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研究課題名（和文）Precision-Targeted Nanotherapy for Ischemic Stroke and Related CNS Disorders

研究課題名（英文）Precision-Targeted Nanotherapy for Ischemic Stroke and Related CNS Disorders

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研究成果の概要（和文）：神経変性、特に虚血性脳卒中の患者に臨床的に使用可能なニューロトロフィンを設計した。血液脳関門に侵入し、神経変性を緩和する能力を持つ神経栄養因子をカプセル化する特定のペプチド-ターゲット複合固体脂質ナノ製剤（HSLNs）を合成して使用した。
本研究にはMCAOモデルを採用し、HSLNは高い生体適合性を示した。RDPターゲットを付加したHSLNsはin vivo/ex vivoイメージングによって脳の虚血領域での蓄積の増加が確認された。虚血誘発マウスを用いた実験においても、RDP/BNDF-HSLN処置したマウスの大部分で、虚血帯が完全に、または大部分が減少することが示された。

研究成果の学術的意義や社会的意義
本プロジェクトで開発したナノ製剤は、血液脳関門を効率的に通過し、疾患部位に神経保護剤を送達することにより、虚血性脳卒中によって引き起こされる神経変性を効果的に軽減できることを明らかにしている。特に近年急速に進む世界人口の高齢化を考慮すると、本研究結果で示した高レベルの治療効率の達成は、科学的および社会的の双方において重要な意義を有するとともに大きなインパクトをもたらすと考える。

研究成果の概要（英文）：This project was designed to make neurotrophins, a clinical reality for patients with neuronal degeneration, particularly ischemic stroke. Specific peptide-targeted hybrid solid lipid nanoformulations (HSLNs) encapsulating neurotrophic factors with ability to trespass the blood brain barrier (BBB) and alleviate neuronal degeneration were utilized.
The HSLNs had excellent in vitro/vivo biocompatibility. Successful MCAO models were established for the study. HSLNs loaded with NGF &/or BDNF, either non-targeted or RDP/RMP-7 targeted were tested. For targeting investigations ICG dye was encapsulated instead of neurotrophins. RDP targeted HSLNs showed excellent results with increased accumulation, determined by in vivo/ex vivo imaging, in the ischemic region of the brain. This was reflected in the therapeutic investigations as well with majority of the ischemia induced-RDP/BDNF HSLN treated mice showing highly reduced ischemic zone. Overall, the research was a great success.

研究分野：Nano Drug Delivery

キーワード：Nano Drug Delivery　Ischemic Stroke　Neurotrophins　Blood Brain Barrier　Neurodegeneration

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１．研究開始当初の背景

Neurodegenerative diseases, depression, dementia and stroke (Fig. 1), account for approximately 40% of annual incidences resulting in human disability and death. Neurological disorders are caused by a multitude of reasons which can be induced by molecular and environmental stress factors related to diverse mechanisms of neuronal cell death (e.g. neurotrophin deficiency, oxidative stress, DNA damage, altered gene expression, protein fibrillation, aggregation, hyperphosphorylation, or unknown structural and metabolic conditions). Although neurodegenerative and psychiatric diseases are a main priority for healthcare delivery systems, most of the existing therapies focus on modifying disease progression and the symptoms of the disease (i.e. symptomatic therapy) without treating the neuronal cell loss. Neuronal survival would require activation of the signaling pathways underlying neuroprotection and neurogenesis, which could rescue diseased neurons.

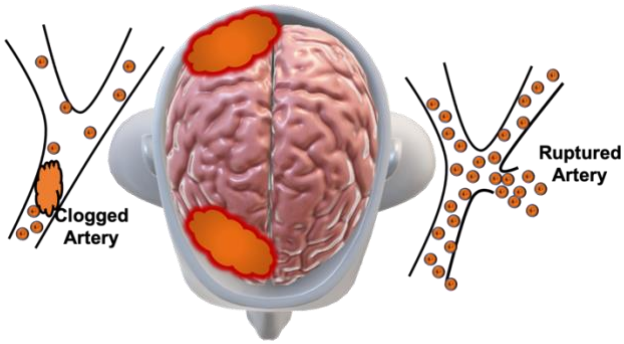


Fig.1. Major cause of Ischemic Stroke

Neurotrophic actions are exerted by four members of the neurotrophin family: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and NT-4/5. Among them, BDNF is the most abundant neurotrophin in the mammalian CNS and displays trophic effects on a range of neuronal cells, including cortical, hippocampal, cerebellar and motor neurons. Given that BDNF-induced signaling is crucial for neuronal survival, function, morphogenesis and plasticity, it has been suggested that the neurotrophin receptors constitute potential therapeutic targets for several neurodegenerative and psychiatric diseases. Increasing the BDNF levels in particular areas of the CNS can rescue the damaged neurons. The nano drug delivery systems, which have now become an indispensable part of scientific research, and which have reached the clinic for actual patient applications, are the most optimal choice to address this situation.

２．研究の目的

The purpose of this research proposal was to develop an efficient strategy employing nanoparticles (NPs) for delivering neutrophins such as NGF & BDNF across the BBB to the brain.

⇒ NP-based platforms have attracted much attention for the delivery of drugs/molecules with neuroprotective/regenerative activities that, under normal conditions, cannot pass through BBB.

Making neurotrophins a clinical reality for patients with neuronal degeneration and physical trauma (i.e., age-related/accidents/sports-related injuries) is of prime essence.

３．研究の方法

To explore treatment strategies that can be utilized for ischemic strokes and other CNS disorders.

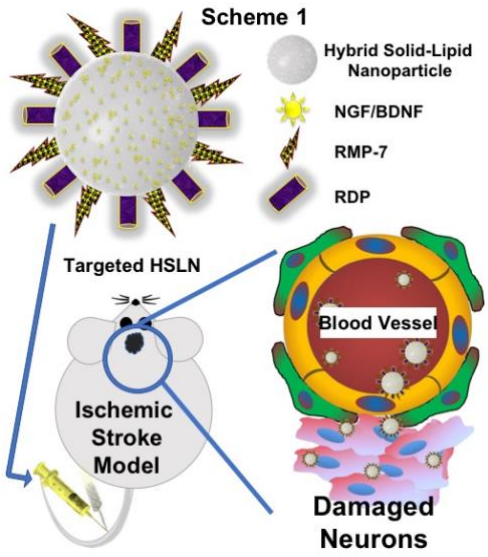
The brief details of the strategy (**Scheme 1**) are:

- **Nanocarrier:**
  - A biocompatible polymeric-lipid shell: with stealth properties (for enhanced blood-life); enhanced drug sink (stable and extended shelf-life) synthesized via simple thin-film hydration technique. The

components of the nanoparticle were DSPE PEG 2000-Amine, stearic acid and L-α-Phosphatidylcholine in equal proportions.

➢ **Cargoes:**

- ***Therapeutics***: brain-derived neurotrophic factor (BDNF) and/or nerve growth factor (NGF).
- ***Imaging***: Rhodamine-B for in vitro & Indocyanin Green (ICG) dye for in vivo imaging.



➢ **Loading:** The loading of the neurotrophins/dye was done by the thin film hydration technique. The thin film of the HSLN was hydrated with aqueous solutions of the desired neurotrophins or the dye. For biocompatibility studies, the thin film was hydrated with PBS.

➢ **Targeting:** RMP-7/RDP peptides, individually (single-targeted) and in conjunction (dual-targeted). The conjugation of the peptides to the HSLNs was done by utilizing the EDC-NHS chemistry. The final suspension was centrifuged at 50,000rpm for 10 min and the supernatant collected for further analysis. Non-targeted HSLNs were devoid of the peptides.

➢ **Mice Models:** 8 week old male C57BL/6J mice were used for the induction of the mid-carotid artery occlusion (MCAO) as models for ischemia.

➢ **Therapeutic Regime:** Within 1h of MCAO induction, the mice were injected with the nanoformulations (therapeutic/imaging). Post 24h of injection the mice were assessed for the ischemic zone (TTC staining) and accumulation of HSLNs (NIR imaging at different time points till 24h).

4．研究成果

➢ **HSLN Preparation:** A simple thin film hydration methodology was utilized to prepare self-assembling HSLNs (Fig. 2). An average particle size of approximately 125±20 nm was observed, a size desirable for receptor mediated BBB transcytosis. A slight increase in size was observed with the peptide conjugated versions [RDP (145±20nm), RMP-7 (150±20nm), Dual (150±20nm)]. TEM observations revealed the formation of monodispersed, spherical to ovoid particles, without any aggregation. HSLNs exhibited a net negative surface charge, with lower values of ζ potential for non-targeted [NT (-28±5mV)] formulations as compared with peptide-conjugated [RDP (-15±5mV), RMP-7 (-19±5mV), Dual (-8±5mV)] versions. The HSLNs remained stable for more than 24 months in suspension and retained their physicochemical properties even after treatment with serum, a proof of their long shelf life and stability.

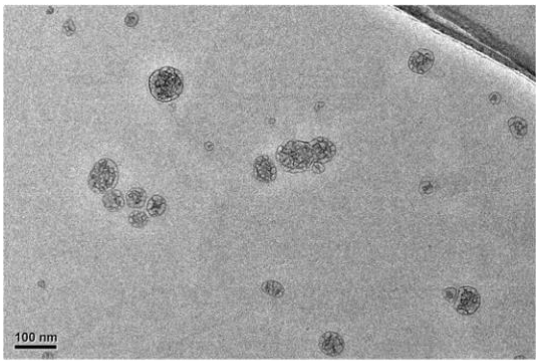


Fig. 2. HRTEM micrograph of HSLNs

➢ **Neurotrophin Loading and Release:** The therapeutic agents-BDNF and NGF were encapsulated into the HSLNs as previously described. The loading efficiency was very high with approximately 85% of the cargo being encapsulated. The release

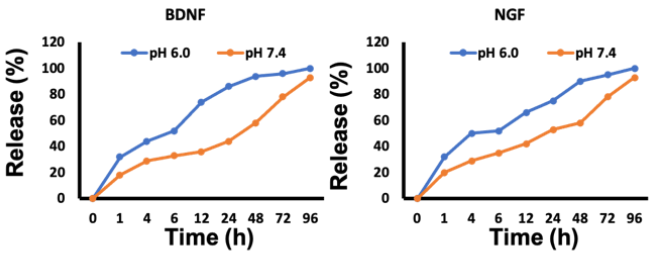


Fig. 3. BDNF & NGF Release from HSLNs

profile was assessed at two different pH conditions (6.0 & 7.4) (Fig. 3). The acidic pH was tested considering the lower pH (5.7-6.5) environment in hypoxic regions such as ischemia. The observations were very encouraging as the release profile for both NGF and BDNF was accelerated at lower pH, with more pronounced release in the case of BDNF.

- **Cyto & Biocompatibility:** The HSLNs were tested for their effect on the normal human brain cell lines namely Human cortical neurons (HCN-1A), Human astrocytes (HA), Human brain pericytes (HBPC) and Human brain microvascular endothelial cells (HBMEC). The tested concentrations ranged from 0.1mg/ml to 0.5mg/ml. Though there was a decrease in the cellular viability at the highest concentration tested, the cellular viability of all the cells under study remained above 80% after a period of 72h (Fig. 4). Additionally, the ROS generation was extremely low, remaining below 2% in all cell lines at all concentrations tested. Under in vivo conditions as well the HSLNs displayed excellent compatibility with the no deleterious effects on the mice. The bodyweight measurements revealed no observable changes from the HSLN administered and control mice. Additionally, the blood biochemistry and histopathology results also confirmed no damage to the vital organs or elevation/decrease in blood parameters when compared with control mice.

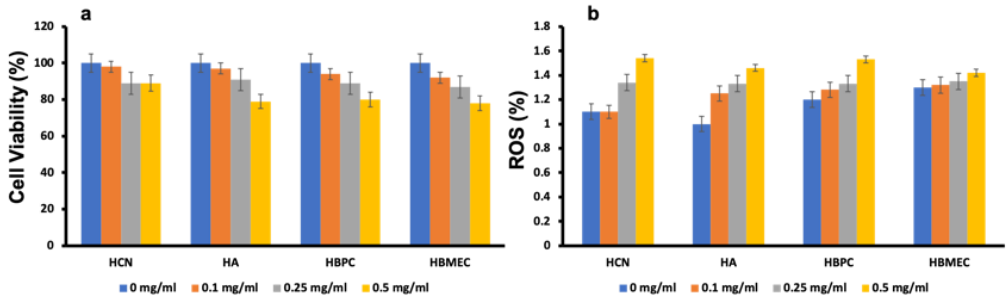


Fig. 4. Cytocompatibility of HSLNs

- **Biodistribution:** One of the major hurdles for any therapeutic agent or even nanoformulations is their ability to bypass the body's clearance systems and reach and accumulate specifically at the desired site. The mice were injected with ICG-loaded HSLNs and imaged at different time points ranging from 0h to 24h. It was observed that the HSLNs were predominantly present in the blood stream even after 6 hr of injection (Fig. 5). This is a welcome sign since this provides ample opportunities for the circulating HSLNs to reach the target site. Additionally, a small percentage of the NPs were present in the brain as well. In the case of ischemia induced mice, a significant accumulation was observed in the brain, specifically in the region of ischemia. This could be attributed to the compromised nature of BBB due to the induction of a hypoxic environment, facilitating the entry of the HSLNs. Additionally, it was observed that the RMP-7 and RDP peptide conjugated HSLNs were more efficient in penetrating the BBB and accumulating at the ischemic zone. These results provide evidence of the specificity of the targeted HSLNs.

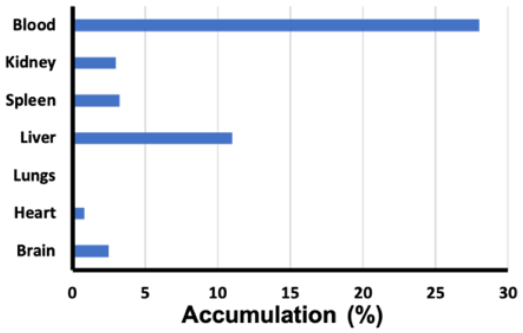


Fig. 5. Biodistribution of HSLNs

- **Therapeutic Outcome:** Firstly, the HSLNs were tested on in vitro BBB constructs which had been exposed to hypoxic conditions. The Phospho TrkA & TrkB and Phospho CREB levels were highly elevated when compared with controls in the NGF and BDNF loaded HSLNs (both non-targeted and targeted). These markers are essential for cell survival, proliferation and especially in the case of neurons

they play a vital role in neuronal survival/development and differentiation. The BDNF loaded formulations expressed higher levels of these markers when compared with NGF. Further, once the targeting potential of the HSLNs was established, the therapeutic formulations were tested to see if they were as

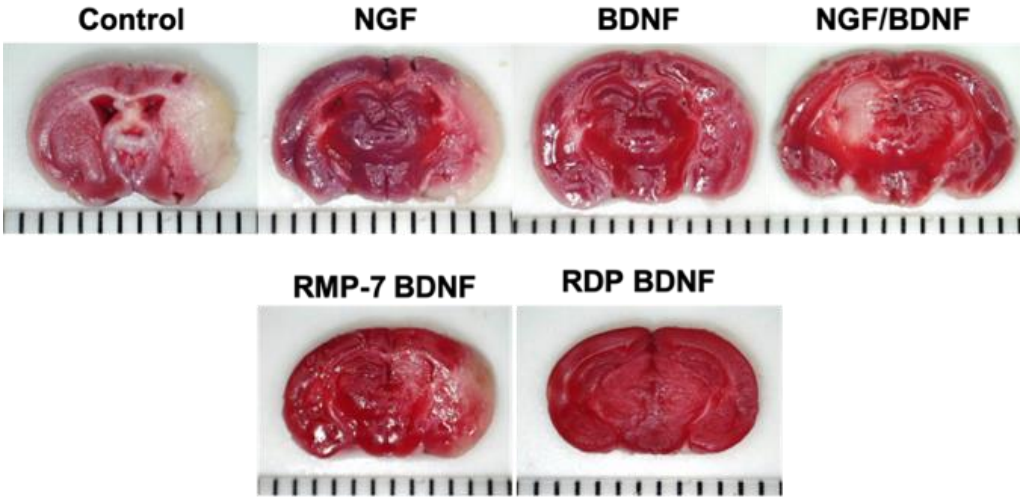


Fig. 6. Therapeutic activity of HSLNs

efficient in alleviating the neurodegeneration induced by the MCAO. The control mice had an average ischemic zone volume of 25-50%. It was observed that the BDNF was proactively better in reducing the ischemic zone (18.5%) when compared with NGF (23.6%) (Fig. 6).

The combination of NGF & BDNF was not as effective (24.8%), possibly due to the low concentration of each neurotrophin, especially BDNF. Also, in the case of the targeting moieties, RDP was found to have better accumulation and effect (0-9.5%) than RMP-7 (17.5-23.3%). Though RMP-7 was slightly inferior in its therapeutic effects, nevertheless it was successful in reducing the ischemic zone to an extent as well. Overall, the volume calculations after TTC staining revealed that there was significant reduction in the RMP-7 and RDP peptide targeted BDNF loaded HSLNs when compared to the control mice (no treatment). Since the therapeutic potential of RDP targeted HSLNs was excellent and that of RMP-7 was not, the dual targeted HSLNs (RDP/RMP-7 HSLNs) were not tested during the current research period.

- **Conclusion:** Highly biocompatible shape and size tuned HSLNs were synthesized with a high loading efficiency for the neurotrophins NGF & BDNF. A sustained release at physiological pH and further accelerated release at low pH was observed which was beneficial in the hypoxic environment of the ischemic region. The HSLNs were efficient in trespassing the BBB and accumulating preferentially at the ischemic zone. High accumulation was observed with RDP followed by the RMP-7 peptide targeted nanoformulations. These observations were reciprocated in the therapeutic outcome as well where the RDP peptide conjugated HSLNs showed high effectiveness in reducing the ischemic region thereby exerting neuroprotective and anti-apoptotic activities. These results are highly significant since the administration of the therapeutic formulations was only once. It is speculated that if the injected dose is increased or the HSLNs are administered at regular intervals, for ex. once every 2-3 days, the therapeutic outcome may be far higher than the current observations. Further analysis of motor functions and survival statistics will be elucidated as a future scope of the present work.

With a rapidly aging population both globally and especially in Japan, the incidence of neurodegenerative diseases is expected to increase significantly. The current research results aims to a provide a solution to this menacing issue.

５．主な発表論文等

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2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関