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Intranasal Administration of Chitosan-Nanoparticles Conjugated with Imipramine and its Effect on Stroke-Induced Secondary Neurodegeneration: A Research Protocol

Sarthak Kakar, BSc Student [1]*, James N. Smorenburg, BSc Student [1]

[1] Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

*Corresponding Author: sarthakkakar37@gmail.com

Abstract

Introduction: Stroke is the second leading cause of death and the third leading cause of permanent disability worldwide. Notably, the recovery period post-stroke is crucial as there is a risk of stroke-induced secondary neurodegeneration. Stroke-induced secondary neurodegeneration is the inevitable loss of viable brain tissue at sites distal from the initial infarct. It shares similarities with neurodegenerative diseases and results in neurological deficits, further complicating stroke recovery. Intranasal administration of chitosan-nanoparticles conjugated with imipramine will be tested to determine if they elicit a synergistic effect in mitigating disease processes associated with stroke-induced secondary neurodegeneration.

Methods: Treatment and testing will be conducted in 30 male Wistar rats aged 12 months. Stroke will be induced by occluding the middle cerebral artery. Rats will be divided into three groups of 10 and will receive an intranasal dose of either saline (Control, C), 20mg/kg of imipramine (Treatment 1, T1), or 20mg/kg of chitosan-nanoparticles conjugated with imipramine (Treatment 2, T2). Statistical analysis using analysis of variance will determine if chitosan-nanoparticles conjugated with imipramine can mitigate the effects of stroke-induced secondary neurodegeneration determined by the proposed tests.

Results: T2 given 20mg/kg of chitosan-nanoparticles conjugated with imipramine is proposed to spend more time exploring the unfamiliar object in the novel object recognition test. Lesser evidence of Alzheimer's disease in T2 is expected, as measured by the fludeoxyglucose positron emission tomography imaging. A higher serum brain-derived neurotrophic factor measured by an enzyme-linked immunosorbent assay is also expected to be present in T2.

Discussion: It is anticipated that chitosan-nanoparticles conjugated with imipramine will exhibit a synergistic effect in mitigating disease processes accompanying stroke-induced secondary neurodegeneration because of properties associated with neuroplasticity and enhanced drug targeting efficacy.

Conclusion: This research protocol aims to elucidate a novel treatment that can be applied to stroke recovery to mitigate stroke-induced secondary neurodegeneration, which tends to complicate this crucial period. Our proposal could have implications in the prognosis and management of stroke and post-stroke recovery, respectively, and inspire a framework for the discovery of novel post-stroke therapeutic interventions.

Keywords: stroke recovery; stroke-induced secondary neurodegeneration; chitosan-nanoparticles; imipramine; Alzheimer's disease

Introduction

According to the World Health Organization, stroke is the second leading cause of death and the third leading cause of permanent disability worldwide [1]. Ischemic stroke occurs due to the occlusion of blood vessels supplying the brain and consequently depriving it of oxygen [2]. Although stroke mortality continues to decline [3], those who survive the initial infarct may often face inevitable progressive loss of neurons, a phenomenon known as stroke-induced secondary neurodegeneration (SND) [4]. SND results in neuronal loss at sites distal but connected to the initial site of infarction, damaging viable brain tissue [4]. Notably, the burden on families and the healthcare system due to SND is overwhelming as extensive damage leads to cognitive symptoms (post-stroke cognitive impairment, PSCI) and physical impairments that are detrimental to quality of life as they hinder activities of daily living [5]. Therefore, despite the considerable progress in managing stroke and care provided to patients, new interventions to help improve neuronal reorganization and promote plasticity post-stroke are critically needed to improve the treatment and recovery of patients.

SND shares similarities with other neurodegenerative diseases, such as Alzheimer's disease (AD), because it also results in the accumulation of beta-amyloid plaques in the brain [4]. This characteristic may explain the high incidence of dementia post-stroke [6,7]. Although significant progress has been achieved concerning the biology of

neurodegenerative disorders, the ability to treat these conditions remain limited due to poor regenerative capabilities of the human central nervous system (CNS) and the difficulty associated with delivering pharmacological treatment across the blood-brain barrier (BBB) [8]. To overcome this problem, biomaterials have been used to transport drugs across the BBB. Chitosan, a polysaccharide biomaterial composed mostly of D-glucosamine obtained from chitin, has a variety of biomedical applications [9]. Usage of chitosan-nanoparticles (C-NP) supports the CNS, averting its weak regenerative capacity by providing a scaffold for reconstructing lost tissue and allowing for the reconnection of neuronal processes [9]. Although commonly used as a drug delivery vector, C-NP administration has also exhibited neuroprotective effects against diseases such as AD by suppressing the formation of beta-amyloid [9]. Since SND shares similarities with neurodegenerative diseases like AD, using C-NP for SND may mitigate associated neuronal loss.

Moreover, C-NP exhibits similar neuroprotective effects to those observed with the tricyclic antidepressant imipramine. Traditionally, imipramine's clinical purpose is to prevent the reuptake of norepinephrine and serotonin, thereby relieving symptoms of depression [10]. However, it has also been shown to improve cognition in mice after traumatic brain injury and has promising effects on neurogenesis, neural remodelling, and hippocampal synaptic plasticity [10]. These are thought to be related to the upregulation of brain-derived neurotrophic factor (BDNF) [10,11]. BDNF signalling is crucial for neurons and promotes neuronal plasticity, which may positively affect patients with AD [11]. As highlighted above, SND and AD share similar pathological processes, and C-NP and imipramine potentially offer therapeutic effects in AD.

Based on these findings, it is hypothesized that the intranasal administration of a C-NP-imipramine conjugate can be applied to the disease processes that accompany SND, as both promote neuroplasticity in the CNS. Thus, this research protocol aims to test whether the intranasal administration of a C-NP-imipramine conjugate can provide synergistic effects to mitigate the hindrances of stroke recovery caused by SND. The results of this study may provide a basis for future research of novel treatments to better the quality of life of patients and decrease the implications of stroke on families and the healthcare system.

Methods

Animal Model and Treatment Groups

This research protocol was informed by the ARRIVE guidelines. The use of 30 male (*n=30*) Wistar rats aged 12 months will be used in this experiment (see Figure 1). All rats will be given the same diet throughout the study and will be caged in conditions controlled for temperature and humidity. Additionally, nesting material and shelter will be provided in their home cage. Light/dark cycles will be in 12-hour increments, with lights on during the hours of 8:00 PM-8:00 AM due to the nocturnal nature of rats. A period of seven days before the start of the study will be used to acclimate the rats to their cage to reduce any associated travel anxieties and become familiar with the lab and cage setting. On day one (one week after rats are obtained), all 30 Wistar rats will be randomly distributed evenly into one of the three treatment groups. The control group will receive an intranasal saline spray (C, *n1 = 10)*, while the Treatment 1 group will be administered imipramine intranasally (T1, *n2= 10)*. Finally, the Treatment 2 group will be administered a C-NP-imipramine conjugate intranasally (T2, *n3=10*).

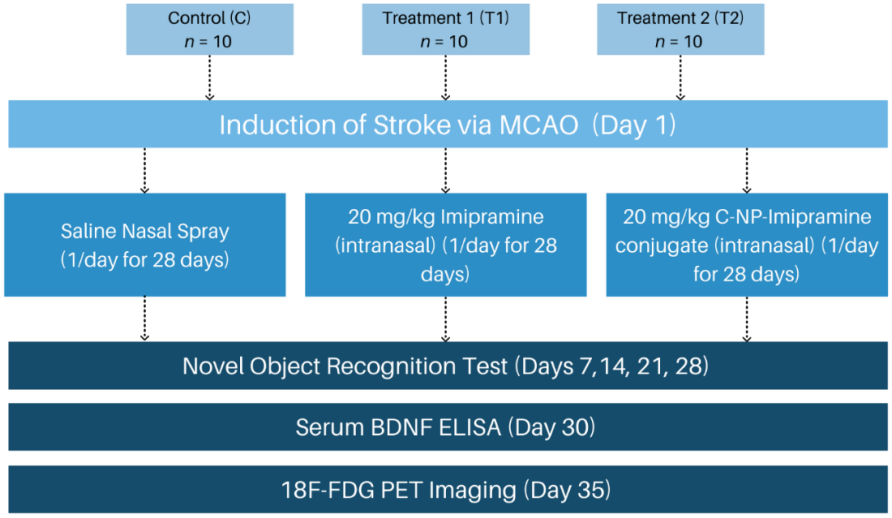

Figure 1. An outline of the methodology presented in this research protocol. This figure was created by the authors using Canva.

Induction of Stroke

Approximately three hours after each rat has been placed into their respective group, an ischemic stroke will be replicated in all rats by occluding the middle cerebral artery (MCA). Prior to stroke induction, buprenorphine will be administered two hours pre-surgical intervention at a dosage of 0.03 mg/kg injected intraperitoneal [12]. At the time of surgery, rats will be induced with 5% isoflurane and maintained at 2% [12]. All rats will have their body temperature maintained through a heating pad. Once anesthetized, the common carotid artery will be temporarily occluded, and a laser Doppler-guided suture coated with silicone will be introduced to the internal carotid artery and advanced until the MCA is occluded [12,13]. A 22 mm suture (3/0 diameter) will be used to ensure consistent reproducibility of middle cerebral artery occlusion (MCAO) in each rat [12]. The MCA will be occluded for a total of 60 minutes. After the 60-minute interval is completed, the MCAO will be reversed. All rats will be continuously monitored to ensure the return of mobility for one hour post reversal, before being returned to their home cage.

Treatment Regimen Post-Stroke

Dosing regimens will begin once mobility has returned, and rats have returned to their home cage. All treatment interventions will be administered daily for 4 weeks (a total number of 28 treatments). The control group will be administered saline daily via the intranasal spray, while rats in T1 will be administered a daily dose of 20mg/kg of imipramine intranasally. Rats in T2 will be administered a C-NP-imipramine conjugate equivalent to 20mg/kg once per day intranasally for a total of 28 treatments. The preparation of C-NP follows methodology by Tian-Yuan *et al*. [14]. All interventions will be administered at the same time throughout the length of the study.

Cognitive Function Evaluation

A novel object recognition test (NOR) adapted from Han *et al.* [10], will be used to evaluate cognitive ability in C, T1, and T2 groups (see Figure 2). NOR closely resembles human conditions to study cognition, proving a valuable way to enhance translatability of behavioural outcomes across species [15]. Each rat will undergo the NOR 4 times throughout the course of the study, with the first test on day 7, second on day 14, third on day 21, and fourth on day 28. On the days before testing (days 6, 13, 20 and 27), each rat will be placed in an empty, transparent PVC testing chamber for one hour and then placed back into their respective cage (habituation phase) [10,15]. On test days, either a Lego pyramid or a 50mL cone-shaped tube (fixed to the chamber's floor) is placed in opposite quadrants of the testing chamber (training phase). Each rat is placed in the middle of the chamber and is video recorded for a total of five minutes. A blinded observer to the treatment groups will record the cumulative time exploring each object. Exploration of an object is defined as the rat's head being approximately 2-3cm away and facing the object [10,15]. After the five-minute interval has ended, rats will be placed back into their housing cage. Five hours later**,** each rat will be reintroduced into the same testing chamber, with one of the objects replaced by another of similar size and complexity (testing phase). Again, rats will be placed in the middle of the chamber and recorded for a total of five minutes. A blinded recorder will then determine the cumulative time spent exploring each object. Each testing chamber is carefully washed with water and 70% v/v ethanol between each trial to remove any odours. Humidity and temperature in the testing chambers are maintained similarly to housing conditions. The center of the testing chamber is illuminated at 20 lux. Total time spent exploring each object is noted, and a discrimination ratio is calculated for all rats. Discrimination ratio is equal to the time spent exploring the new object minus the time spent exploring the old object divided by the total time spent exploring both new and old objects. Rats that spend more time exploring unfamiliar objects are typically seen as healthier [10,15].

^{18}F-Fludeoxyglucose Positron Emission Tomography Imaging

On day 35 (a week after the last treatment), all rats will undergo positron emission tomography (PET) with fludeoxyglucose (^{18}F-FDG) to identify changes in hypothalamic and cerebellar glucose metabolism associated with AD as concluded by Lu *et al.* [16]. Rats will fast for a total of 24 hours after arrival at a PET-CT centre. All rats' blood sugar measurements will be determined, and then rats will be rested in a dark room for 30 minutes. 1.5 MCi/500g of ^{18}F-FDG will be administered via the tail vein, and again rats will be given 30 minutes of rest. Rats will then be anesthetized with 5% isoflurane, and PET imaging will be conducted, covering the head and neck region. Statistical Parametric Mapping 2.0 will be used to process the imaging.

Quantitative ELISA of Serum Brain-Derived Neurotrophic Factor

All animals in C, T1 and T2 will be tested on day 30. Blood will be collected from each rat and allowed to clot. Serum is obtained via centrifugation at 1000 x *g* for 15 minutes. Enzyme-linked immunosorbent assay (ELISA) preparation and protocol are outlined in Dalise *et al.* [17]. ELISA kits obtained from Aviscera-Bioscience will be used to provide the best reproducibility [18]. Optical density is measured at 450nm and averaged over two measurements. A curve of average optical density at 450nm of each reference standard solution vs. the respective concentration of each standard solution is plotted, and the BDNF concentration of the sample is interpolated.

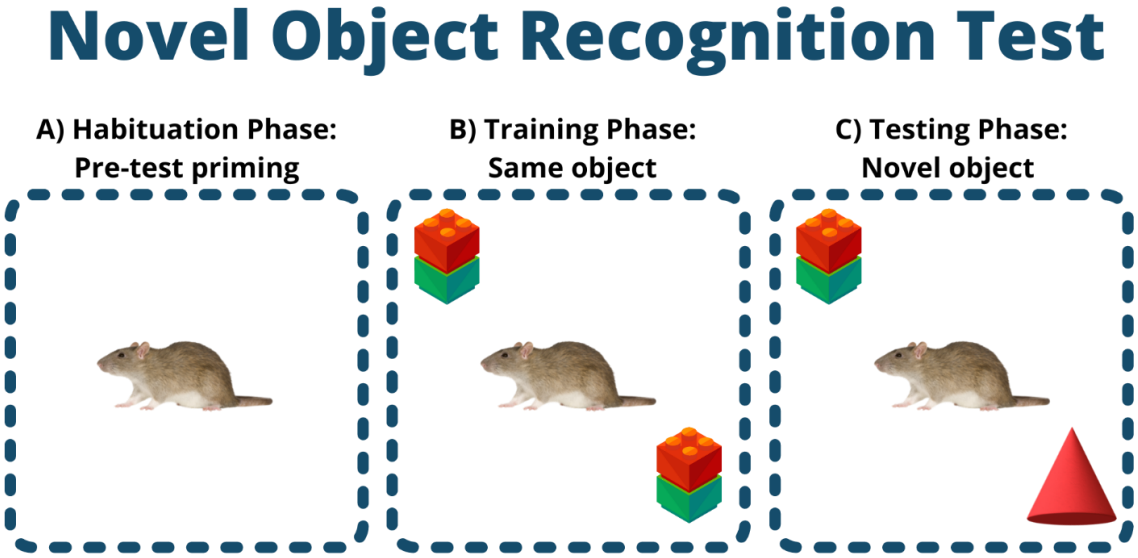

Figure 2. The novel object recognition test (NOR). On days before NOR testing depicted by (A), rats are placed in an empty testing chamber for a total of one hour. On days of testing, rats are placed in a testing chamber with similar objects placed in opposite quadrants and recorded for five minutes (B). Five hours later on the same day, one object is replaced with a new object of similar shape and complexity and rats are again recorded for five minutes (C). This figure was created by the authors using Canva.

Statistical Analysis

All 30 animals will be included in the statistical analysis for the NOR test. Mean discrimination ratios previously obtained from each group (C, T1, T2) will be analyzed using a one-way analysis of variance (ANOVA). Statistical significance will be determined if $p<0.05$. If there is a significant difference found, then further analysis will be conducted by a two-way post-hoc comparison. Statistical Parametric Mapping 2.0 analysis of ^{18}F-FDG PET imaging is conducted in C, T1, and T2, and a voxel-wise comparison and analysis of the PET images is completed. Five rats from each of C, T1 and T2 will be used to analyze serum BDNF concentrations statistically. BDNF concentrations will be presented as mean ± SEM, and a one-way ANOVA will be used to measure the significance of serum BDNF concentrations between C, T1 and T2. Statistical significance is determined if $p<0.05$.

Results

Higher discrimination ratios are indicative of rats who spend more time exploring the novel object. Thus, it is postulated that the results of the NOR will show that T2 will have a higher discrimination ratio, while the control will have a ratio that is lower than both T2 and T1. T2 will potentially show the least amount of glucose hypometabolism in the hypothalamus and/or hypermetabolism in the cerebellum as shown on the ^{18}F-FDG-PET, which are associated with the progression of AD. C and T1 will likely exhibit greater evidence of altered glucose metabolism than T2. ELISA is estimated to show T1 and T2 as having the highest serum BDNF concentration, and C having significantly less than both the treatment groups.

Discussion

This research protocol aims to understand the effects of the intranasal administration of a C-NP-imipramine conjugate during stroke recovery to mitigate the hindrances associated with SND. The treatment groups proposed are used to study whether imipramine (T1) or a C-NP-imipramine conjugate (T2) can reduce SND symptoms, including PSCI and the potential development of neurodegenerative diseases such as AD.

The use of an NOR will allow researchers to compare the cognitive abilities of rats between groups. Han *et al.* [10] concluded mice treated with imipramine after cognitive brain injury had better cognitive outcomes due to neurogenesis in the hippocampus. C-NP provides enhanced efficacy in targeting drugs to the brain [9], and therefore the C-NP imipramine conjugate should show that rats in T2 spend more time exploring the new object than those in T1, indicating better cognitive abilities [10].

^{18}F-FDG PET imaging provides information about altered hypothalamic and cerebellar glucose metabolism that are associated with AD. Lu *et al.* [16] concluded that glucose hypometabolism was present in the hypothalamus, and glucose hypermetabolism was seen in the cerebellum of rat models induced to have AD. It is likely that both control and treatment groups will have altered glucose metabolism

to some extent [16], however, previous literature shows that both C-NP and imipramine have features of neuroplasticity which could provide protection from this alteration [9,10]. Absence of such alterations suggests a therapeutic effect in preventing the onset of neurodegenerative diseases such as AD, that accompany SND.

BDNF is shown to be important in post-stroke recovery [19]. BDNF has important roles such as promoting neuroplasticity and facilitating neurogenesis which are key factors for stroke rehabilitation [19]. Therefore, an ELISA will allow for quantification of serum BDNF levels between control and treatment groups. As previously mentioned, imipramine upregulates BDNF levels which should be reflected in T1 and T2, but higher in T2 due to enhanced drug targeting efficacy of C-NP [9,10]. Although BDNF may not be a clinically reliable biomarker, the ELISA will provide correlational information when analysing its results in accordance with the results from the NOR and ^{18}F-FDG-PET [18].

There are some limitations that are present in this research protocol. Only male rats were used in this protocol because of age. Typically, at 12 months of age female rats enter a period of irregular reproductive cycles termed estropause which alters levels of hormones such as estrogen and progesterone [20]. The effects of estrogen are apparent in stroke recovery [21], and for this reason, female rats were excluded. However, it is acknowledged that using only male rats also poses limitations on the generalizability of the study and will therefore not account for potential sex differences in response to the treatment groups. Therefore, if positive results were obtained, it cannot be guaranteed that the novel treatment of imipramine conjugated with C-NP would show similar results in female rats. It is also important to note the difficulty attributed to translating drug efficacy and safety from rodent models to humans. Novel treatments may prove efficacious in animal trials, but conclusions cannot be drawn about efficacy in humans until evidence from clinical trials is obtained.

Conclusions

Stroke rehabilitation is of utmost importance when trying to mitigate stroke-induced morbidities, but current projections suggest the need for novel treatments to combat such morbidities. The use of nanoparticles for neurodegenerative diseases has recently received significant attention and has shown promise in mitigating and treating such diseases. To our knowledge, this is the first research protocol aimed at conjugating C-NP with the tricyclic antidepressant imipramine to study the possible combinative effect they provide after stroke in a rat model. This research protocol aims to broaden the scope of research in mitigating SND by using novel treatments. Future research should focus on dosing regimens and sex differences if results are shown to be promising. Moreover, they should aim to study both acute and long-term responses to the interventions mentioned in this protocol. Finding efficacious treatments that enhance post-stroke recovery should be researched, as novel treatments may provide a better understanding of functional and structural changes in the CNS related to SND.

List of Abbreviations Used

C, T1, T2: control, treatment 1, treatment 2
SND: stroke-induced secondary neurodegeneration
PSCI: post-stroke cognitive impairment
AD: Alzheimer's disease
CNS: central nervous system
BBB: blood-brain barrier
C-NP: chitosan-nanoparticles
BDNF: brain-derived neurotrophic factor
MCA: middle cerebral artery
MCAO: middle cerebral artery occlusion
NOR: novel object recognition test
PET: positron emission tomography
^{18}F-FDG: fluorodeoxyglucose
ELISA: enzyme-linked immunosorbent assay
ANOVA: analysis of variance

Conflicts of Interest

The authors declare they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This is a proposed protocol therefore it has yet to gain ethics approval. Considering the use of live animals prior to execution of this experiment, ethics approval is essential.

Authors' Contributions

SK: collaborated in conceiving the hypothesis and made substantial contributions to the abstract and introduction, created figures, critically revised and edited the manuscript, formulated manuscript in accordance with guidelines and template, gave approval for this version to be published, and agreed to be accountable for all aspects of their work.
JS: collaborated in conceiving the hypothesis and made substantial contributions to the abstract and introduction, formulated the methodology, results, discussion, conclusion and critically revised and edited the manuscript, gave approval for this version to be published, and agreed to be accountable for all aspects of their work.

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