Synopsis of the Ph.D. thesis on

STUDYING THE ROLE OF EXOSOMAL miRNAs IN INTERCELLULAR COMMUNICATION IN PARKINSON'S DISEASE

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FATEMA CURRIM

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UNDER THE GUIDANCE OF

PROF. RAJESH SINGH
DEPT OF BIOCHEMISTRY
FACULTY OF SCIENCE
THE M. S. UNIVERSITY OF BARODA
VADODARA- 390 002

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain. This involves death and cumulative loss of neurons from regions of the central nervous system. The main pathological hallmark of this disease is the formation of Lewy bodies, which are cytoplasmic inclusions of α-synuclein that are not degraded by the proteasomal system or lysosomal system in the cell. The molecular mechanisms involved in the disease, is the impairment of proteasome, mitochondrial dysfunction, and inflammation. The spread of neuronal dysfunction in different parts of brain and other tissue is not well understood. The intercellular communication between neurons and glial cells may play an important role in the spread of the disease. The exosome is emerging as specific mode of intercellular communication in patho-physiological conditions, however its implication in neuronal loss during pathogenesis of PD is not well understood. Exosome cargo contains proteins along with small non-coding miRNAs from the cell of origin. miRNAs are 18-22 nucleotide small, non-coding RNAs that regulate gene expression at post-transcriptional level, their role has been implicated in the pathogenesis of PD. The project aims to understand the transfer of miRNAs through exosomes, to the bystander neurons, leading to cell death. The modulation of mitochondrial functions of the affected neurons by the miRNAs will provide better insight on the role of exosomes in this disease condition.

INTRODUCTION

Parkinson's Disease (PD) is a chronic, progressive, neurodegenerative movement disorder including motor as well as non-motor symptoms. This disease affects 1% of the population of over 60 years of age, and 3% of people over 80 years of age, and an estimated seven to ten million people are affected worldwide [1,2]. The motor symptoms are attributed to the progressive death of the dopaminergic neurons in the compact portion of the substantia nigra in the basal nuclei. The death of the neurons in the substantia nigra nerve cells leads to a decrease in the dopamine levels in the corpus striatum, which leads to the motor symptoms, namely, tremor, rigidity, and bradykinesia. The pathogenesis of PD includes alpha synuclein protein aggregation, mitochondrial dysfunctions, intracellular protein and membrane trafficking, and protein disposal by the ubiquitin-proteasome and lysosome-autophagy systems [3].

Although it is known that dopaminergic neurons are specifically vulnerable in PD, however, neuronal degeneration and α-synuclein aggregation is observed in non-dopaminergic parts of the brain too, like, neocortex, brain stem and olfactory bulb [4]. This suggests that the pathology spreads to other types of brain cells including microglia and astrocytes. Emerging studies suggest that exosomes play a major role in inter-neuronal and neuron-glia communication in the brain [5]. Exosomes are a class of extracellular vesicles ranging in the size approximately 30-150nm, which are released from almost all cell types including neuron, glial and astrocytes [6]. A variety of mRNA, small non-coding RNA, DNA and proteins, lipids are selectively uptaken into the

ILVs of the MVBs and all this cargo is released from the cell when the MVB fuses with the plasma membrane and releases the ILVs in the form of exosomes [7]. In PD insulted neurons, α-synuclein is predominantly taken up in exosomes and are transferred to nearby neurons and leads to neuronal dysfunction [8]. These α-synuclein loaded exosomes are uptaken by the glial cells and induce inflammation [9]. This evidence suggests that exosomes play a major role in neuron-glial communication, however the intracellular pathway leading to the exosomal release in neuronal and glial cells in the PD stress conditions has not well elucidated.

Given its vital role in the maintenance of the cell homeostasis and viability, mitochondrial dysfunction and abnormalities play a central role in PD pathogenesis. The dysfunction is mainly characterized by a decrease in the complex I activity of the OXPHOS assembly, generation of ROS (Reactive Oxygen Species), ATP depletion, and release of cytochrome c. The turnover of mitochondria through selective process of autophagy called as mitophagy is important neuronal survival including dopaminergic neurons [10]. On one hand, MVBs are known to fuse with the lysosome where their content is degraded and recycled back for the cell's use [11]. On the other hand, MVBs may also fuse with the plasma membrane, releasing the ILV content in the form of exosomes [12]. Lysosomal function may be crucial to this balance hence it is critically regulated through mitochondria and lysosomal crosstalk for the process of autophagy and cellular homeostasis. This suggests a crosstalk between the lysosome and mitochondria and exosome release in PD [13] which may play important role in pathogenesis of PD in bystander cells. This cross talk between mitochondria, lysosome and exosome in neuronal cells have not been yet investigated systematically in PD stress conditions.

Exosomes are also known to contain signaling molecules that are transferred to the nearby recipient cells which modulate the functional outcome of the recipient cells [14,15,16]. MicroRNAs (miRNAs) are small non-coding RNAs of about ~22 nucleotides in length, which bind to the 3`UTR region of coding mRNAs and bring about inhibition of translation of the mRNA [17]. Abnormal expression of some selective miRNAs has already been detected in Parkinson's Disease, which play a role in regulation of PD risk genes and may contribute to the pathogenesis of the disease [18,19,20]. Potential of exosomal miRNAs derived from various biofluids is being explored as biomarkers for early detection of PD [21,22,23], however their role in pathogenesis and intercellular crosstalk in brain is less understood. Neuronal exosomes modulate complement component 3 (C3) in microglia which stimulates microglial phagocytosis to clear out degenerating neurites [24]. Similarly, microglia modulates neuronal growth and functions in an exosome-dependent manner [25]. The potential role of exosomes and exosomal miRNAs in the modulation of the pathophysiology in Parkinson's Disease has been appreciated however yet to be systemically explored.

In this study, we analyzed the mitochondrial-lysosome crosstalk in PD stress conditions and its role in exosome release from the cell in PD stress conditions. Further, we have demonstrated that

exosomes from neuronal cells are readily internalized by bystander neuronal and glial cells. We also identified differentially enriched exosomal miRNAs from dopaminergic neuronal cell line in PD stress conditions and elucidated the role of identified miRNAs in cellular functions, including cell death and mitochondrial functions.

OBJECTIVES

- **Objective 1:** To study the pattern of exosomes in different PD stress conditions and its transfer to the bystander neurons.
- **Objective 2:** Identify the exosomal miRNAs in exosome and identify the targets of identified miRNAs during PD stress conditions.
- **Objective 3:** Study the role of identified miRNAs in regulation of mitochondrial functions and cell death in PD stress conditions as well as bystander cells.

RESULTS: KEY POINTS (OBJECTIVE WISE)

Objective 1.
To study the pattern of exosomes in different PD stress conditions and its transfer to the bystander neurons.

The first objective was the isolation and characterization of exosomes in neuronal and glial cells.

- The exosomes were successfully isolated by affinity purification method and were successfully characterized by Nanoparticle-Tracking analysis (NTA), which shows the size range of exosomes, and by immunoblotting against exosomal marker, CD-63, which is a well-characterized exosome marker.

The further objectives were to understand the release of exosomes in Parkinson's Disease (PD) stress condition, and the role of the inter-organellar crosstalk between mitochondria and lysosomes in the potentiation and release of exosomes in PD.

Exosome release is enhanced in Parkinson's Disease stress conditions:

- Both neuronal, SK-N-SH, SH-SY5Y and glial cell line U-87 MG, when treated with 6-OHDA and Rotenone, both of which induce PD stress conditions, showed increased exosome release in PD stress conditions checked by western blotting against CD-63.
- Exosome release was also checked by acetylcholine esterase activity of the exosomes isolated from PD stress conditions from neuronal as well as glial cells. Further confirmation was obtained by NTA which gives the concentration of number of particles present per ml of solution.

Parkinson's Disease stress conditions lead to mitochondrial dysfunctions:

- Mitochondrial dysfunctions affect several cellular pathways leading to cell death; hence, we monitored mitochondrial functions in PD stress conditions. Confocal microscopy of U-87 MG showed a fragmented mitochondrial morphology in PD stress conditions. Both neuronal and glial cells showed a decrease in complex I spectrophotometric activity as well as a decline in ATP levels under PD stress conditions.

Lysosomal functions are altered in Parkinson's Disease stress conditions:

- Staining of lysosomes using Lysotracker blue and analysis by confocal microscopy showed that all the cell lines showed a considerable reduction in average number of lysosomes per cell.
- Lysosomal acid phosphatase activity was checked using p-nitro phenyl phosphate. All the cell lines show a decrease in the acid phosphatase activity in 6-OHDA and Rotenone conditions. U-87 MG showed a comparatively more compromised lysosomal acid phosphatase activity in the PD stress conditions as compared to SK-N-SH and SH-SY5Y.
- Western blot analysis of the nuclear fraction indicated that there is decreased nuclear localization of TFEB in 6-OHDA conditions in both SH-SY5Y and U-87 MG, while no change could be observed in Rotenone treated conditions.

6-OHDA alters autophagy which in turn alters exosome release:

- Autophagy flux was monitored by fluorescent microscopy using tandem mCherry-GFP-LC3 construct. The yellow-green puncta (red and green merge) indicate autophagosomes whereas red puncta indicate autophagosomes fused with lysosomes also called as autophagolysosomes. All the cell lines were transfected with mCherry-GFP-LC3 and subsequently treated with 6-OHDA and red/yellow-green puncta were monitored. The number of red puncta decreased in 6-OHDA treated cells as compared to untreated, indicating the reduced autophagic flux.
- Autophagy was monitored in all cell lines by western blotting against the autophagy markers in PD stress conditions. In SH-SY5Y, enhanced conversion of LC3-I to LC3-II was observed in 6-OHDA treated cells, and further accumulation was observed when the autophagy pathway is blocked by Bafilomycin. Similarly, NDP52 and p62 levels in SK-N-SH were accumulated when treated with 6-OHDA and the autophagy pathway was blocked. NDP52 accumulation was observed in U-87 MG cells treated with 6-OHDA and Bafilomycin.

There are emerging evidences to show a molecular and functional crosstalk between autophagy pathways and exosome release. Exosome release may serve as a cellular mechanism to partially bypass the autophagic defect that occurs during pathological situations. Several lines of evidence point to a close relationship between the different autophagy pathways and the biogenesis and secretion of exosomes.

- In all the cell lines, enhanced exosome release was observed when autophagy pathway is blocked by Bafilomycin, and even more enhanced when treated with 6-OHDA as well as Bafilomycin.

Enhancement of autophagy flux by Rapamycin decreases the release of exosomes in PD stress conditions:

The above experiments showed that mitochondrial and lysosomal dysfunction can modulate the autophagic flux and hence also modulate the exosomal release in PD stress conditions. We hypothesized that enhancing the basal autophagic flux may decrease the exosomal release. Rapamycin is an allosteric inhibitor of mammalian target of rapamycin (mTOR), and inhibition of mTOR activity enhances autophagy. Hence, we treated the cells with PD stress conditions in presence and absence of rapamycin and checked the exosome release.

- SH-SY5Y cells treated with 6-OHDA showed enhanced level of LC3-II form in western blotting which decreased when cells were co-treated with rapamycin. Similarly, enhanced degradation of accumulated LC3-II forms was observed in U-87 MG glial cells. These evidences suggested that rapamycin enhances the autophagy flux both in neuronal and glial cells in the presence of PD stress conditions.
- We analyzed the exosomal release in presence of PD stress conditions and rapamycin also through western blotting. In SH-SY5Y and U-87 MG, the level of CD63 marking the exosome release is enhanced in the presence of 6-OHDA and Rotenone and PD stress conditions. Interestingly, the level of CD63 is decreased in cells co-treated with 6-OHDA and Rotenone with rapamycin both in SH-SY5Y and U-87 MG cells.

Objective 2.
Identify the exosomal miRNA in exosome and identify the targets of identified miRNAs during PD stress conditions.

Emerging studies suggest that exosomes play a major role in inter-neuronal and neuron-glia communication in the brain. Recent reports have shown that glial cells and neurons communicate via exosomes.

PD-stressed exosomes are internalized by bystander neuronal/glial cells and localize to mitochondria:

To investigate this, exosomes isolated from U-87 MG cells were incubated with SK-N-SH (neuronal) cells and their uptake was observed by confocal microscopy. Different stains selective for cell membrane and RNA molecules, were used to track the uptake of exosomes in recipient cells.

- It was shown through confocal microscopy that neuron-glial communication takes place through exosomes, which are taken up by the recipient cells as intact vesicles, implicating its key role in intercellular communication in Parkinson's disease.
- We further explored the subcellular localization and the functional effect of the exosomes upon uptake in the recipient cells. SH-SY5Y neuronal cells were treated with PD stress inducers, 6-OHDA and Rotenone, and the exosomes isolated from the treated cells respectively and referred as 6-OHDA-exo and Rotenone-exo (Both exosomes sometime referred as PD-exo) were incubated with healthy bystander SH-SY5Y neuronal cell. Analysis by confocal microscopy indicated that 6-OHDA-exo and Rotenone-exo localizes to the mitochondria in the recipient neuronal cells transfected with MT-RFP. We observe that the degree of colocalization is higher in case of 6-OHDA and Rotenone exo as compared to the untreated exo.

PD-stressed exosomes alter the mitochondrial functions and induces cell death:

The exosomal cargo can alter mitochondrial functions sensitizes neuronal cells to stress induced cell death. To study the effect of exosomes on mitochondrial functions, SH-SY5Y neuronal cells were treated with 6-OHDA and Rotenone, and the exosomes isolated from the treated cells (PD-exo) were incubated with healthy bystander SH-SY5Y neuronal cells and/or U-87 MG glial cells.

- Analysis indicated that PD-exo increases the mitochondrial ROS levels in recipient neuronal and glial cells. Rotenone-treated cells were used as a positive control for the study.
- We also checked the cellular ROS levels by DCFDA staining and found that PD-exo increased the ROS levels in recipient SH-SY5Y cells at 24h, while in U-87 MG glial cells, an increase in cellular ROS levels was detected at 48h, using H_2O_2 treated cells as a positive control.
- TMRM staining indicated that the PD-exo reduces the mitochondrial membrane potential in bystander SH-SY5Y cells at 24h and in U-87 MG cells at 48h.

Since, mitochondria are the major source of reactive oxygen species (ROS), these oxidants can lead to cytotoxicity hence we analyzed neuronal/glial cell death upon treatment with PD-exo.

- MTT assay indicated that significant loss of cell viability is observed after 48h and 72h post-treatment of PD-exo in SH-SY5Y and U-87 MG cells. Further, SK-N-SH PD-stressed exosomes were incubated with healthy SH-SY5Y cells and cell death markers were checked by western blotting. The band of 89 kDa corresponding to cleaved PARP increased and 110 kDa band corresponding to pro-form decreased after treatment with PD-exo. Moreover, an increase in cleaved form of caspase-3 was also detected in the cells incubated with PD-exo.

PD stress conditions induces differential enrichment of miRNAs in neuronal exosomes:

The exosomes are generated within multivesicular bodies (MVBs) that contain intra-luminal vesicles (ILVs) in the endosomal system. A variety of mRNA, small non-coding RNA, DNA, and proteins, are selectively enriched into the ILVs of the MVBs and all this cargo is released from the cell when the MVB fuses with the plasma membrane and releases the ILVs in the form of exosomes. The role of the transfer of miRNAs from one neuron to another neuron or glial

cells in PD conditions is not understood. We wanted to check the selective enrichment of miRNAs in exosomes released in PD stress conditions which may play a physiological role in the pathogenesis of the disease in the bystander cells.

- Exosomes were isolated from SK-N-SH cell line in PD stress conditions (6-OHDA) and RNA was isolated from the exosomal samples.
- The RNA samples (6 samples; 3 biological replicates) were sent for small RNA sequencing. NGS data indicated around 170 miRNAs differentially expressed in 6-OHDA treated exosome samples.
- We sorted the miRNAs that were upregulated and downregulated in exosomes in PD stress conditions according to their read counts. We categorized the total miRNAs in three different categories; read count from 0-100, secondly read counts from 100-1000, and third read counts above 1000. The miRNAs were sorted according to their normalized read counts in upregulated and downregulated categories. NGS analysis report showed a few miRNAs that were differentially expressed in exosomes in PD stress conditions.
- The differentially expressed miRNAs were categorized into up- and downregulated columns based on the NGS results. The target pathways of selected miRNA function were determined by selecting all miRNAs of each category, the combination of all five target prediction tools, and ClipSeq with low stringency and corrected p value <0.05. The GO terms and pathways were retrieved for each category and tabulated.
- The subcellular compartment modulated by the differentially enriched exosomal miRNAs targets were nucleus and cytoplasm, but some targets also localize to endosomal system. The targets of the identified upregulated miRNAs show proteins localized in outer membrane of the mitochondria.
- The biological process mostly regulated by most targets in upregulated and downregulated miRNAs is transcription dependent.
- The other critical biological processes modulated by identified miRNAs are related to apoptosis and ageing, as well as processes related to neuronal differentiation and morphogenesis. The major pathways involved by both classes of targets include PI3K-AKT and MAPK signaling pathways. Downregulated exosomal miRNA targets are involved in endocytosis pathways as well.
- Around 10% of the upregulated targets, including FBXO8, WFDC2, ACBD5, ATXN10 and 13% of downregulated targets, like, PTEN, SYN2, DNAJB1 are involved in neurological diseases.

Validation of identified exosomal miRNAs in neuronal cells:

- The miRNAs identified through NGS analysis were validated in SK-N-SH, SH-SY5Y neuronal cells as well as exosomes in 6-OHDA as well as Rotenone conditions through RT-qPCR. The levels of two miRNAs: hsa-miR-30a-5p and hsa-miR-181c-5p were downregulated and the results corroborated with that of NGS analysis.
- We further validated the two candidate miRNAs by using the absolute quantification by droplet digital PCR (ddPCR). The ddPCR of SK-N-SH untreated as well as Rotenone and 6-

OHDA exosomes indicated the same results as obtained by RT-qPCR. Similarly, ddPCR of SH-SY5Y exosomes also showed that miR-181c-5p decreases in both Rotenone and 6-OHDA exosomes, and miR-30a-5p increases in Rotenone exo but decreases in 6-OHDA exo.

Expression of miR-30a-5p and miR-181c-5p in neuronal/glial cells show selective sorting in exosomes:

To determine whether selective enrichment of identified miRNAs in exosome is observed in neuronal cells, SH-SY5Y neuronal cells were transfected with control mimic (CM), miR-30a-5p mimic and miR-181c-5p mimic and subsequently exosomes were isolated from the same.

- Both miR-30a-5p and miR-181c-5p were highly expressed in the cells and enrichment of the miRNAs was also observed in the exosomes.
- We analyzed if exosomes enriched with identified miRNAs were internalized by the recipient cells. For this, exosomes isolated from SH-SY5Y cells expressing identified miRNAs were incubated with fresh SH-SY5Y cells. After the incubation period, the recipient cells were collected, and levels of both miRNAs were checked. RT-qPCR showed that at 6h, levels of miR-30a-5p was significantly higher in the cells incubated with 30a-5p loaded exosomes as compared to control exosomes. Similarly, miR-181c-5p was significantly higher in 181c-5p mimic exo incubated cells as compared to control exosomes.

Objective 3.
Study the role of identified miRNA in regulation of mitochondrial functions and cell death in PD stress conditions as well as bystander cells.

Since miR-30a-5p and miR-181c-5p mimic loaded exosomes were readily internalized by the neuronal cells, we further analyzed the mitochondrial functions in neuronal and glial cells which were pre-exposed to PD stress inducers, 6-OHDA and Rotenone in presence of miRNA enriched exosomes.

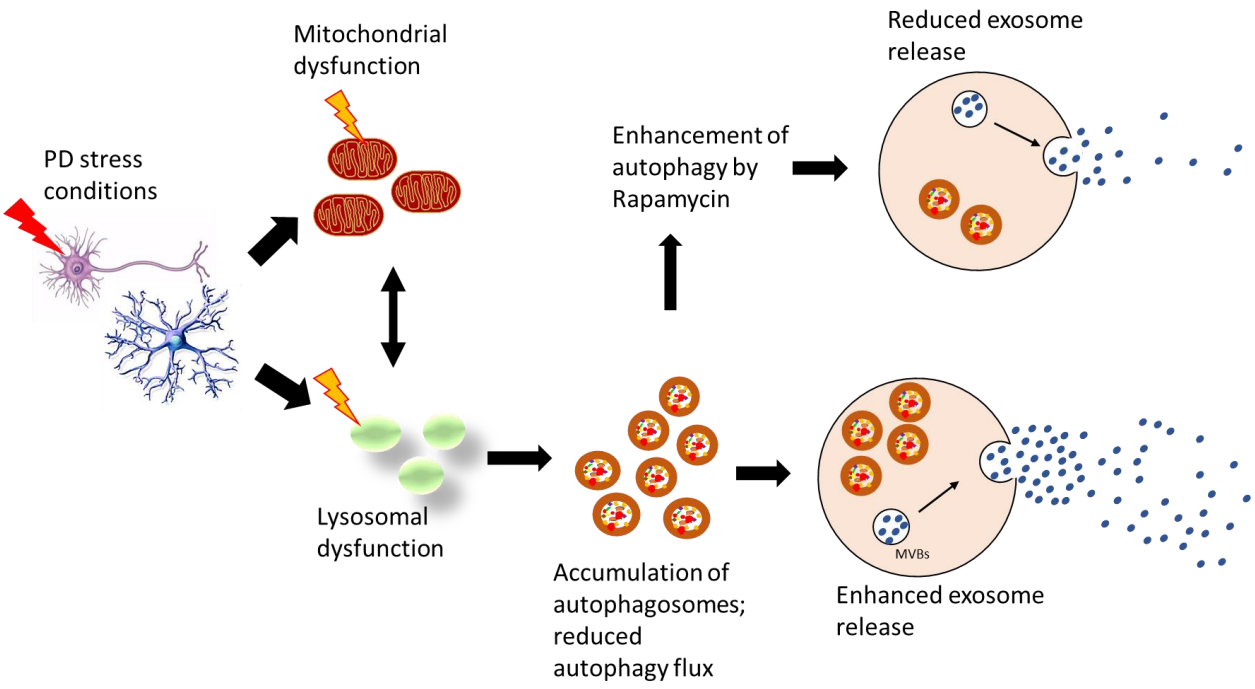
miR-30a-5p and miR-181c-5p mimic loaded exosomes rescue mitochondrial functions and cell death in PD stressed neurons and glial cells:

- Total cellular ROS increased in 6-OHDA, and Rotenone treated SH-SY5Y cells. Interestingly, following incubation with 30a-5p and 181c-5p mimic loaded exosomes, ROS levels in the PD stressed cells reduced as compared to control mimic (CM) exosomes. Similarly, in U-87 MG glial cells, 181c-5p mimic exosomes rescued ROS levels in presence of PD stress inducers, and 30a-5p mimic exosomes also rescues the 6-OHDA treated cells by ameliorating the ROS levels as compared to the CM exosomes.
- Microscopic analysis indicated that 30a-5p and 181c-5p loaded exosomes ameliorated mitochondrial ROS levels in PD stressed SH-SY5Y cells as compared to the control exosomes.

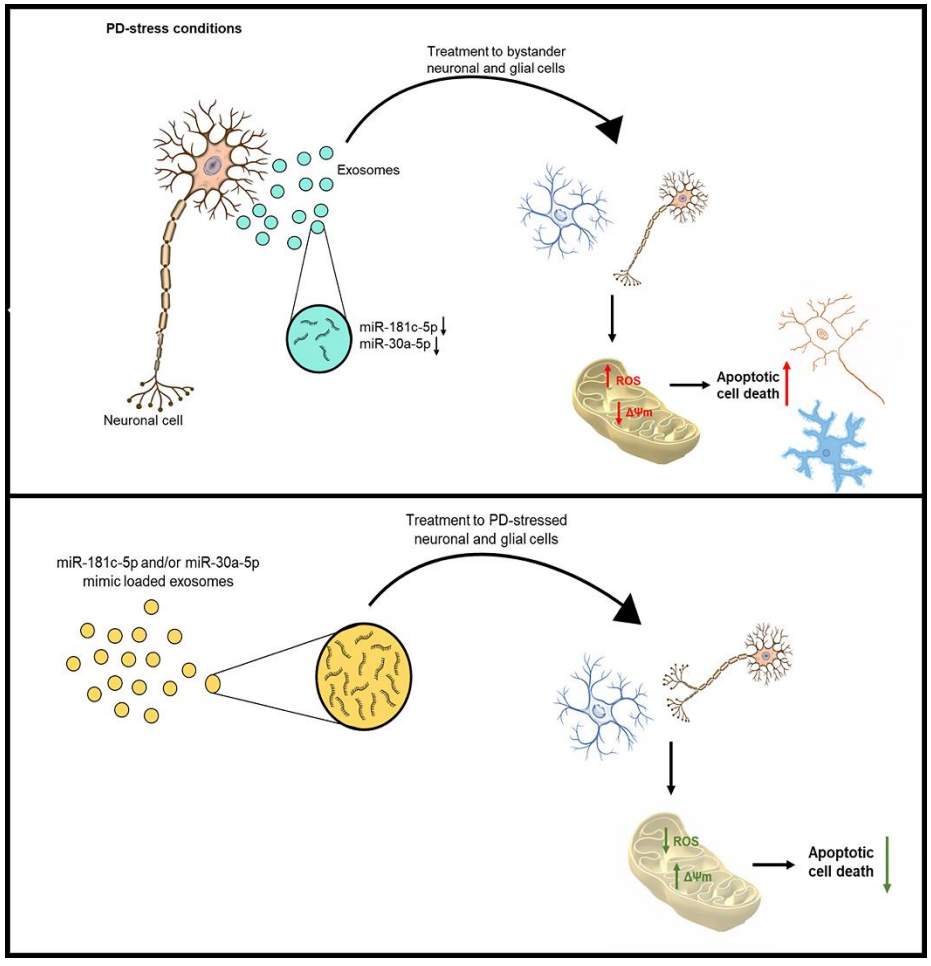
- Surprisingly, when U-87 MG glial cells, which were pre-exposed to 6-OHDA and Rotenone, were incubated with the miRNA mimic loaded exosomes, significant difference was not observed. This indicated that total cellular ROS levels are rescued; however, no difference was observed in the mitochondrial ROS levels in U-87 MG glial cells.
- Next, we analyzed cell death via MTT assay, which indicated that miRNAs 30a-5p and 181c-5p loaded exosomes incubated with both SH-SY5Y and U-87 MG cells; showed an increase in cell viability as compared to the CM exosomes and ameliorated the cell death induced by 6-OHDA and Rotenone.
- Further, we also analyzed caspase 3/7 activity by luminescence method in the same indicated conditions and observed results concordant with the cell viability assay. The caspase activity was higher in the CM-exo incubated PD stressed cells and reduced when the cells are incubated with miRNA mimic loaded exosomes.

SUMMARY

- Inter-organellar cross talk between mitochondria, lysosomes and MVB is now emerging important in PD pathogenesis.
- Understanding of the inter-organellar cross talk may provide unique opportunity to modulate the combinatorial strategy to enhance the autophagy flux and prevent exosomal release hereby reducing the spread of PD and helping ameliorate the disease.



- Exosomes are mediators of intercellular communication between neurons and neuronal and glial cells during PD stress conditions.
- Exosomes are actively internalized by the recipient neuronal and glial cells and further localize to the subcellular organelle mitochondria.
- Exosomal miRNAs from neuronal cells in 6-OHDA condition showed that selected miRNAs are differentially sorted into the exosomes in PD stress conditions. Exosome mediated transfer of identified miRNAs: miR-30a-5p and miR-181c-5p, rescued the mitochondrial functions and cell death in PD stress conditions in both neuronal and glial cells.
- The study suggests that exosome mediated transfer of miRNAs play an important role in the progression of PD and delivery of miR-30a-5p and miR-181c-5p through exosomes may yield novel strategies to combat the spread of PD.



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- **Currim, F.,** Singh, J., Shinde, A., Gohel, D., Roy, M., Singh, K., ... & Singh, R. (2021). Exosome Release Is Modulated By The Mitochondrial-Lysosomal Crosstalk In Parkinson's Disease Stress Conditions. *Molecular Neurobiology*, *58*(4), 1819-1833.

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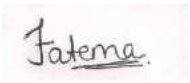
POSTER PRESENTATIONS

- **Fatema Currim**, Anubhav Srivastava, Kritarth Singh, Milton Roy, Dhruv Gohel, Minal Mane, Meenakshi Iyer, Anjali Shinde, Hitesh Vasiyani, Rajesh Singh. The Exosome Release Is Enhanced During Parkinson Disease Stress Conditions To Regulate Intercellular Communication. *Xlii All India Cell Biology Conference And 2nd International Conference On Trends In Cell And Molecular Biology*. Bits Pilani, Goa, India, December 21-23, 2018.

- **Fatema Currim**, Shatakshi Shukla, Jyoti Singh, Dhruv Gohel, Anjali Shinde, Shani Goyani, Milton Roy, Minal Mane, Hitesh Vasiyani, Saranga Mv, Nisha Chandak, Rajesh Singh. Exosomal Mirnas Play A Role In Modulation Of Mitochondrial Functions And Cell Death In Parkinson's Disease. *Mitochondrial Medicine – Therapeutic Development 2021- Vitrual Event.* Wellcome Connecting Science, Wellcome Genome Campus, Uk, November 30 – December 02, 2021.



Signature of Candidate Signature of Guide