COMPARATIVE ANALYSIS OF DRUG INDUCED PARKINSONISM LIKE BEHAVIORS: THE STUDY OF RODENT SPECIES EFFECT USING A FORCE PLATE ACTIMETER

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ABSTRACT

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that mainly affects the motor ability of body. Various agents have been used to study PD for *in vivo* model with the hope to enlighten the pathogenesis and treatment strategies of the disease. The aim of this study is to quantitatively analyse the Parkinsonism characteristics induced by MPTP, tacrine, and rotenone in rodents (mice and rats) and compare their behaviors. 15 and 30mg/kg MPTP were administered intraperitonealy to induce typical motor parkinsonism features such as tremor, rigidity and bradykinesia in mice whereas 2.5 mg/kg rotenone was injected subcutaneously to produce the same behavior in rats. Involuntary lateral movement of jaw is another symptom exhibited by the patients of PD. 5mg/kg tacrine was administered intraperitonealy to both mice and rats to induce lateral movement of jaws. Rodents were kept inside force plate actimeter (FPA) for behavioral quantification after neurotoxin induction. FPA is a modern technological device used to study neurological behaviors of small animals under the influence of toxins inducing neurological problems. All of the neurotoxins used in this study (MPTP, tacrine, and rotenone) significantly induced bradykinesia and reduced locomotion in both mice and rats, as compared with control group. Oral treatment of rodents with 10mg/kg Sinemet® (levodopa: carbidopa 4:1) improved their motor ability. Power spectra analysis revealed that in mice MPTP induced tremor and rigidity at frequency of 7-12 Hz and rotenone produced the same behavior at the frequency of 0.5-2 Hz and 4-12 Hz. Intraperitoneal administration of tacrine to mice generated one significant peak at 10-12Hz while the peak in rats was at 0.5-3 Hz. Oral administration of 10mg/kg Sinemet® lowered power intensity of neurotoxin-induced force peaks, indicating the antagonistic effect of Sinemet® in neurotoxin-induced Parkinsonian symptoms. This model is possibly useful to study anti-parkinsonian potency of newly discovered drugs.

KEY WORDS: PARKINSON’S DISEASE / MPTP / TACRINE / ROTENONE / FPA

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
BLM	Bout of Low Mobility
ELISA	Enzyme-linked Immunosorbent Assay
FPA	Force Plate Actimeter
hr	hour
Hz	Hertz
i.p.	Intraperitoneal
MPTP	1-methyl, 4-phenyl 1,2,3,6 tetrahydropyrolidine
mm	millimeter
NSS	Normal Saline Solution
PBS	Phosphate Buffer Solution
PD	Parkinson’s disease
s.c.	Subcutaneous

CHAPTER I

INTRODUCTION

Parkinson’s disease (PD) is a complex neurodegenerative disorder that mostly affectselderly population. It is characterized by movement related disorders such as resting tremor predominating only one side of body, bradykinesia, muscle stiffness, postural instability**(1-2)**. Apart from these, patients with PD also experience the difficulty in swallowing, reduction of eye blinking and involuntary jaw movement **(1,3-4)**.PD is the second most common neurodegenerative disorder after Alzheimer's disease and is thought to be more common in men than in women **(3,5)**.

In spite of being common, the etiology of PD is not completely understood, but many researchers believe that it may be due to the combination of various genetics and environmental factors. Cumulative incidence of PD revealed that individuals with PD affected parents or siblings have higher risk (about 4-9%) of getting Parkinson’s disease as compared to general population (extra).

PD is highly specific to neuromelanin containing dopaminergic neurons inSubstantianigra pars compacta (Snpc), a structure of brain that is found deep within the midbrain (mesencephalon) **(6-7)**.It is one of the major parts of brain where the synthesis of dopamine, a neurotransmitter, is initiated. Nigrostriatal pathway is the dopaminergic pathway that connects Snpc to striatum. It has a major role in controlling smooth movement of body and muscle coordination under normal physiological condition **(8)**.Currently, it is clear that PD is resulted from the degeneration of dopaminergic neurons. The specific pathway leading to degeneration is still unclear but post mortem study of PD brain has suggested that the combination of mitochondrial stress and oxidative damage would be partly responsible for the development of this disease**(2-5)**. Neuronal function mediated by dopamine is disturbed and the connection between Snpc and striatum becomes irregular leading to the difficulty in movement and perform ordinary daily work (**8**).

Abnormal mutation of α-synuclien is frequently associated with protein aggregation and formation of Lewy body (LB) **(9)**. LB is the pathological hallmark of PD mostly found in the dopaminergic neurons of Snpc and striatum. Formation of LB is linked with nigrostriatal degeneration **(9-10)**.

MAO-B is an enzyme involved in metabolism of endogenous dopamine by the process of oxidative deamination **(11)**. As the age of individual is increased, the level of MAO-B in brain is also increased that may lead to oxidative stress and formation of highly toxic oxygen free radicals **(12-13)**. Under normal physiological condition, dopamine from presynaptic terminal is released to synaptic cleft where it is rapidly reuptake by dopamine transporters **(11-13)**. In case of PD, most of the presynaptic terminals are degenerated and the dopamine level is decreased but the level of MAO-B is high. Thus, inhibition of MAO-B in PD brain could be an effective target for the treatment of PD **(12)**.

Regarding to the current management of PD, after years of its clinical use, levodopa still remains the gold standard for the treatment of PD **(22)**. As compared to other drugs used in PD therapy, it has the maximum efficacy in symptomatic relief of motor related disorders of PD. Levodopa is the natural precursor of dopamine and is generally administered orally. Since it can easily cross the blood brain barrier, it is used as an effective agent to increase the level of dopamine inside the brain. It is commonly given in combination with carbidopa or benserazide to prevent peripheral decarboxylation of levodopa **(22-23).** Sinemet® (levodopa + carbidopa in the ratio of 4:1) is commercially found as tablet and is widely used as the standard treatment of PD.

Despite of the fact that there are many medications available to treat PD, none of them can reverse or cure PD. They can only provide symptomatic relief of motor and non-motor symptoms associated with PD such as depression, anxiety, sleep disorders, and dementia **(26)**. Long term use of dopamine replacement therapy is associated with side effects such as rapid and involuntary jerking of muscles and increased addiction of dopamine. Therefore, a new class of drugs that can improve the motor effects and also reverse symptoms resulted from long term use of dopamine replacement therapy is still the subject of interest in the field of neurology.

Use of animal model is an important approach to study experimental medical science because it provides us a close idea about the mechanism, possible etiology, and therapeutic principles of treating human diseases **(62)**. Monitoring behavior of animals is an essential tool to screen the effectiveness and safety of the potential new drugs. Administration of neurotoxic agents such astacrine,1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), and rotenonehas been shown to induce parkinsonism like symptoms in rodents.

Tacrine (9-amino-1, 2, 3, 4-tetrahydroaminoacridine hydrochloride hydrate) belongs to parasympathomimetics and acetyl-cholinesterase inhibitor group. It induces extrapyramidal motor side effects identical to tremor, bradykinesia and rigidity in humans **(16-17)**. Animal studies have shown that drugs that prolong the action of acetylcholine can induce different movement of facial muscles with lateral movement of jaw being the most common. The vertical deflection of jaw is not stimulated by any factor and is purposeless in nature **(17-18).**

MPTP is a lipid soluble neurotoxin that exhibits its lethal action by activating mitochondria dependent apoptotic molecular mechanisms. It is highly selective to dopaminergic neurons of Snpc .The biochemical changes that occur after MPTP administration is quite similar to idiopathic PD. Since its discovery in the early 1980’s, it has been used successfully to design animal model of PD in laboratory **(14-15)**. It can induce rigidity, tremor, akinesia, and posture alternation which are symptoms of Parkinsonism.

Rotenone is naturally available in roots and stem of herbal species belonging to *Lonchocarpus* or *Derris*. It fits to the family of naturally occurring cytotoxic compounds, and is used as broad spectrum pesticides and herbicides in commercial use **(19)**. It can freely cross cell and mitochondrial membrane.Epidemiological studies have shown that people who are highly exposed to herbicides or pesticides have a high risk of PD **(20)**. Similar to MPTP, it acts as complex I inhibitor of mitochondria and mimics the entire Parkinsonian syndrome like akinesia, byadyskinesia, and tremor. Besides, it can also enhance the aggregation of α-synuclien to form LB, which is the pathological hallmark. Due to this quality, rotenone has now become the new drug of interest to design models of PD in laboratory **(21)**.

As mention above, nowadays there are several neurotoxins proved to be effective for inducing parkinsonian syndrome. The availability of these agents encourages researchers to explore better anti-parkinsonism agents by using these neurotoxins to design animal models of PD. However, the methods for monitoring drug effects are absolutely important and may require complicatedinstruments. One of the equipment that have been used widely to quantify animal behavior is Force Plate Actimeter. **Force Plate Actimeter** (FPA) is especially constructedfor behavior quantification of rodents and other small laboratory animals. This instrument is used to assess the behavioral attributes such as locomotor activity, tremors, distance travelling, gait disturbances, and rhythmicity following neurotoxin administration. Hence, it has been very useful for studying the neurological effects of drugs in the animals. It can record both force of whole body tremor and locomotion of same animal at same time. Therefore, we can quantitatively describe the behavior of rodents after the injection of MPTP, tacrine, and rotenone using a FPA.

Another well-known method that has been used for decades in neurotoxicity studies is Staircase experiment. **Staircase experiment** provides a simple, sensitive, and quantitative measure of skilled reaching and grasping ability of rodents **(65)**.Smooth and coordinated muscle movement is essential for the normal motor ability like maintenance of body posture and grasping of food. These behaviors are critically important for the assessment of neurotoxicity and can be used as the model of fine motor skills in humans **(67)**. Similar to Staircase experiment, Grip test experiment is another simple and non-invasive method for monitoring muscle strength in animals.

Grip test experiment measures grip strength (peak force) of forelimbs in rats. Rodents are allowed to catch the trapezoid bar which is connected to automatic peak detector. The maximum force applied by rodents on bar is shown in meter **(68-69)**. It is based upon the natural characteristics of rodents to grab the bar as hard as they can when they are mildly pulled towards the back. The main advantage of this behind grip test is that it is a non-invasive method; the measurement does not of grip strength without causing any damage to muscles **(69)**.

Aims and Objectives:

Currently, there is effort to develop new drugs for the management of PD as well as new animal models for the exploration of the mystery behind the pathogenesis of this disease. In drug development process, animal studies play an important role in the discovery of new pharmacological active agents. Observing and quantitating the motor performance of animals in respond to the treatment of neurotoxins, such as MPTP, tacrine and rotenone may provide evidence regarding PD. Biochemicalanalysis of the level of dopamine and MAO-B form brain may provide information regarding the extent of damage generated in the brain. Thus, the aims and objective of our study is are

- To quantitatively analyse the Parkinsonism characteristics induced by MPTP, tacrine, and rotenone in different species of rodents (mice and rats).
- To determine any tolerance effects to neurotoxin treatment as assess from on tremor or locomotor movements.
- To differentiate between the characteristics shown by two species of rodents on the neurotoxins administered.
- To quantitatively determinethe amount of brain dopamine and MAO-B level.
- To design an experimental model for PD using FPA

The results obtained from these experiments offer an alternative animal model which is more precise and are appropriate for the study and development of new drugs for the treatment of PD.

CHAPTER II

LITERATURE REVIEW

2.1 Animal as an important model

Animal model is an important approach to elucidate the possible causes, progressions, pathological changes and therapeutic strategies in treatment of human diseases. They are used to design an *in vivo* model that can reflect almost all symptoms similar to diseased state in humans. However, the invented animal model should be capable to exhibit similar pathological, histological and biochemical features in comparison to those occur in humans **(62-64)**. Animal models of PD have been curiously explored in order to establish more evidence regarding this abnormality. Symptoms of PD can be induced by the administration of neurotoxic agents like MPTP, tacrine, and rotenone through various routes. But, different species of animals possess varied degree of sensitivity towards these toxins. For example, horses, dogs and mice aremore sensitive to MPTP than rats and guinea pigs **(62-63)**.

2.2 Mitochondrial dysfunction in the development of Parkinson's disease

PD is a neurodegenerative disorder highly specific to Snpc area of brain. Currently, it is clear that PD is caused by the progressive degeneration of nigrostriata ldopaminergic neurons **(107-108).** Mitochondria have a conspicuous value in energy metabolism of the body, regulating via electron respiratory chain (ETC) **(109-110)**. ETC is a mechanism of cell respiration, and is responsible for ATP generation **(111)**.It is operated by the movement of H+ ions from matrix to intramembranous space and generates concentration gradient, and aids to produce ATP. During this process, the electrons may leak from complex I and react with oxygen to form superoxide anions (O_2-) **(112)**.

Under normal physiological condition, these O_2- is detoxified or removed by Manganese superoxide dismutase (MnSOD) to form H_2O_2, which is converted to water by the interaction with glutathione (GSH) **(113-114)**. ETC helps to regulate many physiological cellular steps, such as calcium homeostasis, stress response and cell death pathways. Dysfunction of mitochondria could be generated from various factors and is also proposed to be accelerated with age. This defect leads to neurodegeneration and cell death **(115-116).** The mitochondria in neurons of Snpc are thought to be more vulnerable to be damaged **(29-31)**.However, it is still no obvious explanation on the association of mitochondrial dysfunction and neurodegeneration, but it may possibly be related through the generation ofreactive oxygen species, free radicals, and excitotoxicity **(115-116)**.

2.3 Dopamine synthesis and metabolism

Dopamine released from Snpc that connects to striatum is proposed to be the major pathway for controlling normal and coordinated movement especially the smooth and coordinated movement of muscles. Thus, the flow of dopamine from snpc to striatum should be strictly regulated. Any disturbance in the flow may lead to motor defects like tremor, rigidity and akinesia. In case of PD, the dopaminergic neurons become~~s~~ degenerated in a remarkable amount, and the normal flow of dopamine in nigrostriatal pathway is affected, leading to some motor disorders **(117-119)**.

Synthesis of dopamine is shown is figure 2.1.The synthesis is initiated by the hydroxylation of tyrosine to levodopa (L-dopa). It requires the presence of tyrosine hydroxylase and TH cofactor 6-tetrahydrobiopterin (BH4). L-dopa is converted to dopamine under the action of enzyme aromatic amino acid decarboxylase (AADC) **(120-121)**. The newly formed dopamine is sequestered and stored in vesicles via vesicular monoamine transporter 2 (VMAT2) **(122)**. Upon the action of any stimulatory signals, this stored dopamine are released to the synaptic cleft, and binds with the dopamine receptors of post synaptic terminals to deliver message throughout the motor ganglia and generate effects. The dopamine that fails to bind to post synaptic dopamine receptors are reuptaken towards presynaptic terminal and again stored in synaptic vesicles via VMAT2 **(120-122)**.

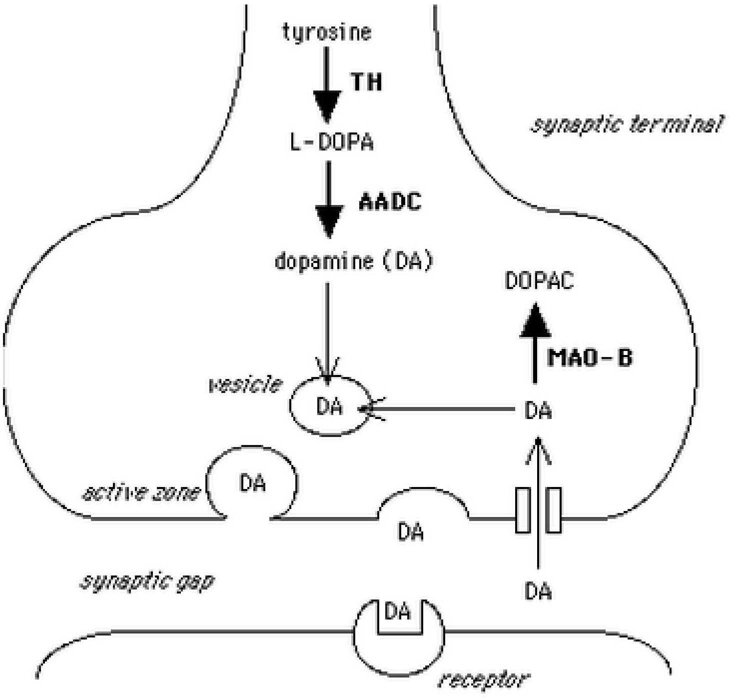


Figure 2.1 Synthesis pathway of Dopamine(123).

Breakdown process of endogenous dopamine is shown is figure 2.2. Metabolism is initiated by the transformation of dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC) a substrate for alcohol dehydrogenase. DOPAC diffuses out from the cell and are either conjugated to glucuronides, or changed to homovanillic acid (HVA) by cathchol-*O*-methyltransferase (C*O*MT) **(120-121)**.

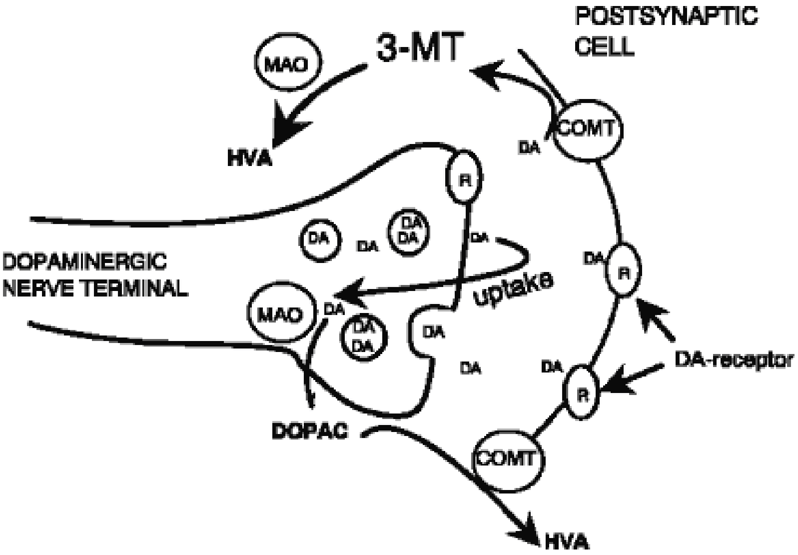


Figure 2.2 Metabolism of dopamine **(124)**

2.4 Function of dopamine in movement

For the normal functioning of brain, the synthesis and metabolism of dopamine should go simultaneously and is thought to be in balance. However, in case of PD, the metabolism of dopamine exceeds synthesis, causing imbalance in dopamine level and leads to motor abnormality of the body **(124)**. During the process of metabolism, dopamine undergoes autoxidation or enzyme catalyzed oxidation to producea large amount of endogenous neurotoxin such as free radicals and reactive oxygen species. These neurotoxins may implement oxidative stress and aggravates mitochondrial dysfunction **(125)**. However, some dopamine derivatives possess protective anti-oxidative effects that may overcome the oxidative stress. Hence, dopamine metabolism is a series of redox reaction that promotes cell homeostasis and viability (125-126).

Post mortem studies of Snpc obtained from PD brain has revealed the presence of inclusions of α synuclein that are linked to nigrostriatal degeneration. The result of oxidative damage or mutations in gene encoding α synuclien (SNCA gene) renders changes in the normal physiology of α synuclein. It is predominantly localized at presynaptic terminals in the CNS, where it is roughly associated with synaptic vesicles **(33-35)**. In normal condition, α-synuclein is supposed to be found in single fibrils like structure that act independently to each other. Under mutation of SNCA gene, α-synuclien tangled with each other and forms a cluster of oligomers called the LB. ThisLBis deposited in affected neurons of PD. Therefore, the main pathological hallmark of PD is oligomerization and aggregation of α-synuclein and the presence of LB **(36-37)**.

Despite the fact that there are many medications available to treat PD, none of them can permanently restore the depleted dopamine or replace the degenerated nigrostriatal dopaminergic neurons. They can only alleviate motor and non-motor symptoms associated with PD similar to drugs used in the management of depression, anxiety, sleep disorders and dementia **(26).** Even after years of its clinical use, Levodopa still remains the gold standard for the treatment of PD.After oral administration of levodopa, it is actively transported from the digestive tract into the circulatory system. Circulatory conversion of levodopa to dopamine produces peripheral effects like nausea and vomiting.Due to its distribution and metabolism throughout the body, only a small portion of drug enters the brain by active transport across blood brain barrier. Administration of levodopa and carbidopa, an inhibitor which blocks the peripheral inactivation of levodopa, and preserves it for the action in brain. Inside the brain, Levodopa is converted to dopamine by enzyme aromatic L-amino acid decarboxylase (AAAD). Although conversion to dopamine is the major mechanism leading to the pharmacologic effect of levodopa, the drug also has direct neuromodulatory and neurotransmitter action that may contribute to its parkinsonian outcome **(22-23)**.

Nevertheless, long term use of levodopa and other dopamine replacement therapies is associated with side effects such as rapid and involuntary jerking of muscles and increased addiction of dopamine **(27)**.Thus, searching for an effective anti-parkinsonian drug that can increase dopamine levels for a long period of time is one of the interests in the field of neuropharmacology. A new class of drugs that can reverse symptoms of PD could definitely improve the patients' quality of life.

2.5 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)

MPTP is a selective neurotoxin used to induce an in vivo animal model of PD. It has been proved to be an important model of PD because it can cause almost all pathology similar to those seen in human patient with PD **(64)**. Structure of MPTP resembles to rotenone **(38,64)** but their mechanisms of action to induce Parkinsonism are slightly different **(55, 64, 69).**

Figure 2.3 shows the possible pathological mechanism of MPTP. Blood brain barrier acts as the primary defense mechanism against foreign objects that enters the circulatory system. It contains a series of tight junction of endothelial cells that has monoamine oxidase **(14)**. A certain portion of MPTP is converted to MPP+, but they cannot be transported through blood brain barrier. The remaining MPTP can easily cross the blood brain barrier because of its lipophilic nature **(14-15)**.Once inside the brain, it moves towards astrocyte and glial cells where itis metabolized to MPP+ by MAO-B. MPP+ is a polar compound so they cannot be freely transported by themselves. They need transporter to move towards the extracellular space **(14,40).** From extracellular space, MPP+ enters dopaminergic neurons via dopamine transporter (DAT) and exerts its toxic effect by inhibiting Complex I of electron transfer chain (ETC). Inhibition of Complex I cut down the synthesis of ATP. Lack of ATP formation leads to cell deathas the result of energy insufficiency. Inhibition of Complex I can also trigger the formation of reactive oxygen species that initiates DNA strands breakage leading to neuronal cell death **(14-15)**. Besides, MPP+ inside neurons can be reused by Vesicular Mono anime Transporter (VMAT) and be stored in vesicles. This process will diminish the toxicity of MPP+ and it ejects out dopamine into intracellular space for further metabolism **(14, 38-39).**

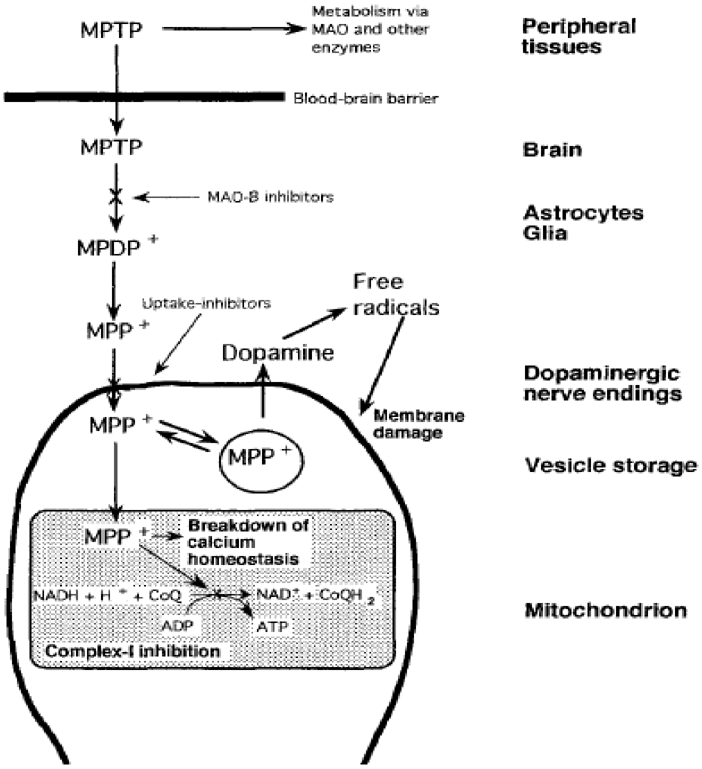


Figure 2.3 Possible pathological mechanism of MPTP **(14)**.

Regarding to the animals used for MPTP induction of PD symptoms, there is evidence indicated that different species of animalsare not equally sensitiveto horses, dogs, sheep and mice are found to be more sensitive to MPTP than rats and guinea pigs **(62-63)**.This wide variation in MPTP sensitivity may be affected by the difference in pharmacokinetics, distribution, and excretion of MPP+ among animal species. Other possible factors that may contribute to this variation are concentration of neuromelanin, differences in distribution and localization of MAO subtypes in brain.In addition, variation in dopamine metabolism and anti-oxidant content in nigrostriatal system may also be responsible for the diversity of MPTP sensitivity in different species of animals **(62-63)**.

Former studies showed that MPTP can significantly reduce motor behavior in both C57BL/6 but not in BALB/c mice. BALB/c mice were reported to be resistant to MPTP whereas C57BL/6 mice were found to be sensitive **(45-47)**.Susceptibility of mice is also dependent on age. Old mice were found to be more vulnerable towards MPTP toxicity than the young ones **(46-47)**.

Intraperitoneal doses of MPTP (10, 30, 60, and 90mg/kg)have showna wide range of response in Wistar rats. No effect was observed on treatment with 10mg/kg MPTP; 30mg/kg MPTP induced ataxia and tremor, while 60 and 90mg/kg MPTP produced seizures and rigidity with high mortality rate. Despite of this variation in motor behavior, MPTP failed to reduce dopamine levels from the brains of these animals **(47)**. On the contrary, infusion of MPTP into substantia nigra of rats caused a partial loss of dopaminergic neurons and a depletion of striatal dopamine level **(48)**. This finding reflected the importance of routes of administration in MPTP sensitivity. Intracranial administrationwas found to be an effective route to induce parkinsonian syndrome in rats. Systemic injection of 3.2-3.9mg/kg MPTP in African green monkey for 5-15 days produced tremor and rigidity with a frequency ranging from 9-13 Hz **(49,57)**. MPTP induced resting tremor only in African Green monkeys while in other species of monkey, it caused tremor and postural instability **(57)**. However, the frequency of parkinsonian syndrome induced by MPTP in rodents has not been reported elsewhere. By using FPA, we may be able to define motor behaviors and determine the frequency of tremor and rigidity. Moreover, we should be able to compare the sensitivity of C57BL/6 mice and Sprague Dawley rats to intraperitoneal injection of MPTP.

2.6 9-amino-1,2,3,4-tetrahydroaminoacridine hydrochloride hydrate (Tacrine)

Tacrine is a centrally acting, reversible noncompetitive inhibitor of acetylcholineesterase. It was initially used as a drug toprevent the progression of dementia in Alzheimer’s disease. However, it’s gastrointestinal and hepatotoxicity terminated the use of tacrine in Alzheimer’s patients. Systemic administration of cholinomimetic drugs, tacrine, can induce monotonous vertical deflection of lower jaw or chewing effect without any stimuli inside mouth, a similar appearance usually appear in PD patients. The rigidity of orofacial muscles in patients with PD causes the patient to manifest non oscillatory chewing involuntarily **(17-18)**. Even though the clinical significance of drug induced oral movement is stillinconclusive, jaw movement in rats treated with tacrine is suggested to correlate with human Parkinsonism symptom **(41)**.The explanation for this phenomenon was drawn from the fact that jaw deflection could be induced by dopamine receptor antagonist which reflected a pharmacological approach to induce the existence of low quantity of dopamine **(17)**. Jaw movement is resulted from the interaction of dopamine and acetylcholine system in brain **(18)**. Tacrine is noncompetitive inhibitor of cholinesterases and acts by reversibly binding and inactivating cholinesterases **(41)**. It blocks the hydrolysis of acetylcholine from cholinergic neurons and elevatesthe endogenous level of acetylcholine, the neurotransmitter at cholinergic synapse, and prolongsthe effects of acetylcholine **(17-19)**.

Former studies revealed the dose dependent reduction of locomotion, and the induction of lateral movement of jaw in rats after the intraperitoneal administration of tacrine **(3,44)**. 2.5mg/kg tacrine (i.p.) caused lateral movement of jaw ranging frequency of 3-6 Hz. This experiment was analyzed by slow motion video and electromyography **(42-43)**. We can compare these findings with the use of modern technology, FPA, where we analyze motor parameters and power spectra induced by 5mg/kg Tacrine. Intraperitoneal injection of Levodopa was able to relievesymptoms induced by administration of tacrine **(44)**. In our experiment, 10mg/kg of Sinemet ® (Levodopa + Carbidopa in the ratio of 4:1) is orally administered 30min prior to i.p. injection 5mg/kg tacrine.

2.7 Rotenone

Rotenone is naturally available in roots and stem of herbal species belonging to *Lonchocarpus* or *Derris* **(19)**. Epidemiological studies reveal an association of environmental toxin and pesticides to aggravate mitochondrial complex I defect.

In the study of Parkinson’s disease, using rotenone to induce Parkinsonian syndrome is the most recent approach in designing PD models in rat. Among cause of

PD, high exposure to environmental toxins like rotenone is one of them that have been proposed. Individuals exposed to rotenone have 2.5 times higher risk of PD, compared to those that are not exposed to this toxin **(50)**.In concord with MPTP, rotenone is also highly selective to Snpc, and thus produce tremor and rigidity similar to that was obtained. In despite of their structural similarity, rotenone is not transported by dopamine transporters to mitochondria as that of MPTP **(64)**. Even though rotenone affects mitochondrial complex I throughout the brain uniformly, it was highly selective to Snpc and brought about the major hallmarks of PD such as the aggregation of α synuclein, ATP exhaustion, and motor defects **(51-53)**. Other factors leading to Rotenone toxicity may be accounted for oxidative stress and the reduction of glutathione level **(51, 54).**

Although MPTP and rotenone is structurally similar and eventually cause ATP exhaustion, and cell death by blocking complex I of electron transport chain, their mechanisms of action are slightly different. The actual mechanism of Rotenone to initiate Parkinsonism symptoms still remains unknown. Figure 2.4 illustrated the possible mechanism of rotenone compared with MPTP and other parkinsonian model used in laboratory. Rotenone being highly lipophilic can easily cross the blood brain barrier and can readily penetrate inside cell membrane and astrocytes. If it enters the mitochondrial environment, then they block complex I of electron transport chain causing deficiency of ATP and generateexcess of reactive oxygen species and other proapoptotic molecules that accelerates apoptosis of cells. The generated reactive oxygen species attacks proteins and DNA, causing specific alternation. They can also initiate the aggregation and accumulation of α synuclien and form LB **(51,55).**

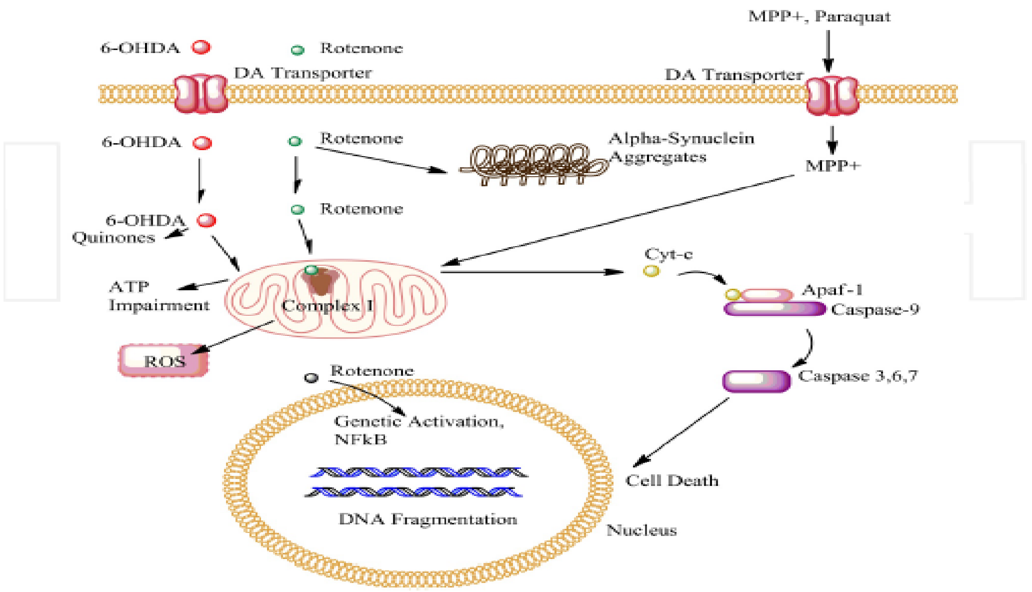


Figure2.4 Possible mechanism of action of Rotenone and comparison with MPTP**(55)**.

Both *in vitro* and *in vivo* studiesclearly revealed that rotenone promotes accumulation and aggregation of alpha synuclein and ubiquitin which indicatedthe existence of cell death caused by oxidativeand endoplasmic stress **(56)**. These events lead to the experimental induction of PD like symptoms and the occurrence of cytoplasmic inclusions resembling Lewy bodies. Injection of 8 and 12µg rotenone into themedial forebrain bundle induced motor defects in rats **(58)**. 2mg/kg rotenone injected subcutaneously (s.c) significantly lowered the dopamine level in striatum of rat brain **(59)**. Intraperitoneal injection of rotenone resulted in approximately 33% mortality of rats **(60)**.Chronic treatment with 2.5mg/kg rotenone injection for 48 days successfully reduced the neurobehavioral parameters e.g. distance travelled (m), number of rears, and increased the inactive sitting (time in seconds). All these parameters were found to be reversed by intraperitoneal injection of levodopa in combination with benserazide for 10days **(61)**.

Although there is some research attempted to evaluate the motor parameters and neurochemical changes in brain after the administration of rotenone in rodents, the frequency of tremor and rigidity which are characteristic of PD has not been quantified. Therefore, we expected that FPA could enable us to monitor motor behavioral changes as well as analyzethe power spectra induced by rotenone in rats.

2.8 Biochemical analysis of dopamine and MAO B from brain

Currently, various quantitative methods which are specific to determine the protein of interest from the given sample have been introduced i.e. High Performance liquid chromatography (HPLC), Immunohistochemistry, Enzyme linked immunosorbant assay (ELISA), Western Blot etc.The principle of ELISA method is the application of enzymes to enhance interaction of antigen and antibody, that promotes change in color, and quantitatively determine a specific protein **(96)**.

Depending upon the type of research, and antigen and antibody type, ELISA can be either competitive or non-competitive method. There are four major steps in ELISA: coating, blocking, interaction of antigen-antibody, and analyzing optical density after the development of color **(97-98)**.

2.8.1 Principle of Competitive ELISA

Competitive ELISA requires the use of Immunoglobin G (IgG) antibodies that are extracted from animals, and acts against the specific antigen. These antibodies can be either monoclonal or polyclonal antibodies. Monoclonal antibody has specific binding site while polyclonal has multiple binding sites **(97-98)**. Figure 2.5 depicts the competitive nature of sample and labeled antigen. Initially, secondary antibody (blue) that is specific to target protein is pre-coated on the walls of ELISA kitand incubated overnight to ensure well settled coating. Detergent was used to prevent any kind of non-specific binding **(98-100)**. Major event in competitive ELISA is the competitive binding of sample antigen and labeled antigen with antibody that is coated on the walls of ELISA kit **(99)**. In the final step, adding of substrate develops color with intensity depending upon the antigen concentration in sample. The concentration of sample antigen and the color elicited is inversely proportional; because higher concentration of the protein of interest leads to lesser labeled antigen being captured and thus, weaker signal is released **(99-100)**. If the sample antigen concentration is low, high intensity of color will be obtained. The optical density of final solution can be measured by plate reader.

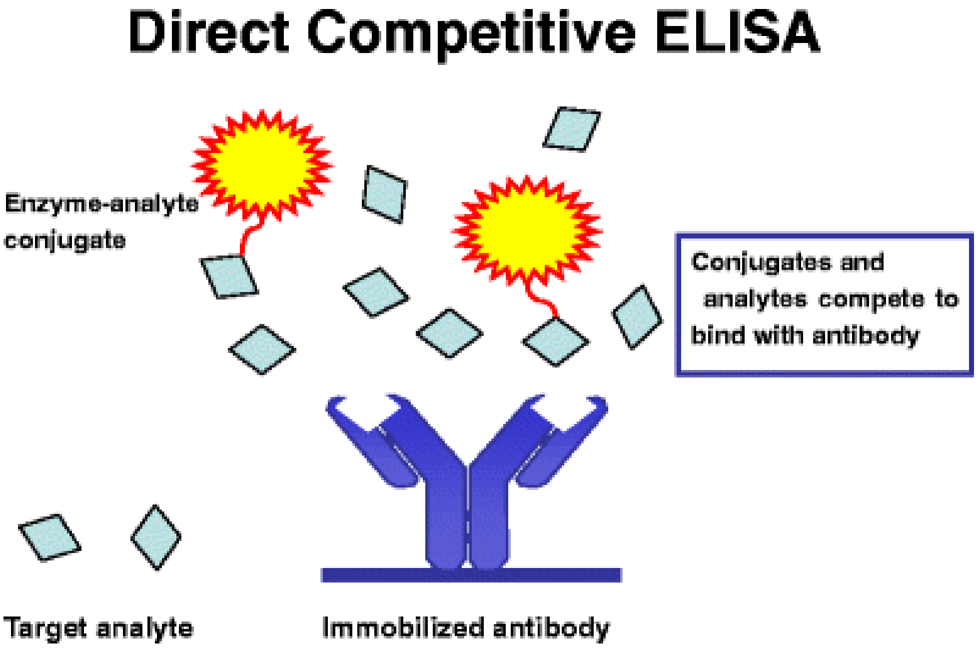


Figure 2.5 Competitive natures of sample and labeled antigen **(101)**.

2.8.2 Advantages of Competitive ELISA (96-97,100):

- Highly specific and sensitive towards the protein of interest.
- Can be applied for complex samples without sample purification.
- Ease of use and speedy result.

Former studies have revealed the quantitative determination of dopamine usinghigh performance liquid chromatography–mass spectrometry (HPLC–MS) **(102,106)** and flow injection chemiluminescence (FL-CL) and HPLC–CL **(98)**. Due to the important role of dopamine in movement, and MAO B in degradation of dopamine, quantitative determination of dopamine and MAO B is of the issue of concern during the study of PD. Several previous studies have demonstrated the reduction of dopamine and escalation of MAO B levelsin the brain sample after the treatment with neurotoxins, such as MPTP and rotenone **(104-105)**. HPLC is a reliable method for the detection of target protein but it is tedious and requires experienced personnel to conduct the experiment. ELISA is relatively simpler and more cost effective than HPLC and the degree of sensitivity is relatively similar to that of HPLC.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

Animals used for this study were C57-BL/6, Sprague Dawley Rats, and Wistar Rats. All animals were purchased from National Laboratory Center, Salaya, Nakorn Pathom. They were individually housed and kept in standard condition with 12 hr light/dark cycle, temperature controlled to 22±2 ºC and humidity of 40-80%. Both rat and mice are allowed free access to a standard diet and water *ad libitum* until the staircase training period started. The protocols of animals handling and procedure were approved by the Animal Care and Use Committee, Thailand Institute of Scientific Technological Research (TISTR) and Animal Ethic Committee of Faculty of Pharmacy, Mahidol University.

3.1.2 Chemicals

- MPTP (Sigma−Aldrich®; USA)
- Normal Saline Solution (Klean&Kare®; Thailand)
- Phenyl methyl sulfonyl fluoride (PMSF) (Sigma−Aldrich®; USA)
- Rotenone (Sigma−Aldrich®; USA)
- Sinemet® (levodopa + carbidopa) (M&H; Thailand)
- Sodium deoxycholate (Sigma−Aldrich®; USA)
- Sodium dodecyl Sulfate (Sigma−Aldrich®; USA)
- Sodium Fluoride (APS Ajax Finechem®; Australia)
- Soybean Oil (Sigma−Aldrich®; USA)
- Tacrine(Sigma−Aldrich®; USA)

3.1.3 Apparatus

- Force plate actimeter (Basi ®; USA)
- ELISA Kit (eBioscience®–Bender MedSystems GmbH)
- Tissue homogenizer (Miccra; Germany)
- Freezer -80^0C (Panasonic, Japan)
- Grip strength meter (UgaBasil, Italy)
- PH30 pH Tester (Clean Instruments; USA)
- Spectrophotometer (Thermo Fisher Scientific; Finland)
- 18 G feeding needles (Nipro; Thailand)
- 26 G needles with 1 ml syringes (Nipro; Thailand)
- Micropipettes (1-10µl, 100-200µl, 1000µl) (BioHitProline; Finland)
- Weighing Balance (GF-300, AND)
- Hot plate (Thermolyne; USA)
- Surgical Set
- Staircase Set

3.1.4 Force Plate Actimeter (FPA) and its mechanism of operation.

FPA is a combination of mechanical, electronic, and computing element that works together to extract measurement of whole body behavioral attributes that serves as a basic to neuroscience research.

A sensitive rigid, horizontal force plate is supported by four force transducers. The animal is placed over the force plate and left freely. When it moves on the plate, the movement is sensed by transducers whose signal is further processed by computer to give quantified data of behavior shown by the animal during the recording **(24-25)**. It is square in shape and the animal enclosure is made up of Plexiglas. Only one animal is kept in the plate and the door is closed for recording behavior of animal. Transducers can be adjusted in a horizontal line by rotating transducer adjuster in clockwise or anti clockwise direction in order to keep the plate in balance and in horizontal plane **(24-25)**. The entire cage is enclosed within a sound attenuating chamber that limits the amount of sound and light entering inside it and thus, minimizing the risk of disturbance by environmental factors in animal. Inner and outer view of FPA along with the labeling of their parts is shown in the figure 3.1.

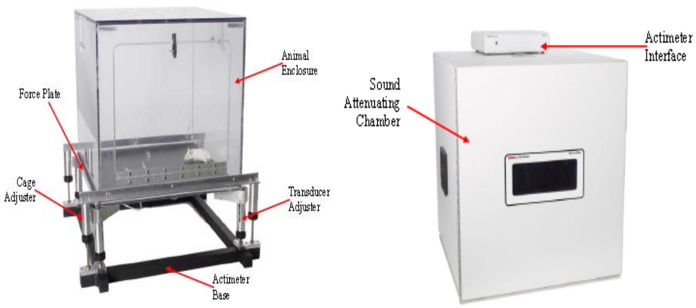


Figure 3.1 Pictorial description of inner and outer view of FPA

Factors to quantitate from FPA:

- Distance travelled by rodents.
- Frequency (Hz) of Parkinsonism symptoms resembling tremor, chewing, rigidity.
- Bout of Low mobility (BLM) of rodents.

FPA has a highly sensitive plate that is connected to transducers which relay signals to computer for further processing. Graphite plate of FPA is influenced by the coordination system of mathematics. Four transducers are placed on all corners of the plate and the forces on every corner of plate are represented by f1, f2, f3 and f4. Combination of all four transducers makes a coordinate system. The four component forces are arranged in counterclockwise direction, starting with f1 in Quadrant 1 (where both X and Y are positive), f2 in Quadrant 2 (where X axis is negative and Y is positive), and so on **(24-25)**.

Significance of using FPA in compare to other traditional devices **(24-25)**:

- Well ventilated sound attenuating chamber for sensitive experiments that requires fine motor activity.
- Much finer temporal and spatial resolution than other previous devices.
- Quantify locomotion, ataxia, tremor, and other animal behavior.
- Works better than rotomoters for quantifying rotational activity.

- Can be used for various small laboratory animals.
- Observational method can be difficult when conducting with small laboratory animals, like mice, where fine movements are difficult to quantify using other rating methods.

Besides behavioral assessment with FPA, mice treated with 15 and 30mg/kg MPTP (i.p.) and rats treated 2.5mg/kg rotenone (s.c.) were tested in some simpler and preliminary device such as staircase and grip test. Since PD is a neurodegenerative disease of motor behavior, it becomes quite important to monitor motor behaviors in animal model **(66)**.

3.2 Methods

3.2.1 Preparation of buffers:

3.2.1.1 Radio immune precipitation assay buffer (RIPA buffer)

Recipe of RIPA buffer is shown as follows:

- 1% Triton
- 0.1% Sodium dodecyl sulfate (SDS)
- 0.5% Sodium deoxycholate
- 1mmol/L Ethylene diamine tetra acetic acid (EDTA)
- 150mmol/L Sodium Cholride (NaCl)
- 20mmol/L Tris
- 10mmol/L Sodium Fluoride (NaF)
- 0.1mmol/L Phenyl methyl sulfonyl fluoride (PMSF)

These chemicals were dissolved in around 700ml of distilled water. The pH was maintained afrom 7.2-7.6 and the volume was adjusted to 1000ml. It was then stored in 2-8ºC.

3.2.1.2 Phosphate buffer saline (PBS)

Recipe of PBS buffer:

- 137 mmol/LSodium Chloride (NaCl)
- 2.7 mmol/LPottasium Chloride (KCl)
- 10 mmol/LSodium Hydrogen Phosphate (Na_2HPO_4)
- 2 mmol/LPottasium Hydrogen Phosphate (KH_2PO_4)

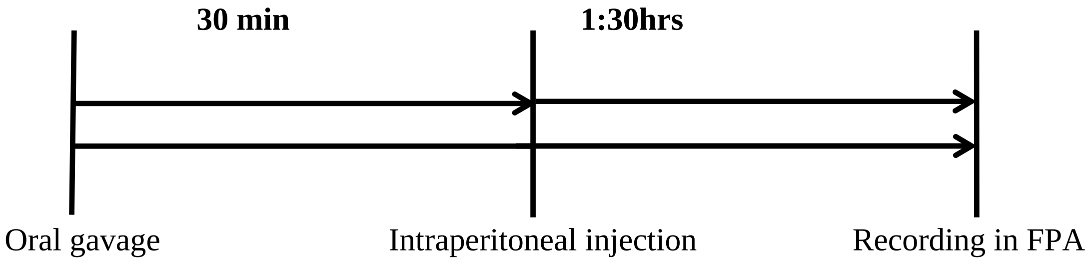
These chemicals were dissolved in 500ml of distilled water to make a stock solution of 1M PBS. In order to make 0.1M PBS, 100ml of this stock solution was dissolved in 600ml distilled water. The pH was maintained to 7.2, and volume was adjusted to 1000ml. It was then stored in 2-8ºC.

3.2.2 5mg/kgTacrine

Protocol of Tacrine in both mice and rats was influenced by the former studies conducted by Carriero et al (1997) (3). 18 Wistar rats and C57BL/6 mice were used in the experiment. They were all fasted for 16 hours prior to the experiment. They were equally divided into three groups with sample size (n) =6 as the following:

- Control (C1): Oral 3% (w/v) acacia and intraperitoenal (i.p) injection of normal saline solution (NSS).
- 5mg/kg Tacrine (T1): Oral 3% (w/v) acacia + 5mg/kg tacrine(i.p.) dissolved in NSS
- Treatment (T2): 10mg/kg Sinemet® mixed with 3% (w/v) acacia + 5mg/kg tacrine(i.p.) dissolved in NSS

Rodents were orally fed with their respective treatment, and after 30 min, i.p. injection of tacrine was administered to all groups. They were immediately kept inside FPA for 1 h and 30 min. They were then euthanized and their brain was extracted and stored in -80°C for further biochemical analysis. Simplified diagram of tacrine experiment is shown in the chart below.



C1: 3% (w/v) acacia in distilled water (vehicle)	C1: NSS
T1: 3% (w/v) acacia	T1: 5mg/kg Tacrine
T2: 10mg/kg Sinemet®	T2: 5mg/kg Tacrine

Figure 3.2 Simplified diagram to describe 5mg/kg Tacrine protocol in rodents.

3.2.3 15mg/kg and 30mg/kg MPTP

3.2.3.1 Behavioral assessment of mice by Staircase experiment

Staircase experiment of our work was influenced by the previous work of Pagnussat et al. (2009) and Schaar et al. (2010) **(65,128)**. Small pellets of cheese were kept on the steps of stairs that allows animal to pass through the platform and grab cheese. Animals were firstly well trained in this experiment to grab cheese before the start of experiment. In order to obtain food, animal must stretch their forepaws to reach the food, grasp it, and retrieve it.

The apparatus is made up of Plexiglas and contains a chamber with stairs **(65)**. Mice took around 8-9 months to become perfectly trained.During the training session; mice were restricted with limited amount of food for 16-18 hrs.in order to make them feel hungry, so that they can perform well in staircase experiment. Due to the fact that limited amount of food is given, the mice were weighed weekly, to ensure that there is no complications in mice. A reduction of body weight by more than 10% in one week is considered to be fatal. After being well trained, the experiment with administration of MPTP is started. This training was done daily 5 days per week for 15 minutes and limited food is given 24 hours prior to the experiment. Limb used by the mice to fetch cheese was observed and recorded.

Rodents injected with MPTP may not be able to grasp and retrieve the food. This experiment gives us an idea about finely controlled motor skills of forelimbs **(65- 67).** The main objective is to compare the amount of food grasped before and after the administration of neurotoxins, which gives an idea about fine motor controlled behavior. After the i.p. administration of MPTP, the mice were kept inside staircase for 15 minutes. As both doses of MPTP exhibited tremor in fore limbs, they were able to grasp cheese in a less amount as compared with baseline. Staircase method is done to monitor any kind of motor disabilities in animals after the administration of toxins that restricts them to grab and retrieve cheese.

3.2.3.2 Intraperitoneal administration of 15mg/kg and 30mg/kg MPTP.

Protocol of MPTP was influenced by the previous study performed by Liang et al (2007) **(129).** After the mice were successfully trained instaircase experiment, they were subjected to two doses of MPTP administration intraperitoneally.

Animals were randomly divided into five groups with each of sample size n= 6:

- Control (C1): Oral 3% (w/v) acacia + i.p. injection of0.1M Phosphate Buffer Saline (PBS) of pH 7.4.
- MPTP 1 (M1): Oral 3% (w/v) acacia + 15mg/kg MPTP (i.p.) dissolved in 0.1M PBS.
- MPTP 2 (M2): Oral 3% (w/v) acacia + 30mg/kg MPTP (i.p.) dissolved in 0.1M PBS.
- Treatment 1 (T1): Oral 10mg/kg Sinemet® suspended in 3% (w/v) acacia + 15mg/kg MPTP (i.p.) dissolved in 0.1 M PBS.
- Treatment 2 (T2): Oral 10mg/kg Sinemet® suspended in 3% (w/v) acacia+30mg/kg MPTP (i.p.) dissolved in 0.1 M PBS.

Mice were injected with their corresponding doses of MPTP once a week. First 5 doses of i.p. injection of 0.1M PBS, 15mg/kg or 30mg/kg MPTP were injected to all mice. After injection, they immediately kept torecording inside FPA for 2 hours. After the end of 5th dose on the 5th week, 2 week interval was kept as wash out phase. After 2 week interval, these mice were again injected with their 6th dose with corresponding i.p administration of 0.1M PBS, 15mg/kg or 30mg/kg MPTP.After injection, they were monitored in staircase experiment for 15 min to observe their grasping skill and recorded in FPA for 2 hours. On the 7th and 8th dose, mice were initially treated orally with 3% (w/v) acacia or 10mg/kg Sinemet®suspended in 3% (w/v) acacia depending upon their group. After 30 min of oral treatment, they were again intraperitoneally treated with respective doses of 0.1M PBS, 15mg/kg or 30mg/kg MPTP, kept in staircase for 15 min and kept inside FPA for 2 hours. After the end of 8th dose of treatment, mice were euthanized and their brains were removed and then washed with ice cold NSS. All brains were stored in RIPA buffer at -80°C. Simplified diagram of MPTP experiment is shown in the timeline below.

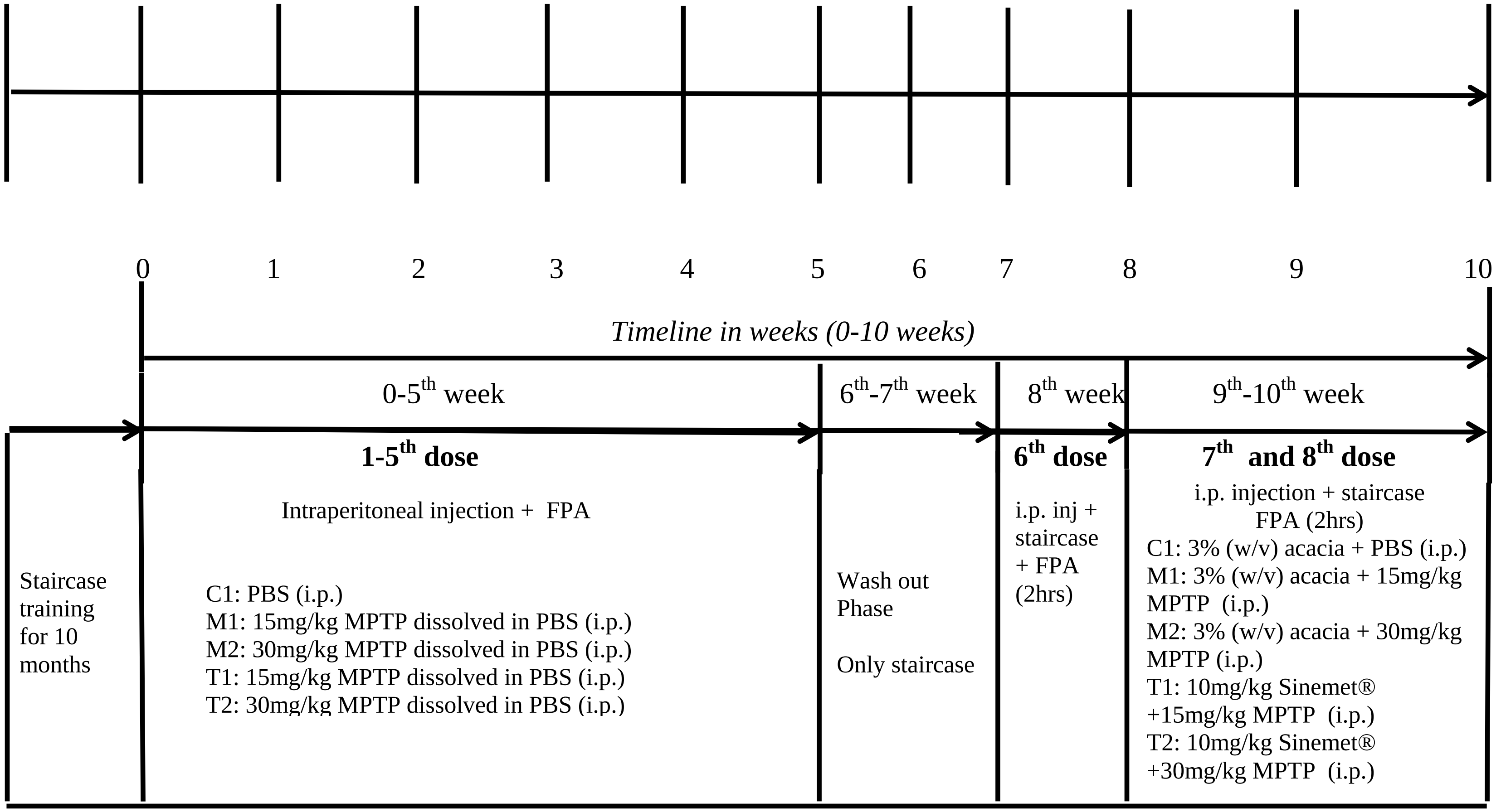


Figure 3.3 Chart of MPTP Protocol in mice

Sprague Dawley rats were used in the same protocol as that of mice for MPTP experiment but due to the high mortality rate of rats on treatment with 15 and 30mg/kg MPTP, the neurotoxin was changed to 2.5mg/kg Rotenone. However, the dose of MPTP was reduced to 10mg/kg and continued till the end of experiment with the remaining rats. 2.5g/kg Rotenone experiment was started with new sets of Wistar rats.

3.2.4 2.5mg/kg Rotenone

3.2.4.1 Behavioral assessment of Wistar Rats by Staircase experiment

Staircase experiment of our work was influenced by the previous work of Pagnussat et al. (2009) and Schaar et al. (2010) **(65,128)**. Wistar rats were primarily well trained to grab cheese before starting experiment. Rats took only around 4-5 months to get trained to retrieve cheese from staircase. The protocol is similar to the staircase of C57 BL/6 mice. 2.5mg/kg Rotenone is started only after the rat becomes well trained to grab cheese. This training was done daily 5 days per week for 10 minutes and limited food is given 24 hours prior to the experiment. Limb used by the mice to fetch cheese was observed and recorded. Due to the increasing weight of rats, the number of cheese pellets per experiment was limited till 15.

After the subcutaneous (s.c.) administration of 2.5mg/kg rotenone, the rats were kept inside staircase for 10 minutes. As 2.5mg/kg rotenone was able to exhibit tremor in fore limbs, rats were able to grasp cheese in a less amount as compared with baseline. Staircase method is done to monitor any kind of motor disabilities in animals after the administration of toxins that restricts them to grab and retrieve cheese.

3.2.4.2 Behavioral assessment of Wistar Rats by Grip test

The grip test method was based upon the previous research done by Coq et al. (2009) where they evaluated motor behavioral deficits in peripheral and central leisons (130). Grip Strength meter measures grip strength (peak force) of forelimbs in rats. Rotenone (s.c.) reduces muscle strength and coordination due to the induction of parkinsonian syndrome. Grip strength meter contains an automatic peak detector which gives the maximum force used by rats on trapeze or bar.

Peak force done by rats on trapezoid before and after the treatment of rotenone was recorded. The data was compared to determine any significant difference in muscle strength and coordination on baseline and after treatment of toxins. Rats were allowed to grab the trapezoid by forelimb, which was connected to automatic peak detector. Then, the rats were slowly pulled back and peak force from grabbing the trapezoid was measured by an automatic peak detector. Grip test can be used to assess the effects of drugs on muscular strength and coordination. However, this test should not be done daily as the rodents may get habituated to it and develop stronger muscular strength which can increase the grip strength. Rotenone can impair the muscle strength and coordination due to the induction of parkinsonian syndrome.

3.2.4.3) Subcutaneous administration of 2.5mg/kg Rotenone

Protocol of rotenone was influenced from the past studies performed by Xiong et al (2011) and Alam et al (2002) **(53,131)**.After the animal passed the staircase test, rotenone experiment was started. 2.5 mg/kg Rotenone was prepared in soybean oil, with a concentration of 2mg/ml, and was injected subcutaneously daily on weekdays to rats. Rats were then kept inside FPA once a week. Animals were equally divided into three groups with sample size of 6 on each group.

Control (C1): 3% (w/v) acacia oral +s.c. injection of Soybean oil; n=6

Rotenone 1 (R1): 3% (w/v) acacia oral + 2.5mg/kg rotenone (s.c.) dissolved in soybean oil; n=6

Treatment 1 (T1): 10mg/kg Sinemet® suspended in 3% (w/v) acacia + 2.5mg/kg rotenone (s.c.) dissolved in soybean oil ; n=6

Rats were subcutaneously injected daily during the weekdays with their corresponding agent for 9 days. Rats were kept for recording in FPA for 2 hrs in 1st, 6th11th and 16th day of experiment. After every injection, staircase experiment was done to check if there is development of any parkinsonian symptoms.

Grip test was performed once a week before keeping inside FPA. One day gap was kept to monitor animal’s behavior. Because some rats developed severe parkinsonian symptoms like postural instability, rigidity and secretion from eyes and nose, oral treatment was given for 5 days without s.c. induction. After this series of oral treatment, rats were treated orally with their respective treatment and after 30 min; they were injected subcutaneously with rotenone or soybean oil depending upon the group they belong. After injection, staircase and griptest were done to monitor their motor behavior. Rats were then kept under record in FPA for 2 hrs. After the end of experiment, rats were euthanized and their brain was removed, washed with ice-cold NSS, and stored in RIPA buffer in the ratio of 1:9 at -80°C for further biochemical analysis to determine dopamine and MAO-B levels.

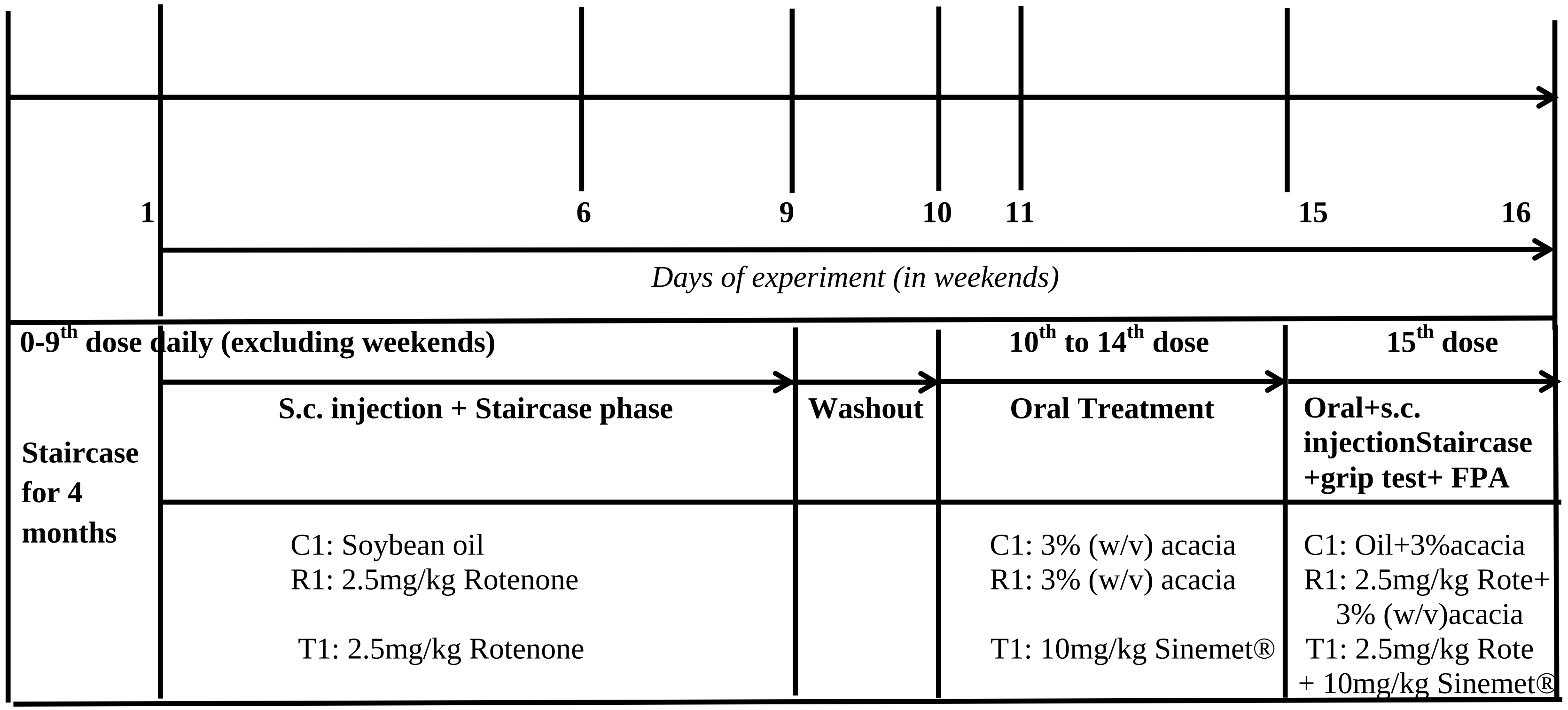


Figure 3.4 Chart of Rotenone protocol in Wistar rats.

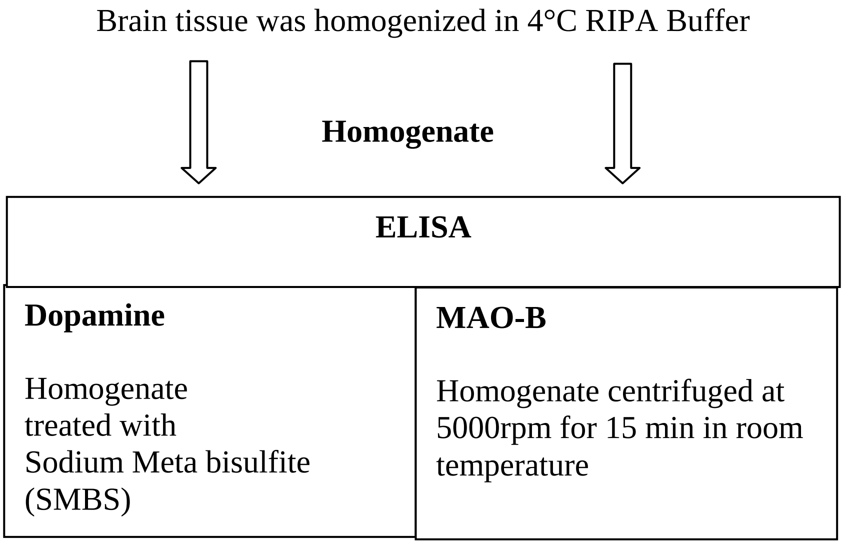
Staircase: Daily till the end of experiment.
Grip test: 1st, 6th, 11th and 16th day of experiment.
FPA: 1st, 6th, 11th and 16th day of experiment

3.2.5 Power Spectra Analysis by using FPA

Quantitative determination of Parkinsonism features exhibited by animal under the response of neurotoxin injected is an important parameter in our study. The power spectra are computed by FPA. Presence of significant peak which was obviously noticeable from the others is supposed to be the desired behavior and we check if the oral administration of 10mg/kg Sinemet® could improve this behavior or not. When inside FPA, the time interval is divided into frames with each frame containing 80 sec.

3.2.6 Quantitative determination of dopamine and MAO-B level.

3.2.6.1 Preparation of brain sample.



Brain tissue was homogenized in 4°C RIPA Buffer

Homogenate

ELISA

Dopamine

Homogenate treated with Sodium Meta bisulfite (SMBS)

MAO-B

Homogenate centrifuged at 5000rpm for 15 min in room temperature

Supernatants were stored at -80°C and used as samples in ELISA.

3.2.6.2 Quantitative determination of Dopamine by ELISA

The protocol of Dopamine Kit (Dopamine Research ELISA^{TM}) was followed. Determination of dopamine by ELISA was divided into two steps:

<u>Extraction and Acylation</u>

10µl of standards, controls and 500µl of supernatant was pipetted in respective wells of Extraction Plate. 490µl of distilled water was added in wells of standards and controls.

⇩

25 µl of TE Buffer was pipetted.

⇩

Plate was kept in shaker for 60 min at room temperature.

⇩

Plate was properly washed with wash buffer.

⇩

150µl of Acylation buffer and 25µl of Acylation reagent was pipetted into all wells.

⇩

Plate was properly washed with wash buffer.

⇩

100µl of HCl was pipetted into all wells.

Figure 3.5 Protocol of dopamine determination by ELISA

Enzymatic Conversion

90µl of extraction was pipetted into corresponding wells of microtiter plate.

⇩

25µl of Enzyme Solution was added into all wells.

⇩

Plate was incubated at 37 °C for 2 hrs.

⇩

100µl of standards, controls and samples was transferred from microtiter plate to precoated Dopamine microtitersrips.

⇩

50µl of Dopamine Antiserum was added and incubated for 15-20hrs at 2-8 °C

⇩

Plate was properly washed with wash buffer.

⇩

100µl Enzyme Conjugate was added in all wells and placed in a shaker for 30 min at 600 rpm.

⇩

Plate was again washed properly with wash buffer and 100µlsubstrate was pipetted in all wells.

⇩

Plate was again placed in shaker for 20 min at 600 rpm.

⇩

100µl of stop solution was added into all wells and absorbance was read at 450nm using Micro plate reader.

Figure 3.5 Protocol of dopamine determination by ELISA (cont.)

3.2.6.3 Quantitative determination of MAO-B by ELISA

The protocol was followed according to the instruction given by MAO ELISA kit.

100 µl of standards and samples were added in their corresponding well. 100 µl of PBS was added in blank wells.

⇩

10µl of Balance solution was added into all wells followed by addition of 50µl of conjugate to each well except blank control wells.

⇩

Plate was incubated for 1 hr at 37°C and washed properly.

⇩

50µl of substrate A and 50 µl of substrate B was added into each wells and incubated at 37°C for 10 min.

⇩

50µl of stop solution was added and optical density was determined at 450 nm.

Figure 3.6 Protocol of MAO-B determination by ELISA

3.3 Data analysis

Data will be expressed as means ± SEM (standard error of mean). The outcomes will be analyzed by using the one way analysis of variance (ANOVA). Statistical analysis will be performed by Student’s paired *t* test as appropriate, and means will be considered significantly different when $P < 0.05$.

CHAPTER IV

RESULTS

4.1 Behavioral assessment of animal

4.1.1 Staircase experiment

4.1.1.1 15mg/kg and 30mg/kg MPTP in C57BL/6 mice

Result of staircase experiment in C57BL/6 mice is shown in Figure 4.1. After the mice became perfectly trained in staircase method, they were subjected to this experiment. Y-axis shows the number of cheese pellets fetched by mice in staircase experiment. Number of cheese fetched in staircase experiment was significantly reduced ($p=0.00018$ and $p=0.00028$ respectively) when mice were treated with 15mg/kg or 30mg/kg of MPTP intraperitoneally. Oral administration of 10mg/kg Sinemet® prior to 15 and 30 mg/kg MPTP administration significantly ameliorate the grasping ability of mice ($p=0.00$ and $p=0.00$ respectively) as compared with i.p. injection only. No significant change was shown in the control group, when the mice were orally fed with 3% acacia followed by i.p. injection of PBS.

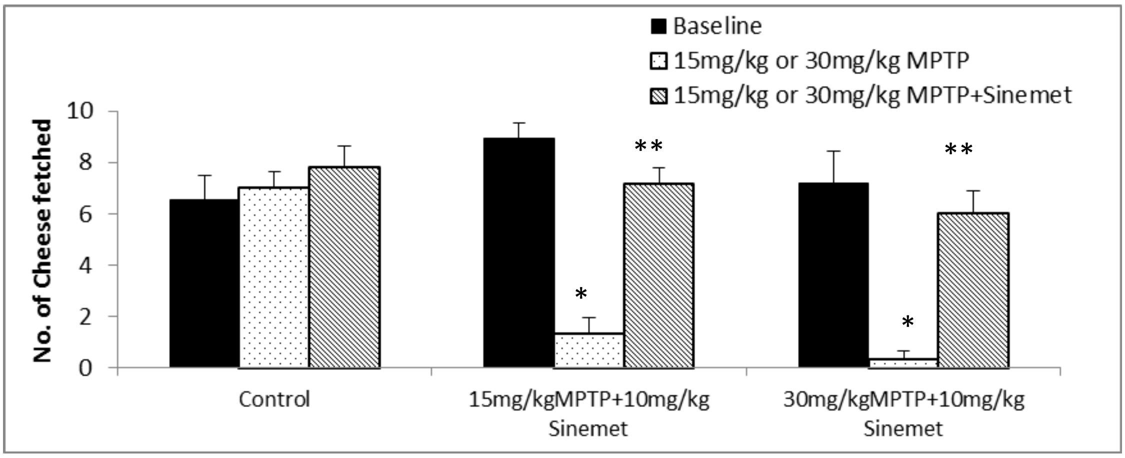


Figure 4.1 Staircase experiment of C57BL/6 mice treated with 15 and 30mg/kg MPTP ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E. of the number of cheese fetched from staircase.

*p value<0.05 as compared with baseline,

**p value<0.05 as compared with i.p. injection group

4.1.1.2 2.5mg/kg Rotenone in Wistar rats.

Result of staircase experiment in Wistar rats is shown in Figure 4.2. Y-axis shows the number of cheese pellets fetched by mice in staircase experiment. Number of cheese fetched in staircase experiment was significantly reduced (p=0.00) when rats were orally fed with 3% acacia and injected with 2.5mg/kg rotenone. Oral administration of 10mg/kg Sinemet ® prior to s.c. injection of 2.5mg/kg rotenone improved the performance of rats in staircase experiment significantly (p=0.001) as compared with 2.5mg/kg rotenone group.

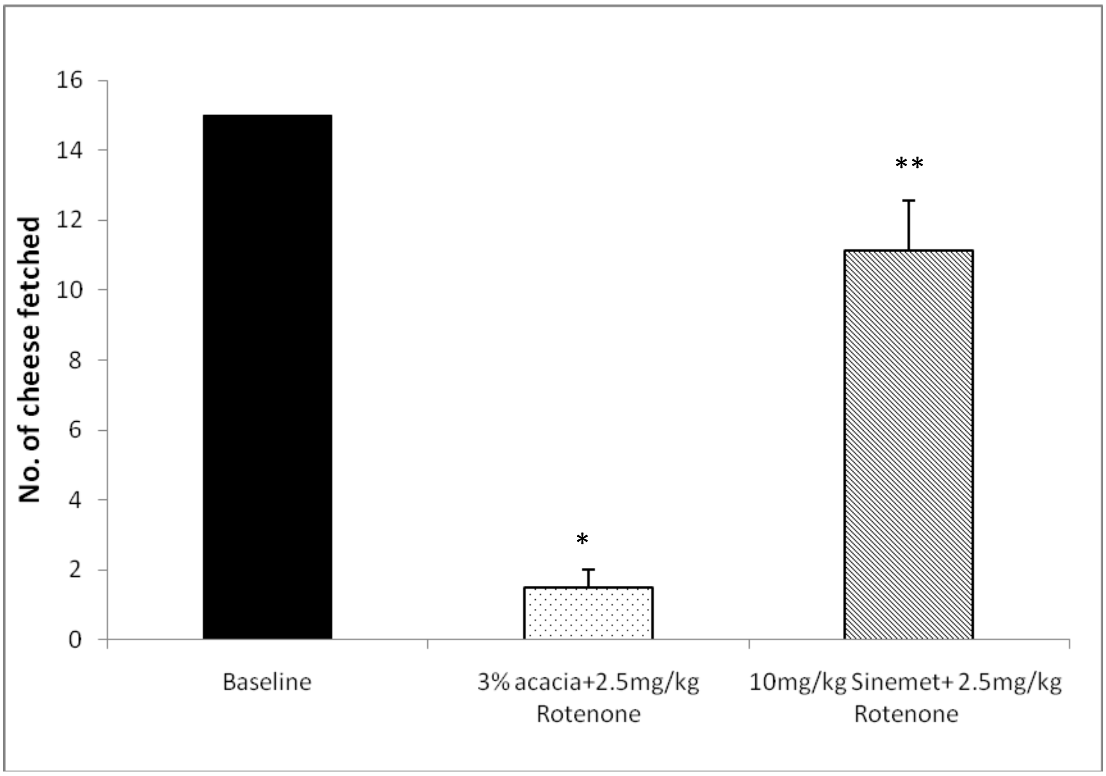


Figure 4.2 Staircase experiment of Wistar rats treated with 2.5mg/kg Rotenone ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E. of the number of cheese fetched from staircase.

*p value<0.05 as compared with baseline

**p value<0.05 as compared with 2.5mg/kg rotenone

4.1.2 Grip test

4.1.2.1 2.5mg/kg Rotenone in rats

Result of grip test in Wistar rats treated with 2.5mg/kg rotenone is shown on Fig 4.3. Y axis of the graph represents the peak force applied by rats on trapezoid bar when it was gently pulled backwards. The peak force was significantly reduced (p=0) as compared with peak force of baseline. Oral gavage of 10mg/kg Sinemet® significantly improved the grip force (p=0) as compared with 2.5mg/kg rotenone group.

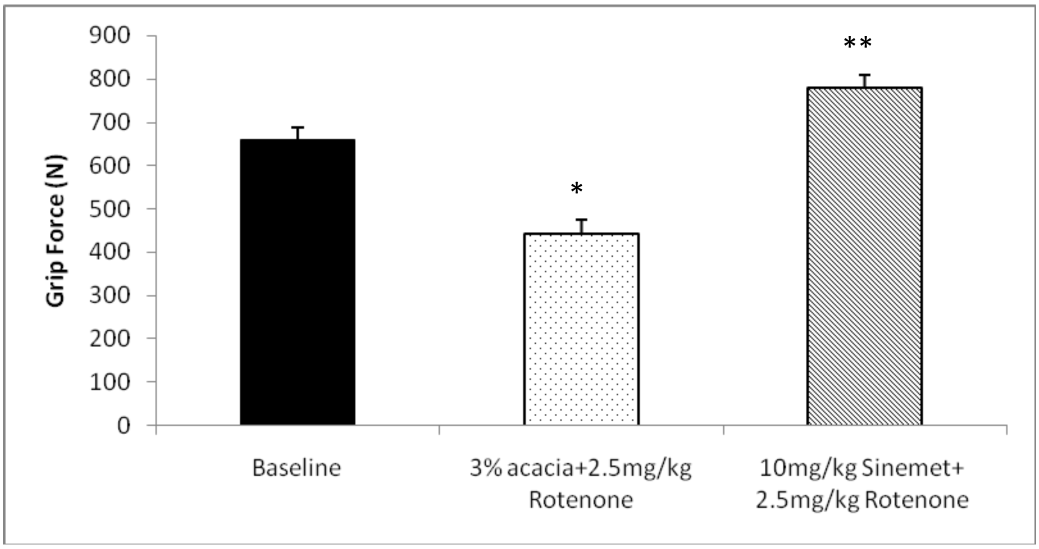


Figure 4.3 Grip test in Wistar rats treated with 2.5mg/kg rotenone ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of grip force.
*p value<0.05 as compared with baseline
**p value<0.05 as compared with 2.5mg/kg rotenone

4.2 Motor activity monitored by using FPA

Locomotion reflects the motor function of the body. Under the influence of certain drugs such as tacrine, MPTP and rotenone, the animal should experience some motor abnormality and may not move at all. Distance travelled and Bout Low Mobility (BLM) were used as parameters of locomotor activity. BLM was used to calculate motor activities in cases when the animal stays in one place and performs other activities like head bobbing, grooming and rearing. BLM scale ranges from 0-8, 0 indicates high motor activity while 8 represents the case in which the animal is not showing any kind of movement.

4.2.1 15mg/kg and 30mg/kg MPTP in C57BL/6 mice

Y axis represents distance travelled by mice during the experiment, measured in meters. Fig 4.4 illustrates that i.p. injection of 15mg/kg MPTP and 30mg/kg MPTP can significantly reduce distance travelled (p=0.000 and p=0.000

respectively) as compared with control. Administration of 10mg/kg Sinemet® prior to i.p. injection of 15mg/kg MPTP non-significantly improve the distance travelled (p=0.293) when compared with control. However, significant improvement was obtained in mice treated with 30mg/kg MPTP (p=0.007).

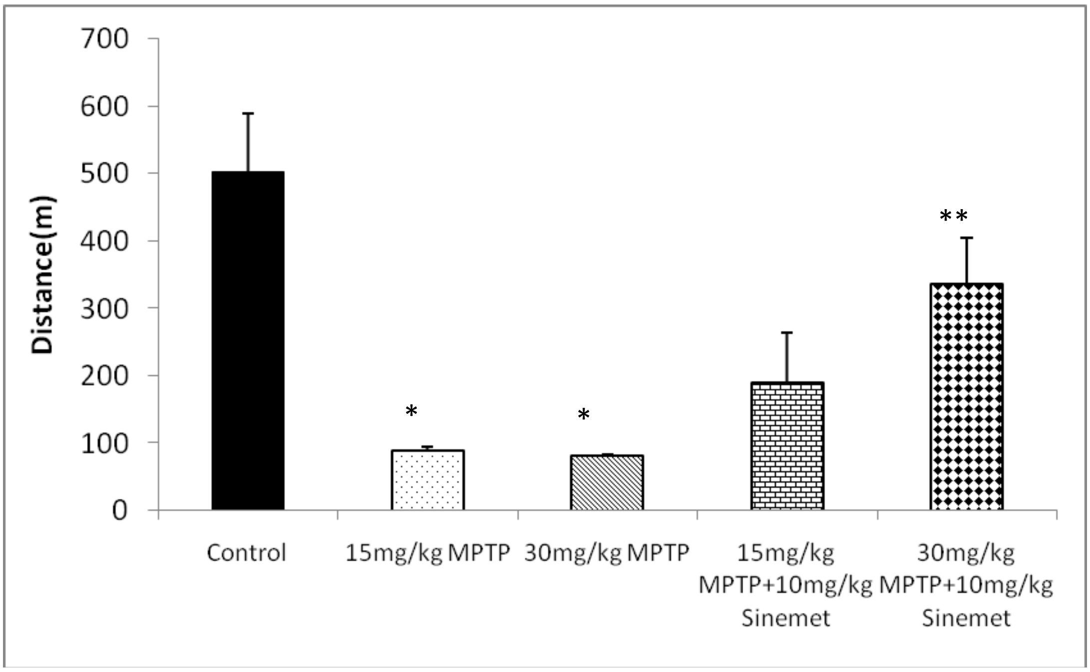


Figure 4.4: Distance travelled (m) by C57BL/6 mice treated with 15mg/kg MPTP and 30mg/kg MPTP ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of the distance travelled.
*p value<0.05 as compared with control
**p value<0.05 as compared with 30mg/kg MPTP

Fig 4.5 shows the result of BLM experiment in C57BL/6 mice treated with 15mg/kg and 30mg/kg MPTP. BLM was significantly reduced in both MPTP-treated groups (p=0.00, p=0.00) respectively as compared with control. Oral administration of 10mg/kg Sinemet® significantly improve BLM (p=0.005, p=0.005, respectively) in both 15 and 30mg/kg MPTP treated mice.

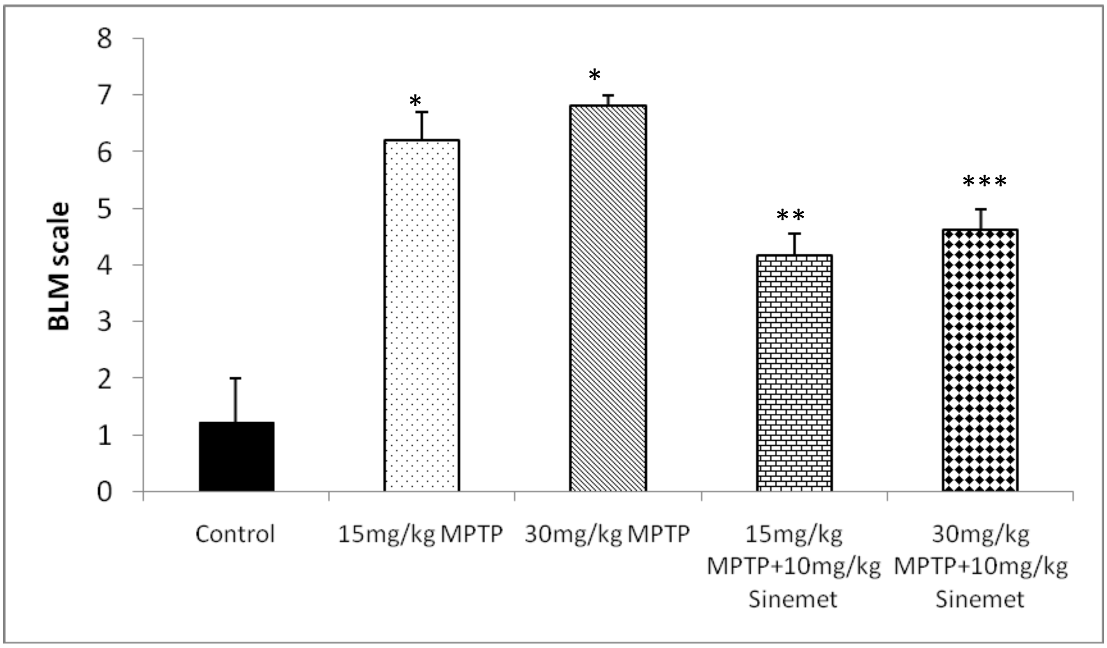


Figure 4.5 BLM scale obtained from C57BL/6 mice treated with 15mg/kg MPTP and 30mg/kg MPTP ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of BLM scale.
*p value<0.05 as compared with control.
**p value<0.05 as compared with 15mg/kg MPTP
***p value<0.05 as compared with 30mg/kg MPTP

4.2.2 5mg/kg Tacrine in C57BL/6 mice

Fig 4.6 presents the distance travelled (m) by mice treated with 5mg/kg tacrine. Y axis of graph shows distance travelled in meters. The distance travelled by mice was significantly reduced with 5mg/kg tacrine treatment (p= 0.009) as compared with control. Oral administration of 10mg/kg Sinemet® improved the locomotion significantly (p=0.039) as compared with 5mg/kg tacrine group.

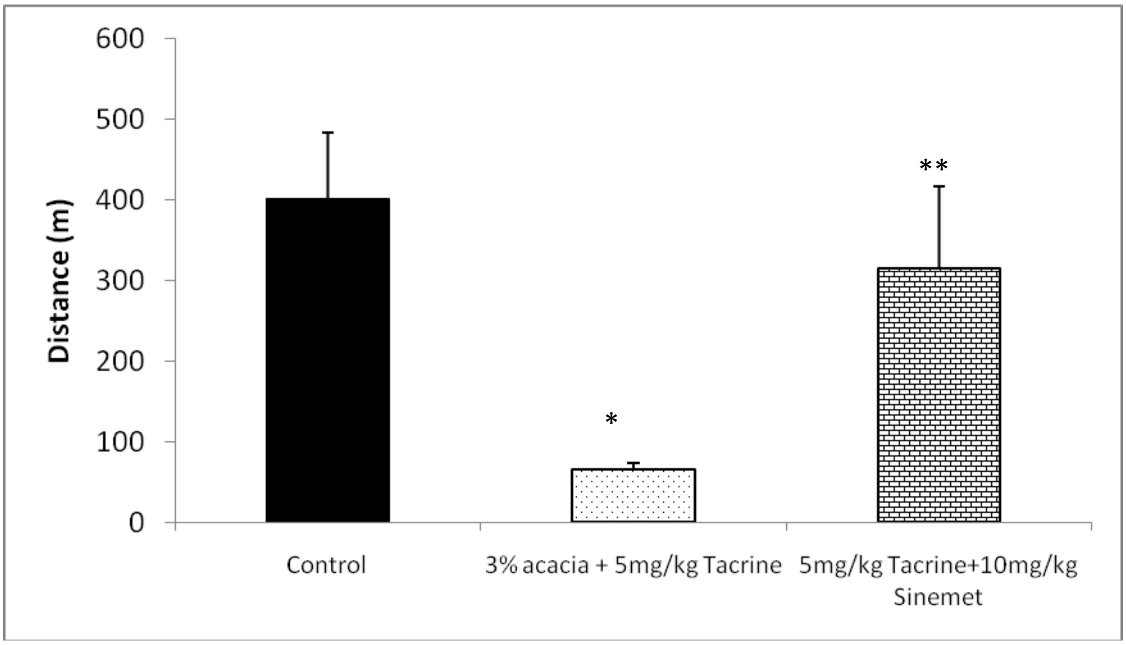


Figure 4.6 Distance travelled (m) by C57BL/6 mice treated with 5mg/kg Tacrine ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of the distance travelled.
*p value<0.05 as compared with control.
**p value<0.05 as compared with 5mg/kg tacrine.

Fig 4.7 shows the result of BLM experiment in C57BL/6 mice treated with 5mg/kg tacrine. Y axis of graph shows BLM scale which ranges from 0-8. BLM of mice injected with 5mg/kg tacrine was significantly reduced (p= 0.009) as compared with control. Oral administration of 10mg/kg Sinemet® also improved BLM significantly (p=0.039) as compared with 5mg/kg tacrine group. This result suggests that 10mg/kg Sinemet® can remarkably ameliorate the motor symptoms induced by tacrine in mice.

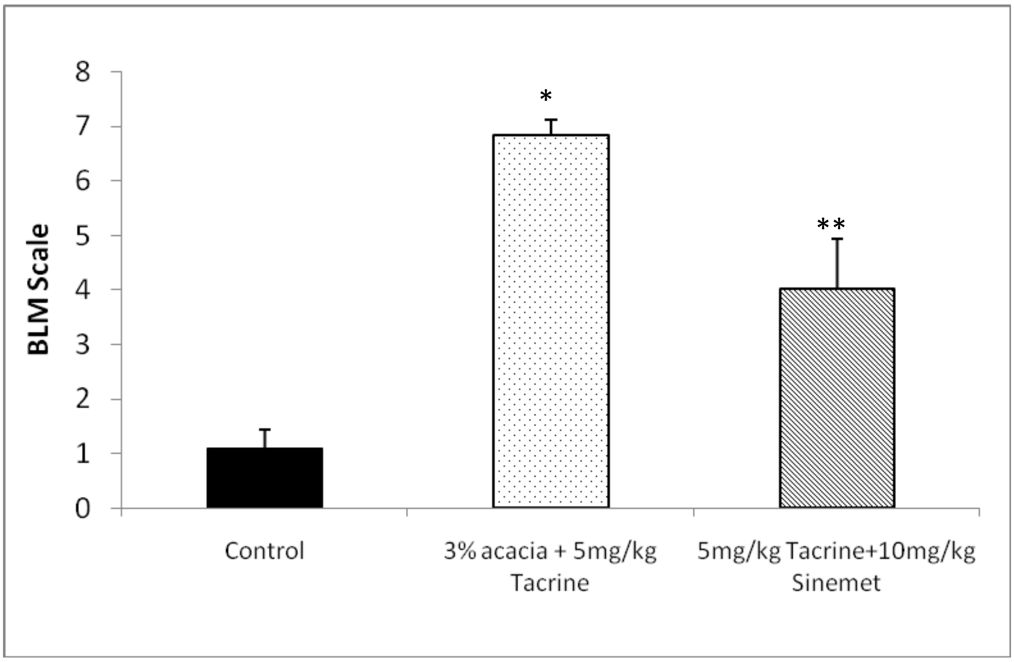


Figure 4.7 BLM scale obtained from C57BL/6 mice treated with 5mg/kg tacrine 10mg/kg Sinemet®. Data expressed as Mean ± S.E of BLM scale.
*p value<0.05 as compared with control,
**p value<0.05 as compared with 5mg/kg tacrine.

4.2.3 5mg/kg Tacrine in Wistar rats

Fig 4.8 presents the distance travelled (m) by rats treated with 5mg/kg tacrine. Y axis shows distance travelled in meters by rats. Rats of 5mg/kg tacrine group showed significant reduction in distance travelled (p=0.00) as compared with control rats who received only vehicles. Oral feeding of 10mg/kg Sinemet® prior to injection of tacrine was able to significantly improve the distance travelled (p=0.006) as compared with 5mg/kg tacrine group.

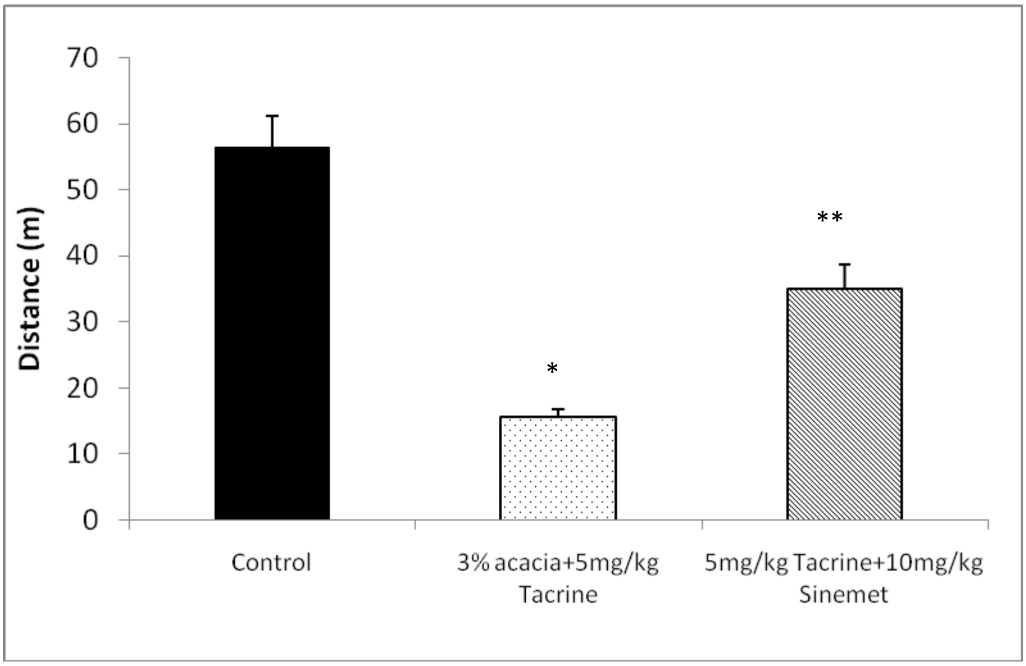


Figure 4.8 Distance travelled (m) by rats treated with 5mg/kg Tacrine ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of the distance travelled.
*p value<0.05 as compared with control,
**p value<0.05 as compared with 5mg/kg tacrine.

Fig 4.9 shows the result of BLM experiment in rats treated with 5mg/kg tacrine. Y-axis represents the BLM scale that ranges from 0-8. Rats injected with 5mg/kg tacrine showed significant increase in BLM (p=0.01) as compared with control, who received only vehicles. Oral feeding of 10mg/kg Sinemet® prior to injection of tacrine significantly reduce BLM scale (p=0.032) as compared with 5mg/kg tacrine group.

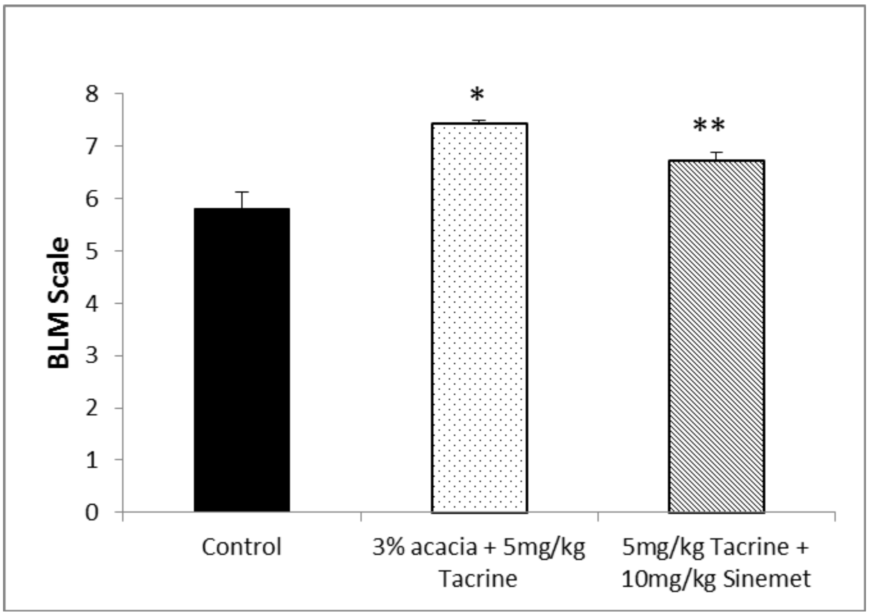


Figure 4.9 BLM scale obtained from rats treated with 5mg/kg tacrine ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of BLM scale.
*p value<0.05 as compared with control,
**p value<0.05 as compared with 5mg/kg Tacrine.

4.2.4 2.5mg/kg Rotenone experiment in Wistar rats

4.10 presents the distance travelled (m) by rats treated with 2.5mg/kg rotenone. Y axis shows distance travelled in meters by rats. The injection of 2.5mg/kg rotenone in rats showed significant reduction in distance travelled (p=0.00) as compared with control rats who received only vehicles. Oral feeding of 10mg/kg Sinemet® prior to injection of rotenone significantly improve the distance travelled (p=0.012) as compared with 2.5mg/kg rotenone group.

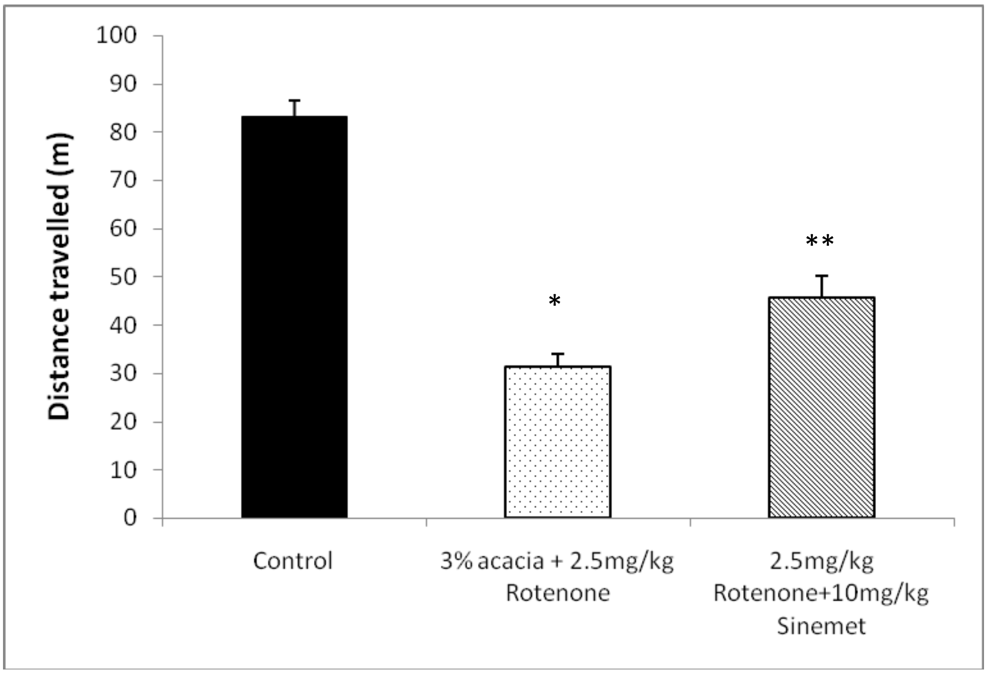


Figure 4.10 Distance travelled (m) by rats treated with with 2.5mg/kg rotenone ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of the distance travelled.
*p value<0.05 as compared with control
**p value<0.05 as compared with 2.5mg/kg Rotenone

Fig 4.11 shows the result of BLM experiment in rats treated with 2.5mg/kg rotenone. Y-axis represents the BLM scale that ranges from 0-8. Rats injected with 2.5mg/kg rotenone showed significant increase in BLM scale (p=0.00) as compared with control, who received only vehicles. Oral feeding of 10mg/kg Sinemet® prior to injection of rotenone significantly reduce BLM scale (p=0.0017) as compared with 2.5mg/kg rotenone group.

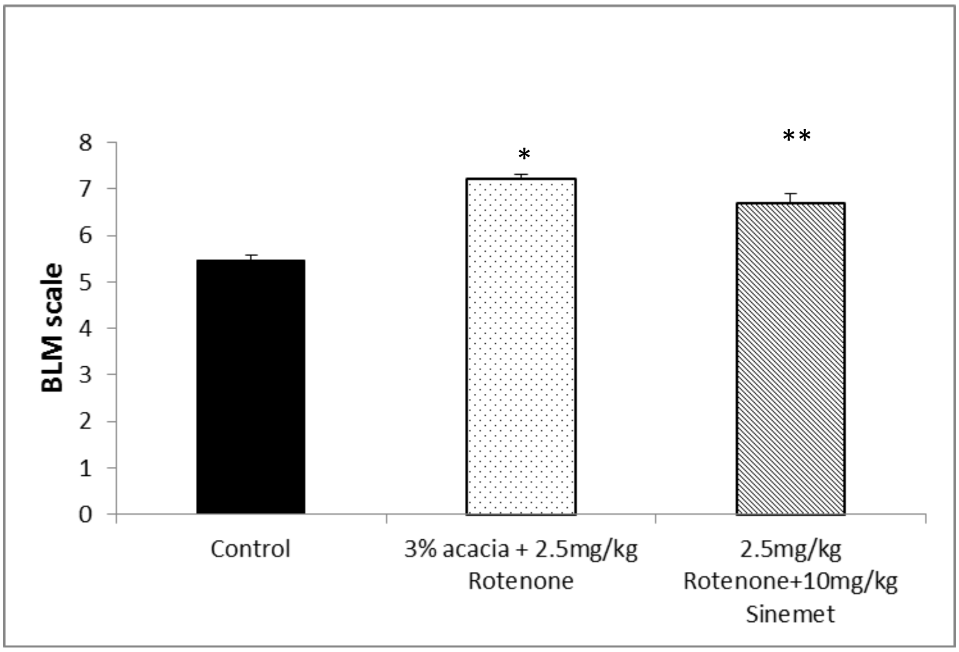


Figure 4.11 BLM scale obtained from rats treated with 2.5mg/kg rotenone ± 10mg/kg Sinemet®. Data expressed as Mean ± S.E of BLM scale.
*p value<0.05 as compared with control,
**p value<0.05 as compared with 2.5mg/kg rotenone

4.3 Power Spectra Analysis

Fig 4.12 shows the power spectra of a control mouse along with its trajectory of movement. Trajectory motion picture exhibits the position of mouse during that particular frame in which the spectrum was being analyzed. Due to the paw force while moving around, there was no peak that was distinctly noticeable from the rest peaks. The figure given below is frame 52. Since one frame has 80 sec, frame 52 will be around 56 min (3360 sec) after the i.p. injection of 0.1M PBS.

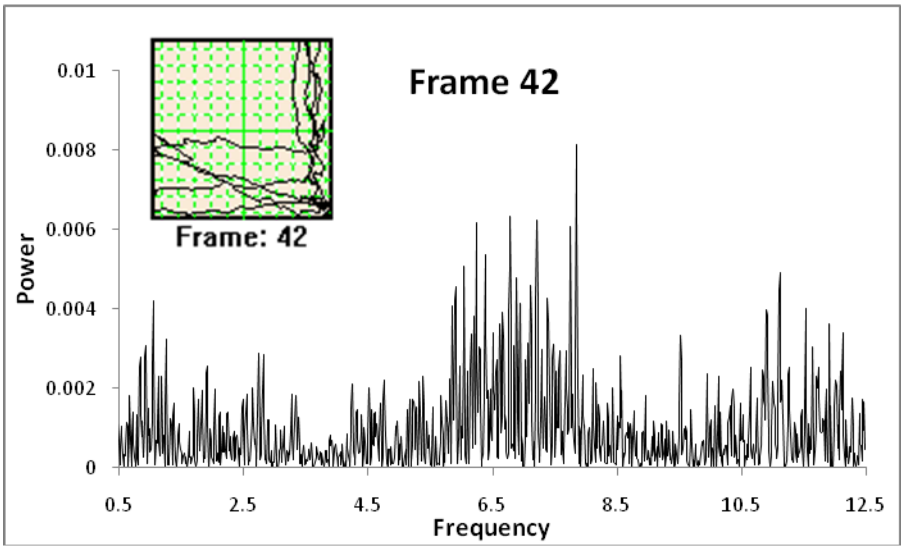


Figure 4.12 Trajectory motion and power spectrum of control mouse injected with 0.1M PBS.

4.3.1 Analysis of Power Spectra Obtained from C57BL/6 mice treated with 15 and 30mg/kg MPTP.

Figures4.13 and 4.14shows the power spectra of different frames produced by mice injected with 15mg/kg MPTP and 30mg/kg MPTP respectively. Choosing of power spectra for analyzing is dependent upon the trajectory of movement pictures of each mice. If the animal was moving in motion picture, that frame was discarded to reduce noise produced by paw force while walking. Maximum number of frames is 88 and the spectra taken for analysis were those in which the animal was not moving and there was one dot in motion picture. The result showed that there was some significant peaks at a range of 8.5Hz to 10.5 Hz. Therefore, we kept the frequency from 7-12 Hz. Even 30mg/kg MPTP showed similar kind of behavior. Hence the power spectra was condensed to 7-12 Hz to make a clear understading with spectra and frequency.

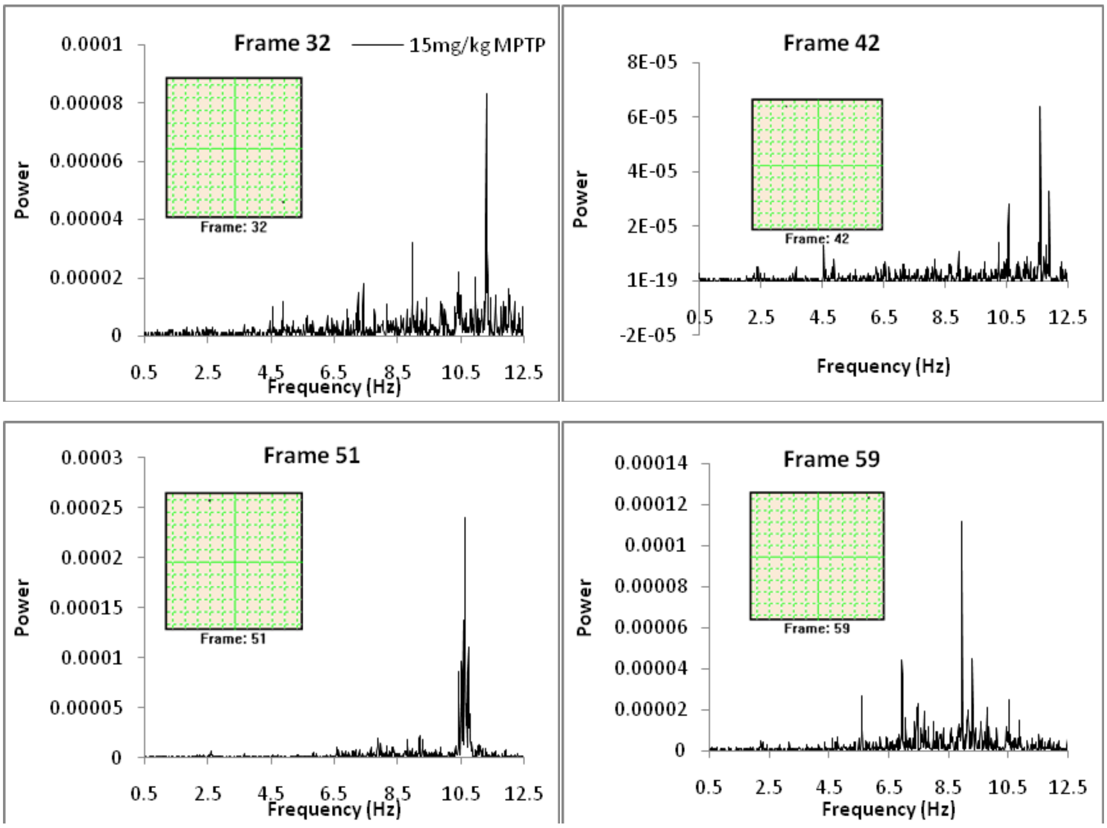


Figure 4.13 Power spectra obtained from mouse treated with 15mg/kg MPTP along with their trajectory motion picture.

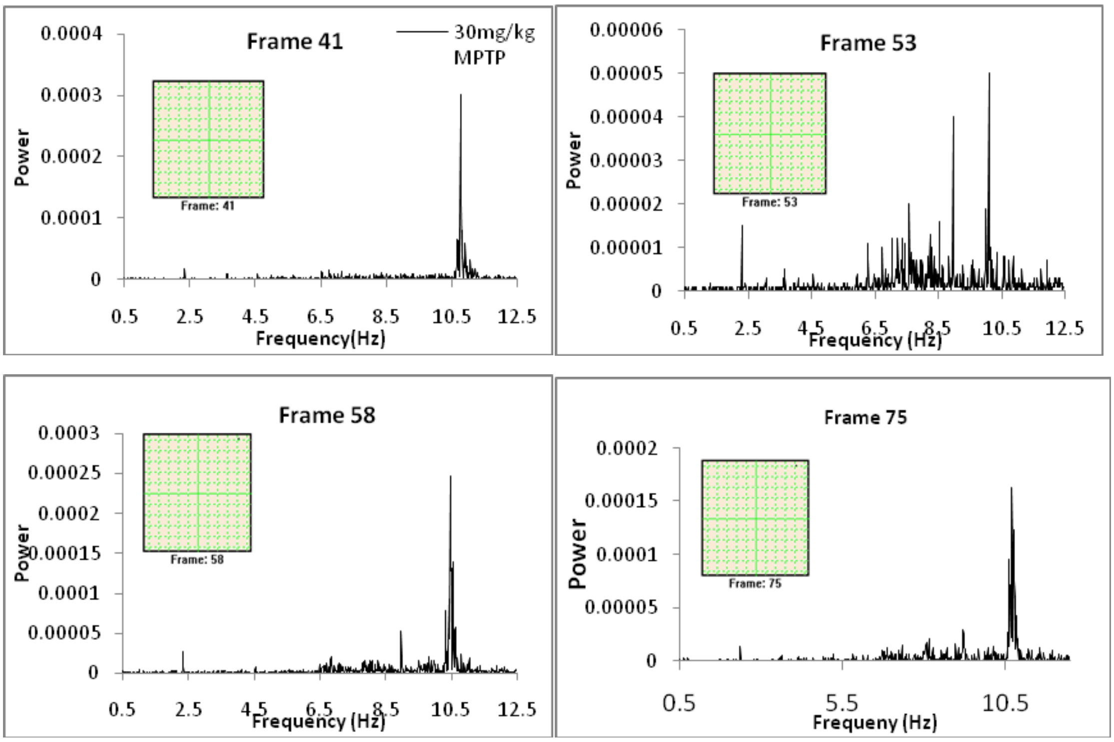


Figure 4.14 Power spectra obtained from mouse treated with 30mg/kg MPTP along with their trajectory motion picture.

4.3.1.1 Comparison of power spectra between control and 15mg/kg MPTP treatment, and between control and 30mg/kg MPTP treatment.

Comparison of power spectra between control mice and 15mg/kg MPTP treated mice, and between control mice and 30mg/kg MPTP treated mice are shown in fig 4.15 and fig 4.16. As for 15 mg/kg MPTP, we selected those frames in which the animal was not moving to reduce any kind of noise that makes our peak of interest insignificant. However, control are those mice that only administered vehicle. So, they acted normally and hoovered around while mice of 15mg/kg MPTP group stayed in one place. This phenomenon is also similar with 30mg/kg MPTP treatment.

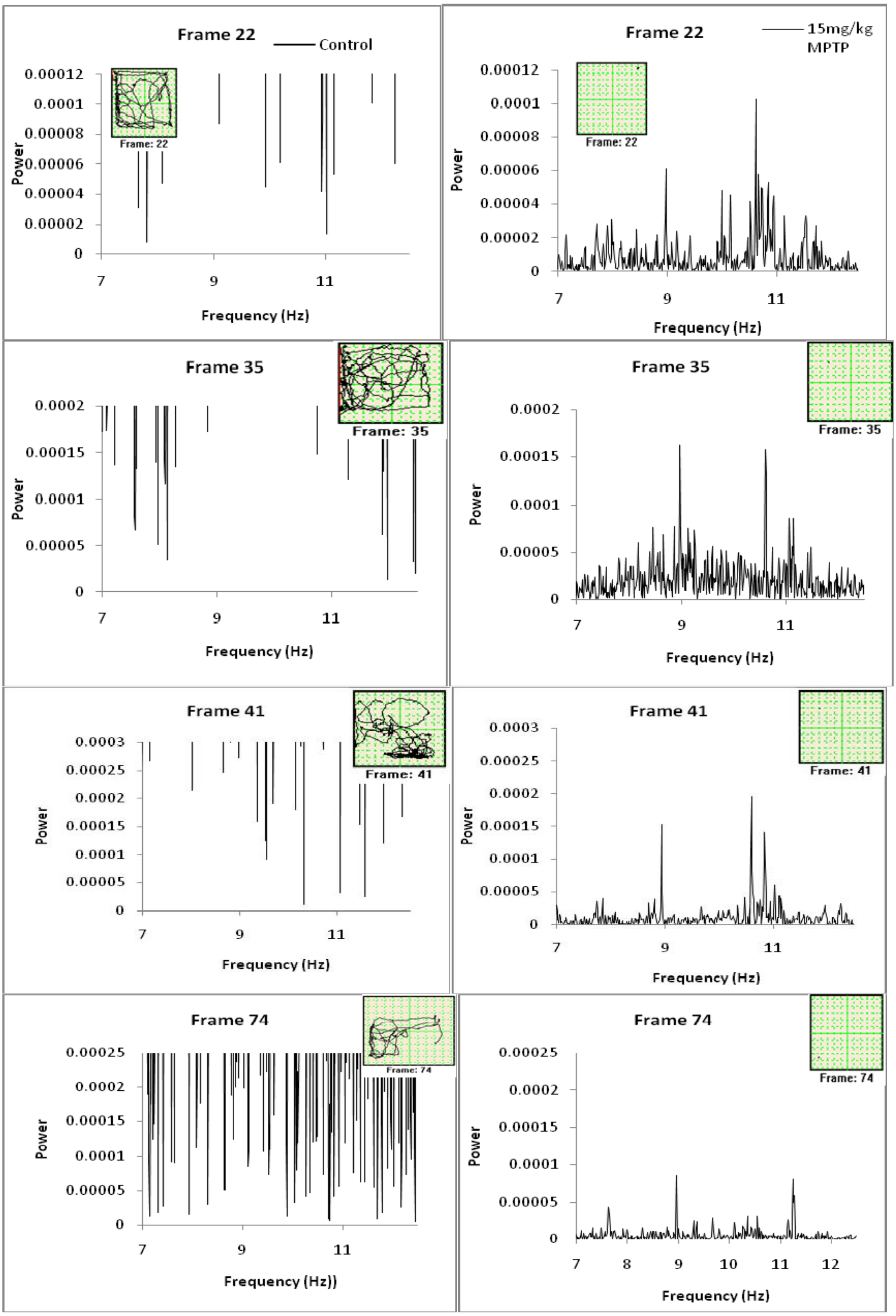


Figure 4.15 Comparison of power spectra between Control and 15mg/kg MPTP treated groups along with their trajectory motion picture.

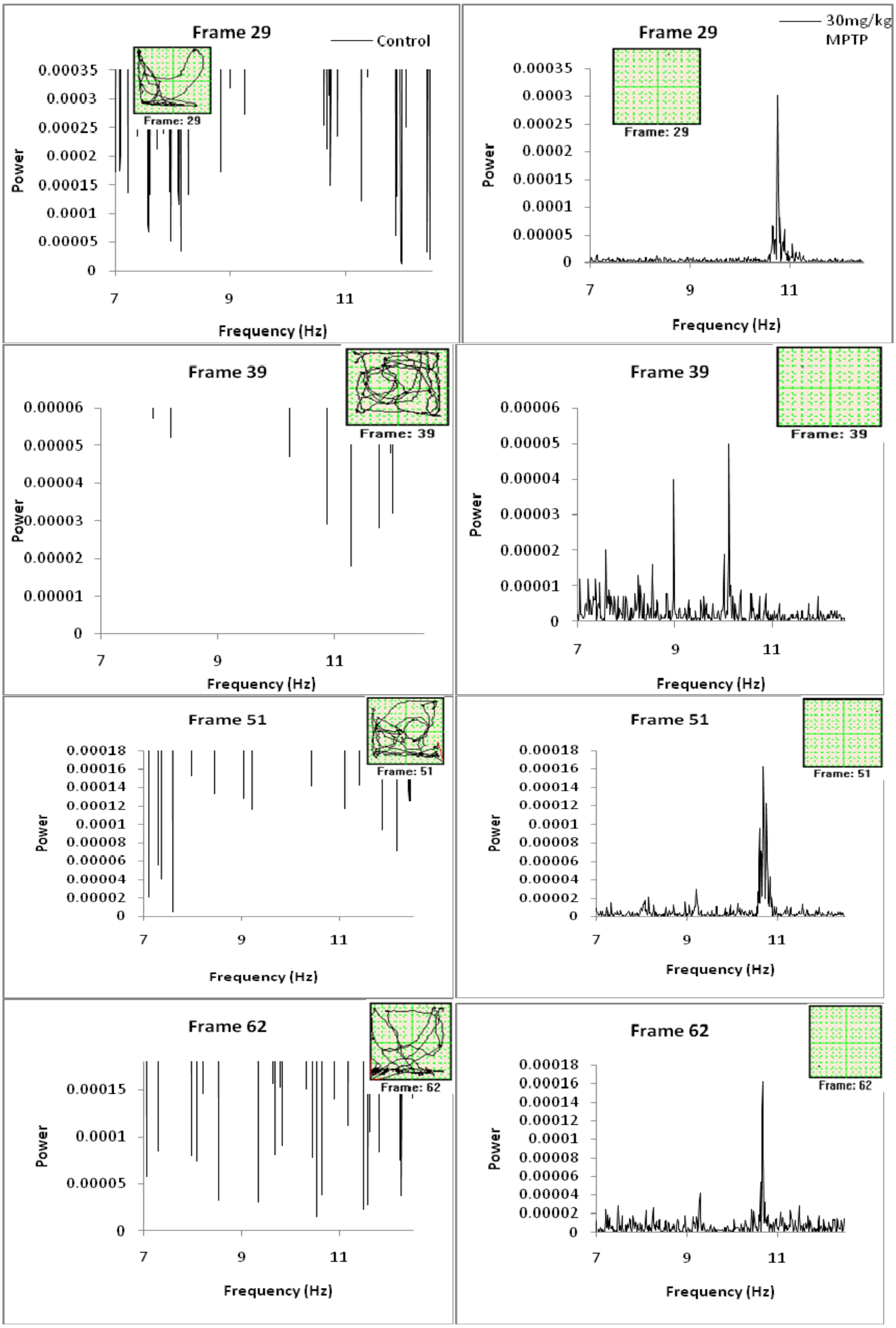


Figure 4.16 Comparison of power spectra between Control and 30mg/kg MPTP treated groups along with their trajectory motion picture.

4.3.1.2 Comparison of power spectra between 15mg/kg MPTP treated and 15mg/kg MPTP+10mg/kg Sinemet® treated groups.

Figure 4.17. shows the comparison of power spectra between 15mg/kg MPTP treated and 15mg/kg MPTP+10mg/kg Sinemet® treated groups. Those power spectra peaks were analysed by selecting from those frames in which the mice were not moving at all. If the peaks were significant, then we expect it to be the parkinsonian symptoms. As the neurotoxin injected was MPTP, we anticipate the peaks to be tremor and rigidity. Oral administration of 10mg/kg Sinemet® reduced the intensity of those peaks induced by 15mg/kg MPTP. Results of analysis of power spectra were choosen and kept in chronological order according to their frame numbers.

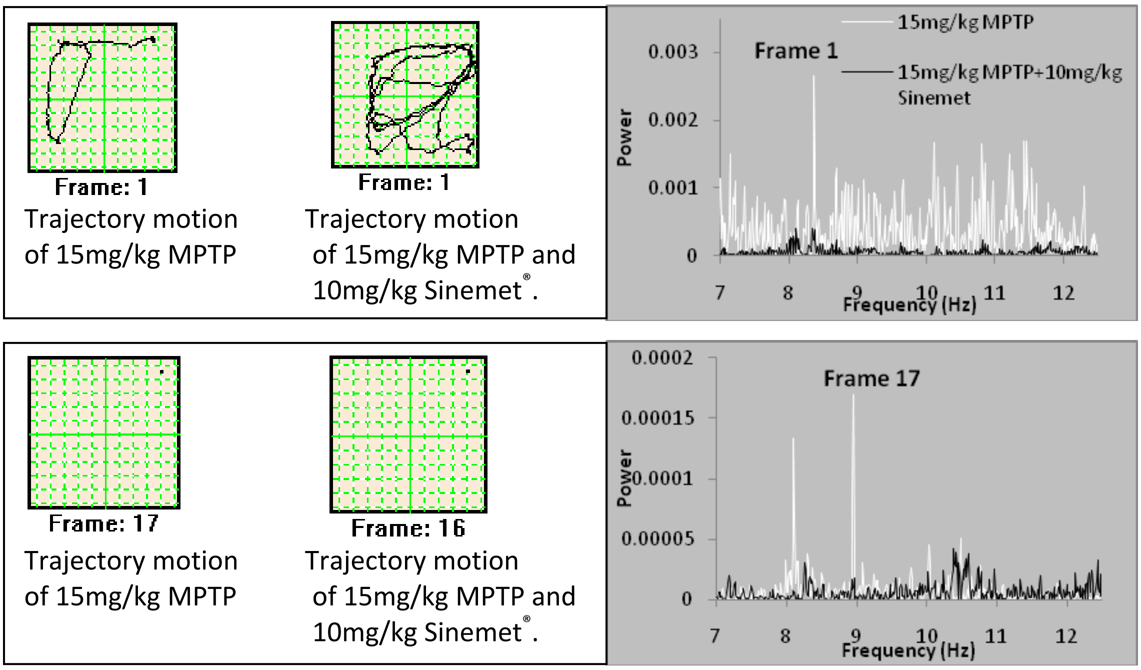
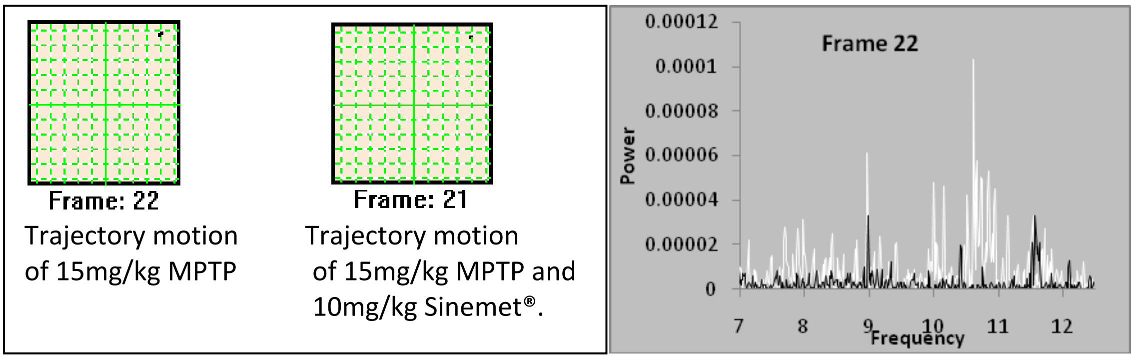
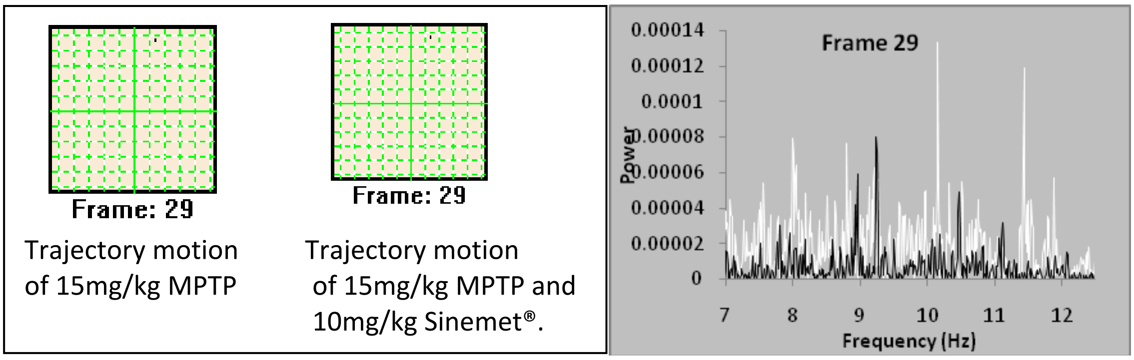


Figure 4.17 Comparison of power spectra between 15mg/kg MPTP treated and 15mg/kg MPTP+10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



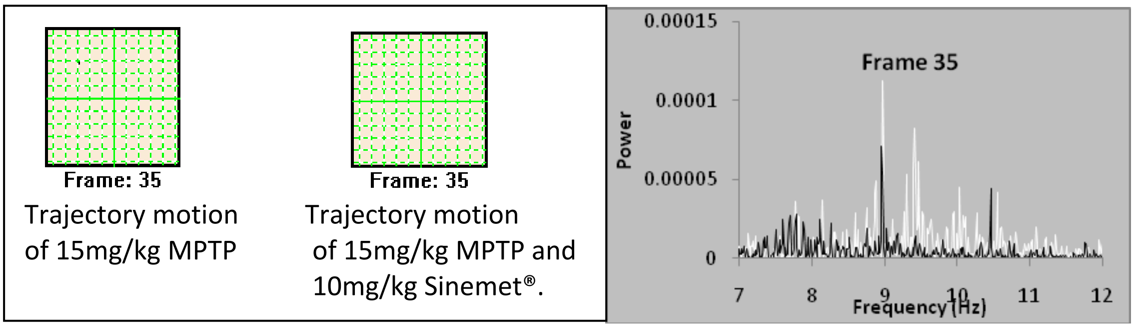
Trajectory motion of 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 15mg/kg MPTP

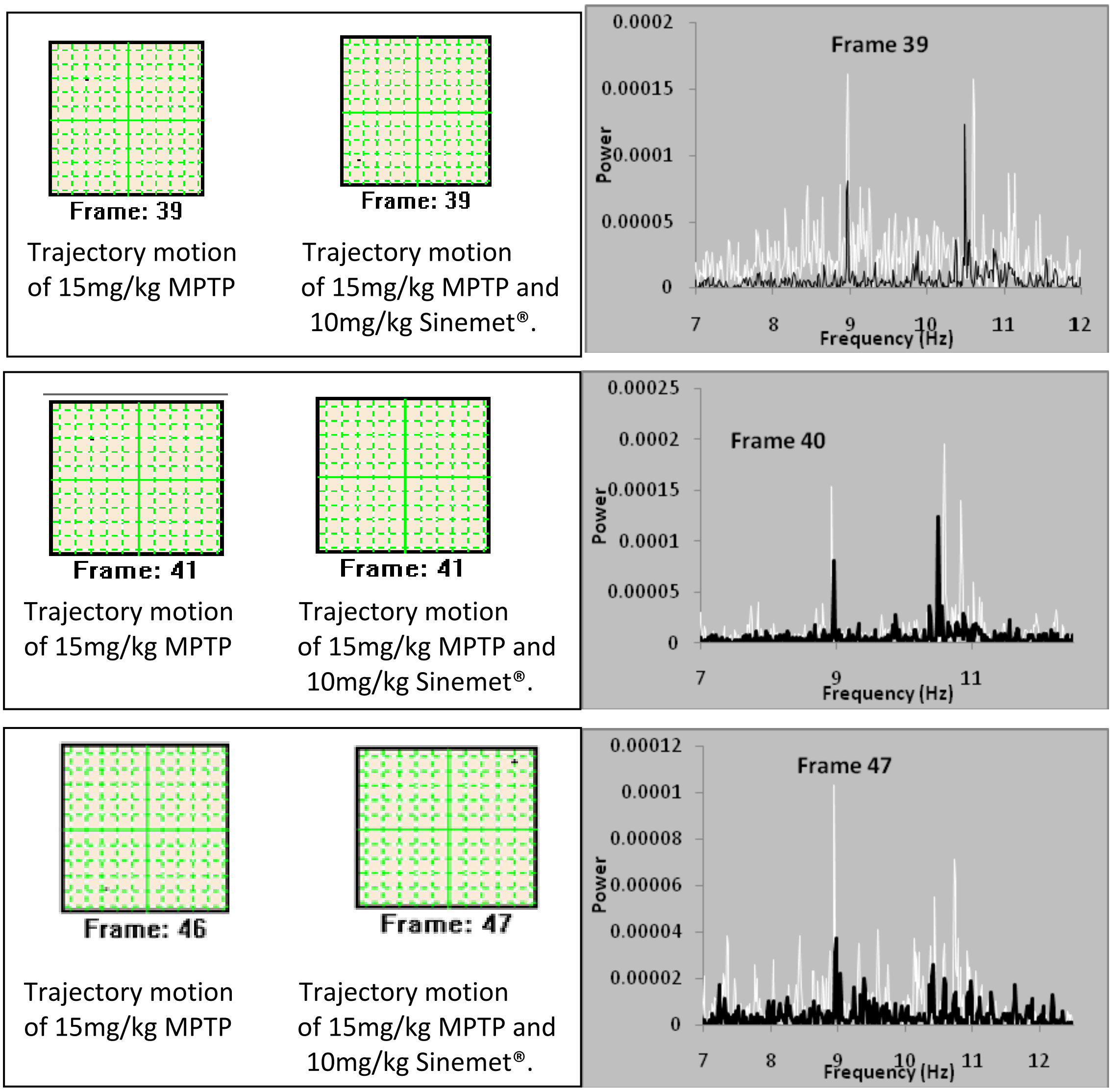
Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.17 Comparison of power spectra between 15mg/kg MPTP treated and 15mg/kg MPTP+10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



Trajectory motion of 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

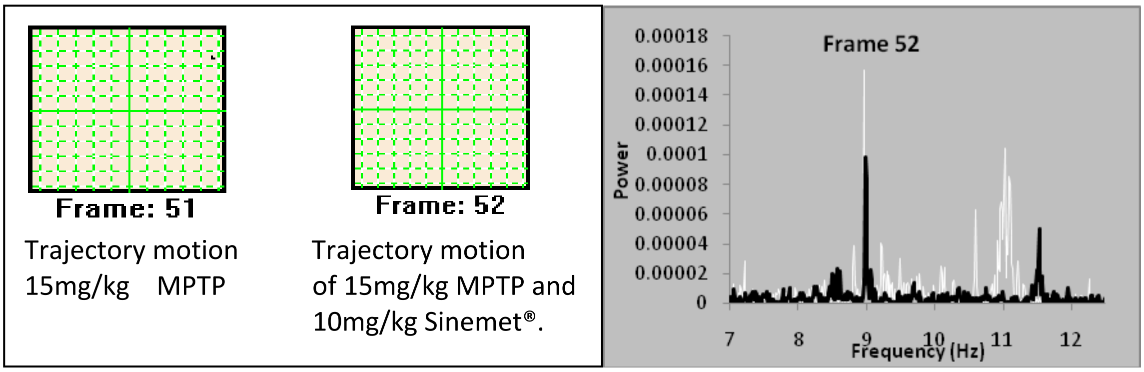
Trajectory motion of 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

Trajectory motion of 15mg/kg MPTP

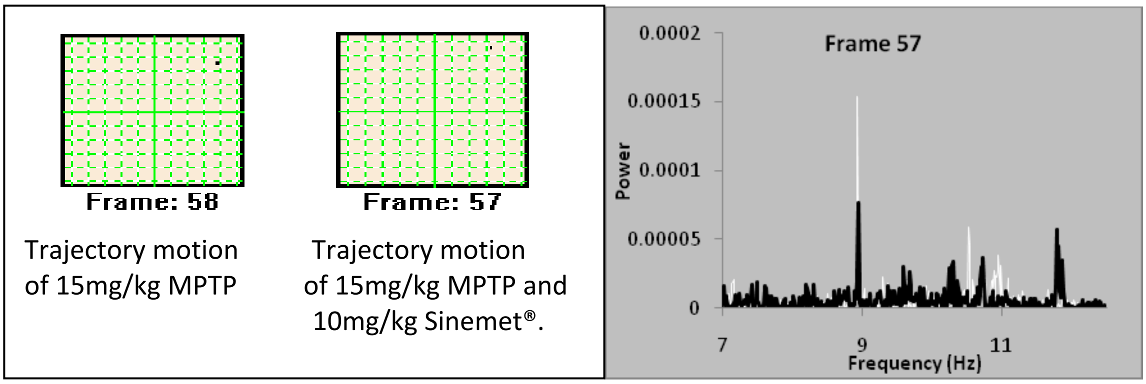
Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.17 Comparison of power spectra between 15mg/kg MPTP treated and 15mg/kg MPTP+10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



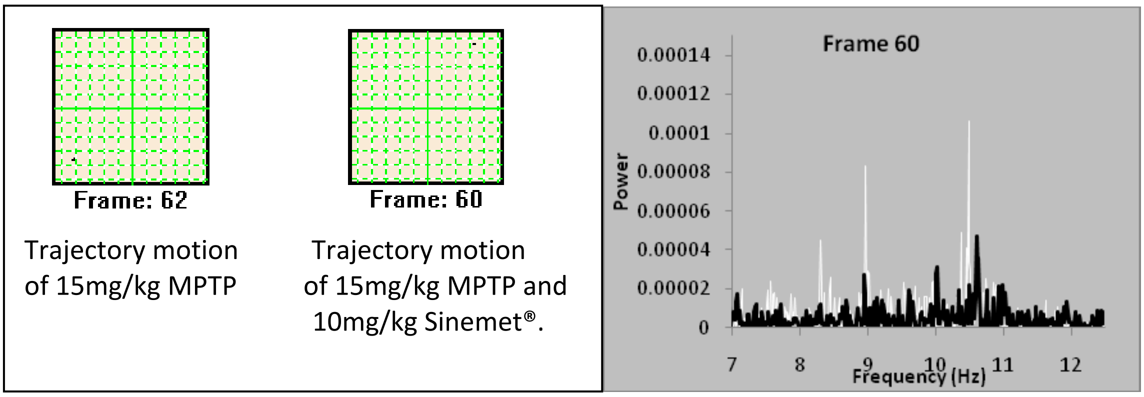
Trajectory motion 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 15mg/kg MPTP

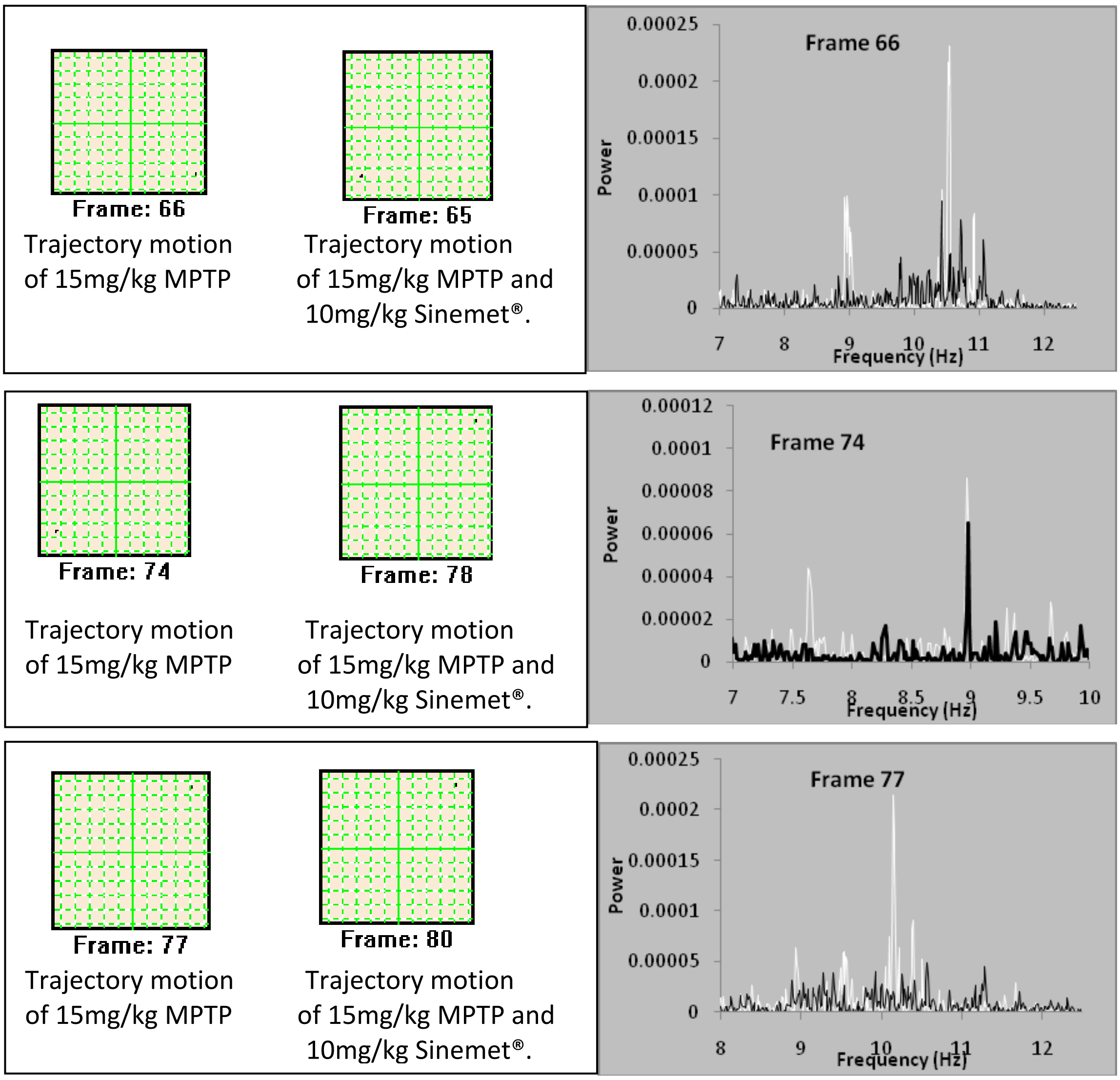
Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.17 Comparison of power spectra between 15mg/kg MPTP treated and 15mg/kg MPTP+10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



Trajectory motion of 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

Trajectory motion of 15mg/kg MPTP

Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

Trajectory motion of 15mg/kg MPTP

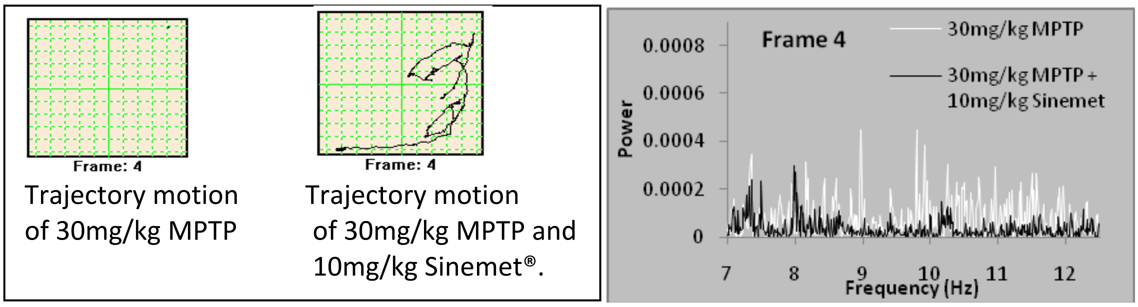
Trajectory motion of 15mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.17 Comparison of power spectra between 15mg/kg MPTP treated and 15mg/kg MPTP+10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session.

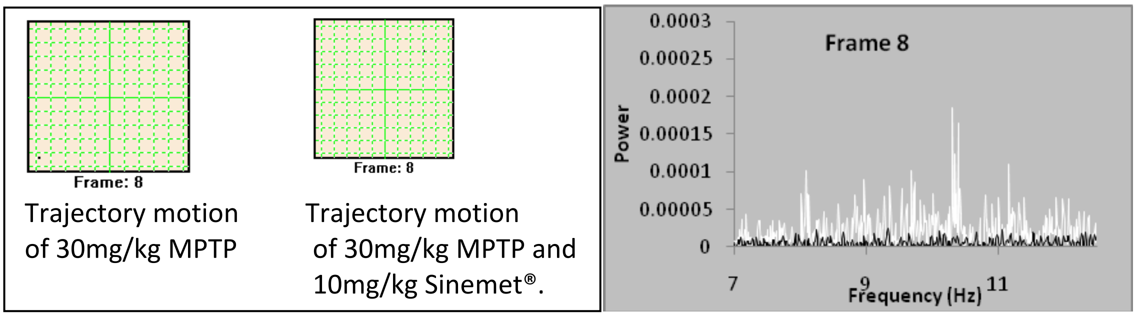
4.3.1.3 Comparison of power spectra between 30mg/kg MPTP treated and 30mg/kg MPTP+10mg/kg Sinemet® treated groups.

Figure 4.18. shows the comparison of power spectra between 30mg/kg MPTP treated and 30mg/kg MPTP +10mg/kg Sinemet® treated groups. Peak of spectra of 30mg/kg MPTP and 30mg/kg MPTP+10mg/kg Sinemet® groups were

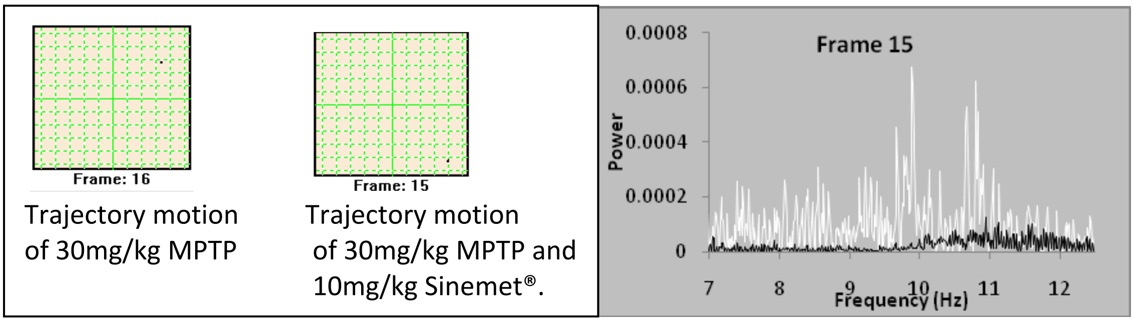
analysed using those frames in which the mice were not moving at all. If the peaks were significant, then we considered it do be the parkinsonian symptoms. As the neurotoxin injected is MPTP, we considered the peaks to be tremor and rigidity. Oral administration of 10mg/kg Sinemet®® was shown to reduce the peaks induced by 30mg/kg MPTP injection.


Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

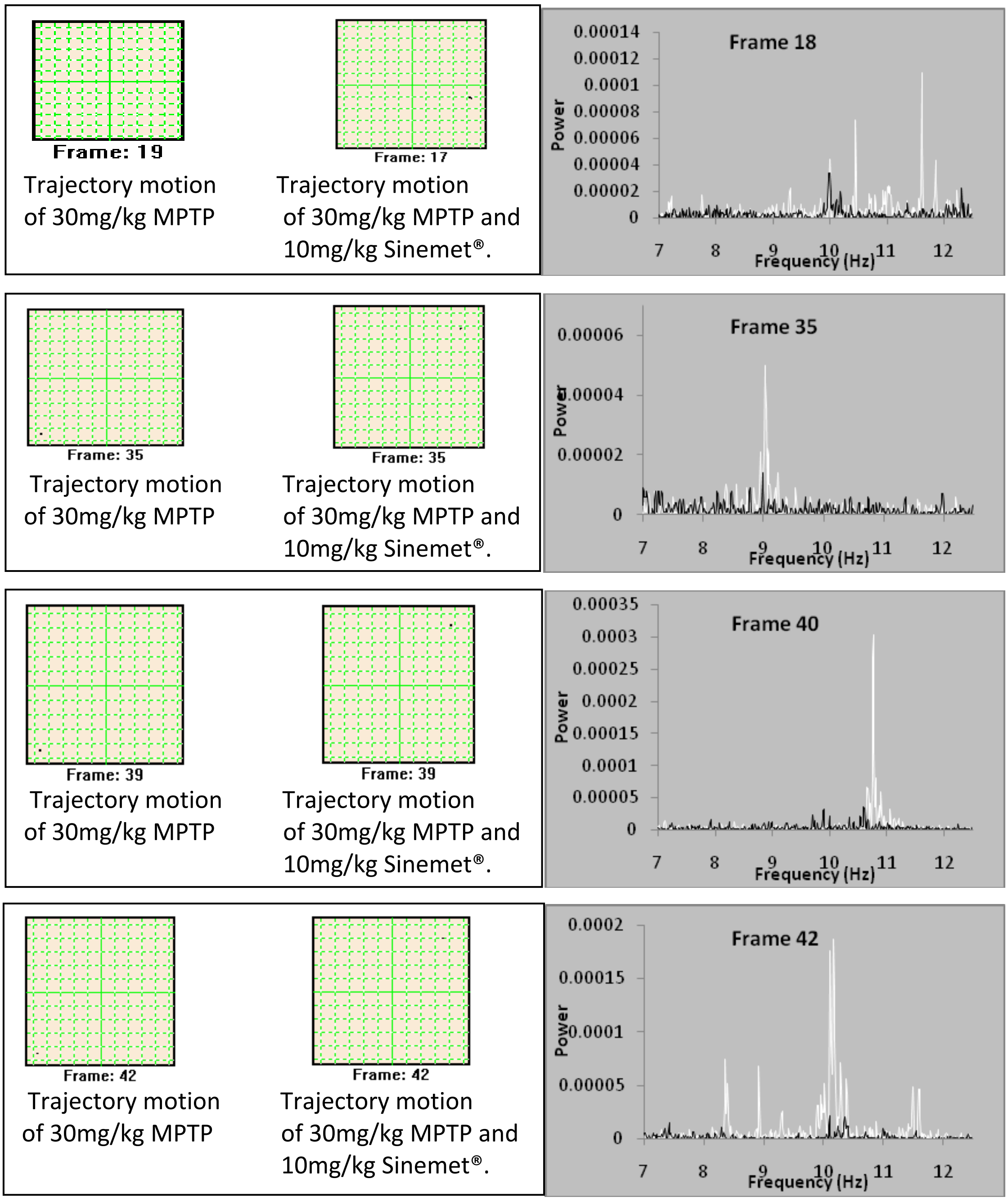

Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.


Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.18 Comparison of power spectra between 30mg/kg MPTP treated and 30mg/kg MPTP+10mg/kg Sineme® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

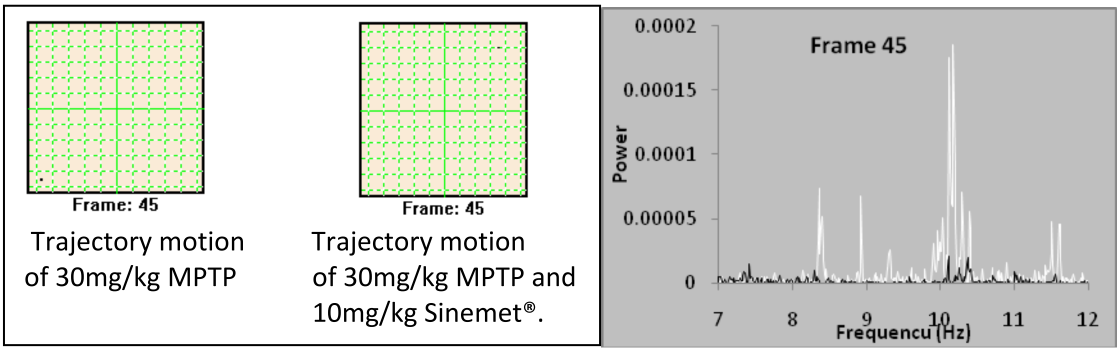
Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Trajectory motion of 30mg/kg MPTP

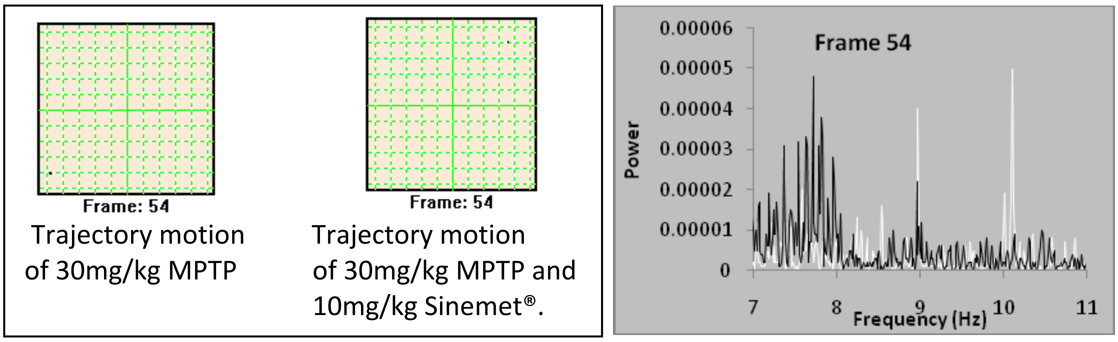
Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.18 Comparison of power spectra between 30mg/kg MPTP treated and 30mg/kg MPTP+10mg/kg Sineme® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



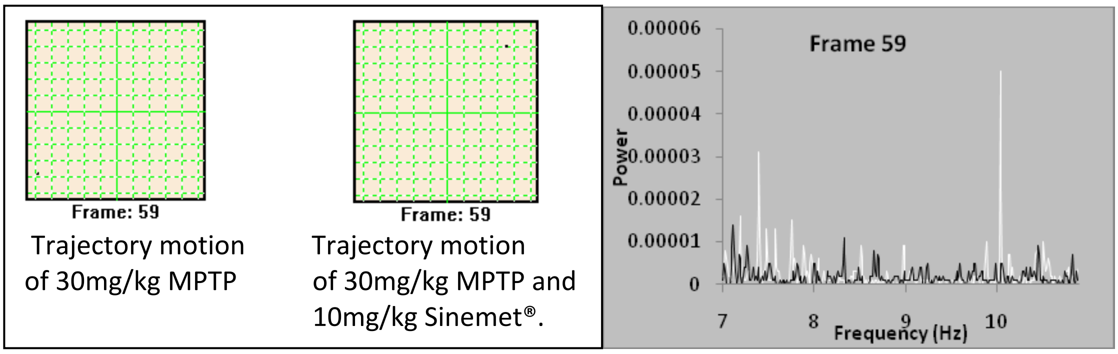
Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 30mg/kg MPTP

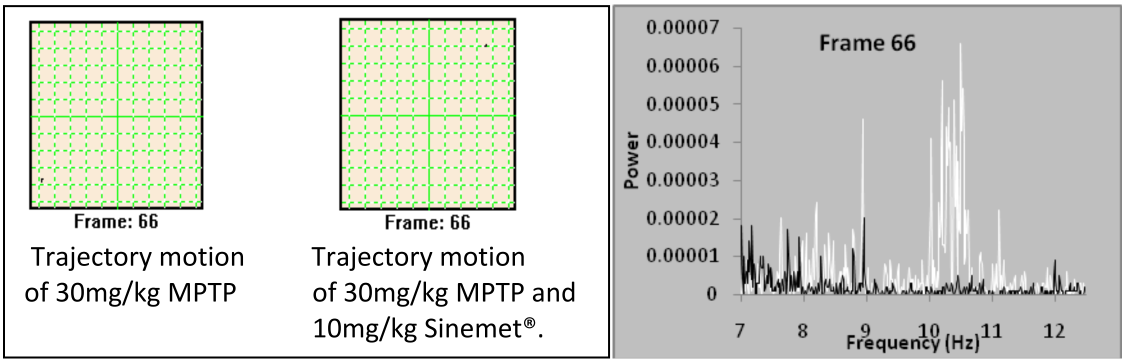
Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 30mg/kg MPTP

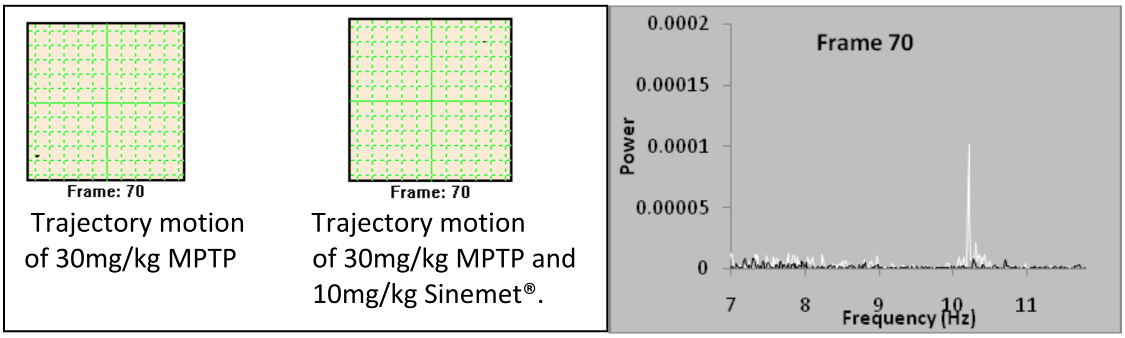
Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.18 Comparison of power spectra between 30mg/kg MPTP treated and 30mg/kg MPTP+10mg/kg Sineme® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



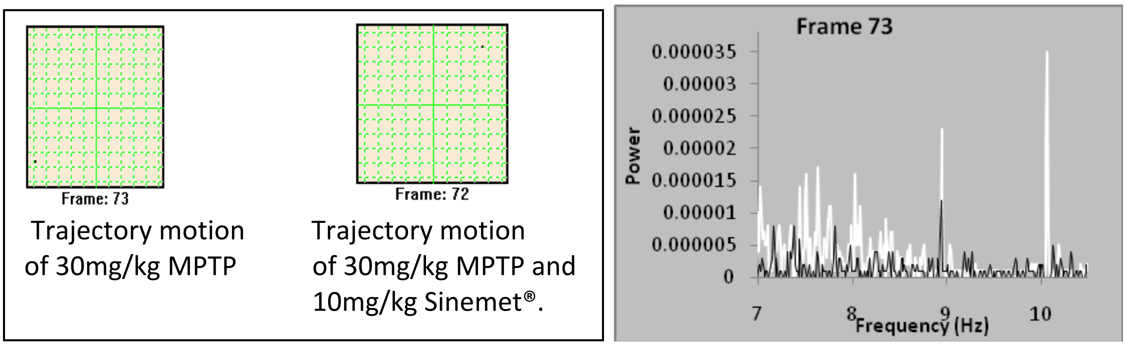
Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.



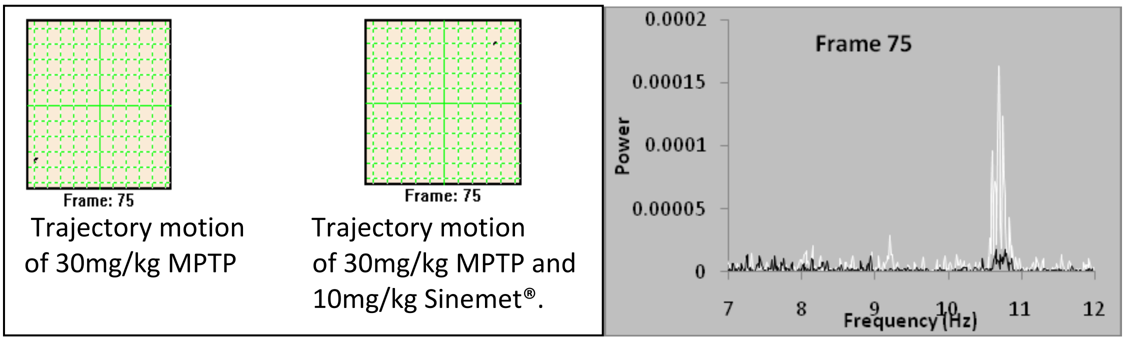
Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 30mg/kg MPTP

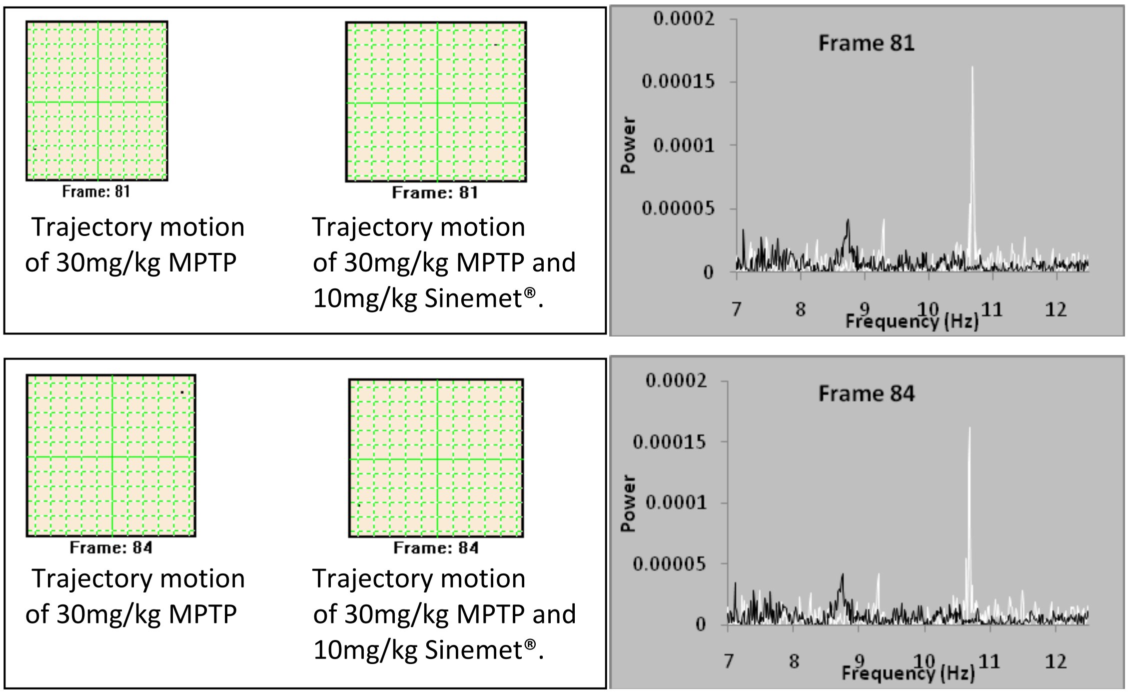
Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.



Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.18 Comparison of power spectra between 30mg/kg MPTP treated and 30mg/kg MPTP+10mg/kg Sineme® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Trajectory motion of 30mg/kg MPTP

Trajectory motion of 30mg/kg MPTP and 10mg/kg Sinemet®.

Figure 4.18 Comparison of power spectra between 30mg/kg MPTP treated and 30mg/kg MPTP+10mg/kg Sineme® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session.

4.3.2 Power Spectra of 5mg/kg Tacrine treated mice.

As the animals were kept inside FPA for 1 hr and 30 min for tacrine expermiment in both mice and rats, the maximum number of frames was 66. Figure 4.19 shows the power spectra of mice treated with 5mg/kg tacrine. We found some significant peaks at around 11-12 Hz. Thus, we kept the frequency from 10-12 Hz to make a clearer understading with spectra and frequency.

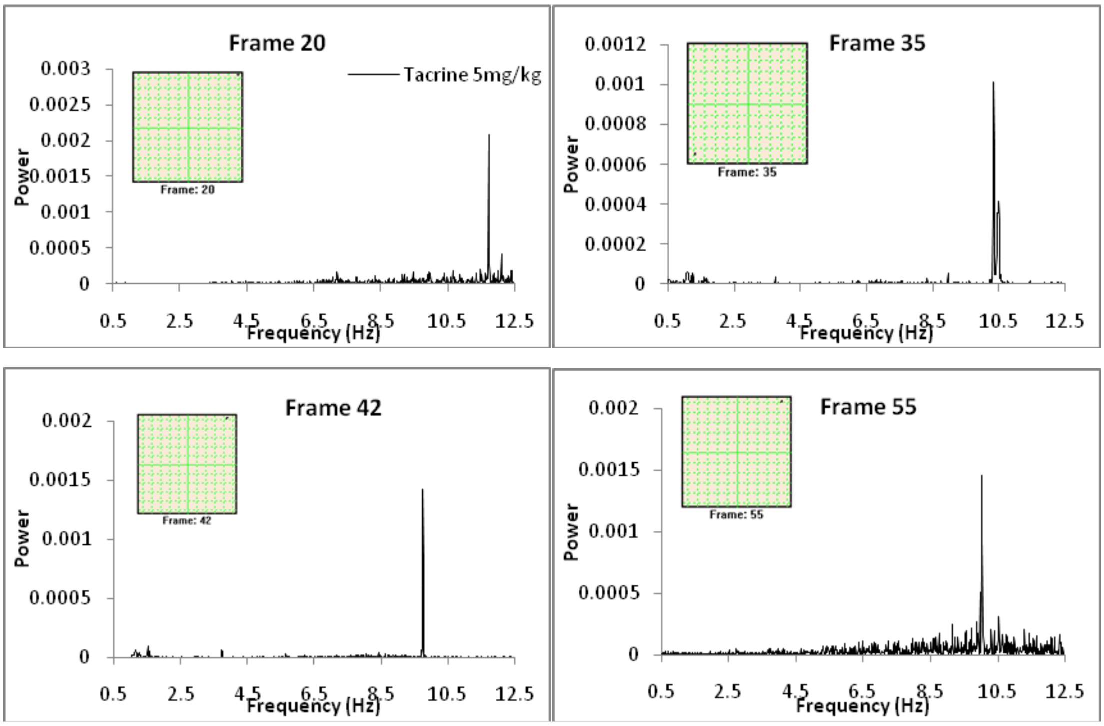


Figure 4.19 Power spectra of mice treated with 5mg/kg Tacrine.

4.3.2.1 Comparison of power spectra of Control Group vs 5mg/kg Tacrinetreated group

Figure 4.20 compares the power spectra obtained from mice of the control group vs 5mg/kg Tacrine treated group in different frames. Mice of the control group received only vehicle, So they moved around much more than mice injected with 5mg/kg Tacrine.

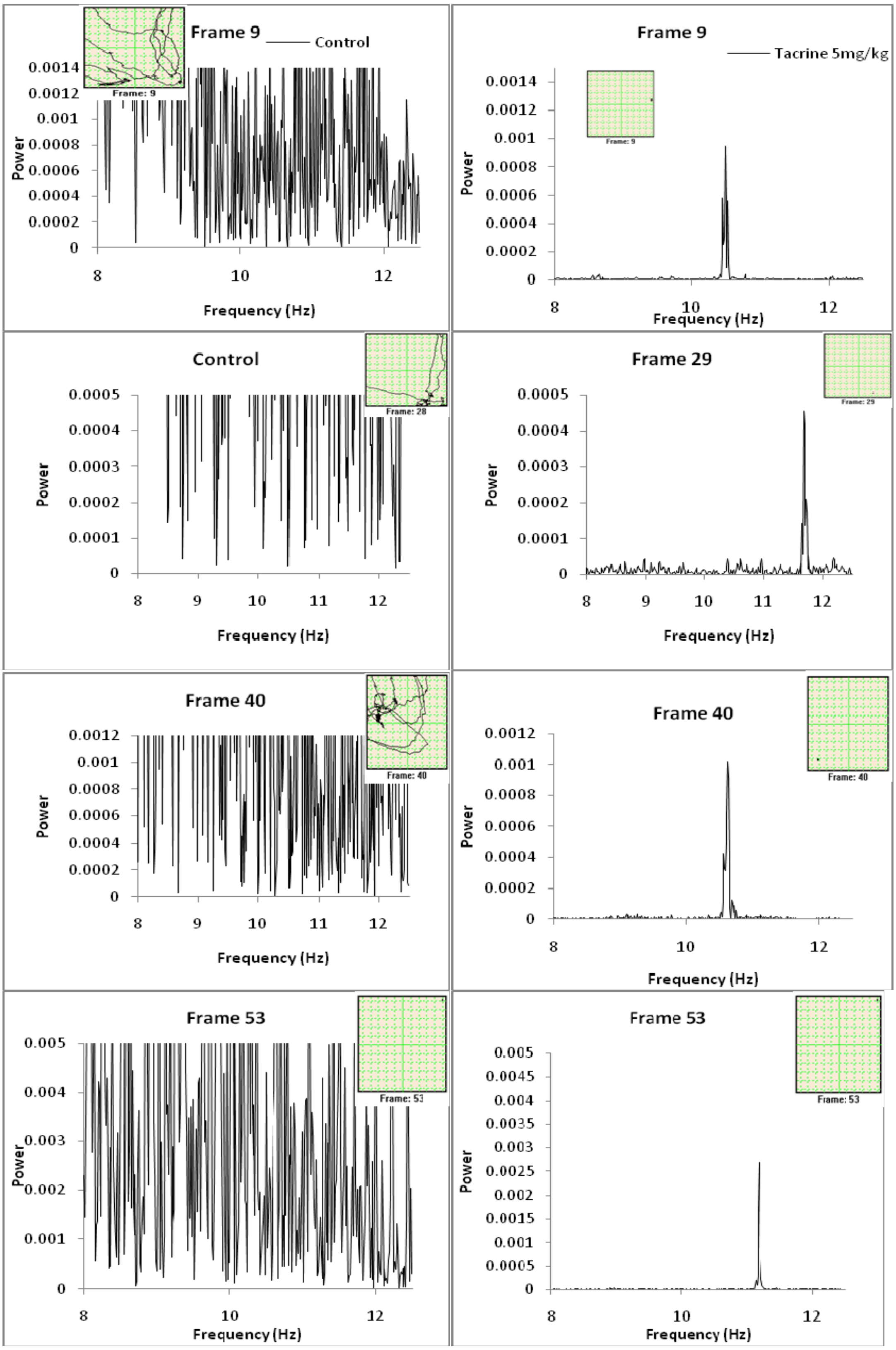


Figure 4.20 Comparison of power spectra of mice in Controlgroup vs 5mg/kg Tacrine treated group along with their motion pictures.

4.3.2.2 Comparison of power spectra between 5mg/kg tacrine treated and 5mg/kg tacrine + 10mg/kg Sinemet® treated groups.

Figure 4.21. shows the comparison of trajectory motion pictures and power spectra between 5mg/kg tacrine group and the power spectra of mice treated with 10mg/kg Sinemet® followed by intraperitoneal administration of 5mg/kg tacrine. Peaks of spectra of 5mg/kg tacrine and 5mg/kg tacrine + 10mg/kg Sinemet® was analysed using those frames in which the mice were not moving at all. If the peaks are significant, then we expect it to be the parkinsonian symptom. As the neurotoxin injected is tacrine, we assume the peaks to represent lateral movement of jaws. Oral administration of 10mg/kg Sinemet® was shown to reduce the peaks induced by 5mg/kg tacrine. Best analysis of power spectra were choosen and kept in chronological order according to their frame numbers.

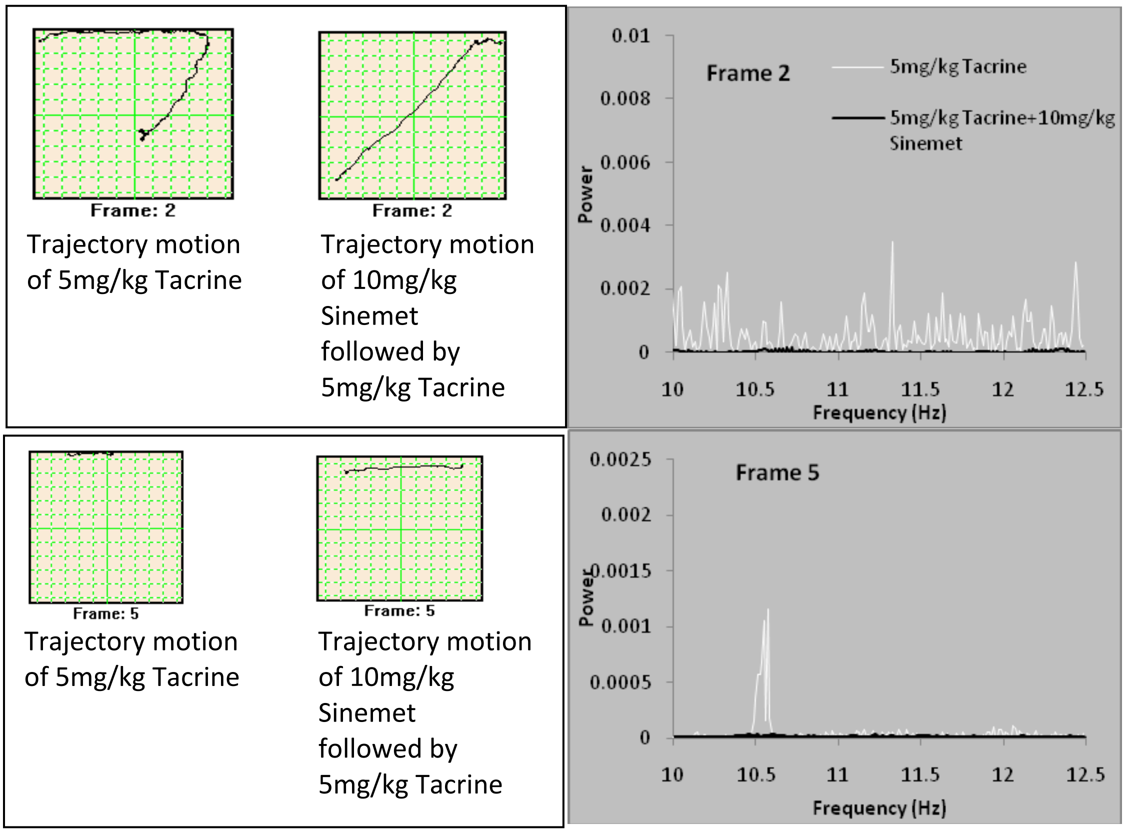
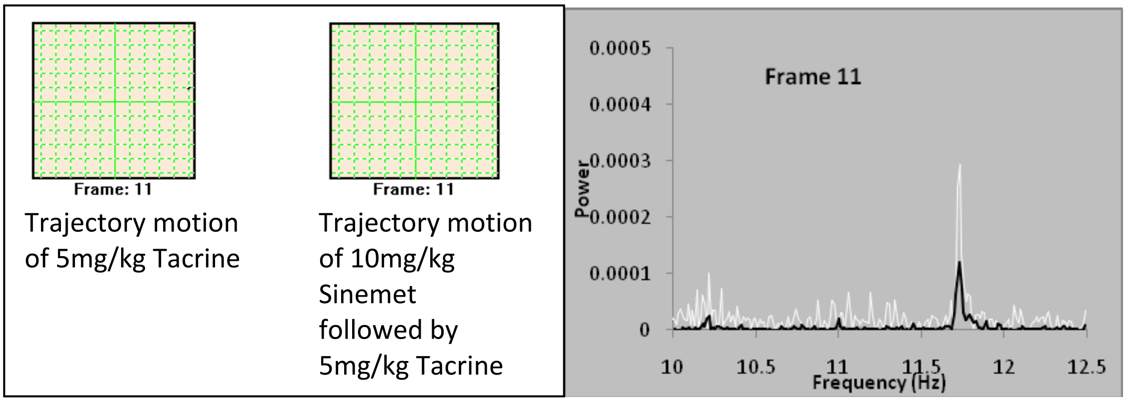
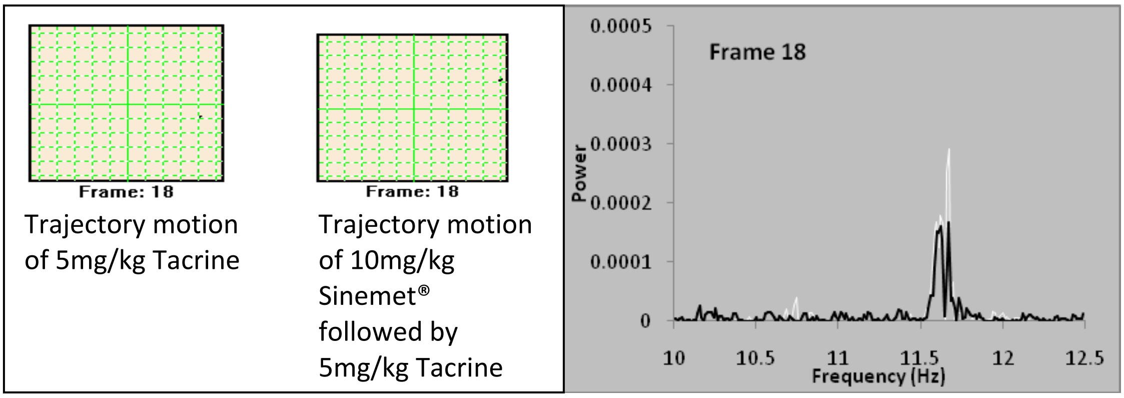


Figure 4.21 Comparison of power spectra of mice in 5mg/kg tacrine group vs 5mg/kg Tacrine + 10mg/kg Sinemet® treated group along with their motion pictures. (cont)



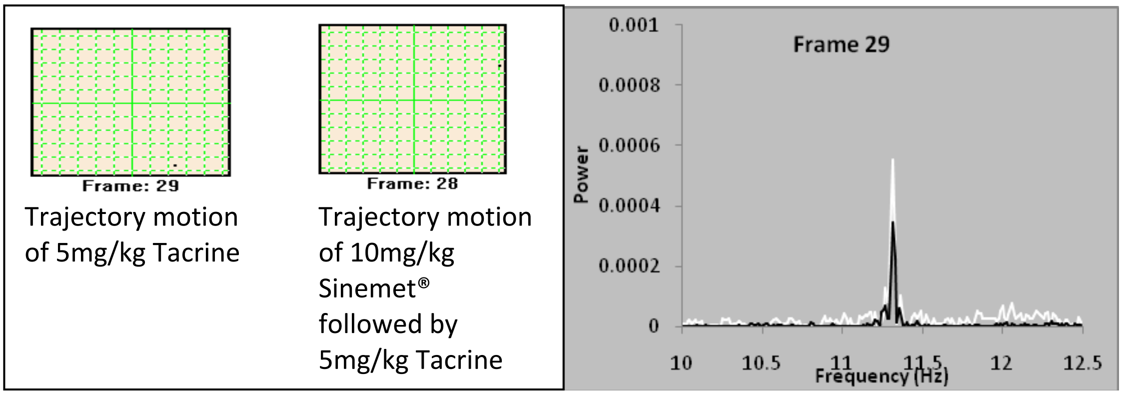
Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 10mg/kg Sinemet followed by 5mg/kg Tacrine



Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 10mg/kg Sinemet® followed by 5mg/kg Tacrine



Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 10mg/kg Sinemet® followed by 5mg/kg Tacrine

Figure 4.21 Comparison of power spectra of mice in 5mg/kg tacrine group vs 5mg/kg Tacrine + 10mg/kg Sinemet® treated group along with their motion pictures. (cont.)

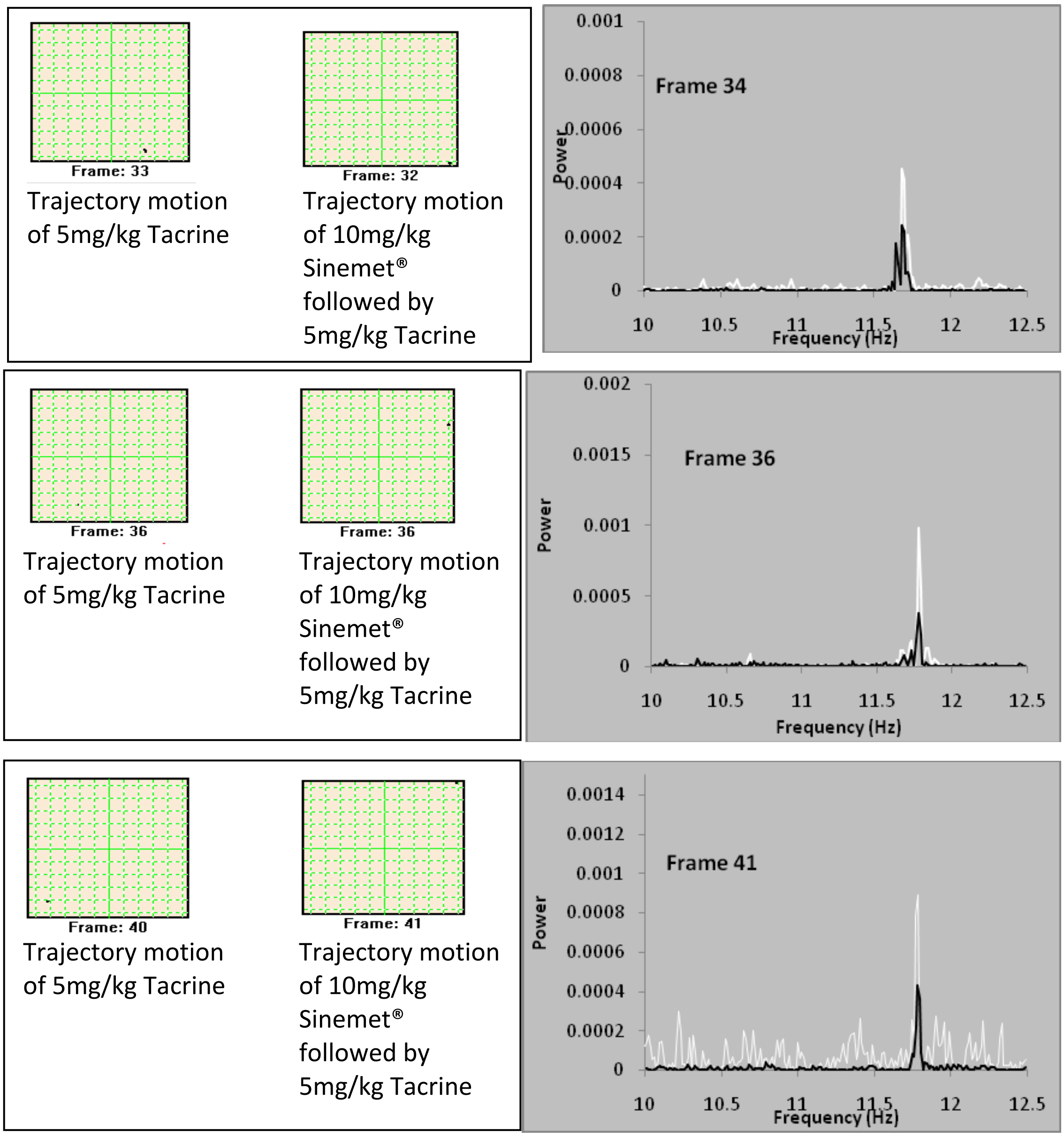


Figure 4.21 Comparison of power spectra of mice in 5mg/kg tacrine group vs 5mg/kg Tacrine + 10mg/kg Sinemet® treated group along with their motion pictures. (cont.)

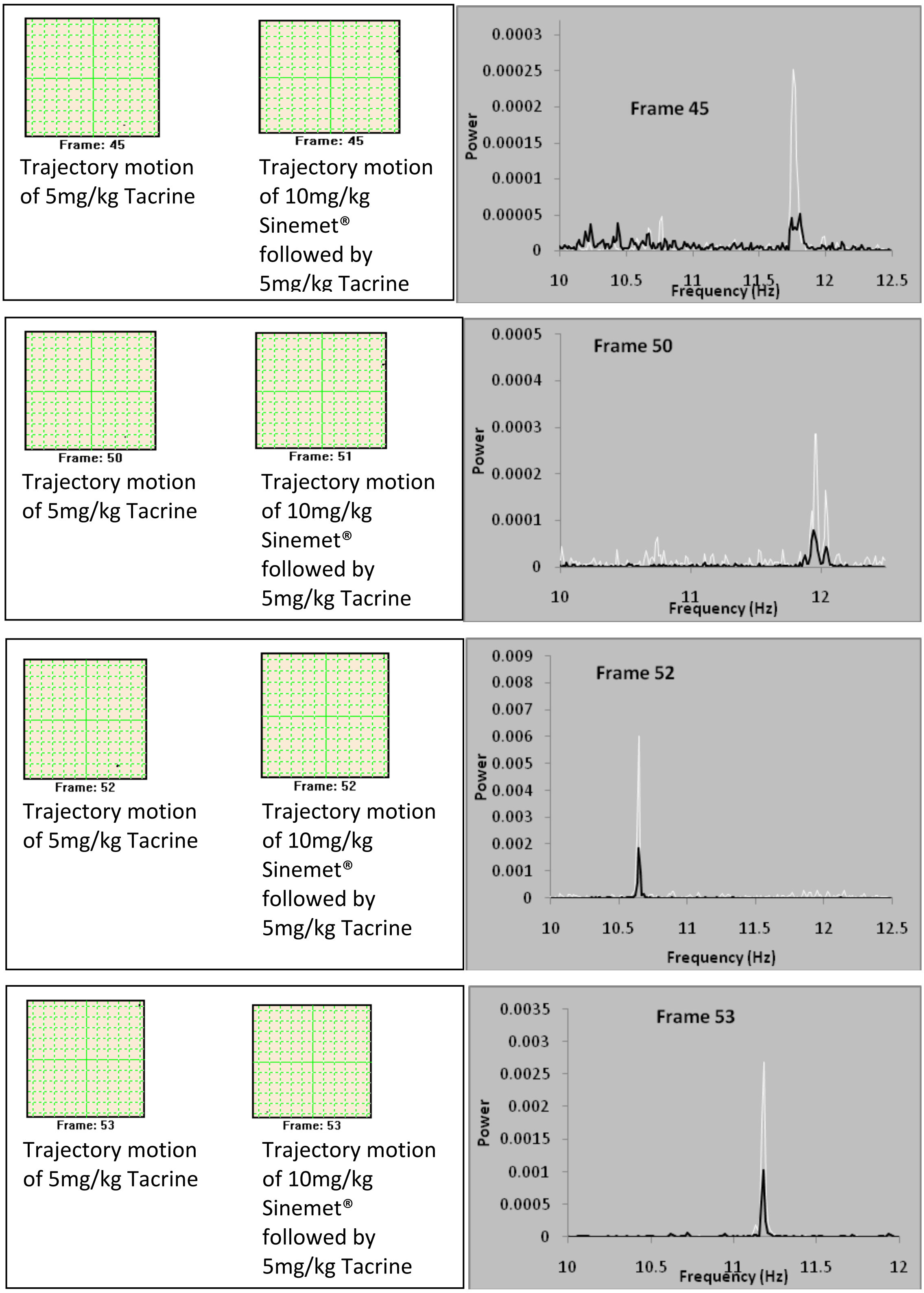


Figure 4.21 Comparison of power spectra of mice in 5mg/kg tacrine group vs 5mg/kg Tacrine + 10mg/kg Sinemet® treated group along with their motion pictures.

4.3.3 Power spectra of 5mg/kg Tacrine treated rats

As the animals were kept inside FPA for 1 hr and 30 min for tacrine expermiment in both mice and rats, the maximum number of frames was 66. Figure 4.22 shows the power spectra of rats treated with 5mg/kg tacrine. We found some significant peaks at around 0.5-2.5 Hz. Thus, we kept the frequency from 0.5-3 Hz to make a stronger understading with spectra and frequency.

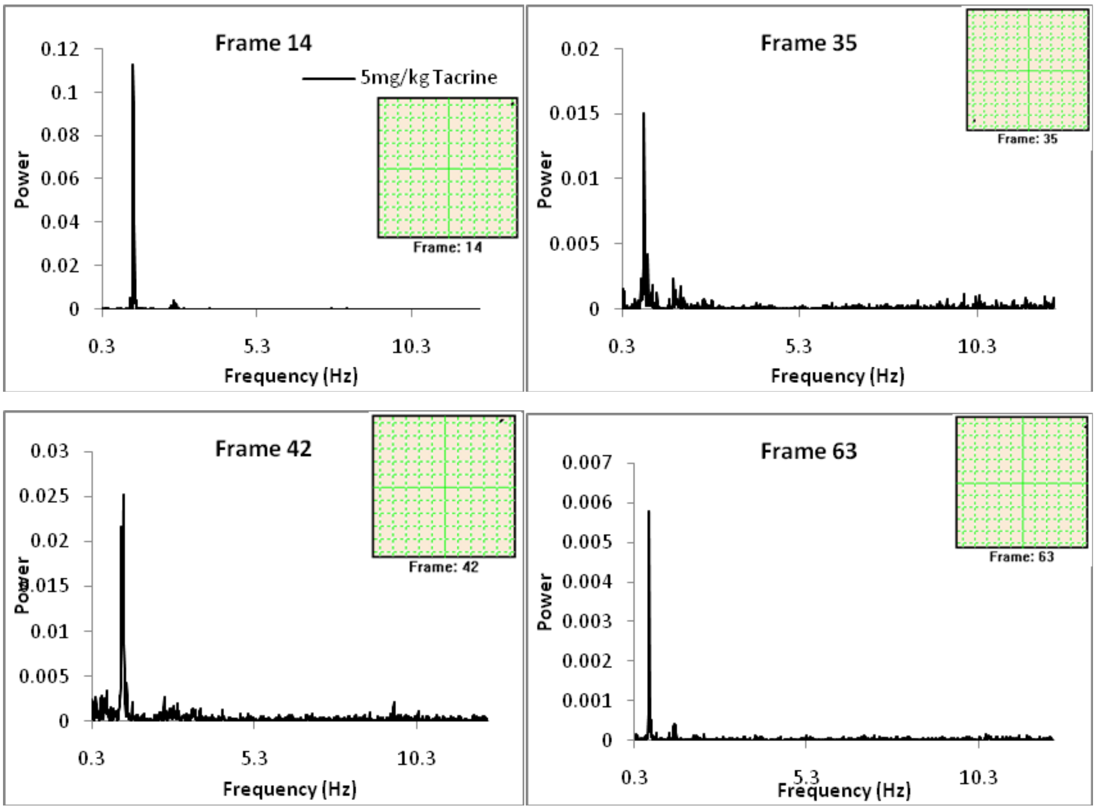


Figure 4.22 Analysis of power spectra of rats treated with 5mg/kg tacrine.

4.3.3.1 Comparison of power spectra of rats in control group and 5mg/kg tacrine treated group.

Fig 5.23 compares the power spectra obtained from rats of the control group vs 5mg/kg tacrine treated group in different frames. Rats of the control group received only vehicle, so they moved around very much as compared with rats injected with 5mg/kg tacrine. Because of this reason, the peak of lateral jaw was relatively insignificant as compared with the spectra of control rats.

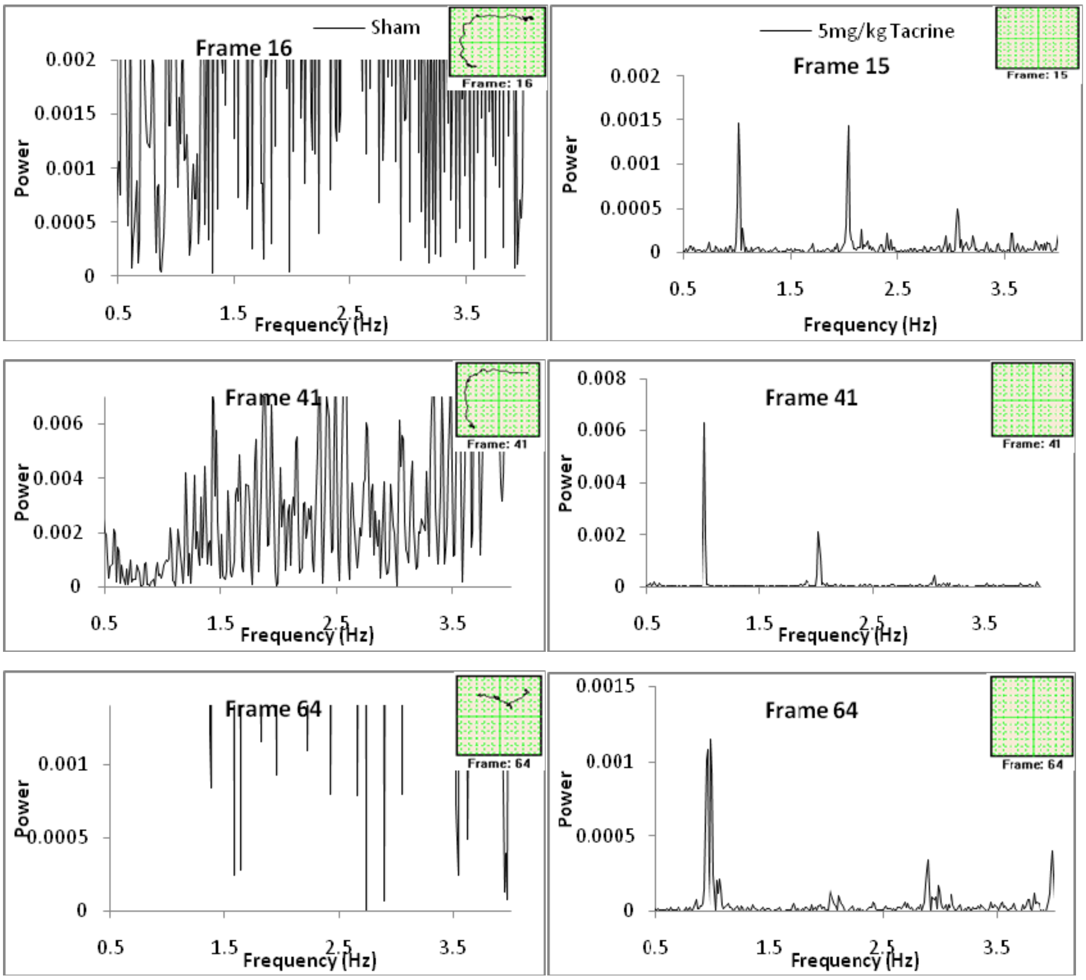
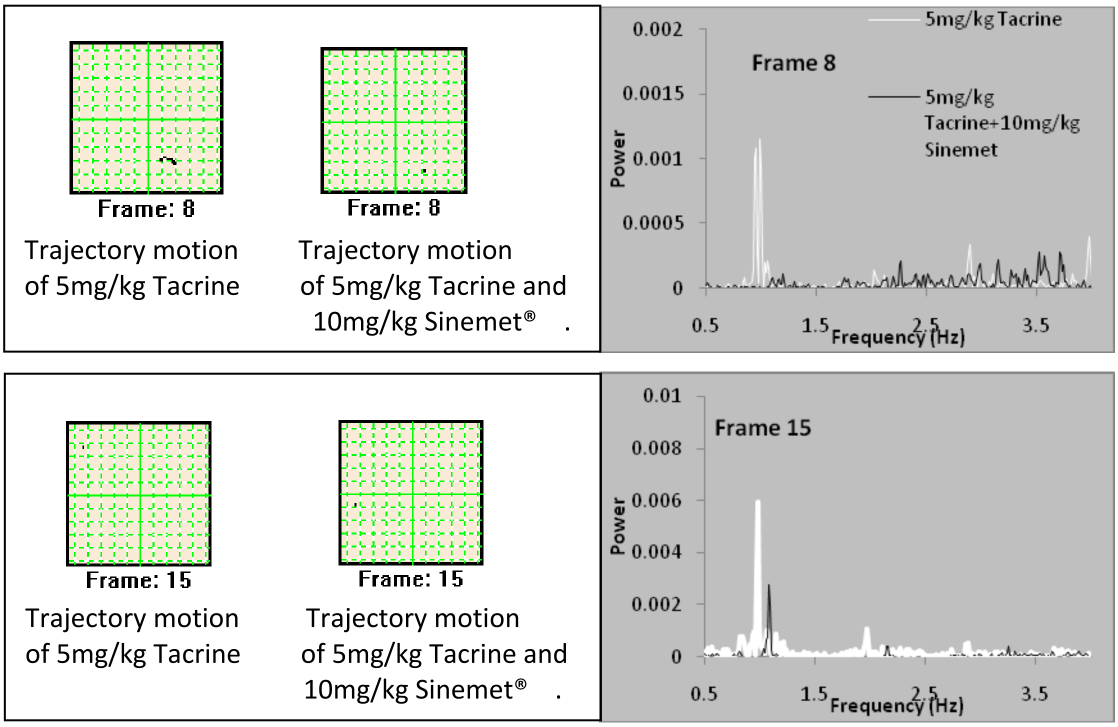


Figure 4.23 Comparison of trajectory motion pictures and power spectra of rats in control group and 5mg/kg tacrine group.

4.3.3.2 Comparison of power spectra of rats in 5mg/kg Tacrine treated with 5mg/kg Tacrine + 10mg/kg Sinemet® treated groups.

Figure 4.24 compares the power spectra obtained from rats of the 5mg/kg tacrine treated group and 5mg/kg tacrine + 10mg/kg Sinemet® treated group.Peaks of spectra of 5mg/kg tacrine and 5mg/kg tacrine + 10mg/kg Sinemet® were analysed using those frames in which the rats were not moving at all. As the neurotoxin injected is Tacrine, we suppose the peaks to represent lateral movement of jaws. Oral administration of 10mg/kg Sinemet® was shown to reduce the peaks induced by 5mg/kg tacrine. Analysis of power spectra were choosen and kept in chronological order according to their frame numbers. Lateral jaw movement induced by tacrine in rats were considered to be represented by a noticeable peak ranging from 0.5-3 Hz.



Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 5mg/kg Tacrine and 10mg/kg Sinemet® .

Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 5mg/kg Tacrine and 10mg/kg Sinemet® .

Figure 4.24 Comparison of power spectra of rats between 5mg/kg tacrine treated and 5mg/kg tacrine + 10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont)

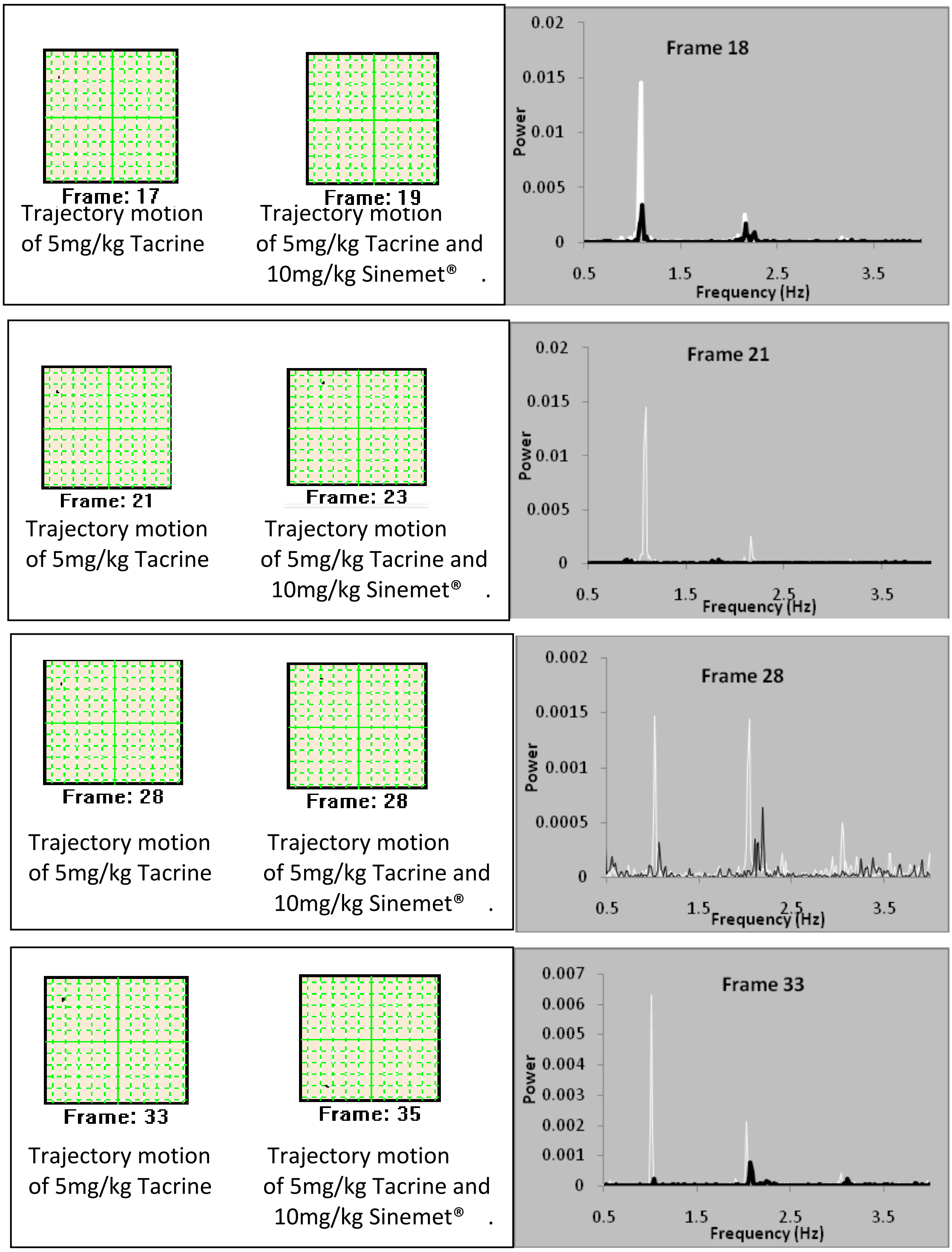
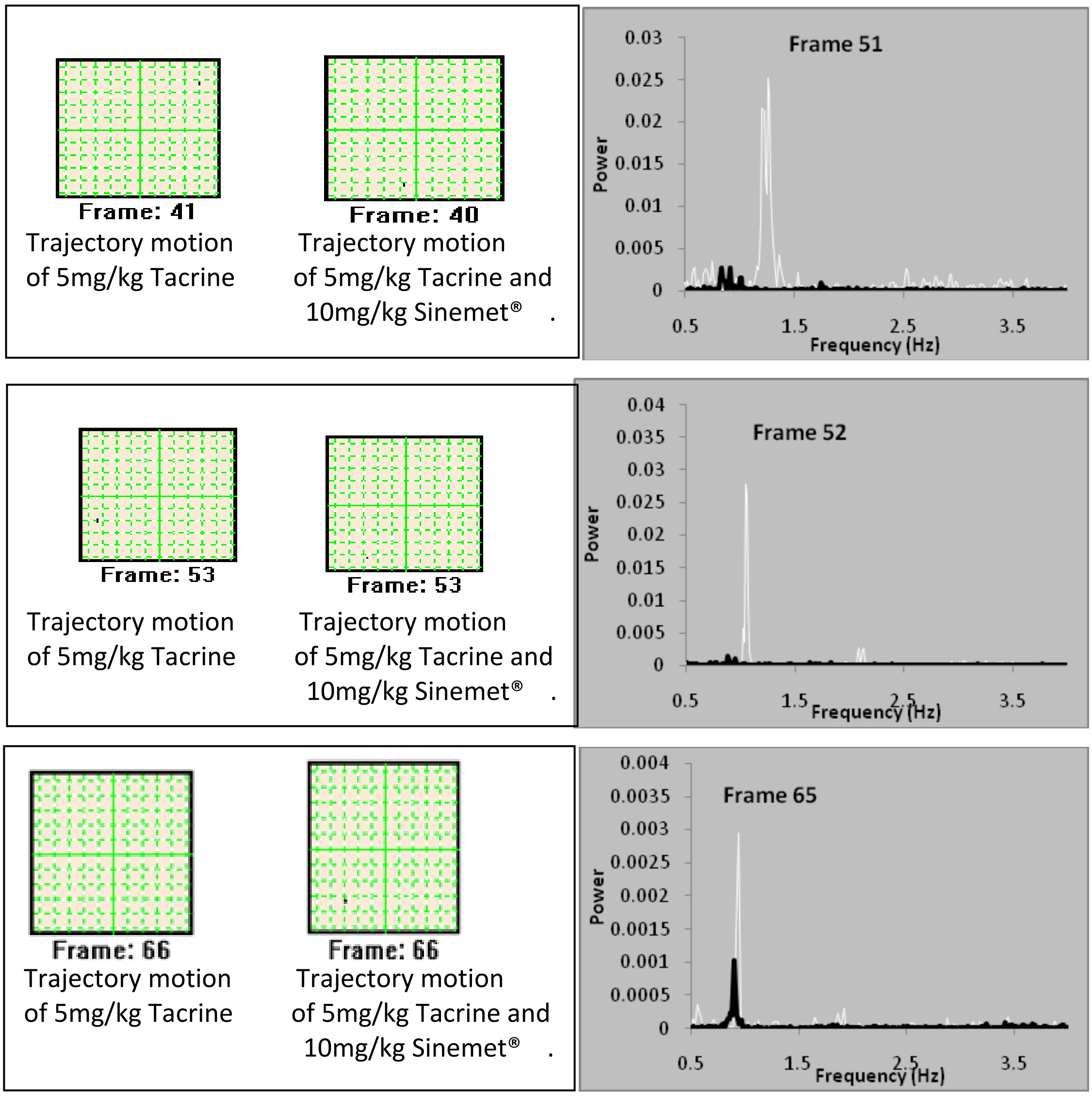


Figure 4.24 Comparison of power spectra of rats between 5mg/kg tacrine treated and 5mg/kg tacrine + 10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session. (cont.)



Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 5mg/kg Tacrine and 10mg/kg Sinemet® .

Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 5mg/kg Tacrine and 10mg/kg Sinemet® .

Trajectory motion of 5mg/kg Tacrine

Trajectory motion of 5mg/kg Tacrine and 10mg/kg Sinemet® .

Figure 4.24 Comparison of power spectra of rats between 5mg/kg tacrine treated and 5mg/kg tacrine + 10mg/kg Sinemet® treated groups along with their trajectory motion pictures. Total frames were 88 per session. These frames were selected to represent each interval from the beginning until the end of session.

4.3.4 Power spectra of 2.5mg/kg Rotenone treated rats.

Parkinsonian symptoms were induced by 7 to 8 doses of rotenone injection. Rats were kept inside FPA for 2 hrs. Therefore, the total frames of experiment were 88. Main peaks were observed at 0.5-2 Hz and 4-12 Hz. Figure 5.25 shows the whole frame analysis of 2.5mg/kg rotenone as compared with 2.5mg/kg rotenone+10mg/kg Sinemet®. As illustrated, oral treatment of Sinemet® reduced some peaks induced by 2.5mg/kg rotenone. Parkinsonian symptoms expected from s.c. injection of rotenone were lateral movement of jaws, tremor and rigidity.

4.3.4.1 Comparison of power spectra between 2.5mg/kg rotenone treated and 2.5mg/kg rotenone+10mg/kg Sinemet® treated groups, at frequency 0.5-12 Hz.

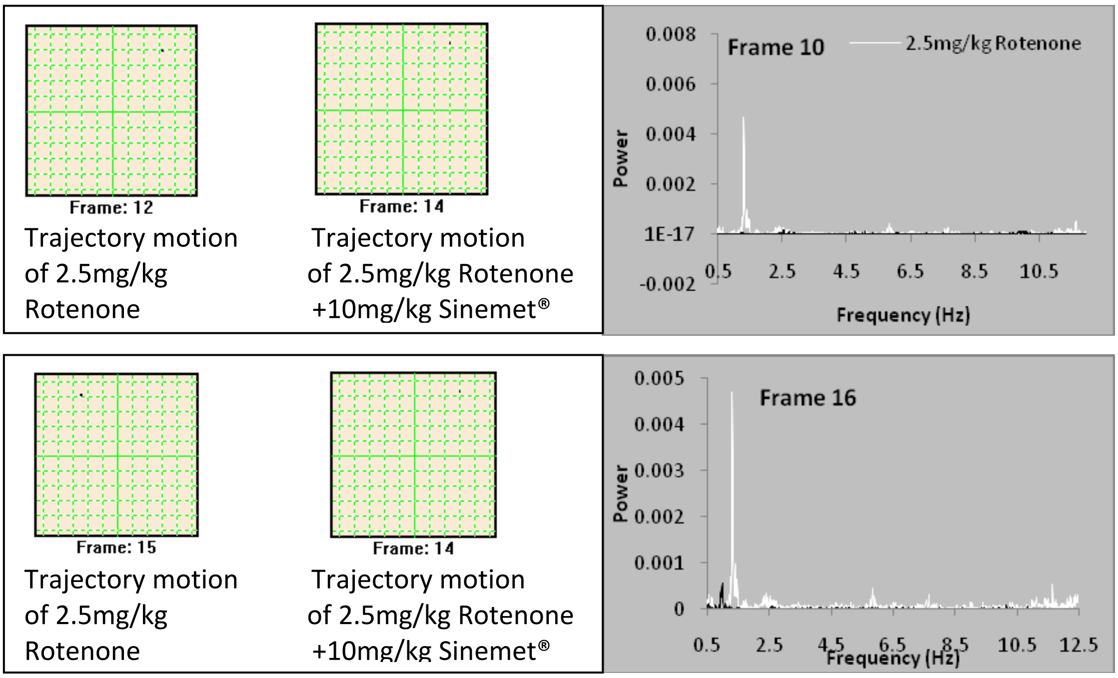


Figure 4.25 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-12 Hz) along with their motion pictures.

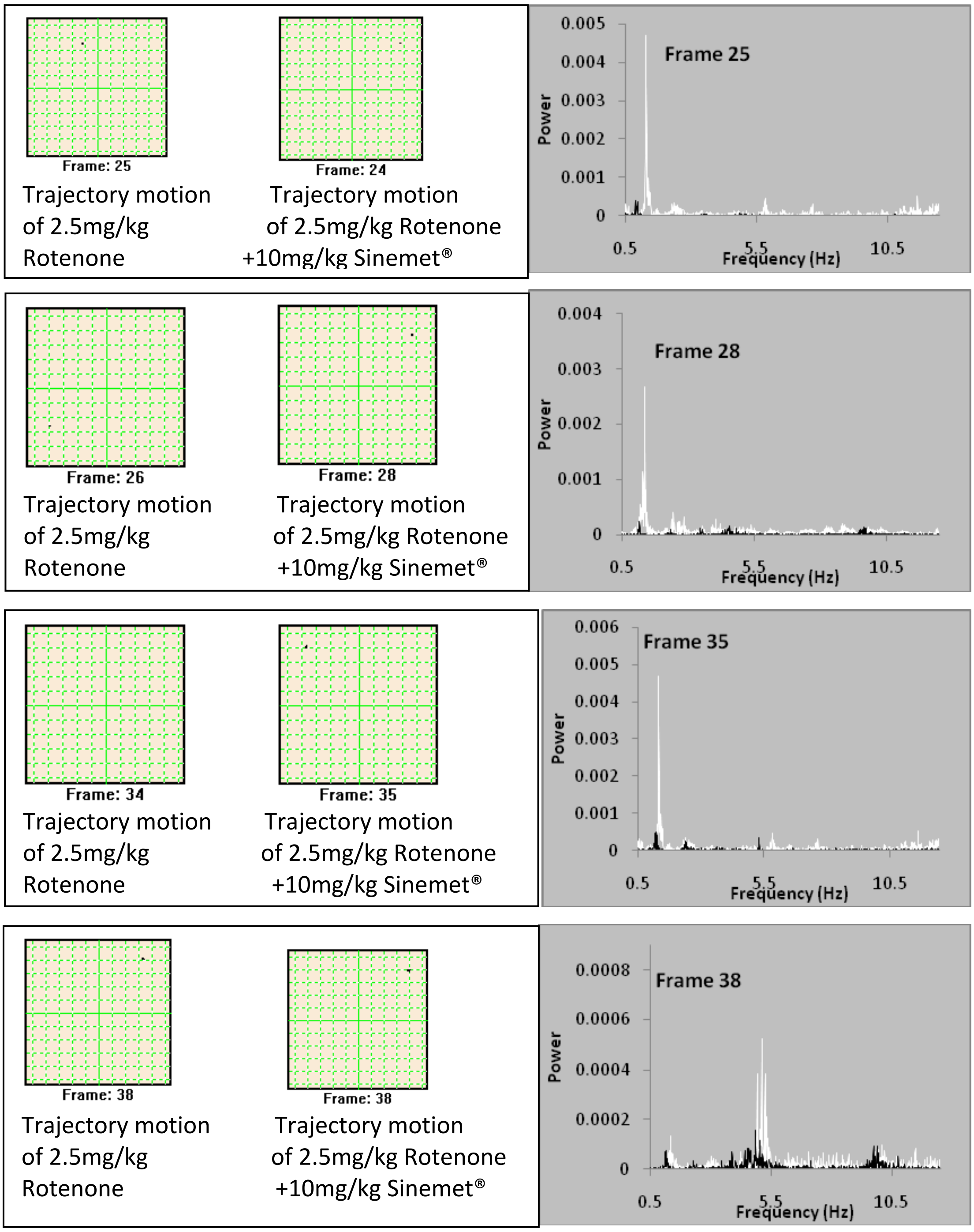
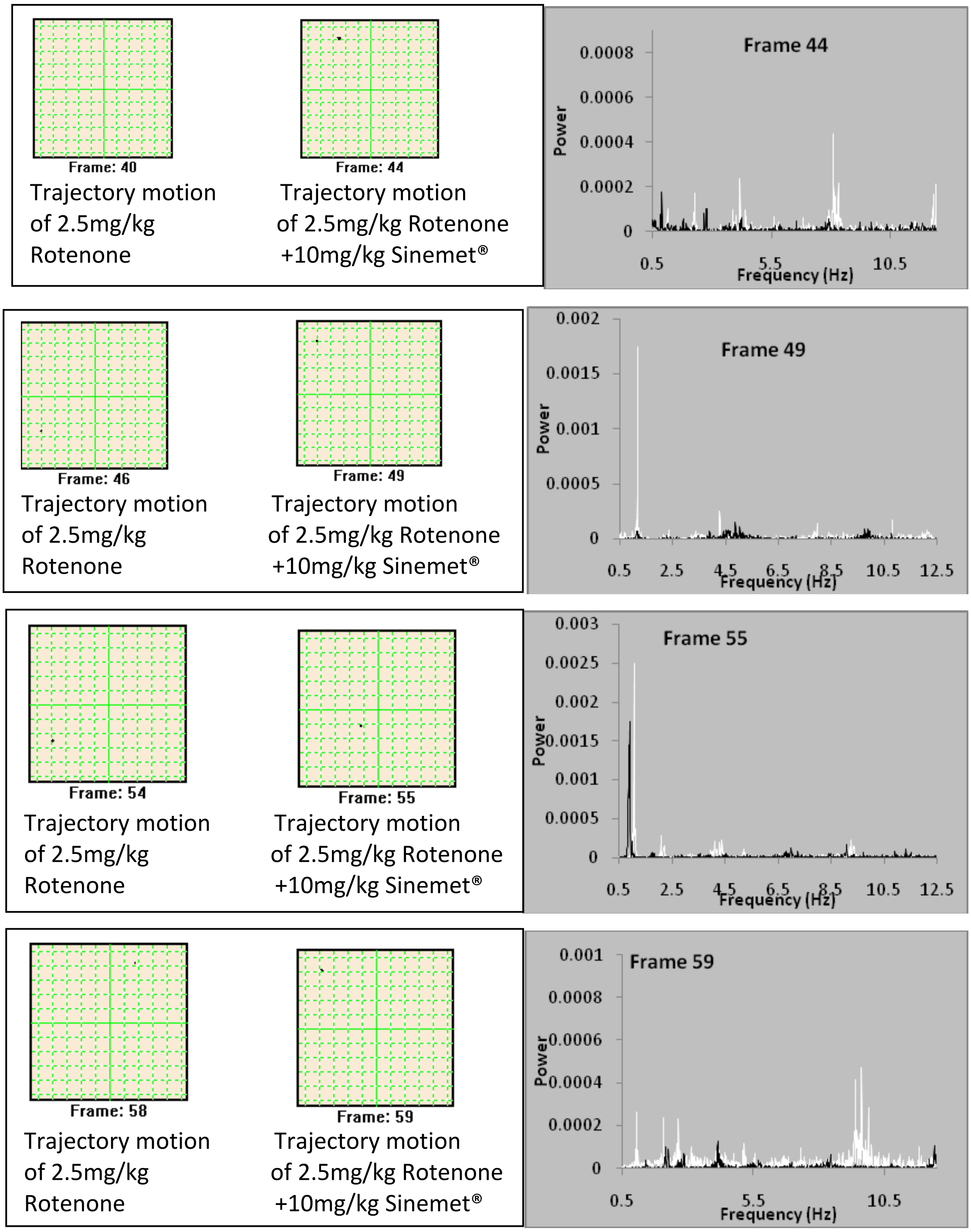


Figure 4.25 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-12 Hz) along with their motion pictures. (cont.)



Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Figure 4.25 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-12 Hz) along with their motion pictures. (cont.)

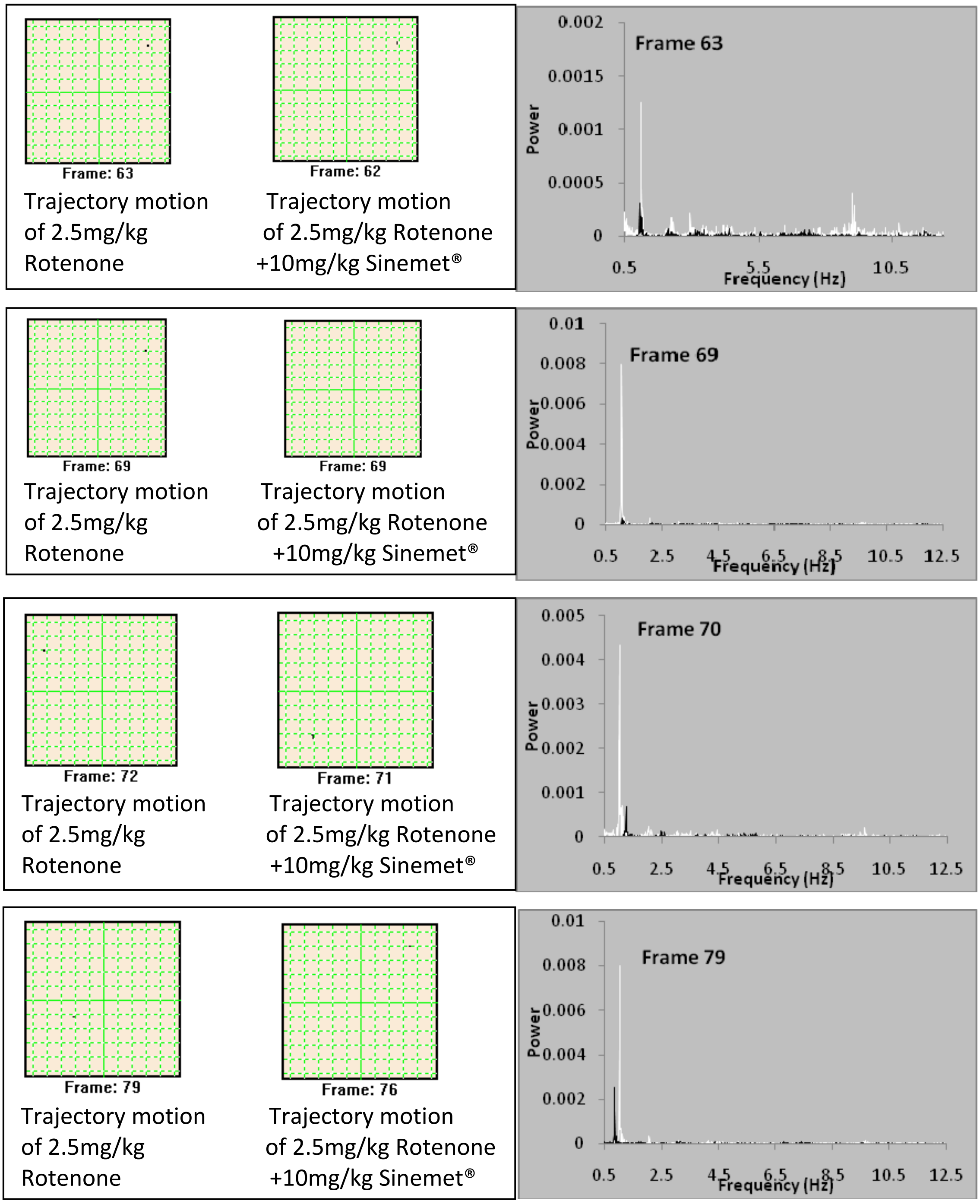


Figure 4.25 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-12 Hz) along with their motion pictures. (cont.)

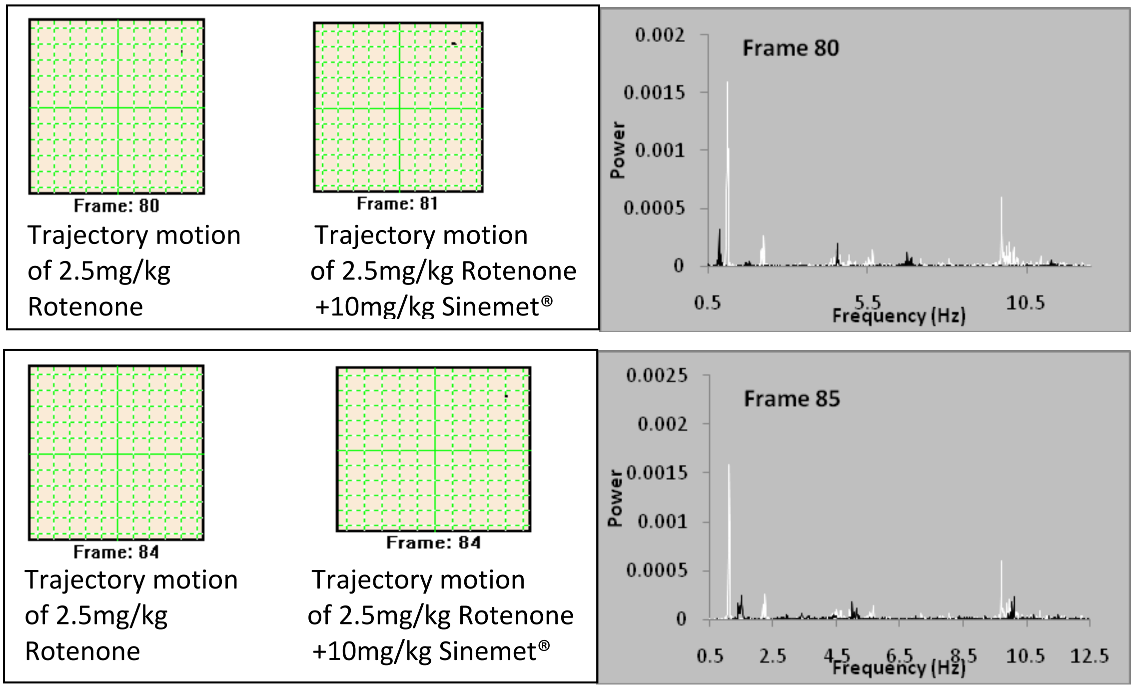
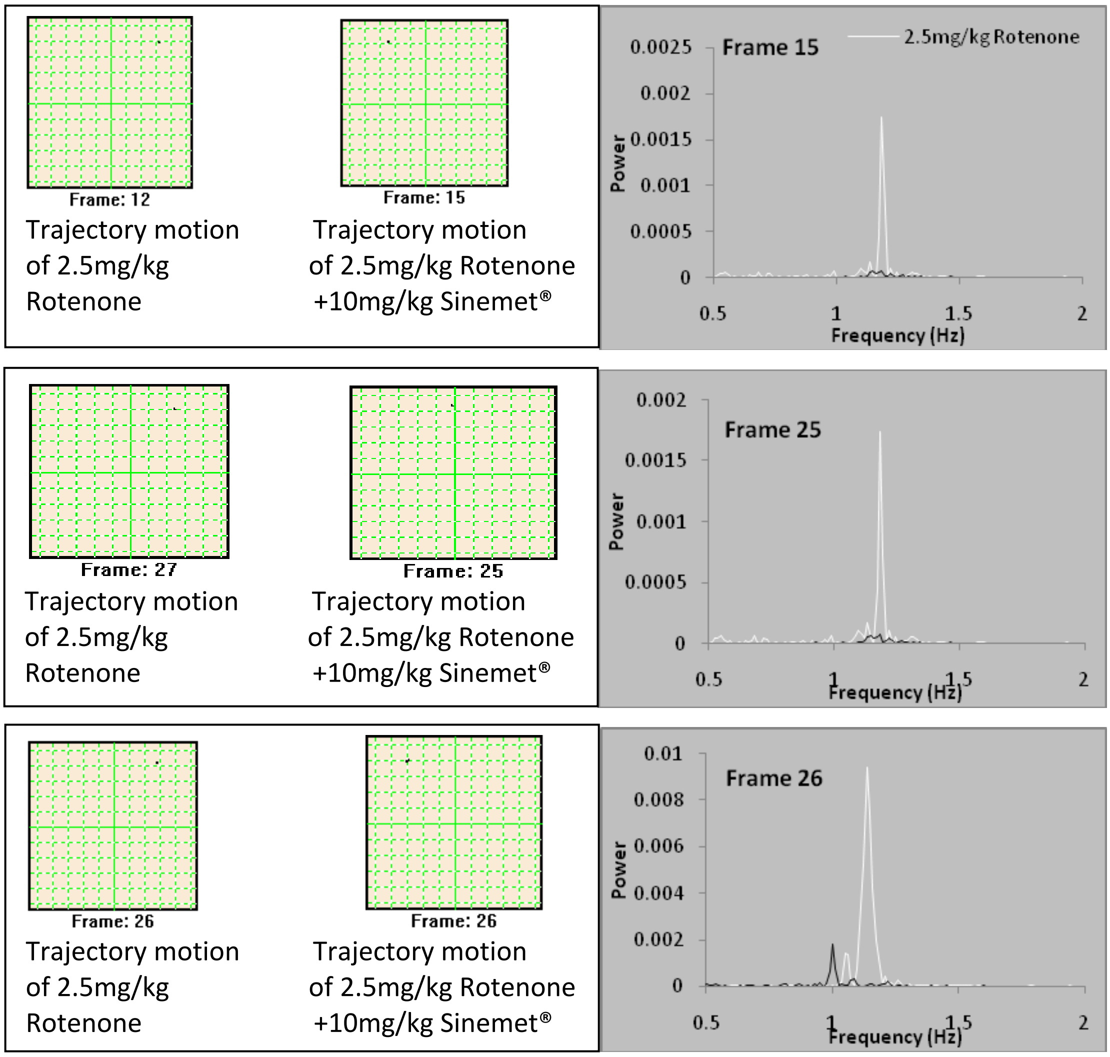


Figure 4.25 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-12 Hz) along with their motion pictures.

From preliminary studies, we observed some lateral movement of jaws that was similar to jaw movement induced by tacrine on rats. As the frequency of jaw movement ranges from 0.5-3 Hz, we suppose the peak that we see at 0.5-2.5 Hz to be the jaw movement induced by rotenone. Peaks seen from 4-12Hz may be body tremor and rigidity induced by rotenone. For better quantification, the frequency ranges were divided into two parts: 0.5-2.0 Hz (fig 4.26) and 4-12 Hz (fig 4.27).

4.3.4.2 Comparison of power spectra of rats between 2.5mg/kg rotenone treated and 2.5mg/kg rotenone+10mg/kg Sinemet® treated groups, at frequency 0.5-2.0 Hz.



Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

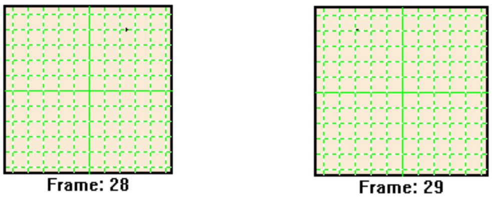
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Trajectory motion of 2.5mg/kg Rotenone

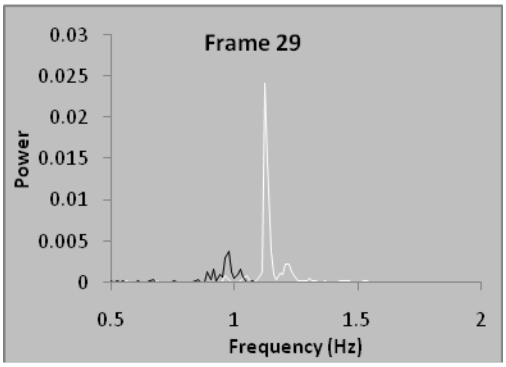
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

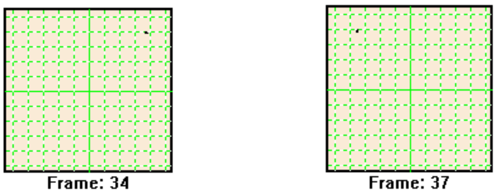
Figure 4.26 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-2.0 Hz) along with their motion pictures.



Trajectory motion of 2.5mg/kg Rotenone

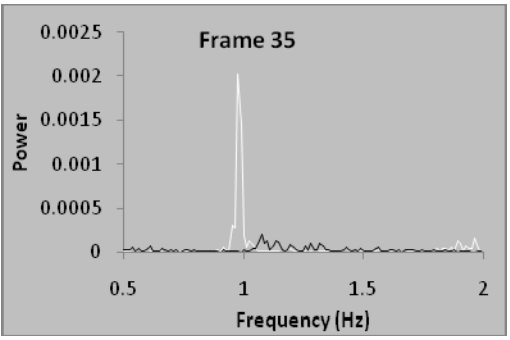
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

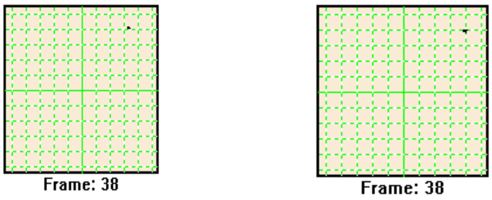




Trajectory motion of 2.5mg/kg Rotenone

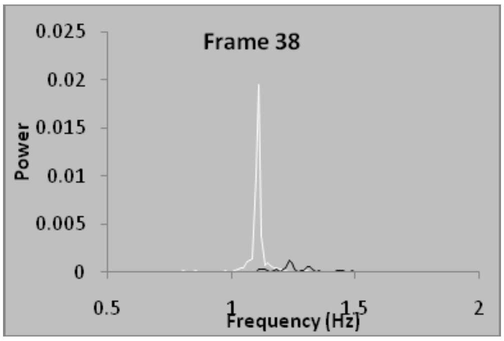
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

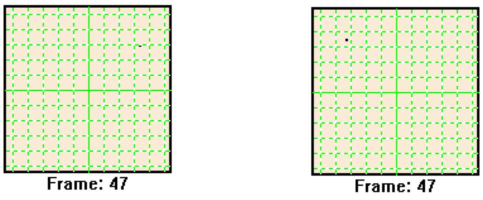




Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®





Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

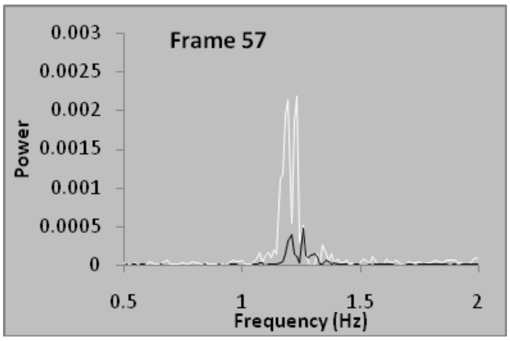
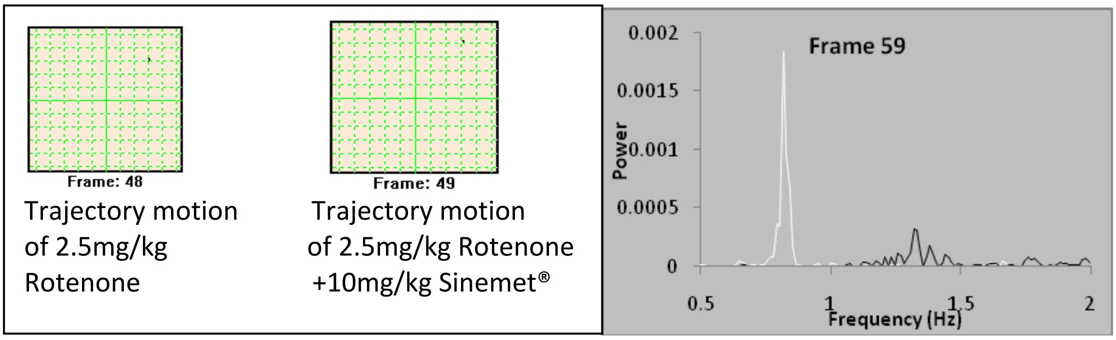
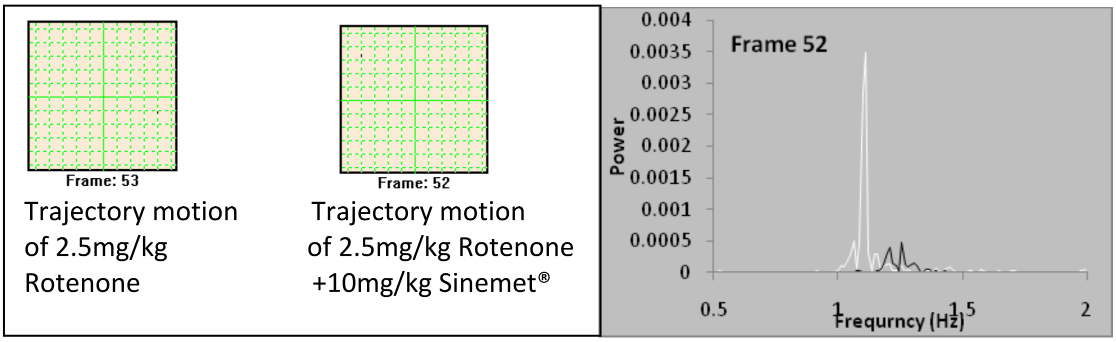


Figure 4.26 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-2.0 Hz) along with their motion pictures. (cont.)



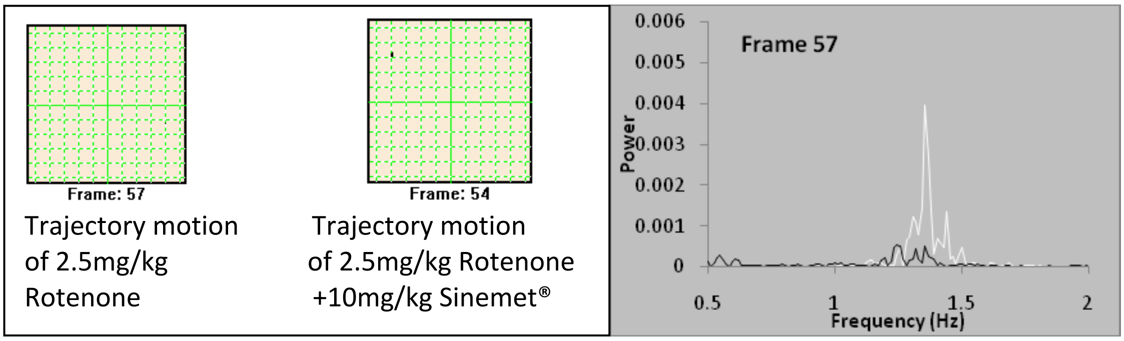
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



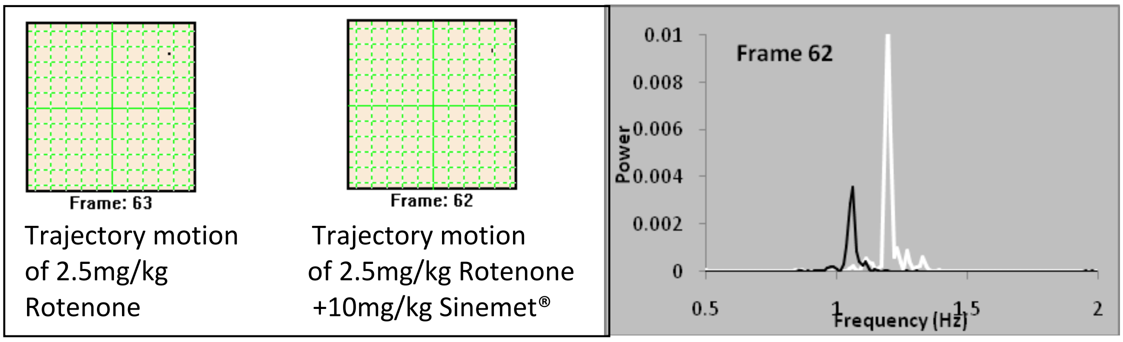
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

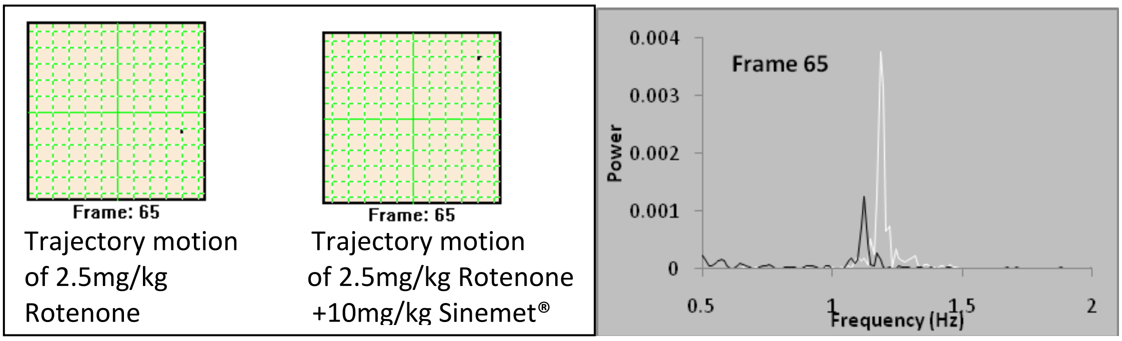
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

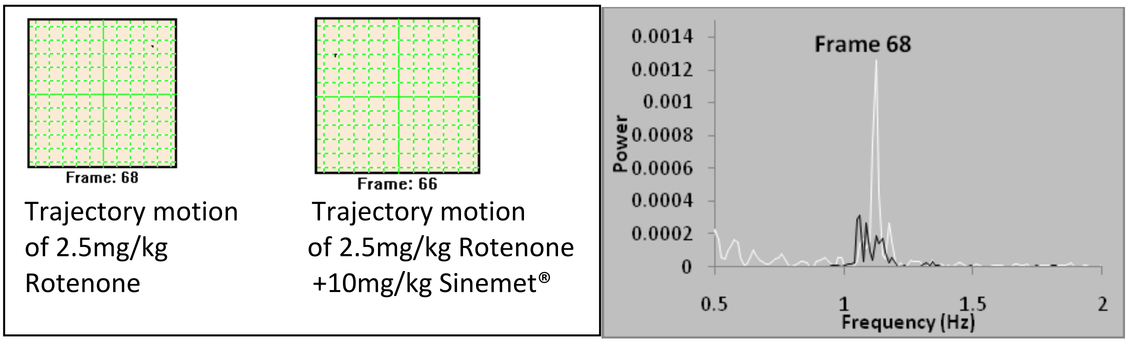
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Figure 4.26 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-2.0 Hz) along with their motion pictures. (cont.)



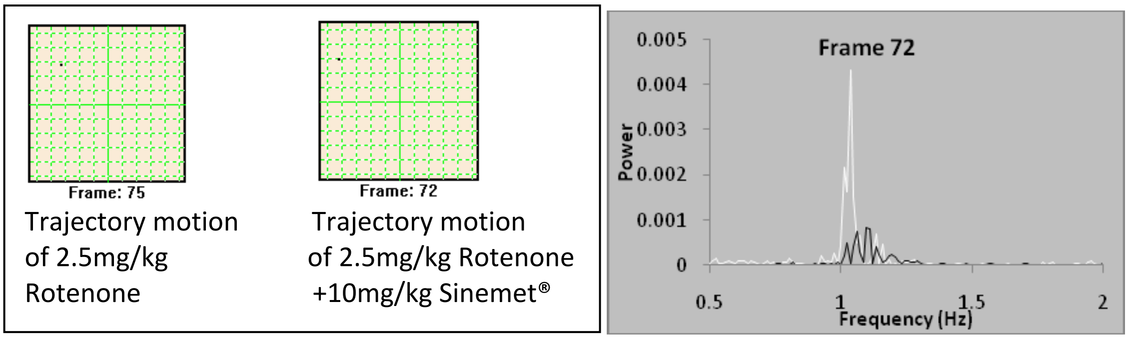
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



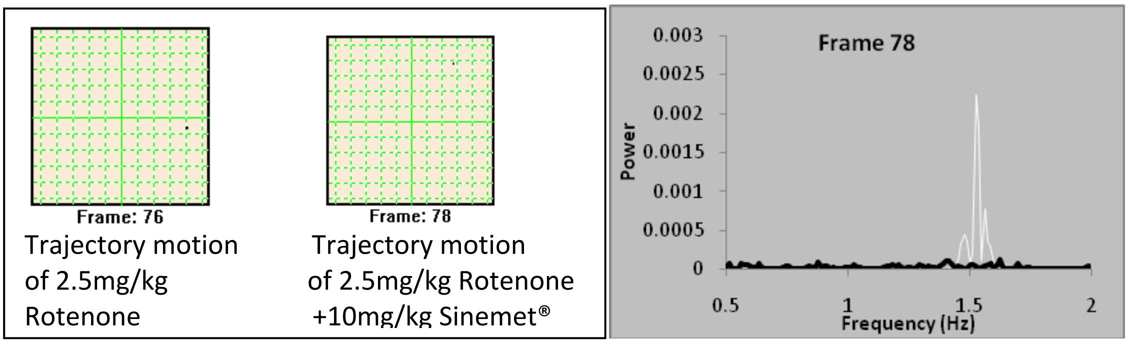
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

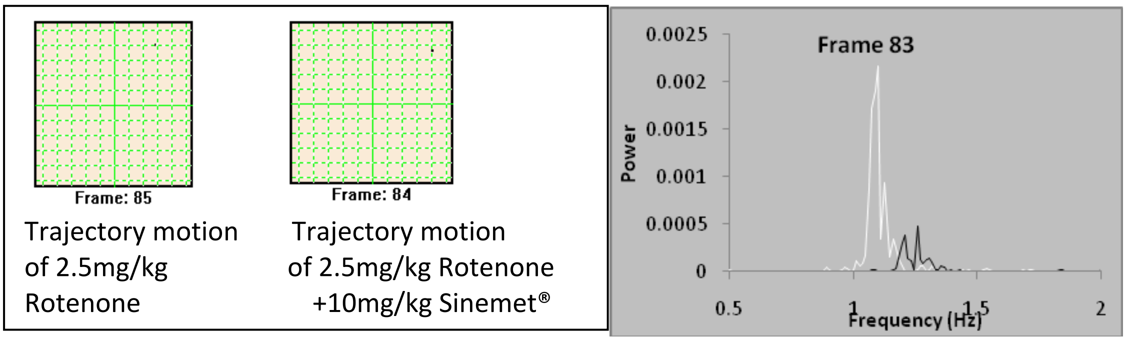
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Figure 4.26 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-2.0 Hz) along with their motion pictures. (cont.)

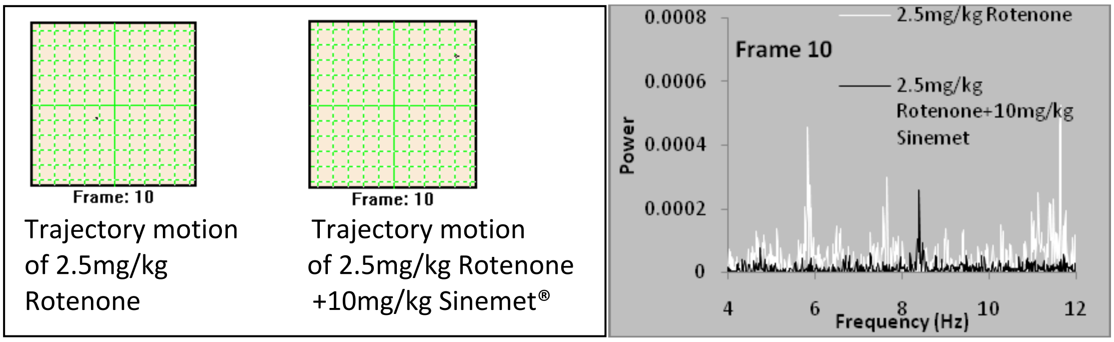


Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Figure 4.26 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 0.5-2.0 Hz) along with their motion pictures.

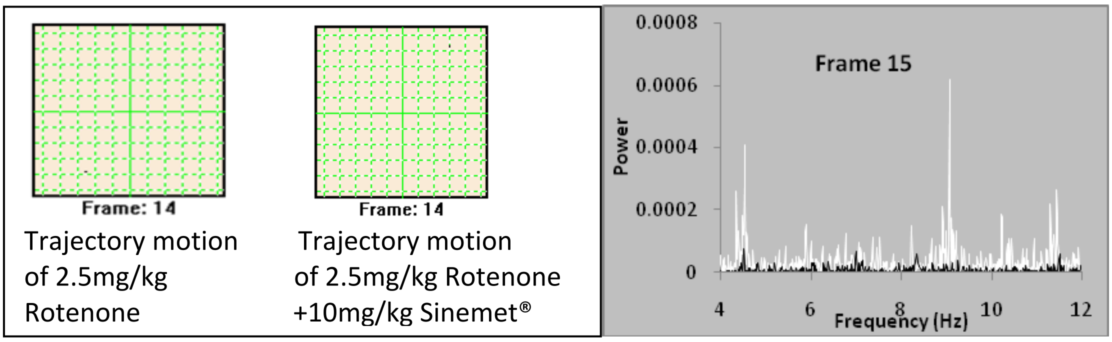
4.3.4 Comparison of power spectra of rats between 2.5mg/kg rotenone treated and 2.5mg/kg rotenone+10mg/kg Sinemet® treated groups, at frequency 4-12 Hz.



Trajectory motion of 2.5mg/kg Rotenone

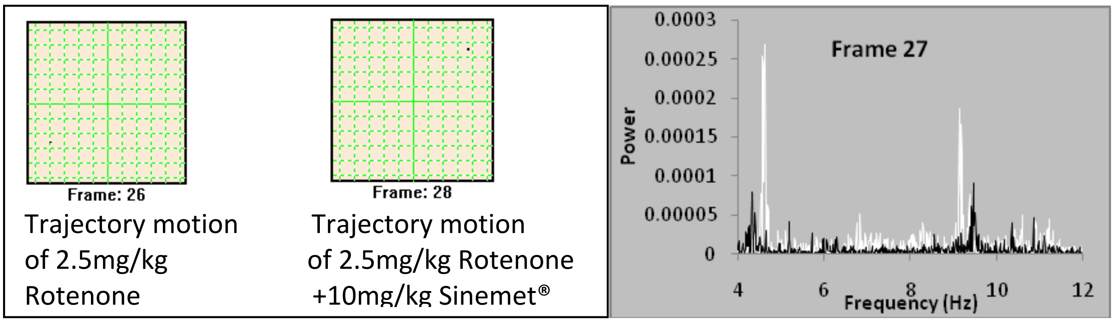
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Figure 4.27 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 4-12 Hz) along with their motion pictures. (cont.)



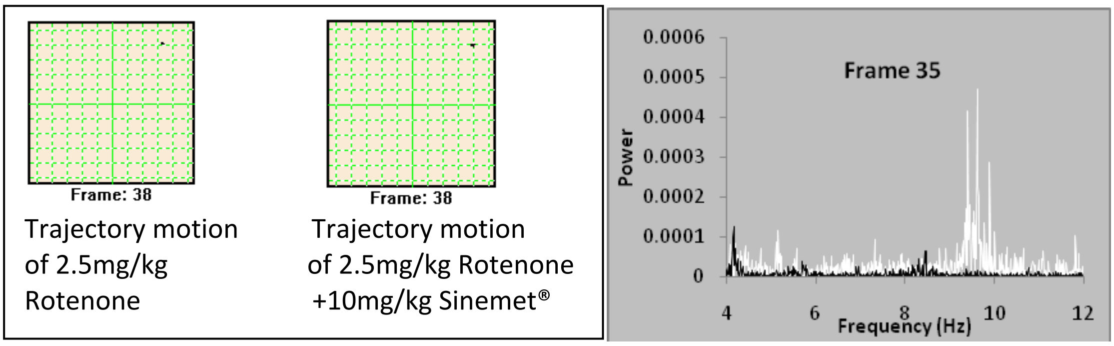
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



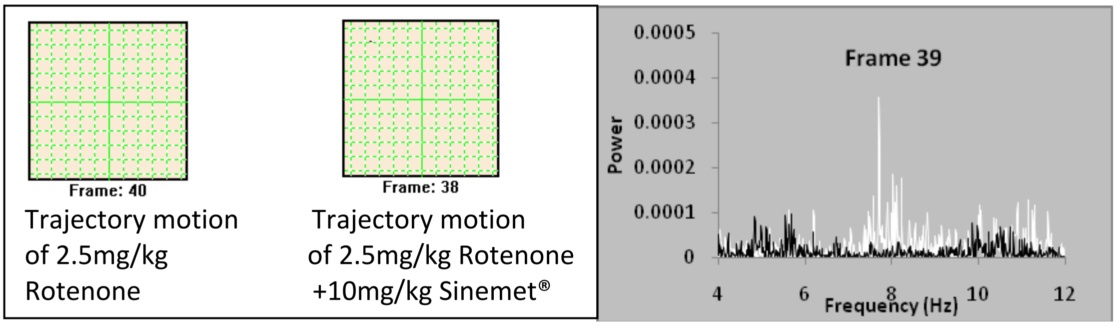
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

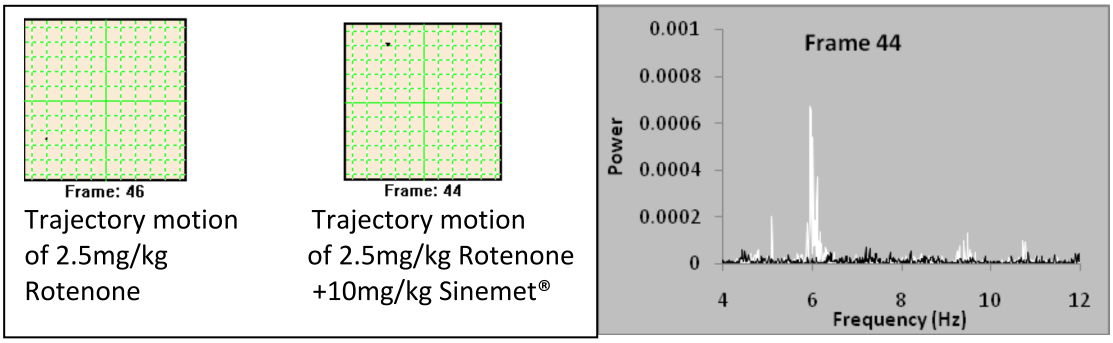
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

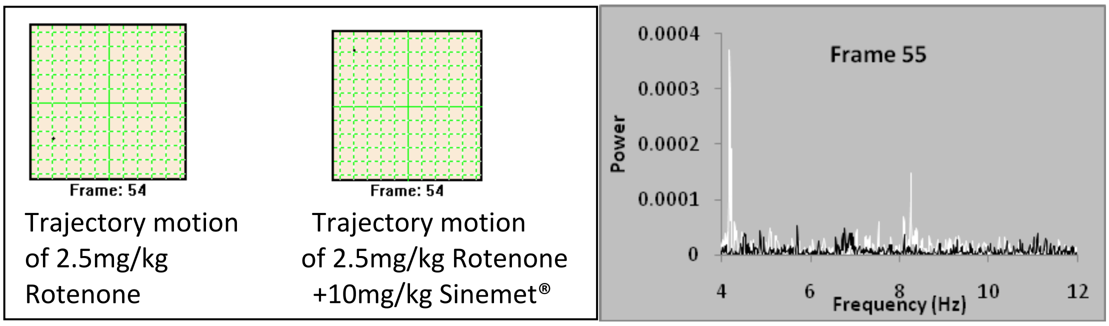
Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Figure 4.27 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 4-12 Hz) along with their motion pictures. (cont.)



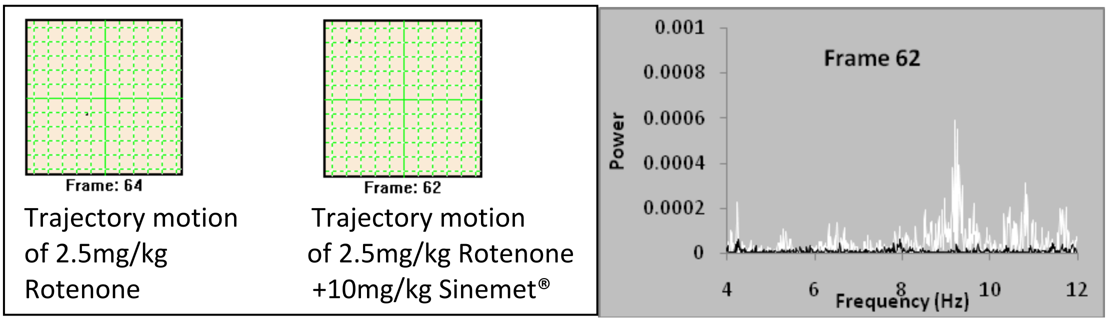
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



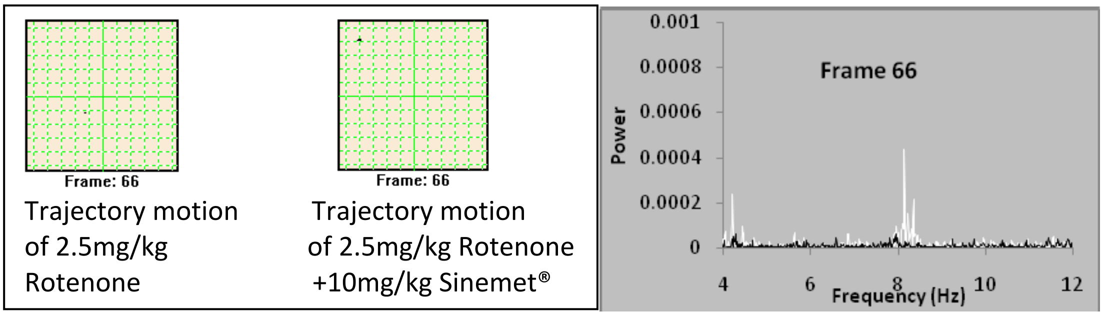
Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®



Trajectory motion of 2.5mg/kg Rotenone

Trajectory motion of 2.5mg/kg Rotenone +10mg/kg Sinemet®

Figure 4.27 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 4-12 Hz) along with their motion pictures. (cont.)

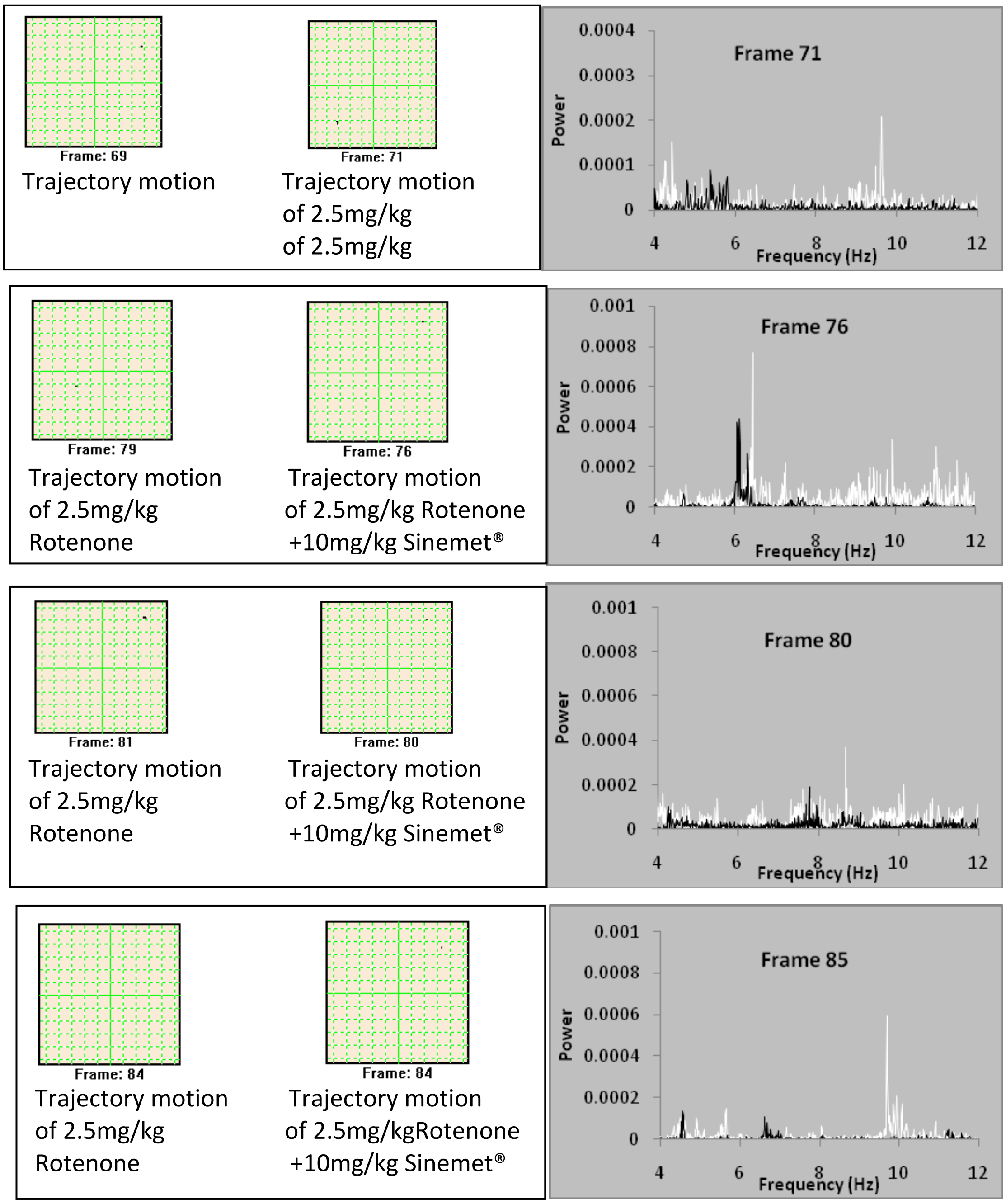


Figure 4.27 Comparison of power spectra of rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group (at frequency 4-12 Hz) along with their motion pictures.

4.4 ELISA test of Dopamine and MAO-B from brain sample

4.4.1 Dopamine levels from brain of C57BL/6 mice, treated with 15 and 30mg/kg MPTP

Figure 4.28 demonstrates the dopamine levels from brain sample of C57BL/6 mice treated with 15 and 30mg/kg MPTP. Since MPTP is selectively toxic to dopaminergic neurons of substantia nigra and striatum, it is hypothesized that MPTP should reduce the dopamine level at this brain area. Y axis represents dopamine level in picogram (pg) and x-axis represents the group of mice. 15 and 30mg/kg MPTP significantly reduced the dopamine level (p=0.057 and p = 0.017, correspondingly) as compared with control. Oral administration of 10mg/kg Sinemet® significantly compensate the damage caused by 15 and 30mg/kg MPTP (p=0.05 and p=0.036) as compared with 15 and 30mg/kg MPTP respectively.

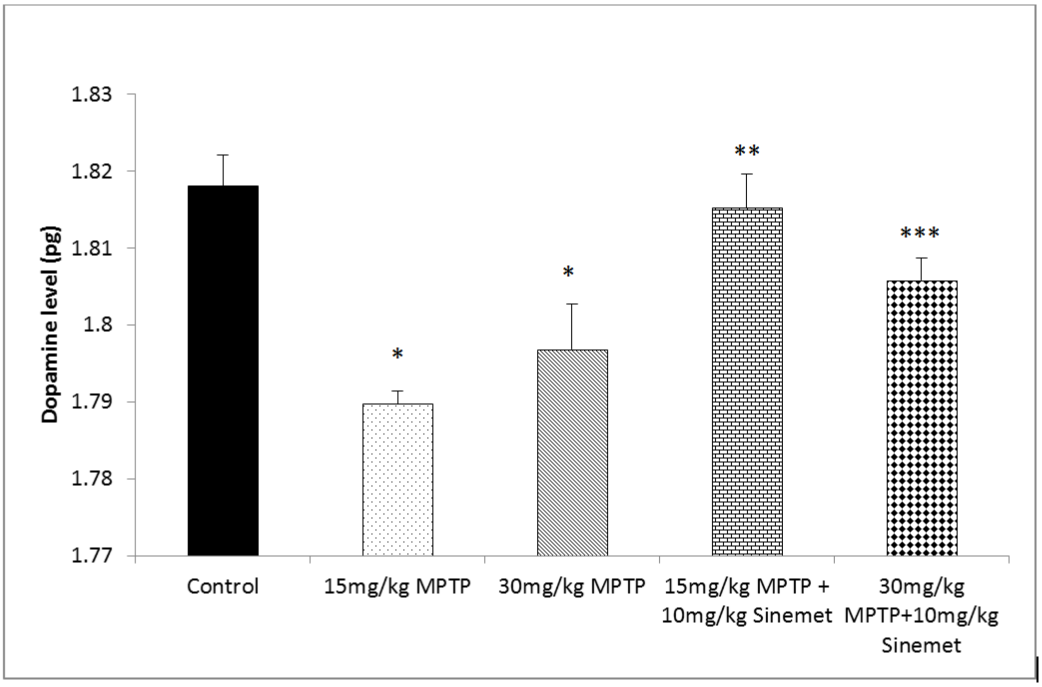


Figure 4.28 Dopamine levels in brain of C57BL/6 mice treated with 15 and 30mg/kg MPTP.

*p value < 0.05 as compared with control,

**p value < 0.05 as compared with 15mg/kg MPTP.

***p value < 0.05 as compared with 30mg/kg MPTP.

4.4.2 MAO B level from brain of C57BL/6 mice, treated with 15 and 30mg/kg MPTP

Figure 4.29 presents the MAO-B level of C57BL/6 mice treated with 15 and 30mg/kg MPTP. Y axis represents MAO-B level in nanogram (ng) and x-axis represents the group of mice. 15 and 30mg/kg MPTP significantly increased the MAO-B level (p=0.025 and p = 0.015, respectively) as compared with control. Oral administration of 10mg/kg Sinemet® successfully compensate the damage induced by 15 and 30mg/kg MPTP (p=0.015 and p=0.028) as compared with 15 and 30mg/kg MPTP, respectively.

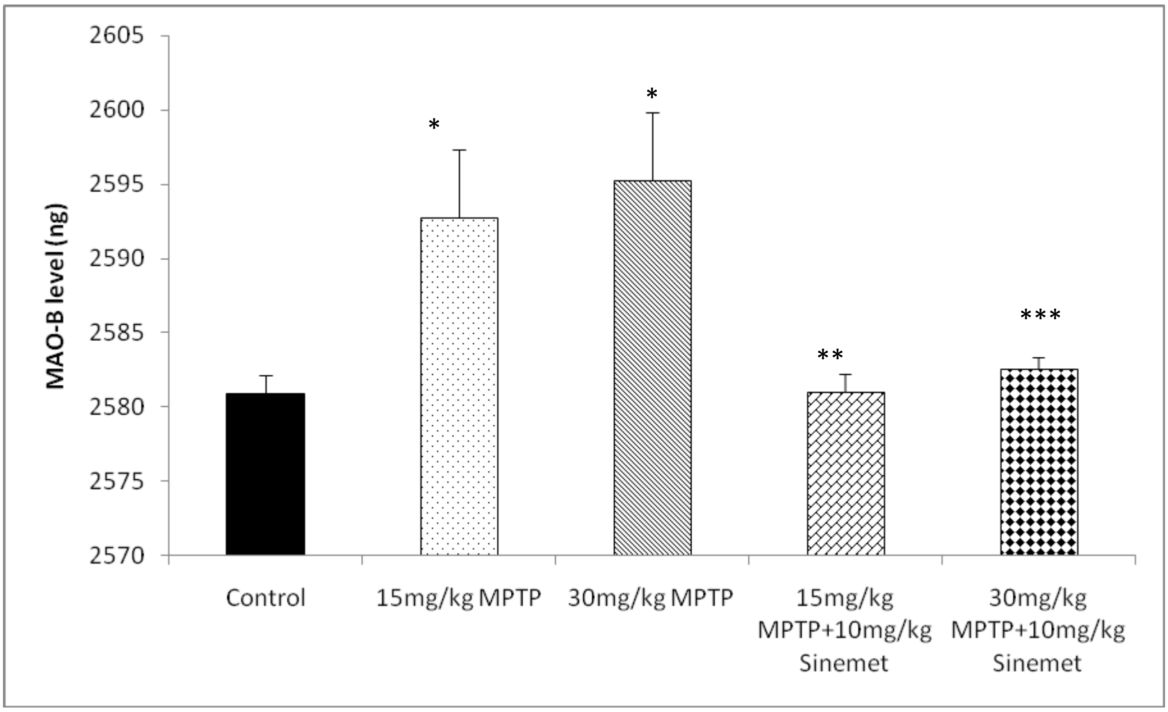


Figure 4.29 Brain MAO B level of C57BL/6 mice treated with 15 and 30mg/kg MPTP.

*p value < 0.05 as compared with control,

**p value < 0.05 as compared with 15mg/kg MPTP.

***p value < 0.05 as compared with 30mg/kg MPTP.

4.4.3 Dopamine levels from brain of Wistar rats treated with 2.5mg/kg Rotenone.

Figure 4.27 shows the dopamine levels from brain sample of Wistar rats treated with 2.5mg/kg rotenone. Rotenone is hypothesized to reduce dopamine level. Y axis represents dopamine level in pictogram (pg) and x-axis represents the group of mice. Rotenone significantly reduced rat brain dopamine level (p=0.022) as compared with control. Oral administration of 10mg/kg Sinemet® significantly compensate the damage caused by rotenone (p=0.002).

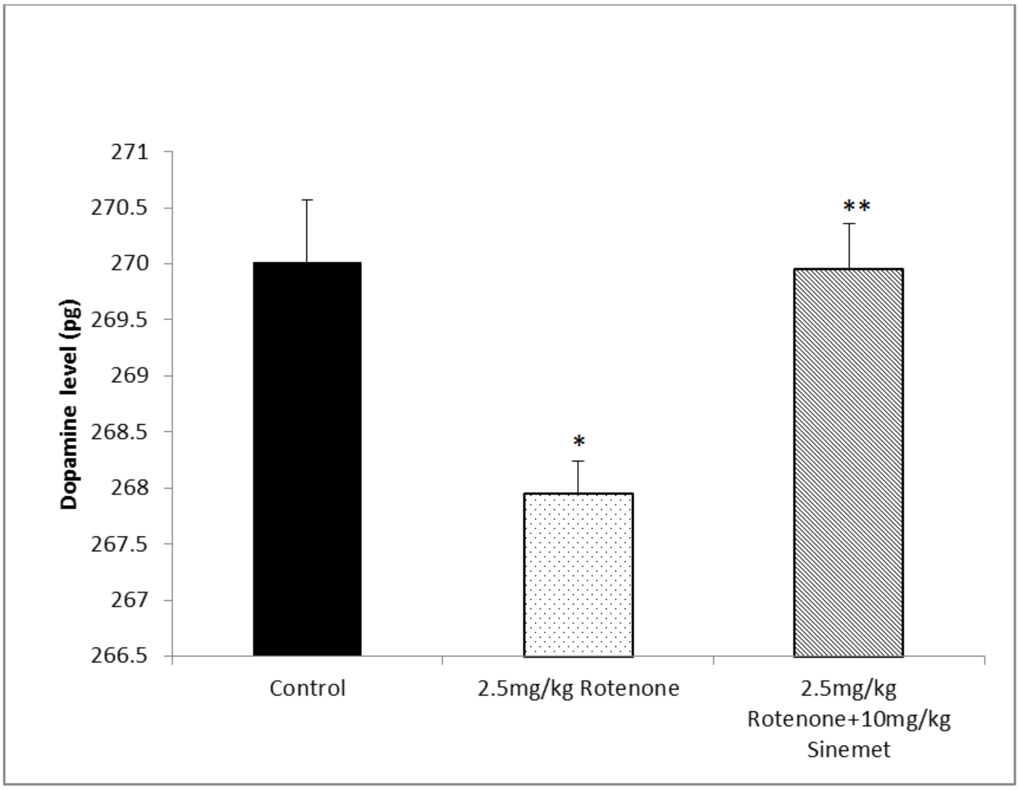


Figure 4.30 Dopamine levels in brain of rats treated with 2.5mg/kg rotenone.
p value < 0.05 as compared with control,
**p value < 0.05 as compared with 2.5mg/kg rotenone.

4.4.4 MAO B levels from brain of Wistar rats treated with 2.5mg/kg Rotenone.

Figure 4.28 shows the MAO-B level from brain sample of Wistar ratstreated with 2.5mg/kg rotenone. Y axis represents MAO-B level in nanogram (ng) and x-axis represents the group of rats. 2.5mg/kg rotenone significantly increased the MAO-B level in rat brain (p=0.10) as compared with control. Oral administration of 10mg/kg Sinemet® successfully abolished the damage induced by 2.5mg/kg rotenone (p=0.004) as compared with 2.5mg/kg rotenone.

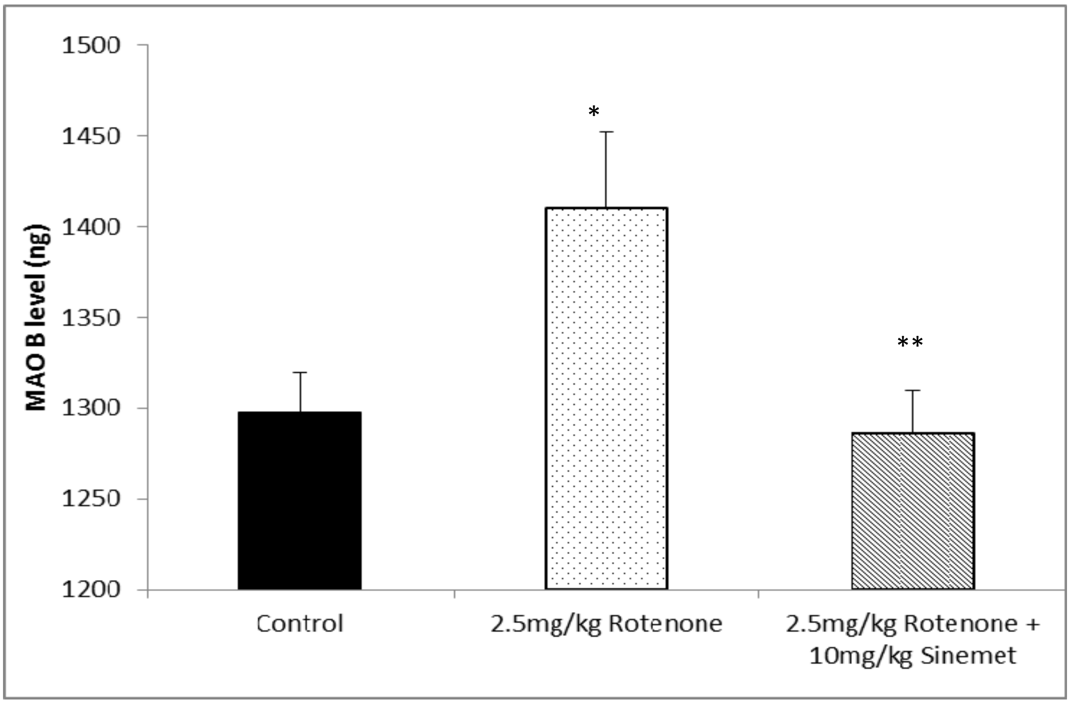


Figure 4.31 MAO-B levels in brain of rats treated with 2.5mg/kg rotenone.

*p value < 0.05 as compared with control,

**p value <0.05 as compared with 2.5mg/kg rotenone.

CHAPTER V

DISCUSSION

Neurotoxic agents such as MPTP, tacrine and rotenone were used to induce Parkinsonism features in rodents. PD is one of the most common neurodegenerative disorders with increasing probability as the age advances. Therefore, designing of a virtual Parkinsonism model in laboratory to explore various aspect of PD is an important approach in improving the treatment strategy of PD. As PD is a primarily motion related disease and major symptom includes motor incapability of patient, methods used to evaluate the motor function of rodents would be valuable for the development of anti-parkinsonian agents as well as to evaluate the possible causative factors/agents of parkinsonism . In this study, staircase test, grip test, distance travelled inside the FPA and BLM were used to evaluate the motor performances of rodents quantitatively.

5.1 Behavioral assessment of C57BL/6 mice and Wistar rats by Staircase experiment.

Staircase is used to evaluate forelimb extension and grasping capability as well as motor coordination of rodents **(66,70)**. It has been shown to be sensitive towards nigrostriatal lesions, motor cortex and sub thalamic nucleus **(66)**. Therefore, staircase experiment may be used as a preliminary experiment to check any kind of motor abnormalities after the injection of neurotoxins and the improvement of motor performance after the administration of an antidote or antagonist. Before the administration of toxins, both mice and rats were trained to grab and retrieve cheese from stairs. A simple scoop done on the cheese pellet was not counted but the animal should perform a smooth and coordinated extension of fore limbs, reach the food, make grasp and retrieve it to get point **(70)**. The neurotoxins used in this study were

MPTP and rotenone. Both toxins are examined to specifically induce brain lesion towards the nigrostriatal pathway including substantia nigra, striatum and motor cortex. **(14-15,55)**.

5.1.1 15 and 30mg/kg MPTP experiment in C57BL/6 mice.

Result of staircase experiment in C57BL/6 mice treated with 15 and 30mg/kg MPTP is shown in Figure 4.1. Baseline is the number of cheese pellets that the mice fetched after being trained (training phase = 10 months) and before the start of experiment. As expected, prior to neurotoxin injection the mice could fetch a higher number of cheeses as compared to the neurotoxin-treated animals. Performance of mice in staircase was reduced since the first dose of both 15 and 30mg/kg MPTP. The result presented in Figure 5.1 was obtained after the 6th dose of 15 or 30mg/kg MPTP (the dose after washout phase) and the 8th injection of MPTP along with the 2nd oral treatment with Sinemet® (final day of MPTP protocol, mice were euthanized). Besides motor abnormalities such as rigidity and tremor, mice also showed excess salivation, nose and eyes secretion. The number of cheese fetched by mice was significantly reduced which might imply the induction of parkinsonism symptoms by MPTP in mice. Oral administration of Sinemet® was able to improve the staircase performance. The described phenomenon could be simply explain that the injection of MPTP caused death of dopaminergic neurons which led to a permanent reduction of dopamine level in nigrostriatal pathway and Sinemet® works by replacing dopamine and increasing its level, thus, the mice could perform better. Since control group only received only vehicle, the number of cheese retrieved was quite similar with the baseline.

5.1.2 2.5mg/kg Rotenone experiment in Wistar rats.

Rats took shorter time to be trained as compared with mice. Almost every rat could retrieve pellets from both hands by the end of the training phase (4-5 months). Baseline data were taken at the end of training phase. The training was done daily for 10 min and number of pellets retrieved was limited to 15 in order to restrict excessive increase in body weight. The response of rats in staircase test was similar to baseline till the 6th injection of 2.5mg/kg rotenone. After the 6th injection, rats showed some rigidity, postural imbalance, rotation towards one side only, salivation and traces of secretion of red fluid from eyes and nose. One rat died after the 7th injection and 4 rats died after the 9th dose of rotenone. Due to this mortality of rats, the next dose of rotenone was stopped and oral treatment with Sinemet® was started for 5 days. Figure 4.2 demonstrates the staircase result of 2.5mg/kg Rotenone injected subcutaneously in Wistar rats. Rotenone treated rats were compared with baseline and rats with oral treatment of Sinemet®. Number of cheese fetched by rats injected with rotenone was significantly reduced as compared with control, and treatment with Sinemet® improved the staircase performance. Similar to MPTP, rotenone also acts specifically at nigrostrital pathway. All the parkinsonian features occurred ~~of~~ following rotenone administration was resulted from the depletion of dopamine in nigrostriatal pathway. As Sinemet® contains levodopa which is the precursor of dopamine, it restores the level of dopamine and leads to the better performance of rats in staircase experiment.

Pagnussat et al (2009) previously conducted the staircase experiment by doing a surgery and crushing median and ulnar nerves which are the nerves responsible for flexion of finger and grasping in rats (65). Montaya et al (1991) also did the staircase experiment by causing unilateral motor cortex leisons but the number of pellets fetched by rats was similar to that of control (no surgery) (71). Staircase experiment was previously used in rats only after doing a unilateral lesion or some surgery in brain to check the preferred forelimb or both forelimb ability. Samsam et al (2004) performed staircase in Long-Evans rats where various doses of scopolamine (0.1,0.3 and 1.0 mg/kg) and hermaline (1, 3, 10mg/kg) were subcutaneously injected to rats. Staircase performance of rats was reduced in both treatments, hermaline showed visual tremors and muscle weakness whereas scopolamine did not exhibited any signs of tremor and muscle weakness but the pellet grasping ability was reduced. In their work, the drugs were injected subcutaneously without making any lesions in brain, which was similar to our work. As MPTP and rotenone affect areas of brain which control movement, administration of these drugs may reduce the grasping capability of animal.

Potential advantages of staircase test are to enable an evaluation of the motor coordination and grasping capabilities which represents the motor function of individual hand. While other methods for motor function assessment allow examining the function of both fore limbs together.

5.2 Behavioral assessment of Wistar rats of 2.5mg/kg Rotenone experiment by grip test method.

Grip test method is based upon the natural principle of rodents to grab the bar and move forward when they are gently pulled backwards. Like staircase, grip test is also used to evaluate the flexion of fingers, muscle coordination as well as grip strength of rodents. Baseline data were taken before the start of experiment. The rats were gently pulled only after both fore limbs properly grabbed the trapezoid bar connected by grip strength meter. Grip test done in rats injected with 2.5mg/kg rotenone was compared with baseline and oral treatment of Sinemet®. After the 9th injections of rotenone, most of the rats showed severe symptoms of PD e.g. rigidity, postural instability, and muscle weakness with tremor. Therefore, the grip strength was significantly reduced as compared with baseline. Oral administration of 10mg/kg Sinemet® daily for 5 days regained their strength and the animals were recovered from symptoms of PD. Possibly, the replacement of depleted dopamine by levodopa was accomplished. Grip strength of rats was also highly improved as compared with 2.5mg/kg rotenone only.

Dunnet et al (2001) used Sprague Dawley rats injected with 6-OHDA via 30 gauge stainless steel cannula into nigrostratial bundle at the rate of 1µl/min. They found that there was an increase in force applied by rats, which was contrary to their hypothesis that the grip force would be reduced (72). Regarding to our experiment, Rigidity was induced but there was a significant decrease in force as per our hypothesis. Jeyasingham et al (2001) did the grip test with Sprague Dawley rats injected with 6-OHDA on right hemisphere of the brain (73). They found an increase in grip force on contralateral side (left forelimb).

5.3 Motor assessment by the use of FPA

FPA is an innovative approach to detect any kind of motor abnormalities and quantify rhythmic behaviors such as tremor in terms of frequency (Hz). As it provides us to assess the coordinated muscle movement and detect~~s~~ motor abnormalities, this device is important for neurological studies in laboratory animal.

5.3.1 Quantification of distance travelled and BLM using FPA.

Rodents administered with vehicle only were expected to have high locomotion tendency. BLM is also a parameter that reflects locomotion. In some cases, the animals stay in one place and perform other motor activities such as grooming, scratching. If an animal stays in a virtual circle of radius for more than 5 sec, then BLM scale is increased. For control animals, they may not stay in one place as they are highly mobile which reduces the BLM. Hence, the control animal should have a higher figure of the distance travelled and a lower BLM scale than the toxin-treated animals.

5.3.1.1 Effect of 15 and 30mg/kg MPTP treatment in C57BL/6 mice.

Figures 4.4 and 4.5 illustrate the effect of MPTP on distance and BLM of mice. As expected, mice receiving 15 and 30mg/kg MPTP showed a significant reduction in distance travelled and an increase in BLM as compared with control. Mice treated with MPTP experienced Parkinsonian features where they have a difficulty in moving, and most of the time stays in only one point of the plate. Therefore, the BLM score was increased and distance travelled was reduced. Oral administration of Sinemet® non-significantly improved the distance travelled in 15mg/kg MPTP-treated mice, whereas mice injected with 30mg/kg MPTP, have their the BLM score and distance travelled improved significantly. Reduction of BLM was significant in both groups.

These doses of MPTP were given once a week until the 8th injection; after that the mice were euthanized. In contrary to our results, previous researches has shown that chronic administration of MPTP increase the locomotion similar to that of control **(89-91)**. This finding probably is resulted from the development of tolerance against MPTP when the animals were given MPTP repeatedly. The hyperactivity might also be due to the serotonergic alternation **(90)**. After a single dose of MPTP, mice exhibits Parkinsonian features such as straub tail (bowed still tail), sialorrhoea (increased salivation and secretion from nose), hyperpnea (increased respiration), muscular hypotonia and teeth chatter possibly due to tremor **(90-91,93)**. Noradrenergic and serotonergic alternation possibly results in sialorrhoea and piloerection because the level of serotonin in striatum is increase after the MPTP injection **(93)**. Consistent with previous report, MPTP treated mice of our experiment also showed similar type of response, but it was usually vanished after 3-4 hours of injection. As the dose of MPTP injected was only once a week, treatment with 8 doses did not cause tolerance effect against MPTP. This could be explained that the rate of MPTP doses injected was too low to induce tolerance in mice.

5.3.1.2 Effect of 5mg/kg Tacrine treatment in C57BL/6 mice and Wistar rats.

Figures 4.6 and 4.7 present the effect of 5mg/kg tacrine on distance travelled and BLM of mice, whereas Figure 4.8 and 4.9 illustrate the effect of 5mg/kg tacrine on distance travelled and BLM of rats. Drug was administered by i.p. injection. The purpose of injecting tacrine was to induce lateral movement of jaws in rodents. Previous studies discovered the motor effects of cholinomimetic drugs e.g. pilocarpine **(79)**, haloperidol **(79)**, tacrine **(3,44)**. All these cholinomimetics are reported to be capable of inducing extra pyramidal dysfunction, catalepsy and reduction of locomotion. Another major motor effect of cholinomimetic drugs is the induction of vertical deflections of jaw which is rapid and is not stimulated by any stimulus. Tacrine acts as a noncompetitive inhibitor of acetyl cholinesterase. It is thought to interact with the hydrophobic site which is adjacent to the catalytic site of the enzyme. Since it binds with a weaker force with its target in rat brain, its binding is reversible with shorter effect in rat brain than compared with mice **(4)**.

Carriero et al (1997) demonstrated dose dependent induction of tremolous jaw movement and dose dependent suppression of locomotor activity of tacrine. The doses of tacrine used were 1.25, 2.5 and 5.0 mg/kg tacrine, and it was administered i.p. to rats **(3)**. In concord with the above finding, Hunter et al (1989) reported that tacrine induced a dose dependent tremor, salivation and chewing in rats and mice. The dose of tacrine administered ranged from 5 –20mg/kg Tacrine **(4)**. Both rats and mice showed an increase in tremor, chewing and salivation when the dose of tacrine was increased from 5 to 20mg/kg. Rearing in both animals was seen to reduce in higher doses. Consistent with their research, i.p. injection of 5mg/kg tacrine reduced locomotion activity and increased BLM in both mice and rats in a significant extent as compared with control. Both animals exhibited salivation and chewing. Chewing was found for one and a half hour, and salivation persisted for 4 hr.

In addition, Cousins et al (1999) presented that the jaw movements induced in rats were directly dependent on the level of Acetylcholine (Ach) in ventrolateral striatum. Administration of 2.5-5mg/kg tacrine increased both jaw movement and extracellular levels of ACh in ventrolateral striatum. As the dose of tacrine increased, the levels of ACh was also increased in a linear correlation in the first 30 minutes post injection **(4)**. Hence, there is a direct relationship between the level of ACh in ventrolateral striatum and the induction of chewing effect in rodents.

Nevertheless, no tremor was found as reported by previous studies **(3-4)**. Balance of dopamine and ACh in brain is an important factor for normal and coordinated movement. Disruption in any of this neurotransmitter may result in motor abnormality **(76-77)**. As tacrine is a cholinomimetic drugs, it increases the level of ACh by blocking Acetylcholine esterase. This mentioned effect alters the normal balance of dopamine and ACh leading to some disruptions in normal brain function as well as motor abnormality. But, the dose of tacrine used was too low to induce tremor in rodents. Higher doses of tacrine induced prominent tremor in rodents **(3-4)**.

6mg/kg Levodopa (i.p) was able to improve motor activity and reduce abnormal involuntary movement induced by 2.5mg/kg tacrine **(44,95)**. Consistent with these results, oral administration of 10mg/kg Sinemet® also improved the locomotion and BLM in both species.

5.3.1.3 Effect of 2.5mg/kg Rotenone treatment in Wistar rats.

Figures 4.10 and 4.11 presents the effect of 2.5mg/kg rotenone on distance travelled and BLM of Wistar rats. Rotenone is expected to induce Parkinsonism symptoms by inhibition of mitochondrial complex I and produces selective degeneration of nigrostriatal system, which is responsible for normal movement and coordination. In contrast to the other Parkinson inducing agents, rotenone causes protein aggregation and forms Lewy body in dopaminergic neurons **(51-52)**. The onset of Parkinsonism symptoms was observed from 5th or 6th dose of rotenone. At the final injection of rotenone, motor activity was significantly reduced as compared with control. Oral treatment of 10mg/kg Sinemet® improved both distance travelled and BLM in rats. Cannon et al (2009) found that i.p. injection of 2-2.5mg/kg rotenone was able to induce Parkinsonism features such as rigidity, bradykinesia, postural instability and decrease in number of rears **(21)**. Animals injected 3.0mg/kg Rotenone (i.p.) was found to have specific lesion in dorsolateral striatal dopamine terminal **(21)**. Alam et al (2002) demonstrated that injection of 3µg/kg Rotenone in right and left medial fore brain bundle reduced locomotion and induced Parkinsonism symptoms and i.p. injection of 10mg/kg levodopa was able to reverse the effect of rotenone **(78)**. This experiment revealed that rotenone acted specifically to dopaminergic neurons. Injection of levodopa was able to replace the degenerated dopamine and restored the normal body movement **(78)**. Peripheral administration of 2.5mg/kg rotenone could also induce Parkinsonism symptoms and the level of dopamine in striatum and substantianigra was found to be significantly reduced. This effect of rotenone was reversed by i.p. injection of Sinemet® **(61)**. In accord with our study, daily treatment of Sinemet® for 5 days was able to reverse the effects of rotenone and reduced parkinsonian effect to a certain extent.

5.4 Power Spectra analysis by the use of FPA.

FPA is used for detecting any kinds of muscular abnormality such as tremor and twitches. The force plate is a horizontal square shaped low mass plate supported by four transducers in every corner of the plate. These transducers sense animal behavior and send the information to computer for data processing. Basically, FPA is a combination of mechanical, electronic and computing element and uses the principle of physics and mathematics to quantify motor abnormalities in terms of frequency (Hz). Power spectra analysis is accomplished for a behavior of rodents, which are periodic and repetitive in nature. Detection of any Parkinsonism like symptom in power spectra analysis is performed by analyzing peak which is finely significant from other peaks in the rest of the spectrum. The behaviors of interest (tremor and lateral movement of jaw) are periodic in nature and are properly quantitated when the animal stays still. Y-axis of power spectrum represents power of Parkinsonism features exhibited by rodents when they are not moving. These features have higher intensity which makes them more significant in the spectrum. Locomotion can be recorded by their respective trajectory motion pictures.

5.4.1 Comparison of power spectra of control and rodents injected with toxins (5mg/kg tacrine, 15mg/kg and 30mg/kg MPTP and 2.5mg/kg rotenone)

Power spectra generated by control animals showed virtually no peaks that indicate the presence of neurotoxicity. Since control mice received only vehicles, they were perfectly fine and according to the natural behavior of rodents, they possessed high degree of locomotion. Therefore, power spectra of control displayed spectra with many peaks of high power that is not significant and resembles noise. Probably, the higher insignificant peaks are generated from the paw force of control rodents applied on the force plate.

5.4.1.1 Effect of 15mg/kg and 30mg/kg MPTP treatment in C57BL/6 mice

Power spectra generated byC57BL/6 mice treated with 15mg/kg and 30mg/kg MPTP showed significant peaks ranging from 7-12 Hz. The spectrum was cutoff and data presented was only from 7-12Hz. Minor peaks were also seen beyond the range in some frames, but were considered to be a noise or other unwanted behavior.

As the outcome expected form MPTP treatment is tremor, rigidity, akinesia and bradykinesia. With the symptom of rigidity, the muscle of limbs as well as tail became stiff and straight. Tremor could occur in any parts of the body starting from oral facial area, abdominal to tail portion. Mostly, tremor was visible in limbs, tail, trunk and head (whiskers). Therefore, MPTP then may induce one or more peaks within the desired range of 7-12 Hz. Oral administration of 10mg/kg Sinemet® reduced the intensity of peaks. In spectra analysis, if the intestity peaks of power between MPTP and Sinemet® coincided or were positioned very close to each other, it was considered to be the behavior induced by MPTP that was ameliorated by Sinemet®.

Bergman et al (1994) performed MPTP experiment in African Green Monkeys with 13 systematic injections of MPTP within 5-15 days. The monkeys developed akinesia and rigidity along with tremor which were detected at 4-8 Hz region of power spectra. Power spectra were quantitated by accelerometer **(49)**. Harmaline has been used as a tremoregenic agent in laboratory. It produces tremor detected at 8-10 Hz region in monkeys and 11-14 Hz in mice **(79)**. Injection of harmaline (i.p. or s.c.) induced tremor within a short interval of time and it was assessed by electromyography recordings or digitally quantified with systems that detected locomotion through force or magnetic field induction **(28,79-80)**. Wang et al (2001) used FPA for the quantification of tremor caused by harmaline and physostigmine in rats. They revealed that harmaline induced narrow band within the range of 10-12 Hz region of power spectra in rats **(28)**. In our study, mice with MPTP treatment generate peaks of tremor at 7-12 Hz region.

5.4.1.2 Effect of 5mg/kg Tacrine treatment in C57BL/6 mice and rats.

Despite the cardinal motor features of PD, patients also suffer from some involuntary or unconscious movement of lower jaw. Tacrine is used to mimic this lateral movement of lower jaw in rodents. Power spectra analysis on the effect of 5mg/kg tacrine displayed one significant narrow peak ranging from 10-12 Hz in mice and 0.5-3Hz in rats. Hence, the spectrum was cutoff and data presented were only from 10-12Hz in mice and 0.2-3 Hz in rats. The presence of this peak reflected the induction of lateral movement of jaws, persistent only in oral part of body. Oral administration of Sinemet® is found to reduce the power of this peak. Cousins et al (1999) performed an experiment in rats, where 2.5mg/kg tacrine was injected. Rats were subjected in electromyography and were recorded by video. Tremulous jaw movements were analyzed by computerized slow motion video tape analysis. Most of the jaw movements ranged from 3-5 Hz **(43)**. In our study where the movement of animals was monitored quantitatively by FPA, the jaw movements rendered peaks at frequency ranged from 0.5-3 Hz in rats; which was a little deviated from the result reported by Cousins et al. This slightly was possibly due to the difference in instrument used. Other parasympathomimetic drugs, physiostigmine and pilocarpine were found to induce lateral movement of jaws at 4-6 Hz.

The frequency that indicates the presence of lateral movement of jaws in mice and rats were different, possibly because they belong to different species, even if they are both rodent. Jaw movements exhibited by both species were supposed to be correlated with the parkinsonism jaw movement in humans. Henceforth, pharmacological approach for treatment of this characteristic in rodents may have positive effects on humans too.

5.4.2 Effect of 2.5mg/kg Rotenone treatment in Wistar rats.

Using Rotenone to induce Parkinsonism features is the most recent approach in animal studies of PD. Rats treated with rotenone have~~s~~ shown the presence of α synuclien protein aggregates in cell body of dopaminergic neurons. Daily s.c. injection of 2.5mg/kg rotenone in weekdays resulted in the induction of Parkinsonism features in the 6th or 7th injection. Major peaks in power spectra appeared at 0.5-2 Hz and 5-12 Hz. Figure 4.25 compared the whole frame of power spectra obtained from rats in 2.5mg/kg rotenone group vs 2.5mg/kg rotenone + 10mg/kg Sinemet® treated group. Rotenone is expected to act by increasing oxidative stress, depleting ATP and complex I in mitochondria. These effects ultimately leads to dopaminergic neurons cell death specific to striatum and Snpc causing disturbance of dopamine flow in nigrostriatal pathway. Oral treatment of Sinemet® can reduced the intensity of some peaks induced by 2.5mg/kg Rotenone. Parkinsonism symptoms expected from s.c. injection of rotenone were lateral movement of jaws, tremor and rigidity.

As rotenone is a new agent in inducing Parkinsonism features, no studies on the quantification of tremor have been done. From preliminary studies, we observed some lateral movements of jaws that were similar to jaw movement induced by tacrine in rats. As the frequency of jaw movement ranged from 0.5-3 Hz, we expected the peak that we found around 0.5-2.5 Hz to be the jaw movement induced by rotenone. Peaks observed from 4-12 Hz might be body tremor and rigidity. For better quantification, the frequency ranges were divided into two parts; 0.5-2.5 Hz and 4-12 Hz. As the behavior of interest was expected to induce when the animal stayed still, we selected to analyze those frames in which the mice were not mobile. Presence of multiple peaks from 4-12 Hz might be body tremor of rats along with rigidity.

5.5 Effect of intraperitoneal injection of 15mg/kg and 30mg/kg MPTP in rats.

MPTP experiment in rats followed the same protocol as that in mice but the mortality rate was very high. Therefore, the dose of MPTP was reduced to 10mg/kg after the 3rd injection and continued till the end of the protocol of experiment. Due to high mortality rate, this experiment was considered as unsuccessful and the toxin was later changed to rotenone to induce Parkinsonism features in rats. However, rats that received 10mg/kg MPTP, showed some Parkinsonism features such as postural instability, tremor and rigidity.

Shortly after the discovery of MPTP induced parkinsonian behavior in human, there was report indicated that rats were not sensitive to MPTP while mice responded with an intermediate sensitivity between humans and rats **(82,85)**. As discussed previously, MPTP needs MAO to be converted to its oxidized form, MPDP+ or MPP+. MPP+ is the toxic free radical that is responsible for inducing almost all the Parkinsonism features found in mice as well as in rats **(14-15)**. After s.c. injection of 60mg/kg MPTP, the concentration of MPP+ in rat brain was almost 10 times higher than in mice brain, and MPP+ remained in rat brain longer than in mice **(83)**. The higher concentrations of MPP+ in rat brain may be explicated to be the result of higher MAO-B activity in rat brain than in mice. However, dopaminergic neurons of rats need higher concentration of MPP+ than those in mice for exhibition of neurotoxicity.

Infusion of MPTP directly into substantianigra caused damage of dopaminergic neurons and degeneration of striatal dopamine levels **(87-88)**. Klaus W Lange (2005) emphasized that there was a variation in the extent of response to unilateral intranigral injection of 50µg/µl MPTP and 4µg/µl MPP+ between young (4-5 months) and old (22-24 months) rats. Old rats were more susceptible to toxicity. After one week of MPTP injection, old rats still manifested ipsilateral rotation while young ones presented ipsilateral rotation for a short period of time. However, MPP+ still can inducedipsilateral rotation in young rats. They concluded that the neurotoxic effect of MPTP was age dependent and it was more efficient to attack nigrostriatal dopaminergic system in striatum of old rat than the young ones **(85)**.

Systemic administration of MPTP exhibited a wide variation of response in rats and mice. MPTP might be metabolized by MAO in capillaries of rat brain and the enzyme barrier at blood-brain barrier which elevated levels of MPP+ in brain. MPP+ possibly either be dispersed in circulatory system or came in contact with endothelial cells **(82-83)**. According to Chiueh et al (1984), systemic administration of 5-10mg/kg MPTP caused parkinsonism features such as immobility, salivation, pilo erection but no permanent destruction of motor function **(86)**. Subcutaneous injection of 40mg/kg MPTP reduced dopamine levels in neostriatum in mice but did not alter this level in rats **(83)**. Therefore, rats might be more resistant to the systematic treatment of MPTP as compared with mice.

However, repeated subcutaneous injection of 60mg/kg MPTP at an interval of 8 hrs caused high mortality in rats **(83)**. Pretreatment with guanethidine, a sympatholytic agent administered before subcutaneous injection of 60mg/kg MPTP improved the longevity of rat. Guanethidine acts by reducing peripheral catecholamines and thus prevents peripheral conversion of MPTP. As guanethidine is structurally bigger, it is not allowed to pass through blood brain barrier **(83)**. This suggested that MAO level in brain capillaries may possibly have some role in MPTP toxicity, MPTP administered systemically may be peripherally converted to toxic metabolite in organs which led to high degree of mortality in rats **(83)**.

The purpose behind MPTP to be highly specific towards contents of motor cortex despite the fact that there are other parts of brain where the concentration of MAO-B is relatively higher also remains to be studied.

5.6 ELISA test of Dopamine and MAO-B from brain sample of C57BL/6 mice treated with 15 and 30mg/kg MPTP.

According to the mechanism of action of MPTP, MAO-B is an important enzyme for the conversion of MPTP to MPP+. Accumulation of MPP+ in dopaminergic neurons via dopamine transporter is the pathological reason behind the induction of Parkinsonism features in humans and rodents. Therefore, it is important to determine the levels of dopamine and MAO-B from the brain sample. We proposed that there should be a significant reduction of dopamine and remarkable increase in MAO-B level in the brain of mice treated with 15 and 30mg/kg MPTP as compared with control. In addition, oral treatment with 10mg/kg Sinemet® should increase the levels of dopamine and reduce MAO-B levels in mice brain as compared with 15 and 30mg/kg MPTP treatment.

Fig 4.28 illustrates the result of dopamine level quantification using ELISA process. As expected, brain sample from control animals had the highest levels of dopamine as compared to those with 15 and 30mg/kg MPTP treatment and oral administration of Sinemet® restored the depleted dopamine and increased its value. Sinemet® contains levodopa which is the precursor of dopamine. Levodopa crossed the blood brain barrier and being converted to dopamine and replaces the depleted dopamine that resulted from MPTP. Since nigrostriatal pathway is essential for normal movement and function of body, replacement of dopamine might help rodents to regain their normal movement to some extent.

Fig 4.29 presents the result of MAO-B level quantification using by ELISA technique. Level of MAO-B increased significantly in 15 and 30mg/kg MPTP treated mice as compared with control mice. MAO-B is an important enzyme involved in *in vivo* endogenous metabolism of dopamine by oxidative deamination (11) (12). Higher level of MAO-B reveals the fact that a higher amount of dopamine could be degraded. Moreover, level of MAO-B has been reported to be associated with MPTP-induced Parkinsonism and Parkinson's disease. Level of MAO-B may increase with acceleration of age. In addition, the activity of MAO-B has been demonstrated to be increased upon the response of increased oxidative attack. Raise of MAO-B level in the brain results in depletion of dopamine as well as the selective and progressive loss of dopaminergic neurons in substantianigra, along with distress in mitochondrial complex I activity and increased mitochondrial stress. Increase in MAO-B levelmay also involve the generation of H2O2, which can induce some dysfunction in mitochondrial complex, I and generates mitochondrial superoxide **(11) (12) (127)**. The activity of MAO-B is doubled in substantianigra and correlates with the extent of dopaminergic cell loss **(11)**. Introduction of MPTP within the glial cells or astrocytes of brain might enhance the activity of MAO-B, increasing their level when compared with control mice **(14)**. Increase in levels of MAO-B may also correlate directly with the production of reactive oxygen species, aggravating the oxidative stress. Oral treatment of Sinemet® reduced the level of MAO-B as compared with 15 and 30mg/kg MPTP.

5.7 ELISA test of Dopamine and MAO-B from brain sample of Wistar rats treated with 2.5mg/kg Rotenone.

Rotenone treatment is a recent approach to generate an animal model for studies in PD. Even though rotenone is not a selective dopaminergic neurotoxin, systemic administration of rotenone in rats has been revealed to be an important approach to study human Parkinsonism **(89)**. Rotenone is lipophilic in nature; it does not require any transporters or carrier to reach the site of action **(64)**. Like MPP+, it inhibits cell respiration by attacking complex I and causes ATP depletion causing oxidative damage **(51-52) (54)**.

Systemic injection of rotenone continuously was shown to induce α-synucleopathy along with selective dopaminergic neuro-degeneration and motor dysfunction **(53,89)**.

The exact site of action of rotenone is still controversial. Betabert et al (2000) demonstrated that continuous intravenous infusion of rotenone is highly specific to dopaminergic neurons of substantianigra **(90)**. Ferrante et al (2005) revealed that it doesn’t attack dopaminergic neurons of substantianigra but acts at globuspallidus and striatum of the brain. Alam et al (2002) demonstrated that rotenone reduced the dopaminergic level from pre frontal cortex and striatum. These results provided an obvious evidence that nigrostriatal dopaminergic pathway was at least the area of brain that showed susceptibility when rotenone was infused in rats. Reduction of dopamine level directly may correlate to the high level of MAO-B because MAO-B was involved in the metabolism of dopamine.

Chronic subcutaneous injection of rotenone might induce the production of high levels of reactive oxygen species. These high amounts of free radicals probably act by inhibiting the complex I of mitochondria, causing oxidative stress and cell death **(11-12)**. Rats treated with 2.5mg/kg rotenone (s.c.) were shown to have a reduced level of dopamine and an increase in MAO-B level. Sinemet® administration replaced the dopamine and inhibited its metabolism at peripheral compartments. Thus, the level of dopamine was increased, reduced oxidative stress and reduced MAO-B level.

CHAPTER VI
CONCLUSION

15mg/kg and 30mg/kg MPTP and 5mg/kg tacrine were used to induce Parkinsonism features in mice whereas 2.5mg/kg rotenone and 5mg/kg tacrinewere used for the same purpose in rats. Due to their difference in solubility, MPTP and tacrine were injected intraperitoneally while rotenone was injected subcutaneously.

Power spectra analysis revealed that 5mg/kg tacrine was able to induce lateral movement of jaws in both mice and rats at a frequency of 10-12 Hz and 0.5-3 Hz respectively. Locomotion ability of both mice and rats was reduced by i.p. administration of 5mg/kg tacrine which was ameliorated by oral administration of 10mg/kg Sinemet®at 30 min prior to i.p. injection.

15mg/kg and 30mg/kg MPTP was able to reduce locomotion and induced tremor in mice at a frequency of 7-12 Hz as detected by using Power spectra analysis. MPTP was found to be effective for inducing parkinsonism features in mice to but not in rats. Rats were shown to be extremely sensitive tothe toxicity of systemic injection of 15 and 30mg/kg MPTP and resulted in high mortality rate. Thus, the drug used to induce PD in rats was changed to rotenone.In our preliminary study,rotenone caused lateral movement of lower jaws as well as body tremor in rats. When analyzing the power spectra, we found significant peaks at 0.5-3Hz and 4-12 Hz. As the first peak lied on the range of peak showed by tacrine in rats, we expected that this peak should be the frequency induced by movement of lower jaws; whereas 4-12 Hz might represent the tremor induced by rotenone in rats. Oral administration of 10mg/kg Sinemet® was able to improve locomotion in mice induced by MPTP and in rats induced by rotenone.

Staircase results of mice treated with15 and 30mg/kg MPTP and rats injected with 2.5mg/kg rotenone showed significant lowering in capability of grabbing and retrieving cheese, suggesting the disturbance of smooth movement of muscles and tremor induced by MPTP and rotenone respectively. While the animalswere manifesting the symptom of tremor, they were not able to perform normal activity such as grasping and retrieving cheese. Oral administration of Sinemet®; however improved their performance in grasping and hand motor skills.

Grip test was done to measure the maximum grip strength force applied by rotenone treated ratson the trapezoid bar when their tails were gently pulled backwards. Administration of rotenone reduced the ability of rats to grasp the bar and reduce the strength applied on bar when being pulledbackwards, which reflected the muscle weakness and induction of tremor by rotenone. Some of the rats experienced higher degree of postural instability and were not able to grasp or grasp with very light strength on the bar. Oral administration of Sinemet® improved their grasping ability and peak strength force applied on bar.

ELISA analysis revealed significant reduction of dopamine level in brain ofboth MPTP treated mice and rotenone treated rats.As PD is a movement disorder and is highly specific to nigrostriatal pathway, degeneration of dopamine neurons is a major aggravating factor in the pathogenesis of this disease in both humans and rodents. Oral administration of Sinemet® has shown to improve the dopamine level possibly because it supplies levodopa, the precursor of dopamine and thus restores the depleted dopamine in brain.

ELISA analysis quantitatively demonstrated the higher level of metabolizing enzyme, MAO-B, upon the treatment with MPTP and rotenone. Higher level of MAO-B reveals the fact that a higher amount of dopamine could be degraded. Moreover, it has been reported previously that the activity of MAO-B is increased upon the response of increased oxidative attack. Thus, this observation indicatedthe generation of free radical and oxidative stress as well as the increase in dopamine metabolism following MPTP treatment. These effects of MPTP lead to the decrement of dopamine in brain and are supposed to be associated with the induction of parkinsonian symptoms. Oral ingestion of 10mg/kg Sinemet® was able to reduce the MAO level back to the same amount as that of control rats.

High mortality rate of rats injected with MPTP wasone of the major problemsduring the work. This led us to conclude that rats were not suitable for the administration of MPTP and new experiment was conducted in a new set of rats with a new toxin, rotenone.Our experimental model suggests that mice can be a good model of MPTP to induce and study Parkinsonism but not in case of rats. Rotenone can be used to induce parkinsonismmodel in rats.But, these effects of rotenone have not been quantitativelyanalysed. Administration of MPTP in mice and rotenone in rats also supports the proposed hypothesis of neurotransmitter imbalance in PD. Levodopa which was considered as the best drug for the management of PD showed a favorable response in both mice and rats injected with MPTP and rotenone respectively. Due to its side effects in case ofchronic administration, levodopa induced dyskinesia and other movement related disorder. Therefore,there is a need of new drugs for the treatment ofPD with more effective and with fewer side effects. Our result suggested that the efficacy of any new drugs in the treatment of PD can be evaluated by using this model and FPA can be used for behavioral quantification.

Further studies:

Neurochemical quantification of tyrosine hydroxylase andhomovanillic acid can also provide some knowledge about the extent of dopamine degradation in brain. Therefore, quantitative analysis of these chemicals in brain can also be a part of new study.Our rotenone model in mice could be a new experimental option to study the behavioral effect of compound that could induce parkinsonian features as well as to evaluate any anti-parkinsonian agent. Analysis of dopamine and MAO-B using other biochemical tools such as Western blot might enable a further exploration to the mystery of this disease. Neurochemical quantification of Ach and Acetylcholine esterase could be studied on the brains of rodents treated with tacrine.

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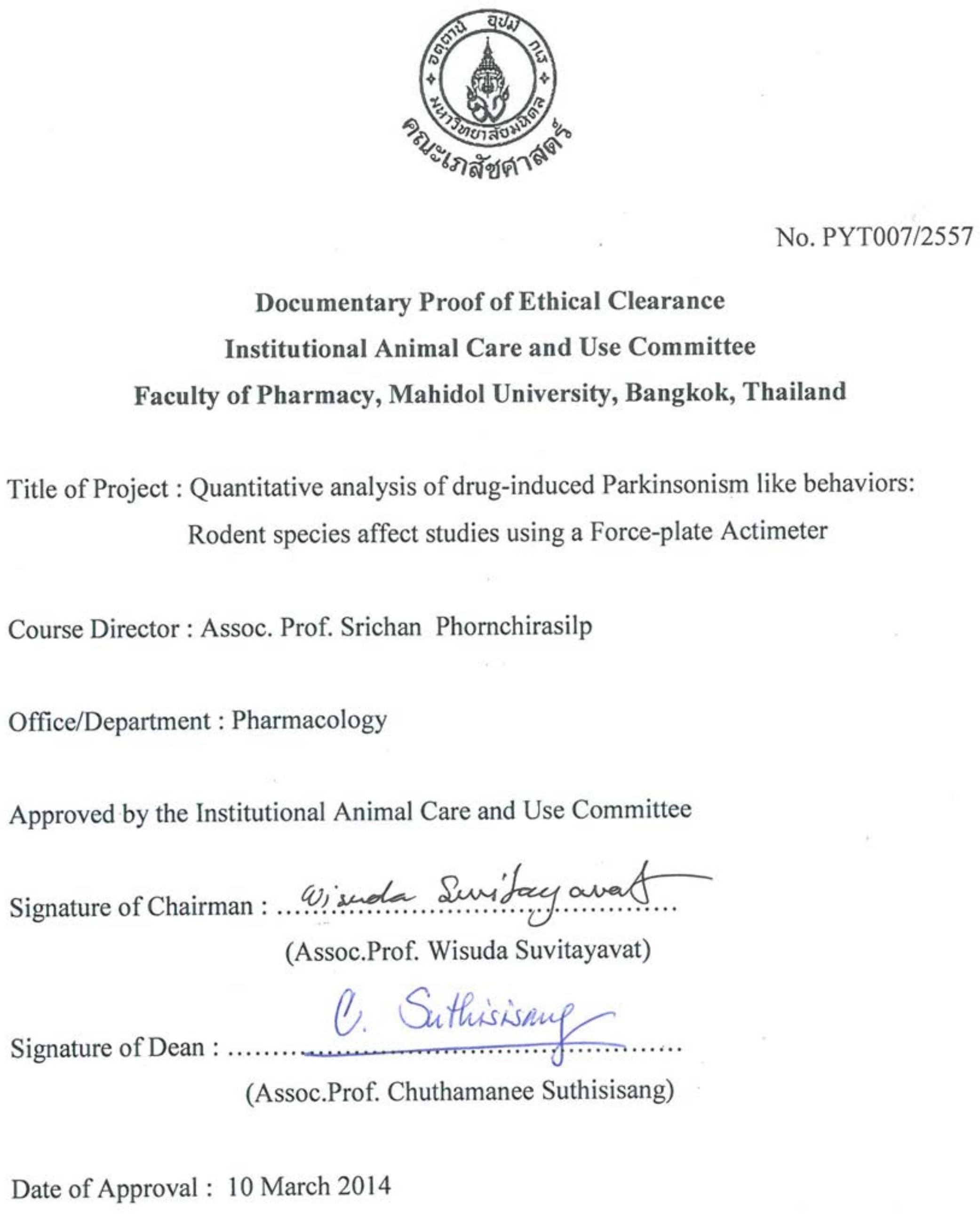
APPENDICES

APPENDIX A



No. PYT007/2557

Documentary Proof of Ethical Clearance
Institutional Animal Care and Use Committee
Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

Title of Project : Quantitative analysis of drug-induced Parkinsonism like behaviors: Rodent species affect studies using a Force-plate Actimeter

Course Director : Assoc. Prof. Srichan Phornchirasilp

Office/Department : Pharmacology

Approved by the Institutional Animal Care and Use Committee

Signature of Chairman : Wisuda Suvitayavat
(Assoc.Prof. Wisuda Suvitayavat)

Signature of Dean : C. Suthisisang
(Assoc.Prof. Chuthamanee Suthisisang)

Date of Approval : 10 March 2014

APPENDIX B

DATA FROM FORCE PLATE ACTIMETER

Distance travelled by mice after the injection of 5mg/kg tacrine and Oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from mice of 5mg/kg tacrine group					
	1	2	3	4	5	Mean±SE
Control	407.12	714.77	335.70	267.74	279.41	400.95±82.2
5mg/kg tacrine	40.97	54.959	71.3667	71.359	87.8165	65.294±7.99
5mg/kg tacrine+10mg/kg Sinemet ®	79.99	132.88	363.54	333.75	662.61	314.56±102.

BLM of mice after the injection of 5mg/kg tacrine and Oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from mice of 5mg/kg Tacrine group					
	1	2	3	4	5	Mean±SE
Control	2.36	0.78	0.36	0.86	1.1	1.09±0.33
3% acacia + 5mg/kg tacrine	7.21	6.91	7.6	6.46	5.91	6.82±0.29
10mg/kg Sinemet ®+5mg/kg tacrine	4.68	7.16	3.7	2.38	2.11	4.01±0.915

Distance travelled by rats after the injection of 5mg/kg tacrine and Oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from Rats of 5mg/kg Tacrine group						Mean±SE
	1	2	3	4	5	6	
Control	58.81	55.38	71.38	47.96	65.99	38.39	56.32±4.89
3%acacia+5mg/kg tacrine	12.71	13.66	19.25	13.70	17.69	16.88	15.65±1.08
10mg/kg Sinemet ®+5mg/kg tacrine	27.98	39.30	26.81	37.84	28.70	49.42	35.00±3.61

BLM of rats after the injection of 5mg/kg tacrine and Oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from Rats of 5mg/kg Tacrine group						Mean±SE
	1	2	3	4	5	6	
Control	5.62	5.70	5	6.38	5.08	7.02	5.80±0.31
3%acacia+5mg/kg tacrine	7.60	7.25	7.60	7.39	7.42	7.25	7.42±0.06
10mg/kg Sinemet ®+5mg/kg tacrine	7.04	6.56	7.01	6.60	7.06	6.09	6.72±0.15

Distance travelled by mice after the injection of 15mg/kg and 30mg/kg MPTP along with oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from Mice of 15 and 30mg/kg MPTP						
	1	2	3	4	5	6	Mean±SE
Control	513.29	610.10	550.09	173.65	667.29		502.89±86.38
15mg/kg MPTP	76.15	86.57	113.57	74.08	96.04	76.57	89.28±6.27
30mg/kg MPTP	75.67	88.74	78.525	85.33	76.57		80.97±2.57
15mg/kg MPTP+10mg/kg Sinemet ®	98.82	118.68	547.06	83.88	100.85	83.60	189.86±75.16
30mg/kg MPTP+10mg/kg Sinemet ®	214.34	335.32	552.09	180.63	404.16		337.31±67.18

BLM of mice after the injection of 15mg/kg and 30mg/kg MPTP along with oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from Mice of 15 and 30mg/kg MPTP						
	1	2	3	4	5	6	Mean±SE
Control	0.42	0.51	4.38	0.54	0.20		1.21±0.79
15mg/kg MPTP	6.97	6.26	4.13	6.27	7.44	7.01	6.21±0.48
30mg/kg MPTP	6.60	6.85	6.37	6.93	7.36		6.82±0.16
15mg/kg MPTP+10mg/kg Sinemet ®	3.73	4.65	5.34	2.79	4.34	5.24	4.17±0.39
30mg/kg MPTP+10mg/kg Sinemet ®	3.33	4.96	5.24	5.20	4.38		4.62±0.35

Distance travelled by rats after the injection of 2.5mg/kg rotenone and Oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from rats of 2.5mg/kg rotenone					
	1	2	3	4	5	Mean±SE
Control	92.58	89.27	76.22	81.63	76.76	83.29±3.29
3% acacia + 2.5mg/kg rotenone	27.65	28.21	34.27	40.56	26.13	31.36±2.68
10mg/kg Sinemet ® + 2.5mg/kg rotenone	48.19	34.54	55.13	35.44	54.63	45.59±4.49

BLM of rats after the injection of 2.5mg/kg Rotenone and Oral administration of 10mg/kg Sinemet ® compared with Control.

	Data from rats of 2.5mg/kg Rotenone					
	1	2	3	4	5	Mean±SE
Control	5.08	5.66	5.49	5.56	5.52	5.44±0.1
3% acacia + 2.5mg/kg rotenone	7.27	7.37	7.03	7.03	6.95	7.21±0.09
10mg/kg Sinemet ® + 2.5mg/kg rotenone	6.56	7.09	6.37	6.37	7.20	6.68±0.19

APPENDIX C

DATA FROM DOPAMINE ELISA KIT

Dopamine level from the brain of mice treated with 15 and 30mg/kg MPTP group.

	Dopamine level (pg) of 15 and 30mg/kg MPTP mice group						
	1	2	3	4	5	6	Mean ± SE
Control	1.814	1.831	1.811	1.814	1.818		1.81 ±0.003
15mg/kg MPTP	1.817	1.823	1.805	1.725	1.738	1.827	1.789 ±0.001
30mg/kg MPTP	1.753	1.798	1.810	1.803	1.801	1.813	1.7968 ±0.005
10mg/kg Sinemet ®+15mg/kg MPTP	1.816	1.811	1.830	1.798	1.823		1.8152 ±0.004
10mg/kg Sinemet ®+30mg/kg MPTP	1.812	1.799	1.809	1.798	1.8135		1.805 ±0.0028

Dopamine level from the brain of rats treated with 2.5mg/kg rotenone.

	Dopamine level (pg) of 2.5mg/kg rotenone group						
	1	2	3	4	5	6	Mean ± SE
Control	268.427	268.30	271.29	271.15	270.83	270.08	270.01±0.549
2.5mg/kg rotenone	266.843	267.867	268.505	268.851	267.575	268.051	267.94±0.288
10mg/kg Sinemet ®+2.5mg/kg rotenone	269.687	270.756	270.564	268.496	269.026	271.207	269.95±0.401

APPENDIX D

DATA FROM MAO-B ELISA KIT

MAO-B level from the brain of mice treated with 15 and 30mg/kg MPTP group.

	MAO-B level of mice of 15 and 30mg/kg MPTP group						
	1	2	3	4	5	6	Mean ± SE
Control	2579.68	2584.206	2577.985	2581.798			2580.917±1.2034
15mg/kg MPTP	2587.45	2582.85	2607.108	2607.034	2587.866	2584.216	2592.75±4.593
30mg/kg MPTP	2602.742	2606.897	2584.362	2587.194	2576		2595.299±4.565
10mg/kg Sinemet ® + 15mg/kg MPTP	2584.157	2584.367	2581.13	2579.432	2580.627	2576.703	2581.069±1.188
10mg/kg Sinemet ® + 30mg/kg MPTP	2580.886	2584.615	2583.864	2580.852			2582.554±0.8043

MAO-B level from the brain of rats treated with 2.5mg/kg rotenone.

	MAO-B level of rats of 2.5mg/kg rotenone						
	1	2	3	4	5	6	Mean±SE
Control	1237.89	1333.13	1365.743	1235.261	1307.592	1308.728	1298.057±21.27
2.5mg/kg rotenone	1201.74	1462.963	1458.874	1432.947	1435.607	1468.804	1410.156±42.113
10mg/kg Sinemet ®+2.5mg/kg rotenone	1356.364	1220.171	1320.864	1332.806	1186.943	1277.998	1286.407±23.466

APPENDIX E
ABSTRACT OF PRESENTATION

Poster Presentation, JSPS-NRCT Follow-Up Seminar 2015 and 31st International Annual Meeting in Pharmaceutical Sciences (JSPS-NRCT 2015 and IAMPS31)

QUANTITATIVE ANALYSIS OF TACRINE INDUCED PARKINSONISM IN C57BL/6 MICE USING FORCE PLATE ACTIMETER.

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Abstract

Parkinson’s disease (PD) is a common neurodegenerative disorder mainly characterized by the progressive loss of dopaminergic neurons in Substantia nigra pars compacta (SNpc). It is generally specific to nigro-striatal dopaminergic pathway which is involved in normal and coordinated movement. Apart from their cardinal motor features like tremor, rigidity, bradyskinesia and postural instability, patient with PD also displays hypokinesia and rigidity of orofacial muscles. Due to this, patient shows symptoms like difficulty in swallowing, reduced blinking of eyes and involuntary jaw movement. Systematic administration of cholinomimetic drugs like tacrine in rodents induce lateral movement of lower jaw which is supposed to be tremulous and repetitive and are not stimulated by any purpose. It has also been

suggested that these deflections of lower jaw can be used to mimic parkinsonian tremor in laboratory.

This experiment aims to study behavior and frequency characteristics of parkinsonian symptoms induced by Tacrine, an anticholinesterase. Force Plate Actimeter (FPA) was used for the quantification of results. Goal of using FPA is objective quantification of animal’s behavior like locomotion, tremor, ataxia when the animal is kept inside it. The main significance of FPA is the light and sound attenuating chamber that can shield environmental impact on mice up to some extent.

18 C57BL/6 mice were equally divided into three groups: Sham, Control and Treatment. Sham and control group received 3% acacia orally while treatment group received 10mg/kg Sinemet (Levodopa + Carbidopa in the ratio of 4:1). After 30 minutes sham received normal saline solution (NSS) intraperitoneally (i.p.), control and treatment group received 5mg/kg Tacrine i.p. Mice were immediately kept inside FPA for 1 hour and 30 min to record their behavior. Motor parameters like distance travelled, Bout Low Mobility (BLM) and power spectra of parkinsonian symptoms shown by tacrine were computed. Time interval inside FPA was divided into frames with 1 frame consisting of 80 seconds. Only the frame in which the mouse was not moving was taken for further analysis of power spectra. Movement of mouse can be detected by picture of trajectory motion.

Distance travelled by control animal inside FPA was significantly reduced (p= 0.004) as compared with sham. In some cases, the animal may stand still and perform other activities like head bobbing, grooming. Therefore, we need to compute another parameter, the BLM. BLM scale ranges from 0 to 8 and has lower scale when the animal shows high mobility. BLM of control group increased significantly as compared with sham group (p= 0.00). Oral administration of 10mg/kg Sinemet was seen to ameliorate both distance travelled (p= 0.017) and BLM (p= 0.004) as compared with control group. According to power spectra, frequency in Hertz (Hz) of lateral jaw movement as induced by tacrine is indicated by a peak that is easily noticeable from the rest of the spectra. This peak was seen to range from 10-12 Hz of spectra. Sinemet was seen to reduce the power of this peak at around same frequency as that of tacrine. Tacrine was found to start its effect from about 10-15 min of i.p. injection (around frame 10).

Henceforth, Sinemet was seen to improve movement of lower jaw as well as increase motor capabilities of mice that were suppressed by tacrine. It is possible to use these results to create parkinsonian model in laboratory.

Keywords:Parkinson’s disease, Power spectra, Tacrine, FPA.

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