated release mechanism of CNT from the nanocapsule is attributed to the breakage of lecithin's phosphodiester bonds by pheophorbide-produced singlet oxygen.

Analgesic effects of the CNT nanocapsules

The hot plate test was a common model of thermal-induced nociception, which was used to evaluate the central action.[7,29] Hot plate test in mice indicated that the latency of response gradually increased with time in the medicated groups (Figure 5). There were obvious differences among the groups of CNT nanocapsules with and without irradiation. The medicated group with CNT nanocapsule plus 30 min illumination not only had greater pain threshold than others but also reached the maximum at interval quickly at 6 h. This suggests that irradiation of CNT nanocapsule by red light expedites the permeation of CNT across the BBB into the brain and promotes its central analgesic activity.

Acetic acid-induced writhing test in mice also showed that CNT group could inhibit the writhing number of mice, but no significant ($P>0.05$) changes took place during illumination (Table 2). Only CNT nanocapsule group had significant ($P<0.01$) changes with dose dependence in writhing number after illumination. It suggests that CNT nanocapsules with illumination can cause an analgesic effect.

The two pain models in animals have different mechanisms. Hot plate test is a model of heat nociception, which is mainly related to central action.[30] Acetic acid-induced writhing test is a model of chemical nociception, which has combined action on both central and peripheral nerves.[31] The CNT nanocapsules are effective in both pain models, suggesting both central and peripheral analgesic effects, but act with more power on the central nervous system. It proves that the red light illumination of CNT nanocapsules enhances its central analgesic effects. Nasal cavity irradiation is convenient and causes no injury. It is reported that drugs absorbed by nasal mucosa can be easily transported to the brain,[32] and intranasal route is the alternative route to parenteral administration.[33] Nasal cavity irradiation helps CNT go across the BBB and brings better central analgesic effects.

Conclusion

The CNT nanocapsules made by double emulsification of soybean lecithin, cholesterol, CNT and pheophorbide with a 1% lecithin concentration have the maximum EE and DLC of CNT. The pheophorbide is well scattered in the outer shell, and CNT is encapsulated in the nanocapsules with higher stability. The release of CNT from the

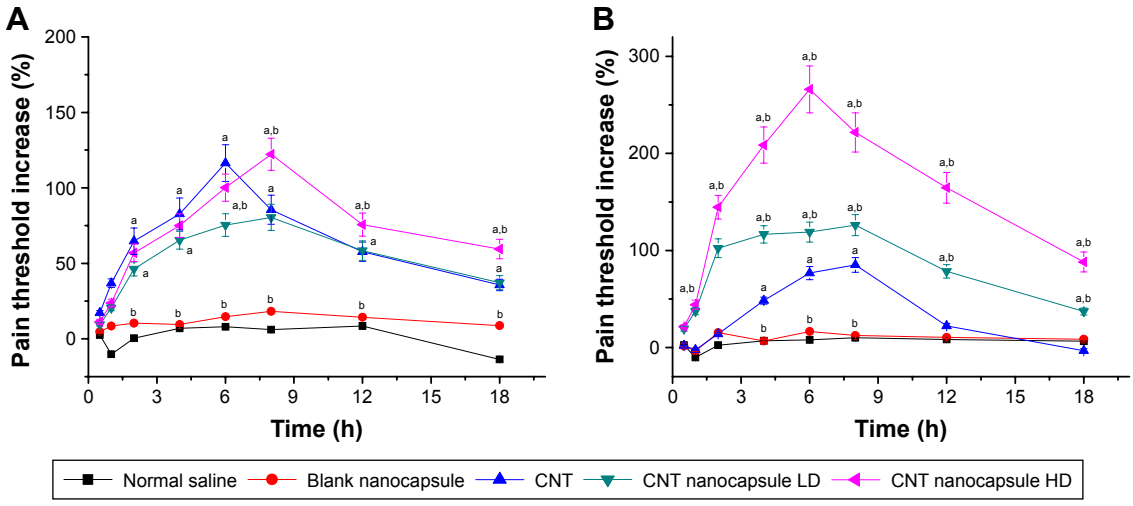

Figure 5 Analgesic effects of the CNT nanocapsules on mice by hot plate test. Mice groups were administered a dose of 50 μg·kg^{-1} of CNT and blank nanocapsules ip mice groups were also administered CNT nanocapsules LD and HD 25 μg·kg^{-1} and 50 μg·kg^{-1} ip, respectively. (**A**) No illumination, (**B**) 30 min illumination of nasal cavity by 100 mW 650 nm laser. Data were represented as $\bar{x} \pm s$, n=8. (a) $P<0.01$, compared with blank nanocapsule group; (b) $P<0.01$, compared with CNT group.
Abbreviations: CNT, cobra neurotoxin; HD, high dose; ip, intraperitoneal; LD, low dose.

Table 2 Writhing number of mice before and after illumination

Groups without illumination	Writhing number	Inhibition/%	Groups with illumination	Writhing number	Inhibition/%
Normal saline	62.2±5.4	/	Normal saline	65.7±6.4	/
Blank nanocapsule	64.3±6.7	–3.4	Blank nanocapsule	63.7±6.7	3.0
CNT	46.8±5.3	24.8	CNT	45.8±6.2	30.3
CNT nanocapsule LD	42.5±4.8	31.7	CNT nanocapsule LD	19.4±3.6**	70.5
CNT nanocapsule HD	35.7±4.6	42.6	CNT nanocapsule HD	13.7±3.4**	79.1

Notes: Mice groups were administered CNT and blank nanocapsules at dose of 50 μg·kg^{-1} ip; mice groups were also administered CNT nanocapsules LD and HD at 25 μg·kg^{-1} and 50 μg·kg^{-1} ip, respectively. Illumination of the nasal cavity was performed by 100 mW 650 nm laser for 30 min. Data were represented as $\bar{x} \pm s$, n=8. **$P<0.01$, compared with corresponding groups without illumination.

Abbreviations: CNT, cobra neurotoxin; HD, high dose; ip, intraperitoneal; LD, low dose.

nanocapsules is significantly enhanced by 650 nm irradiation. The central analgesic effect of CNT nanocapsules improves when the nasal cavity is irradiated by red light. The mechanism is related to excitation of pheophorbide by red light and production of singlet oxygen to increase CNT release from the nanocapsules, which enter the brain. This research provides a new type of red light-responsive nanopreparation of CNT, which is more effective for treatment of central pain.

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Disclosure

The authors report no conflicts of interest in this work.

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