

Inhalable Nanoagglomerate Powder of Cholecalciferol for Nose-to-Brain Delivery: A Promising First-Aid Therapy for Ischemic Stroke

Zitong Shao^{1,2}, Jiahui Deng^{1,2}, Ho Wan Chan¹ & Shing Fung Chow^{1,2}

¹Department of Pharmacology and Pharmacy, The University of Hong Kong, Hong Kong SAR China

²Advanced Biomedical Instrumentation Centre, Hong Kong Science Park, Shatin, Hong Kong SAR China

Summary

Ischemic stroke causes rapid neuronal damage within seconds, particularly during the hyperacute phase, leading to high mortality and long-term disability. Current reperfusion therapies, such as tissue plasminogen activator and endovascular thrombectomy, are limited by issues of accessibility and the urgency of administration. Despite extensive neuroprotective research, the blood-brain barrier (BBB) remains a major obstacle to effective neurotherapeutic delivery. Nose-to-brain delivery offers a promising alternative by bypassing the BBB through direct olfactory pathways, especially when combined with nanotechnology. Engineered nanoparticles can facilitate rapid mucosal transport and targeted cellular uptake. When formulated into stable micronized aerosol powders, these nanoparticles can achieve consistent olfactory deposition and controlled drug release, enhancing delivery efficacy.

Recent studies identified cholecalciferol (Vitamin D3), a safe endogenous prohormone, as an effective neuroprotective agent due to its multi-target, multi-phase mechanisms against stroke pathology. To ensure efficient nose-to-brain delivery, cholecalciferol nanosuspension was spray-dried with methylcellulose (MC) and polyvinylpyrrolidone K30 (PVP) to produce a nanoagglomerate powder. The resulting formulation preserved nanoparticle integrity, evidenced by a redispersibility index (RdI) of 1.36 ± 0.05 , and achieved a satisfactory olfactory deposition fraction of $18.47 \pm 1.55\%$. *In vivo* efficacy evaluation in a mouse stroke model demonstrated significant restoration of neurological functions following a single intranasal administration of 2 mg/kg.

These findings indicate that cholecalciferol nanoagglomerate powders delivered via the intranasal route represent a rapid, non-invasive, and effective therapeutic strategy for ischemic stroke. This innovative approach has the potential to address current clinical challenges, particularly in prehospital settings as a first-aid therapy, thereby improving patient outcomes.

Key Message

The cholecalciferol nanoagglomerate powder was successfully produced via flash nanoprecipitation and spray drying, exhibiting satisfactory redispersibility and *in vitro* aerosol performance for nose-to-brain delivery. The powder demonstrated promising neuroprotective efficacy against acute ischemic stroke in animal models, highlighting its potential as a novel first-aid therapy.

Introduction

Stroke is the second leading cause of death worldwide, with its prevalence rising due to aging populations and demographic growth [1]. Ischemic stroke, caused by blood clots blocking brain vessels, accounts for 87% of cases. In the hyperacute phase of acute ischemic stroke, each minute of delay results in the loss of 1.9 million neurons, leading to cognitive and motor impairments or death, revealing the need for rapid treatment [2]. Current therapies, including intravenous thrombolysis with tissue-type plasminogen activator and endovascular thrombectomy, face limitations such as narrow time windows, bleeding risks, access challenges, and delays in emergency response. Importantly, these reperfusion approaches do not address irreversible damage to the BBB [3], uncovering an unmet need for innovative and user-friendly first-aid therapies.

Neuroprotection aims to preserve brain tissue by targeting damaging processes like neuroinflammation and BBB disruption. An ideal neuroprotective therapy should be safe, easy to administer early, and compatible with existing therapies [4]. Cholecalciferol has demonstrated neuroprotective effects in stroke models, including anti-inflammatory, antioxidative actions and BBB preservation. Its established safety profile and multi-target mechanisms make it a promising candidate for adjunctive or standalone therapy [5]. Nose-to-brain delivery route offers a non-invasive way for brain-targeted drug delivery and bypasses the BBB; while nanotechnology helps overcome the limitations of intranasal administration such as protecting the drugs against degradation and enhancing drug mucosal permeation [6]. This study focuses on developing a nasally delivered nanoagglomerate powder of cholecalciferol, leveraging

nanotechnology and nose-to-brain delivery to provide a speedy, effective, accessible, and advanced treatment for ischemic stroke.

Methods

Cholecalciferol was purchased from Hefei Hirisun Pharmatech (Hefei, China). D- α -tocopheryl polyethylene glycol 1000 succinate (TPGS), cholesterol (CLT), MC and PVP were obtained from Sigma-Aldrich (Poole, UK).

The cholecalciferol nanosuspension was fabricated using flash nanoprecipitation using a four-stream multi-inlet vortex mixer (MIVM) [7, 8]. TPGS and CLT were employed as stabilizers in a cholecalciferol : TPGS : CLT ratio of 50:1:0.2 (w/w/w). The Z-average particle size and polydispersity index (PDI) were measured by a particle analyzer (Delsa Nano C, Beckman Coulter, CA, USA) using dynamic light scattering (DLS). The encapsulation efficiency (EE) of cholecalciferol was determined using a high-performance liquid chromatography method (HPLC, Agilent 1260 Infinity, Agilent Technologies, CA, USA) [9]. To produce the cholecalciferol nanoparticle-based dry powder formulations, a laboratory-scale spray dryer (Mini Spray Dryer B-290 with Dehumidifier B-296, Flawil, Switzerland) was used. The freshly prepared cholecalciferol nanosuspension was mixed with the polymer solutions in a nanosuspension: MC : PVP ratio of 1:10:0.1 (w/w/w). The mixed feed solution was spray-dried under the following parameters: inlet temperature 80 °C; nitrogen atomization flow rate 742 L/h; liquid feed rate 1.7 mL/min. The morphology of the nanoagglomerate dry powder was observed using the Scanning Electron Microscopy (SEM, S-4800 FEG, Hitachi, Tokyo, Japan). The drug content (DC) was assessed by HPLC. The Z-average particle size of the reconstituted powder solution was measured by DLS and the Rdl was calculated by particle size of the reconstituted sample referred to the initial particle size of nanosuspension. The *in vitro* aerosol performance of the nanoagglomerate powder was assessed with an uncoated Alberta Idealized Nasal Inlet (AINI) coupled to a silicon-coated Next Generation Impactor (NGI) (Copley, Nottingham, UK). Inspiratory flow rate was controlled at 15 L/min for 2 s during dispersion of 8 mg nanoagglomerate powder with a Unidose (UDS) Powder Nasal Spray System (Aptar Pharma, Le Vaudreuil, France). *In vivo* efficacy was evaluated in C57BL/6 mice using the permanent middle cerebral artery occlusion (MCAO) model induced by electrocoagulation. All animal studies followed the ethical policies and guidelines of the ARRIVE guidelines 2.0 and National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Animal Experimentation of Jinan University (Approval No.: 20230901-0012). Two treatment groups of mice (MCAO+NS and MCAO+NP) received intranasal liquid instillation of the nanosuspension or intranasal powder insufflation of the nanoagglomerate powder (2 mg/kg of cholecalciferol) at 30 minutes post-surgery (n=5). For comparison, a sham-operated group (sham) and an untreated group (MCAO) were included. Neurological recovery was assessed after 24 hours post-surgery using a variety of behavioral tests, including the adhesive removal test, balance beam test, and rotarod test. Briefly, the adhesive removal test quantifies the time taken for mice to remove adhesive tapes placed on their bilateral paws, the cross beam test quantifies the time taken for mice to walk across a tall beam, and the rotarod test quantifies the duration of time mice were able to remain on a constantly accelerating rotarod.

Results

The cholecalciferol nanosuspension exhibited a Z-average particle size of 145.7 ± 0.80 nm, a PDI of 0.149 ± 0.036 , and an EE exceeding 99.99%. After spray drying, the nanosuspension was formulated into the nanoagglomerate dry powder, with a percentage yield of 49.90%. The cholecalciferol nanoagglomerate powder exhibited a DC of $5.78 \pm 0.72\%$. According to the SEM image (Figure 1A), the nanoagglomerate powder exhibited an irregular spherical structure with external void space in buckles and dimples. The reconstituted powder suspension exhibited a Z-average particle size of 197.2 ± 6.90 nm, a PDI of 0.179 ± 0.013 and the Rdl of 1.36 ± 0.05 (Figure 1B). The *in vitro* aerosol performance of nanoagglomerate powder was shown in Figure 1C. The cholecalciferol nanoagglomerate powder formulation was fully emitted from the nasal spray device. More than 18% and 35% of nanoagglomerate powder were deposited in the olfactory region and the turbinate region in the AINI, respectively. Only around 14% of the powder reached the NGI stages after dispersion.

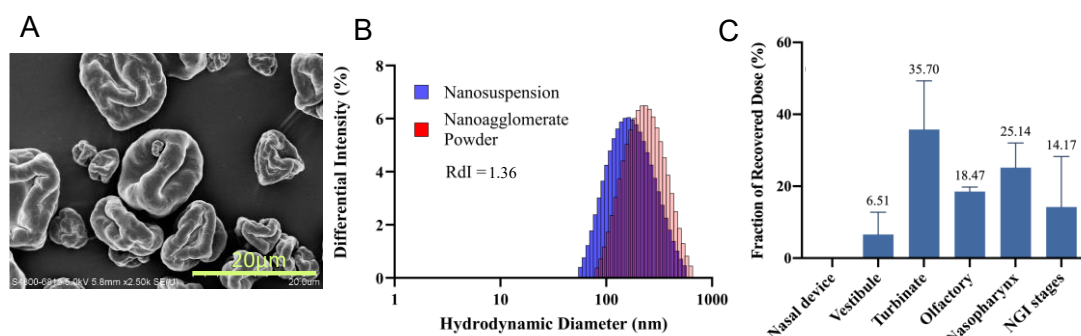


Figure 1 - A) SEM image of cholecalciferol nanoagglomerate powder; B) Differential intensity with size distribution of cholecalciferol nanosuspension and reconstituted nanoagglomerate powder ($n=1$) and C) *In vitro* aerosol performance of cholecalciferol nanoagglomerate powder in AINI-NGI ($n=3$, UDS Powder Nasal Spray System, 15 L/min, 2s). Data are presented as means $\pm$ S.D.

The *in vivo* neuroprotective effects of the cholecalciferol nanosuspension and nanoagglomerate powder were evaluated through the mice behavioral tests (Figure 2). In both the adhesive removal test and the balance beam test, the mice in MCAO+NS and MCAO+NP groups removed the adhesive tapes and traversed the beam in a time comparable to the sham group and significantly shorter than the MCAO group ($p < 0.0001$, One-way ANOVA). In the rotarod test, the mice in two treatment groups remained on the rotarod for a significantly longer duration than the MCAO group ($p < 0.001$, One-way ANOVA). No statistically significant differences were observed between two treatment groups.

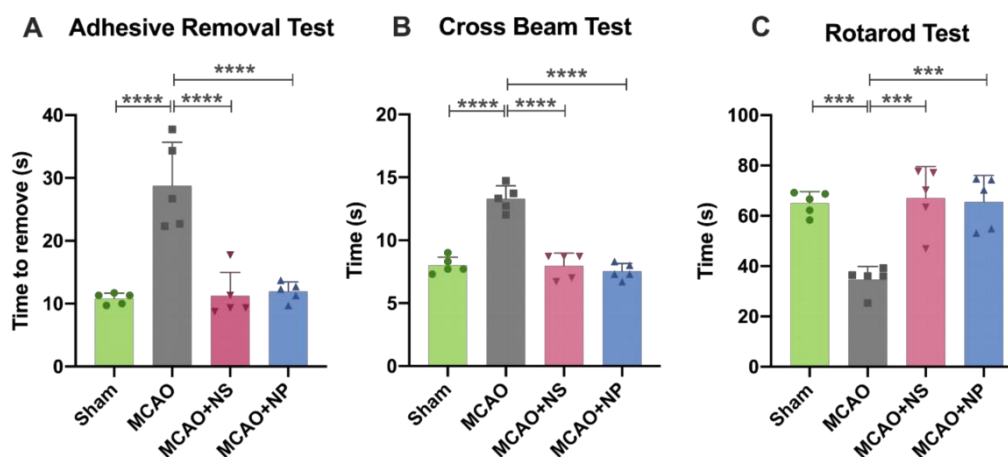


Figure 2 - Scatter bar plot of mice behavioral tests ($n = 5$). A) Adhesive removal test, B) Balance beam test and C) Rotarod test. Data are presented as means $\pm$ S.D. *** $p < 0.001$, **** $p < 0.0001$.

Discussion and Conclusion

The Z-average particle size of cholecalciferol nanosuspension ranged from 100 to 200 nm, which is optimal for effective cellular uptake [10] and efficient transport to the brain via olfactory route [11]. The PDI was below 0.3, indicating a narrow size distribution; values above this threshold could result in heterogenous distribution, potentially leading to variability in bio-performance. The high EE suggests that cholecalciferol was effectively encapsulated into the polymeric nanoparticles. Hypothetically, this can be attributed to TPGS, an amphiphilic block copolymer that self-assembles around the hydrophobic cholecalciferol core, and CLT, a hydrophobic co-stabiliser that strengthens the interaction with block copolymer hydrophobic moiety through the difference in solubility. This affords a stronger anchorage of the surface block copolymer around the hydrophobic drug core, thereby reorienting nanoparticles into a more stable micelle-like configuration during the fabrication [12].

During spray drying, MC and PVP served as excipients. MC as a gel-forming polymer with T_{gel} of 40 – 50°C [13], formed a turbid gel upon heating during spray drying. This gel facilitated the agglomeration of nanoparticles by thermo-reversible cross-linking, protecting nanoparticles against heat-induced degradation and collapse. However, its viscosity could retard the migration of nanoparticles towards the droplet core during drying, causing nanoparticles to remain at the surface. To protect the nanoparticles at the droplet surface, PVP was incorporated. As a homopolymer, PVP formed strong interactions (e.g. H-bonds) between the drug molecules and polymer chains [14], thereby preserving nanoparticle integrity during spray drying. PVP also inhibited nanoparticle growth and aggregation by enhancing the aqueous

redispersibility of the surface polymer coating as a water-soluble stabilizer. The resulting nanoagglomerate powder demonstrated acceptable Rdl, implying that nanoparticles could readily redisperse with a similar particle size distribution to that of the fresh nanosuspension.

The nanoagglomerate powder formulation was fully emitted from the nasal spray device in all runs, indicating excellent powder dispersibility and compatibility with the nasal device. Minimal deposition in non-target nasal regions (NGI stages) reduced off-target effects. The olfactory region, directly connected to the olfactory bulb, is the primary target for effective nose-to-brain delivery. Achieving a high olfactory deposition rate ensures the satisfactory targeting performance, surpassing most published studies reporting olfactory deposition rates below 10% in the AINI model. Moreover, high drug deposition in the nasal turbinate can facilitate brain access via secondary pathways (the trigeminal nerves, vasculature, and perineural pathways), further enhancing central nervous system drug absorption [15].

Intranasal administration of nanosuspension or dry powder formulation significantly improved motor coordination, balance, and neurological recovery in the MCAO mice. No significant differences between the nanosuspension and dry powder formulation suggest that the biological activity of the cholecalciferol nanoparticles was well-preserved after spray drying and agglomeration. Cholecalciferol likely intervenes directly in multiple signalling pathways involved in the ischemic cascade, mitigating neuronal destruction before hospitalization. The significant neuroprotectant effect observed after a single-dose administration highlights the tremendous potential of this formulation as an easy-to-administer, non-invasive and rapid-acting neurotherapy suitable for first-aid and prehospital use.

In conclusion, the cholecalciferol nanoagglomerate powder formulation was successfully fabricated, exhibiting acceptable redispersibility and favourable nasal aerosol deposition profiles. The nose-to-brain delivery of cholecalciferol nanoparticles effectively protected neurons in diseased mice, preserving their motor function. This innovative approach holds significant clinical potential for ischemic stroke treatment.

Acknowledgments

This study was supported by the Health@InnoHK initiative of the Innovation and Technology Commission of the Hong Kong Special Administrative Region Government.

References

- [1] Feigin V L, Brainin M, Norrving B, Martins S O, Pandian J, Lindsay P, M F G, Rautalin I: World Stroke Organization: Global Stroke Fact Sheet 2025, *Int J Stroke* 2025; 20: pp 132-144.
- [2] Hasan T F, Hasan H, Kelley R E: Overview of Acute Ischemic Stroke Evaluation and Management, *Biomedicines* 2021; 9: pp 1486.
- [3] Mosconi M G, Paciaroni M: Treatments in Ischemic Stroke: Current and Future, *Eur Neurol* 2022; 85: pp 349-366.
- [4] Haupt M, Gerner S T, Bähr M, Doeppner T R: Neuroprotective Strategies for Ischemic Stroke-Future Perspectives, *Int J Mol Sci* 2023; 24: pp 4334.
- [5] Lasoń W, Jantas D, Leśkiewicz M, Regulska M, Basta-Kaim A: Vitamin D3 and Ischemic Stroke: A Narrative Review, *Antioxidants* 2022; 11: pp 2120.
- [6] Formica M L, Real D A, Picchio M L, Catlin E, Donnelly R F, Paredes A J: On a highway to the brain: A review on nose-to-brain drug delivery using nanoparticles, *Applied Materials Today* 2022; 29: pp 101631.
- [7] Markwalter C E, Prud'homme R K: Design of a Small-Scale Multi-Inlet Vortex Mixer for Scalable Nanoparticle Production and Application to the Encapsulation of Biologics by Inverse Flash NanoPrecipitation, *Journal of Pharmaceutical Sciences* 2018; 107: pp 2465-2471.
- [8] Maaz A, Blagbrough I S, De Bank P A: A Cell-Based Nasal Model for Screening the Deposition, Biocompatibility, and Transport of Aerosolized PLGA Nanoparticles, *Molecular Pharmaceutics* 2024; 21: pp 1108-1124.
- [9] Weng J, Shao Z, Chan H W, Li S P Y, Lam J K W, Tsang C K, Chow S F: Mediating bio-fate of polymeric cholecalciferol nanoparticles through rational size control, *Biomaterials Advances* 2022; 140: pp 213074.
- [10] Xu A, Yao M, Xu G, Ying J, Ma W, Li B, Jin Y: A physical model for the size-dependent cellular uptake of nanoparticles modified with cationic surfactants, *Int J Nanomedicine* 2012; 7: pp 3547-3554.
- [11] Lee D, Minko T: Nanotherapeutics for Nose-to-Brain Drug Delivery: An Approach to Bypass the Blood Brain Barrier, *Pharmaceutics* 2021; 13: pp 2049.
- [12] Wan K Y, Wong S N, Wong K W, Chow S F, Lum Chow A H: Interplay between Amphiphilic Stabilizers and Cholesterol in the Stabilization of Itraconazole Nanoparticles Prepared by Flash Nanoprecipitation, *Mol Pharm* 2019; 16: pp 195-204.
- [13] Coughlin M L, Liberman L, Ertem S P, Edmund J, Bates F S, Lodge T P: Methyl cellulose solutions and gels: fibril formation and gelation properties, *Progress in Polymer Science* 2021; 112: pp 101324.
- [14] Chan H W, Zhang X, Chow S, Lam D C L, Chow S F: Inhalable paclitaxel nanoagglomerate dry powders for lung cancer chemotherapy: Design of experiments-guided development, characterization and in vitro evaluation, *International Journal of Pharmaceutics* 2024; 653: pp 123877.
- [15] Thorne R G, Pronk G J, Padmanabhan V, Frey W H, 2nd: Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration, *Neuroscience* 2004; 127: pp 481-496.