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Exploring the in-vitro models for Parkinson's Disease: Differentiation and Limitations

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ABSTRACT

An extensive synopsis of the several in-vitro models used in Parkinson disease (PD) research is given in the summary. The main features of Parkinson's disease (PD) pathology are discussed, such as the degeneration of dopaminergic neurons in the substantia nigra, the role of basal ganglia in motor function, and the complexity of both motor and non-motor symptoms in PD. This paper examines in vitro models; it covers primary cultures, organotypic cultures, induced pluripotent stem cells (iPSCs), genetic models, neurotoxin-based models, and cell lines (such SH-SY5Y, LUHMES, PC12, MN9D, N27, and Neuro2a). It delves into the differentiation methods, advantages, limitations, and applications of each model. Notably, it discusses the potential of these models in mimicking PD pathology, their use in screening potential treatments, and their significance in elucidating molecular mechanisms underlying PD pathogenesis. The document also addresses limitations of these models, including challenges in replicating the complexity of the central nervous system and the difficulty in simulating early disease stages. Ultimately, the review underscores the importance of in-vitro models in studying PD pathophysiology while acknowledging that no single model fully encapsulates all aspects of the disease. It emphasizes the need for combined models and highlights recent advancements in technologies like iPSCs and organoids derived from PD patient cells, offering new avenues for investigating neurodegenerative diseases.

1. INTRODUCTION

The loss of dopaminergic neurons in the midbrain region's substantia nigra pars compacta (SNpc) is what characterizes Parkinson disease (1). The Lewy body is connected to this as well. The loss of striatal dopaminergic neurons is linked to Parkinson's disease (PD) motor symptoms, although non-motor symptoms can also arise from non-dopaminergic locations. Motor and cognitive abilities are affected by distinct effects on the basal ganglia during the neurodegenerative process in Parkinson's disease (PD). The dorsal motor nucleus of the vagus nerve, the olfactory bulbs and nucleus, the locus coeruleus, and finally the loss of neurons from the SNpc are the first areas where Parkinson's disease (PD) pathology arises. The cortical regions and susceptible nuclei are the areas most affected (2). Lewy bodies, which are composed of neurofilaments and clumps of α-synuclein and ubiquitin immunoreactivity, are

seen in degenerating neurons.

There are 5/100,000 to 35/100,000 new instances of Parkinson's disease (PD) annually. Instances of these grow five to ten times during the sixth and ninth decades of life. According to reports, the prevalence of Parkinson's disease (PD) rises from 1% to 3% in those over 80 years of age. The neuroprotective effects of estrogen on the nigrostriatal dopaminergic pathway are responsible for the gender difference in incidence, which is reflected in a 3:2 ratio of males to females. End-stage and advanced disease can cause major side effects, such as pneumonia, which is frequently fatal (2).

2. PATHOPHYSIOLOGY

The extrapyramidal system ailment known as Parkinson's disease affects the basal ganglia's motor structure. Reduced motor functions resulting from dopaminergic function loss are characteristic of Parkinson's disease (PD) (3). Although the primary cause of Parkinson's disease (PD) motor symptoms is the loss of dopaminergic neurons in the striatum, other neurotransmitters from the glutaminergic, cholinergic, serotonergic, and adrenergic systems are also likely involved, as evidenced by the occurrence of non-motor symptoms (4). The motor activity in the extrapyramidal system is influenced by two types of dopamine receptors: D1 (excitatory type) and D2 (inhibitory type). The basal ganglia, which involve the pars reticulata section of the substantia nigra (SNpr) and the globus pallidal segment (GPi) of the ventral striatum, are components of this system.

Larger circuits found in the thalamus and cortex contain these parts. When gamma aminobutyric acid (GABA) is disrupted due to loss of dopamine in the striatum of Parkinson's disease (PD) patients, the GPi/SNpc circuits become more active and the thalamus is inhibited. Reduced motor activity, a hallmark of Parkinson's disease (PD), is ultimately caused by the thalamus's diminished capacity to activate the frontal cortex (5).

Because the human brain is not easily accessible for study, little is known about the early stages of Parkinson's disease. The majority of research has utilized postmortem brain tissues to examine the molecular mechanisms behind the pathogenesis of the disease, however this approach is limited to the end stage of the disease and does not reveal the critical early stages of the disease etiology. Regarding the prenatal diagnosis PD models, It is employed both in vitro and in vivo. They are able to mimic the illness phenotype and replicate the essential components of dopaminergic systems (6).

3. IN-VITRO MODELS

In vitro models offer a regulated setting that makes it easier to investigate the pathophysiology of dopaminergic degeneration in Parkinson's disease. This can also be applied to the research that screens possible treatments. However, they also have certain drawbacks, such as the challenge of accurately simulating the in vivo complexity of the central nervous system (7).

The DA is produced in the SNpc by synthesizing tyrosine. The production of 3,4-dihydroxy-L-phenylalanine (L-DOPA), which is the rate-limiting step, is catalyzed by tyrosine hydroxylase (TH). L-amino acid decarboxylase converts L-DOPA into DA (8). An action potential is triggered upon synthesis, storing dopamine in the synaptic cleft. After being transported to the presynaptic terminal, DA is transferred into vesicles by vesicular monoamine transporters (VMAT) 1 and 2. Following its release into the synaptic cleft, dopamine (DA) binds to receptors on post-synaptic neurons (D1, D2, D3, D4, D5). The presynaptic neurons' reuptake of neurotransmitters from the synaptic cleft is primarily carried out by the dopamine transporter (DAT). The enzymes catechol O-methyl transferase and monoamine oxidase, which are produced by glial cells and neurons, can also metabolize dopamine (DA) in the synaptic cleft (9).

4. CELL LINES

They originate from the cell population of a multicellular organism. These are either naturally or artificially immortalized, which results in the normal senescence of cells and the loss of some checkpoint pathways in the cell

cycle. It is appropriate for testing neuroprotective substances for the treatment of Parkinson's disease. Large drug panels can be quickly screened, and the most promising drug can be chosen for additional research (10). To replicate the genetic or toxin-based PD pathology in cell lines models are applicable. Genes linked to Parkinson's disease are either highly efficiently knocked out or overexpressed. In cell lines that produce DA, cell death can be induced using MPP^+ or 6-OHDA.

The advantages of using these models are as follows:

Since these cells represent a homogenous population, their endless proliferation enables high throughput experimentation utilizing a wide range of experimental techniques and endpoints. Additionally, some cell lines express crucial enzymes for DA metabolism and synapse formation, making them highly reproducible when compared to primary and organotypic cultures.

Additionally, cell lines have certain drawbacks: In stark contrast to neurons, which do not divide, they are in a highly proliferative state. in comparison to primary neurons and organotypic cultures, do not express synaptic proteins at significant levels. Continuous proliferation creates a selection pressure that favors mutations that increase survival and proliferation, leading to the loss of dopaminergic phenotype in subsequent generations of cell lines relative to their parent lines (11).

Following cell lines are used: **SH-SY5Y, LUHMES, PC12, MN9D, N27, Neuro2a**

(1) SH-SY5Y

These are the SK-N-SH subclone, which were first created from a bone marrow biopsy of a patient diagnosed with neuroblastoma, and which have sympathetic adrenergic ganglia origins. Because these cell lines have many of the biochemical and functional characteristics of neurons, they are used as models of neurons. They are classified into three distinct phenotypes: intermediate (I type), Schwannian (S type), and neuronal (N type). They express one or more filamental proteins, show specific uptake of norepinephrine (NA), and show activity of neuronal marker enzymes (tyrosine and dopamine-β-hydroxylase). They are able to multiply in culture for extended periods of time without becoming contaminated. They also display a stem cell characteristic (12). The cell line is appropriate for the PD model due to a number of factors. First off, because the cells express tyrosine and dopamine-β-hydroxylases, they can synthesize dopamine (DA) and NA. They exhibit dopamine transporter (DAT) expression, which is restricted to dopaminergic neurons in the central nervous system. DAT controls dopamine homeostasis by selectively absorbing and securing dopamine. These cells can be used to test DA agonists, which may have neuroprotective effects, because they exhibit low levels of DA receptor expression. When present, this can be distinguished into a more prominent DAergic neuronal phenotype. Primary mesencephalic neurons and some differentiated SH-SY5Y cells are comparable to certain agents (13).

Numerous agents, such as retinoic acid (RA), phenol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), brain-derived neurotropic factor (BDNF), dibutyryl cyclic AMP (dBcAMP), purine, or stautrosporine, can be used to differentiate these (14).

Limitations of SHSY-5Y undifferentiated model

It becomes more challenging to determine whether neuroprotective or neurotoxic substances affect the rate of cell death or proliferation as the number of cells rises. There is uncertainty because these cell cultures are not synchronized and do not show the typical signs of mature neurons. They do not express DAT or DA synthesis enzymes at high levels. Both neurotoxins and neuroprotective agents have less of an effect on them. It is challenging to investigate the

therapeutic benefits of DA agonists in Parkinson's disease due to the low expression of DA receptors. Their tyrosine hydroxylase and dopamine-β-hydroxylase activity are low.

Following differentiation, cells cease to divide, stabilize, and exhibit the development of neuritis. These resemble neurons more in terms of biochemistry, ultrastructure, morphology, and electrophysiology. They demonstrate the existence of markers unique to neurons. Classical markers of mature neurons include synaptophysin, NeuN (neuronal nuclei), MAP (microtube associated protein), and GAP-43 (growth associated protein). These cells have a high membrane potential and are more excitable. They exhibit high levels of DAT, TH, and dopamine-β-hydroxylase activity. They have more D2 and D3 dopamine receptors. It is possible to examine the neuroprotective properties of DA agonists in differentiated cells (15).

Various phenotypes induced by different agents

Depending on the media conditions, differentiation can result in a cholinergic, adrenergic, or DAergic phenotype.

Differentiation induced by RA

Retinoic acid receptors (RAR) and retinoid X receptors (RXR) are two classes of non-steroid nuclear hormone receptors that RA binds to. causes transcriptional activation by causing RAR to heterodimerize with RXR and RAR/RXR heterodimers to attach to the RA response element (RARE). Regulation of the neurotrophin receptor's transcription promotes differentiation genes, type II protein kinase A (PKA), and the Wnt signaling pathway. Reductions in neuropeptide tyrosine (NYP) expression were brought on by RA. The DAergic neuronal phenotype can be induced by RA in combination with other substances like cholesterol or TPA (16).

Differentiation induced by Phorbol esters

TPA is one example of a biologically active phorbol esters that influences cell growth and differentiation. In nanomolar concentrations, TPA facilitates differentiation. They display a decline in the serum's growth-promoting activity. Additionally, they eliminate SHSY5Y cells' mitogenic response to human insulin-like growth factors I and II (IGF-I and IGF-II). Isoforms of protein kinase C (PKC) mediate differentiation. PKC increases Ca+2 channel activity, which in turn increases NA release. TPA causes a 200-fold increase in NA at optimal concentration, whereas RA causes a 4-fold increase. After being exposed to RA and TPA, cells express high amounts of TH and DAT and take on a DAergic phenotype. The density of D2 and D3 receptors in them is high (16).

Differentiation induced by Neurotrophins

Neurotrophins affect neurons' ability to survive, differentiate, form synapses, and function. Nerve growth factor (NGF), BDNF, neurotropin-4 (NT-4/5), and neurotrophin-3 (NT-3) make up the majority of them. Tyrosine kinase (TRK) receptors and neurotrophins receptors (p75) are the two types of cell surface receptors to which they bind (NTR). The actions that promote survival are mediated by these. The upregulation of neurotrophins receptors brought on by RA increases the sensitivity of the cells to neurotrophins. The cells undergo apoptotic cell death and make an attempt to reenter the cell cycle once the neurotrophins are removed from the culture media (17).

Differentiation induced by other agents

The non-selective PKC inhibitor stautrosporine causes an advanced adrenergic neuronal phenotype. Cell cycle arrest is induced by guanosine and guanosine-5'-triphosphate, which also raises the levels of DAT and TH. The

DAergic/adrenergic phenotype is induced by these purines. Both genetic and toxin-based methods can be used to replicate the pathology of Parkinson's disease. 6-OHDA is frequently used. 6-OHDA causes oxidative stress by entering dopaminergic neurons through DAT (17).

(2) LUHMES

The subclone of mesencephalic cell lines MESC 2.10 obtained from the ventral mesencephalic brain tissues of an 8-week-old fetus is known as Lund human mesencephalic cells. Tetracycline regulates these cells, and Tet-off promotor controls the overexpression of v-myc. Neurotrphins and antibiotics induce dopaminergic differentiation. Important dopaminergic markers like DAT,TH, VMAT, and D2 receptors are expressed by differentiated cells (18). It displays characteristics of electrophysiology. Neurotoxin and genetically based models are used to mimic the pathology of Parkinson's disease. MPP+ and toxin-based models are the principal uses. When they are proliferating and undifferentiated, genetic manipulation is applied, and differentiation is then induced (13). A gene of interest can continue to express in post-mitotic neurons. These cell lines originate from somewhere other than the metastasis or tumor. However, these grow very slowly and have a lengthy doubling cycle. Significantly more supplementation is required for the culture medium than for the standards. N2 supplements and fibroblast growth factor (FGF) must be added to them. Human fibronectin and poly-L-ornithine must be coated on the plates. Cell culture is therefore more expensive and time-consuming (19).

(3) PC12

The transplantable rat pheochromocytoma of the adrenal medulla is the source of these cell lines. When these cells are cultured on type IV collagen coated coverslips, NGF induces differentiation in the cells. Axon development, electrical property acquisition, and neurotransmitter release from vesicles are all possible in differentiated cells (20). They release NA and have DA and other catecholamines in them. These cell lines can be employed in amperometry experiments to investigate endocytosis. This can also be applied to assess the pathological property of α-synuclein (21). However, the cellular models used to obtain these results are oncogenic. By administering neurotoxins like MPP+, 6OHDA, and rotenone to them, they can be utilized as an in vitro model for Parkinson's disease (22).

(4) MN9D

These cells were produced by the somatic fusion of neuroblastoma cells (N18TG2) with mouse embryonic mesencephalic cells (23). They are able to express TH, produce sodium currents, and synthesize catecholamines. GDNF, butyric acid, and/or additional factors such as retinoic acid (24), are the ones that differentiate them.

(5) N27

These come from mesencephalic cells from immortalized rats. They exhibit low TH and DAT expression. N27 cell lines that were cloned again and now have greater TH and DAT levels. Additionally, under normal and depolarizing circumstances, express VMAT and release DA (25). Studies on neurotoxicity, neurodegeneration, and other cellular processes employ these (26).

(6) Neuro 2a

The neural crest of mice is the source of these cell lines. Dibutyryl cyclic adenosine monophosphate (dbcAMP) is responsible for differentiating these into DAergic cells (27). They function as a useful PD model because they express the expression levels of TH and DA. These are employed in the assessment of neuronal differentiation, axonal growth, and neurotoxicity of signaling pathways (28).

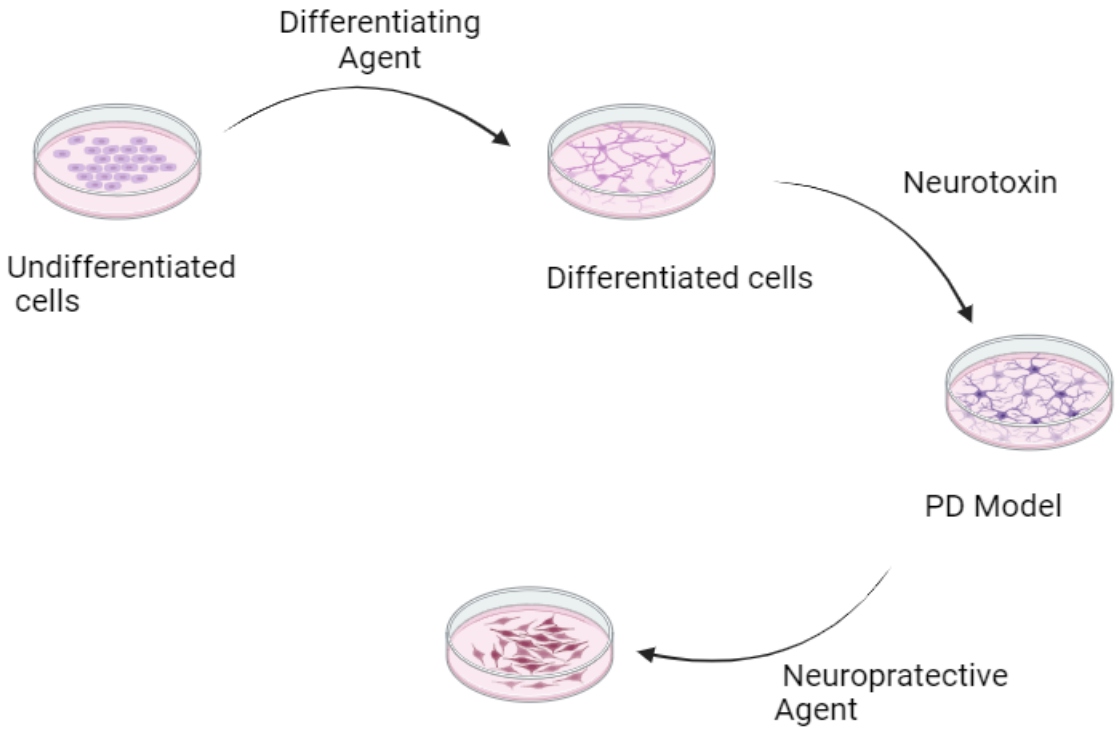

Figure 1: Differentiation of the Cell line Models

S.No.	Cell line	Differentiating Agent
1.	SHSY5Y	Retinoic Acid, TPA (14).
2.	LUHMES	Tetracycline**,** GDNF (glial cell line-derived neurotrophic factor), and dibutyryl cyclic AMP (29).
3.	PC12	Nerve Growth Factor (NGF) (30).
4.	MN9D	Butyric acid, Glial cell line-derived neurotrophic factor (31)
5.	N27	dibutyryl cyclic AMP (32)
6.	Neuro2a	dibutyryl cyclic AMP (33)

5. PRIMARY CULTURES

Murine brain cells are used to create primary dopaminergic cultures. Intrauterine horns from a gestating mother at a particular developmental stage—E14 in rats and E13 in mice. The brain is removed, the fetus is beheaded, and the central midbrain region is removed. By means of enzymatic digestion, differential centrifugation, and culture in a particular medium, the dopaminergic cells are isolated (34). They quickly give rise to synapses and neurites. However, there are a lot of glia cells in them. Toxins like MPP+ and 6-OHDA can cause Parkinson's disease pathology. They are used sparingly because they are extremely sensitive. Genetic manipulation once these cells are cultured, or the use of PD transgenic animals, can be used to induce the genetic models. .. The main source of culture is murine. Because VMA can replicate the morphology and physiology of neuronal cells, they are the most effective at replicating the dopaminergic neuron phenotype. They express high levels of DAT and TH and do not go through mitosis (35). Just five to ten percent of neurons are TH positive overall. This presents a challenge for the viability assays. However, there are some variations in the physiology, anatomy, behavior, regulation of gene expression, and drug metabolism. In these primary cultures, gene transfer is more challenging. While viral gene transfer is beneficial in most cases, calcium phosphate transfection is effective in certain situations. The glass or plastic support occupies one side of the cell, while the liquid cell culture medium occupies the other. The specific culture conditions play a major role in determining which cell types survive. When plated without serum, the primary cultures are nearly exclusively neuronal; however, when plated with serum, glial cells proliferate and aid in the survival of neurons. Higher density cell plated primary cultures exhibit superior survival (36).

While adenovirus and adeno-associated virus infect GABAergic neurons but not dopaminergic neurons, lentivirus is a useful tool. It takes a lot of time and effort to prepare the primary cultures. Because VMA isolation is a small region, it requires experience and skill. The extremely diverse cell populations make up VMA. Compound libraries cannot be screened extensively using primary cultures. Genetic manipulation is a challenging task (37).

6. ORGANOTYPIC CULTURES

These are slices of the brain taken from postnatal rat pups and cultured on coverslips in "roller tubes" or on membranes that are in contact with the incubator's medium and air. They blend the characteristics of intact animal and cell culture. They preserve the three-dimensional structure and in vivo cellular interactions. Transverse dissection of the VMA is done in 110μm slices. For ten to twelve days, they are kept in the designated culture medium (38). Toxin models containing both 6-OHDA and MPP+ have the ability to cause PD pathology (39). The viral vector is used to transfer genes. They are either mixed into the culture medium or injected locally into the slice. In order to maintain significant neural projections, it is crucial to select the appropriate slicing planes (40). NeuN and TH markers are expressed by neurons in tissue slices. They offer adaptability for long-term survival, such as continuous medication administration. They are employed in the investigation of neurogenesis, electrophysiology, and the relationships among the striatum, SNpc, and cortex. However, it is very challenging to replicate them. Dissecting the brain takes a skilled researcher. The ethical issues surrounding the use of animals are involved (41).

7. INDUCED PLURIPOTENT STEM CELLS

Any type of human body cell can be differentiated from human embryonic cells. Preimplantation embryo destruction is necessary for their isolation. Consequently, the process of creating induced pluripotent stem cells, which are nearly as capable as embryonic cells (42). These cells can be established irrespective of the genetic background and health status of the donor thanks to their protocol. Four genes (Oct4, Sox2, Kif4, and c-Myc) can be introduced into mature human cells to achieve this (43).A neutralized rosette and neural tube-like structures form as a result of the induction of the dopaminergic phenotype through specific steps like ventralization (sonication hedgehog-SHH) and caudalization (fibroblast growth factor 8-a). The cells can be treated with BDNF, Camp, and GDNF to undergo maturation and produce differentiated dopaminergic neurons (44). It took four to ten weeks for the culture to produce the mature neurons. Those exhibit high levels of DA as well as TH and DAT expression (45). They develop impulsive synaptic activity. Gene models are used to replicate these. Fibroblasts from PD patients or healthy individuals can be used to create iPSCs. The PD patients with mutations linked to the disease (SNCA, LRRK, etc.) are utilized. Analysis is possible for genetic and pathological conditions. These PD patient-derived cells' mitochondria exhibit decreased basal oxygen consumption, changed motility, and increased susceptibility to pesticide toxicity.
Genome editing is required for cells derived from healthy individuals, such as overexpressing or knocking out genes linked to Parkinson's disease. The use of toxins to treat PD pathology (46).

The physiology of dopaminergic neurons can be more precisely replicated by these cells. They may be able to produce cells unique to each patient (47).

They have certain drawbacks, such as the need for seasoned researchers to execute the techniques. Because their culture lasts for four to ten weeks, their isolation and in vitro neuronal maturation take a very long time. Both written consent and ethics commission approval are needed for this. It's also important to take into account the biological limitations of the system, such as the incapacity to recapitulate the effects of aging and the loss of epigenetic influence during the reprogramming process (48).

8. GENETIC MODELS

The α-synuclein protein is encoded by the SNCA gene, which was the first genetic mutation linked to Parkinson's disease (PD). The PD-related genes LRRK2 (PARK8), VPS35 (PARK17), and SNCA (PARK1 and 4) all have autosomal dominant inheritance. The initial description of PARK 1 involved a missense mutation (Ala53Thr). The most common mutation in familial Parkinson's disease (PD) is LRRK2, which increases kinase activity and confers toxic function. The altered vesicle formation and trafficking may be caused by VPS35 (49).

Three genes make up the recessive inheritance: DJ-1 (PARK7), PINK1 (PARK6), and Parkin (PARK2). The missense, nonsense, or structural mutations are in Parkin and PINK1.Early-onset PD is strongly correlated with DJ-1. The PD target gene is manipulated by either overexpressing the wild-type gene for autosomal dominant genes (SNCA) or transgenically expressing a mutant. An additional technique is autosomal recessive gene knockout (DJ-1, PINK-1) (50).

9. NEUROTOXIN BASED MODEL – MODELS FOR TESTING PD?

1-methyl-1,2,3,4-tetrahydropyridine (MPTP), 6-hydroxy-dopamine (6-OHDA), rotenone, and paraquat can all be used to simulate Parkinson's disease pathology.
(51).

9.1 MPTP

It is a very lipophilic substance that easily penetrates the blood-brain barrier (BBB). Because of its strong affinity for the transporter, monoamine oxidase B (MAOB) in glial cells converts MPTP to 1-methyl, 1-4-phenylpyridinium ions (MPP+), which are then taken up by dopaminergic neurons via DAT. The foundation of MPP+ uptake and neurotoxicity is DAT. MPP+ binds to the VMAT inside the dopaminergic neuron and is trapped in a dopamine vesicle. The mitochondria absorb a small but significant amount, which inhibits complex I activity. This disrupts the respiratory chain's electron transport, which raises reactive oxygen species from Complex I's Fe-S clusters and lowers ATP levels. These result in the activation of signaling pathways linked to cell death, including p38 mitogen-activated kinase (51).

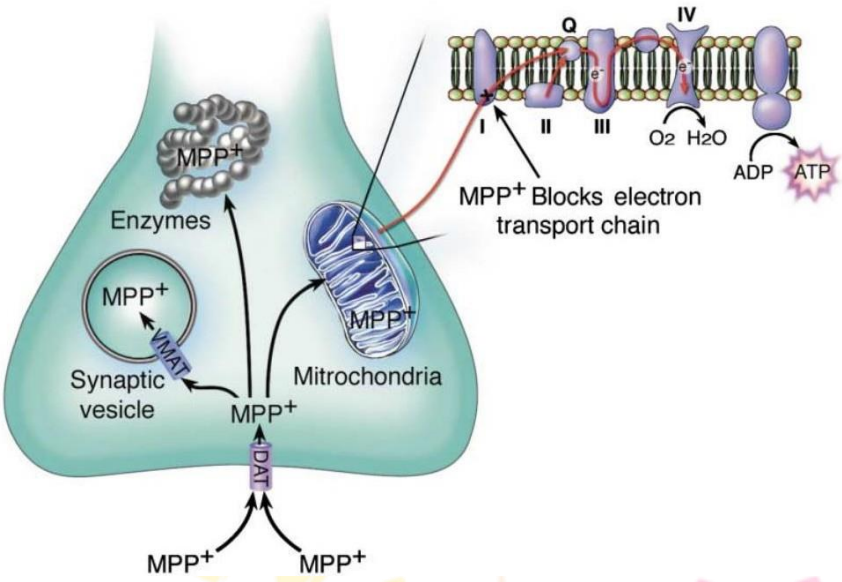

Figure 2: MPP^+ model for PD

9.2 6-OHDA

It is the hydrolyte metabolite of DA sharing structural similarity such as affinity to DAT resultingIt is the hydrolyte metabolite of DA that selectively binds to catecholaminergic neurons due to structural similarities and affinity. It builds up in the cytosol, causing harm to the neurons. When 6-OHDA is auto-oxidized, hydroxyl radicals, hydrogen peroxide (H2O2), and quinones linked to molecular instability caused by cytotoxicity. Ascorbic acid and other antioxidants are therefore used with it (52). The auto-oxidation of toxins in extracellular medium is demonstrated by cellular models expressing high doses of 6-OHDA in conjunction with low levels of DAT. The oxidants produced by this mechanism cause cell death. Excessive DAT expression demonstrated that oxidative stress occurs in the cytosol and that DAT antagonists can stop 6-OHDA-induced cell death. This builds up within the mitochondria and inhibits Complex 1 (53).

10. CONCLUSION

Palliative care for Parkinson's disease solely targets its symptoms. To date, there is no treatment that can alter the disease or halt the progression of Parkinson's disease. This is because the pathogenic process of dopaminergic degeneration is not well understood. The pathological mechanisms of Parkinson's disease can be discovered with the aid of in vitro models. They present the data and theories regarding the preliminary stages of Parkinson's disease pathology. The research conducted using these models produced the following findings: (1) α-synuclein modulation in DA toxicity (54), (2) PINK1, PARKIN, and DJ-1 study in mitochondrial dysfunction (55), (3) DJ-1's neuroprotective mechanism and leading to toxicity (56), (4 mutations in the LRRK2 gene causing proteosome stress and apoptosis (57). As a result, studying PD pathology can benefit from the use of in-vitro models.

In addition to their significance and function in Parkinson's disease research, data indicate that these models are not entirely trustworthy experiments. They don't know the etiology of Parkinson's disease. There is still no ideal model. Combinations of models may be necessary since no single model can fully explain all of the pathogenic aspects of Parkinson's disease (57).

Utilizing cells derived from Parkinson's disease patients, research on midbrain dopaminergic neurons has been made possible by the recent advancements in organoids, iPSCs, and technology. The molecular, functional, maturation, and morphological characteristics of these human-derived cells allow them to be

utilized for studying neurodegenerative diseases (6).

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