UNIVERSITI PUTRA MALAYSIA

COMPARISON STUDY ON HISTOPATHOLOGICAL CHANGES OF THE GASTROINTESTINAL TRACT AFTER TREATMENT OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) BETWEEN BALB/C AND C57BLK/6 MICE

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A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE (BIOMEDICAL SCIENCES)

DEPARTMENT OF BIOMEDICAL SCIENCES
FACULTY OF MEDICINE AND HEALTH SCIENCES, UNIVERSITI PUTRA MALAYSIA

2022

ABSTRACT

Comparison study on histopathological changes of the gastrointestinal tract after treatment of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) between BALB/C and C57BLK/6 Mice

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Introduction: The neurotoxin 1-methyl,4-phenyl-1,2,3,6-tetrahydropiridine (MPTP) is commonly used in rodents to induce experimental parkinsonism. Among various administration protocols, it has been reported that continuous or chronic exposure to small amounts of MPTP better mimics cell pathology resembling Parkinson's disease (PD). PD can give effects both motor (tremor, rigidity, bradykinesia, and posture instability) and non-motor symptoms (depression, olfactory impairment, and sleep disturbance). Apart from it, PD is also often presented with various gastrointestinal symptoms. Administration of neurotoxin (MPTP) in this study to different strains of mice BALB/c and C57BLK/6) to create a Parkinson's disease model and have a better understanding of the effects of MPTP on the gastrointestinal tract. However, due to different susceptibility to the MPTP in both strains, the differences in histopathological changes of the gastrointestinal tract because of MPTP administration has not yet been compared. It is also still controversial and not clear since previous studies showed MPTP works well in C57BLK/6 but still there are finding showing it also can cause an effect on BALB/c. **Objective**: To compare the effects of MPTP on the gastrointestinal tract between two inbred-mice strains (BALB/c and C57BLK/6). **Methodology**: 32 adult males of BALB/c and C57BLK/6 at age of 12 weeks were randomly divided into four groups, with each group comprised of four mice. The four groups were administered with normal saline, 15mg/kg, 30mg/kg, and 60mg/kg of MPTP respectively. After five days of treatments, all mice were sacrificed. The stomach, ileum, and descending colon were collected and fixed in formalin, dehydrated, and embedded in paraffin wax prior to sectioning for histological examination. The samples were stained with hematoxylin-eosin (H&E) and examined. Each photomicrograph taken was examined and scored based on three different categories of necrosis. The score was analyzed by using SPSS software version 25.0. **Results**: Increasing doses of MPTP can cause enhancement of necrosis formation in the stomach, ileum, and colon of C57BLK/6 if compared with BALB/c. It is proven by three different categories of necrosis show increasing trends in graph means and histopathology examination of three different gastrointestinal organs are more severe with increasing doses of MPTP in C57BLK/6 in comparison to BALB/c. **Discussion:** Our findings yielded MPTP also can cause effects of toxicity in both strains including BALB/c although it has the characteristics of resistance. However, it is only reasonable

to see the effects of MPTP histologically in the gastrointestinal organs right after the administration of MPTP. The reason is that the BALB/c mice are expected to recover and reach control levels on a subsequent day, with a similar recovery observed in CBLK/6 on day 3. As the dose of MPTP is increased, the effect of MPTP toxicity is enhanced in C57BLK/6 compared to BALB/c. The possible mechanisms of differential sensitivity between those two strains are due to coat color, the different activity of MAO enzyme, a different expression of TH as well as a different expression of inflammatory mediators and oxidative markers. **Conclusion**: In short, the present study signifies that MPTP can cause effects of toxicity in both strains including BALB/c. Nonetheless, necrosis formation is enhanced with increasing doses of MPTP in C57BLK/6 compared to BALB/C.

Keywords: Parkinson's disease (PD), 1-methy1,4-phenyl-1,2,3,6-tetrahydropiridine (MPTP), BALB/C, C57BLK/6, gastrointestinal tract (GIT), necrosis

ABSTRAK

Kajian perbandingan mengenai perubahan histopatologi dalam saluran pencernaan selepas rawatan 1-metyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) antara jenis tikus BALB/C dan C57BLK/6

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Pengenalan: Sebatian neurotoksin 1-methyl,4-phenyl-1,2,3,6-tetrahydropiridine (MPTP) biasanya digunakan untuk mendorong parkinsonisme dalam eksperimen melibatkan tikus. Diantara pelbagai protocol pemberian sebatian tersebut, sejumlah kecil MPTP melalui pendedahan yang berterusan atau bersifat kronik adalah lebih baik dalam menyerupai patologi sel yang dapat dilihat dalam penyakit Parkinson (PD). PD boleh memberi kesan kepada fizikal atau motor simptom (gegaran, ketegaran, bradykinesia, dan ketidakstabilan postur badan) dan symptom bukan motor (kemurungan, gangguan pernafasan and gangguan tidur). Selain daripada itu, PD juga sering menghadapi pelbagai gejala yang berkaitan saluran pencernaan. Pemberian MPTP dalam kajian ini kepada dua strain tikus yang berbeza iaitu BALB/c dan C57BLK/6 untuk mencipta model penyakit Parkinson dan mendorong para saintis untuk mempunyai pemahaman yang lebih baik tentang kesan MPTP terhadap saluran pencernaan. Walau bagaimanapun, disebabkan oleh kerentanan yang berbeza terhadap MPTP dalam kedua-dua strain tikus, perbezaan dalam perubahan histopatologi dalam saluran pencernaan masih belum dibandingkan dalam kajian-kajian lepas. Ia juga masih menjadi kontroversi dan tidak jelas kerana terdapat kajian terdahulu menunjukkan bahawa MPTP berfungsi dengan baik dalam menunjukkan perubahan histologi terhadap C57BLK/6 tetapi masih terdapat penemuan yang menunjukkan ia juga boleh menyebabkan kesan kepada BALB/c. **Objektif**: Untuk membandingkan kesan MPTP pada saluran pencernaan antara dua strain tikus (BALB/c dan C57BLK/6). **Metodologi**: 32 ekor tikus jantan dewasa BALB/c dan C57BLK/6 pada usia 12 minggu dibahagikan secara rawak kepada empat kumpulan, dengan setiap kumpulan terdiri daripada empat ekor tikus. Empat kumpulan masing-masing telah diberikan dengan air garam biasa, 15mg/kg, 30mg/kg, dan 60mg/kg MPTP. Selepas 5 hari pemberian MPTP, kesemua ekor tikur dikorbankan. Perut, ileum, dan kolon dikumpulkan dan direndam dalam formalin, dehidrasi, dan dibenamkan dalam lilin parafin sebelum pemotongan untuk pemeriksaan histologi. Sampel telah diwarnai dengan hematoxylin-eosin (H&E) dan diperiksa. Setiap fotomikrograf yang diambil telah diperiksa dan diberi skor berdasarkan tiga kategori nekrosis yang berbeza. Skor dianalisis menggunakan perisian IBM SPSS versi 25.0. **Keputusan**: Peningkatan dos MPTP boleh menyebabkan peningkatan pembentukan nekrosis dalam perut, ileum, dan kolon C57BLK/6 berbanding dengan BALB/c. Ia dibuktikan oleh tiga kategori nekrosis yang berbeza menunjukkan tren peningkatan dalam graf purata dan pemeriksaan histopatologi tiga organ sistem pencernaan yang berbeza adalah lebih teruk dengan peningkatan dos MPTP dalam C57BLK/6 berbanding BALB/c.

Perbincangan: Penemuan kami menunjukkan bahawa MPTP juga boleh menyebabkan kesan ketoksikan dalam kedua-dua strain tikus termasuklah BALB/c walaupun ia mempunyai ciri-ciri kerintangan. Walau bagaimanapun, adalah munasabah untuk melihat kesan MPTP secara histologi dalam organ sistem pencernaan sejurus selepas pemberian MPTP. Hal ini kerana, tikus BALB/c dijangka pulih dan mncapai tahap kawalan pada hari berikutnya, dengan pemulihan yang sama diperhatikan dalam tikus C57BLK/6 pada hari ketiga. Di samping itu, apabila dos MPTP meningkat, kesan ketoksikan MPTP meningkat dalam C57BLK/6 berbanding BALB/c. Mekanisme perbezaan sensitiviti yang mungkin berlaku antara kedua-dua strain tersebut adalah disebabkan perbezaan warna bulu, aktiviti enzim MAO yang berbeza, ekspresi TH yang berbeza serta ekspresi molekul keradangan dan oksidatif yang berbeza. **Kesimpulan:** Secara ringkasnya, kajian ini menunjukkan bahawa MPTP juga boleh menyebabkan kesan ketoksikan dalam kedua-dua strain termasuk BALB/c. Walau bagaimanapun, pembentukan nekrosis adalah tinggi dengan peningkatan dos MPTP dalam C57BLK/6 berbanding BALB/c.

Kata kunci: penyakit Parkinson (PD), 1-methy1,4-phenyl-1,2,3,6-tetrahydropiridine (MPTP), BALB/c, C57BLK/6, saluran pencernaan, nekrosis

ACKNOWLEDGEMENT

"In the name of Allah, The Most Gracious and The Most Merciful"

First and foremost, all thanks to the Almighty God for the grace of God for honoring me with His aid to complete my Final Year Project (FYP). My utmost gratitude to Dr. Tengku Azam Shah Tengku Mohamad for his supervision throughout my journey directly or indirectly and also to my co-supervisor, Associate Prof. Dr. Che Norma Mat Taib for her unwavering support. Her amiable disposition, explanation, earnest assistance, and valuable information to every question I have can make my learning process easier.

My appreciation also extends to my fellow project team member, Dr. Musa Mustapha, and all staffs from the Human Anatomy Laboratory, Faculty of Medicine and Health Sciences, UPM for lending their precious strength to my project. Without their diligent help, I would not be able to complete my work in such a short period. Although our time together was short, it was the most engaging and passionate period of learning, where I constantly find inspiration from their help. Moreover, I am also grateful to my classmates for their unwavering support in every manner. Their suggestions, ideas, and moral support were both reassuring and helpful. They gave me a lot of confidence and motivation to finish my task by sticking together during difficult circumstances. Despite the obstacles, I was able to complete my task with their support and encouragement.

Finally, a big thank to my parents and siblings for their support. They are my greatest pillars of strength, and they will forever be. Even though I am always away, their confidence in me never wavers. As a result, I can concentrate and excel in my studies because I know they will support me wholeheartedly with their unconditional love. Without the cooperation of the whole tea, I would not be able to do all the tasks assigned to me. This dissertation stands as a testament to everyone's support.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
B6	Black 6/C57 black 6
BBB	Blood-Brain Barrier
C57BLK/6	C57 Black 6
CNS	Central Nervous System
DAergic	Dopaminergic
DAT	Dopamine Active Transporter
DPX	Dibutylphthalate Polystrene Xylene
ENS	Enteric Nervous System
ETC	Electron Transport Chain
GBA	Gut-Brain axis
GBD	Global Burden of Disease, Injuries, and Risk Factors
GIT	Gastrointestinal Tract
H&E	Hematoxylin and Eosin
H_2O	Dihydrogen Monoxide/Water
IP	Intraperitoneal Injection
LD	Lethal Dose
MAO	Monoamine Oxidase
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MPP+	1-Methyl-4-Phenylpyridium ion
NaCl	Natrium Chloride
NBF	Normal Buffer Formalin

ND	Neurodegenerative Disease/Disorder
NIEHS	National Institute of Environmental Health Science
PD	Parkinson’s Disease
PNS	Peripheral Nervous System
ROS	Reactive Oxidative Species
SNpc	Subtantia Nigra pars compacta
TH	Tyrosine Hydroxylase

CHAPTER 1

INTRODUCTION

1.1 Background

Since cells in the brain are closely related and connected, disruption in one area can affect the activities of the other brain parts, meaning that any brain disorders can lead to various widespread problems in the brain and also to the whole body (systemic responses). There are so many disorders that can affect the brain; however, the most complicated disease suffered by millions of people throughout the world is known as neurodegenerative disorder. According to the National Institute of Environmental Health Sciences (NIEHS) (2018), neurodegenerative disorders (ND) occur when neuron cells in the Central Nervous System (CNS), particularly in the brain, or Peripheral Nervous System (PNS) cannot function properly and eventually die. As a 2021 report in the same study by NIEHS, Alzheimer's disease (AD) and Parkinson's Disease (PD) are the most common types of neurodegenerative diseases and by 2030, the numbers are expected to rise to 6.2 million among Americans and 1.2 million in the United States, respectively. Among these two neurodegenerative disorders, as reported in the Global Burden of Disease, Injuries, and Risk Factors Study (GBD) (2015), PD was the fastest-growing neurological disorder in terms of prevalence, disability, and fatalities and is currently the major cause of disability worldwide. They also add PD epidemiology for various parts of the world revealed that the number of people affected by the disease had more than doubled globally between 1990 and 2015. According to a news report based on the Department of Statistics Malaysia 2018, the number of people with PD in Malaysia is expected to increase more than fivefold by 2040, from an estimated 20000 to 120000.

PD is defined as a progressive neurological illness characterized by tremors, stiffness, bradykinesia, and postural instability caused by degeneration or loss of dopaminergic (DAergic) neurons in the brain structure called substantia nigra pars compacta (SNpc). According to Chiang and Lin (2019), PD is a type of prevalent neurodegenerative disorder caused by the interaction of hereditary and environmental variables. During the last two decades, tremendous progress has been made in understanding the etiology and pathophysiology of PD. However, until now the specific cause of it remains unknown but several major factors that contribute to this type of disease include genetics and triggers from the environment (Mayo Clinic, 2022). In addition, most researchers are aware of the changes that occur in people with PD, but it is not clear why those changes occur. Nevertheless, most of the previous studies have been done are focused more on the degeneration of dopaminergic neurons in SNpc of the brain which is part of the coordinator for the body's movement, and most effects that can be seen are related to the movement. Besides, ENS can mediate behaviour independently of the CNS; however, the gut and the CNS generally communicate and influence with one another. Therefore, there are also non-motor symptoms that often present with gastrointestinal tract (GIT) symptoms and the patients with PD may experience those symptoms from the entire enteric nervous system (ENS). Hence, understanding the pathology of PD in relation to gastrointestinal symptoms histologically would be the primary focus of research in this area.

Here comes the main compound which believed in the creation of PD animal models called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is an analog of meperidine produced as a by-product of the synthesis of 1-methyl-4-phenyl-propionoxy-piperidine (Mat Taib & Mustapha, 2021). It has been used as a drug to create the best PD animal models of parkinsonism in rodents since ancient times. This

drug is known to induce DAergic neurons degeneration and affects the areas of the brain that are most vulnerable to MPTP-induced neurotoxicity. Any disruption in the brain can cause systemic response indirectly such as symptoms related to GIT. Induction of MPTP into animals used in this study will allow the rapid onset of parkinsonism as it is very practicable by which it can transverse the blood-brain barrier (BBB) and CNS easily because of its lipophilic characteristic (Sian et al., 1999).

For the purpose of studying the various effects of MPTP in causing the pathophysiology of PD, this study focused more on two different inbred strains of mice which are BALB/C and C57BLK/6. However, MPTP sensitivity varies between mouse strains and species. The C57BLK/6 mouse strain is said to be the most vulnerable to MPTP, while BALB/C mice show resistance to the MPTP but as they age, they will lose their resistance (Filipov et al., 2009). Since C57BLK/6 is the most sensitive strain of mice, this neurotoxin-based animal model in comparison with BALB/C has been used in this study as a disease gene-based model to have a better understanding of the characteristics of PD after induction of MPTP. Previous studies show that MPTP will work well in C57BLK/6; however, it also can cause effects on BALB/C mice although they have characteristics of resistance. Therefore, the present study is to confirm this finding and also compare the histopathological changes primarily focused on GIT after treatment of MPTP between BALB/C and C57BL/6 mice.

To summarize, MPTP mouse models have been among the most widely used in PD research because they have the advantages of ease of use, low cost, fewer ethical considerations, and higher clinical correlation (Mat Taib & Mustapha, 2021) as induction of MPTP in mice allow to mimic Parkinson's disease symptoms in both inbred mice. Hence, this paper is aim to differentiate the effects of MPTP by

comparing the histopathological changes primarily focused on GIT between BALB/C and C57BL/6 mice.

1.2 Objectives

1.2.1 General Objective

This study generally aimed to compare the effect of MPTP on the gastrointestinal tract between 2 inbred-mice strains (BALB/C and C57BLK/6).

1.2.2 Specific Objectives

The specific objectives of this study are to differentiate the necrosis formation with increasing doses of MPTP between BALB/C and C57BLK/6 and also to confirm that MPTP also give effects on the GIT of BALB/c histologically.

1.3 Hypothesis

It is hypothesized that necrosis formation is enhanced with increasing doses of MPTP in C57BLK/6 compared to BALB/C. MPTP also causes histopathology of GIT in BALB/c although it is said to be resistant.

CHAPTER 2

LITERATURE REVIEW

2.1 Parkinson's disease (PD)

2.1.1 Statistics of PD

Parkinson's Disease (PD) is categorized as a prevalent disorder as PD keeps rising in aged people over the age of 65, and 4-5 percent of those over the age of 85. (Pagano et al., 2016). According to Garza-Ulloa (2019), Parkinson's disease is the world's second most common neurological disease, after Alzheimer's disease. 70% of people with PD die as a result of complications from the disease, also making it the 14th leading cause of death in the United States among people the age of 60 years and above (Katz et al., 2018). According to The Health of America in 2020, PD was diagnosed in roughly 90,000 commercially insured adult people between the ages of 30 and 64 in 2017. While this is a small figure, their research shows that the prevalence rate has increased by higher than 50% in the last five years and as reported by Dorsey et al. (2018), the prevalence rate will be increased up to 12 million in 2040. This increasing number globally allows the disease to have huge health importance in all aspects of quality of life, as well as significant economic and institutional costs to the family and society (Dorsey et al., 2018)

2.1.2 Introduction of PD

Dopamine is a type of neurotransmitter that allows neurons in the brain responsible for communicating and control body movement. In patients with PD, the neurons that release this type of dopamine neurotransmitter called dopaminergic

(DAergic) neurons in subtantia nigra pars compacta (SNpc) steadily degenerated. As a result, no signal will be transmitted anymore and any chemical imbalance can lead to physical or motor symptoms characterized by tremors, stiffness, bradykinesia, and postural instability (Lai et al., 2018). In their study, they also added patients with PD frequently experience non-motor symptoms associated with gastrointestinal (GI) dysfunction, such as constipation and delayed stomach emptying, which appear before the motor symptoms of the disease. Although motor symptoms are the major effects that can be seen in patients with PD due to neurodegeneration, other parts of the Central Nervous System (CNS) as well as the Enteric Nervous System (ENS) are also affected and can disrupt the life quality of the patients (Paul et al., 2011). The mechanism that causes the effects on non-motor symptoms is still poorly understood, but rising evidence proposes that changes in the histology of the gastrointestinal system might be linked to the etiology of PD. When considering the causes of PD, it remains unknown; but it is thought to be caused by a complex interplay between mitochondrial dysfunction, oxidative stress, apoptotic cell death, protein aggregation and misfolding, neuroinflammation, excitotoxicity, loss of trophic factors, and other cell death pathways, all of which lead to the degeneration of dopaminergic (DA) nigrostriatal neurons in the brain (Fujimaki et al., 2014). However, several factors might play a role such as genetics and environmental factors (Mayo Clinic, 2022). In addition to that, research suggests that genetic factors may only contribute to PD cases by about 10% roughly (Thomas & Beal, 2007). In some studies, exposure to toxic chemicals such as pesticides also can be an environmental factor that increases the risk of people developing PD.

2.2 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

2.2.1 Introduction to MPTP

(a) (b)

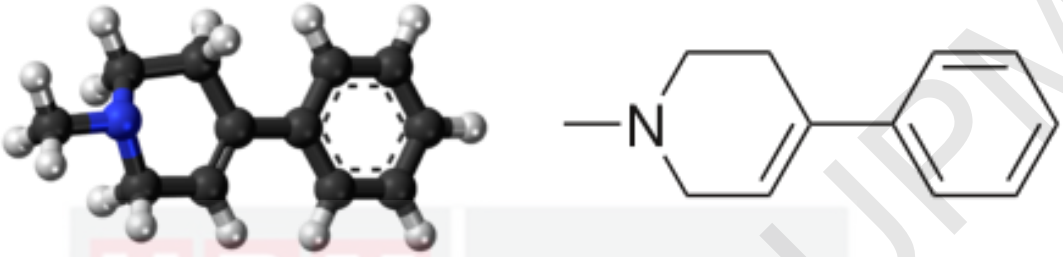

Figure 2.2.1: **(a)** The ball-and-stick model of MPTP. Colour coded: Carbon (C) – Black, Hydrogen (H) – White, Nitrogen (N) - Blue **(b)** 2D structure of MPTP

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a type of lipid-soluble compound that can penetrate the blood-brain barrier (BBB) and can pass through the cells inside the brain (Sian et al., 1999). The substitution of a methyl group at position 1 and substitution of a phenyl group at position 4 gives the ability to a neurotoxin (Figure 2.2.1). According to a study done by Davis et. al. (1979), MPTP was discovered in 1976 by a chemistry student who was trying to make synthetic heroin but ended up with MPTP, which kills DAergic neurons (Meredith & Rademacher, 2011). They further added regarding the study done by Dr. Langston and his colleagues detected the effects of MPTP injection in non-human primates which are squirrel monkeys and characterized the impairments that mirrored idiopathic PD motor difficulties. Further investigation was done by Sonsalla and Heikkila (1986) who demonstrated in mice that MPTP might have many of the same effects.

2.2.2 Bioactivation mechanism of MPTP

In the study by Sian et al. (1999), they added the MPTP itself does not appear to be a toxic compound, but the bio-activation of MPTP by an enzyme called Monoamine Oxidase (MAO) to the 1-methyl-4-phenylpyridinium ion (MPP+) make it toxic (Figure 2.2.2). This results in the travel of this toxic oxidation product to the extracellular fluid before it is transported by Dopamine Active Transporter (DAT) to dopaminergic neuron terminals (Sian et al., 1999) (Figure 2.2.3). In a study by Smeyne & Jacson-Lewis (2005), MPP+ will interfere with the mitochondria component of metabolism called mitochondria complex I in the electron transport chain (ETC) which then results in the production of free radical molecules or particularly in the form of reactive oxidative stress molecules (ROS) and then cause abnormalities to the cells including DAnergic neurons (Mau & Jadavji, 2017). Consequently, energy production is hampered. As for that, increasing stress from the production of ROS is the most common mechanism for the destruction of DAergic neurons in PD, and complex I in ETC is known to be the primary source of ROS production (Subramaniam & Chesselet, 2014).

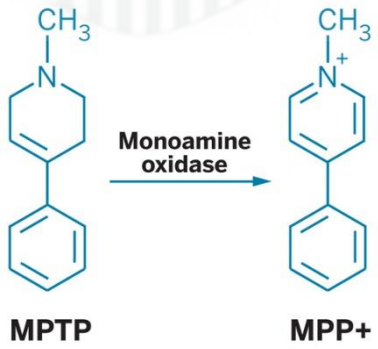

Figure 2.2.2: The bioactivation process of MPTP to toxic molecule MPP+

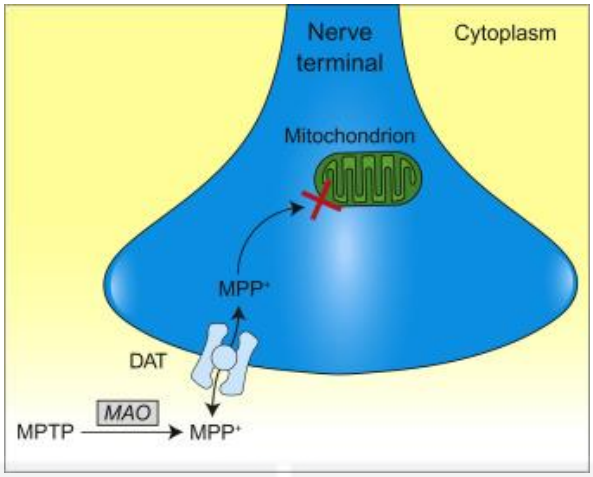

Figure 2.2.2: Transportation of MPP+ via DAT. The MPP+ produced interfere mitochondrial system and produce ROS resulting in cells destruction.

2.2.3 The Relation between PD and Gastrointestinal pathology

The ENS also known as the 'second brain in the gut' regulates GIT functions such as motility and secretion independently of the CNS (Harsanyiova et al., 2020). The ENS, on the other hand, is bidirectionally related to the CNS, and both together form the gut–brain axis (GBA) at the autonomic nervous system level (Lebouvier et al., 2009). Because of this connection, neurodegenerative CNS diseases manifest in ENS abnormalities and thus in gastrointestinal problems. As mentioned earlier, PD is associated with the destruction of dopaminergic neurons specifically in the CNS brain structure called subtantia nigra pars compacta (SNpc). Although ENS function independently of CNS, but still, they are intercorrelated to each other. Like CNS, ENS contains a small fraction of dopaminergic neurons, which are more abundant in myenteric and submucosal plexuses of the proximal regions of the GIT and have a lower tendency in its distal parts (Anlauf et al., 2003). Thus, the ENS can mediate

behavior independently of the CNS; however, the gut and the CNS generally communicate and influence one another (Harsanyiova et al., 2020; Rao & Gershon, 2016)

Apart from motor symptoms that might be experienced by the patients with PD, there are also non-motor symptoms are frequently associated with GIT symptoms, and people with PD may feel those symptoms throughout the ENS. Before neuropathological alterations in the CNS, the development of the disease is thought to be restricted to peripheral organs, primarily the GIT (Harsanyiova et al., 2020). The chronic low-dose MPTP model was used to assess the progression of intestinal pathology in PD. As described in the bioactivation process of MPTP to a toxic molecule above in Figure 2.2.2, the reaction is catalyzed by an enzyme called MAO. The MAO enzymes are localized mainly in CNS and can be classified into two subtypes; MAO-A and MAO-B. MAO-A is located largely in the neurons in the brain (Tong et al., 2017) and expressed primarily in the gastrointestinal tract (Aljanabi et al. 2021; Ostadkarampour & Putnins, 2021) while MAO-B is considered to be localized primarily to astrocytes which are produced by astroglial cells (Tong et al., 2017) and is mostly found in the kidney, platelets, granulocytes, and lymphocytes, with a more evenly distributed expression in the lungs, spleen, and liver Ostadkarampour & Putnins, 2021). In this study, we believed that the activity of MAO-A in the brain and gastrointestinal tract, as well as MAO-B, might also be related to the concurrent production of ROS and destroy the cells within the gastrointestinal organs resulting in abnormalities that can be seen histologically. To support the evidence, the pathogenesis of this type of neurodegenerative disorder is mediated by higher-level expression and activity of both subtypes of MAO in the gastrointestinal tract (Aljanabi et al. 2021).

2.2.4 Administration of MPTP

MPTP is also known as a dopaminergic neurotoxic compound in which it will produce pathological alterations that somehow mimicking to those found in PD. Meanwhile, the different regimen of MPTP administration produces different effects on nigrostriatal dysfunction. MPTP generally be delivered systematically via repeated intraperitoneal (IP) injection. A single injection of 14 to 20mg/kg of MPTP 4 times a day at 2 hours interval (acute dosing) will result in 20-90% of DAergic neurons depletion and if 30 mg/kg of MPTP is given daily for 5 subsequent days (subacute or chronic dosing), it will result in 40-50% of DAergic depletion (Mustapha & Mat Taib, 2020). In addition to that, acute dosing only can mimic cell destruction or loss in the early stage of PD while subchronic or chronic dosing results in more robust neurodegeneration in later stages of PD (Bezard et al., 1997). This is the reason why we use subacute or chronic dosing as our main regimen in this study to keep reproducing the slow, steady, and progressive neurodegeneration process in the MPTP mouse models. In the comparison of MPTP to the other neurotoxins in the creation of PD models in animals, the simplicity of MPTP by IP injection allows quick absorption as its ability to transverse BBB into CNS and cause the rapid onset of PD symptoms (Mustapha & Mat Taib, 2021). Additionally, MPTP mouse models not only can reveal motor symptoms of PD but also reveals non-motor symptoms related to it. It is the main reason for its usage due to it has a very greater clinical correlation similar to what we can see in PD patients.

2.3 Animal Experimentation

2.3.1 MPTP Mouse Model

Animal experimentation or also known as in-vivo testing is defined as the use of animals in experiments that focus on the fundamental knowledge development about certain diseases including answering questions of clinical importance such as searching for a cure for a disease. An example of applied research in an animal trial done in this study is in which we create the Parkinson's disease (PD) models in a laboratory setting with experimental protocols including MPTP administration, transgenic strain development, and some behavioral tests (Konnova & Swanberg, 2018). Among all neurotoxins used in the creation of PD models, MPTP is the most widely used by researchers for understanding PD in more depth. Moreover, MPTP mouse models have led to a better understanding of how MPTP affects the mitochondria system within the cells and then destroys the cells. Although in acute or chronic MPTP models, it is considered very practicability and can cause at least less than half of dopaminergic neurons destruction in SNpc. This shows that administration of MPTP either acute or chronic regimen able to induce neuronal damage and further recapitulate PD symptoms in the mouse. Hence, the MPTP mouse model has a very greater clinical correlation as it can produce neurotoxic effects that same as what we can see in PD patients. The greater a model's resemblance to PD, the greater its predictive value for clinical trials (Paul et al., 2011). That is why MPTP is mostly like to be used in mice to give more light on the illness's origin and pathology of PD. The study by Mustapha & Mat Taib (2021), believed that with continuous research, this MPTP mouse model can be improved to the point where it can replicate all of the pathogenic and phenotypical aspects of human Parkinson's disease. In addition to that, by merging the MPTP neurotoxic and genetic mice models, a superior model may be

created, allowing for a better understanding of the gradual neurodegeneration associated with PD.

2.3.2 BALB/c

According to Johnson (2012), BALB/C is known as immunodeficient inbred and can be bred easily as well as they have minimal weight variations between males and females. In terms of its application, BALB/C mice are commonly used as research models for cancer therapy and are also used in various immunological studies. This albino mouse strain is lacking or absence in melanin pigments production resulting in the point mutation that occurred in the enzyme that catalyzes the melanin synthesis (Meredith & Rademacher, 2011) making it suitable for this study and it is the main reason for its characteristic of resistance toward MPTP. Other than that, BALB/c is very sensitive to carcinogens although tumor incidence is low. As stated in the same study by Johnson (2012), BALB/c mice play a very important role in oncology type of research. This can be proved in the study done by Mauffrey et al., which used BALB/c from Charles River Laboratories to study neurogenesis in prostate cancer.

2.3.3 C57BLK/6

C57BLK/6 or also known as “Black 6” is the inbred black pigmented strain and this strain is said to have the highest sensitivity to MPTP due to the presence of a susceptible gene located on chromosome 7 (Meredith & Rademacher, 2011). According to Johnson (2012), C57BLK/6 is frequently used for general purposes including for the production of transgenic and congenic with both spontaneous and induced mutations. C57BLK/6 has the characteristics of easily breeding and strain

stability. The unique finding of this type of strain is that C57BLK/6 is the first mouse strain whose genome was fully sequenced in 2002, right after the human genome (Johnson, 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) were kindly provided by Associate Professor Dr. Che Norma Mat Taib from the Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. MPTP was from Sigma Aldrich, USA. Normal saline (NaCl) was used to dissolve the MPTP compound. Generally, the amount of MPTP administration should be less than 1% of mice's body weight (weight range of mice used between 18 to 25g) to avoid any fluid overload.

3.1.2 Experimental Animals

A total of 32 healthy adult males of BALB/C and C57BLK/6 mice at the age of eight weeks purchased from a reliable supplier (Interscience Sdn Bhd) in Selangor were used in this study. The mice were housed in 12 hours of light and dark cycle to imitate the normal circadian cycle, at a controlled temperature of 23-25ºC and with constant humidity. The mice were acclimatized for one month with free access to food and water until it reaches the age of three months old (12 weeks). After one month of acclimatization, the mice were randomly divided into four groups, with each group comprised of four mice respectively (n=4). The respective groups were labeled accordingly:

Strain	*Group*	*Mice*
BALB/c	1	4
	2	4
	3	4
	4	4
C57BLK/6	1	4
	2	4
	3	4
	4	4
Total	**8**	**32**

Table 3.1.2: Table of mice grouping

3.1.3 Other Reagents and Instruments

These reagents and instruments were provided by the laboratory of Anatomy and Histology Laboratory 1 in the Faculty of Medicine and Health Sciences, UPM.

No	*Materials and Instruments*	*Country*
1	Dissection Kits	-
2	Terumo Syringes and Needles	Japan
3	Leica TP1020 Automatic Benchtop Tissue Processor, Semi-Enclosed	Germany
4	Leica RM2255 Fully Automated Rotary Microtome	Germany
5	Feather Microtome Blade High Profile	Japan
6	Microscope Frosted Glass Slide	China
7	Mounting Bath Leica HI1220	Germany

8	Cold Plat Leica HI1130	Germany
9	Oven Memmert ULM400	Germany
10	Leica ST5010 Autostainer XL	Germany
12	Leica EG1160 Tissue Embedding Station	Germany
13	Coverslip	Germany
14	Sigma-Aldrich DPX Mountant for microscopy	Germany
15	Olympus BX51 Fluorescence Microscope	Japan
16	Toup Camera and Toup View Software by Touptek Photonics	China
17	Fume Hood	-
18	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	USA
19	Xylene	-
20	70% alcohol	-
20	10% normal buffer formalin (NBF)	-

Table 3.1.3: List of Reagents and Instruments

3.2 Methodology

3.2.1 Animal trial to establish an MPTP mouse model of Parkinson's disease.

All groups were given treatment with different doses for 5 days in a row (Cao et al., 2017). A saline injection (1 ml/100g BW) was be given to the control group. Throughout the 5 days of the treatment phase, the body weight and body temperature were measured and recorded.

Strain	Group	Administration
BALB/c	1	Normal saline
	2	15mg/kg of MPTP
	3	30mg/kg of MPTP
	4	60mg/kg of MPTP
C57BLK/6	1	Normal saline
	2	15mg/kg of MPTP
	3	30mg/kg of MPTP
	4	60mg/kg of MPTP

Table 3.2.1: Table of treatment groups. Throughout the treatment phase, the mice were divided into groups. All of the mice were given a standard pellet and water.

3.2.2 Grossing/Sample Collection

At the end of treatment, behavioral tests were performed before all the surviving mice were sacrificed to perform the sample collection through the dissection process for histopathology purposes. The main parts of the gastrointestinal tract were extracted from the mice such as the stomach, ileum, and colon. All the samples were fixed in 10% normal buffer formalin (NBF) to avoid any destruction of the sample before the histopathological examination routine.

3.2.3 Routine Histopathological Techniques

All the tissue samples were fixed in formalin, dehydrated, and embedded in paraffin wax using Leica EG1160 Tissue Embedding Station. The sectioning process

was done in which 5 µm thick sections of each sample were obtained using a Leica RM2255 Fully Automated Rotary Microtome and the cutting sections were placed on the glass slides. The samples were stained with hematoxylin-eosin (H&E) using Leica ST5010 Autostainer XL before the mounting process. A drop of mounting fluid (DPX medium) was placed on top of the coverslip to be mounted with the sample. The air bubbles were removed by gently pressing down the coverslip with mounting fluid and the sample on the glass slide. The samples were then dried at room temperature for one to two days before being examined. Photomicrographs were taken by using a Fluorescence Microscope at low (10x) to high magnification (40x) and captured with the Toup Camera.

3.3 Semi-Quantitative Scoring Assessment

Parameters	**Score**			
	0	**1**	**2**	**3**
Extent damage to the epithelial lining	No damage	Mild	Moderate	Extensive
Inflammation	No	Mild	Moderate	Extensive
Goblet cells (exception for stomach)	>25	11-25	1-10	0
Parietal cells (only for stomach)	>10	6-10	1-5	0

Table 3.3: Semi-quantitative scoring assessment of histology sections used to assess damage in the stomach, ileum, and colon tissues (Modification from Jayatilake et al., 2014)

Each photomicrograph taken was analyzed and lesion scoring was made based on **Table 3.3**. Table 3.3 summarized the semi-quantitative scoring assessment that was assigned to assess damage in each sample based on four different parameters. ± 20 spots in total for each treatment group were used for microscopic examination with 5 spots ($Area \pm 3165 \mu m^2$ represent each spot) for each sample per mouse. The mean of 5 different spots was recorded for further statistical analysis.

3.4 Statistical Analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) and Dunnett's post hoc test in IBM SPSS version 25.0. A *p*-value of less than 0.05 ($p<0.05$) was regarded as statistically significant.

CHAPTER 4

RESULT

4.1 Toxicity effects of MPTP on the stomach of BALB/c and C57BLK/6

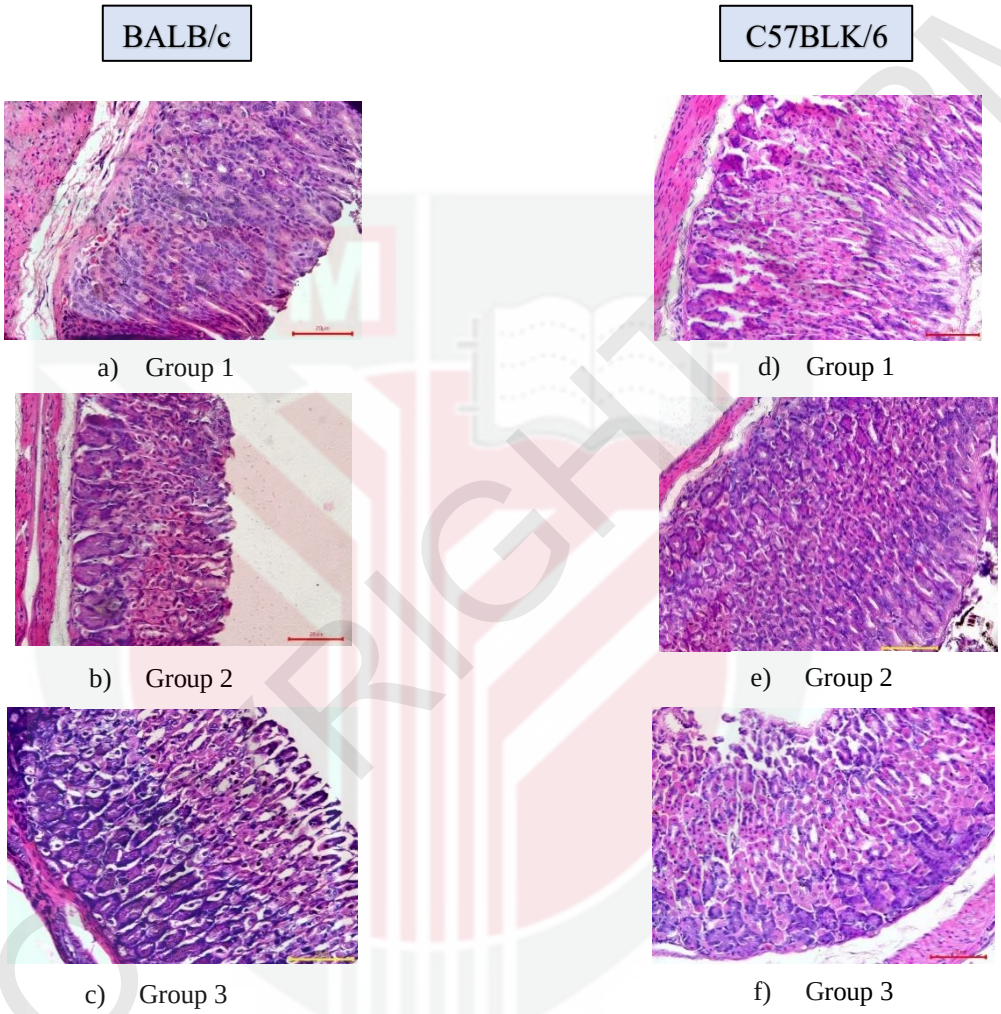

Figure 4.1.1: Histology of stomach of BALB/c and C57BLK/6 at a magnification of 20X. Representative images of the stomach of **a.** Control BALB/c mice, **b.** 15mg/kg MPTP-treated BALB/c mice, **c.** 30mg/kg MPTP-treated BALB/c mice **d.** Control C57BLK/6, **e.** 15mg/kg MPTP-treated C57BLK/6, **f.** 30mg/kg MPTP-treated C57BLK/6. Paraffin-embedded 5µm sections from the stomach of BALB/c and C57BLK/6 were stained with H&E staining and imaged at X20 magnification.

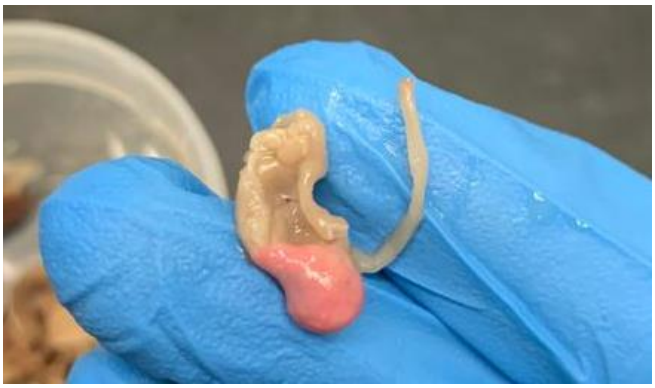

Figure 4.1.2: Stomach ulceration due to the effects of MPTP toxicity. MPTP damages both central and peripheral dopaminergic system (ENS) resulting in decreased gastric motility with a compensatory increase in gastric hormone and consequently more gastric acid secretion. Hence, stomach ulceration because of gastric stasis can be observed.

The toxicity effect of MPTP on the stomach was compared between 2 inbred strains of mice. The necrosis scoring is represented as shown in Figure 4.1 after being treated with MPTP at a concentration of both 15mg/kg and 30mg/kg. Data are presented as mean ±S.E.M in three different categories of necrosis which are a) extent damage of epithelial lining, b) inflammation, and c) destruction of parietal cells. The one-way ANOVA analysis of MPTP toxicity on the damage to stomach epithelial lining determined that there were no statistically significant differences between strains ($F(1,22)=2.055$, $p=0.166$). However, Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p=0.012$) and 30 mg/kg ($p=0.000$) had a significant effect on the stomach epithelial lining as compared to the control group in both strains. Furthermore, the one-way ANOVA analysis of MPTP toxicity on the stomach inflammation showed that there were no statistically significant differences between strains ($F(1,22)=0.660$, $p=0.425$). Nevertheless, Dunnett's test revealed that administration of 30mg/kg of MPTP ($p=0.048$) had a significant effect on the stomach inflammation as compared to 15mg/kg of MPTP ($p=0.100$) and the control group in BALB/c. Besides, the one-way ANOVA analysis of MPTP toxicity on the stomach parietal cells showed that there were statistically significant differences between strains ($F(1,22)=14.807$, $p=0.001$). In addition to that, Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p= 0.024$) and 30 mg/kg ($p=0.04$) had a significant effect on the stomach parietal cells of BALB/c mice as compared to the control group.

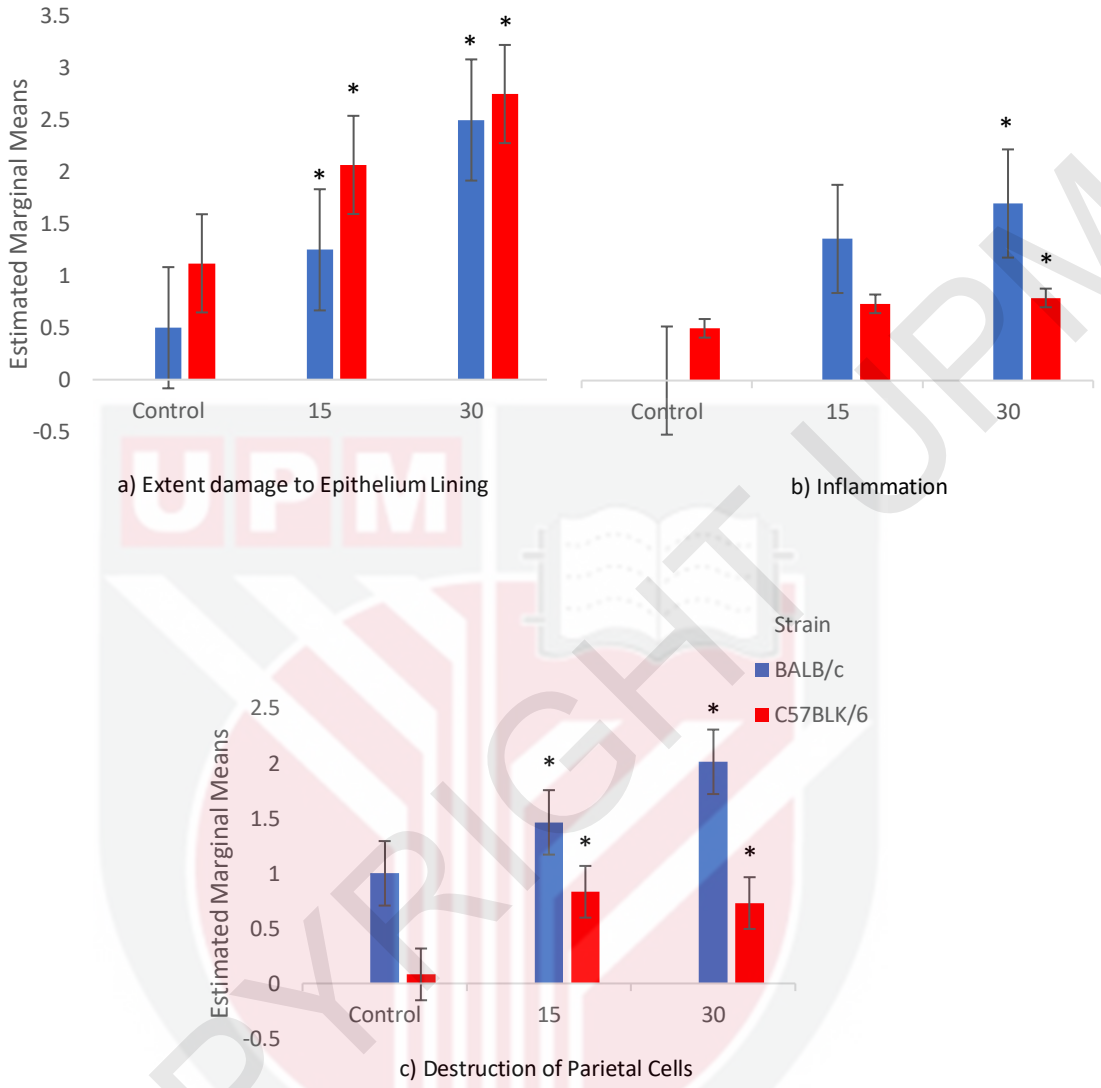

Figure 4.1.3: The graph means of necrosis scoring in the stomach of BALB/c and C57BLK/6 due to MPTP administration. Data are presented as mean ±S.E.M represented in three categories of necrosis **a.** Extent damage of epithelium lining, **b.** Inflammation, **c**. Destruction of parietal cells. *p<0.05

4.2 Toxicity effects of MPTP on the ileum of BALB/c and C57BLK/6

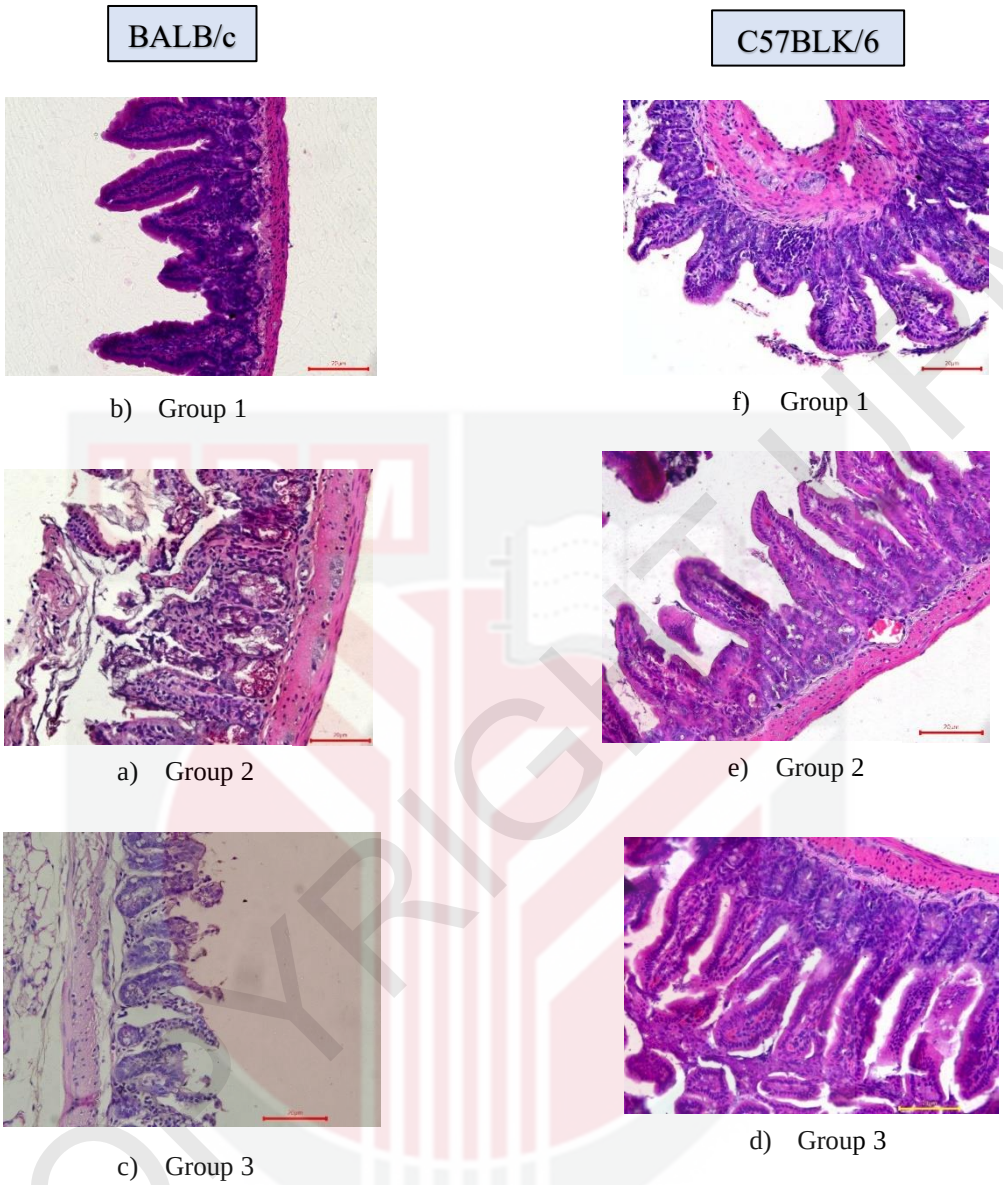

Figure 4.2.1: Histology of ileum of BALB/c and C57BLK/6 at a magnification of 20X. Representative images of the stomach of **a.** Control BALB/c mice, **b.** 15mg/kg MPTP-treated BALB/c mice, **c.** 30mg/kg MPTP-treated BALB/c mice **d.** Control C57BLK/6, **e.** 15mg/kg MPTP-treated C57BLK/6, **f.** 30mg/kg MPTP-treated C57BLK/6. Paraffin-embedded 5µm sections from the ileum of BALB/c and C57BLK/6 were stained with H&E staining and imaged at X20 magnification.

The toxicity effect of MPTP on the ileum was compared between 2 inbred strains of mice. The one-way ANOVA analysis of MPTP toxicity on the damage to ileum epithelial lining determined that there were no statistically significant differences between strains ($F(1,22)=1.273$, $p=0.271$). However, Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p=0.031$) and 30 mg/kg ($p=0.000$) had a significant effect on the stomach epithelial lining as compared to the control group in both strains. Furthermore, the one-way ANOVA analysis of MPTP toxicity on the ileum inflammation showed that there were statistically significant differences between strains ($F(1,22)=6.385$, $p=0.019$). Nevertheless, Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p=0.031$) and 30 mg/kg ($p=0.000$) had no statistically significant effect on inflammation as compared to the control group in both strains. Besides, the one-way ANOVA analysis of MPTP toxicity on the goblet cells of the ileum showed that there were no statistically significant differences between strains ($F(1,22)=0.923$, $p=0.347$). Whereas Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p=0.001$) and 30 mg/kg ($p=0.000$) had a significant effect on the goblet cells of ileum as compared to the control group in both strains.

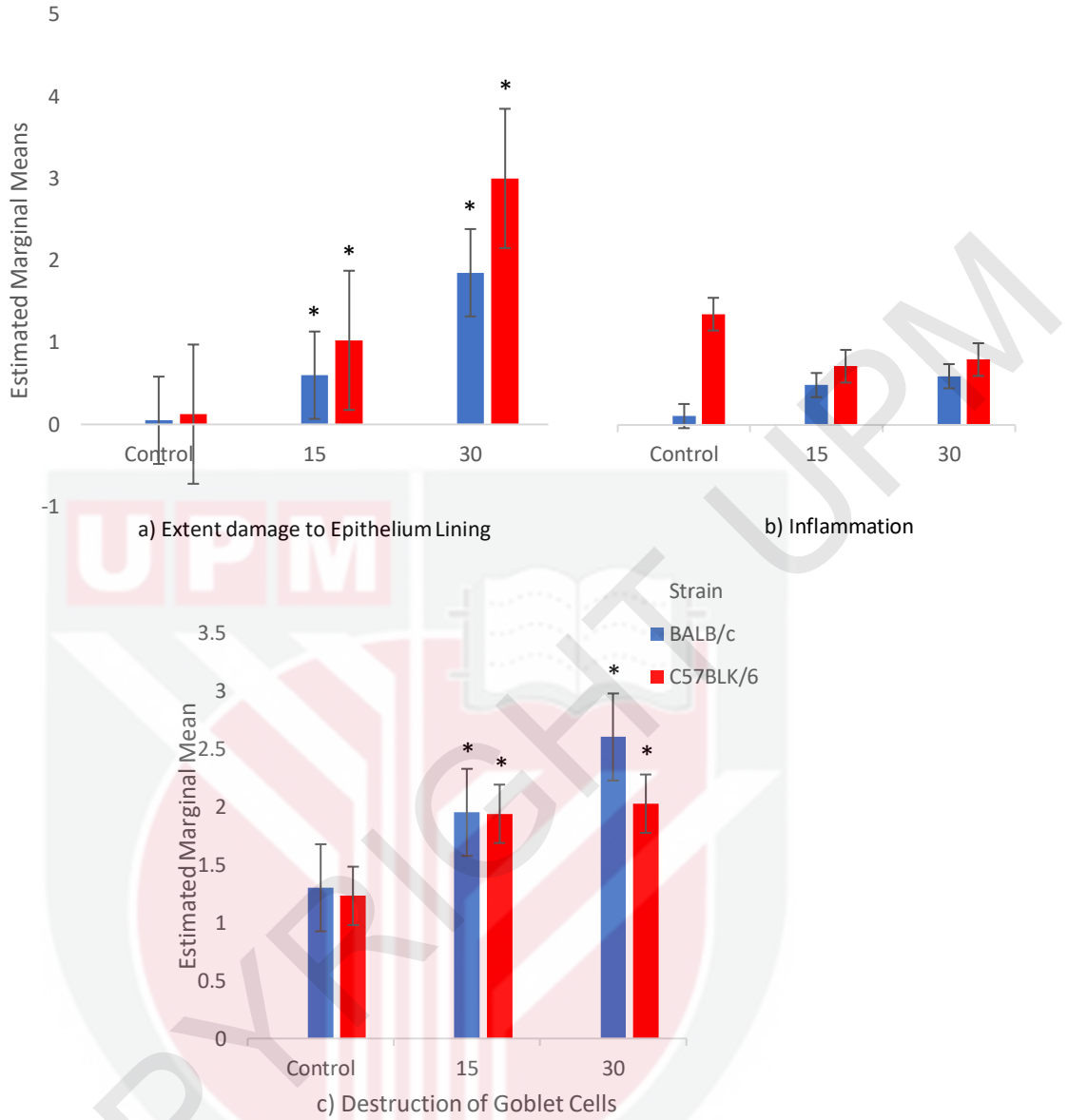

Figure 4.2.2: The graph means of necrosis scoring in the ileum of BALB/c and C57BLK/6 due to MPTP administration. Data are presented as mean ±S.E.M represented in three categories of necrosis **a.** Extent damage of epithelium lining, **b.** Inflammation, **c**. Destruction of goblet cells. *p<0.05

4.3 Toxicity effects of MPTP on the colon of BALB/c and C57BLK/6

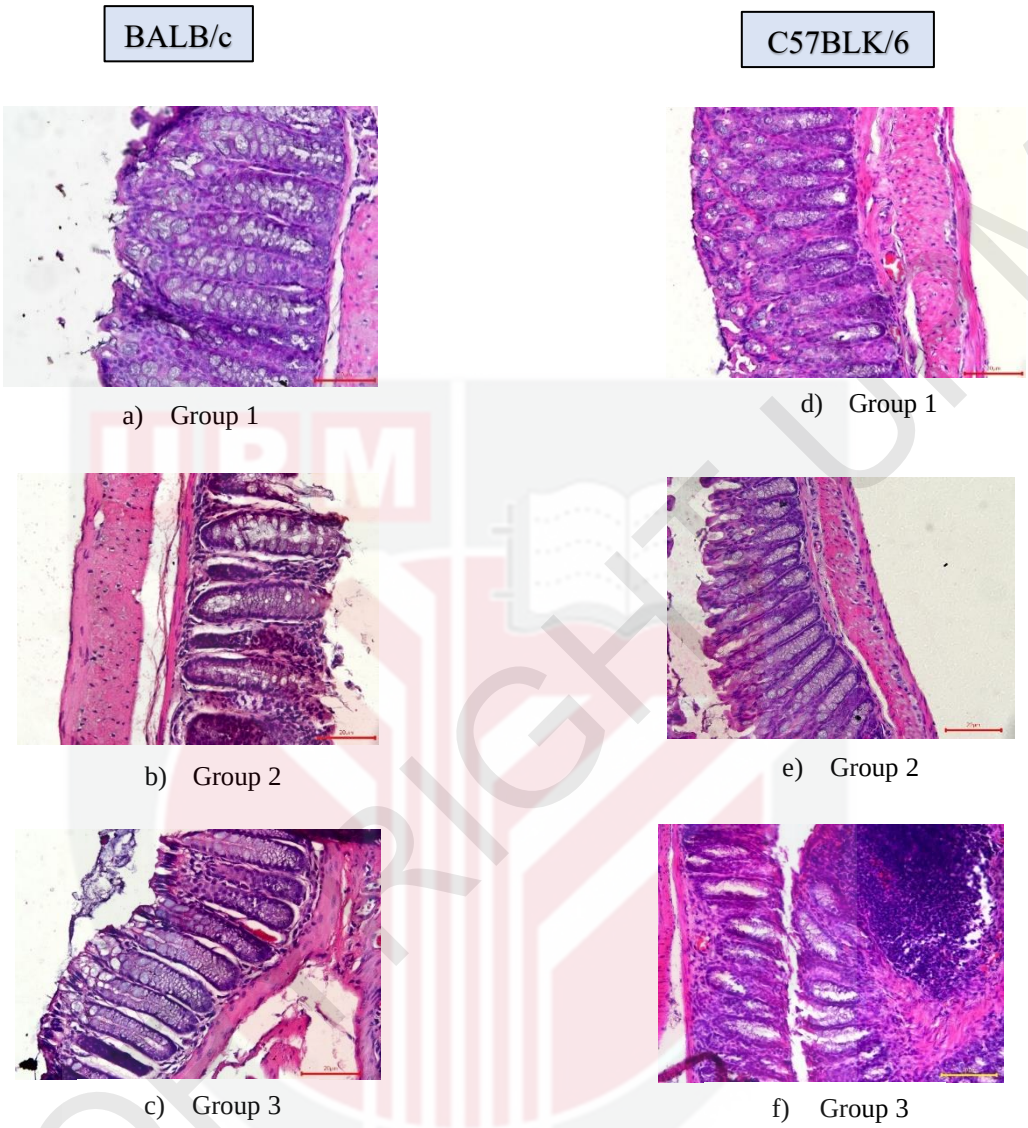

Figure 4.3.1: Histology of colon of BALB/c and C57BLK/6 at a magnification of 20X. Representative images of the stomach of **a.** Control BALB/c mice, **b.** 15mg/kg MPTP-treated BALB/c mice, **c.** 30mg/kg MPTP-treated BALB/c mice **d.** Control C57BLK/6, **e.** 15mg/kg MPTP-treated C57BLK/6, **f.** 30mg/kg MPTP-treated C57BLK/6. Paraffin-embedded 5µm sections from the colon of BALB/c and C57BLK/6 were stained with H&E staining and imaged at X20 magnification.

The toxicity effect of MPTP on the colon was compared between 2 inbred strains of mice. The one-way ANOVA analysis of MPTP toxicity on the damage to colon epithelial lining determined that there were no statistically significant differences between strains ($F(1,22)=0.128$, $p=0.724$). However, Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p=0.001$) and 30 mg/kg ($p=0.000$) had a significant effect on the colon epithelial lining as compared to the control group in both strains. Furthermore, the one-way ANOVA analysis of MPTP toxicity on the colon inflammation showed that there were no statistically significant differences between strains ($F(1,22)=0.190$, $p=0.667$). Although, Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p=0.027$) and 30 mg/kg ($p=0.002$) had a statistically significant effect on inflammation as compared to the control group in both strains. Besides, the one-way ANOVA analysis of MPTP toxicity on the goblet cells of the colon showed that there were no statistically significant differences between strains ($F(1,22)=0.923$, $p=0.347$). However, Dunnett's test revealed that administration of both concentrations of MPTP, 15 mg/kg ($p=0.026$) and 30 mg/kg ($p=0.005$) had a significant effect on the goblet cells of ileum as compared to the control group in both strains.

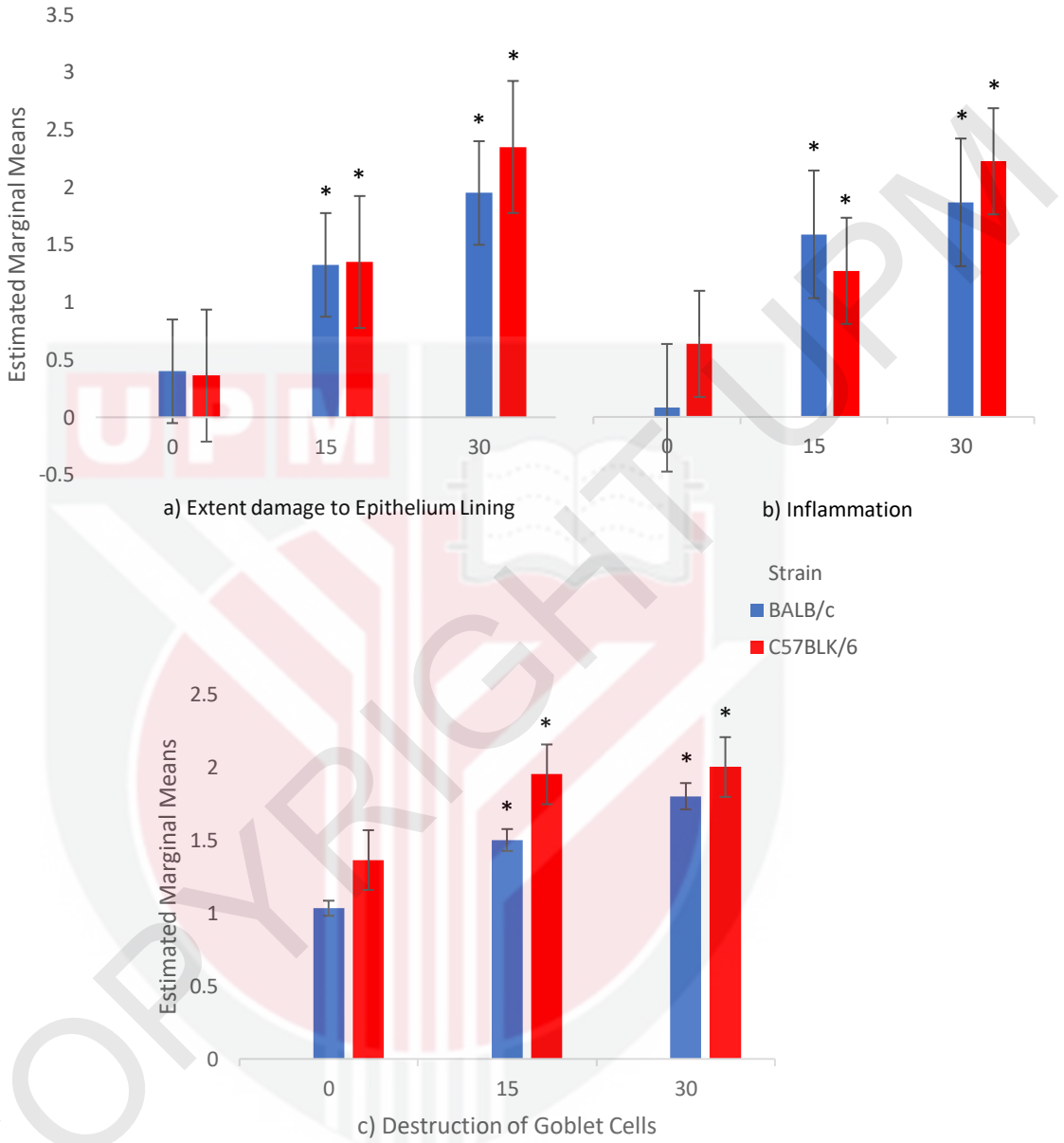

Figure 4.3.2: The graph means of necrosis scoring in the colon of BALB/c and C57BLK/6 due to MPTP administration. Data are presented as mean ±S.E.M represented in three categories of necrosis **a.** Extent damage of epithelium lining, **b.** Inflammation, c. Destruction of goblet cells. *p<0.05

4.4 Additional Finding

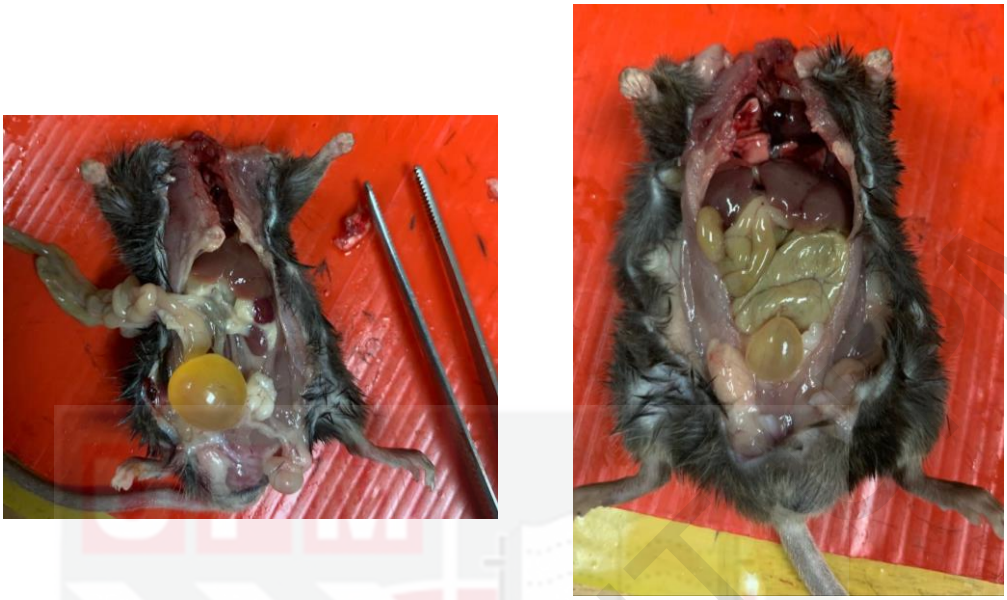

Figure 4.4: Images of bladder enlargement in a few 30mg/kg treated C57BLK/6 mice. Bladder problems can also occur with the administration of MPTP, though this is not as common among mice.

CHAPTER 5

DISCUSSION

This study had two major aims. For one, we wanted to determine that MPTP also can give effects toxicity to the BALB/c although they are said to be resistant to MPTP. Secondly, and the most important part of this study, we wished to differentiate and make a comparison histologically regarding the effects of MPTP on the gastrointestinal organs between two inbred strains of mice which are BALB/c and C57BLK/6. We used the stomach, ileum, and colon as representatives of GIT to study its pathology related to PD. Therefore, the intraperitoneal route of subacute or chronic infusion of MPTP ranging from 15mg/kg to 60mg/kg in a single daily injection over several days was used to evaluate the progression of gastrointestinal pathology in PD mouse models. Among most PD animal models, MPTP is the best compound that can mimic the pathology of PD as well as can determine the gastrointestinal histopathology that occurs in the early stage of PD after administration of MPTP.

With respect to the first aim, our results yielded MPTP also can cause effects of toxicity in both strains including BALB/c although it has the characteristics of resistance. However, it is only reasonable to see the effects of MPTP histologically in the gastrointestinal organs right after administration of MPTP. The reason is that the BALB/c mice are expected to recover and reach control levels on a subsequent day, similar recovery observed in C57BLK/6 on day 3 (Sedelis et al., 2000). Therefore, we assumed that the day after the last treatment is the acceptable day to show that MPTP also works well in BALB/c and make a comparison with C57BLK/6. This technique was applied in this study in which we sacrificed the mice and did routine

histopathological techniques right after the last treatment to avoid any recovery process in all BALB/c mice.

Previous research has demonstrated that the histological susceptibility to the neurotoxin MPTP varies between mouse strains (German et al., 1996). In a study done by Sedelis et. al. (2000), most of their analysis consistently revealed that the C57BLK/6 mouse strain is more vulnerable to MPTP than the BALB/c strain. Besides confirming this, our findings of this study show increased sensitivity to systemic injection of MPTP in C57BLK/6 with increasing dose of MPTP administration compared to BALB/c. Due to the facts that support these findings, there are many possible mechanisms of their differential sensitivity such as the activity of the enzyme that is responsible for the bioactivation of MPTP which is known as monoamine oxidase (MAO) (Sedelis et. al, 2000; Meredith & Rademacher, 2011). In other words, MPTP's neurotoxicity is reliant on MAO activity. MAO activity is increased with age, but since we use the same age of mouse models, we considered age of mice as our constant variable. In this case, the activity of MAO-A in gastrointestinal organs and MAO-B in the brain was greater resulting in the increased susceptibility of C57BLK/6 to the MPTP compared to BALB/c.

Another possible explanation to discuss strain differential sensitivity to MPTP is a change in the expression of the rate-limiting enzyme in dopamine production called Tyrosine Hydroxylase (TH) and the numbers of DAergic neurons in SNpc (Filipov et. al, 2009). TH is an enzyme that important for the generation of all catecholamines, including dopamine. The activity of TH and the number of DAergic neurons are said to be higher in BALB/c in comparison with the C57BLK/6. Hence, a greater number of DAergic neurons and greater striatal TH can be the reasons why BALB/c is minimally affected by MPTP since those positive feedback mechanisms

only can be applied in BALB/c mice if the response of DAergic neurons is depleted in this strain. This evidence is strong enough to support our first aim in which BALB/c can recover more rapidly to the control levels rather than C57BLK/6.

Another reason to explain the degree of sensitivity to MPTP across different mouse strains may be related to the coat color (Meredith & Rademacher, 2011). Pigmented mouse strain which is C57BLK/6 is very sensitive rather than albino strain BALB/c. This is might due to the presence of a susceptibility gene in C57BLK/6 located on chromosome 7 that does not protect the mice's MPTP-induced behavioral impairments, striatal DA depletion, and SNpc neuronal death. According to the same study by Meredith & Rademacher (2011), further research is needed to determine whether the increased sensitivity to MPTP reported in pigmented mice is mediated by that susceptibility gene. Furthermore, the differential in the expression of inflammatory mediators and oxidative markers also can be one of the reasons for differential sensitivity to MPTP among those two mice strains (Filipov et. al, 2009). Inflammatory mediators and oxidative markers are important key players in the induction of neuronal damage and neurodegeneration. As such, C57BLK/6 mice have higher sensitive due to their capability to produce greater key players and actively contribute to the pathogenesis of the neurodegenerative disorder.

Three different categories were used to represent necrosis that happened within the tissues of control and MPTP-treated mice. Necrosis was chosen as part of tissue damage instead of apoptosis because it is frequently associated with neurodegenerative disorders which involve the formation of toxic molecules called ROS and contribute to further cell destruction (Negroni et al., 2015). Necrosis is defined as an uncontrolled process that is initiated by an external factor such as toxins, which in this study refers to the MPTP compound that can create a certain pathological process such as rapid

breakdown of the cell membrane. We choose three categories of necrosis which are; a) extent damage to epithelium lining, b) inflammation, and c) destruction to the cells. Among all types of necrosis, those three are considered as the damage that are commonly presented within the tissues of BALB/c and C57BLK/6 mice. Semi-quantitative scoring assessment of histology sections was done to assess damage in the stomach, ileum, and colon to indicate the effects of MPTP toxicity on the gastrointestinal organs.

The findings of this study show that the overall trends of three different categories represent necrosis in three organs of the gastrointestinal tract which are the stomach, ileum, and colon showing that administration of MPTP gives more toxicity effects to the C57BLK/6 compared to BALB/c. However, there are some inconsistent trends of necrosis happened such as in the stomach shown in Figure 4.1 where we could see the increasing trend of stomach inflammation and destruction of parietal cells of BALB/c is higher than C57BLK/6. This finding is contrary to the main theory, and we assumed that C57BLK/6 gradually recover to the control levels since we have dissected and processed C57BLK/6 mice a bit later than BALB/c. Supposedly, process of dissection and routine histopathological techniques of C57BLK/6 should be the same as what we did for previous BALB/c mice to avoid any recovery process in C57BLK/6 mice. However, the overall extent of damage to epithelial lining in the stomach of C57BLK/6 mice is more obvious and enhanced with increasing doses of MPTP compared to BALB/c. Since the C57BLK/6 mice are the most vulnerable strain, MPTP can cause more severe damage to the stomach mucosa including blood vessels in C57BLK/6 mice, causing ischemia and ulceration of the stomach epithelial lining. MPTP affects both the central and peripheral dopaminergic systems of ENS, resulting in decreased gastric motility with a compensatory increased in gastric hormone and

consequently more gastric acid secretion, leading to decreased acid neutralization capacity (Szabo et al., 1985; Szabo & Cho, 1988). As a result of gastric stasis, stomach ulceration has been observed shown in Figure 4.1.2.

Despite inflammation playing a role in the development and progression of PD, we also observed significant pathological damage to the three gastrointestinal organs in both strains related to the epithelial lining and parietal cells in the stomach as well as goblet cells in the ileum and colon. Representative images of the stomach in Figure 4.1 from control mice, 15mg/kg MPTP-treated, and 30mg/kg MPTP-treated mice were chosen to show differences in histopathology in both strains. Stomach histology of the control mice in both strains of mice shows a normal structure of stomach mucosa with well-positioned of the epithelial lining, centrally nuclei of stomach cells (parietal cells and chief cells), tall columnar epithelium lines the glandular stomach and gastric pits. While stomach histology in 15mg/kg and 30 mg/kg MPTP-treated mice of BALB/c appeared moderate to severe inflammation with the aberrant architecture of stomach and hyperchromatic nuclei in stomach epithelial cells compared to the control mice. In 15mg/kg and 30mg/kg MPTP-treated mice of C57BLK/6, stomach histology shows that the stomach cells start to not be in an aligned position or significantly irregular arrangement of stomach cells, irregularities of glandular structure, and disruption of the epithelial lining and more severe can be seen in 30mg/kg of MPTP. Meanwhile, Figure 4.2 shows disruption of epithelial lining in 15mg/kg MPTP-treated BALB/c mice and severe epithelial destruction and erosion of epithelial lining that can be seen in 30mg/kg MPTP-treated BALB/c mice. Nothing much changes can be seen in the 15mg/kg and 30mg/kg MPTP-treated C57BLK/6 mice, only a few areas with mild predominant inflammation can be observed. Furthermore, Figure 4.3 shows an observation of the colon of 15mg/kg and 30mg/kg MPTP-treated BALB/c mice with

loss of normal epithelial architecture which results in epithelial erosion. In comparison with 15mg/kg and 30mg/kg MPTP-treated C57BLK/6, photomicrograph shows moderate to more severe epithelial and colonic crypt erosion. It might be due to mucin erupting out of crypts forming an inflammatory membrane of crypts (Moore et al., 2020). Severe accumulation of predominant inflammatory cells also can be seen to show that severe inflammation happened within 30mg/kg MPTP-treated C57BLK/6 mice. We hypothesize that inflammation, epithelial damage, parietal cells as well as goblet cells destruction are caused by the toxicity of MPTP administration in both strains, specifically more severe in the C57BLK/6 strain of mice.

It is important to mention that MPTP poisoning causes neurodegeneration not only in the substantia nigra, but also in other brain areas as well as in other body parts including gastrointestinal organs. The regimen that we used in this study for the administration of MPTP is a subacute or chronic regimen in which MPTP is given at doses of 15mg/kg, 30mg/kg, and 60mg/kg for 5 consecutive days because it can show approximately 40-50% of dopamine depletion (Mustapha & Mat Taib, 2020) and to maintain in reproducing slow, constant, and gradual neurodegenerative process characteristics of PD (Lai et al., 2018); however, there is some mortality associated with this chronic method (Meredith & Rademacher, 2011). BALB/c and C57BLK/6 mice treated with a single 60mg/kg dose of MPTP intraperitoneally display severe signs of neuronal degeneration with acute tremor and the mice died right after the first day of treatment (data not shown). We assume that 60mg/kg of MPTP is a lethal dose (LD) for the mouse and it is not suitable for us to observe the progress of the disease. The possible mechanism involved might be the higher production of oxidative stress molecules known as reactive oxygen species (ROS) by high activity of MAO. This condition causes severe disruption to the key cellular components involved in several

biological processes in subtantia nigra of the mice including the metabolism of dopamine itself and mitochondrial dysfunction, making the mice cannot cope with that kind of situation, ultimately leading to death (Dias et al., 2013).

Another additional finding, one of the most prevalent nonmotor symptoms of Parkinson's disease is urinary bladder dysfunction, which includes urine frequency, urgency, and incontinence. MPTP-induced Parkinsonian-like symptoms in mice could be employed as a model in this study to observe if there is any bladder dysfunction in Parkinson's disease. According to our findings shown in Figure 4.4, few C57BLK/6 mice can develop bladder enlargement when they are given 30mg/kg of MPTP, although it is not as common among all mouse models. This condition might be known as neurogenic bladder or neurogenic lower urinary tract dysfunction due to the problems of muscle and nerves failing to work together (Kitta et al., 2020). Nerves damaged due to the administration of MPTP will fail the bladder to expel out the urine, subsequently leading to urinary bladder enlargement or bladder hypertrophy (Young, 2018). An enlarged bladder has become larger than normal because it grows when it became overstretched as a result of the high volume and pressure of urine inside the bladder. The mice keep producing a large volume of urine and their bladders are never empty.

CHAPTER 6

LIMITATIONS, RECOMMENDATIONS AND CONCLUSION

6.1 Limitations and Recommendations

Although we could create the MPTP animal models to recapitulate symptoms in human PD, we cannot deny the anatomy and biomechanics of various animal models and humans differ significantly. Therefore, we cannot expect 100% similarities from what we can see between MPTP animal models and those patients with PD. Besides, 60mg/kg dose of MPTP is quite high for a mouse, and it is proven in present study which administration of 60mg/kg of MPTP cause mortality to all mice in both strains. Obviously, animal studies must be refined or improved in any way that reduces the possibility of suffering for all participating animals. If possible, reduction of dose from 60mg/kg to 40mg/kg could be an appropriate way to reduce fatal in the mice. Despites all limitations, the current animal models can serve as a useful platform to study pathophysiology and etiology that related to PD.

6.2 Conclusion

We concluded that necrosis formation is enhanced with increasing doses of MPTP in C57BLK/6 compared to BALB/C. It is proven by three different categories of necrosis showing increasing trends of mean graph and histopathology examination of three different gastrointestinal organs are more severe in C57BLK/6 in comparison to BALB/c. Besides, MPTP also causes histopathology of GIT in BALB/c although it is said to be resistant. However, it is only reasonable to observe the effects of MPTP

histologically in the gastrointestinal organs immediately following MPTP administration. The reason is that the BALB/c mice are predicted to recover and return to control levels on the subsequent day, while CBLK/6 animals recovered similarly on day 3.

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APPENDICES

Necrosis scoring of BALB/c and C57BLK/6

A) Extent damage to epithelium lining

I) Colon

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	0	0	0	0	0.2	0.400
	G1M2	0	0	0	0	1	0.2	
	G1M3	0	1	1			0.6	
	G1M4	0	0	1	1	1	0.6	
2	G2M1	2	2	2	0	0	1.2	1.325
	G2M2	1	1	1	0	1	0.8	
	G2M3	2	1	1	2		1.5	
	G2M4	2	2	1	1	3	1.8	
3	G3M1	1	2	2	1	1	1.4	1.575
	G3M2	1	2	2	3	3	2.2	
	G3M3	1	3	1	1	3	1.8	
	G3M4	1	3	2	3	3	2.4	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	1	0		0.25	0.35
	G1M2	0	0	0	1	1	0.4	
	G1M3	0	0	0	0	1	0.2	
	G1M4	1	1	1	0	0	0.6	
2	G2M1	0	0	2	1	1	0.8	1.35
	G2M2	1	1	0	1	0	0.6	
	G2M3	2	2	2	1	1	1.6	
	G2M4	3	3	2	2	2	2.4	
3	G3M1	3	2	3	2	2	2.4	1.85
	G3M2	3	3	3	3	3	3.0	
	G3M3	2	2	2	1	1	1.6	
	G3M4	2	2	3	3	2	2.4	

II) Ileum

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	0			0.0	0.05
	G1M2	0	0	0	0	1	0.2	
	G1M3	0	0	0	0	0	0.0	
	G1M4	0	0	0	0	0	0.0	
2	G2M1	0	0	0	0	0	0.0	0.60
	G2M2	1	1	0	0	0	0.4	
	G2M3	0	0	1	1	0	0.4	
	G2M4	3	3	1	1	0	1.6	
3	G3M1	1	2	3	0	1	1.4	1.85
	G3M2	1	1	1	0	1	0.8	
	G3M3	1	2	3	3	2	2.2	
	G3M4	3	3	3	3	3	3.0	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	0			0.0	0.125
	G1M2	0	0				0.0	
	G1M3	1	0	1	0		0.5	
	G1M4	0	0	0	0		0.0	
2	G2M1	0	0	0	0		0.0	1.025
	G2M2	1	0	0	1		0.5	
	G2M3	1	1	3	3	3	2.2	
	G2M4	3	3	1			1.4	
3	G3M1	3	3	3	3		3.0	3.000
	G3M2	3	3	3	3	3	3.0	
	G3M3	3	3	3	3		3.0	
	G3M4	3	3	3	3		3.0	

III) Stomach

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	1	1	1	1	1.0	0.500
	G1M2	1	1	1	1	1	1.0	
	G1M3	0	0	0	0	0	0.0	
	G1M4	0	0	0	0		0.0	
2	G2M1	0	0	0	0	0	0.0	1.250
	G2M2	2	2	2	2	2	2.0	
	G2M3	1	0	2	2	0	1.0	
	G2M4	2	2	2			2.0	
3	G3M1	2	2	2			2.0	2.500
	G3M2	3	3	2	2	2	2.4	
	G3M3	2	3	2	3	3	2.6	
	G3M4	3	3	3	3		3.0	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	1	1	2		1.25	1.12
	G1M2	2	1	1			1.33	
	G1M3	0	0	1	1		0.50	
	G1M4	1	1	2	2	1	1.40	
2	G2M1	3	3	3	3	3	3.00	2.07
	G2M2	2	2	2	1	1	1.60	
	G2M3	2	2	2	2	2	2.00	
	G2M4	1	2	2			1.67	
3	G3M1	3	3	3	3	3	3.00	2.75
	G3M2	2	2	3	1		2.00	
	G3M3	3	3	3	3	3	3.00	
	G3M4	3	3	3			3.00	

B) Inflammation

I) Colon

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	0	0	0	0.0	0.050
	G1M2	1	0	0	0	0	0.2	
	G1M3	0	0	0			0.0	
	G1M4	0	0	0	0	0	0.0	
2	G2M1	1	0	2	2	3	1.6	1.000
	G2M2	2	0	0	1	1	0.8	
	G2M3	3	2	1	1		1.4	
	G2M4	1	0	0	0	0	0.2	
3	G3M1	2	2	1	1	2	1.6	1.175
	G3M2	1	1	1	1	1	1.0	
	G3M3	0	0	1	1	1	0.5	
	G3M4	2	3	1	1	1	1.6	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	0	0		0.0	0.4
	G1M2	1	1	1	0	0	0.6	
	G1M3	1	1	0	0	0	0.4	
	G1M4	1	1	1	0	0	0.6	
2	G2M1	2	1	1	1	0	1.0	0.8
	G2M2	1	1	1	0	0	0.6	
	G2M3	1	1	1	1	1	1.0	
	G2M4	1	1	0	0	1	0.6	
3	G3M1	1	1	0	0	2	0.8	1.4
	G3M2	3	3	3	3	3	3.0	
	G3M3	2	2	1	0	0	1.0	
	G3M4	0	0	1	2	1	0.8	

II) Ileum

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	0			0.0	0
	G1M2	0	0	0	0	0	0.0	
	G1M3	0	0	0	0	0	0.0	
	G1M4	0	0	0	0	0	0.0	
2	G2M1	1	1	0	0	0	0.4	0.45
	G2M2	0	0	0	0	0	0.0	
	G2M3	1	0	0	0	0	0.2	
	G2M4	0	2	2	2	0	1.2	
3	G3M1	1	1	0	0	0	0.4	0.55
	G3M2	1	1	0	0	0	0.4	
	G3M3	0	0	1	1	0	0.4	
	G3M4	2	2	1	0	0	1.0	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	2	2	2			2.00	1.075
	G1M2	2	0				1.00	
	G1M3	2	2	1	1		1.50	
	G1M4	1	1	0	0		0.50	
2	G2M1	1	1	0	0		0.50	0.663
	G2M2	1	0	0	0		0.25	
	G2M3	2	2	2	2	0	1.60	
	G2M4	0	0	1			0.30	
3	G3M1	1	1	0	0		0.50	0.738
	G3M2	2	2	0	1	1	1.20	
	G3M3	0	0	1	0		0.25	
	G3M4	2	1	1	1		1.00	

III) Stomach

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	0	0	0	0.0	0
	G1M2	0	0	0	0	0	0.0	
	G1M3	0	0	0	0	0	0.0	
	G1M4	0	0	0	0	0	0.0	
2	G2M1	1	1	1	2		1.0	1.2
	G2M2	0	0	1	0	0	0.2	
	G2M3	1	1	1	0	0	0.6	
	G2M4	3	3	3			3.0	
3	G3M1	2	2	2			2.0	1.5
	G3M2	0	1	1	0	1	0.6	
	G3M3	1	1	0	0	0	0.4	
	G3M4	3	3	3	3		3.0	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	1	0	0		0.50	0.44
	G1M2	1	1	0			0.67	
	G1M3	0	0	0	0		0.00	
	G1M4	0	0	1	1	1	0.60	
2	G2M1	2	2	2	2	3	2.2	0.65
	G2M2	0	0	0	0	0	0.00	
	G2M3	1	1	0	0	0	0.40	
	G2M4	0	0	0			0.00	
3	G3M1	1	1	1	0	0	0.50	0.70
	G3M2	1	1	1	1		1.00	
	G3M3	1	1	1	1	1	1.00	
	G3M4	1	0	0			0.30	

C) Destruction of Goblet Cells

I) Colon

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	2	2	2	1	0	1.40	1.03
	G1M2	2	2	2	2	2	2.00	
	G1M3	1	0	0			0.33	
	G1M4	0	1	1	0	0	0.40	
2	G2M1	2	2	2	1	2	1.80	1.50
	G2M2	2	2	2	2	1	1.80	
	G2M3	1	2	2	2		1.80	
	G2M4	1	1	0	1	0	0.60	
3	G3M1	1	1	3	2	1	1.6	1.80
	G3M2	2	1	2	2	2	1.8	
	G3M3	3	2	2	2	1	2.0	
	G3M4	2	2	1	2	2	1.8	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	1	2	2		1.50	1.36
	G1M2	1	2	2	2		1.75	
	G1M3	1	1	1	1	1	1.00	
	G1M4	2	1	1	1	1	1.20	
2	G2M1	2	3	2	1	2	2.00	1.95
	G2M2	2	2	2	2	2	2.00	
	G2M3	2	2	2	2	2	2.00	
	G2M4	2	1	2	2	2	1.80	
3	G3M1	2	2	2	1	2	1.80	2.75
	G3M2	2	2	2	2	2	2.00	
	G3M3	2	3	1	2	2	2.00	
	G3M4	2	2	3	2	2	2.20	

II) Ileum

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	1	1			1.0	1.30
	G1M2	1	2	1	1	1	1.2	
	G1M3	2	2	1	1		1.5	
	G1M4	1	1	2	2		1.5	
2	G2M1	1	1	1	2	2	1.4	1.95
	G2M2	2	2	2	2	2	2.0	
	G2M3	2	2	2	2	2	2.0	
	G2M4	2	2	2	3	3	2.4	
3	G3M1	3	3	2	2	2	2.4	2.60
	G3M2	2	3	3	2	2	2.4	
	G3M3	3	2	3	3	2	2.6	
	G3M4	3	3	3	3	3	3.0	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	0	1			0.67	1.230
	G1M2	1	1				1.00	
	G1M3	2	2	2	1		1.75	
	G1M4	2	1	1	2		1.50	
2	G2M1	2	2	2	1		1.75	1.938
	G2M2	2	2	3	1		2.00	
	G2M3	2	2	2	2	2	2.00	
	G2M4	2	2	2			2.00	
3	G3M1	2	3	2	2		2.25	2.025
	G3M2	2	2	2	1	1	1.60	
	G3M3	2	2	2	2		2.00	
	G3M4	3	2	2	2		2.25	

D) Destruction of Parietal Cells

I) Stomach

BALB/c								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	1	1	2	2	2	1.60	1.00
	G1M2	1	1	0	0	1	0.60	
	G1M3	0	0	1	1	2	0.80	
	G1M4	0	1	1	1	2	1.00	
2	G2M1	1	2	2	2		1.75	1.46
	G2M2	1	1	1	0	0	0.60	
	G2M3	1	1	1	1	2	1.20	
	G2M4	2	2	3			2.30	
3	G3M1	2	2	1			1.67	2.01
	G3M2	2	3	3	3	3	2.80	
	G3M3	1	3	3			2.33	
	G3M4	1	1	1	2		1.25	

C57BLK/6								
GROUP	**LABEL**	**SCORE**					**MEAN SCORE PER MICE**	**MEAN SCORE PER GROUP**
1	G1M1	0	0	0	0		0.00	0.082
	G1M2	0	0	1			0.33	
	G1M3	0	0	0	0		0.00	
	G1M4	0	0	0	0	0	0.00	
2	G2M1	1	1	0	0	1	0.60	0.833
	G2M2	0	0	0	1	1	0.40	
	G2M3	1	1	1	1	1	1.00	
	G2M4	1	2	1			1.33	
3	G3M1	1	1	1	1	1	1.00	0.730
	G3M2	0	0	0	1		0.25	
	G3M3	1	1	1	1	1	1.00	
	G3M4	1	1	0			0.67	