KULLIYYAH OF MEDICINE
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RESEARCH PROPOSAL

HIGH-MOBILITY GROUP BOX 1 (HMGB1) PROTEIN EVALUATION IN MPTP-INDUCED ZEBRAFISH MODEL OF PARKINSON'S DISEASE

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1. INTRODUCTION

1.1. Study background

Parkinson's disease (PD) is a progressive neurodegenerative disease that attacks dopaminergic neurons in substantia nigra pars compacta (SNpc), a brain region that is typically involved in motor control (DeMaagd & Philip, 2015; Harris *et al.*, 2020). According to the 2015 Global Burden of Disease, the prevalence and death rates of PD had increased significantly over the past 25 years and currently, there are more than 10 million reported PD cases worldwide (Feigin *et al.*, 2017; Ray Dorsey *et al.*, 2018). Parkinson's disease affects the quality of life as it causes movement impairments as well as deteriorates psychological and cognitive functioning (Yang *et al.*, 2016; Maiti *et al.*, 2017). The main hallmarks of PD pathology are substantial loss of dopaminergic (DA) neurons in SNpc and intracellular aggregation of α-synuclein protein or also known as Lewy bodies (LBs) formation (Stefanis, 2012; Barnhill *et al.*, 2020). In molecular context, PD is often associated with mitochondrial dysfunctionality, oxidative stress and chronic neuroinflammation, all of which are believed to trigger subsequent neurodegeneration. At the moment, there is no cure to this disease and researchers are still overwhelmed by its complexity, despite extensive studies have been done.

When neuronal homeostasis is disturbed, the central nervous system (CNS) elicits a defence mechanism in the form of inflammatory response to counter the disturbance. Although it is neuroprotective, however, prolonged inflammation can cause detrimental effects to the neurons (Morales *et al.*, 2016; Sochocka *et al.*, 2017). Chronic neuroinflammation is believed to be one of the biggest factors that contribute to the pathogenesis of PD (Wang *et al.*, 2015). High-mobility group box 1 (HMGB1) protein, or also known as amphoterin, is a DNA-binding nuclear protein that in normal condition, plays a role in nucleosome structural maintenance and transcription regulation (Magna & Pisetsky, 2014; Paudel *et al.*, 2018). Nevertheless, in pathologic condition, HMGB1 is released to the extracellular matrix as damaged-associated

molecular protein (DAMP) and triggers downstream inflammatory response through binding with RAGE (receptor for advanced glycation end-products) and toll-like receptors (TLRs) (Santoro *et al.*, 2016). HMGB1 has been implicated in PD due to its involvement with chronic neuroinflammation, oxidative stress, autophagy disorders and consequently, neuronal apoptosis (Angelopoulou *et al.*, 2018). In an animal study of PD, HMGB1 was shown to be upregulated following neurotoxin induction and caused dopaminergic neuronal death through inducing neuroinflammation, exacerbating α-synuclein aggregation and disturbing autophagy process (Huang *et al.*, 2017). Inhibition of HMGB1 on the other hand, was reported to suppress inflammatory activity and protect dopaminergic neurons against degeneration (Sasaki *et al.*, 2016). With current evidences, HMGB1 has emerged as a novel target for the treatment of PD. Though proven to be involved in PD pathogenesis, but its molecular mechanism is not yet fully understood.

Since decades ago, zebrafish has been an ideal animal model for PD molecular studies due to its close homology to human (Shehwana & Konu, 2019) and easy genetic manipulations (Koster & Sassen, 2015). Zebrafish also expresses HMGB1, which is involved in forebrain development (Zhao *et al.*, 2011), and like humans, in immune response mechanism (Fang *et al.*, 2014). Since the function of this protein is conserved in both species and zebrafish is suitable to be used for PD molecular studies, this provides a promising platform in investigating the involvement of HMGB1 in PD pathogenesis. By using anti-sense HMGB1 morpholino oligonucleotides to knockdown HMGB1 expression and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin to induce parkinsonism in adult zebrafish, this current study aims to delineate the role of HMGB1 protein in MPTP-induced zebrafish model of PD. Findings from this study will pave the way in designing potential therapies targeting molecular docking for the inhibition of HMGB1 protein. In longer term, this study will help in addressing

fundamental role of HMGB1 as a novel target for PD that may open the venue for proper treatment of this chronic progressive neurodegenerative disease.

1.2. Problem statement

Molecular studies have proven beneficial in understanding the pathophysiology of PD. Besides, advancing application of zebrafish as PD model have enabled researchers to conduct molecular studies with more promising outcomes and significance. Current evidences reported on the association of HMGB1 protein with neuroinflammation-induced PD pathogenesis (Sasaki *et al.*, 2016; Huang *et al.*, 2017; Angelopoulou *et al.*, 2018). However, to the extent of our knowledge, the molecular mechanism pertaining to HMGB1 involvement in PD are still elusive.

Hence, we propose to conduct a study on HMGB1 protein, to elucidate its role in the pathogenesis of MPTP-induced zebrafish model of PD. This study will answer the question pertaining to the involvement of HMGB1 in PD development and whether the knockdown of this protein can improve PD symptoms in zebrafish, particularly MPTP-induced motility disorders.

1.3. General objective

To investigate the role of HMGB1 in the development of parkinsonian motility disorders using MPTP-induced zebrafish model of PD.

1.4. General hypothesis

HMGB1 is involved in the development of parkinsonian motility disorders of MPTP-induced zebrafish model of PD by mediating chronic neuroinflammatory response.

1.5. Specific objectives

1.5.1. To establish and validate zebrafish model of PD via intraperitoneal injection of MPTP neurotoxin.

1.5.2. To knockdown the expression of HMGB1 in the neurons of zebrafish brain using anti-sense HMGB1 morpholino oligonucleotides.

1.5.3. To evaluate the effect of HMGB1 knockdown at behavioural, histopathological, and molecular levels.

2. LITERATURE REVIEW

2.1. Parkinson's disease aetiology and pathophysiology

Parkinson's disease affects the dopaminergic neuronal population, particularly in SNpc. Since the SNpc is the origin of nigrostriatal dopaminergic system, gradual loss of DA neurons in this brain region will perturb dopaminergic neuronal projection to the striatum and result in subsequent loss of dopamine input (Sarath Babu *et al.*, 2016; Barnhill *et al.*, 2020). Striatum, apart from the SNpc, is mainly associated with movement regulations, which is why PD is often linked to motility disorders. Apart from substantial dopamine loss, another hallmarks of PD pathology are α-synuclein protein aggregations inside neuronal cells, or also known as Lewy bodies (LBs) formation (Stefanis, 2012; Barnhill *et al.*, 2020), as well as chronic neuroinflammation (Troncoso-Escudero *et al.*, 2018). The former causes toxicity to the neurons thus triggers neuronal damage (Meade *et al.*, 2019) while the latter is the result of increased DAMPs population and other pro-inflammatory components (Bajwa *et al.*, 2019). Besides that, molecular studies on PD also revealed mitochondrial dysfunctionality inside the brain. PD patients were reported to have deficient level of mitochondrial complex I, an enzyme that is responsible for the initiation of mitochondrial respiratory chain (Mimaki *et al.*, 2012). Significant loss of mitochondrial complex I leads to failure in ATP synthesis and increased

production of reactive oxygen species (ROS), which subsequently contributes to the death of DA neurons (Kouli *et al.*, 2018).

Although the absolute cause of PD is still largely unknown, this disease is regarded as a multifactorial disease because it is believed to be influenced by both environmental and genetic factors. Increased longevity is the most known aetiology of PD and it is considered to be the biggest contributing factor to the development of this disease (Hindle, 2010; Reeve *et al.*¸2014; Ball *et al.*, 2019). In fact, only four per cent of reported PD cases came from individuals under the age of 50 (Lee *et al.*, 2019). Other than that, some known synthetic compounds are also evidenced to possess neurotoxin properties that can induce PD-like symptoms in humans, such as MPTP, 6-hydroxydopamine (6-OHDA), as well as several herbicides and pesticides like rotenone and paraquat. In the context of genetic factor, researchers identified several mutations to certain genes that can lead to either autosomal dominant or recessive PD development. For example, mutated *snca* and *lrrk2* gene particularly causes autosomal dominant PD while mutated *prkn* or *pink1* gene triggers the development of autosomal recessive PD (Billingsley *et al.*, 2018; Pang *et al.*, 2019). In terms of inheritance, only a small percentage of individuals diagnosed with PD has a family history of PD symptoms. According to Tran *et al.* (2020), more than 85% of PD cases are reported to be sporadic, or in other words, not inherited. From the above findings, it can therefore be concluded that, since PD is a multifactorial disease and majority of PD cases are sporadic, it is definitely challenging to find the cure to completely treat this disease.

As previously mentioned, PD is often linked to motor impairments, hence the main pathology of PD is progressive deterioration of movement ability. Individuals diagnosed with PD are usually presented with symptoms such as resting tremor (tremor during relaxation), bradykinesia (slowness of movement), dyskinesia (uncontrolled, involuntary movements), postural imbalance, and muscular stiffness (Moustafa *et al.*, 2019; Cilia *et al.*, 2020). Besides

motor symptoms, PD patients can also develop non-motor symptoms, which typically involve psychological and cognitive functioning, for example, executive dysfunction, working memory impairment, sleep dysregulation and psychiatric disturbances (Hermanowicz *et al.*, 2019; Peball *et al.*, 2020). Having said that, as this disease progresses, PD does not only restrict locomotor activity but it also influences mental health, all of which can severely affect the quality of life.

Despite extensive studies, there is still no available treatment that can cure PD completely. Current management involve giving patients medications that can only elevate motor symptoms without actually curing it. The most popular medication for PD is levodopa, a dopamine precursor that helps in replenishing dopamine population in the brain. Levodopa improves motor functions and slows down the progression of PD but at the same time elicit adverse side effects to the patients. When the disease progresses and patients need higher dose of levodopa to elevate tremors, this increases the chance of them experiencing hallucinations and delusions (Chen, 2017). Few other available PD medications include dopamine agonists and monoamine oxidases (MAO) inhibitors, which are also used to restore dopamine level and dopaminergic neurotransmission in the brain (Krishna *et al.*¸ 2014; Hajj, 2018). In some cases where patients respond poorly to medications, it is suggestive to do deep brain stimulation (DBS, if they are deemed fit) in which abnormal motor signals are interrupted by external electrical impulses (Hickey & Stacy, 2016; Sveinbjornsdottir, 2016). However, similar to other medications, DBS can only alleviate uncontrolled tremors without actually curing the dopamine loss.

In view of all that has been mentioned so far, accumulating evidences understood the underlying pathophysiological mechanisms of PD, which include neuroinflammation, mitochondrial dysfunctions in dopaminergic neurons, α-synuclein aggregations and autophagy

disturbances, however, the molecular changes in each of these pathophysiological perturbations are still largely inclusive.

2.2. High-mobility group box 1 (HMGB1) protein

Several studies thus far have linked chronic neuroinflammation with PD development (Sasaki *et al.*, 2016; Huang *et al.*, 2017; Angelopoulou *et al.*, 2018). High-mobility group box 1 protein is a DNA-binding nuclear protein that is actively secreted following cytokine stimulation and passively released during cell death or insult (Angelopoulou *et al.*, 2018). In normal condition, HMGB1 is expressed inside the nucleus of a cell playing its role as architectural binding factor and transcription regulator. However, in a condition where cells are damaged or insulted, HMGB1 is released as DAMPs from the nucleus into the cytoplasm and subsequently to the extracellular matrix. HMGB1 acts as alarmin, a danger signal capable of stimulating the immune system to elicit inflammatory response (Frank *et al.*, 2015). It has been demonstrated that following an immune response, HMGB1 can be secreted by degenerating or damaged neurons, as well as by various immune cells such as macrophages, monocytes, and natural killer (NK) cells (Yang *et al.*, 2013). Overexpression of HMGB1 results in uncontrolled neuroinflammation, that will eventually lead to cell damage and cell death.

Analysis on post-mortem human PD brain indicated that HMGB1 level is upregulated in the substantia nigra as well as the cerebrospinal fluid and serum (Santoro *et al.*, 2016). Following PD development, dysfunctions of several mechanisms such as mitochondrial respiratory chain and protein autophagy as previously mentioned, cause continuous neuronal insults, thus, more HMGB1 proteins are being secreted from inflammatory cells to perform its function as alarmin. When this happens over a prolonged time, it will result in chronic neuroinflammation, which will potentially worsen the disease even more. Gao *et al.* (2011) investigated on inflammation-induced PD when dopaminergic neurons are co-cultured with

microglial cells. The study revealed that the presence of microglial cells with dopaminergic neuronal culture upregulates nuclear factor-κB (NFκB) to stimulate downstream inflammatory response and trigger neurodegeneration. Interestingly, such observations were not seen when HMGB1 activity is neutralized, which may suggest probable involvement of HMGB1 protein in the heightened risk of developing PD (Gao *et al.*, 2011).

In a more current study, Lee *et al.* (2014) in their thorough analysis were able to show that HMGB1 provokes microglia-mediated neuroinflammation and suppression of this protein exerts neuroprotective effect on the neurons. Consistent with above findings, neutralization of HMGB1 expression inhibits dopaminergic neuronal loss in an MPTP-induced rodent model of PD (Santoro *et al.*, 2016). Collectively, there seems to be some evidence to indicate that although beneficial at some level, HMGB1 plays a detrimental role in the immune response that may lead to the progression of PD. Therefore, this protein represents a promising target in the future treatment of PD that needs to be carefully investigated. Whilst some research has been carried out on the mediation of HMGB1 on neuroinflammatory process, the mechanism by which it is involved in PD development has not yet been well established.

2.3. MPTP-induced zebrafish as PD model

The past three to four decades have witnessed astounding development on zebrafish application in scientific research. This species is an ideal model for the study of the nervous system due to its conserved homology and synteny with human (Barbazuk *et al.*, 2000; Howe *et al.*¸2013). Ever since its establishment as a model organism in late 1900s, zebrafish has been used in various research on neurodegenerative diseases, including PD, Alzheimer's disease, and Huntington's disease (Freeman *et al.*, 2007; Eisen, 2020). Parkinson's disease affects the production of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and striatum. In zebrafish, the equivalences of these two regions are posterior tuberculum of ventral

diencephalon (vDn) and ventral telencephalon (vTn), respectively (Figure 1) (Correia *et al.*, 2017; Vijayanathan *et al.*, 2017).

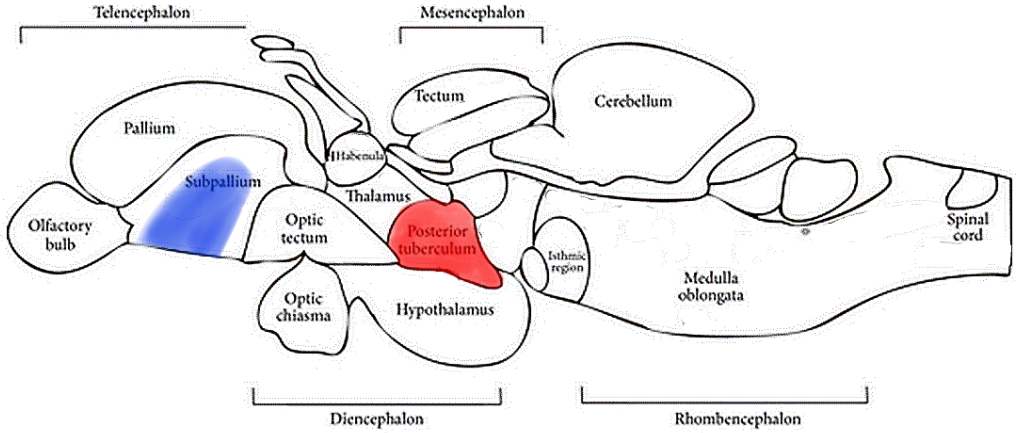


Figure 1 Illustration of the zebrafish brain in sagittal view. Posterior tuberculum of ventral diencephalon (red) and ventral telencephalon (blue) are equivalent to human SNpc and striatum, respectively (adapted from Toledo-Ibarra *et al.*, 2013).

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is the most widely used neurotoxin to simulate PD in zebrafish. This compound is a dopaminergic neurotoxin that was accidentally discovered in 1980s when a number of drug users developed acute PD-like symptoms upon injecting themselves with MPTP-containing heroin (Stepens & Taba, 2016; Langston, 2017). It was then found that besides human, MPTP is able to trigger parkinsonism in other animal species like primates, rodents, and zebrafish as well (Vaz *et al.*, 2018). Animal models of MPTP-induced PD were then developed to meet research demand of studying PD.

As noted by Barnhill *et al.* (2020), zebrafish metabolizes MPTP in a similar way as human. MPTP easily crosses the blood brain barrier (BBB) into the CNS and is taken up by glial cells (Zeng *et al.*, 2018). Inside the glial cells, MPTP is metabolized by MAO B enzyme to its active form, 1-methyl-4-phenylpyridinium (MPP+) (Bajpai *et al.*, 2013; Robea *et al.*, 2020). Structurally, MPP+ is analogous to dopamine (Fernagut, 2016), hence, it is readily

transported into dopaminergic neurons by dopamine transporter (DAT) that is located at the presynaptic membrane. MPP+ inside the neurons is toxic as it concentrates around mitochondria and disturbs mitochondrial respiratory chain by inhibiting the action of mitochondrial complex I enzyme (Zawada *et al.*, 2011). This action then results in mitochondrial dysfunction and oxidative stress (Perier & Vila, 2012, Robea *et al.*, 2020), which consequently leads to dopaminergic neuronal death, closely mimicking PD pathology.

Following MPTP administration, adult zebrafish displays behavioural alterations such as reduced swimming speed and aberrant swimming behaviour, which are reflected as bradykinesia in humans. In addition, MPTP-induced zebrafish also exhibit increased freezing bouts and poor response to tactile stimuli (Lam *et al.*, 2005; Wasel & Freeman, 2020), indicating significant reduction in locomotor activities and sensory deficit. Weakened touch sensory was also seen following neurotoxin administration in rodent model of PD, which is thought to be influenced by striatal dysfunction due to reduction of striatal dopamine population (Ketzef *et al.*, 2017). Based on the abovementioned findings, the genetic similarity of zebrafish to human and its relatable behaviours have conclusively been shown that MPTP-induced zebrafish is a genetically and phenotypically ideal model to be used in investigating PD pathology in both molecular and behavioural levels.

2.4. Significance of current study

Despite extensive research has been carried out investigating the hallmarks of PD pathophysiology, most studies in this area have only focused on the pathology and possible aetiology that lead to the neuro-behavioural alterations. However, there is still very little scientific understanding pertaining to the molecular mechanism of actions that influence the observed pathology and changes. Since chronic neuroinflammation is one of the hallmarks of PD pathogenesis and HMGB1 is believed to be involved in mediating inflammatory response,

hence, investigating the role of HMGB1 and what lies in between HMGB1/neuroinflammation activation pathway and PD development are highly reasonable to be put as major discussion.

Therefore, the main aim of this study is to evaluate the role of HMGB1 protein in the development of PD, especially in terms of motility disorders, using MPTP-induced zebrafish as model organism. At the end of this study, it is hoped that the functional role of HMGB1 in mediating PD development can be well evaluated. By providing experimental evidence on the role imposed by HMGB1 in inflammatory response mechanism, knowledge gap between chronic neuroinflammation and PD pathogenesis can be eliminated. In longer term, future studies can benefit from this finding in the quest of uncovering promising treatment or discovering drugs that may improve motility disorders and potentially alleviate PD completely.

3. METHODOLOGY

3.1. Animal subject and husbandry

Adult wild type AB zebrafish (*Danio rerio*) both male and female with body length of approximately 3-4 cm will be used in this study. All zebrafish will be bred and housed in 9 litre acrylic tanks located at the Central Research and Animal Facility (CREAM), International Islamic University Malaysia (Kuantan Campus), Pahang, Malaysia. Each tank will consist of 30-35 adult zebrafish with mixed sex groups and they will be fed with dry feed diet (Zeigler Bros Inc, PA, USA) twice per day. The water inside the tanks will be filtered and equipped with air pump to ensure sufficient oxygenation, and the housing systems will be kept maintained at 26-28°C and pH level of 6.5-7.5, with 14:10h light and dark cycle (Benchoula *et al.*, 2019; Murugesu *et al.*, 2019).

Prior to the start of the experiment, all zebrafish will be monitored and maintained in standard conditions to ensure that they are in good health and are not distressed. Zebrafish exhibits shoaling behaviour and aggression in stressful condition (Aleström *et al.*, 2020;

Kleinhappel *et al.*, 2019). Any stress inducers will be avoided by minimizing changes in water composition and quality (such as temperature, salinity, pH level and oxygen content) as well as light intensity. All considerations will be taken to minimize zebrafish suffering and number of zebrafish necessary.

3.2. Experimental design

(Corresponding to Specific Objective 1)

3.2.1. Induction of MPTP on zebrafish

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin compound capable of inducing PD-like symptoms in zebrafish. Powdered MPTP hydrochloride manufactured by Sigma-Aldrich, MO, USA (Cat# M0896) will be purchased and a fresh working solution (10µg/µL) will be prepared by dissolving the powder in sterile water (Sarath Babu *et al.*, 2016). Two doses of 50µg MPTP working solution will be injected into the zebrafish via intraperitoneal injection (i/p) with 24h interval between doses (total MPTP dose will be 100µg). According to Sarath Babu *et al.* (2016), one dose of 50µg per day is adequate to exert parkinsonism effect and does not cause lethality to zebrafish. Sterile water will be administered to control group. Prior to the i/p injection, zebrafish will be anesthetized in 0.1% tricaine solution. The entire procedure will be done accordingly and zebrafish will be monitored upon recovery for any side effects or injuries.

3.2.2. Validation of MPTP-induced zebrafish model

The validation procedure will be done following the methods described by Sarath Babu *et al.* (2016), which include behavioural, gene expression (real-time-qPCR), and protein (western blotting) analyses.

3.2.2.1. Behavioural analysis

After 24h following MPTP induction, zebrafish will be transferred into a transparent 3 litre tank to observe their behaviour. Cameras will be mounted on top and in front of the tank to record parameters such as freezing duration (in sec), number of freezing bouts, distance travelled (in mm), and swimming speed (in mm/s). Each zebrafish will be allowed to swim in the tank for 2 mins and its behaviour will be recorded throughout the swimming period (Sarath Babu *et al.*, 2016). Recorded videos will be evaluated and measured using EthoVision software (Noldus Information Technology, NL). All parameters will be statistically analysed to compare the behaviours between MPTP-induced and control groups.

3.2.2.2. Gene expression analysis using RT-qPCR

RT-qPCR assay will be conducted to quantify and compare targeted gene expressions between control and MPTP-induced zebrafish. Zebrafish will be anesthetized with 0.1% tricaine solution prior to harvesting the brain. Harvested brain tissues from MPTP-induced and control groups will be rinsed with isotonic solution and prepared for total RNA extraction using TRIzol reagent (Invitrogen, CA, USA; Cat# 15596026). Subsequently, cDNA synthesis will be performed using reverse transcriptase following manufacturer's protocol (SuperScript III; Invitrogen, CA, USA; Cat# 11732020). RT-qPCR will be conducted using specific primers (Table 1) to analyse several PD-associated genes including *lrrk2*, *park2*, *park7*, *sncga* and *sncgb* genes. β-actin and GAPDH genes will be used as internal controls. Quantitative comparison will be made between MPTP-induced and control groups to estimate relative expression of the selected genes.

Table 1 Forward and reverse primers for each target gene.

No	Target gene	Forward primer	Reverse primer
1.	*lrrk2* (Leucine-rich repeat kinase 2)	ACTCGGATTAAGTTCCACCAGA	CAGTGAGGGTTGATGGTCTGTA
2.	*park2* (Parkin RBR E3 ubiquitin protein ligase)	ACAGACATCATGACTCCAGTGC	ACACGGAAATGATGAACCTCTT
3.	*park7* (Parkinson protein 7, DJ-1)	GAAAGAGGTGTTGAAGGACCAG	TGATCACGTTACCATCCTTCTG
4.	*sncga* (Synuclein gamma a)	ATGCACTGAAGAAGGGATTCTC	AGATTTGCCTGGTCAGTTGTTT
5.	*sncgb* (Synuclein gamma b)	GACTAAAGCTGGGGTTGAAGAG	CGTTCTCCAGTCCCTCTACTGT
6.	β-actin	GCCGGGACCTGACTGACTAC	TTCTCCTTAATGTCACGCACGAT
7.	GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	ACCCACTCCTCCACCTTTGAC	TGTTGCTGTAGCCAAATTCGTT

3.2.2.3. Protein expression analysis using western blotting

Western blotting analysis will be performed to validate and compare the expression of targeted proteins between control and MPTP-induced zebrafish. Total proteins will be extracted from the pooled brain tissue samples using radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, MA, USA; Cat# 89900) with sonication for homogenization. Extracted total proteins will be quantified using bicinchoninic acid assay (BSA assay) before proceeding to the next step.

50µg of the total protein from each group will be gel electrophoresed in SDS-PAGE and consequently transferred to polyvinylidene fluoride (PVDF) membrane via electroblotting. Following that, membrane will be incubated with the following primary antibodies: anti-α-synuclein antibody (1:1000; Cell Signaling Technology, MA, USA; Cat# 4179), anti-tyrosine hydroxylase antibody (TH; 1:1000; Merck Millipore, MA, USA; Cat# MAB318), anti-HMGB1 antibody (1:500; Abcam, UK; Cat# ab233236), anti-Iba1 antibody (1:100; Wako Pure Chemicals Industries, Germany; Cat# 019-19741) and anti-NFκB p65 antibody (1:1000; Novus

Biologicals, CO, USA; Cat# NBP2-67352) at respective optimum dilution for 1 to 2 hours at room temperature. β-tubulin expression will be used as internal control. Next, the membrane will be incubated with their respective HRP-conjugated secondary antibody and processed following manufacturer's protocol for the chemiluminescent detection and quantification.

(Corresponding to Objective 2)

3.2.3. HMGB1 knockdown using morpholino oligonucleotides

Morpholino oligonucleotides (MOs) are synthetic oligonucleotides used to inhibit RNA transcripts from being translated to proteins (Yuan *et al.*, 2013). HMGB1 anti-sense MOs (5'-GATCCTTCCCCATCTTTGCCTAAAT-3') will be utilized in this study to knockdown the expression of HMGB1 protein. In addition, a standard control MOs (5'-CCTCTTACCTCAGTTACAATTTATA-3') will be used as negative control (Fang *et al.*, 2014).

Zebrafish will be first anesthetized with 0.1% tricaine solution. Then, they will be carefully fixed with forceps and a small incision will be created into the skull using a needle. Zebrafish will be microinjected with 1µl of aliquot containing 500µM morpholino solution mixed with fluorescent dye (for confirmation of MOs delivery) using a microinjection capillary oriented towards the ventral diencephalon (vDn). To reach the target region, the microcapillary will be set to an injection angle and depth of 60° and 1200µm, respectively (Vijayanathan *et al.*, 2017).

Validation process will be done through western blotting (to quantify HMGB1 protein expression) and immunohistochemistry (to validate the location of HMGB1 knockdown) analyses. 12 hours after confirmation, zebrafish will be intraperitoneally injected with MPTP

neurotoxin to induce PD following the procedures explained earlier (Section 3.2.1). Zebrafish will be monitored post injection for any surgical side effects or injuries.

(Corresponding to Objective 3)

3.2.4. Analyses on the effects of HMGB1 knockdown on MPTP-induced zebrafish

Following the knockdown of HMGB1 and induction of MPTP neurotoxin, analyses will be conducted to investigate the effects of HMGB1 knockdown on MPTP-induced zebrafish. Similar analyses will be performed as in Section 3.2.2, which include behavioural, gene expression, and protein expression analyses, the only difference will be, for this section onwards, another group will be added (HMGB1 knocked down group), making a total of three experimental groups, and an additional immunohistochemistry analysis will be conducted as well.

3.2.4.1. Behavioural analysis

The procedures for behavioural analyses will be performed similar to Section 3.2.2.1. All parameters will be analysed statistically to compare the behaviours between normal MPTP-induced, HMGB1-deficient and control groups.

3.2.4.2. Gene expression analysis using RT-qPCR

The procedures for gene expression analysis will be performed similar to Section 3.2.2.2. Comparisons will be made on the targeted gene expressions between normal MPTP-induced, HMGB1-deficient and control groups.

3.2.4.3. Protein expression analysis using western blotting

The procedures for protein expression analysis will be performed similar to Section 3.2.2.3. Comparisons will be made on targeted protein expressions between normal MPTP-induced, HMGB1-deficient and control groups.

3.2.4.4. Immunohistochemistry analysis

Zebrafish will be anesthetized in 0.1% tricaine and fixed in 4% paraformaldehyde (PFA) for 2 days in 4°C (Sarath Babu *et al.*, 2016). After that, zebrafish brain will be harvested and stored in phosphate buffered saline (PBS). Brain tissues will be processed and embedded in paraffin wax. Sliced brain tissue sections will be immunostained following standardized in-lab protocols (Sathe *et al.*, 2012).

Samples will be incubated separately with the following primary antibodies: anti-HMGB1 antibody (1:1000; Abcam, UK; Cat# ab233236), anti-glial fibrillary acidic protein antibody (GFAP; 1:1000; Dako, CA, USA; Cat# Z0334), anti-TH antibody (1:100; Merck Millipore, MA, USA; Cat# MAB318), and anti-Iba1 antibody (1:100; Wako Pure Chemicals Industries, Germany; Cat# 019-19741). Correspondingly, anti-rabbit (Cy5-AffiniPure; 1:500; Jackson ImmunoResearch Laboratories, UK; Cat# 111-175-144) or anti-mouse (Alexa Fluor 488; 1:1000; Thermo Fisher Scientific, MA, USA; Cat# A-11001) fluorescein-conjugated secondary antibodies will be added for visualization using confocal microscopy.

Immunohistochemistry will be carried out as described and the sections will be counterstained with Nissl (Thionin; Sigma-Aldrich, MO, USA; Cat# 861340). HMGB1-, TH-, GFAP-, Iba1-, and Nissl- positive cells in the ventral diencephalon (zebrafish equivalence of human SNpc) will be counted by blinded examiners using optical fractionator method (Sathe *et al.*, 2012). Ventral telencephalic density of TH immunoreactivity will be measured as described (Sathe *et al.*, 2012). For double immunofluorescence staining of microglia, sections will be incubated with isolectin B4 conjugated with DyLight 594 (1:200; Vector Laboratories, CA, USA; Cat# DL-1207) (Lai *et al.*, 2017). The relative expression of each protein in two regions of interest, vDn and vTn, will be compared between groups using Fiji version of ImageJ 1.52p software (Java 1.8.0_172) (NIH, Bethesda, MD) (Sarath Babu *et al.*, 2016).

3.3. Statistical analyses

The obtained results from all analyses will be evaluated statistically using Prism 7.00 for Windows (GraphPad Software, CA, USA). The data will be tabulated and represented as mean (SEM). The statistical significance of the data will be analysed through parametric independent t-test (for two experimental groups) or one-way ANOVA with post-hoc Tukey's test (for three experimental groups). P-values of less than 0.05 ($p < 0.05$) will be considered as significant for all statistical analyses. All results from this study will be tabulated and/or graphically plotted.

3.4. Sample size calculation and groupings

A total of 94 zebrafish will be used in this study (47 males and 47 females), with consideration of 20% dropouts. Reasons of considering 20% dropouts is due to possible performance outliers and potential adverse events during surgical procedures (MPTP induction and HMGB1 knockdown).

The sample size of this study is calculated using G*Power software Version 3.1.9.4. (Germany). As this study will be using two types of statistical analyses, two sample sizes are calculated with reference to past literatures. With a 90% power, 5% significance level and an effect size of 1.5, the sample sizes required are listed in Table 2. Detailed calculation from G*Power software is attached as Appendix 1.

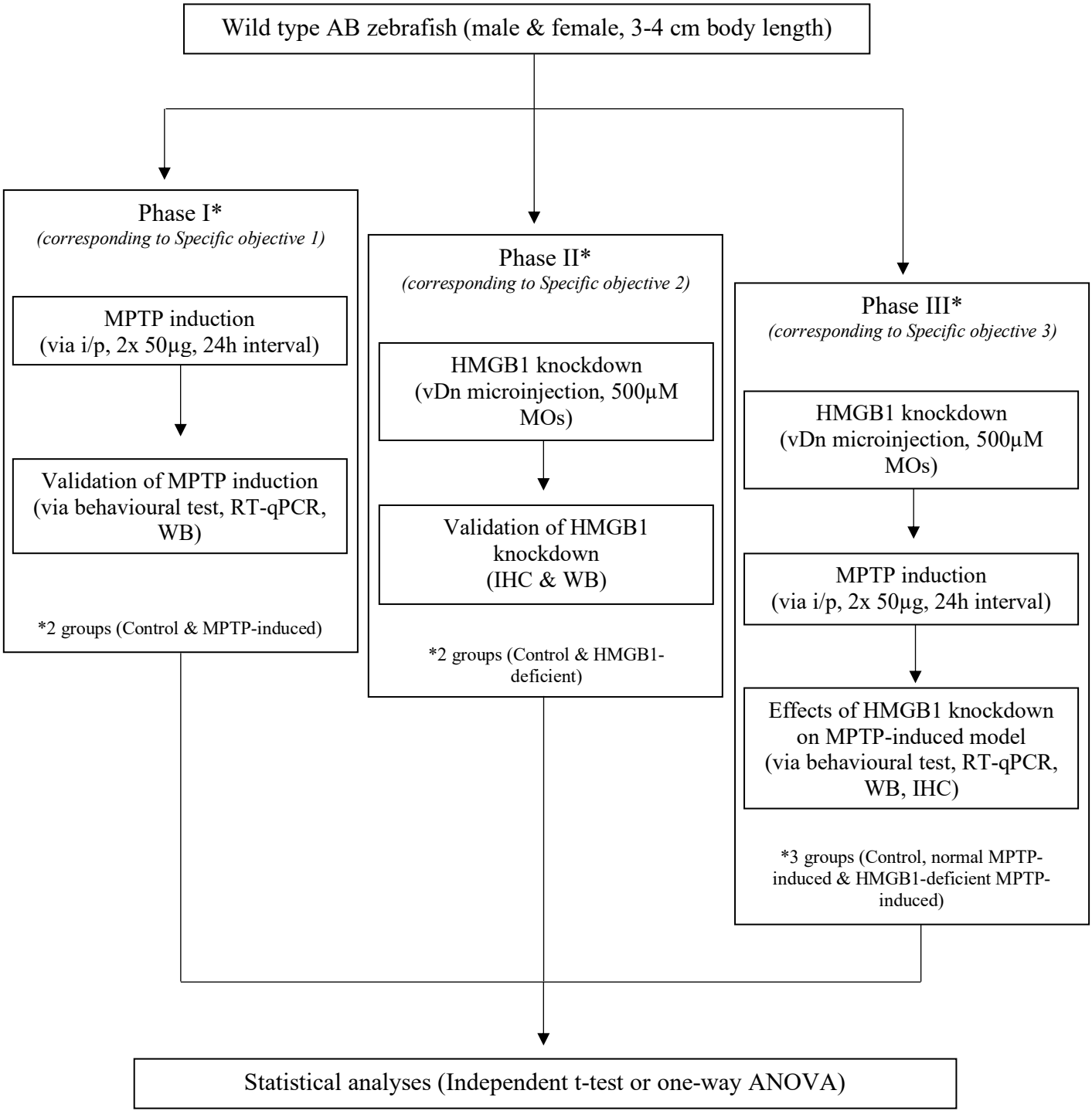
Based on Table 2, to evaluate Specific Objective 1, zebrafish will be randomly grouped into two groups: 1) Control, and 2) MPTP-induced groups (n = 12). Likewise, for Specific Objective 2, zebrafish will be randomly grouped into two groups: 1) Control, and HMGB1-deficient groups (n = 12), whereas for Specific Objective 3, zebrafish will be randomly allocated to three groups: 1) Control, 2) normal MPTP-induced, and 3) HMGB1-deficient groups (n = 10).

Table 2 Sample size required for each statistical analysis.

Specific objective	Statistical analysis	[a]Overall sample size (*n* per group)	[b]Required sample size (*n* per group)	Reference
To establish and validate zebrafish model of PD via intraperitoneal injection of MPTP neurotoxin	Independent t-test	22 (11 per group)	24 (12 per group)	(Sarath Babu *et al.*, 2016)
To knockdown the expression of HMGB1 in the neurons of zebrafish brain using anti-sense HMGB1 morpholino oligonucleotides.	Independent t-test	22 (11 per group)	24 (12 per group)	(Zhao *et al.*, 2011)
To evaluate the effect of HMGB1 knockdown at behavioural, histopathological, and molecular levels.	One-way ANOVA	12 (4 per group)	30 (10 per group)	(Sarath Babu *et al.*, 2016)

[a] Overall sample size is the number of zebrafish needed as calculated by G*Power software.
[b] Required sample size is the number of zebrafish required to satisfy all analyses (behaviour, RT-qPCR, WB and/or IHC) in a particular Specific Objective.

3.5. Flowchart of methodology



4. EXPECTED RESULTS

4.1. Validation of MPTP-induced zebrafish model of PD

4.1.1. Behavioural analysis

Graphs representing the performance of the two groups (control and MPTP-induced groups) in behavioural test [parameters: freezing duration (in sec), number of freezing bouts, travelled distance (in mm) and swimming speed (in mm/s)] will be plotted.

It is expected that MPTP-induced group will have significantly longer freezing duration, higher freezing frequency, shorter travelled distance, and slower swimming speed compared to normal group.

4.1.2. Gene expression analysis

A graph representing the relative expression of target genes (*lrrk2*, *park2*, *park7*, *sncga* and *sncgb* genes) obtained from RT-qPCR will be plotted comparing MPTP-induced and control groups.

It is expected that MPTP-induced group will have significantly higher expression levels of all target genes compared to control group.

4.1.3. Protein expression analysis

A graph representing the relative expression of target proteins (SNCGA/B, TH, HMGB1, Iba1 and NFκB p65 proteins) obtained from WB will be plotted comparing MPTP-induced and control groups.

It is expected that MPTP-induced group will have significantly higher expression levels of SNCGA/B, HMGB1, Iba1 and NFκB p65 proteins and lower level of TH proteins compared to control group.

4.2. Validation of HMGB1-knockdown zebrafish

4.2.1. Western blot and immunohistochemistry analyses

Graphs representing the expression level of HMGB1 protein obtained from WB and IHC will be plotted comparing HMGB1-deficient and control groups.

It is expected that the expression level of HMGB1 protein will be significantly lowered down than normal level following the knockdown, specifically at the targeted region (ventral diencephalon, vDn).

4.3. Effects of HMGB1-knockdown on MPTP-induced zebrafish model of PD

4.3.1. Behavioural analysis

Graphs representing the performance of the three groups (control, MPTP-induced and HMGB1-deficient groups) in behavioural test [parameters: freezing duration (in sec), number of freezing bouts, travelled distance (in mm) and swimming speed (in mm/s)] will be plotted.

It is expected that MPTP-induced group will have significantly longer freezing duration, higher freezing frequency, shorter travelled distance, and slower swimming speed compared to control group, while knockdown of HMGB1 will restore the performance back to normal.

4.3.2. Gene expression analysis

A graph representing the relative expression of target genes (*lrrk2, park2, park7, sncga* and *sncgb* genes) obtained from RT-qPCR will be plotted comparing control, MPTP-induced and HMGB1-deficient groups.

It is expected that MPTP-induced group will have significantly higher expression levels of all target genes compared to control group, and HMGB1-knockdown will restore the level back to normal.

4.3.3. Protein expression analysis

A graph representing the relative expression of target proteins (SNCGA/B, TH, HMGB1, Iba1 and NFκB p65 proteins) obtained from WB will be plotted comparing MPTP-induced and control groups.

It is expected that MPTP-induced group will have significantly higher expression levels of SNCGA/B, HMGB1, Iba1 and NFκB p65 proteins and lower level of TH proteins compared to control group, while HMGB1-deficient group will have similar expression levels as control.

4.3.4. Immunohistochemistry analysis

Graphs representing the expression level of target proteins (HMGB1, GFAP, Iba1, and TH) in specific regions (vDn and vTn) will be plotted comparing the three groups.

It is expected that MPTP-induced group will have significantly higher expression level of HMGB1, GFAP and Iba1, and lower level of TH proteins than control group in both vDn and vTn, while knockdown of HMGB1 will revert the expressions back to normal level.

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6. GANTT CHART AND MILESTONES

6.1. Gantt chart

Year	2020		2021												2022							
Activity/month	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A
Proposal defence and ethics approval																						
Breeding and maintenance of zebrafish																						
Induction of MPTP via i/p injection																						
Validation of MPTP-induced model																						
HMGB1 knockdown via MOs microinjection																						
Validation of HMGB1-deficient model																						
Induction of MPTP on HMGB1-knocked down model																						
Analyses on the effects of HMGB1 knockdown on MPTP-induced model																						
Statistical analyses and report writing																						

6.2. Milestones

Completed task	Completion date	Percentage of completion (%)
Induced MPTP on zebrafish via i/p injection	31st May 2021	10%
Validated MPTP-induced zebrafish model	31st July 2021	20%
Knocked down HMGB1 on zebrafish via MOs microinjection	30th September 2021	30%
Validated HMGB1-deficient zebrafish model	30th November 2021	40%
Induced MPTP on HMGB1-deficient zebrafish model	28th February 2022	50%
Analysed the effects of HMGB1 knockdown on MPTP-induced zebrafish model	30th June 2022	70%
Completion of statistical analyses and report writing	31st August 2022	100%

APPENDIX 1

a)

Thursday, November 26, 2020 -- 11:05:53

t tests - Means: Difference between two independent means (two groups)

Analysis:	A priori: Compute required sample size		
Input:	Tail(s)	=	Two
	Effect size d	=	1.5
	α err prob	=	0.05
	Power (1-β err prob)	=	0.9
	Allocation ratio N2/N1	=	1
Output:	Noncentrality parameter δ	=	3.5178118
	Critical t	=	2.0859634
	Df	=	20
	Sample size group 1	=	11
	Sample size group 2	=	11
	Total sample size	=	22
	Actual power	=	0.9168991

b)

Thursday, November 26, 2020 -- 11:05:38

F tests - ANOVA: Fixed effects, omnibus, one-way

Analysis:	A priori: Compute required sample size		
Input:	Effect size f	=	1.5
	α err prob	=	0.05
	Power (1-β err prob)	=	0.9
	Number of groups	=	3
Output:	Noncentrality parameter λ	=	27.0000000
	Critical F	=	4.2564947
	Numerator df	=	2
	Denominator df	=	9
	Total sample size	=	12
	Actual power	=	0.9785871

Sample size for each statistical method as calculated by G*Power software. a) independent t-test for Specific Objective 1 and 2, b) One-way ANOVA for Specific Objective 3.