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Experimental Research Article

Salsolinol toxicity in SH-SY5Y cells: PINK1/PARKIN and receptor-mediated mitophagy

Shi Hong Zhang[1], Chooi Ling Lim[2], Yong Qi Leong[3], Soi Moi Chye[2], Yin Yin Ooi[4,5], Anna Pick Kiong Ling[2], Kenny Gah Leong Voon[6], Rhun Yian Koh[2*]

1. School of Clinical Medicine, UNSW, Sydney, New South Wales, Australia
2. School of Health Sciences, IMU University, Bukit Jalil, Kuala Lumpur, Malaysia
3. School of Pharmacy, Monash University Malaysia, Subang Jaya, Selangor, Malaysia
4. School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, Subang Jaya, Selangor, Malaysia
5. Digital Health and Medical Advancements Impact Lab, Taylor's University, Subang Jaya, Selangor, Malaysia
6. School of Pharmacy, University of Nottingham, Semenyih, Selangor, Malaysia

ABSTRACT

Introduction: Parkinson's disease (PD) is commonly characterised by motor movement deterioration and cognitive impairment. Salsolinol, a dopamine-derived endogenous neurotoxin, may contribute to PD pathogenesis due to its structural and chemical similarity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). However, the precise molecular mechanisms of the neurotoxin remain unexplored. Hence, this study aimed to evaluate the toxic effects of salsolinol on SH-SY5Y neuronal cells, focusing on mitophagy and its associated pathways.

Methods: SH-SY5Y cells were exposed to salsolinol for toxicity assessment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and inhibitory concentrations (ICs) were determined for the following assays. Induction of autophagy and mitophagy in the salsolinol-treated SH-SY5Y cells was detected with acridine orange and Mtphagy Dye, respectively. Additionally, an enzyme-linked immunosorbent assay (ELISA) was performed to determine the protein levels of the PINK1/PARKIN-mediated mitophagy pathway and the receptor-mediated mitophagy pathway in salsolinol-treated SH-SY5Y cells.

Results: Results revealed that SH-SY5Y cells, when treated with salsolinol (0 to 400 µM) for 24 and 48 hours, significantly elicited dose-dependent neurotoxicity. The upregulation of autophagy and mitophagy coincided with increased levels of proteins related to the PINK1/PARKIN-mediated mitophagy pathway and the receptor-mediated mitophagy pathway when treated with IC_{50} and IC_{75} as compared to untreated (UT) and IC_{25}.

Conclusion: Our findings suggest that salsolinol induces mitophagy via the PINK1/PARKIN-mediated mitophagy and receptor-mediated mitophagy pathways.

Keywords:
Parkinson Disease
Salsolinol
Mitophagy
PINK1
PARKIN

* Corresponding author: Rhun Yian Koh , rhunyian_koh@imu.edu.my
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Introduction

Salsolinol, a derivative of tetrahydroisoquinoline, was initially discovered in the urine of PD patients undergoing l-dihydroxyphenylalanine (L-DOPA) treatment (Sandler et al., 1973). A notable elevation in salsolinol levels in both cerebrospinal fluid and urine has been seen in individuals diagnosed with PD who are undergoing L-DOPA treatment, as well as in those with chronic alcoholism (Sandler et al., 1973; Moser et al., 1992). Endogenously, regions with elevated dopamine production and turnover, such as the ventral midbrain and striatum, have higher concentrations of these substances. Exogenously, salsolinol has been found in fruits and vegetables such as bananas, lettuce, celery, and grapes (DeCuypere et al., 2008). The enzyme salsolinol synthase catalyses the formation of salsolinol through a reaction between dopamine and acetaldehyde or pyruvic acid. Salsolinol can also be generated through a non-enzymatic process known as the Pictet-Spengler reaction, which involves dopamine and acetaldehyde.

Despite various proposed hypotheses surrounding its physiological and pathophysiological roles, especially in the context of PD, much remains to be understood. The structural similarity of salsolinol to the neurotoxin MPTP has led to speculation about its potential role in the development of idiopathic PD, as MPTP, a synthetic compound used in industrial processes, is a known neurotoxin that induces Parkinsonism in both humans and animals (Langston et al., 1983). Studies have shown that salsolinol strongly decreases mitochondrial complex I activity, increases reactive oxygen species (ROS), induces apoptosis, and enhances the release of cytochrome c from the mitochondria (Morikawa et al., 1998; Wanpen et al., 2007; Takahashi et al., 1997; Akao et al., 1999, 2002; Maruyama et al., 2001; Storch et al., 2000). Maruyama et al. (1995) suggested that salsolinol and its derivatives can have both harmful and protective effects on neurons. They found that giving exogenous salsolinol reduced free radical formation and dopamine breakdown. However, salsolinol at certain doses increased hydroxyl radicals in the striatum and promoted free radical formation during autoxidation (Maruyama et al., 1995). The dual effects of salsolinol were further confirmed by Możdżeń et al. (2015). In rat hippocampus cell cultures, low concentrations of salsolinol reduced apoptosis, while high concentrations increased apoptosis (Możdżeń et al., 2015). However, the precise molecular mechanisms underlying salsolinol-induced neurotoxicity, particularly regarding mitophagy induction, remain poorly understood.

Coined by Lemasters in 2005, "mitophagy" refers to the targeted removal of excess or damaged mitochondria through autophagy (Kim et al., 2007), a critical process for maintaining a healthy mitochondrial population. Mitophagy selectively eliminates dysfunctional or depolarised mitochondria, including those generating harmful ROS (Scherz-Shouval et al., 2007). The two primary pathways in mitophagy are the PINK1/PARKIN-mediated pathway and the receptor-mediated pathway. Investigations into MPTP's impact on autophagy and mitophagy explored the roles of autophagy in MPTP-induced neurotoxicity *in vivo*. The study focused on mitochondria and α-synuclein aggregation, revealing that autophagy not only contributes to cell death but also engulfs mitochondria, a process known as mitophagy (Hung et al., 2014). PD is undeniably linked to mutations in the PINK1 and PARKIN genes, influencing mitophagy regulation (Chu 2019; Liu et al., 2019). In neurons, maintaining a delicate balance between mitochondrial production and degradation is crucial for overall cellular health. Insufficient mitochondrial production, coupled with excessive mitophagy, can lead to the depletion of mitochondria, resulting in cell death (Subramaniam 2020; Palikaras et al., 2014). While mitophagy is indispensable for cellular and organismal health, it can become detrimental in various pathological conditions, including mitochondrial disorders, ischemic stroke, chronic cerebral hypoperfusion, and diabetes (Shi et al., 2014; Sharma et al., 2019; Yu-Wai-Man et al., 2010; Zaninello et al., 2020). Uncontrolled elimination of mitochondria in these situations leads to the death of neuronal cells. These findings highlight the dual nature of mitophagy, acting as both a guardian of neuronal balance and viability and a potential contributor to neurodegeneration in specific pathological contexts.

The SH-SY5Y cell line, derived from a metastatic neuroblastoma biopsy obtained from a four-year-old female patient, has undergone clonal selection on three separate occasions (Biedler et al., 1978). The neuroblastoma cell line, which originated from the SK-N-SH cell line developed in 1970, is often used as a model in PD research due to its ability to produce both dopamine and norepinephrine (Xicoy et al., 2017; Xie et al., 2010).

Despite existing observations, the relationship be-

tween the neurotoxic effects of salsolinol and mitophagy has not been exhaustively investigated. This study seeks to clarify the influence of salsolinol on the SH-SY5Y cell line, specifically examining its neurotoxicity and its potential to induce mitophagy. Furthermore, the research explores the participation of the PINK1/PARKIN-mediated pathway and the receptor-mediated pathway in the mitophagy triggered by salsolinol.

Material and Methods

Cell Culture

SH-SY5Y (ATCC, USA), a popular cell model for PD research (Xie et al., 2010), was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics at 37°C in an atmosphere containing 5% carbon dioxide (CO_2). The compound (R/S)-salsolinol hydrobromide (Sigma Aldrich, UK) was provided in a powdered form and dissolved in water to prepare a solution of 5 mg/mL. With a molecular weight of 260.13 g/mol, it is represented by the chemical formula $C_{10}H_{13}NO_2 \cdot HBr$.

MTT Assay

The MTT assay assessed salsolinol toxicity and ICs on SH-SY5Y cells. Cells were seeded in poly-l-lysine-coated 96-well plates at 2.5×10^4 cells/well in 100 µL DMEM (10% FBS) and cultured for 24 hours. Salsolinol was prepared in six concentrations (0 µM, 25 µM, 50 µM, 100 µM, 200 µM, and 400 µM) via two-fold serial dilution. The medium was replaced with 100 µL salsolinol diluted with DMEM (1% FBS), with 0 µM as control, and incubated for 24 hours. Next, 20 µL of 5 mg/mL MTT solution (Bio Basic, Canada) was added to each well and incubated for four hours. The medium was discarded, and 100 µL dimethyl sulfoxide (DMSO) dissolved the formazan crystals. Absorbance was measured at 570 nm with a reference at 630 nm using the Infinite M Plex Microplate Reader (TECAN, Switzerland). The experiment was performed in triplicate and independently repeated three times. Statistical analysis determined IC_{25}, IC_{50}, and IC_{75} for the subsequent assays. The assay was repeated for 48 hours, as IC_{75} was not achieved in 24 hours. Cell viability percentage relative to the control was calculated as:

$$\text{Relative Cell Viability Percentage} = \frac{\text{Absorbance of samples at 570 nm with reference at 630 nm}}{\text{Absorbance of control at 570 nm with reference at 630 nm}} \times 100\%$$

Acridine Orange Staining

Acridine orange staining evaluated autophagy in salsolinol-treated SH-SY5Y cells. Cells were seeded in poly-l-lysine-coated 24-well plates at 6×10^4 cells/well in 400 µL DMEM (10% FBS) and cultured for 24 hours. Wells were treated with 400 µL salsolinol at IC_{25}, $IC_{50,}$ and IC_{75} diluted with DMEM (1% FBS), while untreated cells (UT) served as controls, and incubated for 48 hours. After incubation, the medium was removed, and wells were washed twice with 200 µL phosphate -buffered saline (PBS). Cells were fixed with 400µL 4% formaldehyde for 20 minutes, followed by two 200 µL PBS washes. Next, cells were stained with 400 µL of 5 µg/mL acridine orange diluted with PBS and incubated in the dark for 10 minutes. Green and red fluorescence were confirmed under the Eclipse Ti-U Inverted Fluorescence Microscope (Nikon, Japan) at 495 nm and 550 nm, respectively. Wells were washed twice with 200 µL PBS, and 200 µL PBS was added for imaging. Micrographs of green and red fluorescence were taken at 100 times magnification using NIS-Elements AR (Version 5.4, Japan). Images were merged into composite fields, and red and green vesicles were quantified using NIH ImageJ (Version 1.53, USA). Three fields per well were analysed, with three independent experiments performed. Statistical analysis generated a bar graph, and autophagosome to nuclei percentages relative to control were calculated as:

$$\text{Relative Autophagosomes to Nuclei Percentage} = \frac{\frac{\text{Number of red vesicles in sample}}{\text{Number of green vesicles in sample}}}{\frac{\text{Number of red vesicles in control}}{\text{Number of green vesicles in control}}} \times 100\%$$

Mtphagy Dye Staining

Mtphagy Dye and Lyso Dye evaluated mitophagy in salsolinol-treated SH-SY5Y cells. Cells were seeded in poly-l-lysine-coated 24-well plates at 6×10^4 cells/well in 400 µL DMEM (10% FBS) and cultured for 24 hours. Wells were washed twice with 200 µL serum-free DMEM before adding 400 µL of 200 nmol/L Mtphagy Dye (Dojindo Molecular Technologies, Japan), diluted with serum-free DMEM. After a 30-minute incubation, the supernatant was discarded, and wells were washed twice with 200 µL serum-free DMEM. Wells were treated with 400 µL salsolinol at IC_{25}, IC_{50} and IC_{75} diluted with DMEM (1% FBS), with untreated cells (UT) serving as controls, and incubated for 48 hours. After incuba-

tion, 400 µL of 2 µmol/L Lyso Dye (Dojindo Molecular Technologies, Japan), diluted with serum-free DMEM was added and incubated for 30 minutes to observe the co-localisation of Mtphagy Dye and lysosomes. The supernatant was discarded after confirming green and red fluorescence under the Eclipse Ti-U inverted fluorescence microscope with wavelengths of 495 nm and 550 nm, respectively. Wells were washed twice with 200 µL serum-free DMEM, fixed with 400 µL of 4% formaldehyde for 20 minutes, and washed twice again with 200 µL serum-free DMEM. Finally, 200 µL serum-free DMEM was added for imaging. Micrographs of green and red fluorescence were taken at 100 times magnification using NIS-Elements AR. Images were merged into composite fields, and red and green vesicles were quantified using NIH ImageJ. Three fields per well were analysed, with three independent experiments performed. Statistical analysis generated a bar graph, and damaged mitochondria to lysosomes percentage relative to control was calculated as:

$$\text{Relative Damaged Mitochondria to Lysosomes Percentage} = \frac{\frac{\text{Number of red vesicles in sample}}{\text{Number of green vesicles in sample}}}{\frac{\text{Number of red vesicles in control}}{\text{Number of green vesicles in control}}} \times 100\%$$

ELISA

ELISA detected mitophagy protein levels in salsolinol-treated SH-SY5Y cells. SH-SY5Y cells were seeded in two poly-l-lysine-coated 96-well plates at 2.5×10^4 cells/well in 100 µL DMEM (10% FBS) and cultured for 24 hours. One plate was designated for MTT assay, and the other for ELISA. After 24 hours, wells were treated with 100 µL salsolinol at IC_{25}, $IC_{50,}$ and IC_{75} diluted with DMEM (1% FBS), with UT serving as controls, and incubated for 48 hours. For the MTT plate, 20 µL of 5 mg/mL MTT was added to each well and incubated for four hours. The medium was discarded, and 100 µL DMSO was used to dissolve formazan crystals. Absorbance was measured at 570 nm with a reference at 630 nm using the Infinite M Plex Microplate Reader. For the ELISA plate, cells were fixed with 100 µL ice-cold methanol at -20°C and incubated at 4°C with agitation for 20 minutes. Wells were washed thrice with 100 µL Tris-buffered saline with 0.1% Tween ® 20 detergent (TBST) for five minutes per wash. Next, wells were treated with 100 µL of 0.6% hydrogen peroxide (H_2O_2) diluted with Tris-buffered saline (TBS) for 30 minutes in the dark with agitation, followed by three similar TBST washes. Wells were blocked with 100 µL of 3% bovine serum albumin (BSA) diluted with TBS for one hour with agitation, then incubated with 100 µL of 0.1% primary antibodies (Cell Signaling Technology, USA) diluted with BSA overnight at 4°C on a belly dancer. After three similar TBST washes, wells were incubated for one hour with agitation with 100 µL of 0.1% secondary horseradish peroxidase-linked antibody (Cell Signaling Technology, USA) diluted with BSA. Table 1 lists the primary and secondary antibodies used in ELISA, all purchased from Cell Signaling Technology. After three similar TBST washes, 75 µL 3,3',5,5'-tetramethylbenzidine (TMB) solution was added for blue color development, followed by 75 µL of 500 mM sulfuric acid (H_2SO_4). Absorbance was measured at 490 nm using the Infinite M Plex Microplate Reader. Triplicates and three independent experiments were performed for each protein. Statistical analysis generated bar graphs, and protein level percentage relative to control was calculated as:

$$\text{Relative Protein Level Percentage} = \frac{\frac{\text{Absorbance of samples at 490 nm (ELISA)}}{\text{Absorbance of samples at 570 nm with reference at 630 nm (MTT)}}}{\frac{\text{Absorbance of control at 490 nm (ELISA)}}{\text{Absorbance of control at 570 nm with reference at 630 nm (MTT)}}} \times 100\%$$

Statistical Analysis

Normality of data distribution was assessed using the Shapiro–Wilk test. As the assumptions of normality and homogeneity of variance were met, measurements obtained from triplicates and three independent experiments were analysed using one-way ANOVA, followed by Tukey's Honest Significant Difference post-hoc test, employing IBM SPSS (Version 29.0, USA). Statistical significance was set at $p < 0.05$ (*) in comparison with the negative control. The results are presented as mean ± standard deviation in either a line graph or bar graph, created using Microsoft Excel (Version 16.78, USA).

Results

Toxicity of salsolinol on SH-SY5Y cells and the inhibitory concentrations (ICs)

In a 24-hour timeframe (Figure 1a.), it is evident that as the concentration of salsolinol increased from 0 µM to 400 µM, a corresponding decrease in the viability was observed in SH-SY5Y cells, and this difference was statistically significant ($p < 0.05$), indicating dose-dependent toxicity of salsolinol on SH-SY5Y cells. It's worth noting that IC_{25} and IC_{50} values were successfully determined, although IC_{75} remained undetermined (Table 2.).

TABLE 1: Details of primary antibodies and secondary antibodies used in ELISA

Protein	Product	Quantity	Mol. Wgt. (kDa)	Isotype
Primary Antibodies				
SQSTM1/P62 (D5E2) Rabbit mAB	8025	20 µl	62	Rabbit IgG
NDP52 (D1E4A) Rabbit mAB	60732	20 µl	52,60	Rabbit IgG
OPTINEURIN (D2L8S) Rabbit mAB	58981	20 µl	75	Rabbit IgG
PARKIN (PRK8) Mouse mAB	4211	20 µl	50	Rabbit IgG
PINK1 (D8G3) Rabbit mAB	6946	20 µl	60,50	Rabbit IgG
BNIP3 (D7U1T) Rabbit mAB	12396	20 µl	22-28, 50-55	Rabbit IgG
LC3B (D11) Rabbit mAB	3868	20 µl	38,76	Rabbit IgG
PHOSPHO-UBIQUITIN (SER65) (E2J6T) Rabbit mAB	62802	20 µl	14,16	Rabbit IgG
Secondary Antibodies				
Anti-Rabbit IgG HRP-Linked	7074			Goat
Anti-Mouse IgG HRP-Linked	7076			Horse

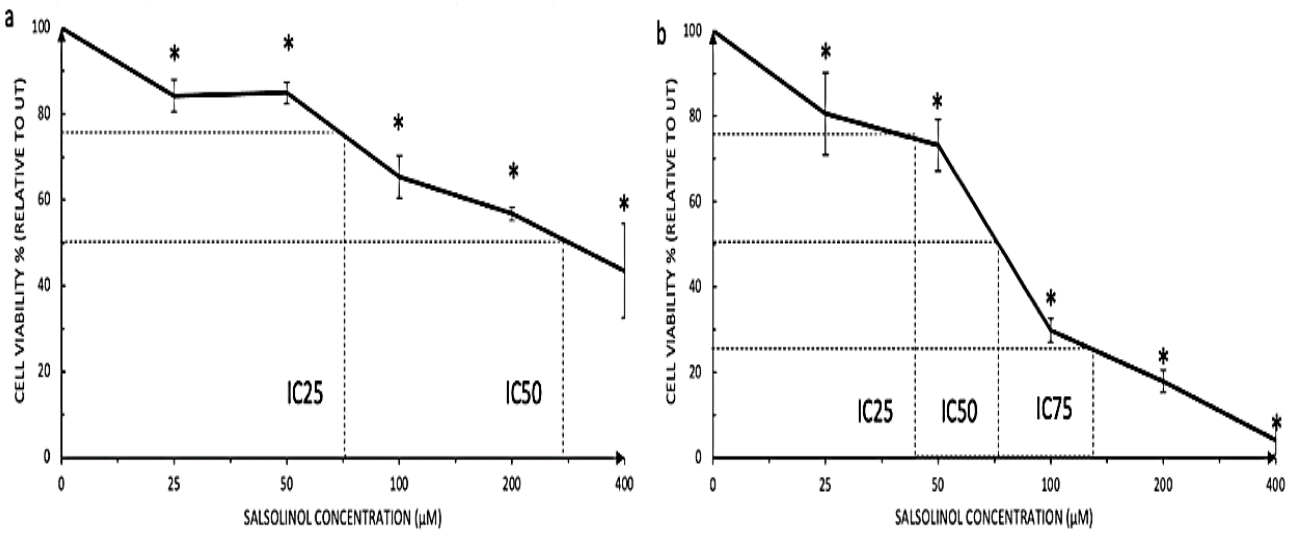

FIGURE 1. Viability of salsolinol-treated SH-SY5Y cells. a Results in 24 hours. b Results in 48 hours. SH-SY5Y cells were treated with salsolinol at increasing concentrations from 0 to 400 µM for 24 hours. Cell viability was detected by using an MTT assay. Data were expressed as mean ± standard deviation of three independent experiments. IC_{25}, IC_{50} and IC_{75} were plotted. * indicates a significant difference in comparison to control (0 µM) with $p < 0.05$. IC, Inhibitory Concentration.

TABLE 2: ICs of salsolinol on SH-SY5Y cells in 24 hours and 48 hours. Data were expressed as mean ± standard deviation of three independent experiments. IC, Inhibitory Concentration.

IC	Values in 24 Hours (µM)	Values in 48 Hours (µM)
25	76.22 ± 9.98	47.83 ± 8.63
50	279.71 ± 42.21	76.50 ± 2.95
75	Undetermined	139.55 ± 22.88

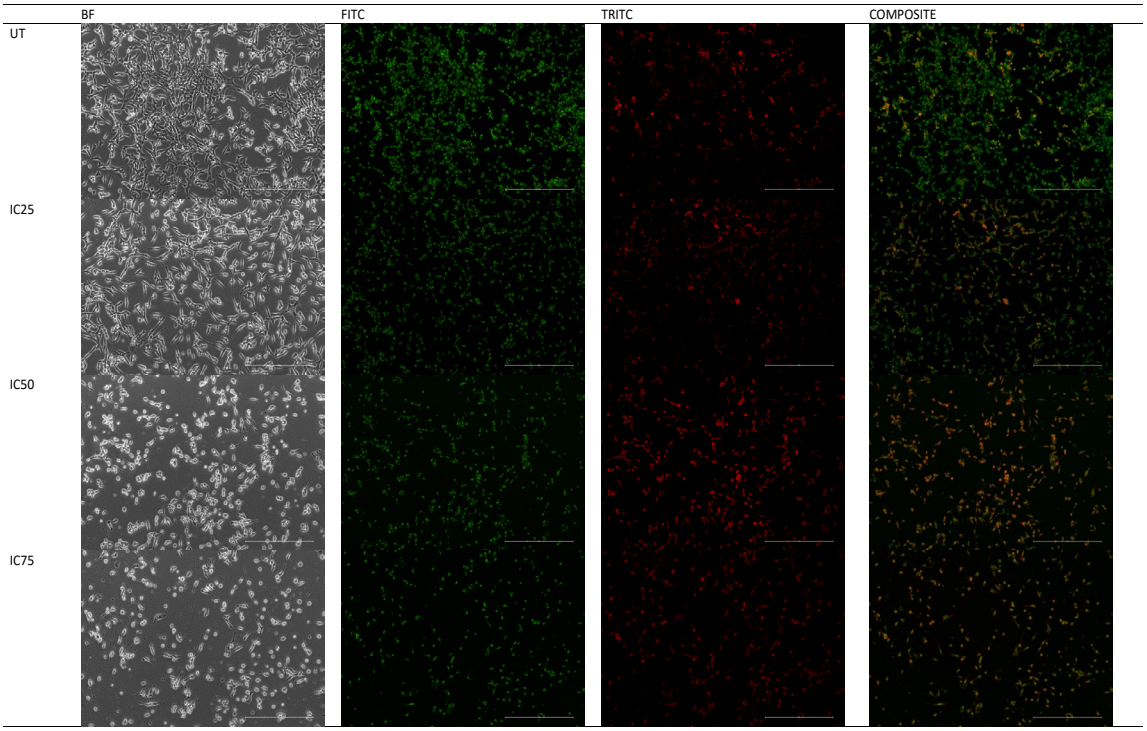

FIGURE 2. Induction of autophagy in salsolinol-treated SH-SY5Y cells in 48 hours. SH-SY5Y cells were treated with salsolinol at UT, IC_{25}, IC_{50} and IC_{75} for 48 hours. Red vesicles and green vesicles were detected by using acridine orange staining. Brightfield, green fluorescence, red fluorescence, and composite are shown. Pictures are taken under 100 times magnification using an inverted fluorescence microscope. IC, Inhibitory Concentration. UT, Untreated.

In the context of a 48-hour period (Figure 1b.), a similar pattern emerged, as the escalating concentration of salsolinol from 0 µM to 400 µM led to a downtrend in the viability of SH-SY5Y cells, and this difference was statistically significant ($p < 0.05$), indicating dose-dependent toxicity of salsolinol on SH-SY5Y cells. Remarkably, IC_{25}, IC_{50}, and IC_{75} values were successfully ascertained within the 48-hour timeframe (Table 2.).

Induction of autophagy in salsolinol-treated SH-SY5Y cells

The examination of the brightfield under the inverted fluorescence microscope (Figure 2.) unveiled a progressive decline in the cell count and a deterioration in neuronal morphology, transitioning from UT to IC_{25}, then to IC_{50}, and finally to IC_{75}, indicating dose-dependent toxicity of salsolinol on SH-SY5Y cells from the qualitative aspect.

In a 48-hour treatment of SH-SY5Y cells with salsolinol (Figure 3.), a notable upward trend in the autophagosomes/nuclei percentage was evident, indicating the upregulation of autophagy in salsolinol-treated SH-SY5Y cells. Only IC_{50} and IC_{75} values exhibited significant differences when compared to UT and IC_{25} ($p < 0.05$). Conversely, IC_{25} did not exhibit a significant difference from UT ($p > 0.05$).

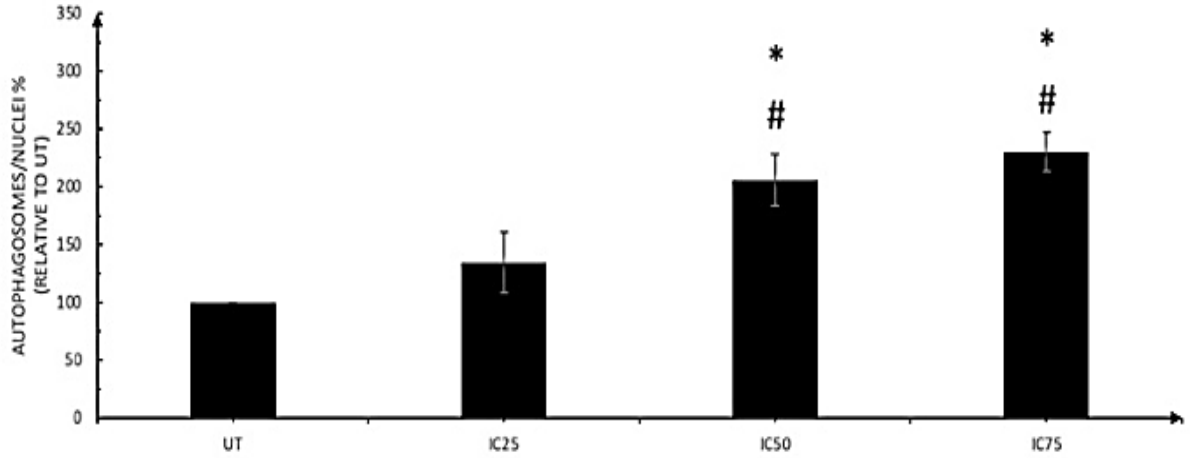

FIGURE 3. Induction of autophagy in salsolinol-treated SH-SY5Y cells in 48 hours. SH-SY5Y cells were treated with salsolinol at UT, IC_{25}, IC_{50} and IC_{75} for 48 hours. Autophagosomes and nuclei were detected by using acridine orange staining. Data were expressed as mean ± standard deviation of three independent experiments. * indicates a significant difference in comparison to control (UT) with $p < 0.05$. # indicates a significant difference in comparison to IC_{25} with $p < 0.05$. IC, Inhibitory Concentration. UT, Untreated.

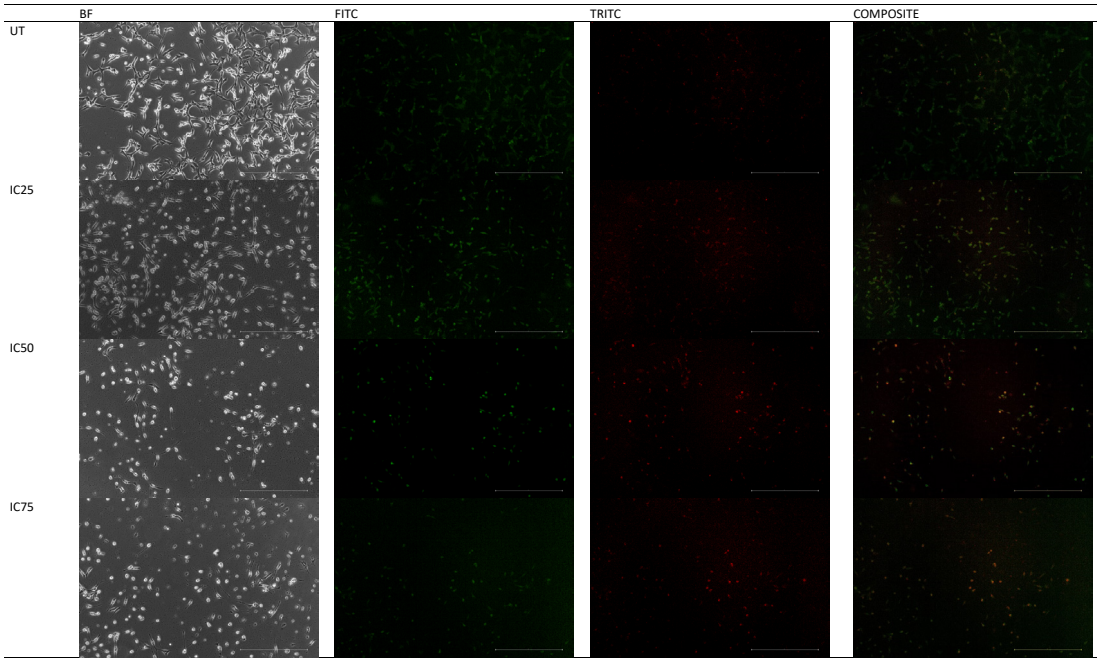

FIGURE 4. Induction of mitophagy in salsolinol-treated SH-SY5Y cells in 48 hours. SH-SY5Y cells were treated with salsolinol at UT, IC_{25}, IC_{50} and IC_{75} for 48 hours. Red vesicles and green vesicles were detected by using Mtphagy Dye. Brightfield, green fluorescence, red fluorescence, and composite are shown. Pictures are taken under 100 times magnification using an inverted fluorescence microscope. IC, Inhibitory Concentration. UT, Untreated.

Induction of mitophagy in salsolinol-treated SH-SY5Y cells

When examining the brightfield images using the inverted fluorescence microscope (Figure 4.), a gradual decrease in the number of cells and a deterioration in neuronal morphology became apparent moving from UT to IC_{25}, followed by IC_{50}, and ultimately to IC_{75},

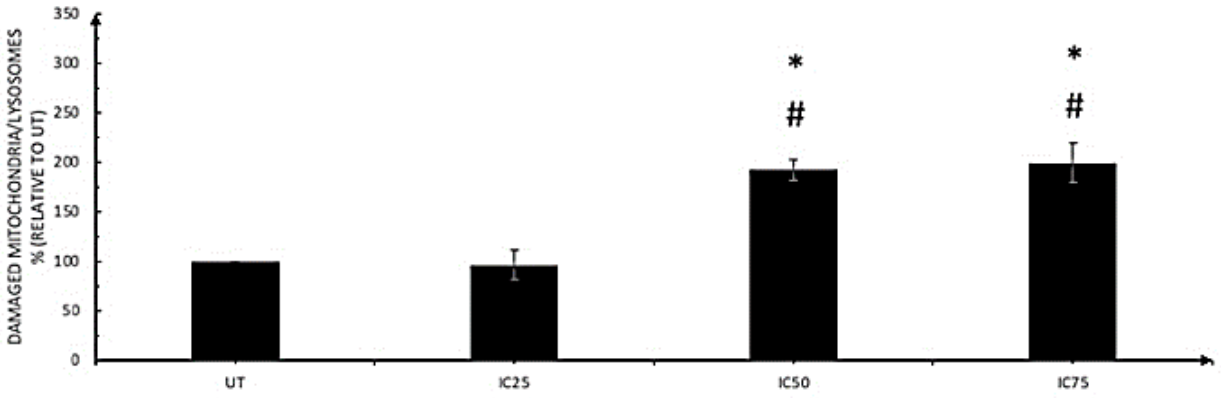

FIGURE 5. Induction of mitophagy in salsolinol-treated SH-SY5Y cells in 48 hours. SH-SY5Y cells were treated with salsolinol at UT, IC_{25}, IC_{50} and IC_{75} for 48 hours. Damaged mitochondria and lysosomes were detected by using Mtphagy Dye. Data were expressed as mean ± standard deviation of three independent experiments. * indicates a significant difference in comparison to control (UT) with $p < 0.05$. # indicates a significant difference in comparison to IC_{25} with $p < 0.05$. IC, Inhibitory Concentration. UT, Untreated.

indicating dose-dependent toxicity of salsolinol on SH-SY5Y cells from the qualitative aspect.

In a 48-hour treatment of SH-SY5Y cells with salsolinol (Figure 5.), an upward trend in the damaged mitochondria/lysosomes percentage was observed, indicating the upregulation of mitophagy in salsolinol-treated SH-SY5Y cells. Only the IC_{50} and IC_{75} values displayed significant differences when compared to UT and IC_{25} ($p < 0.05$). Conversely, the IC_{25} value did not exhibit a significant difference from UT ($p > 0.05$).

Protein levels in PINK1/PARKIN-Mediated Mitophagy Pathway (Upstream)

In a 48-hour treatment of SH-SY5Y cells with salsolinol, a noticeable upward trend in the protein levels of PINK1 (Figure 6a.), Ser65 (Figure 6b.), and PARKIN (Figure 6c.) was evident. Significant differences in the protein levels of PINK1, Ser65, and PARKIN were only observed with IC_{50} and IC_{75} when compared to UT and IC_{25} ($p < 0.05$). In contrast, IC_{25} did not exhibit any significant difference from UT ($p > 0.05$).

Protein levels in SH-SY5Y cells PINK1/PARKIN-Mediated Mitophagy Pathway (Downstream)

During a 48-hour treatment of SH-SY5Y cells with salsolinol, a distinct upward trend in the protein levels of OPTINEURIN (Figure 7a.), NDP52 (Figure 7b.), p62 (Figure 7c.), and LC3B (Figure 7d.) became evident. Significant differences in the protein levels of these proteins were only observed with IC_{50} and IC_{75} when compared to UT and IC_{25} ($p < 0.05$). In contrast, IC_{25} did not manifest any significant difference from UT ($p > 0.05$).

Protein levels in Receptor-Mediated Mitophagy

During a 48-hour treatment of SH-SY5Y cells with salsolinol, a clear increment in the protein levels of BNIP3 (Figure 8a.) and NIX (Figure 8b.) became evident. Significant differences in the protein levels of these proteins were only observed with IC_{50} and IC_{75} in comparison to UT and IC_{25} ($p < 0.05$). Conversely, IC_{25} did not exhibit any significant difference from UT ($p > 0.05$).

Discussion

The cytotoxicity assay findings demonstrated a dose-dependent increase in the toxicity of salsolinol on SH-SY5Y cells. A rise in salsolinol concentration from 0 μM to 400 μM in 24-hour and 48-hour experiments corresponded to a proportional decrease in cellular viability. This aligns with prior studies that have already substantiated salsolinol's toxicity on SH-SY5Y cells by affecting mitochondrial complex I activity, increasing reactive oxygen species (ROS), inducing apoptosis, and enhancing the release of cytochrome c from the mitochondria (Morikawa et al., 1998; Wanpen et al., 2007; Takahashi et al., 1997; Akao et al., 1999, 2002; Maruyama et al., 2001; Storch et al., 2000). Phase contrast microscopy revealed a sequential decline in cell count, accompanied by

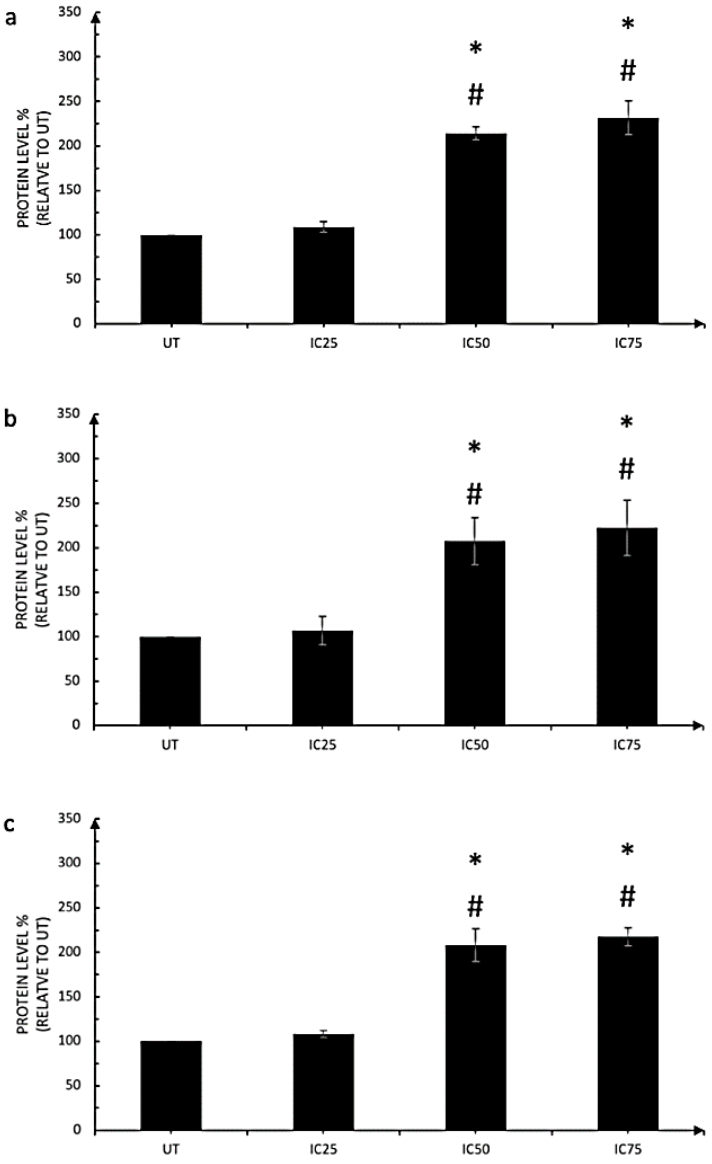

FIGURE 6. Protein level in salsolinol-treated SH-SY5Y cells in 48 hours. a PINK1 protein level. b Ser65 protein level. c PARKIN protein level. SH-SY5Y cells were treated with salsolinol at UT, IC_{25}, IC_{50} and IC_{75} for 48 hours. Protein levels were detected by using ELISA. Data were expressed as mean ± standard deviation of three independent experiments. * over data point indicates a significant difference in comparison to control (UT) with p value less than 0.05. # over data point indicates a significant difference in comparison to IC_{25} with p value less than 0.05. IC, Inhibitory Concentration. UT, Untreated.

a noticeable deterioration in neuronal morphology. This deterioration became increasingly pronounced from the UT group to the IC_{25}, IC_{50}, and finally the IC_{75} treatment groups. These qualitative observations provide further evidence of salsolinol's toxicity on SH-SY5Y cells, supporting the conclusions from a qualitative perspective.

This study provides evidence of the significant upregulation of autophagy and mitophagy in salsolinol-treated SH-SY5Y cells when compared to UT and IC_{25}, observed upon treatment with IC_{50} and IC_{75} concentrations. The current study is the first to explore the connection between salsolinol and autophagy or mitophagy. As indicated in the introduction, Hung *et al.*'s study (2014) revealed the role of autophagy and mitophagy in MPTP-induced neurotoxicity in an *in vivo* setting. Drawing parallels to this current research, it's important to note the structural and chemical similarities between salsolinol and MPTP. Notably, the N-methyl group has

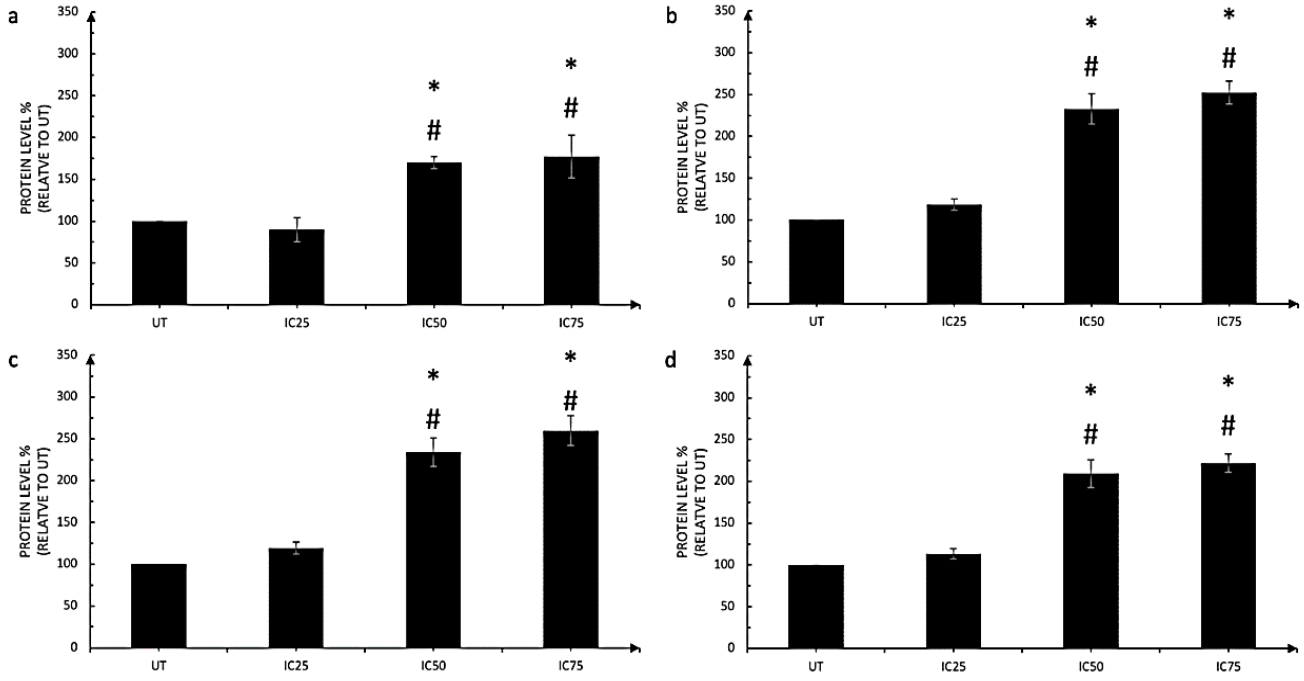

FIGURE 7. Protein level in salsolinol-treated SH-SY5Y cells in 48 hours. **a** OPTINEURIN protein level. **b** NDP52 protein level. **c** p62 protein level. **d** LC3B protein level. SH-SY5Y cells were treated with salsolinol at UT, IC_{25}, IC_{50} and IC_{75} for 48 hours. Protein levels were detected by using ELISA. Data were expressed as mean ± standard deviation of three independent experiments. * indicates a significant difference in comparison to control (UT) with $p < 0.05$. # indicates a significant difference in comparison to IC_{25} with $p < 0.05$. IC, Inhibitory Concentration. UT, Untreated.

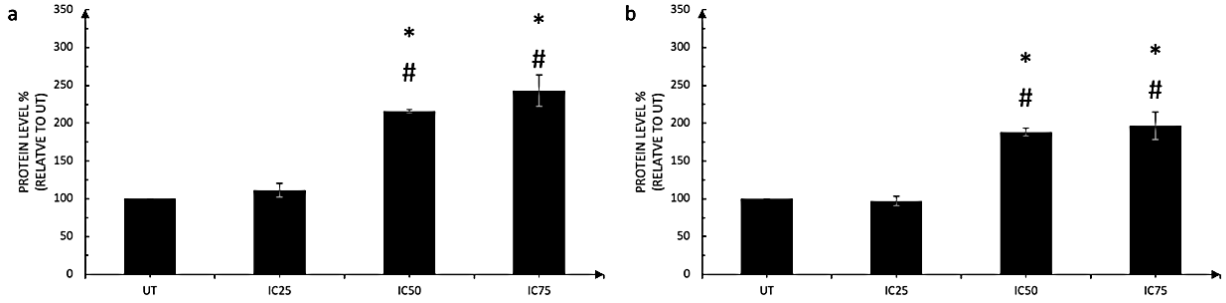

FIGURE 8. Protein level in salsolinol-treated SH-SY5Y cells in 48 hours. **a** BNIP3 protein level. **b** NIX protein level. SH-SY5Y cells were treated with salsolinol at UT, IC25, IC50 and IC75 for 48 hours. Protein levels were detected by using ELISA. Data were expressed as mean ± standard deviation of three independent experiments. * indicates a significant difference in comparison to control (UT) with $p < 0.05$. # indicates a significant difference in comparison to IC25 with $p < 0.05$. IC, Inhibitory Concentration. UT, Untreated.

been identified as a critical factor in MPTP's toxicity (Javitch et al., 1985), and interestingly, salsolinol possesses a similar N-methyl group, a characteristic attributed to N-methyltransferase. These shared features suggest that salsolinol, given its structural and chemical resemblance to MPTP, could induce autophagy and mitophagy, akin to MPTP.

The mitophagy pathway under investigation involves a series of proteins, starting with the upstream components associated with the PINK1/PARKIN-mediated mitophagy pathway, which include PINK1, Ser65, and PARKIN (Figure 9a.). In the 48-hour treatment of SH-SY5Y cells with salsolinol, significant upregulation was noted in the levels of these proteins in those treated with IC_{50} and IC_{75} concentrations when compared to UT and IC_{25}. PINK1, typically subjected to cleavage by mito-

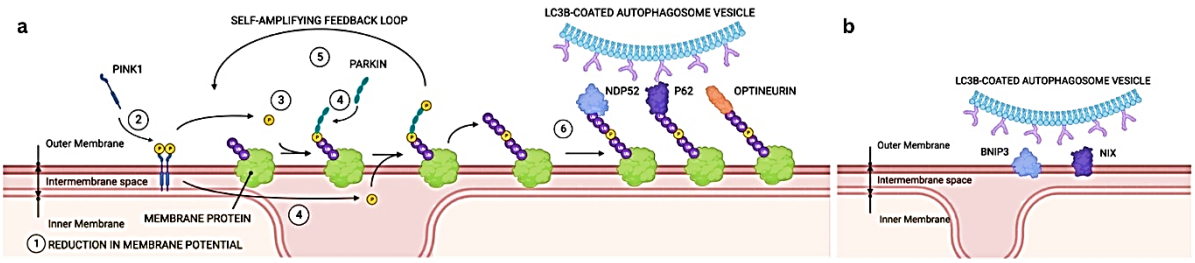

FIGURE 9. Primary mechanism of mitophagy pathways. a PINK1/PARKIN-dependent pathway. (1) Reduction in the mitochondrial membrane potential (2) PINK1 stabilises at the outer mitochondrial membrane (OMM) where it dimerises and autophosphorylates (3) Activated PINK1 phosphorylates ubiquitin chain at Ser65 on OMM protein (4) Phosphorylated ubiquitin chains allow the recruitment of PARKIN from cytoplasm to OMM and PARKIN is phosphorylated at Ser65 by PINK1 and becomes fully activated (5) Activated PARKIN ubiquitinates OMM proteins generating a self-amplifying feedback loop with PINK1 (6) Proteins such as NDP52 or OPTINEURIN or p62 bring together ubiquitin chains with LC3-coated vesicles b Receptor-mediated pathway. The linking of mitochondria to autophagosome is achieved in the absence of PARKIN, with the help of BNIP3 and NIX.

chondrial proteases and degradation by proteasomes under normal conditions (Jin et al., 2010; Yamano et al., 2013; Okatsu et al., 2012), accumulates on the outer mitochondrial membrane (OMM) in response to mitochondrial stress or damage (Okatsu et al., 2012). The observed upregulation of PINK1 in salsolinol-treated SH-SY5Y cells implies that salsolinol-induced mitochondrial stress leads to the accumulation of PINK1. Furthermore, Ser65, phosphorylatable by PINK1, plays a pivotal role in recruiting PARKIN from the cytoplasm to the mitochondria. Specifically, the phosphorylated Ser65 activates PARKIN and anchors it to the mitochondria (Wauer et al., 2015; Okatsu et al., 2015). The significant upregulation of both Ser65 and PARKIN in salsolinol-treated SH-SY5Y cells aligns with the expectations, as the increased PINK1 levels can phosphorylate Ser65 to facilitate PARKIN recruitment. Hence, the significant upregulation of PINK1, Ser65, and PARKIN provides evidence of the upstream activation of the PINK1/PARKIN-mediated mitophagy pathway.

Yet, the downstream components of this pathway cannot be overlooked. Here, proteins such as OPTINEURIN, NDP52, p62, and LC3B come into play (Figure 9a.). During the 48-hour salsolinol treatment of SH-SY5Y cells, these proteins in the IC_{50} and IC_{75} treatment groups displayed significant upregulation compared to UT and IC_{25}. OPTINEURIN, NDP52, and p62, functioning as autophagy receptors, recognise substrates that have been ubiquitinated by active PARKIN and serve as degradation signals, subsequently being recruited to damaged mitochondria (Kazlauskaite et al., 2015; Lazarou et al., 2015; Shin et al., 2020). The heightened levels of these proteins make sense, as activated PARKIN from the upstream can effectively recruit them to the mitochondria. LC3B, which forms the coat of autophagosomes, can be recruited by OPTINEURIN, NDP52, and p62 to aid in the formation of autophagosomes around the mitochondria (Lazarou et al., 2015; Shin et al., 2020). The upregulation of LC3B, in this context, is entirely consistent, as the elevated levels of OPTINEURIN, NDP52, and p62 are expected to recruit LC3B for autophagosome formation. Hence, the marked upregulation of OPTINEURIN, NDP52, p62, and LC3B constitutes evidence for the downstream activation of the PINK1/PARKIN-mediated mitophagy pathway.

However, the involvement of the receptor-mediated mitophagy pathway must not be neglected. This pathway relies on proteins such as BNIP3 and NIX, both of which are OMM proteins equipped with LC3-Interacting Region (Figure 9b.). They possess the ability to bind to LC3B, effectively inducing mitophagy, even in the absence of PARKIN (Liu et al., 2014; Novak et al., 2010; Naeem et al., 2020). This research has revealed a significant upregulation of BNIP3 and NIX during the 48-hour salsolinol treatment of SH-SY5Y cells in the IC_{50} and IC_{75} treatment groups when compared to UT and IC_{25}. This observed upregulation provides evidence of the activation of the receptor-mediated mitophagy pathway.

In the investigation of autophagy, mitophagy, and pro-

tein levels, a noteworthy contrast was observed between the IC_{25} treatment group and both the UT and IC_{50}/ IC_{75} groups. While IC_{50} and IC_{75} exhibited significant upregulations in comparison to UT, IC_{25} did not display significant differences. This discrepancy raises a question on whether salsolinol at IC_{25} induces toxicity in SH-SY5Y cells without concurrent autophagy/mitophagy induction or altered protein levels compared to UT. One plausible explanation for this inconsistency lies in the stress levels imposed on the cells. When exposed to mild stress, as in the case of IC_{25}, it appears to trigger a response aimed at promoting normal mitochondrial biogenesis (Lee et al. 2002; Miranda et al., 1999). In this scenario, autophagy and mitophagy might play a neuroprotective role by eliminating compromised mitochondria (Zhu et al., 2007, 2012; Tzeng et al., 2010; Singer et al., 1990). Conversely, under more severe stress conditions, such as those induced by IC_{50} or IC_{75}, the heightened autophagy and mitophagy activities could potentially become excessive, resulting in impaired mitochondrial biogenesis (Zhu et al., 2012). This excessive degradation of mitochondria may lead to an insufficient supply of intact mitochondria, ultimately culminating in cell death in the salsolinol-affected SH-SY5Y cells.

Having shown that salsolinol triggers mitophagy, its relevance to PD lies in the balance between mitochondrial biogenesis and degradation. As indicated in the introduction, although PD is often linked to impaired mitophagy due to PINK1 and PARKIN mutations (Chu 2019; Liu et al., 2019), excessive mitophagy can be equally detrimental by depleting mitochondria and leading to cell death (Subramaniam 2020; Palikaras et al., 2014; Shi et al., 2014; Sharma et al., 2019; Yu-Wai-Man et al., 2010; Zaninello et al., 2020). The upregulation of mitophagy observed in dopaminergic SH-SY5Y cells therefore provides insight into how salsolinol may contribute to PD pathogenesis.

While salsolinol's toxicity on SH-SY5Y cells and its ability to induce autophagy and mitophagy through both PINK1/PARKIN-mediated and receptor-mediated pathways have been established, several doubts remain. Salsolinol's effects vary with dose, stereoisomer, route of administration, and species, which may influence its blood–brain barrier permeability (Origitano et al., 1981; Székács et al., 2007; Quintanilla et al., 2014; Lorenc-Koci et al., 2008) and determine whether it acts as neurotoxic or neuroprotective (Maruyama et al., 1995; Możdżeń et al., 2015; Kurnik-Łucka et al., 2018). The debate regarding endogenous versus exogenous levels further complicates extrapolation of in vitro and in vivo findings to clinical relevance (Lee et al., 2010; Bettiol et al., 2015; Ward et al., 2008). In addition, whether salsolinol exerts toxicity through mechanisms beyond autophagy or mitophagy, or activates alternative mitophagy pathways, remains unresolved. Finally, while SH-SY5Y cells provide a useful proliferative model, supplementing SH-SY5Y investigations with other cell types may overcome limitations such as its weak ability to represent actual neurons (Xicoy et al., 2017).

Conclusion

Our study set out to investigate whether salsolinol induces mitophagy in SH-SY5Y neuronal cells and to elucidate the specific pathways involved. Our findings support the hypothesis that salsolinol exerts neurotoxic effects through the induction of mitophagy. Specifically, treatment with salsolinol at IC_{50} and IC_{75} concentrations significantly increased markers of both PINK1/PARKIN-mediated and receptor-mediated mitophagy pathways, as confirmed by ELISA. The dose-dependent rise in autophagic and mitophagic activity, along with reduced cell viability, underscores the capacity of salsolinol to trigger selective mitochondrial degradation. These results suggest that dysregulation of mitophagy may be a mechanism by which salsolinol could contribute to dopaminergic neuron vulnerability, which may be relevant to PD, offering insights into its pathogenesis and potential molecular targets for therapeutic intervention.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

All studies were approved by the Joint Committee on Research and Ethics of IMU University. This article does not contain any studies with human participants or animals performed by any of the authors.

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