Mehrotra et al. Scr Med. 2024 Jan-Feb;55(1):23-32. DOI:10.5937/scriptamed55-47980

ORIGINAL ARTICLE

Quantitative Evaluation of Mitochondrial Dynamics During Maintenance of Cellular Bioenergetics Using *ImageJ*

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Abstract

Background/Aim: Mitochondria are one of the most dynamic organelles essential for maintaining cellular energy demands, including execution of several vital cellular processes. This feature is attributed to rapid adaptation in morphological features which dictates their functionality. Depending on the cellular status, mitochondria can be rod shaped, branched, spherical, inter-connected or can exist as a network. Aim of this study was to analyse mitochondrial morphological appearance under normal vs stress condition in mitochondria.

Methods: The study evaluated mitochondrial morphology under normal and experimentally generated cellular stress condition by utilising *ImageJ* software, a versatile image analysis tool. Live-cell imaging technique was employed to capture high-resolution images of mitochondrial dynamics in SH-SY5Y cells and subsequent ultra-structural changes were evaluated using transmission electron microscopy. The images were later processed using *ImageJ* software, with inbuilt plugins designed for image processing.

Results: The present study identified alterations in mitochondrial morphology ranging from elongated, rod and interconnected mitochondria indicative of healthy mitochondrial network in controls to punctate, large/rounded and fragmented mitochondria in stress induced treated condition. Moreover, transmission electron microscopy confirmed significant abberation of mitochondrial structure with disapperance of outer mitochondrial membrane, decrease in matrix space and increase in mitochondrial size, with concomittant decrease in the cristae length and simultaneous increase in cristae lumen width in treated sections.

Conclusion: The study implicates existence of a mutual association between mitochondrial morphology and execution of cellular functions occurring during several pathological conditions, including neurodegenerative disorders. Furthermore, by utilising such a tool for quantitative analysis, a deeper understanding of mitochondrial dynamics and potential advancement in development of mitochondria-targeted drugs is suggested.

Key words: *ImageJ*; Mitochondria; Mitochondrial dynamics; Mitochondrial network; Mitochondrial morphology; Oxidative stress.

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Citation:
Mehrotra A, Sood A, Kanwar D. Quantitative evaluation of mitochondrial dynamics during maintenance of cellular bioenergetics using *ImageJ*. Scr Med. 2024 Jan-Feb;55(1):23-32.

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Received: 30 November 2023
Revision received: 24 January 2024
Accepted: 24 January 2024

Introduction

The eukaryotic cell is a notable entity which encompasses an intricate interplay between different organelles dedicated for precise functioning *via* tightly regulated processes. Amongst such

organelles, mitochondrion is a pivotal player involved in maintaining cellular bioenergetics, regulation of apoptosis and its associated diverse signalling pathways.[1] Mitochondria are highly dynamic entity, wherein incessant fusion and fission processes, movement along the cytoskeleton network and selective degradation through mitophagy occurs. Collectively, such plethora of mechanisms is referred to as "mitochondrial dynamics" and they characterise an essential aspect of mitochondrial biology.[2] Maintenance of such mitochondrial dynamics is fundamentally significant for regulation of cellular homeostasis. Moreover, by regulating their morphological features and distribution within the cellular environment, mitochondria possess the ability to rapidly provide bioenergetic demands, with concomitant response to diverse environmental factors. Any dysregulation or failure in maintenance of mitochondrial dynamics has been implicated in several pathological conditions, including neurodegenerative disorders.[3] In recent years, a significant advancement in microscopy techniques coupled with state-of-the art image analysis tools such as *ImageJ* software have provided investigators potent analytical tools to quantify mitochondrial dynamics at unprecedented levels of detail and precision. This has indeed provided new opportunities for investigating the molecular mechanisms, helping in regulation of mitochondrial dynamics and their associated functions.[4]

The morphological appearance of a mitochondrion can vary in shapes depending upon the metabolic requirement of the cell at particular localisation, such as: it could be rod, branched, spherical or highly interconnected in the form of a network. This existence of mitochondrial morphology is influenced by constantly occurring fission and fusion events at mitochondrial metabolic sites. These regular events are required to replace damaged mitochondria or to form new mitochondria whenever there is an increased cellular energy demand.[5] Fission leads to initiation of apoptosis, whereas fusion involves mixing of mitochondria leading to the formation of reticular mitochondrial networks, associated with increased bioenergetic demand. Depending upon energy requirements there are hundreds to thousands of mitochondria per cell in humans. Mutation in mitochondria has been shown to cause advancement of several neurological disorders, like Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) etc.[6,7] For instance, the first report of a direct involvement of mitochondrial dysfunction in progression of PD pathology came from brain samples of PD patient. Later, a significantly reduced activity of mitochondrial complex-I in the brain tissue of PD patients was also confirmed.[8,9] Rotenone, a naturally occurring pesticide and insecticide extracted from the roots of plants was first used as a potent inhibitor of mitochondrial complex-I in pre-clinical PD research. Rotenone is highly lipophilic and readily crosses all biological membranes including the blood-brain barrier and leads to the production of free radicals, thus causing oxidative damage.[10]

This research article aimed to provide a succinct overview of mitochondrial morphological appearance under normal vs stress condition in mitochondria, thereby emphasising on the significant role played by mitochondrial dynamics in maintaining normal cellular physiology or rather progression of a pathological condition. Furthermore, the study analysed usage of *ImageJ* software based quantitative analysis in studying such mitochondrial dynamics, highlighting its application in measuring mitochondrial morphological features.

Methods

Cell culture

For the evaluation of mitochondrial dynamics, human neuroblastoma SH-SY5Y cells (ATCC CRL-2266, USA) were used. The cells were cultured in DMEM/F12 media (*Thermo Fischer Scientific*, USA) with 10 % heat-inactivated foetal bovine serum (*Thermo Fischer Scientific*, USA) and a mixture of 0.1 % penicillin + streptomycin antibiotics (*Merck*, Mumbai, India). Cells were seeded on sterile glass coverslip and maintained at 37 °C in 5 % CO_2 incubator for the microscopy experiments.[11]

Confocal microscopy

For visualising mitochondrial morphology, *MitoTracker™ Red CMXRos* dye (*Thermo Fischer Scientific*, USA) was used. Neuronal cells (SH-SY5Y) were cultured in respective culturing media on coverslips till the day of experiment, labelled with *MitoTracker™ Red CMXRos* dye and placed on *Attoflour Cell Chamber* (*Thermo Fisher Scientific*, USA) for live cell viewing under the Nikon A1 plus Ti confocal microscope (*NIKON*, Japan). Im-

aging was done using 60X oil objective lens, with a numerical aperture of 1.45. The *MitoTracker™ Red CMXRos* dye was excited (571 nm excitation) with argon and a helium-neon laser using confocal microscope.[12] Images were captured using *NIS elements* software and analysed using *ImageJ* software (*NIH*, USA).

Transmission electron microscopy

Transmission electron microscopy was done as previously described.[13] The protocols followed were approved by the Institutional Animal Ethics Committee of the University and were in accordance with the guidelines for humane use and care of laboratory animals. Tissue was fixed in 4 % glutaraldehyde solution overnight and later post fixed in osmium tetraoxide solution, dehydrated in a graded ethanol, cleared in propylene oxide at room temperature and finally embedded in an EPON mixture. The 0.5 µm sections were cut using ultra microtone, mounted on Nickel grids and were stained using uranyl acetate and lead citrate. Sections were examined and photographed using *Hitachi H-7500* transmission electron microscope (*ZE155906*, Munich, Germany). The captured images were analysed using *ImageJ* software (*NIH*, USA).

Image processing and analysis using *ImageJ*

A widely accepted method of scoring mitochondrial morphological features was followed in the present study. The analysis of mitochondrial networking was done with the help of *ImageJ* software. Initially, captured images were processed for various parameters by using toolbar kit containing, both build-in and customised plugin/s.[5]

Statistical analysis

All values were expressed as mean ± standard error of mean (SEM) and data was analysed using one way analysis of variance (ANOVA) followed by Student t-test for comparisons between the groups using SPSS 17 software. Values with $p < 0.05$ were considered as statistically significant.

Results

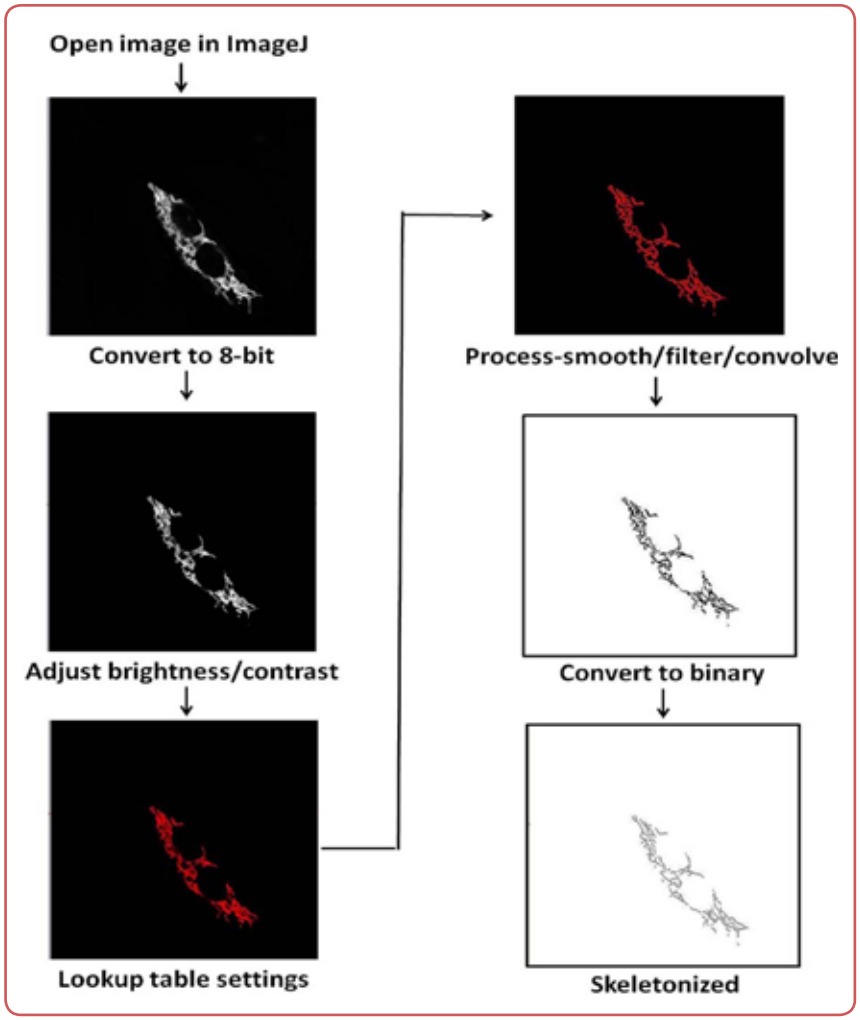
Evaluation of altered mitochondrial dynamics using *ImageJ*

The raw images of control cells were captured using confocal microscope and processed using *ImageJ* software in order to investigate mitochondrial dynamics in control conditions (Figure 1). Processing and analysis of raw images showed significant disruption in mitochondrial networks in treated cells as compared to controls (Figure 2A). The stress-induced rotenone treated cells showed higher punctate morphology followed by large/rod shape with very few mitochondria showing network connectivity, however, control cells showed significantly greater network connectivity. In Figure 2B, the high density of green dots represented a significant perturbation in mitochondrial networking in treated cells, as shown by punctate appearance. Whereas, white dots (representing large/round appearance) showed mitochondria were not well connected to each other due to broken mitochondrial networking with concomitant appearance of very few connected mitochondrial network (represented by red dots) in treated cells.

These mitochondrial observations suggest that mitochondrial network has been significantly interrupted due to hampered dynamics in treated cells. Moreover, due to rotenone treatment the large mitochondrial network broke into small network leading to significantly increased appearance of punctate, large/round and rod observations (Figure 2C). This is in alignment with low score obtained in treated cells as compared to controls due to significantly reduced presence of healthy mitochondrial network (Figure 2D). These results were further supported by correlation analysis performed between the two parameter- punctate vs rod and punctate vs large/round with respect to mitochondrial observations obtained in control and treated groups, respectively (Figure 3). It was found that there was 94 % correlation/similarity in punctate vs rod for controls, whereas it reduced to 82.5 % in treated cells. Similarly, a correlation analysis for punctate vs large/round also showed the same trend, wherein 82 % of correlation was observed in controls, however, this was increased to 94.6 % in case of treated cells.

Accessing mitochondrial ultrastructural changes using *ImageJ*

In order to strengthen the above findings with mitochondrial morphological appearance, the *ImageJ* software was extensively used to measure ultrastructural changes in mitochondrial length, mitochondrial width, mitochondrial cristae length, mitochondrial cristae lumen width in the raw images of control and treated sections,



The original raw image is processed through several filters and plugins, using ImageJ software and then evaluated for desired observations, scale bar = 50 nm.

Figure 1: Processing of a raw image from control cells for analysing mitochondrial dynamics

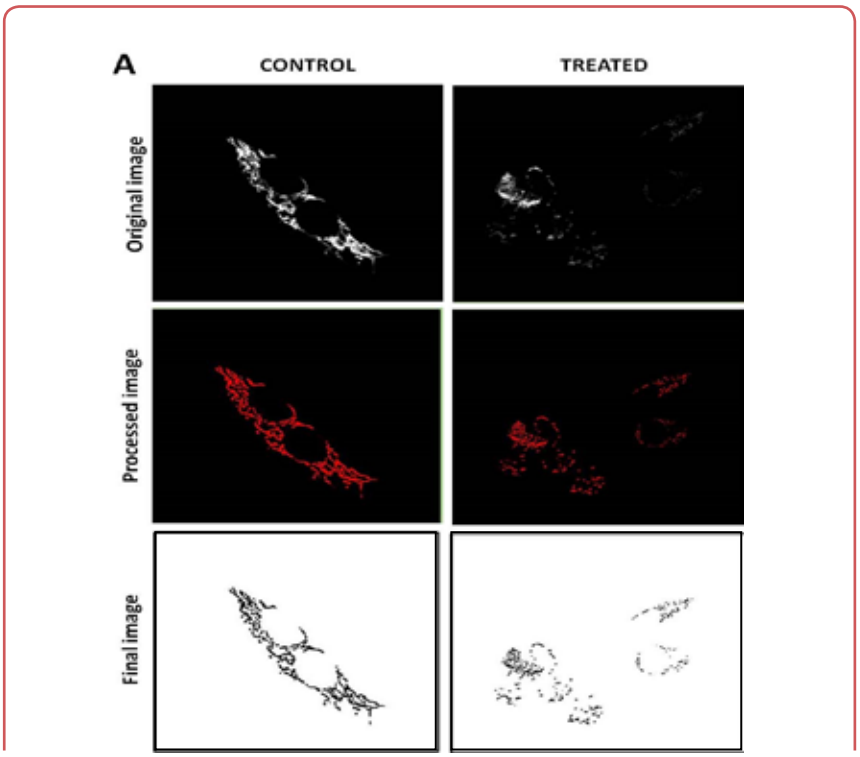


Figure 2: Evalution of changes in mitochondrial dynamics in control vs treated cells.

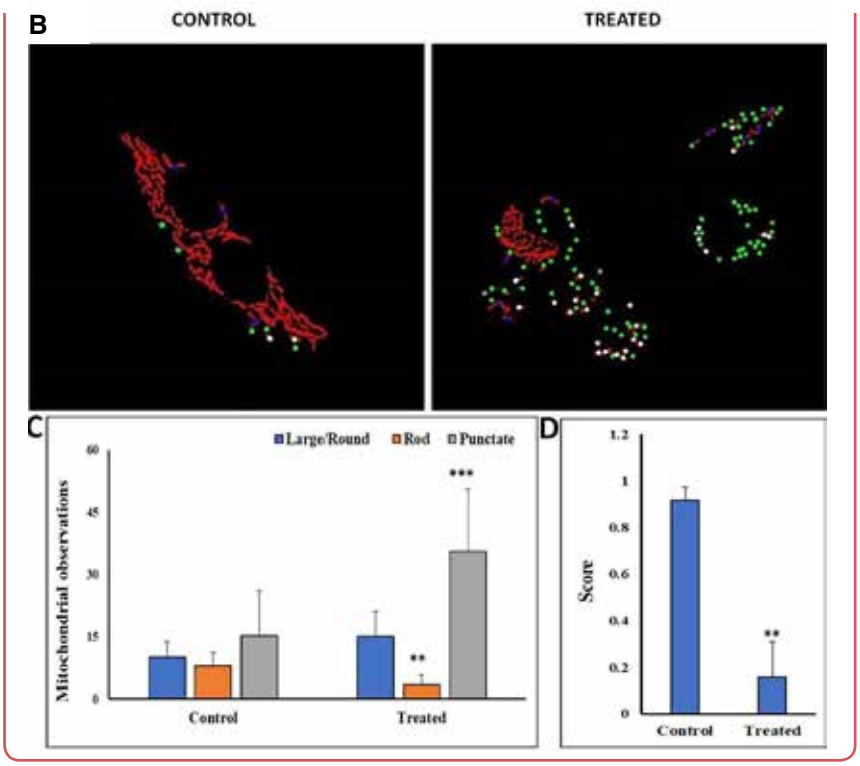


Figure 2: *(continued from previous page)*

The raw images were processed for in-depth analysis of fragmented mitochondrial network using ImageJ software (A). A healthy-intact mitochondrial network was observed in controls in comparison to fragmented mitochondrial network 48 h after retenone treatment (B). The images shows the number of punctate, rod, large/round in control and treated cells represented by green, blue and white colour respectively (C). The graphical data representation shows reduced number of rod and increased number of punctate, large/round mitochondrial network with reduced mitochondrial score in rotenone treated cells (D), scale bar = 50 nm.

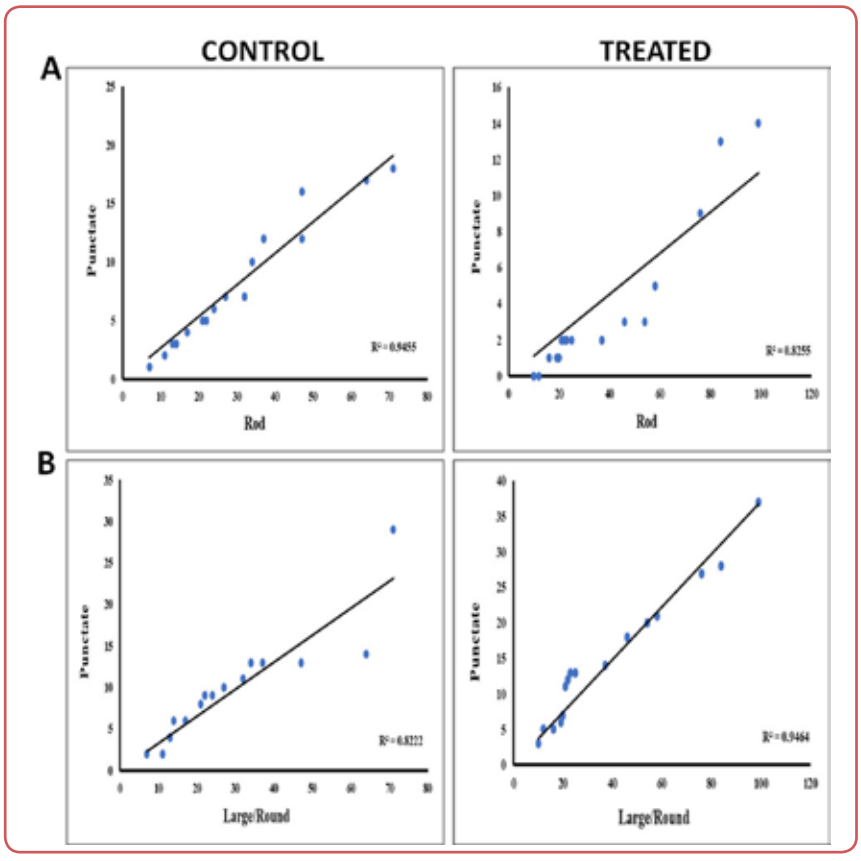
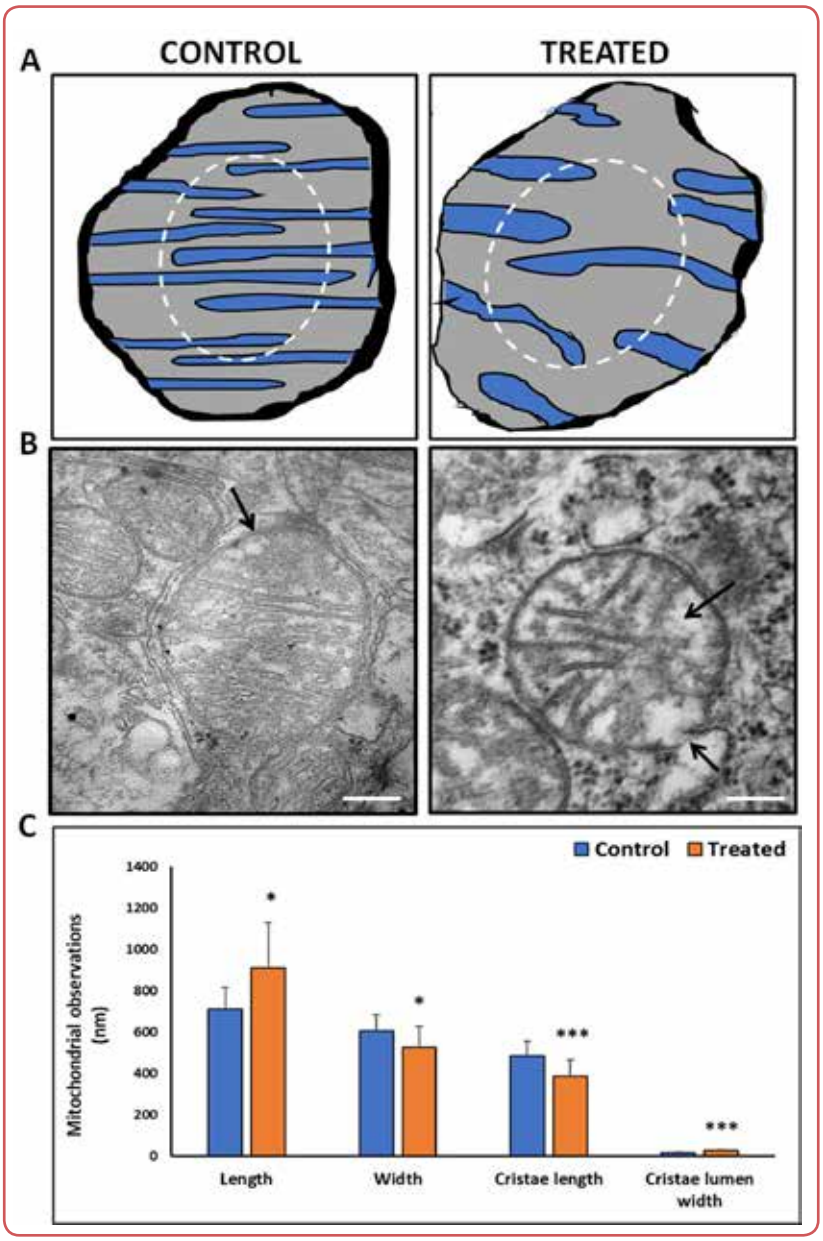


Figure 3: *Correlation analysis for evaluating changes in mitochondrial dynamics*

Correlation analysis for mitochondrial observations between punctate vs rod (A) and punctate vs large/rod (B), in control and treated cells respectively.

using TEM (Figure 4). The hypothetical model showed the difference between cristae length and cristae lumen width in control and treated conditions respectively, wherein presence of intact mitochondrial membranes in control and swollen mitochondrial apperance upon rotenone treatment is shown with dotted circle in Figure 4A. Transmission electron microscopy showed significant abberation of mitochondrial structure with disapperance of outer membrane of mitochondria, decrease in matrix space and increase in the size of mitochondria in treated sections as compared to controls (Figure 4B). Moreover, this was accompained by enlarged and disrupted cristae due to rotenone induced swelling, with concomittant decrease in the cristae length and simultaneous increase in cristae lumen width (Figure 4C). The results were further supported by the correlation analysis performed between two parameters, that is: mitochondrial length vs mitochondrial width (Figure 5A) and mitochondrial cristae length vs



*The hypothetical model representing appearance of mitochondrial morphological features during control and treated conditions (A). Ultra-structural changes in mitochondrial morphology were found to be altered in treated conditions (as shown by arrow) in comparison to controls in images captured at 60,000X (B). Such changes were quantified using ImageJ for calculating the mitochondrial length, mitochondrial width, mitochondrial cristae length and mitochondrial cristae lumen width, as represented by graphical data (C). * Statistically significant at $p < 0.05$, *** Statistically significant at $p < 0.001$; scale bar = 100 nm.*

Figure 4: Ultra-structural appearance of a mitochondrion using TEM

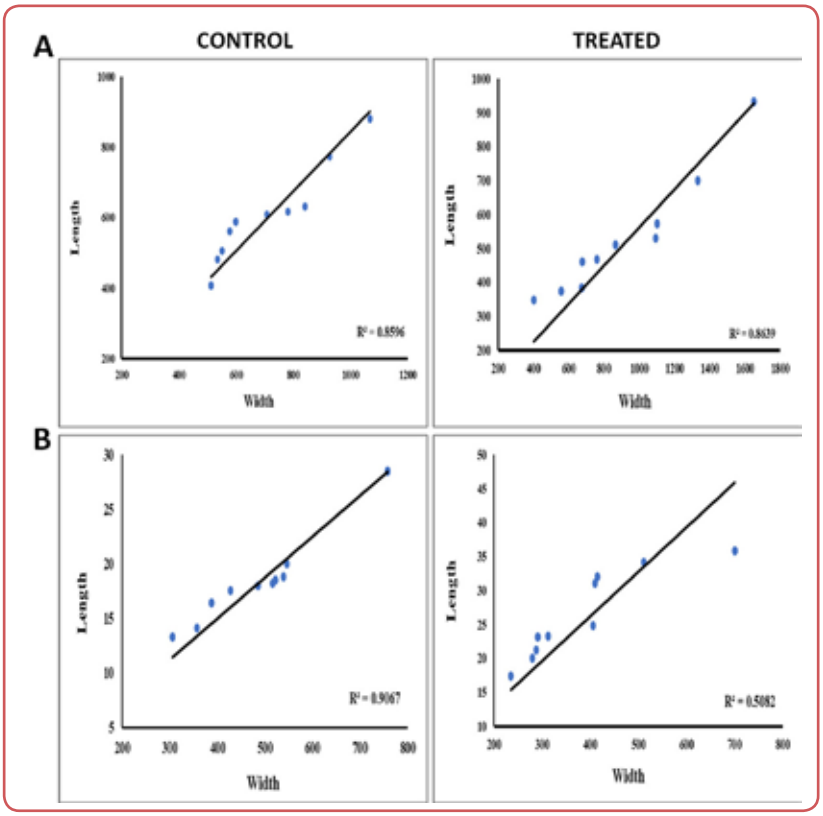


Figure 5: Correlation analysis for evaluating changes in mitochondrial morphology

The correlation analysis showed existence of ~85 % similarity in mitochondrial length vs mitochondrial width (A) for both the conditions, whereas correlation analysis for mitochondrial cristae length vs mitochondrial cristae lumen width was found to be red.

mitochondrial cristae lumen width (Figure 5B) in both control and treated groups, respectively. It was found that there was 85 % correlation in mitochondrial length vs mitochondrial width for control group, whereas it was increased to 86 % in treated group.

Similarly, a correlation analysis for mitochondrial cristae length vs mitochondrial cristae lumen width also showed the same trend, wherein 90 % of correlation was observed in control group. However, the values were reduced to 50 % in case of treated group.

Discussion

The processed images were evaluated for desired observations, such as mitochondrial morphology and mitochondrial network. The evaluation of mitochondrial dynamics changes was performed using *MitoTracker™ Red CMX Ros* dye in control and rotenone treated (to generate mitochondrial stress condition) SH-SY5Y cells. Networks are mitochondrial structures which have single node and are characterised by connected branches. Whereas, individual structures are commonly punctate, rods, or large/round in appearance. Rod structures show that, the mitochondrial network has been interrupted or broken, but may be connected at places and are unbranched. Punctate structures are small dot like and highlights that the mitochondria are destined for degradation, whereas large/round structures reflect the mitochondria are completely disconnected from each other. The punctate and large/round appear more in fragmented mitochondrial network, however the number of rods are more in non-fragmented healthy mitochondrial network.[5] Rotenone is a plant derived toxic compound and a mitochondrial complex-I inhibitor, which has been extensively used to study mitochondrial dysfunctions in pre-clinical or cellular models of neurodegenerative diseases.[14, 15] Whereas, the human neuroblastoma cells (SH-SY5Y) are known to exhibit impairment in mitochondrial functioning upon rotenone treatment due to alteration in several mitochondrial proteins associated in maintaining mitochondrial dynamics.[16, 17] Such an analysis demonstrated

that, due to presence of significantly increased amount of large/round and punctate mitochondrial observations, with reduced rod appearance suggests compromised mitochondrial dynamics and existence of significantly impaired mitochondrial networking in treated cells.

Advances in live cell imaging in the past decade have revealed the dynamic existence of mitochondria and its associated network in a cellular environment. In order to preserve their functional homeostasis, these dynamic organelles are known to form elongated tubules (termed as mitotubes) that constantly divide and fuse and such morphological existence is due to maintenance of equilibrium between fusion and fission processes. Whenever this equilibrium is compromised, mitochondria are expected to lose their characteristic morphological features.[18] In the present study, rotenone induced stress resulted in significant appearance of punctate and fragmented mitochondrial morphology and this finding was further supported by establishment of a positive correlation among such characteristic features in treated cells. In case of normal cellular status, mitochondria are reported to form an elongated and interconnected network, thereby resisting mitophagy with concomitant increase in ATP generation. However, mitochondria exhibit a fragmented network accompanied by smaller fragments (*punctae* or round) in case of failed homeostasis.[19] The mitochondrial cristae morphology was found to be altered in treated sections due to presence of significantly increased cristae length and cristae lumen width in comparison to controls. These changes are suggested to occur due to enhanced reactive oxygen species (ROS) mediated ensuing of mitochondrial swelling in cristae upon stress generated by rotenone treatment.[20]

Overall, such changes are well reported to cause mitochondrial dysfunctioning due to failure in maintenance of mitochondrial biogenesis, less proportion of active mitochondria to total mitochondria, enhanced ROS production, with simultaneous reduction in ATP generation and eventually leading to apoptosis.[21] However, the appearance of normal mitochondrial structure, with intact morphological features was observed in controls.

Conclusion

Mitochondrial dynamics regulating or maintaining mitochondrial structure are widely documented to be involved in smooth functioning of mitochondrial metabolism. Any change or imbalance in the mitochondrial dynamics may be involved in progression of pathological condition. Therefore, it becomes obligatory to analyse any imperfections observed in mitochondrial network which could impart more extensive understanding of mitochondria induced changes observed during such diseases, using high end software tools. One such software employed in the present study was *ImageJ*, which improved the outcome of the dataset from a raw image of mitochondrion by passing through several stages of processing and quantified later. With the help of a special plug-in installed with *ImageJ*, a detail analysis of morphological features of mitochondrial networks in control and treated cells was obtained, thus helping to conclude a clear difference between an intact and fragmented mitochondrial network. In conclusion, the investigations involving elucidation of mitochondrial dynamics using such software based approaches could be utilised in the area of targeted drug development.

Ethics

The ethical approval for the study was obtained from the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh, India (decision No IAEC/11/19, dated 22 October 2019).

Acknowledgement

The authors acknowledge CSIR-Institute of Microbial Technology, Chandigarh, India and Panjab University, Chandigarh, India for providing the infrastructural facility for the study.

Conflicts of interest

The authors declare that there is no conflict of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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Validation: AM, DK
Formal analysis: AM, AS, DK
Investigation: AM, AS, DK
Resources: AM, DK
Data curation: AM, DK
Writing - original draft: AM, AS, DK
Writing - review and editing: AM, AS, DK
Visualisation: AM, DK
Supervision: AM, AS
Project administration: AM
Funding acquisition: None

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