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The Gut-Brain Axis: Assessment of EcNL-DOPA in mice and dogs

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Abstract

The gut-brain axis is becoming a hot topic in the research world for its possible role in many disorders and diseases. As we take a deeper dive in to its role in neurodegenerative disorders like Parkinson's disease, we present evidence of a strategy of using genetically engineered probiotic to help regulate and maintain levels of L-DOPA, the gold standard of treatment used in Parkinson's disease patients. Evidence from trials with mice and dogs present significant findings that prove that a small change like adding a modified E. Coli probiotic may be all the change needed to provide a better, more stable treatment to neurological disorders like Parkinson's disease.

Introduction

Parkinson's disease (PD) is acknowledged as the second most common neurodegenerative disorder, estimated to affect 1-2 people per 1000 of the population worldwide (1). PD is a costly neurodegenerative disease that may pose a significant economic burden on patients, the healthcare system and society. The chronicity of PD and its lack of treatment make its projected economic burden to grow substantially over the next few decades (10), with out-of-pocket costs for neurologic medications has increased considerably over the last 12 years (11). The total cost of PD is $52 billion every year, with $25.4 billion attributable to direct medical costs such as hospitalizations and medication, and $26.5 billion in non-medical costs like missed work, lost wages, early forced retirement, and family caregiver time (12).

To try and alleviate this financial burden, researchers are looking into signs of neurodegenerative disease in earlier stages of life. In the last decade, emerging evidence has revealed the presence of an intense dialogue between the brain and the GI system, the so-called gut-brain axis. The microbiota-gut-brain axis has attracted much attention regarding the pathogenesis of PD, in which GI dysfunction appears about twenty years before motor

impairments. Although PD patients manifest both gut dysmotility and altered microbial composition, it is still unclear which condition comes first and what role the gut and the gut microbiota have in PD progression (2). L-DOPA has proven to be the most reliable and efficacious of the current PD therapeutics for motor deficits in patients with PD. To date, L-DOPA remains the benchmark therapeutic for the restoration of dopamine in PD (3). L-DOPA is not without its negative side effects, however, as increased treatment duration is associated with the occurrence of dyskinesia (4). These effects are believed to be caused by the current pulsatile delivery of L-DOPA, causing L-DOPA peaks, responsible for the L-DOPA-induced dyskinesia, followed by off-periods, responsible for depression and anxiety. There is thus a dramatic need for a therapeutic strategy allowing a more constant delivery of L-DOPA. There are other options than taking L-DOPA orally to minimize side effects or have stable L-DOPA levels. Some options are using drugs like Ondansetron in addition to L-DOPA to reduce L-DOPA induced dyskinesia (13) or use entirely new therapies like stereotaxic surgery injecting a drug cocktail to alter neurons (14) or doing duodenal levodopa infusions for more stable plasma levels and better motor control (15). These alternatives do not treat Parkinson's side effects as well as and/or are much more invasive and expensive than to treat with L-DOPA, meaning that L-DOPA is still considered the best treatment course for patients with Parkinson's disease.
For that purpose, a probiotic using E. Coli has been genetically engineered to produce L-DOPA in a sustained manner (EcNL-DOPA) (5). In order to maximize L-DOPA's diffusion into the brain, we will use Benserazide in combination with EcNL-DOPA. Levodopa is a precursor to the neurotransmitter dopamine which is administered to increase its levels in the central nervous system. However, most Levodopa is decarboxylized to dopamine before it reaches the brain, and since dopamine cannot cross the blood-brain-barrier, this translates to little therapeutic gain with strong peripheral side effects. Benserizide inhibits the aforementioned decarboxylation, and since it itself cannot cross the blood-brain barrier, this allows dopamine to build up solely in the brain instead (5). By using EcNL-DOPA with Benserizide in both mice and dog trials, progress is being made for an effective treatment combating peaks and off-periods with L-DOPA usage.

Methods:

Animal models are essential in a preclinical study (6) because of their ability to replicate symptoms or progression of the disease for drug development. In order to research, discover and understand diseases, test subjects are necessary to figure out what is working and what is not. To enable this, reliable animal models are required (7). Despite the need for animal models, researchers cannot use animals for research without following strict guidelines from the Institutional Animal Care and Use Committee (IACUC). The researchers must ensure the humane care and use of animals while interpreting the regulations in a practical, meaningful, and reasonable manner that facilitates valuable research and teaching (8). Animal models have been used in a large amount of the disease research that has been published, but why use two animal models? This is because no single animal is able to mimic a given human disease, which is itself polymorphic between patients, but the differences between strains or species provides (an) unmatched opportunity to understand disease development and differential host response, and eventually fund new cures (9). Each species displays advantages as well as disadvantages over the other for showcasing a certain aspect of disease that would further test the efficacy of the treatment. Putting the treatment through tests like documenting the similarities and differences from one disease species model to another can be the difference of the treatment working or not working in a clinical trial. With this in mind, we evaluated L-DOPA and its effects on the Parkinson's mouse model and dogs that spontaneously generate a Parkinson's-like disease.

Mice Study

C57B6 mice and Parkinson's disease model mice (Mitopark) were divided into 6 groups; C57B6 treatment group, Mitopark treatment group, C57B6 control group, Mitopark control group, C57B6 BZ only group and Mitopark BZ only group. Both treatment groups of mice (C57B6 and Mitopark) are treated with a single dose of ($2x10^{10}$CFU)—suspended in formulating buffer EcN^{4}-LDOPA-Rham-Re and BZ (40mg/kg Benserazide 100ul). Each of the treatment groups contains 4 mice that will receive these treatments. The control groups of C57B6 and Mitopark receive no treatment and contain 3 mice each. The BZ only groups of C57B6 and Mitopark mice are

treated with a single dose of BZ (40mg/kg Benserazide 100ul) with each group containing 4 mice. Since this trial is done with mice, blood collection is restricted to only 2 blood collections per week, hence why multiple mice are used to perform the study and sacrificed at the last time point. The mice used were aged 18-20 weeks: 2 males and 2 females for Mitopark and C57B6 treatment and BZ groups, 2 males and 1 female for Mitopark control group and 3 females for C57B6 control group. Baseline urine and blood samples were taken before any treatments were given. At each time point during the study, submandibular blood collection in EDTA tubes for plasma preparation, 2 tubes of fecal samples and a urine sample were collected. At terminal time points at 4, 8 and 24 hours; blood collection by cardiac puncture for plasma preparation and brain dissection were collected and taken. All plasma L-DOPA, BZ and urine HVA levels were compared to baseline.

Dog Study

Dogs spontaneously experience similar symptoms to PD, expressed by depression, anxiety, and cognitive dysfunctions, and is thus a very relevant model for this disease (5). Accumulated data shows that the dog is the superior model system over mouse models for investigating the treatment of neurologic disease because of the environmental, genomic, and intestinal physiologic features they share with humans (16) They show a parallel aging process to humans as evidenced by beagles between 5-9 years old showing cognitive dysfunction similar to humans between 40 and 60 years old (17). Additionally, brain vs body size compares favorable between humans and dogs when compared to mice (18). Canine spontaneous disease models offer additional predictive value for treatment before transitioning to humans for clinical trials (19), along with dogs having being adapted to having a starch-rice diet similar to humans, which can improve our understanding of human evolution and disease (20, 21).

In this study, dogs were given EcNL-DOPA orally for up to 21 days, twice a day. Before administration, baseline samples were collected (blood, feces, cerebrospinal fluid [CSF]). During the treatment period (up to 21 days), blood and feces were collected again daily. On days 18-21, CSF was collected again in all dogs. Since this procedure can only be performed on 2-3 dogs per day, 4 days were required to complete the CSF taps on all 10 dogs. After CSP taps were

performed, the dogs no longer received EcNL-DOPA and entered a wash-out period to make sure EcNL-DOPA had indeed been evacuated from the dogs' system. Unlike EcNL-DOPA, Benserizide will continue to be given until the wash out period is completed (up to day 25). The reasoning for this is because most Levodopa is decarboxylized to dopamine very quickly in the system, it will not be found in the dogs' system without Benserizide. In order to be sure the wash-out is completed, we need to be able to obtain undetectable levels of L-DOPA with Benserizide. In addition to all of these measures, twice during the study (once between day -3 – day 0 and once between day 1 – day 21) each dogs' heart rate and ECG will be monitored continuously for 24 hours using Holter monitoring. Holter monitoring is a non-invasive monitoring system designed to remain on the dog for 24-48 hours without impacting dog's activity in any way. This is to give us additional data to ensure the dog's wellness and overall good health during the study. Feces, after being obtained from spontaneous defecation, was sampled on all 10 dogs in order to assess the presence of L-DOPA in the dog's stools, as well as any microbiome changes. Feces was collected once before treatment for baseline values, collected as many times as possible during the day during treatment administration and once a day in the morning during the wash-out period.

Blood collection from all 10 dogs followed a specific schedule, which is displayed on *Table 1*. Day -3 to day 0: up to 10mL of blood was collected all at once to run health screening tests (CBC, Chemistry) and for baseline values. Day 1 and day 15: up to 3mL of blood was collected at up to 10 time points during the day. Each time point was separated by 30 minutes to 2 hours and the total blood volume taken on that day did not exceed 30 mL (5% of total blood volume in a smaller dog). Day 2 and day 16: up to 3mL of blood was collected at up to 4 time points. The time points were separated by 3-4 hours. 3 additional mLs were taken at first time point to run CBC/Chemistry and hemocrit. Total blood volume on that day did not exceed 6mL (2.5% of total blood volume in a smaller dog). Day 3-14 and day 17: up to 2mL of blood was collected at up to 3 time points. The time points were separated by 3-4 hours and the total blood volume taken on that day did not exceed 6mL (1% of total blood volume in a smaller dog). Day 18 to day 21: this group of days has 2 categories of what will happen; dogs undergoing CSF taps that day or on the following days and dogs having already undergone the CSF tap on a previous day.

For the dogs undergoing the CSF tap that day or the following days: up to 3ML of blood will be collected at up to 3 time points with 3-4 hours separating the points. The blood total will not exceed 6mL (1% of total blood volume of a smaller dog). For dogs having already undergone the CSF tap on a previous day: up to 3mL of blood was collected all at once (0.5% of total blood volume in a smaller dog) and day 22 up to day 25: up to 3mL of blood was collected at once (0.5% of total blood volume in a smaller dog).

Day -3 to Day 0	Day 1 and Day 15	Day 2 and Day 16	Day 3-14 and Day 17	Day 18 to Day 21	Day 22-25
Fecal CSF Up to 10mL blood collected (CBC, Chemistry, hemocrit) (Baseline values)	Fecal Up to 3mL blood collected at up to 10 time points	Fecal Up to 3mL blood collected at up to 4 time points Additional 3mls for CBC/Chemistry and hemocrit	Fecal Up to 2mL blood collected at up to 3 time points	Fecal Dogs undergoing CSF on day or following days: fecal and up to 3mL blood collected at 3 time points Dogs that already undergone CSF: fecal and up to 3mL blood collected at once	Fecal Up to 3mL blood collected at once (Wash out period)

Table 1: Dog Study Design showcasing what was collected and how much was collected.

Results

In the mice study, EcNL-LDOPA-Rham-Re increased plasma L-DOPA levels significantly after treatment at the 4 and 6 hour time points when compared to the non-treated and Benserizide treated control groups. In addition to this, homovanillic acid (HVA), a dopamine metabolite that increases when more dopamine is being produced in the brain, was significantly increased in the urine at 6 hours post treatment that further proves the increased plasma L-DOPA metabolism. In the dog study, fecal scores show that the CFU/g values in *Figure 1* are higher during the treatment and the washout showing that the treatment was beneficial because of the EcN_{LDOPA}^{4} levels still being up in the washout. This means that the bacteria in the gut is still

providing some support to PD after treatment has ended. In addition to the fecal EcN_{LDOPA}^{4} quantification, the fecal data shows that the fecal score averages to 2, which means the dogs remained healthy throughout the study. The plasma profile of L-DOPA fluctuates slightly throughout the trail, but stay in a good range for L-DOPA to be effective. Dopamine levels are low throughout the trail with Benserizide levels staying consistent with very little fluctuations. In the washout period, there is a spike in L-DOPA and Benserizide plasma levels at the beginning of the period, but they go back down within the next time point and trail off for the rest of the washout periods. Dopamine plasma levels stay consistent from the treatment to the washout period.

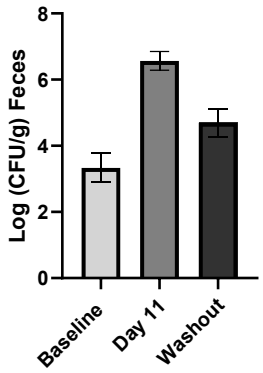

Figure 1: ***Quantification of EcN_{LDOPA}^{4} by qPCR in Dog feces****. All dogs were orally administered EcN_{LDOPA}^{4} induced with 0.5% Rhamnose and Benserazide at a respective doses of $1x10^{11}$CFU and 200mg every 12h (q12h). Moderate/fresh fecal samples were collected ad libitum, followed by extraction and quantification by qPCR. Copy number were quantified and normalized to fecal weight for respective dogs (n = 5-10)* ***A.*** *Logarithmic values for CFU/g for respective days*

Conclusions

Overall, the Levodopa treatment increases stability of the LDOPA concentration in the plasma. In the mice study, EcN^{4}-$_{LDOPA\text{-}Rham\text{-}Re}$ increased plasma L-DOPA level significantly after treatment compared to the non-treated and Benserazide treated control. For the dog study, L-DOPA levels are consistent throughout both trails showing that treatment with the EcN^{4}-LDOPA with Benserizide is a good option for keeping L-DOPA levels up during treatment times. This treatment of using EcNL bacteria to maintain levels of L-DOPA for treatment might be the next go-to therapy used to treat Parkinson's disease

Discussion

Parkinson's affects millions worldwide and the cost of treatment is only increasing with time. To help combat this, researchers are focusing on new ways to catch the disease in early stages of life and treat more effectively. The gut-brain axis is one of the new hot topics to improve Parkinson's treatment. In this study, we used a genetically engineered probiotic E. coli called EcNL-DOPA, which produces L-DOPA in a sustained manner, while also treating with the gold standard treatment of L-DOPA in combination with Benserazide to maximize levodopa's diffusion in the brain. This combination of treatment proved significant in keeping L-DOPA plasma levels consistent though both trails showing its potential to be a useful treatment in future clinical studies. With the positive results of the studies, there are also limitations that are important to state. Due to the small sizes of the samples groups, these studies are not comprehensive. Along with small sample sizes, no test were done with the mucosa microbiome. The data of the microbiome was taken from the fecal collection with each species of animal and feces can only do and show so much about the microbiome. The next step in order to improve these limitations is further studies on dogs can be done with more testing on the microbiome in addition to larger sample sizes in order to be more comprehensive to give a better idea of these effects before moving onto clinical trials in humans. Our prediction is that it will be possible to have a good understanding of the outcome of using EcNL-DOPA probiotic in addition to L-DOPA with Benserazide treatment and use this understanding to move this treatment on to human clinical trials as a new novel therapeutic reducing L-DOPA-inducing dyskinesia.

References

1. Tysnes, Ole-Bjørn, and Anette Storstein. Epidemiology of Parkinson's Disease. *Journal of Neural Transmission (Vienna, Austria : 1996)*, U.S. National Library of Medicine, Aug. 2017.
2. Caputi V, Giron MC. Microbiome-Gut-Brain Axis and Toll-Like Receptors in Parkinson's Disease. *International Journal of Molecular Sciences*. 2018; 19(6):1689.
3. Stansley, Branden J., and Bryan K. Yamamoto. "L-Dopa and Brain Serotonin System Dysfunction." *MDPI*, Multidisciplinary Digital Publishing Institute, 5 Mar. 2015.

4. Barbeau, A.; Mars, H.; Gillo-Joffroy, L. Adverse clinical side effects of levodopa therapy. Contemp. Neurol. Ser. 1971, 8, 203–237.
5. Mochel, Jonathon. “IACUC Protocol.” *PK/PD Assessment of EcNL-DOPA in 10 Healthy Dogs*, 2020.
6. Taguchi, et al. *Animal Model for Prodromal Parkinson's Disease*. 13 Mar. 2020
7. Buhidma, Yazead, et al. “Potential of Animal Models for Advancing the Understanding and Treatment of Pain in Parkinson's Disease.” *NPJ Parkinson's Disease*, Nature Publishing Group UK, 6 Jan. 2020
8. “Animals - Institutional Animal Care and Use Committee (IACUC).” *Animals - Institutional Animal Care and Use Committee (IACUC) | Office for Responsible Research*, Iowa State University
9. Barré-Sinoussi, Françoise, and Xavier Montagutelli. “Animal Models Are Essential to Biological Research: Issues and Perspectives.” *Future Science OA*, Future Science Ltd, 1 Nov. 2015
10. Prado, Mario, and Roland Dominic Jamora. “Cost of Parkinson's Disease among Filipino Patients Seen at a Public Tertiary Hospital in Metro Manila.” *Journal of Clinical Neuroscience : Official Journal of the Neurosurgical Society of Australasia*, U.S. National Library of Medicine, 23 Jan. 2020
11. Callaghan, Brian C, et al. “Out-of-Pocket Costs Are on the Rise for Commonly Prescribed Neurologic Medications.” *Neurology*, Lippincott Williams & Wilkins, 28 May 2019.
12. Horn, Sarah, and Howard Hurtig. “The Many Faces of Parkinson's Disease.” *Cerebrum : the Dana Forum on Brain Science*, The Dana Foundation, 1 Aug. 2019.
13. Kwan, Cynthia, et al. “Ondansetron, a Highly Selective 5-HT3 Receptor Antagonist, Reduces L-DOPA-Induced Dyskinesia in the 6-OHDA-Lesioned Rat Model of Parkinson's Disease.” *European Journal of Pharmacology*, Elsevier, 8 Jan. 2020.
14. Ingallinesi, Manuela, et al. “Knock-Down of GPR88 in the Dorsal Striatum Alters the Response of Medium Spiny Neurons to the Loss of Dopamine Input and L-3-4-Dyhydroxyphenylalanine.” *Frontiers in Pharmacology*, Frontiers Media S.A., 25 Oct. 2019.

15. Jankovic, et al. “Short-Term Cost and Health Consequences of Duodenal Levodopa Infusion in Advanced Parkinson's Disease in Sweden.” *Applied Health Economics and Health Policy*, Springer International Publishing, 1 Jan. 1970.
16. Cummings, B. J., Pike, C. J., Shankle, R., and Cotman, C. W. (1996). β-amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer’s disease. *Neurobiol. Aging* 17, 921–933. doi: 10.1016/s0197- 4580(96)00170-4
17. Patronek, G. J., Waters, D. J., and Glickman, L. T. (1997). Comparative longevity of pet dogs and humans: implications for gerontology research. *J. Gerontol. A Biol. Sci. Med. Sci.* 52, B171–B178. doi: 10.1093/gerona/52a.3.b171
18. Roth, G., and Dicke, U. (2005). Evolution of the brain and intelligence. *Trends Cogn. Sci.* 9, 250–257. doi: 10.1016/j.tics.2005.03.005
19. Schütt, T., Helboe, L., Pedersen, L. Ø., Waldemar, G., Berendt, M., and Pedersen, J. T. (2016). Dogs with cognitive dysfunction as a spontaneous model for early Alzheimer’s disease: a translational study of neuropathological and inflammatory markers. *J. Alzheimers Dis.* 52, 433–449. doi: 10.3233/JAD- 151085
20. Axelsson, E., Ratnakumar, A., Arendt, M.-L., Maqbool, K., Webster, M. T., Perloski, M., et al. (2013). The genomic signature of dog domestication reveals adaptation to a starch-rich diet. *Nature* 495, 360–364. doi: 10.1038/nature 11837
21. Ambrosini, et al. “The Gut-Brain Axis in Neurodegenerative Diseases and Relevance of the Canine Model: A Review.” *Frontiers*, Frontiers, 16 May 2019.