

Neuroprotective effect of *Thuja orientalis* in haloperidol induced animal model of Parkinson's Disease

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Abstract

Introduction: Neuro-inflammation, increased microglial activation & interaction and oxidative stress are the new targets for Parkinson management. Protective effect of NSAIDs and anti-oxidants has been demonstrated in many animal studies with inconclusive epidemiological reports. *Thuja orientalis* (TOFE) a common shrub found widely in India has shown neuro-protective effect against 6-OHDA induced toxicity on SH-SY cells. Aim & Objective: To assess and compare neuro-protective effect of TOFE with Ibuprofen and vitamin E in haloperidol induced rat models of Parkinson.

Methodology: 6 adult male *Sprague Dawley* rats each in 4 groups were given *Thuja orientalis* (500mg/kg) / Ibuprofen (100mg/kg) and Vitamin E (35mg/kg) orally followed 1 hour later by haloperidol. (2mg/kg.IP) for 7 days. Motor activity and rigidity were assessed with actophotometer and common bar test. Catatonia scoring was also done. One way ANOVA and Kruskal-Wallis tests followed by Dunn's multiple comparison test were used for statistical significance of <0.05.

Results: Significant reduction in motor activity was observed in all on 7th day. Mild protection by *thuja orientalis* against motor rigidity was noted with p-value of 0.032 and against catatonia with 2.5 scoring.

Conclusion: Neuroprotective effect shown in-vitro experiments by anti-oxidants and anti-inflammatory drugs did not show any significant effects in our *in vivo* animals study against clinical features as seen in Parkinson's disease. This inconclusive neuroprotective effect of standards & thuja observed signifies that all preclinical data from in vitro studies cannot be effectively extrapolated to *in vivo* animal & human studies due to many variations. Further probe in this aspect is suggested.

Keywords: Anti-inflammatory, Anti-oxidant, Neuroprotection, Parkinson animal model, *Thuja orientalis*

1. Introduction

Parkinson's is the second most common neurodegenerative disease affecting 1% of population including both men and women equally above 55yrs of age. It can be of unknown origin as primary or secondary due to some drugs/ stroke/trauma or purely genetical when it is manifested as early onset before 55 yrs.

Loss of 50-70% of dopaminergic neurons in substantia nigra compacta (SNc) and in striatum associated with the presence of intra-cytoplasmic Lewy bodies due to aggregation of α -synuclein and ubiquitin are the basic pathological features. [1] Four cardinal clinical signs of Parkinson are tremor, rigidity, Akinesia/hypokinesia and postural instability. Other parts of CNS like dorsal motor neuron of Vagus, Nucleus basalis of Mayner, locus ceruleus and Hypothalamus are also affected even extending outside CNS like myentric plexus evidenced by the presence of Lewy bodies. These features account for non motor symptoms like sleep disturbances, depression, cognitive impairment, anosmia, constipation, incontinence and ANS dysfunctions.[2] Death of dopamine neurons has been linked to mitochondrial dysfunction, oxidative stress, nerve inflammation and insufficient autophasic proteosomal degeneration.[2]

Mutation of genes LLRK, PARK-2 (encode parkin), PARK-7, PINK-1 and SNC-A (coding α -synuclein) have been attributed only to 10% of cases. Environmental exposure to organ chlorine pesticides, polychlorinated phenols and herbicides like Paraquat also have been shown to be associated with increase in risk and occurrence of Parkinson's due to their oxidative stress and neurotoxic effects by many epidemiological studies. [3, 4]

Transient Parkinsonism like state has been observed with use of reserpine /haloperidol in animals (mice, rat & rabbits) and humans also, because of their dopamine blocking and toxic effect. This has led to the discovery of L-DOPA for reversal. Use of MAO-B inhibitor like selegiline and rasagiline and COMT inhibitors like Entecapane and Tolcapane, drugs that slow the degradation of dopamine were established as next therapeutic approach. This was followed by the

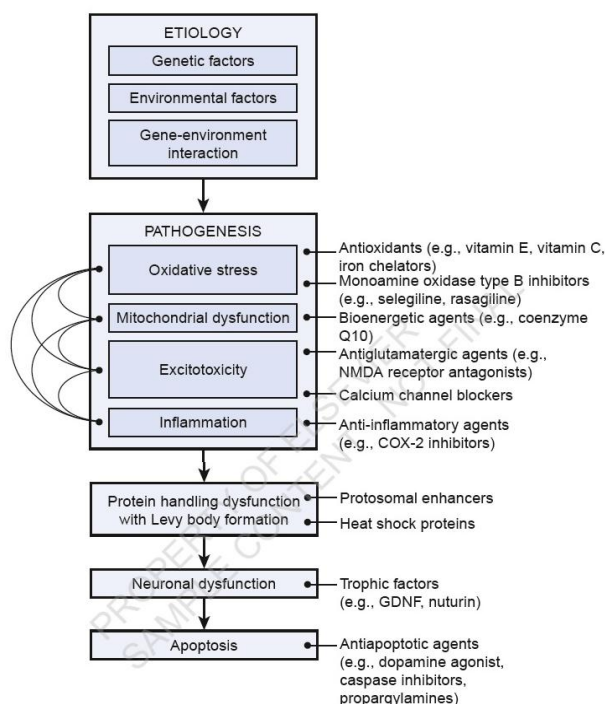
recommendation of ergot and non-ergot preparations having dopamine agonistic action for Parkinson's management. But all these drugs have their disadvantages like significant motor fluctuations like on/off wearing of effect, dyskinesia and freezing on chronic use and with disease progression. [5]

Involvement of neuro-inflammation and oxidative stress being important etiological factors has opened up a newer therapeutic approach. Neuro-inflammation, microglial activation and neuro-glia interactions have been well demonstrated in animal models in 1990 itself. Many indicators of inflammation like up-regulation MHC molecule, NO synthetase, COX-1, COX-2, BDNF, neuronal degeneration with gliosis in brain have been observed in post-mortem examination in animal models of Parkinson's. Increase in TNF α , β 2 microglobulin in CSF in humans is also observed. [3,6] The inflammation activated microglial cells are recruited stick and damage DA neurons by phagocytosis and oxidative stress, especially in SNc as dopaminergic neurons are high in number with high iron content and low in glutathione making SNc more prone for degeneration and oxidative stress. [3]

Hence this led to the hypothesis that both aspirin and non-aspirin NSAIDs and anti-oxidants can protect by preventing or delaying damage to dopaminergic neurons thereby Parkinson's disease.(Figure 1)[3,6] Association of prior use of NSAIDs and reduction in the risk of developing parkinson's later in life has been observed in 5 epidemiological studies done but with varying results. NSAIDs also show scavenging action of reactive oxygen species (ROS) and reactive nitrogen species independent of their anti-inflammatory effect. [3] Increase in COX-2 expression can increase neuronal vulnerability to glutamate induced neurotoxicity as seen in Parkinson's. Protective effect of NSAIDs against Glutamate induced neurotoxicity is also noted. The inhibitory effect of NSAIDs on PGE2 synthesis is also attributed to be beneficial as PGE2 receptors have been identified which play a crucial role in microglial activation. [7] Even though increased COX-2 expression especially in SNc is identified in Parkinson's animal models, there are no supportive animal studies and human epidemiological studies for proper evaluation as they are not in use for long time due to their CVS and other side effects. Among the NSAIDs studied Ibuprofen a non-selective COX inhibitor, is said to have neuro-protective property independent of its NSAID action by inhibiting fibrilization of α -synuclein and by its destabilization action on existing synuclein fibrils in, *vitro* and *vivo* studies done on rat models.[8] Ibuprofen and indomethacin seem to protect nerves via PPAR γ also, a ligand on activation inhibit transcription factor and antagonise activity of NF-kB, AP-1 signal transducer and activator of transcription-1 (STAT-1 and nuclear factor of activated T cells.(NFAT) and inhibition of pro-inflammatory gene expression and thereby IL-1, IL-6 and TNF.[3]

Figure 1: Therapeutic Targets for Parkinson's Disease

NEUROPROTECTION IN PARKINSON'S DISEASE –
CLUES AND TARGETS



Well known side effects of NSAIDs on GIT and CVS on long term use on high dose has prompted for search and the possibility of neuro-protective effect from natural sources. *Thuja orientalis* a shrub grown widely in Asia has been shown to have protective effect against 6OHDA induced toxicity in SH-SY cells by its free radical scavenging and anti-apoptotic effect by an *in vitro* study. [9]

Thuja orientalis (Figure 2) (TOFE) commonly known as Morpanki belonging to Cupressaceae family is an ever green monoecious tree/shrub grown in temperate regions of Asia and America. It is native of China and grows widely in West Himalayas and planted all over India in parks. It is 10-60 feet tall, shoot are flat, and leaves are scale like arranged in flattened fan shape with resin glands. Thuja poles are used for fencing and its wood is used for guitar sound board. Its extract is used in traditional medicine and Homeopathy for various biological properties both topically and orally. It is recommended for conditions like bronchial catarrh, cystitis, cancer, microbial infections and some dermatological conditions like fungus, warts and psoriasis, molluscum etc. Thujone an essential oil extracted, a ketone and monoterpene existing in two diastereomeric forms: α -thujone and β - thujone has been studied for its GABA receptor antagonistic activity.[10,11] Its anti-inflammatory property is confirmed by its inhibiting effect on TNF α and its stimulation on monocyte adhesion to Human Umbilical Vein Endothelial cells. It also inhibits NF κ B and thereby intracellular ROS synthesis. [10]

Figure 2: *Thuja orientalis*



Anti-oxidant effect of TOFE has been demonstrated to be equal to that of vitamin E by its ability to reverse the glutathione level back to normal in alloxan induced rat model of diabetes. [12] It's anti-oxidant effect is also comparable to ascorbic acid another anti-oxidant vitamin.[13] Efficacy of TOFE on 6-OHDA induced neurotoxicity in SH-SY5Y cells has shown strong radical scavenging effect by reducing intracellular ROS level and extracellular nitric oxide synthesis induced by 6-OHDA. TOFE has shown anti-apoptotic action mediated by mitochondrial activation by blocking the reduction in the mitochondrial membrane potential, the release of cytochrome c, and the activation of caspase-3. Moreover, TOFE has been shown to decrease the phosphorylation of extracellular signal-regulated kinase (pERK), which has proapoptotic functions. [9] Anti-inflammatory and anti-oxidant effects of extracts of *Thuja orientalis* have been established in many animal studies at much lower dose compared to LD50 value of its fruits and leaves of 5 and 4.5 gm per kg in rats assessed by Karber method.[12] The role of inflammation and oxidative stress in causing neuronal degeneration resulting in various CNS diseases like Alzheimer's, Amyotrophic lateral sclerosis, Chorea and Parkinson's etc has been well established in animal studies and in human by epidemiological studies. Hence this Placebo controlled comparative study has been taken up to asses and compare the neuro-protective effect of *Thuja orientalis* against Ibuprofen a non selective COX inhibitor and an anti-oxidant vitamin-E in Haloperidol induced Parkinson model using rats.

2. Material and Methods

After obtaining approval from institutional research and animal ethics committee male adult *albino Sprague Dawley* rats weighing 200-250 gms maintained as per CPCSEA guide lines, fed with normal pellet feeds and water *ad libidum* were used. The selected rats were divided in to 4 groups with 6 rats for each group. They were treated with test, standard drugs and saline (control) as per the schedule shown below. *Thuja orientalis* in strength of 100mg/ml was purchased from Mahatma Gandhi Pharmacy Pondicherry. Injection Haloperidol, Ibuprofen syrup and Vitamin-E tablets were procured from the pharmacy of Sri Venkateshwaraa Medical College Hospital Ariyur Pondicherry the study site.

Group I: received normal saline orally as placebo for control followed by Injection Haloperidol intraperitoneally in the dose of 2mg/kg 45 minutes later daily for 7 days.

Group II: received *Thuja orientalis* extract orally in the dose of 500mg/kg followed by Injection Haloperidol intraperitoneally in the dose of 2mg/kg 45 minutes later daily for 7 days.

Group III: received Ibuprofen syrup orally in the dose of 100mg/kg followed by Injection Haloperidol intraperitoneally in the dose of 2mg/kg 45 minutes later daily for 7 days.

Group IV: received Vitamin –E syrup orally in the dose of 35mg/kg followed by Injection Haloperidol intra-peritoneally in the dose of 2mg/kg 45 minutes later daily for 7 days.

2.1. The following parameters were recorded in all the rats of all 4 groups at day-0 before administration of any medicine, 4th day, 8th and 15th day after administration of medicines

2.1.1. Assessment of Akinesia/ hypokinesia:

Akinesia/ hypokinesia was Measured using Actophotometer. Animal was placed in the actophotometer. After allowing for acclimatization for 2 minutes motor activity was measured for 10mts by noting the counts when the beam of light from photocell was cut by the movement of animal. [14,15]

2.1.2. Assessment of motor rigidity:

Common bar test: Descent latency time taken by rat to remove its fore paws was recorded by using Common iron bar test placed at the height of 10 cms. The animal was suspended with its fore paws and the time taken for the animal to fall with the cut-off time of 100 seconds was noted as a measure of rigidity.[5]

2.1.3. Assessment of catatonia:

2 wooden blocks of 3cms and 9cms height were used. The fore paws of animal were placed one after another over the blocks and the time taken to remove was noted and cumulative scoring was done as given below to the maximum of 3.5.[14]

Table 1: Scoring For Catatonia

Stage	Finding	Score
1	Rat moves normally when placed on the table	0
2	Rat moves only when pushed/ touched	0.5
3	Rat placed on table with fore paw set alternatively on a 3cms height block- fails to correct in 10 seconds	0.5 for each-Total-1
4	Rat fails to remove when front paws are placed on 9 cms height block in 10 secs.	1 for each-Total-2

2.2 Statistical Analysis

The results were tabulated and analyzed using Graph Pad Prism-6. One way ANOVA and Kruscal-Wallis tests for statistical significance and Dunn's multiple comparison test were used to compare within and between groups considering p-value of less than 0.05 as statistically significant.

3. Results

Analysis of the data derived from the readings in actophotometer reflects that there was significant decrease in the motor activity on the 7th day compared to 0 day in all the groups, the p-value being 0.0006 for control, 0.0003 for brufen, 0.0104 for vitamin-E and 0.0002 for *thuja orientalis* group. Reduction in motor activity was observed to be less in the rats received vitamin-E compared to that received the investigational drug giving p-value of 0.031. Reduction in motor activity was more in control and *thuja* group than the groups received Ibuprofen and vitamin-E. Motor activity slowly returned almost to the basal level on the 14th day in all the groups but not completely reverted. (Table 2)

Table 2: Motor activity- Counts (Mean value with SD)

Groups	Day-0	7 th -Day	8 th -Day	14 th Day	p-Value	Significance between days
Control	148.5±43.26	26.33±19.76	93.83±20.38	117.2±45.70	0.0006	0 Vs 7 * 7 Vs 14 *
Brufen	226.5±38.77	37.50±24.82	122.8±44.16	185.7±10.23	0.0003	0vs7 * 7 Vs 14*
Vitamin-E	222.3±70.49	79.17±57.85	87.67±60.65	160.7±71.51	0.0104	0 Vs 7* 0 Vs 8 *
Thuja	243.8±48.74	19.83±17.61	59.17±54.71	177.0±42.12	0.0002	0vs 7 * 7 Vs 14 * 0 Vs 8 *
p-Value	±	0.031	0.0536	0.140	0.113	
	Significance within groups	Control Vs Thuja *	Vitamin E Vs Thuja *	N.S	N.S	

One way ANOVA and Kruscal-Wallis tests for statistical significance and Dunn's multiple comparison test were used to compare within and between groups. P value ≤ 0.05 is considered as statistically significant. *Denotes the groups and days that exhibit significant difference between them.

Motor rigidity tested using common bar test was significantly increased in all the 4 groups from the basal value of 8.67 sec, 7.5 sec, 10.2 sec and 12.5 sec to 35.47 sec, 30 sec, 32.2 sec and 33.33 sec on the 7th day with p-value of 0.0007, 0.0006, 0.0007 and 0.03 in control, Ibuprofen, vitamin-E and *thuja* groups respectively. (Figure 3 & Table 3) It was more in control group compared to others. The increased motor rigidity was same in all the groups on 8th day one day after stopping the Parkinson inducing drug haloperidol. No significant difference was observed between the treated and untreated groups

on 7th and on 8th day. On 14th day much more recovery of muscle tone was noted in all the groups evidenced by common bar holding time being 10.3sec, 12.8sec, 16sec and 22sec in control Ibuprofen, vitamin-E and thuja groups respectively. But complete recovery was not observed in all the experimental groups 7 days after stopping the standard and investigational drugs.

Table 3: Motor rigidity-common bar test- duration in seconds (Mean value with STD)

Groups		Day-0	7 th -Day	8 th -Day	14 th Day	p-Value	Significance between days
Control		8.667±0.816	35.47±19.48	18.83±7.935	10.33±1.966	0.0017	0VS 7 * 7VS 14 *
Brufen		7.5±1.049	30±14.24	11.67±2.251	12.83±2.229	0.0006	0VS7 *
Vitamin-E		10.17±2.483	32.17±7.195	18.17±5.419	16±4.733	0.0007	0VS 7*
Thuja		12.50±4.806	33.33±14.28	24±12.02	22.33±1.633	0.0322	0VS 7*
p-Value	±	0.0045					
	Significance within groups	Brufen Vs Thuja*	N.S	N.S	Control Vs Thuja*		

One way ANOVA and Kruscal-Wallis tests for statistical significance and Dunn’s multiple comparison test were used to compare within and between groups. P value ≤0.05 is considered as statistically significant. *Denotes the groups and days that exhibit significant difference between them.

Figure 3: Motor rigidity-common bar test



On analyzing the catatonia scoring by placing the rat’s forelimbs on wooden block of 3cms and 9 cms height, it was found to be more in all the groups on 7th day with the scoring of 3.5(p=0, 0003), 3.25(p=0.0003), 3.08(p=0.0003) in control, Ibuprofen and vitamin-E groups respectively. The scoring in Thuja group was little less with 2.5(0.0026) but no statistical significance was there between the groups. On 8th day recovery from catatonia scoring to that of 0.5-0.99 was observed in all the groups. On 14th day tone has returned to near normal in all the groups. (Figure 4, 5 and 6, Table 4)

Table 4: Catatonia- Scoring (Mean value with STD)

Groups		Day-0	7 th -Day	8 th -Day	14 th Day	P-Value	Significance Between Days
Control		0	3.5±0	0.583±1.429	0.0	0.0003	0 Vs 7* 7 Vs8 * 7 Vs14 *
Brufen		0	3.25±0.612	0.583±1.429	0.0	0.0003	0 Vs 7 * 7 Vs 8 * 7 Vs 14*
Vitamin-E		0	3.083±0.584	0.083±0.204	0.083±0.204	0.0003	0 Vs 7 * 7 Vs8 * 7 Vs 14 *
Thuja		0	2.5±1.549	0.583±1.429	0.250±0.273	0.0026	0 Vs 7* 7 Vs 8 *
p-Value	±	0	0.285	0.999	0.075		
	Significance within groups	N.S	N.S	N.S	N.S		

One way ANOVA and Kruscal-Wallis tests for statistical significance and Dunn’s multiple comparison test were used to compare within and between groups. P value ≤0.05 is considered as statistically significant. *Denotes the groups and days that exhibit significant difference between them.

Figure 4: Catatonia-Stage-3-Score-0.5**Figure 5: Catatonia-Stage-3-Score-1****Figure 6: Catatonia-Stage-4-Score-3.5**

4. Discussion

Out of the available targets for Parkinson's disease like oxidative stress, protein aggregation, inflammation and excito-toxicity (figure 2), protection against oxidative stress and inflammation *in vivo* by the test molecule Thuja, has been attempted by us and compared, to that of Ibuprofen and vitamin-E. Neuroprotective effect on dopaminergic neurons of them have been demonstrated *in vitro* [17], but with inconclusive reports in humans. Systemic deficiency of vitamin-E in humans and mice has been associated with enhanced oxidative stress in brain and evidenced by protective effect on dopaminergic neurons in SNc by pretreatment with vitamin-E. [18]

Reproducible toxin induced nigra striatal lesion will be suitable to study the neuroprotective effects than the transgenic models which will be helpful in evaluating the selective molecular aspects of Parkinson's pathology. [19] Hence haloperidol induced Parkinson model has been taken up in our study to assess the neuroprotective effects of Thuja which is said to have both.

Statistical analysis of observed data has revealed significant decrease in motor activity on 7th day in all the control and treated groups with standard and test molecule. (Table 2) Slightly significant protection was seen with vitamin-E showing p-value of 0.0536 VS thuja. Such reduced antioxidant property may be due to low bioavailability which is not

clearly known to us. The *in-vivo* anti oxidant effect of thuja is also not yet proved and *in vitro* effect only has been evidenced on incubation of 6OHDA induced toxicity in SH-SY cells in TOFE.[9] Motor activity has returned to some extent but not to the basal level on 8th and 14th days in all the groups showing no significant effects of standard anti-oxidant (Vitamin-E) and anti-inflammatory (Ibuprofen) also that were said to possess *in vitro* animal models.(Table 2)

Increased motor rigidity evidenced by increased holding time in common bar test was seen in all the groups on day 7 with p-value of 0.0017, 0.0006 and 0.0007 in control, thuja and vitamin-E received groups, compared to thuja treated rats showing p-value of 0.032 evidencing minimal protection. Slightly significant difference between Ibuprofen and Thuja on day 0 might be due to inherent variation, which stresses the difficulty in assessing behavioral studies in animals. Recovery from increased motor rigidity was observed more in control than in other groups. This may be due to absence of neuroprotection by anti-inflammatory and anti-oxidant drugs *in vivo* even though their effects have been demonstrated by *in vitro* studies.

On scoring the catatonia it was noticed that rats were not able to correct to their original position when their fore limbs were placed on 3cms and 9 cms block due to hypertonia as a result of dopaminergic deficiency induced by haloperidol by blocking D2 receptors. Equal increase in scoring was seen on 7th day compared to that on 0 day in all the groups except in Thuja group which showed less catatonia with that of 2.5 showing the possibility of minimal neuroprotection. (Table 4) Catatonia scoring did not return to 0 (basal value) on 8th day or on 14th day showing absence of expected complete neuroprotection by these *in vitro* proved test drugs. Recovery from catatonia was better than motor rigidity and reduced motor activity. This differential efficacy could not be clearly understood. Whereas water soluble Q10 co-enzyme has demonstrated neuroprotection by absence of degeneration in histo-pathological and bio-chemical parameters and by normal balancing skills similar to that of controls in a Paraquat induced Parkinson's model by its anti-oxidant action. [20] Katharina Faust, Stephan Gehrke, Yufeng Yang *et al* have suggested that D5-1A model produced by inhibiting DJ-1A. The *Drosophila* Homologue of familial PD gene will be valuable for preclinical testing to assess the therapeutic prophylactic potential of drugs.[16]

A nested case control analysis done among healthy male physicians in a Physician health study in USA found no clear cut evidence of reduction in the risk of Parkinson's with aspirin and non-aspirin prior use. [21] A population based study done by Medical record linking has reported strong and significant inverse association of steroidal and non-steroidal NSAIDs use against parkinson's risk among 392 individuals.[3] Regular use of more than 2 tablets of aspirin per day has been said to reduce the risk of Parkinson's disease.[22] Ton and colleagues who have noted no significant association of NSAIDs with P.D in 2006 have suggested that NSAIDs may protect but cannot reverse neuronal damage once started and so use of NSAIDs during pre-symptomatic period will be more beneficial.[3] Hence prophylactic use of NSAIDs anticipating protection in future may not be practically possible due to their known adverse effects on long term use.

Even though pre clinical data may provide starting point for research in Parkinson's it will be difficult to draw firm conclusion as to which substance has more neuro-protective potential as suggested Douna *et al*.[17] Our study molecule(*Thuja orientalis*) has shown slight protection against motor rigidity and catatonia compared to standard drugs. Being the first effort this observation needs further confirmation. The anti-oxidants and anti-inflammatory drugs proved to possess neuroprotective effect *in-vitro* experiments did not show any such effects in our *in vivo* study using whole animals by monitoring their effect on motor activity, motor rigidity and hypertonia as seen in clinical presentation of Parkinson's disease. This might be due to poor bio-availability or insufficient dose or a need for much better model that can focus many targets at a time. Anti-oxidants and anti-inflammatory drugs might have beneficial effect when combined with other anti-Parkinson drugs rather being giving alone. Further probe in this aspect can be beneficial and it is suggested.

6. Conclusion

Even though *Thuja orientalis* has exhibited minimal protection against motor rigidity and catatonia it was not very much significant in our study. This signifies the fact that all preclinical data derived especially from *in vitro* studies cannot be effectively extrapolated to human studies as they may not be equally effective even when done in whole animal. This may be due to wide variations in various etiological factors like genetic mutation, environmental toxicity in humans, which might be entirely different in animals. As translation of preclinical findings to human trials is very difficult due to many factors like difference in etiological features, course of the disease, determination of appropriate dose of the test molecule because of pharmacokinetic and pharmacodynamic variations like bioavailability etc. The possibility of additional effect of anti-oxidants and anti-inflammatory drugs along with already existing anti-Parkinson drugs can be explored. If such a novel molecule which can take care of all the aspects of this degenerative neural disorder could be identified, morbidity, mortality and quality of life can be improved.

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