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Autophagy and Parkinsons Disease– Role of Caffeine as Autophagic Stimulator and Anti Apoptotic Agent

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Abstract

Though, being the second most common neurodegenerative disorder, the socio-economic impacts of Parkinson's Disease (PD) viz its effects on cognitive movements and limited treatment regimens has raised concerns over the decade. Caffeine (1,3,7 trimethyl xanthine), the most common psychoactive substance exerts neuroprotection and cognitive benefits which attracts more research interest. The study focuses on exploring the role of caffeine in neuroprotection targeting different areas of anti-apoptotic function, neurite growth, calcium homeostasis and autophagy. Caffeine underwent cytotoxicity screening on L929 cells and was assessed for its neuroprotective effects on IMR32 cells. Anti-apoptotic effects were evaluated through fluorescent staining and Caspase ELISA analysis. Neurite outgrowth was measured experimentally, while intracellular calcium levels were determined using Alizarin staining and spectrophotometric analysis. The impact of caffeine administration on cellular autophagy was analyzed through LC3 flow cytometry. In the *in vitro* cytotoxic study, administration of caffeine (10 µM) showed a cell viability of about 88% at a 6.25 µg mL $^{-1}$ concentration in rotenone-treated neuronal cells. Further, using the neutral red assay it was observed that caffeine's neuroprotection on rotenone-treated IMR32 cells was about 87.4% at a concentration of 6.25 µg mL $^{-1}$ compared to 49.43 % viability in untreated control cells and after performing FDA/EtBr staining it was clear that caffeine co-administration can reduce apoptotic cell death incited using rotenone, the caspase 9 levels obtained supported this finding. Caffeine showed a tremendous effect in maintaining neurite length, similarly, the Alizarin red staining studies indicated that caffeine treatment can restore calcium levels. Finally from the LC3 Flow cytometry results, it was evident that caffeine could restore autophagy induction confirming the effect of caffeine on neuronal growth.

Keywords: Parkinson's disease, Caffeine, Autophagy, Neurite length, apoptosis

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Introduction

Parkinson's Disease is one of the most common neurodegenerative disorders affecting the central nervous system, is characterized by the concomitant loss of dopaminergic neurons in substantial nigra pars compacta, which deters the function of the midbrain steering the advancement of clinical features like rigidity, resting tremor, bradykinesia in the early stages, and postural instability in the advanced phase [1–3]. As per WHO, the preponderance of Parkinson's disease (PD) has plaited in over 25 years with worldwide assessments in 2019 showing over 8.5 million individuals are living with PD[4].

In most cases, PD is caused by a mutation in different genes like LRRK2, PARK2, PINK1, SNCA, and DJ-1[5–7]expedite the phenomenon of protein misfolding called "Lewy bodies "(LBs) [8], causing marred functioning of neurons. The LBs contain a protein called alpha-synuclein, which pertained to synaptic vesicle functioning, axonal transport, and plasticity of neurons [9]. The impeding of ubiquitin proteasomal and chaperone-mediated autophagic system finally leads to neuroinflammation and neurodegeneration [10]

Neuroinflammation entails the activation of reactive astrocytes and microglial cells within the brain. The aggregation of α-synuclein in the dopaminergic neurons will trigger the microglial cells [11], this happens when the oligomers of the α-synuclein switch on toll-like receptor 2 (TLR2)-mediated signaling [12]. This brings fluctuations in mitochondrial signaling and energy metabolism resulting in neuroinflammation via NO and ROS generation and thereby neurodegeneration [13].

Furthermore, the overproduction of ROS (Reactive Oxygen Species) due to the ineptitude of the body to neutralize the generated ROS residue eventuates a condition called Oxidative stress (OD) [14]. The cytoplasmic dopamine coordinates Fe molecules within the cell to undergo redox reactions and elicit a dark pigment called Neuro-melanin (NM) and ROS [15]. Neuro-melanin itself can generate surplus ROS. This ROS surge results in the deficiency of complex – I which steers the activation of apoptosis and pro-apoptosis factors ending in neuronal death [16]. As per various research,

Dopamine-based drugs have curtailed cogency in ameliorating signs of disease advancement [17].

According to various traditional medicines, plant extracts embodying phytochemical components that are competent enough to target α-syn could be reckoned as shaped by dietary interventions [18]. Caffeine (1,3,7-trimethylxanthine) is used as a psychostimulant with antioxidant, anti-inflammatory, and anti-apoptotic properties, and is recently reported to possess potent neuroprotective effects [19], also it is reported that it can slow down the neuroinflammation caused by activated microglia and can reduce the extracellular glutamate in the brain [20]. There suggest profound studies supporting the effect of caffeine on PD stating that it procures neuroprotection against neurodegeneration caused by different neurotoxins [21]. In this scenario, the present study aimed to assess the impact of caffeine on neuroprotection in human neuroblastoma cells IMR 32 induced with rotenone.

Materials and methods

Materials

The caffeine (CAS 58-08-2) used in this study was obtained from Sigma Aldrich. All other chemicals proposed for the studies were recouped from Invitrogen and HiMedia.

Cell lines

Neuroblastoma (IMR32) and Murine fibroblast (L929) cell lines were obtained from National Cantre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% FBS. After trypsinization, the cells were suspended in a 10% growth medium and cell suspension (5x10^4 cells/well) was seeded in a 96-well tissue culture plate and incubated at 37 C in a humidified 5% CO2 incubator (NBS Eppendorf, Germany).

Cytotoxic effect of caffeine on cultured l929 cells using neutral red assay

Neutral red was enacted in consonance with the method of Borenfreund et al [22]. To assess the nontoxic concentration of caffeine *in vitro* cytotoxicity was determined in L929 cells. Caffeine at a concentration of 1 mg mL^{-1} was dissolved in DMEM and used for the study. L929 cells were treated with different concentrations of caffeine for 24 hours and the cell viability was determined by the neutral red assay as per the following method. Cells are washed with PBS and fixed with a fixing solution (50% ethanol and 1% acetic acid). Later, the fixation solution was discarded, extraction buffer was added, mixed well, and the plates were kept for incubation for about 20 minutes. Further, the absorbance was measured using a microplate reader at 540 nm and the percentage viability was determined.

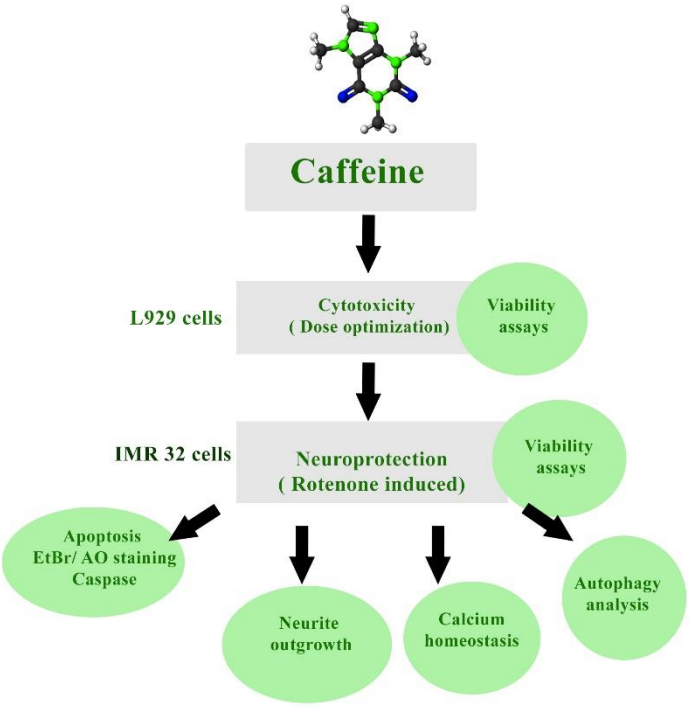

Figure 1. Process flow chart depicting the major experimental procedures

Neuroprotective effect of caffeine by neutral red assay

Cells were divided into groups consisting of rotenone-treated positive control groups, rotenone-induced cells co-treated with different concentrations of caffeine ranging from 6.25 to 100 (µg mL^{-1}), and untreated cells were maintained as the negative control. All treatments were carried out for 24 hours and the cell viability was determined by Neutral red assay in which the quantitative estimation of viable cells is determined based on the ability of viable cells to uptake of the neutral red dye [23].

Anti-apoptotic effects of caffeine on rotenone-induced neurotoxicity

The cultured cell lines were treated with IC 50 concentration of samples and incubated for 24 hours, later the cells were washed with cold PBS and stained with a mixture of fluorescein diacetate (FDA) (100 µg mL^{-1}) and Ethidium Bromide (100 µg mL^{-1}) at room temperature for 10 minutes. The stained cells were washed with PBS and observed under a fluorescence microscope in the blue filter of a fluorescent microscope (Olympus CkX-41 connected with Optika pro 5 camera) and analyzed by ImageJ analysis software.

Indirect ELISA

The supernatant nuclear and cytosolic fractions were collected from the treated and Non-treated cells, and a fraction of it was added to the 96 well plates followed by incubation overnight at 37^0C succeeded by washing and draining using PBS. To this blocking buffer (0.2% gelatin in 0.05% Tween 20: Merck; Germany) in PBS was added and incubated for 1 hour at room temperature and washed twice with PBS TWEEN. Later on primary antibodies (NFkB) was added and left for 2 hours at room temperature, followed by incubation and washing with PBS TWEEN (2 times). Subsequently, a secondary antibody (HRP Conjugate, SantaCruz, USA) was added, kept for 1 hour at room temperature, and then washed with PBS TWEEN twice. Finally, O- dianizdine hydrochloride (methanol + citrate buffer pH 5 + H_2O_2: Sigma Aldrich, USA) was added and incubated at room temperature for 30 minutes. The procedure is terminated by adding 5N HCL and OD read at 415 nm in an ELISA reader. The concentration of protein was also estimated.

Morphology and neurite length differentiation of imr 32 cells

Neurons are differentiated using retinoic acid, and the cells were treated with rotenone for 2 hours, exposed to IC50 concentration of caffeine, and incubated for 24 hours. Then these cells were examined under an inverted microscope (Olympus CKX-41 connected with Optika Pro 5 camera).

For morphological differentiation, fluorescein diacetate (FDA) staining was employed. The staining solution consisting of FDA was added to the cell culture and incubated in dark for 15 minutes eventually washed extensively in PBS; cells were observed under a fluorescence microscope using a blue filter.

Alizarin red staining

Tissue culture plates were washed with PBS three times, the cells were fixed with 2.5% glutaraldehyde, and later washed with distilled water three times. Subsequently added Alizarin red S stain (pH 4.1) was added. The dye was removed and washed with distilled water five times. The morphological images were taken. The plates were tilted and kept in -20^0C for five minutes and then 10% acetic acid was added and kept for 30 minutes. The cells were collected in a centrifuge tube and vortexed. The tubes containing samples were heated at 85^0C and further incubated on ice for five minutes. Then it was centrifuged at 10,000 rpm to settle down the cells. The OD was measured at 405 nm.

Determination of calcium

The intracellular calcium levels post-caffeine treatment was determined spectrophotometrically as per manufacturer instructions. Briefly, the cells were cultured as per previous protocols and the calcium levels were determined in cell lysates. The calcium levels were correlated with standards and were expressed in terms of µg/mg proteins.

Determination of autophagy

CytoID autophagy kit (Enzo Life Sciences, Cat. No. ENZ-51031-K200) was used to measure the autophagic flux. Rotenone-treated IMR32 cells were trypsinized and collected by centrifugation at 1000 rpm for 10 minute and resuspended in 1X assay buffer. Cells were mixed with 250 µl of Cyto ID green detection reagent (1:4000) and incubated for 30 minutes in dark at 37°C. Cells were then resuspended in 1X assay buffer and analyzed with Muse (Muse Cell Analyzer and the Muse analysis software, (Millipore, Billerica, MA, United States). The mean autophagy intensity is based on the estimation of the number of vesicles produced during autophagy.

Results and discussion

Neurodegeneration is one of the common features in many incurable diseases, especially in neurodegenerative diseases like Parkinson's disease, Alzheimer's, Huntington's disease, and amyotrophic lateral sclerosis where the well-being of the central nervous system is being marred, henceforth there is an urgent need to develop new and more effective therapeutic strategies to combat these devastating diseases or to improve the living of patients affected with ND [19].

As per the study conducted by Neil et.al neuronally differentiated IMR32 cells can be used as a model system to investigate possible interactions between APP-processing and PHF formation [24], and in our study, we have used IMR32 cell lines which is a human brain-derived neuroblast cell. The model of PD was generated by inducing rotenone (10µM), which is capable of reproducing two pathological hallmarks of PD [25], and caffeine-treated cells were put through different biological efficiency studies which produced discernible outcomes. Caffeine is non-toxic at lower concentrations: Firstly, the *in vitro* toxicity effect of caffeine was determined in normal cells, and thereafter dosage fixation. Caffeine was found to be nontoxic at lower concentrations and with the increase in concentration reduced cell viability when determined by Neutral red assay in L929 fibroblast cells (**Figure 1** & **2**). This follows the findings of workers like Tiwari et al, 2014 [26]. Rotenone administration produced a marked difference

in cell viability as well as cell morphology. The viability was decreased to 49.3% upon rotenone administration for 24 hours along with morphological changes suggesting apoptosis and membrane damages. Co-administration of caffeine was found to be beneficial at lower concentrations; more precisely 6.25 ug/ml of caffeine administration increased the cell viability to 88% with better morphological changes. This confirms that the co-administration of caffeine can in turn protect the neuronal cells from cell death induced by rotenone.

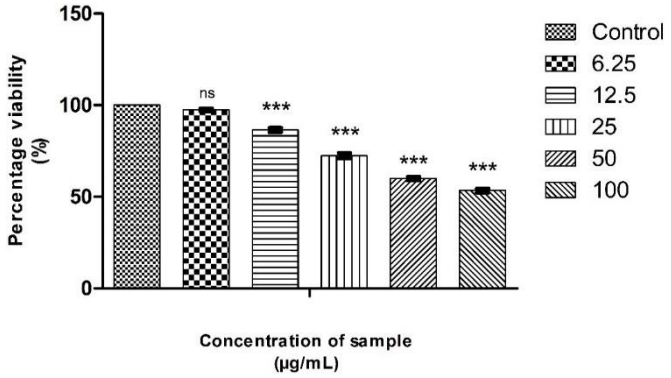

Figure 2. Graphical representation depicting caffeine's *in vitro* cytotoxic effect on cultured L929 cells in terms of percentage viability (%). The results are represented as the mean average of triplicates with SD, the results are compared with untreated control samples, and p <0.05 was accepted as significant.

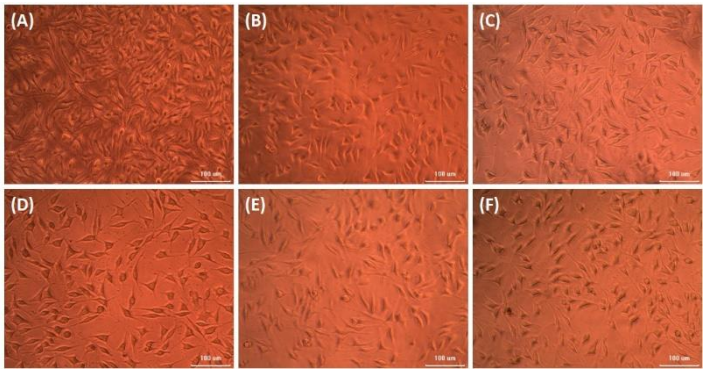

Figure 3. Phase contrast image depicting the *in vitro* cytotoxic effect of caffeine on cultured L929 cells (A) untreated control (B) 6.25 (C) 12.5 (D) 25 (E) 50 and (F) 100 µg.mL-1. (10X magnification-photographed using Olympus CKX-41with Optika pro5 camera)

Caffeine is neuroprotective at a concentration of 6.25 µg mL^{-1}

Furthermore, the neuroprotective effect of the caffeine co-administration on rotenone-exposed IMR32 cells was inferred with the help of a neutral red assay. Our results showed that when the cell line was exposed to rotenone, there is a significant decrease in the cell viability by 49.43 % compared with the untreated control cells. It can be assumed that the cells co-administrated with caffeine showed a significant increase in cell viability of approximately 87.4 % at a concentration of 6.25 µg.mL^{-1}. The results drawn from the neutral red assay confirmed the neuroprotective effect of caffeine (**Figure 3** & **4**) and were subjected to further assays to study the mechanistic approach to caffeine.

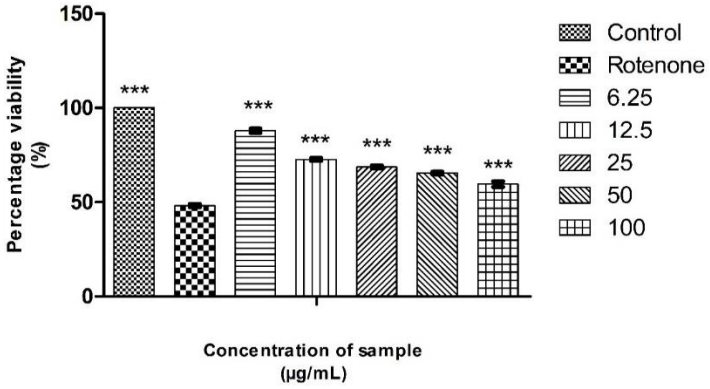

Figure 4. Graphical representation illustrating the *in vitro* neuroprotective effect of caffeine co-administration on rotenone-exposed IMR32 cells. The results are represented as the mean average of triplicates with SD, the results are compared with rotenone, and p <0.05 was accepted as significant.

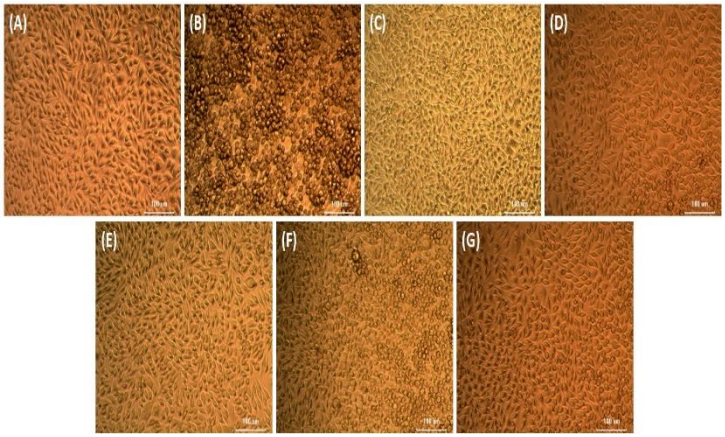

Figure 5. Phase contrast image depicting the *in vitro* cytotoxic effect of caffeine on cultured L929 cells (A) untreated control (B) 6.25 (C) 12.5 (D) 25 (E) 50 and (F) 100 µg.mL-1. (10X magnification-photographed using Olympus CKX-41with Optika pro5 camera).

We checked whether caffeine co-administration can turn down the apoptotic cell death induced by rotenone by dual staining using fluorescent microscopy. FDA/EtBr staining confirmed less number of apoptotic cells in groups co-administrated with caffeine when compared with the rotenone-induced group. This was further re-confirmed by measuring caspase level by ELISA. To access the apoptosis FDA/Et-Br, a double staining method using fluorescence microscopy was performed. (**Figure 5**)

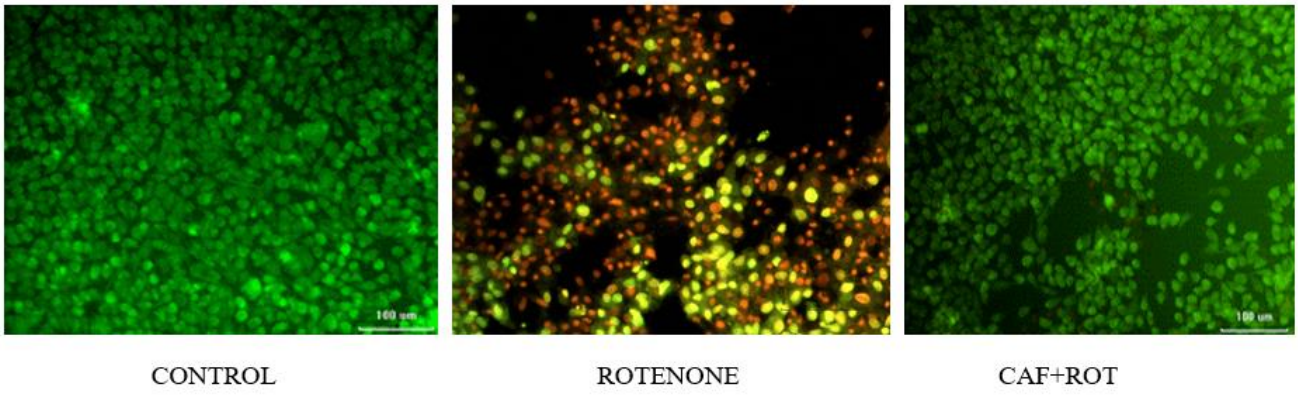

Figure 6. Determination of apoptosis via double staining method (FDA/ Et-Br staining) **(A)** untreated IMR32 cells (**B)** cells exposed to rotenone alone **(C)** cells exposed to rotenone co-administrated with caffeine (Magnification 20X).

Caspases are essential mediators of programmed cell death and are needed for both the induction of apoptosis and the promotion of degradation of cellular structure. Among them is Caspase 9 which is an initiator caspase, which senses and responds to various signals including intracellular stress or binding of the death receptor to external ligands. Upon dimerization, initiator caspases get activated following the cleavage of downstream effector caspases for carrying out the apoptotic program. Our results confirm activation and increase of caspase 9 activity in cells treated with rotenone which confirms the induction of neuronal cell apoptosis by rotenone. Co-administration of caffeine was effective in reducing the caspase 9 levels (fig6) which confirmed the anti-apoptotic activity of caffeine.

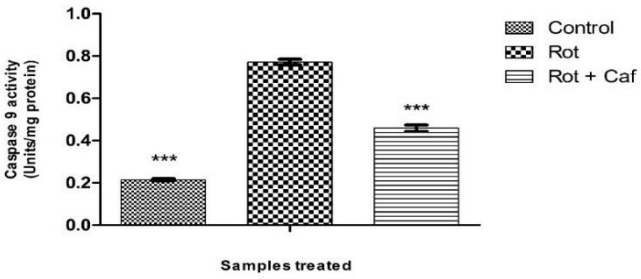

Figure 7. Caspase 9 activity along the X and Y axis. The results are compared with rotenone, and p <0.05 was accepted as significant.

Caffeine is effective in maintaining neural length: The maintenance of axons and dendrites (together called neurites) extending long distances from the cell body, plays a key role in the functioning of neurons. The regulation of neurite length and physiological and pathological remodeling functioning are been incriminated by both autophagy and mitochondrial content. We reconnoitred the effect of caffeine on neuronal outgrowth and neurite length by staining cells with cytoplasmic staining dye fluorescein di acetate and the pictures showed caffeine was effective in maintaining neurite length as that of untreated control IMR 32 cells compared with the decreased length obtained in rotenone treated cells. (**Figure 8 - i, ii, iii)**) .

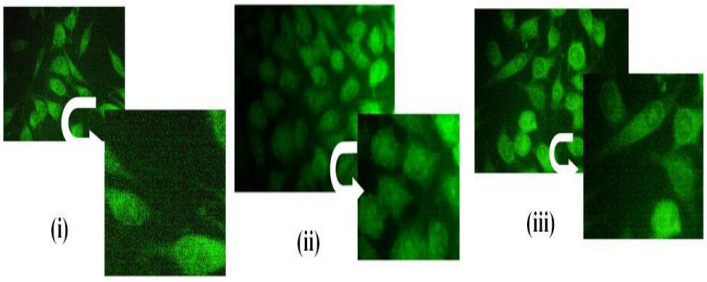

Figure 8. Determination of neurite length by FDA staining i) untreated control cells (ii) Cell treated with rotenone (iii) Cell co treated with caffeine and rotenone.

Caffeine restores cellular calcium levels in rotenone treated cells: In the brain, calcium is fundamental in the control of synaptic activity and memory formation, a process that leads to the activation of specific calcium-dependent signal transduction pathways and implicates key protein effectors, such as CaMKs, MAPK/ERKs, and CREB. Properly controlled homeostasis of calcium signaling not only supports normal brain physiology but also maintains neuronal integrity and long-term cell survival. Emerging knowledge indicates that calcium homeostasis is not only critical for cell physiology and health but also when deregulated, can lead to neurodegeneration via complex and diverse mechanisms involved in selective neuronal impairments and death [27]. We determined the calcium content of cells by alizarin staining and microscopic analysis. Surprisingly it was observed that rotenone produced a distinct decrease in the calcium content of IMR 32 cells. It is already reported that dysregulated calcium signaling can in turn speed up neurodegeneration [28]. Caffeine treatment restored cellular calcium levels which can be beneficial in this context (**Figure 10** & **11**).

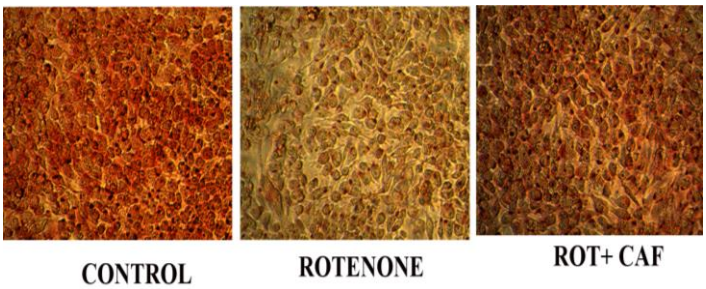

Figure 9. Determination of calcium deposition via alizarin red staining **(A)** untreated IMR32 cells (**B)** cells exposed to rotenone alone **(C)** cells exposed to rotenone co-administrated with caffeine (Magnification 20X).

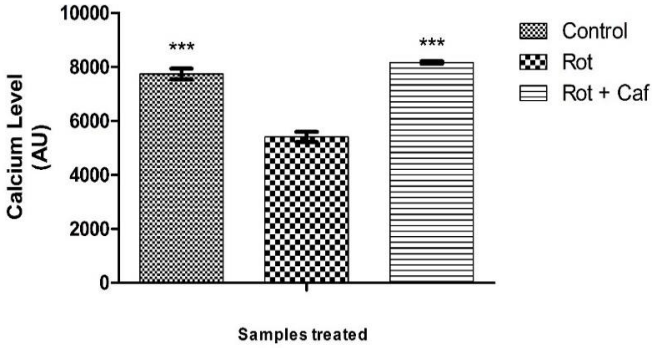

Figure 10. Calcium estimation via alizarin red staining. The results are compared with rotenone, and p <0.05 was accepted as significant.

Calcium estimation indicates that calcium deposition significantly decreased in the rotenone-exposed cells compared with the untreated control. The co-administration of caffeine in rotenone-exposed cells restored the intracellular calcium level reasonably (**Figure 11**).

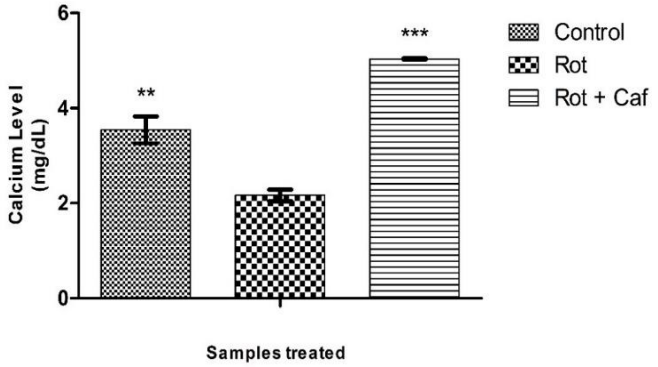

Figure 11. Calcium estimation along the X axis – samples treated and Y-axis - Calcium level (mg dL^{-1}). The results are compared with rotenone, and p <0.05 was accepted as significant.

Caffeine restores autophagy in rotenone treated cells: The degradation of long-lived proteins and dysfunctional organelles in eukaryotic cells is mediated through a catabolic process called Autophagy, impaired protein homeostasis and accumulation of damaged or abnormally modified proteins and loss of degradation pathways are common in Parkinson's disease [29].

From our flow cytometry results, it was evident that rotenone administration produced a notable decrease in autophagy induction when compared to untreated control samples. Caffeine was effective in restoring autophagy induction compared with the `untreated control samples (**Figure 12**). Previous studies have shown that potent autophagy enhancers routinely used in a laboratory setting, resulted in increased clearance of α-syn and neuroprotective effects, which were primarily related to increased autophagy [30].

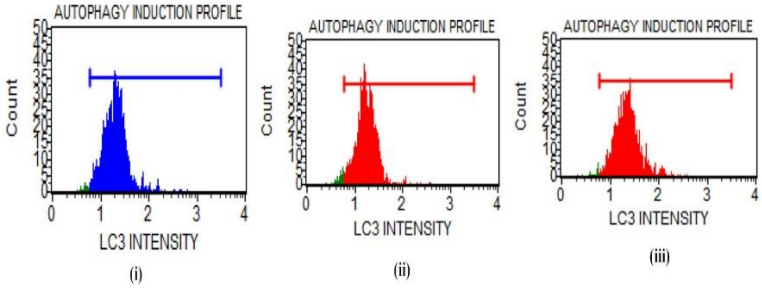

Figure 12. Representative images of autophagy analysis by LC3 flow cytometry **(i)** autophagy induction profile of untreated control **(ii)** autophagy induction profile of rotenone exposed cells **(iii)** autophagy induction profile of rotenone exposed cells co-administrated with caffeine

Conclusions and limitations

Effective treatment of neurodegenerative diseases remains one of the greatest challenges in modern medicine. Parkinson's disease is a life-threatening neurodegenerative disease that results from a progressive loss of nerve cells in the brain. In the present study the anti-Parkinson's effect of caffeine on experimental model IMR32 human neuroblastoma cells were determined. Rotenone was used to induce neurodegeneration. A non-toxic concentration of caffeine was determined by neutral red assay in L929 fibroblast cells. IMR32 cells co-administrated with caffeine and rotenone show significant changes in cell morphology and viability. Caffeine added at a concentration of 6.25µg mL^{-1} showed increased cell viability by about 88% with betterment in the cell and morphology was obtained. The anti-apoptotic activity of caffeine in rotenone-treated cells was confirmed with fluorescent staining and Caspase ELISA. FDA staining confirms neurite outgrowth and length in caffeine co-administrated with rotenone groups when compared with rotenone alone. A significant change in calcium homeostasis was achieved with caffeine treatment. The autophagic flux which was decreased with rotenone treatment was found to be

normalized with caffeine co-administration. The study showed the effect of caffeine on a fibroblast cell line and a neuroblastoma cell line and is a preliminary study on the effect of caffeine on a neuronal cell line. Detailed and thorough experimental analysis on more neuronal cell lines is required for an effective conclusion.

Graphical abstract

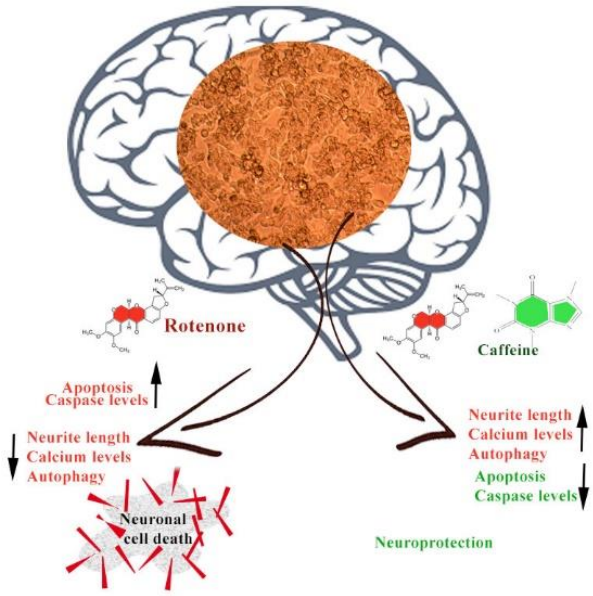

Figure 13. Figure depicted the cellular protective effect of caffeine on neuronal cells induced with rotenone toxicity

Author contributions

Geethu suresh and Swathi Surendran carried out the work, Swathi suresh wrote the manuscript under the guidance of Nithin Vijayakumar and Rajesh Ramachandran, Rajesh Ramachandran conceived the original idea, Rajesh Ramachandran supervised the project

Competing interests

The authors declare no competing interests.

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Data availability

Please contact the corresponding author for access to the data.

Ethical Approval

No ethical approval was required, sought or granted for this study.

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