The Viability of Engineering a Clostridium to Produce DBHB and Enhance Neuroprotection

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***Abstract*- Neurodegenerative diseases provide a unique challenge in a world with an increasingly ageing population. More than 850,000 people have been diagnosed with Alzheimer's disease in the UK. A further 140,000 will experience the hardship that accompanies Parkinson's disease. These neurodegenerative diseases currently have no cure and limited knowledge of causes; with treatments focusing on alleviating symptoms rather than preventing further neurodegeneration. Even if a curative treatment was available for diagnosed patients, fifty percent of the neurons in the substantia nigra are incapacitated by the time Parkinson's disease can be officially diagnosed. This leads to the conclusion that treatments need to focus on preventing neuronal damage before it can occur. D-Beta-Hydroxybutyrate (DBHB) is a ketone body that is usually produced during periods of fasting when glucose levels are low. This molecule has been linked with reducing reactive oxygen species and increasing the transcription of antioxidant genes as well as providing an alternate energy source for neurons. Our project aims to ameliorate the growing restriction on aging by using a Clostridia-based probiotic to increase the amount of DBHB reaching the brain which will help prevent neurodegeneration.**

***Index Terms*-** C.*sporogenes*, DBHB, Ketone bodies, neuroprotection, therapeutic.

I. INTRODUCTION

Our general research goal for this project was to find out if it was possible to engineer a Clostridium to produce DBHB. The DBHB molecules would then reach and generate a positive effect in the brain. Therefore, the hypothesis was that an engineered Clostridium (C. sporogenes) could make enough DBHB to provide significant neuroprotection and delay the onset of neurodegenerative diseases. Research has already shown multiple positive effects of DBHB and ketones in general. This includes its role in neuroprotection and migraine prevention to its promotion of brain-derived neurotrophic factor (BDNF) expression (Yang et al., 2019) (Gross et al., 2019) (Hu et al., 2018). The current landscape for treating neurodegenerative diseases is far from ideal. Diagnosis can only occur after significant neuronal death leading to treatments that help with symptoms rather than a proactive neuroprotectant that reduces the risk of developing a neurodegenerative disease in the first place (Oertel et al, 2016).

II. RESEARCH ELABORATIONS

Unfortunately, we could not perform any lab work this year. Various online resources were utilized to make the project as scientifically sound as possible as well as expertise from our supervisors. This includes reviewing applicable papers on pubmed, building models, and using tools such as snapgene and BLAST.

III. RESULTS AND FINDINGS

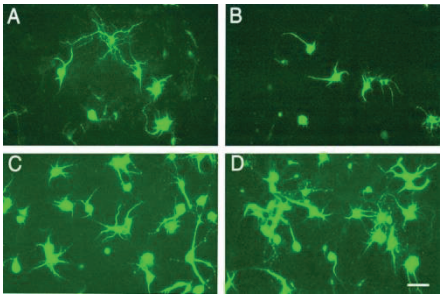

Figure 8: Anti-TH stain on day 7 of a rat mesencephalic neuronal culture. (A) Control culture. (B) Culture after addition of 5 μM MPP+.(C) after addition of MPP+ and 4 mM ketone bodies. (D) after addition of 4 mM ketone bodies alone. The anti-TH stains tyrosine hydroxylase which is an enzyme present in dopaminergic neurons such as the ones affected in Parkinson's disease.

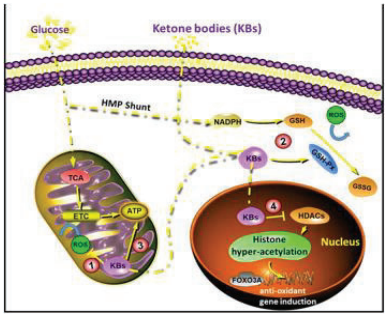

Figure 9: (1) DBHB reduces NAD couples, decreasing ROS production (2) KBs activate glutathione peroxidase, this increases rate of ROS elimination (3)ATP concentration increased (4) Increase anti-oxidant gene expression.

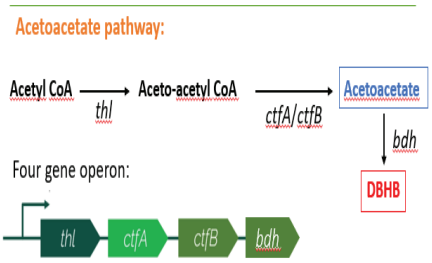

Figure 10: The pathway that will be used to produce DBHB in C.sporogenes as well as the genes that will be transfered to our species. Thl (thiolase), ctfA/ctfB (CoA-transferase subunit A/B) will be utilised from C. Acetobutylicum. Bdh (beta-hydroxybutyrate dehydrogenase) will be taken from S.dysgalactiae subsp. equisimilis.

IV. Discussion

During this project, we wanted to answer 3 main questions: **(1)** Does this ketone body act as a neuroprotectant? **(2)** Could our species produce DBHB? **(3)** Would our therapeutic provide a risk to those who ingest it?

(1) Figure 1 shows the effect that DBHB has on dopaminergic neurons especially in the presence of a toxin (MPP+) that is known to cause neuronal death (Kashiwaya et al., 2000). It is important to notice the difference in parts A and D of Figure 1. The sole addition of ketone bodies to these neurons leads to a greater number of neurons. Comparing B and C also sheds light into the ability of DBHB to protect against this toxin which causes Parkinson's like symptoms. This work on rat mesencephalic neurons shows the potential that DBHB could have in providing neuroprotection. We also must note that rat neurons and brains in general will have differences to human neurons and therefore a future focus should be on the ability to translate this work through to human neurons. Despite this, further papers have shown how a ketogenic diet improves cognitive function in Alzheimer's patients leading to the assumption that DBHBs neuroprotective effects are not just limited to the rodent brain and can be translated effectively (Ota et al., 2019).

Figure 2 highlights the mechanisms by which DBHB could provide increased neuronal survival (Yang et al., 2019). A large proportion of its actions focus on anti-oxidation. These actions counter the oxidative stress that is a common cause of neuronal death (Kim et al., 2015). DBHB works in the neuronal cells to decrease the concentration of reactive oxygen species (ROS') via the activation of glutathione peroxidase, reduction of NAD couples and the increase of anti-oxidant gene expression. Reactive oxygen species are produced naturally in the metabolism of oxygen in mitochondria. A large concentration of ROS' can be toxic to the cell - an excess of these is called oxidative stress. ROS' can cause damage to DNA, deactivate enzymes via the oxidation of their cofactors and oxidise certain amino acid residues in proteins (Kim et al., 2015). Their presence in the cell, especially in high concentrations, can cause apoptosis and are thought to contribute to many neurodegenerative diseases (Nunomura et al., 2006) (Ramalingam & Kim, 2012). Therefore, DBHB could have great potential as a neuroprotectant if it focuses on preventing the oxidation of neurons which would normally lead to excessive neuronal death. Neurodegenerative diseases present themselves due to lack of available neurons able to perform certain tasks. If there was less neuronal death, then there would be a lower likelihood of neurodegenerative diseases overwhelming the brain.

(2) C. *sporogenes* has many useful pathways that could lead to DBHB production. We created a structural model to ascertain the most suitable pathway to produce DBHB. It came down to two pathways in the native C. *sporogenes* Acetone-Butanol-Ethanol (ABE) fermentation pathway (Cooksley et al, 2012). Figure 3 shows the chosen pathway, it is a manipulation of the acetoacetate pathway. During the process of choosing a pathway we examined various complications such as increased ethanol production. These factors were considered giving us a pathway that we believe is suitable to exist in the human gut. DBHB will be produced in the gut and reaches the brain via the bloodstream. DBHB can travel through the blood brain barrier. Further research is needed to ascertain the amount of DBHB being produced and reaching the brain as well as if this creates a worthwhile protective effect. This project is in the very early stage of drug development and if it were to follow that route, further work in C. *sporogenes* and animal models would be used to look at the effectiveness of our therapeutic.

(3) As we progressed through the project, it became clear that a method of control was needed. This control would prevent our bacteria from escaping into the environment or evolving to threaten the health of the patient. Various methods have been thought through. Our vision is to use sporulation as a control mechanism. In short, we would prevent our anaerobic bacteria from sporulating after it reaches the gut. This would prevent its survival outside of this anaerobic environment. We would insert an inducible (tetracycline) promoter just prior to our target genes (Dembek et al, 2017). During production of the bacteria in the factory, anhydrotetracycline would be present so that sporulation could occur. When C. sporogenes is outside of this factory environment and not in the presence of anhydrotetracycline, it will not be able to express our target genes as the promoter cannot be induced. Our target genes are SpoIIIAA, SpoIVA and SpoIID. These were chosen as they are essential to the sporulation process and the lack of their expression prevents sporulation. They are also spread throughout the genome to prevent a horizontal gene transfer event from wiping the control mechanism out at once. This control mechanism limits the ability of our species to survive outside of our target environment preventing any unforeseen damage to the environment or organisms.

V. CONCLUSION

Our work throughout the project has furthered the belief that this solution would be effective. The project is entirely dry lab based and the lack of experiments prevents numerous avenues of exploration that we would have liked to go down. The future of this project would include proof of concept experiments for our

control mechanism and DBHB production. This would involve experiments on E. coli and then C. sporogenes. In the long term, a therapeutic like this would be a regular treatment from early adulthood through to old age giving neuroprotection throughout the lifetime and ultimately decreasing the risk of neurodegenerative diseases.

Our project focuses on a problem that does not yet have a solution. Given the heterogeneity of brain composition in the population; one solution may not be applicable to all. However, using DBHB to increase neuroprotection from early adulthood would diminish this problem as its effects are applicable to neurons in general rather than focusing on one possible cause of neurodegenerative diseases. An example of this are treatments that focus on reducing amyloid beta plaque formation in Alzheimer's patients. Current treatments for Parkinson's disease and Alzheimer's are prohibitive in that they are reactive to when symptoms start presenting themselves rather than being proactive. This means that treatments only focus on alleviating symptoms. A solution like ours would work on preventing neurodegeneration in the first place with the goal of increasing the age of onset of neurodegenerative diseases by decreasing the rate of neuronal death. This delay of onset would not only improve the lives of those that are potentially affected but also decrease the economical and societal burden that these diseases provide.

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