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PARKINSON'S DISEASE GENES EXPRESSION STUDY BY QPCR IN NITROSATIVE STRESS INDUCED PC12 CELL LINE

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ABSTRACT

Nitrosative stress leads to Parkinson's disease through mitochondrial dysregulation. Although Parkinson's disease is caused by several genes such as *Parkin, DJ-1, PINK1, ATP13A2, PLA2G6, FBXO7, SNCA, LRRK2, VPS35* and *GBA* but there is a need for the investigation of sensitivity and expression of Parkinson's disease genes upon nitrosative stress. Hence the current study focused on Parkinson's disease gene expression in PC12 cells upon nitrosative stress. The nitrosative stress was carried out by rotenone, SIN1 and peroxynitrite and the expression pattern of genes were studied using real time PCR. PTEN-Induced putative kinase 1 (*PINK1*), Leucine-rich repeat kinase 2 (*LRRK2*) and Vacuolar Protein Sorting-associated protein 35 (*VPS35*) genes were analysed by real time PCR. Our findings demonstrate that under nitrosative stress the expression of *VPS35* is up regulated by 1.35 fold when treated with lower concentration of peroxynitrite (250 µM); however under higher concentration it is down regulated :10 fold with 1mM peroxynitrite, 16.7 fold with 0.5 µM and 2.5 µM rotenone, 8.3 fold and 7.6 fold with 50 µM and 250 µM SIN1. The *PINK1* found to be down regulated: 50 fold with 250 µM peroxynitrite, 20 fold with 1mM peroxynitrite, 14.3 fold with 0.5 µM rotenone, 16.7 fold with 2.5 µM rotenone, 11.1 fold with 50 µM SIN1 and 10 fold with 250 µM SIN1 under all concentrations; surprisingly *LRRK2* gene did not show any kind of expression during PCR optimisation itself. In conclusion *PINK1* appears to be most sensitive compare to *VPS35* as it is down regulated with lower concentration of peroxynitrite.

KEYWORDS: ***Gene expression, Nitrosative stress, Parkinson's disease, Real Time PCR***

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INTRODUCTION

Neurotoxicity caused by free radicals generated due to overproduction of reactive oxygen and nitrogen species leads to neuronal death and involved in the pathogenesis of various neurodegenerative disorders including Parkinson's disease (PD).[1] PD is second most vulnerable neurodegenerative disorder after Alzheimer disease.[2] PD is a characterised by resting tremor, bradykinesia, muscular rigidity, postural instability, freezing, dysfunction of the autonomic nervous system, micrographia, depression and movement disorder etc. The key symptoms of disease appear after the significant loss of dopaminergic neurons in the substantia nigra pars compacta.[3-4] More than 1% in those over 60 years suffers from PD and the frequency of the disease in general population is about 0.3%. Parkinson's disease is more prevalent in males than in females.[5-6] By 2027, the incidence of Parkinson's disease is expected to be double around the world.[7] But till date, there is no definite therapy for the disease as the molecular mechanism involved in the pathogenesis of disease need to be understood more clearly. PD is sporadic in nature[1]; but mutation in numerous genes and loci associated with PD susceptibility have been identified.[8-9] Second most common cause for the early onset of PD is due to the mutation in *PINK1*, which is autosomal recessive in nature. *PINK1* phosphorylate ubiquitin and activate Parkin gene which leads to enhanced Parkin-mediated elimination of damaged mitochondria (mitophagy). Thus, mutations in *PINK1* may result in dysregulation of mitochondrial homeostasis leading to neurodegeneration.[10] *LRRK2* gene is found to play a role in the pathogenesis of both familial and sporadic PD but till date, the physiological and pathological functions of *LRRK2* are not clearly defined.[11] *VPS35* is a subunit of the retromer complex responsible for autosomal dominant form of PD. According to Wang and team, *VPS35* involve in mitochondrial fission, dysregulation of which is likely involved in the pathogenesis of familial and possibly sporadic PD.[12] Other then PINK1, LRRK2 and VPS35, proteins encoded by genes associated with PD, including α-synuclein, PARKIN and DJ-1, are mostly localized to mitochondria and involved in the regulation of mitochondrial dynamics in the same or parallel pathways. Therefore, an altered balance in mitochondrial fission and fusion is likely a common mechanism leading to impaired mitochondrial quality control and mitochondrial dysfunction critical to PD pathogenesis.[12-13]Nitrosative stress due to reactive nitrogen species (RNS) is widely implicated in various neurodegenerative diseases including Parkinson's disease.[1]Peroxynitrite, one of the well known reactive nitrogen species, formation has been implicated in Parkinson's disease and other neurodegenerative diseases. Once formed in the diseased brain, peroxynitrite may exert its toxic effects through multiple mechanisms, including lipid peroxidation, mitochondrial damage, protein nitration and oxidation, depletion of antioxidant reserves (especially glutathione), activation or inhibition of various signalling pathways, and DNA damage followed by the activation of the nuclear enzyme PARP.[14] Peroxynitrite may also induce nitration of tyrosine hydroxylase, the initial and rate-limiting enzyme in the biosynthesis of dopamine, leading to inhibition of enzyme activity and consequent failure in the synthesis of dopamine which leads to loss of dopaminergic neuron which is hallmark of PD pathogenesis.[15] Rotenone has also been reported to cause detrimental effect on dopaminergic neurons *in vitro* and play significant role in disease onset and progression of PD.[16-18] For the present study rotenone is been used to induce the nitrosative stress in PC12 neuronal cell line. SIN1 produces nitric oxide (NO) and superoxide anion ($O_2^{\cdot-}$) which results in the formation of peroxynitrite ($ONOO^-$).[19] Higher level of peroxynitrite is reported in patient with PD.[20] There are various evidences which show $ONOO^-$ and SIN1 involved in the pathogenesis of PD.[20-23]In spite of understanding the PD in various models, the understanding of the PD genes sensitivity caused by nitrosative stress is less studied with PC12 cells. Thus, there is a need to investigate the expression of PD genes in PC12 cells. Therefore three genes *PINK1*, *LRRK2*, *VPS35* are quantified for their expression levels by inducing PC12 cells with SIN1, rotenone and peroxynitrite by real time PCR

MATERIAL AND METHODS

PC12 cells were purchased from ATCC (#CRL −1721™). Poly-L-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from SRL and dimethyl sulfoxide (DMSO) were purchased from Merck India Pvt. Ltd. F12 Ham's media, horse serum and fetal bovine serum (FBS) were purchased from Gibco. BSA from Sigma. TRIzol Reagent from Life technologies, SuperScript® VILO™ cDNA Synthesis Kit, Platinum® PCR SuperMix High Fidelity Kit, EXPRESS SYBR® GreenER™ qPCR SuperMixes were purchased from Invitrogen. DNA primers were purchased from Shrimpex Biotech Service private Limited, India.

Cell culture maintenance

PC12 cells were procured from ATCC and then they were washed with PBS for three times to remove the cell debris and suspended in growth medium on cell culture dish. Cells were resuspended in growth medium which consist of F12 Ham's media, 15% heat inactivated fetal calf serum and 100 units/ml penicillin G, and 100µg/ml streptomycin on a collagen coated culture dish. Cells were grown as a monolayer in 150 mm tissue culture plates, at 37°C in a humidified atmosphere of 5% CO2 incubator.

Assessment of cell viability by MTT Assay

Cell viability was assessed using MTT assay. Cells were taken for the experiment when they reached 70-80% confluence. Approximately 50,000 cells / well were seeded in a 96 well plate and were incubated for 24 Hr at 37°C with 5 % CO_2. Different concentrations ranging from 0-2000µM, 50-1000 µM, 0-10 µM of peroxynitrite, SIN1 and rotenone were added to the test samples. F12 Ham's media without FBS was added and then it was incubated for 24 Hr. After incubation 100 µl/well (50 µg /well) of the MTT (5 mg/10ml of MTT in 1X PBS) were added to the test samples and were incubated for 3 Hr. The culture medium was removed and 0.1 ml DMSO was added to each well to dissolve the formazan product. Reduced MTT was measured using microplate

reader (Tecan SpectraFluor Plus) at a wavelength of 570 nm.

Treatment with Nitrating Agents

$1.5x10^6$ PC12 cells were seeded in a T-25 flask containing 6 ml of F12 complete media. After 24 Hr, cells were serum deprived for 4Hr. Further the deprived cells were treated with peroxynitrite (250 µM and 1 mM), SIN1 (50 µM and 250 µM) and rotenone (0.5 µM and 2.5 µM) without FBS and further processed for RNA isolation and RT-PCR analysis.

RNA Isolation and Real Time PCR

Real-time PCR analysis was conducted to determine the expression of *LRRK2*, *PINK1* and *VPS35* gene in PC12 cells induced with nitrosative stress. Cells are treated with selected concentration which was determined by dose-response curves in terms of cell viability. The total RNA was extracted using TRIZOL reagent according to the manufacturer's instructions. cDNA synthesis was performed using SuperScript® VILO™ cDNA Synthesis Kit. For cDNA synthesis, reaction was performed on ice by mixing 2 µg RNA to 5X VILO reaction mix (random primers, $MgCl_2$, dNTPs) and enzyme reaction mixture (SuperScript™ III RT, RNaseOUT™ Recombinant Ribonuclease Inhibitor, and a proprietary helper protein). The reaction volume was 20 µl and cDNA synthesis was performed at 42°C for 60 min, followed by reverse transcriptase inactivation at 85°C for 5 min. Amplified products were analyzed using agarose gel electrophoresis to check their specificity. Primers were designed based on known cDNA sequences. The *LRRK2* primer: ATGATGACAGCACAGCTAGGA (forward) and AAACGGTCAAGCAAGATTGTA (reverse); *PINK1* primer: TACCAGTGCACCAGGAGAAG (forward) and GCTTGG GACCTCTCTTGGAT (reverse); and the *VPS35* primer: GTTTTGACTGGCATATTGGAGCA (forward) and TCTGGTGTAACTCAGCACAGG (reverse). Thereafter, the RT-generated DNA was diluted to 40 µl volume with distilled water. The diluted RT-generated DNA (2 µl) was amplified using EXPRESS SYBR® GreenER™ qPCR SuperMixes (Invitrogen™). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as internal control. Real-time PCR was run at 95°C for 10 mins, the experimental reaction consisted of 40 cycles of 95°C for 15 sec and 58°C for 15 sec. Fluorescent reading from real time PCR (ABI-7900HT) were quantitatively analysed by determining the Ct (cycle threshold) difference i.e. delta Ct between treated and control. The mRNA expression level was determined by calculating delta Ct.

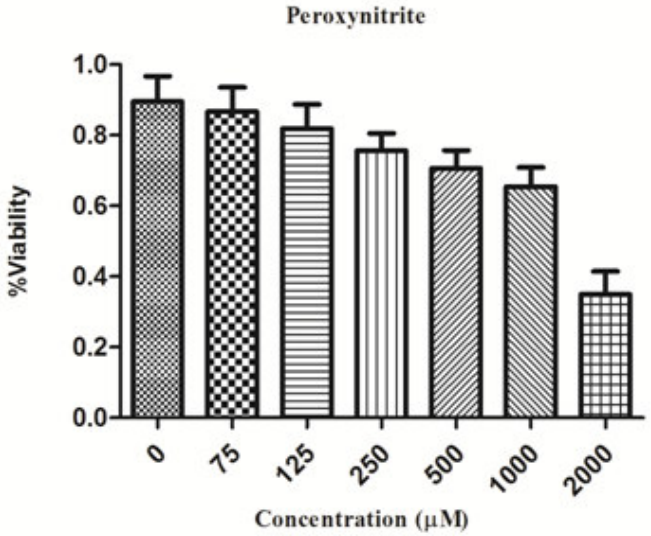
STATISTICAL ANALYSIS

All data were presented as the mean ± standard error of the mean (SEM) derived from three experiments (n=3). Statistical analysis was done by using graph pad prism version 7. Comparison was made between the groups by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison with control. Statistical significance is expressed as P Value ($P \leq 0.001$). Graphs were plotted using graph pad prism version 7.

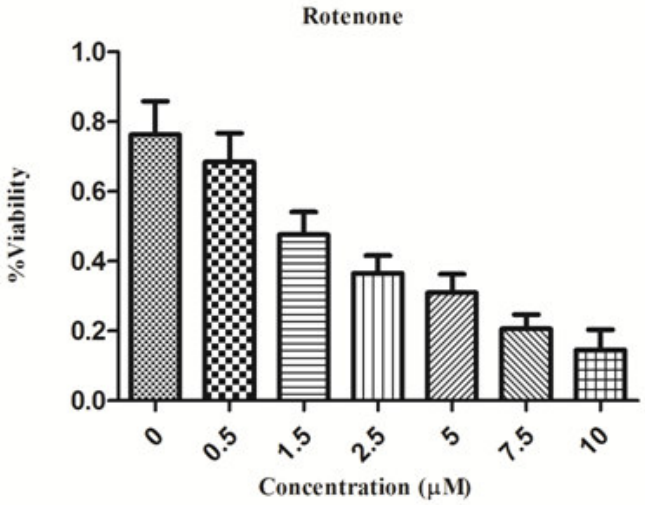
RESULT

MTT Assay

MTT assay for all the three drugs are summarized in Fig. I. A concentration dependent decrease in percentage of cell viability in PC12 cells were observed in case of peroxynitrite, rotenone and SIN1 treatment compared to control. Peroxynitrite treatment did not show any significant inhibition of PC12 cells till 1000 µM but there was a significant decrease in cell viability at 2000 µM only. Rotenone and SIN-1 treatment showed significant dose-dependent inhibition. The IC_{50} values found to be 1.437 and 252.6µM for rotenone and SIN1 respectively.



(a)



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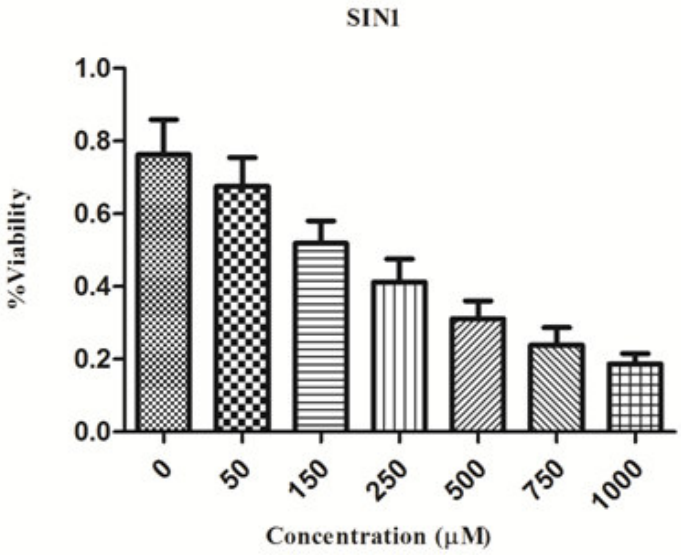


(c)

Figure I

Cell viability assay with different stress inducers: Peroxynitrite (a), Rotenone (b) and SIN 1 (c) with different concentration by MTT assay. The values are presented as percentage cell viability. The data are the mean ± SE of three experiments (n=3).

Real time PCR

PCR was optimized and *LRRK2* expression was not found in the optimized PCR conditions (needs further optimization). The Ct values obtained for individual genes in treated group were compared statistically with that of control (untreated groups). Ct values for *PINK1*, *VPS35* and *GAPDH* is graphically presented in Fig. II and tabulated in Table I. As expected no significant difference in the *GAPDH* expression levels in control and treated groups. The Ct levels of *PINK1* and *VPS35* were observed in treatment groups compared to control. The Ct value decrease linearly with increasing targeted gene.[24-25] Ct value for both *PINK1* and *VPS35* is high as compared to control, which means expression of the genes must be downregulated compared to control. There is not much difference, when Ct value for PINK1 and *VPS35* was compared with each other, except for Ct value of *VPS35* with 250 µM peroxynitrite (Fig. II). So the expression of *PINK1* and *VPS35* genes in different treatment conditions were comparable to each other but was lower as compared to control. This was validated by calculating the relative fold in gene expression for both the genes with all the inducers with different concentration. Relative gene expression of *VPS35* and *PINK1* were determined by comparative Ct method.[26] Results showed that under oxidative or nitrosative stress, the expression of *PINK1* gene down regulated (Fig. III a). It may be because of *PINK1* gene might have mutate guanine residue when treated with SIN1, rotenone and peroxynitrite in vitro. The expression of *VPS35* gene was also down regulated with the treatment compared to but with 250 µM peroxynitrite, up regulated by 1.35 fold (Fig. III b).

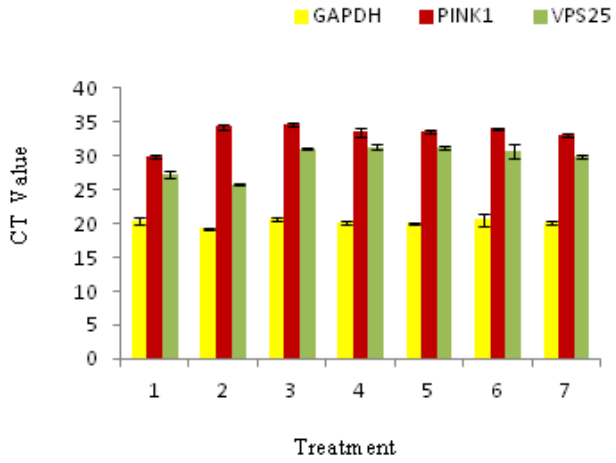


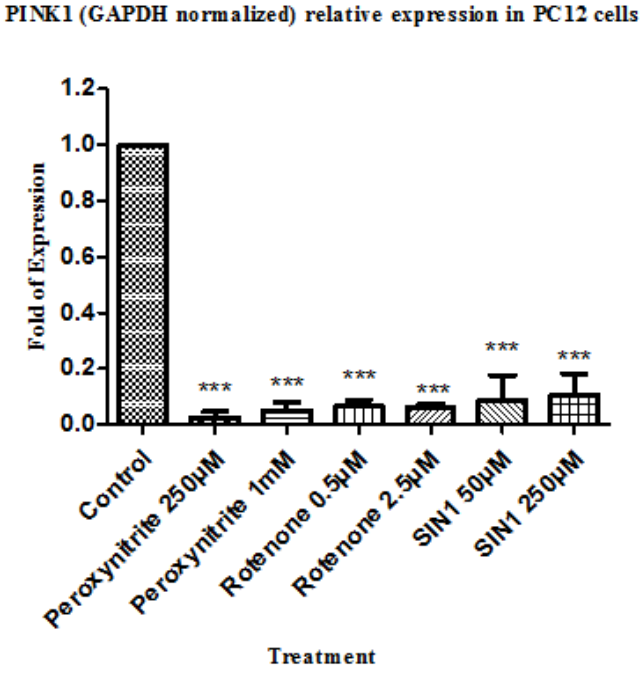
Figure II

Gene expression profile in control and treated cells. Control (1); 250 µM peroxynitrite (2); 1mM peroxynitrite (3); Rotenone 0.5 µM (4); rotenone 2.5 µM (5); SIN1 50 µM (6); SIN1 250 µM (7)

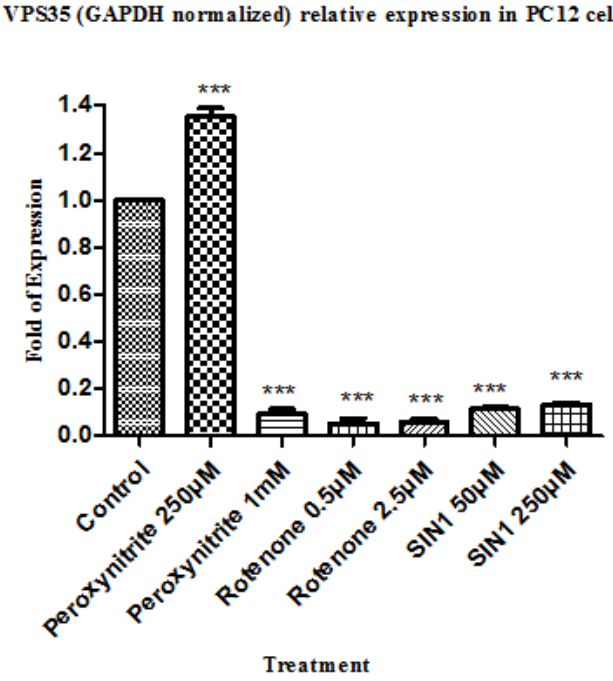
Table I

Ct value for two Parkinson's disease genes (PINK1 and VPS35) with one internal control (GAPDH) in different treatment conditions.

Treatment	*GAPDH*	*PINK1*	*VPS35*
Control	20.41	29.83	27.33
Peroxynitrite 250µM	19.29	34.29	25.77
Peroxynitrite 1mM	20.75	34.68	31.05
Rotenone 0.5µM	20.14	33.52	31.27
Rotenone 2.5µM	20.06	33.58	31.14
SIN1 50µM	20.59	34.05	30.63
SIN1 250µM	20.1	33.09	29.94



(a)



(b)

Figure III

(a)Relative expression of PINK1 (a) and VPS35 (b) compared to control PC12 cells. Relative expression was calculated from Ct value. Expression of both the PD genes was downregulated except for VPS35 with peroxynitrite 250 µM, expression was upregulated by 1.35 fold. Data are expressed as mean ±SD. (n=3). *p< 0.0001

Table II

Mean and standard deviation of PINK1 and VPS35 is provided in tabulated form. *p< 0.0001

Treatment	PINK1		VPS35	
	Mean	Standard Deviation	Mean	Standard Deviation
Control	1	0	1	0
Peroxynitrite 250µM	0.02	0.01	1.35	0.02
Peroxynitrite 1mm	0.05	0.02	0.1	0.01
Rotenone 0.5 µM	0.07	0.01	0.06	0.02
Rotenone 2.5 µM	0.06	0.01	0.06	0.01
SIN1 50 µM	0.09	0.06	0.12	0.005
SIN1 250 µM	0.1	0.05	0.13	0.005

DISCUSSION

In the present investigation, RT-PCR was used for gene profiling of two selected PD genes (*VPS35* and *PINK1*) in PC12 cell lines by treating it with rotenone, SIN1 and peroxynitrite. Cytotoxicity studies such as MTT assay was done and results show the dose dependent decrease in percentage cell viability with different concentration of peroxynitrite, rotenone and SIN1. In PD pathogenesis, the detrimental effects of rotenone are reported for its ability to cross blood brain barrier (BBB) which leads to dysfunction of Complex I of the mitochondrial electron transport chain which ultimately leads to neuronal cell death via oxidative stress.[24-25] 2.5 µM of rotenone induces cell death more than 50%. SIN1 is a potent peroxynitrite ion generator and induces oxidative and nitrosative stress. Peroxynitrite nitrates guanine residues on DNA and causes DNA damage. Guanine is the major site for peroxynitrite treated DNA and as the concentration of peroxynitrite increases the DNA damage products also increases.[27] 500 µM SIN1 can cause more than 50% cell death. Higher concentration of peroxynitrite was required to see the effective cell death because the half life of peroxynitrite is very short (~1s). So to enter the cell and cause its effect higher concentration (~2mM) of peroxynitrite is required.[28] In experiment, the PCR optimization were done with three genes (*PINK1*, *LRRK2* and *VPS35*) with one internal control *GAPDH*, but later narrowed it down to two genes as the results of PCR showed that the *PINK1* and *VPS35* genes are expressed during PCR optimization leaving behind *LRRK2*. PCR data was further validated by real time PCR. For the present investigation genes expression are presented as relative gene expression via. fold change.[24] The fold change was calculated using the formula $2^{-\Delta\Delta Ct}$.[29] Two genes with direct or indirect involvement in the pathogenesis of Parkinson's disease were analyzed. Significantly ($P<0.0001$) elevated Ct levels of *PINK1* and *VPS35*

were observed in treatment groups compared to untreated cells. Higher is the Ct value lower is the targeted gene. Ct value for both *PINK1* and *VPS35* is high as compared to control, which means expression of the genes are being downregulated. The expression of *PINK1* and *VPS35* genes in different treatment conditions are comparable to each other but was lower as compared to control. This was validated by calculating relative gene expression of *VPS35* and *PINK1* by comparative Ct method.[11] Relative gene expression shows that, the expression of genes (*PINK1* and *VPS35*) down regulated compared to control except the expression of *VPS35* with 250 µM peroxynitrite is upregulated by 1.35 fold (Fig. III a, b) *PINK1* and *VPS35* genes are compared, when treated with rotenone (0.5 µM and 2.5 µM) there was nominal difference in gene expression i.e. 14.3 fold *PINK1* and 16.7 fold *VPS35* gene downregulated with 0.5 µM of rotenone and with 2.5 µM rotenone both the genes were downregulated by 16.7 fold. When expressions of *PINK1* and *VPS35* genes were studied with SIN1 (50 µM and 250 µM) again not much difference in downregulation of both the genes i.e. *PINK1* and *VPS35* genes were downregulated by 11.1fold and 8.3 fold with 50 µM SIN1 and 10 fold and 7.6 fold with 250 µM of SIN1 respectively. But, this study shows remarkable difference when these two genes were studied with peroxynitrite. There was big difference in expression pattern of two genes when treated with 250 µM of peroxynitrite. *VPS35* gene got upregulated by 1.35 fold with 250 µM of peroxynitrite but *PINK1* gene expression got downregulated by 50 fold. The expression of *PINK1* gene was downregulated by double as compared to *VPS35* gene when treated with 1 mM of peroxynitrite. The expression of *PINK1* downregulation may be because of peroxynitrite, SIN1 and rotenone. In case of *VPS35* lower concentration (250 µM) of peroxynitrite was not sufficient to induce mutation in *VPS35* gene. Therefore, higher concentration (1mM) of peroxynitrite was used to induce mutation in *VPS35* and hence the expression was down regulated but in case of *PINK1* lower concentration of peroxynitrite is enough to cause mutation and hence the expression of *PINK1* downregulated and mutations in *PINK1* may result in deregulation of mitochondrial homeostasis leads to neurodegeneration.[8]

CONCLUSION

Among the studied PD genes (*VPS35*, *LRRK2*, *PINK1*), *PINK1* appears to be most sensitive towards peroxynitrite compare to *VPS35*, as *VPS35* expression down regulated only at higher concentration. The results of these testing fully corroborated with earlier investigations conducted by Olszewska et al. 2014 and Wang et al. 2016; that these genes are involved in the pathogenesis of Parkinson's disease due to oxidative or nitrosative stress. Therefore, our results provide groundwork for PINK1 sensitivity towards peroxynitrite, upon which future studies can be designed and pursued and further research on *LRRK2* would give more insights into this study.

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CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Gupta SP, Yadav S, Singhal NK, Tiwari MN, Mishra SK, Singh MP. Dose restraining nitric oxide biosynthesis rescue from toxins-induced parkinsonism and sporadic parkinson's disease? Mol Neurobiol. 2014 Feb 1;49(1):262-75.
2. Yang X, Xu Y. Mutations in the *ATP13A2* gene and Parkinsonism: a preliminary review. Biomed Res Int. 2014 Aug;2014:371256. DOI: 10.1155/2014/371256.
3. Singh MP, Patel S, Dikshit M, Gupta YK. Contribution of genomics and proteomics in understanding the role of modifying factors in Parkinson's disease. Indian J Biochem Biophys. 2006 Apr;43(2):69-81.
4. Yadav S, Dixit A, Agrawal S, Singh A, Srivastava G, Singh AK, et al. Rodent models and contemporary molecular techniques: notable feats yet incomplete explanations of Parkinson's disease pathogenesis. Mol Neurobiol. 2012 Jun;46(2):495-512.
5. Cacabelos R. Parkinson's disease from pathogenesis to pharmacogenomics. Int J Mol Sci. 2017 Mar 4;18(3):pii:E551.
6. Riedel O, Bitters D, Amann U, Garbe E, Langner I. Estimating the prevalence of Parkinson's disease (PD) and proportions of patients with associated dementia and depression among the older adults based on secondary claims data. Int J Geriatr Psychiatry. 2016 Aug;31(8):938–943.
7. Liu S, Zhang Y, Bian H, Li X. Gene expression profiling predicts pathways and gene associated with Parkinson's disease. Neurol Sci. 2016 Aug;37(1):73-79.
8. Bonifati V. Genetics of Parkinson's disease-state of the art, 2013. Parkinsonism Relat Disord. 2014 Jan;20:S23–28.
9. Coppede F. Genetics and epigenetics of Parkinson's disease. ScientificWorldJournal. 2012 May;2012:489830. DOI: 10.1100/2012/489830
10. Koyano F, Okatsu K, Kosako H, Tamura Y, Go E, Kimura Y, et al. Ubiquitin is phosphorylated by PINK1 to activate Parkin. Nature. 2014 Jun 5;510(7503):162-6.
11. Migheli R, Giudice MGD, Spissu Y, Sanna G, Xiong Y, Dawson TM, et al. LRRK2 affects vesicle trafficking neurotransmitter extracellular level and membrane receptor localization. PLos One. 2013

Oct 22;8(10):e77198. DOI: 10.1371/journal.pone.0077198.
12. Wang W, Wang X, Fujioka H, Hoppel C, Whone AL, Caldwell MA, et al. Parkinson disease-associated mutant VPS35 causes mitochondrial dysfunction by recycling DLP1 complexes. Nat Med. 2016 Nov;22(1):54-63.
13. Vives-Bauza C, Tocilescu M, Devries RL, Alessi DM, Jackson-Lewis V, Przedborski S .Control of mitochondrial integrity in Parkinson's disease. Prog Brain Res. 2010;183:99–113.
14. Ischiropoulos H, Beckman JS. Oxidative stress and nitration in neurodegeneration: cause, effect, or association? J Clin Invest. 2003 Jan;111(2):163–169.
15. Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, Ischiropoulos H. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Proc Natl Acad Sci U S A. 1998 Jun 23; 95(13):7659–7663.
16. Zhang L, Cen L, Qu S, Wei L, Mo M, Feng J, et al. Enhancing beta-catenine activity via GSK3beta inhibition protects PC12 cells against rotenone toxicity through Nurr1 induction. PloS One. 2016 Apr 5;11(4):e0152931. DOI: 10.1371/journal.pone.0152931.
17. Olanow CW. The pathogenesis of cell death in Parkinson's disease. Mov Disord. 2007 Sep;17:S335–342.
18. Sai Y, Wu Q, Le W, Ye F, Li Y, Dong Z. Rotenone-induced PC12 cell toxicity is caused by oxidative stress resulting from altered dopamine metabolism. Toxicol In Vitro. 2008 Sep;22(6):1461–1468.
19. Wang YJ, Ho YS, Pan MH, Lin JK. Mechanism of cell death induced by nitric oxide and peroxynitrite in Calu-1 cells. Environ Toxicol Pharmacol. 1998 Aug 4;6(1):35–44.
20. Kouti L, Noroozian M, Akhondzadeh S, Abdollahi M, Javadi M, Faramarzi MA, et al. Nitric oxide and peroxynitrite serum levels in Parkinson's disease: correlation of oxidative stress and the severity of the disease. Eur Rev Med Pharmacol Sci. 2013 Apr;17(7):964-70.
21. Ebadi M, Sharma SK, Ghafourifar P, Brown-Borg H, Refaey H. Peroxynitrite in the pathogenesis of Parkinson's disease and the neuroprotective role of metallothioneins. Methods Enzymol. 2005;396:276-98.
22. Jiang H, Wc YC, Nakamura M, Liang Y, Tanaka Y, Holmes S, et al. Parkinson's disease genetic mutations increase cell susceptibility to stress: mutant alpha-synuclein enhances H2O2- and Sin-1-induced cell death. Neurobiol Aging. 2007 Nov;28(11):1709-17.
23. Martinez MC, Andriantsitohaina R. Reactive Nitrogen Species: Molecular mechanisms and potential significance in health and disease. Antioxid Redox Signal. 2009 Mar;11(3):669-702.
24. Heid CA, Steven J, Livak KJ, William PM. Real time quantitative PCR. Genome Res. 1996 Oct;6(10):986-94.
25. Mao YR, Jiang L, Duan YL, An LJ, Jiang B. Efficacy of catalpol as protectant against oxidative stress and mitochondrial dysfunction on rotenone-induced toxicity in mice brain. Environ Toxicol Pharmacol. 2007 May;23(3):314–18.
26. Siddiqui MA, Kashyap MP, Khanna VK, Yadav S, Al-Khedhairy AA, Musarrat J, et al. Association of dopamine DA-D2 receptor in rotenone-induced cytotoxicity in PC12 cells. Toxicol Ind Health. 2010 Sep;26(8):533–42.
27. Anushree, Ambikapathi G, Thimappa GS, Shivanna DK; Bangalore University. Comparative study of UV- Vis spectra and restriction digestion gel patterns of DNA and nitrated DNA. European J Biotechnol Biosci. 2016 Jan;4(1):63-69.
28. Estévez AG, Radi R, Barbeito L, Shin JT, Thompson JA, Beckman JS. Peroxynitrite-induced cytotoxicity in PC12 cells: evidence for an apoptotic mechanism differentially modulated by neurotropic factors. J Neurochem. 1995 Oct;65(4):1543-50.
29. Magalingam KB, Radhakrishan A, Ramdas P, Haleagrahara N. Quercetin glycosides induced neuroprotection by changes in the gene expressionin a cellular model of Parkinson's disease. J Mol Neurosci. 2015 Mar;55(3):609-17.

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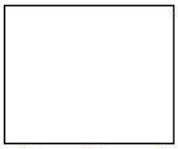

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