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Original Article

Neurotoxicity of salsolinol through apoptosis induction and oxidative stress in BV2 microglial cells

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ABSTRACT

Salsolinol (SAL) is an endogenous neurotoxin identified as a potential aetiological factor in Parkinson's disease (PD). Its neurotoxicity through apoptosis induction and oxidative stress have been extensively studied in dopaminergic cell lines; however the influence of SAL on other cell types have yet to be explored. As microglia hold integral role in neuroprotection and maintenance of brain homeostasis, the present study aimed to investigate the apoptotic effects of SAL on microglial cells. The influence of SAL on cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry was applied to observe the distribution of cell cycle phases following SAL treatment. Caspase assays were used to detect changes in caspase activities, whereas the production of reactive oxygen species (ROS) in BV2 cells was measured using 2',7'-dihydrodichlorofluorescein diacetate (DCFH-DA) assay. SAL was demonstrated to exert a dose- and time-dependent cytotoxicity on BV2 cells. Significant increase in subG_1 population implicated that the cells were undergoing apoptotic cell death. SAL also induced activation of multiple caspases in BV2 cells, such as caspase-3/7, -8 and -9. Interestingly, ROS assay revealed that SAL at lower concentrations significantly reduced intracellular ROS levels; however a sudden surge in ROS production was observed when the cells were exposed to high-dose SAL. These findings suggest that SAL could be neuroprotective at low concentrations; however its accumulation could induce significant apoptotic deaths and oxidative stress. The neurotoxicity of SAL not only affects dopaminergic neurons, but it can also be extended to the microglia population. This provides new insights on the role of microglia in PD pathogenesis.

KEYWORDS: salsolinol, Parkinson's disease, microglia, apoptosis, caspase activation, oxidative stress.

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, especially among aged individuals [1]. It is typically characterised as extensive loss of dopaminergic neurons in the substantia nigra, affecting various dopamine-mediated pathways such as nigrostriatal, mesolimbic, and cholinergic pathways, as a result of severe neurotransmitter depletion [2]. Apart from the typical motor-related symptoms, non-motor symptoms ranging from neuropsychiatric,

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gastrointestinal and autonomic symptoms have also been reported in PD cases and some may even precede disease diagnosis [3]. Although several key processes in PD have been identified, there is still a lack of disease-modifying treatment for PD due to incomplete understanding of its complex pathophysiology [4-7]. Majority of PD cases were sporadic in nature, with reported heterogeneity in disease phenotypes due to their multifactorial origin.

The progressive nature of PD suggests involvement of endogenous neurotoxin in its pathophysiology, such as salsolinol (SAL), that could accumulate over a long period and gradually induce changes in the brain environment. Therefore, the symptoms may not be apparent at initial stage of the disease. SAL is a naturally occurring tetrahydroisoquinoline which results from condensation between dopamine and acetaldehyde. It is predominantly found in brain regions with high dopamine turnover, such as striatum, substantia nigra and frontal cortex [8-11]. Its physiological functions include modulation of neurotransmitters by regulating catecholamines levels and the release of pituitary hormones [12, 13].

Numerous studies performed on dopaminergic cell lines revealed that SAL exerts dose-dependent toxicity, suggesting that the perturbation in physiologic SAL metabolism can lead to accumulation and have damaging effects to neuronal survival [14, 15]. It had been found in elevated concentrations among PD patients, including those who are at early stage of disease [16-19]. Several factors such as dysregulated dopamine metabolism, increased enzymatic formation, chronic alcoholism, and alpha-synuclein pathology were suspected to be the cause of accumulation [16, 20, 21]. SAL also demonstrated commonalities with disease mechanisms in PD, including its selective toxicity towards dopaminergic cells, mitochondrial impairment, caspase-dependent apoptosis induction and oxidative stress [14, 22, 23]. The effects of SAL have been widely studied in dopaminergic cell lines; however the behaviour of surrounding cell types in response to SAL has not been clarified.

Aside from neuronal cells which play key role in cell-cell communication, non-neuronal cells such as microglia are important as they regulate the immunity within central nervous system (CNS). Microglia primarily maintain brain homeostasis through a strict surveillance system and exert neuroprotection by promoting cell survival, secretion of neurotrophic factors, coordination of phagocytotic activities and immunomodulation [24-26]. They are also found to be densely distributed in the substantia nigra, just as dopaminergic cells [27]. This is of particular relevance as the presence of neurotoxins in substantia nigra may not only affect the neuronal cells, but could also influence the surrounding cells. As microglial cells usually have a slow turnover and could live on a person's lifespan, depletion in microglia population can significantly affect their protective functions to the brain environment [28]. Therefore, the present study intended to explore the effects of SAL on other cell population, such as microglia.

MATERIALS AND METHODS

Reagents

Dulbecco′s minimal essential medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin solution, trypsin-EDTA (0.25%), trypan blue stain (0.4%), and phosphate buffered saline (PBS) solution were purchased from Gibco Life Technologies (USA). Salsolinol hydrobromide, sodium bicarbonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2'-7'dichlorofluorescein diacetate (DCFH-DA) and dimethyl sulfoxide (DMSO) in their powder form, together with DMSO solution, were obtained from Sigma-Aldrich Chemicals (USA). Novagen® RNAse A solution was purchased from EMD Millipore Corporation (USA). Invitrogen® Propidium Iodide (PI) solution was purchased from Thermo Fisher Scientific (USA). Caspase-Glo®3/7 assay, Caspase-Glo®8 assay and Caspase-Glo®9 assay kits were purchased from Promega Corporation (USA).

Cell culture and salsolinol treatment

BV2 microglial cells were kindly gifted by Professor Sharmili Vidyadaran from Universiti Putra Malaysia (Selangor, Malaysia). BV2 microglial cells (passage number 20-30) were cultured in DMEM supplemented with 10% FBS,

100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown under humidified condition of 5% carbon dioxide (CO_2) at 37 °C. SAL was prepared by dissolving in distilled water to yield a stock concentration of 5 mM. SAL was further diluted to the required concentrations with media containing 1% FBS in the experiments described below.

Cell cytotoxicity assay

Cell viability was determined using MTT assay. BV2 cells were first seeded in 96-well plate and incubated overnight. Upon treatment with SAL at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µM) and incubation at 3 timepoints (24, 48 and 72 hours), MTT solution (5 mg/mL) was added into each well and further incubated for 4 hours. The media was then removed and replaced with DMSO to dissolve the formazan crystals. The absorbance was measured using a microplate reader (Plate Reader Infinite 200 Pro, Tecan) at wavelengths of 570/630 nm. The inhibitory concentrations (IC) to induce 25%, 50% and 75% reduction in cell viability (also known as IC_{25}, IC_{50}, IC_{75}, respectively) were determined from cell viability graph and used in subsequent assays.

Cell cycle analysis

Cell cycle phases of BV2 cells population were analysed using flow cytometry by measuring fluorescence of DNA-binding dye (PI). BV2 cells were first seeded in 60-mm plates and incubated overnight. Upon SAL treatment (with values of IC_{25}, IC_{50} and IC_{75}) and incubation for 48 hours, the cells were harvested and fixed in ice-cold 70% ethanol overnight. The cells were then washed twice with PBS, resuspended in staining solution containing PI (20 µg/ml) and RNAse (15 µg/ml). The cells were incubated in the dark for 30 minutes before analysis using flow cytometer (FACSCalibur 4 Colour Flow Cytometer, BD Biosciences) with BD CellQuest Pro software.

Caspase assay

The activity of caspases was determined by measurement of cell luminescence upon addition of Caspase-Glo® 3/7 reagent, Caspase-Glo® 8 reagent and Caspase-Glo® 9 reagent. BV2 cells were first seeded in white-bottomed 96-well plate and incubated overnight. Upon SAL treatment (with values of IC_{25}, IC_{50} and IC_{75}) and incubation for 48 hours, 100 µL of caspase reagents were added to respective wells. The cells were further incubated for 30 minutes at 37 ^{o}C before measurement using microplate reader (Plate Reader Infinite 200 Pro, Tecan). The changes in caspase activities upon SAL treatment were compared to untreated control.

Reactive oxygen species assay

The production of intracellular ROS from BV2 cells were measured using DCFH-DA dye. BV2 cells were seeded in black-bottomed 96-well plate and incubated overnight. Upon SAL treatment (with values of IC_{25}, IC_{50} and IC_{75}) and incubation for 48 hours, DCFH-DA (10 µM) was added and the plates were read at every 10-minute interval for 30 minutes at a wavelength of 485 nm and 530 nm using microplate reader (Plate Reader Infinite 200 Pro, Tecan). The level of ROS production was expressed as fold change with respect to control.

Statistical analysis

All experiments were performed in triplicates, and data was reported as mean ± standard error (SE) of 3 independent experiments. Significant differences between mean values of treated groups and untreated control were determined using Student's t-test with significance level set at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS

Salsolinol reduced BV2 cell viability

The changes in cell viability upon SAL treatment over periods of 24, 48, and 72 hours are presented in Figure 1. Overall, SAL exerted dose-dependent toxicity in BV2 cells, where descending trends in cell viability were observed with increasing SAL concentrations across all timepoints. At 24 hours, the toxicity of SAL at concentrations of 6.25 µM and 12.5 µM appeared to be lower than that of 3.125 µM, but the differences were not significant ($p > 0.05$) when compared to control cells. Cells exposed to SAL for a duration of 48 hours showed marked reduction in viability from basal level for all tested concentrations. SAL exhibited the highest toxicity at concentration of 100 µM, in which it inhibited the growth of BV2 cells by 84.18% ($p < 0.001$).

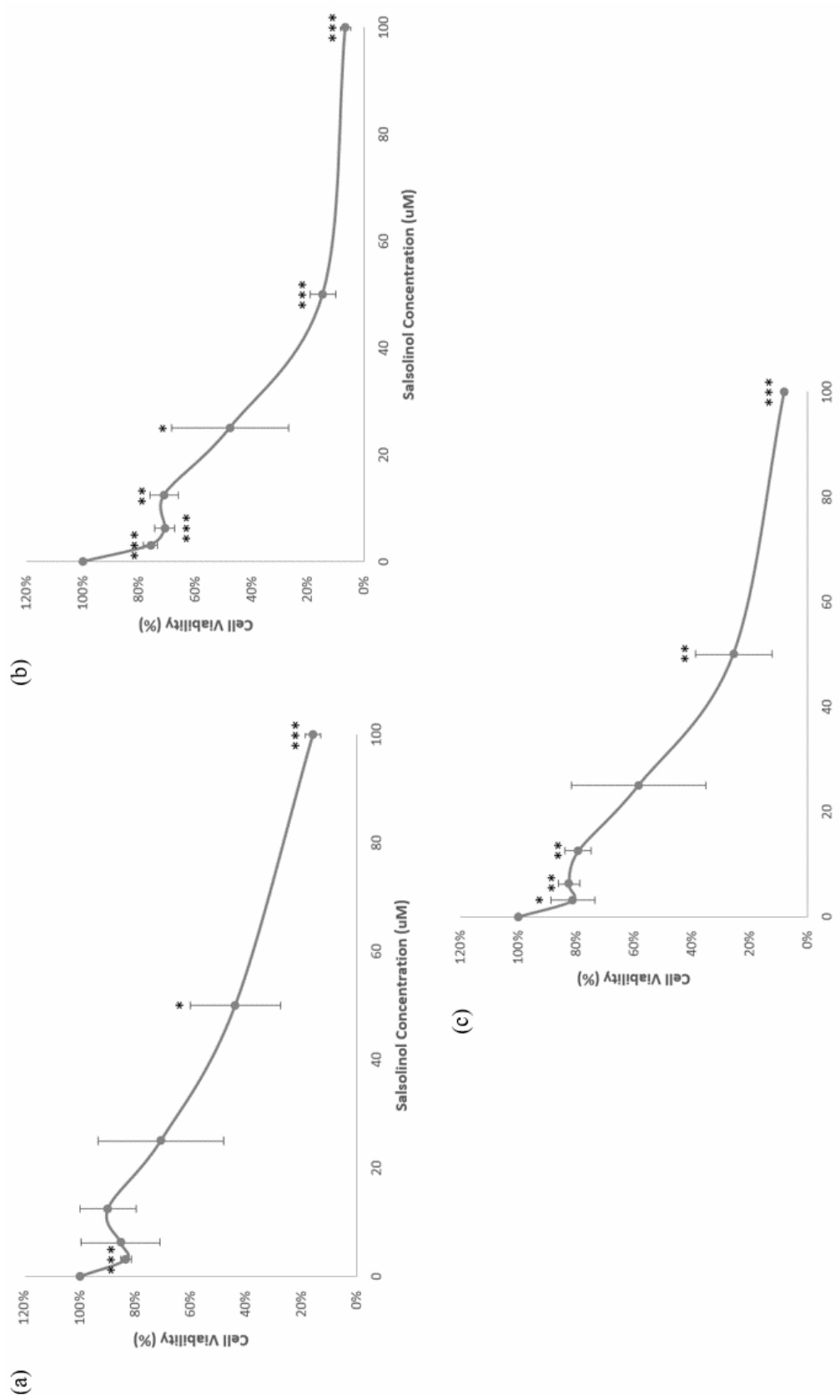

Figure 1. Dose-response curve of MTT assay in BV2 cells after salsolinol (SAL) treatment for 24 hours (a), 48 hours (b) and 72 hours (c). BV2 cells were treated at different concentrations of SAL (3.125, 6.25, 12.5, 25, 50, 100 μM). The results are presented as percentage of cell viability with respect to control cells. Data are expressed as mean ± SE of 3 independent experiments. The symbols *, ** and *** denote significant difference between control and treatment groups at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

The growth inhibitory effects were even greater with longer treatment periods, as higher reduction in cell viability was observed at 48 hours (93.38%; $p < 0.001$) and 72 hours (91.95%; $p < 0.001$). The IC_{50} values for SAL determined at each timepoints, in the order of 24, 48, and 72 hours, were 43 μM, 24 μM and 30 μM, respectively. The minimum inhibitory concentration to induce 50% reduction in cell viability decreased by 1.8-fold when BV2 cells were treated for longer duration. Interestingly, prolonged exposure to SAL treatment from 48 to 72 hours demonstrated only small, additional effect in reduction of BV2 cell viability. Hence, the IC values at 48 hours were chosen to be applied in subsequent assays, as SAL had reached its maximal toxicity at the particular timepoint. The values of IC_{25}, IC_{50} and IC_{75} at 48 hours determined from the dose-response curve were 3.5 μM, 24 μM, and 39 μM, respectively. The subsequent experiments were designed to assess the underlying mechanisms of SAL cytotoxicity.

Salsolinol altered cell cycle distribution in BV2 cells

As illustrated in Figure 2, a concentration-dependent rise in $subG_1$ cell population was observed, indicating that SAL induced an increase of apoptotic cells among BV2 population. Significantly higher $subG_1$ population was observed, with additional 7.14 ($p < 0.001$), 14.58 ($p < 0.001$) and 55.28 ($p < 0.05$) increase in the percentage of population at concentration of IC_{25}, IC_{50} and IC_{75}, respectively. At the same time, BV2 cell populations under G_o/G_1 phase were markedly reduced by 10.28 ($p < 0.01$), 14.34 ($p < 0.001$) and 34.36 ($p < 0.01$) in the percentage of population with increasing SAL concentration (IC_{25}, IC_{50} and IC_{75}, respectively). The flow cytometry results corresponded to the decreased viability of BV2 cells observed in MTT dose-response curves documented above. No significant decrease in S phase was recorded, except for high-dose SAL (IC_{75}) which resulted in 5.0% ($p < 0.05$) reduction in BV2 cells under S phase. BV2 cells treated with low-dose SAL arrested cells in G_2/M phase, with notable increase in G_2/M cell population by 3.78 ($p < 0.05$) in the percentage of population. However, when SAL concentration was further increased, the cell population under G_2/M phase was significant reduced by 17.44 ($p < 0.01$) in the percentage of population. It was also noted that the statistical significance was lowered in high-dose SAL.

Salsolinol induced caspase activation in BV2 cells

As shown in Figure 3, no notable changes in caspase activities were observed in BV2 cells following 48 hours exposure to low-dose SAL. However, when BV2 cells were treated at higher doses of SAL, activities of all caspases (caspase-3/7, -8 and -9) were detected above basal level. The most drastic change in caspase activities was observed with caspase-3/7, where intermediate- and high-dose SAL triggered 3.5-fold ($p < 0.01$) and 21-fold ($p < 0.01$) increase, respectively. SAL appeared to activate both caspase-8 and -9 at a similar rate. Following BV2 cells exposure to intermediate-dose SAL, there was a 1.6-fold ($p < 0.01$) increase in caspase-8 activity, whereas for caspase-9, its activity increased by 1.8-fold ($p < 0.01$). Both caspase-8 and caspase-9 showed similar increase in their activities (12-fold, $p < 0.01$) upon exposure to high-dose SAL.

High-dose salsolinol induced oxidative stress in BV2 cells

To determine whether SAL induces an oxidative stress environment in microglia, BV2 cells were exposed to the drug for a period of 48 hours, and the effects of low-dose (IC_{25}), intermediate-dose (IC_{50}) and high-dose (IC_{75}) were assessed. Figure 4 below depicts the changes in the production of intracellular ROS upon SAL exposure to BV2 cells for 48 hours. When compared to basal ROS level of untreated cells, the production of ROS in BV2 cells treated with low- and intermediate-dose of SAL reduced by 14.52% ($p < 0.001$) and 23.54% ($p < 0.001$), respectively. However, when BV2 cells were exposed to the high-dose SAL, a steep increase in ROS production by 1.64-fold ($p < 0.01$) was observed.

DISCUSSION

The neurotoxicity of SAL is consistently demonstrated across large number of studies. However, majority of the existing studies were centrally focused on their effects in dopaminergic

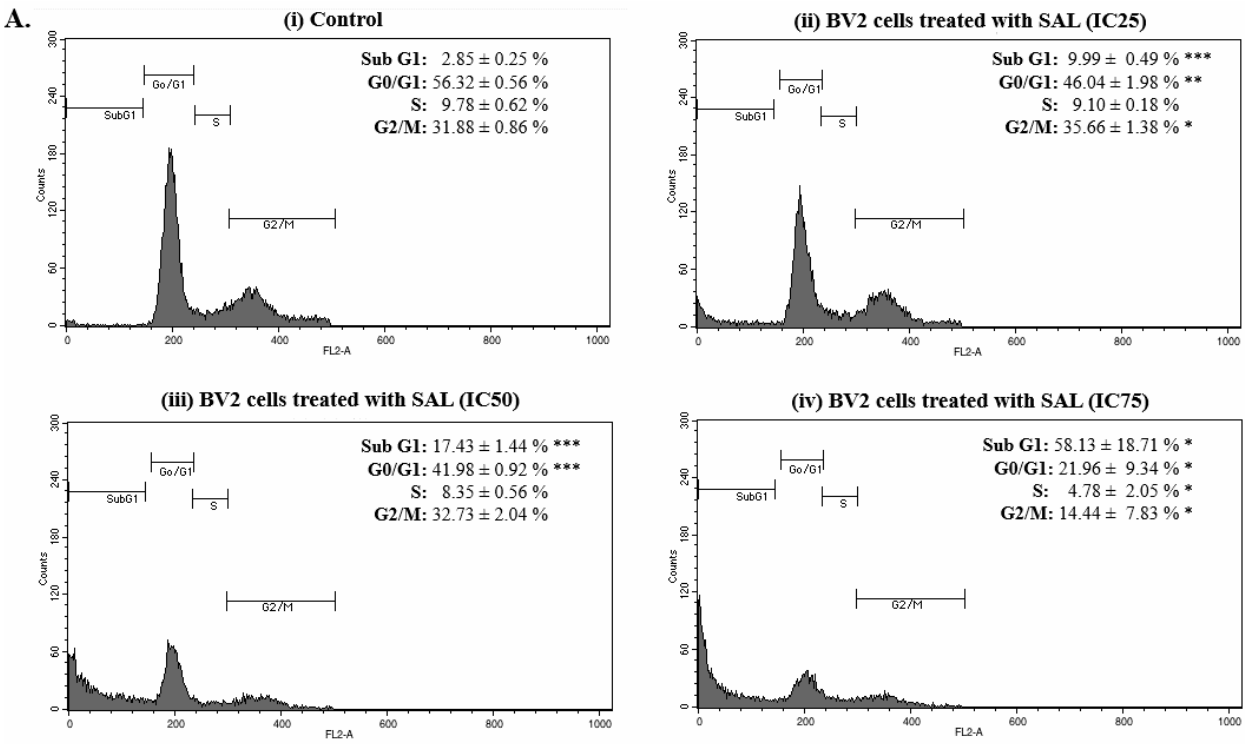

Figure 2. Cell cycle analysis of BV2 cells after SAL treatment for 48 hours. (A) Flow cytometric analysis of SAL-treated BV2 cells at zero (control), low (IC_{25}), intermediate (IC_{50}) and high (IC_{75}) concentrations are represented by figures (i), (ii), (iii), and (iv), respectively.

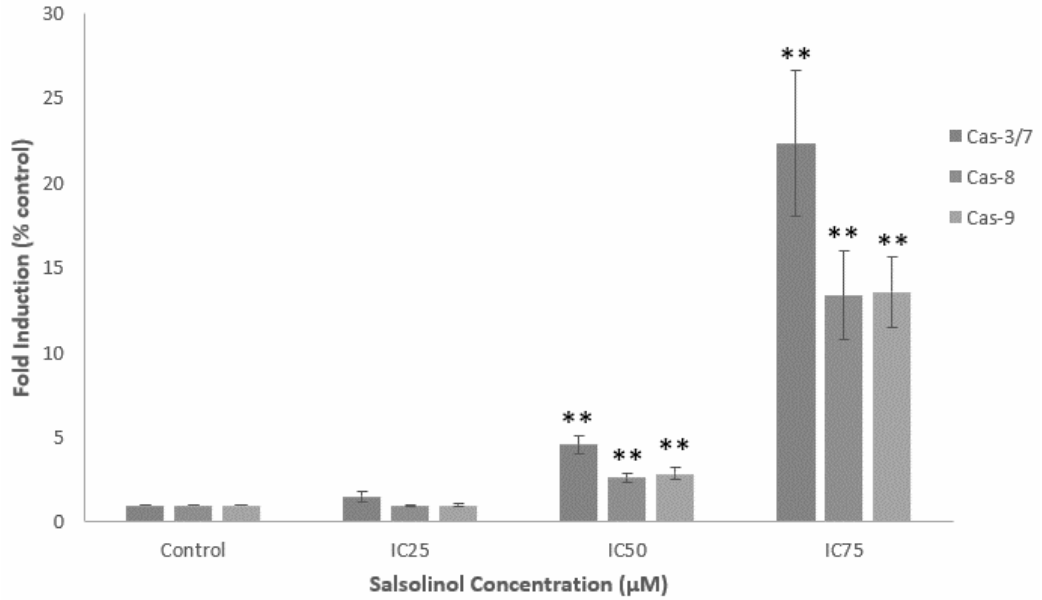

Figure 3. Induction of caspase activities in BV2 microglial cells after salsolinol (SAL) treatment for 48 hours. BV2 cells were treated with SAL at different IC values (3.5, 24, 39 μM) determined from dose-response curve of MTT assay at 48 hours. Data was expressed as mean ± SE of 3 independent experiments. The symbol ** denotes significant difference between control and treatment groups at $p < 0.01$.

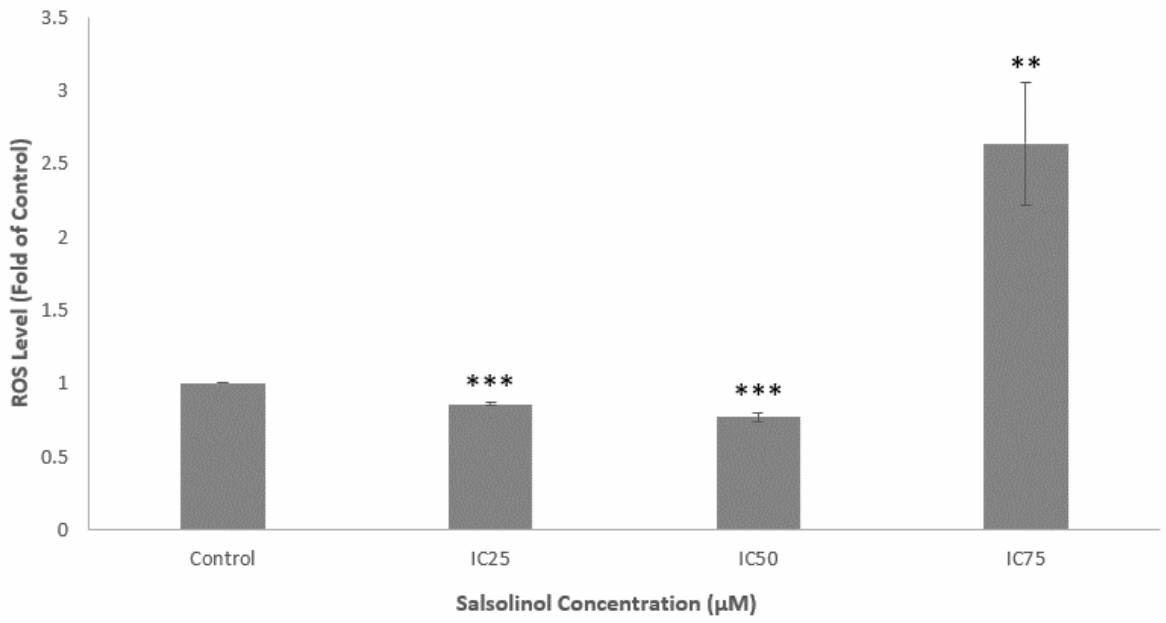

Figure 4. The levels of intracellular reactive oxygen species (ROS) levels in BV2 cells after salsolinol (SAL) treatment for 48 hours. BV2 cells were treated with SAL at different IC values (3.5, 24, 39 μM) determined from dose-response curve of MTT assay at 48 hours. The results were presented as percentage of ROS level with respect to control cells. Data was expressed as mean ± SE of 3 independent experiments. The symbols ** and *** denote significant difference between control and treatment groups at $p < 0.01$ and $p < 0.001$, respectively.

cell lines. The present study revealed that their toxicity is also extended to microglial cell population. As microglial cells express receptors for a variety of neurotransmitters including dopamine, SAL as a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) structural analogue, could also be selectively taken up by these receptors into microglial cells to exert its toxicity [29, 30]. As documented above, SAL exhibited anti-proliferative effects on BV2 microglial cells, and depleted more than 90% of cell viability at high concentration. This is in agreement with existing studies related to SAL, which also displayed dose- and time-dependent toxicity towards other cell types, such as dopaminergic cells, endothelial cells and neural stem cells [14, 15, 31]. Based on current findings, it appeared that microglial cells may be more susceptible to SAL toxicity as exposure to 100 μM for 24 hours reduced BV2 cell viability to 16%, whereas 51% of viable dopaminergic cells still remained following SAL treatment at the same concentration [14]. This may suggest that microglial cell death may even precede dopaminergic neurons in PD condition.

Early depletion of microglial cells can be correlated with the diminished neuroprotection observed in PD condition. The large number of degenerated neurons in PD requires frequent clearance by microglia to prevent overspill of toxic contents that could threaten the surrounding cell populations. However, the presence of mitochondrial toxins, such as SAL, could severely deplete microglial cell population that usually has slow turnover rate, impeding their usual phagocytic clearance function, and exacerbate cell death [28]. Moreover, SAL toxicity was associated with the depletion of brain-derived neurotrophic factor (BDNF) in dopaminergic SH-5YSY cells, which can greatly affect neuronal survival [32]. The impaired energy production can also affect autophagy regulation, facilitating the formation of Lewy bodies.

Furthermore, the cytotoxicity of SAL was linked to drastic increase in $subG_1$ population from flow cytometry analysis, indicating that apoptosis is the primary mode of cell death in SAL toxicity mechanisms. The ability of SAL in apoptosis induction is consistently proven in numerous

studies, and thus, it is often correlated to PD pathology [33, 34]. Small, but significant increase in G_2/M cell population was observed when BV2 cells were treated with low-dose SAL. This is in contrast with the previous report that showed SAL demonstrated no effect on G_2/M phase of cell cycle [35]. In this case, increased proportion of G_2/M cell population may implicate that SAL exerts anti-proliferative effects on BV2 cells, by means of arresting the cells in G_2/M phase as the DNA damage was beyond repair for progression into proliferative stage. To verify cell cycle arrest at G_2/M phase, expression levels of regulatory proteins such as cyclin-dependent kinase-1 (CDK1) and cyclin B should be examined. At high doses however, SAL induced extensive apoptosis without any cell cycle blockade, and therefore marked increase in subG_1 population but decrease in G_2/M population were observed. The lower statistical power at high concentration could be attributed to the low number of living cells in the sample, which affected the sensitivity of assay.

There are 2 pathways that are associated with apoptotic cell death, namely intrinsic and extrinsic pathways. Although they may differ in terms of their initiation events, both pathways can eventually lead to activation of effector caspases such as caspase-3 and caspase-7, which in turn execute various cell degradation process in apoptosis. High level of effector caspase-3 activation has been detected in PD and SAL-treated dopaminergic neurons [35, 36]. Similar observations were implicated in the present study, where the highest caspase activity upon SAL treatment in BV2 cells was noted in caspase-3/7. This signifies that inhibition of key mediator in caspase cascade, such as caspase-3 or -7 could greatly affect the rate of cells undergoing apoptosis, thereby preserving the microglial pool. On the contrary, both caspase-8 and caspase 9 are upstream caspases that induce extrinsic and intrinsic pathways respectively, which will cleave their downstream effector caspases upon activation. In the present study, both initiator caspases appeared to have similar levels of activity in BV2 cells, implicating that SAL triggers activation of both types of apoptotic pathways. Some studies indicate that both intrinsic and extrinsic pathways are active in PD, although intrinsic pathway had been suggested to have a higher prevalence. MPTP, a SAL analogue was also reported to induce activation of both initiator caspases [37]. SAL-activated caspase-9 is associated with perturbation in cytoplasmic Ca^{2+} homeostasis which subsequently initiates apoptotic events [38]. The activation of caspase-8 is initiated by the interaction between Fas-associated death domain (FADD) death receptor and TNF receptor 1 (TNFR1). Both TNFR1 upregulation and caspase-8 activation are evident in PD [39-41]. However, previous study performed on dopaminergic SH-5YSY cells did not report any significant caspase-8 activity upon SAL exposure [23]. This could imply that SAL is able to exert varying caspase-mediated apoptotic mechanism in different cell types.

Additionally, it was noted that SAL induced an oxidative stress environment only when BV2 cells were treated with high concentration. Similar observation was also noted in dopaminergic SH-5YSY cells [35]. When the cells are exposed to low SAL concentration, SAL-mediated ROS production triggers the intracellular antioxidant defence to prevent overaccumulation of ROS. However, the ROS production induced by high-dose SAL was beyond the neutralising capability of intracellular antioxidant mechanism, thereby creating an oxidative stress environment that can be detrimental to cell survival. This could be the reason for greater reduction in BV2 cell viability with increasing SAL concentration, as previously noted in the MTT dose-response curves from Figure 1. Several stimulating agents for oxidative stress in microglia, such as menadione and chromogranin, also led to similar outcome, which is the activation of microglial apoptosis [42, 43]. However, different stimulating agents may activate different type of caspases, for instance menadione-treated microglia only demonstrated activation of caspase-9, but not caspase-8 [43]. These findings are also in agreement with the progressive nature of PD, and thus the symptoms are usually not apparent at initial stage of disease. However, as the neurotoxins accumulate during the course of disease, the brain gradually loses its defence mechanism against oxidative damage, resulting in extensive cell death. Moreover,

microglial cells are known to be activated following exposure to stressful environment [44]. This signifies that SAL not only provokes microglial cell death, but it could also induce oxidative stress and propel the remaining microglial population to switch into pro-inflammatory state, which further exacerbate cell death.

Both activation and cell death in microglia were able to contribute to the onset of neurodegeneration [45]. The high plasticity of microglia allows them to flexibly switch between their neuroprotective and activated phenotypes, depending on the stimuli [46]. In diseased state however, physiologic functions of microglia may be dysregulated, possibly due to impaired mitochondrial regulation of cellular activities. Consequently, microglia are subjected to apoptotic death mediated by mitochondria itself, as a result of disrupted energy metabolism. Remaining microglia that are not removed by apoptosis, contain dysfunctional mitochondria, which could in turn attain an activated state that sustain for prolonged period, leading to microgliosis that is often implicated in advanced-stage PD [47]. The gradual loss in its neuroprotective effects and increase in pro-inflammatory phenotypes could be attributed to aging effects or the presence of neurotoxins. In the current study, SAL was demonstrated to induce activation of caspases in BV2 microglial cells. The activated caspases could contribute to microglial activation mediated by protein kinase C (PKC)-delta pathway [48]. The overactivation of microglial cells can further induce its own apoptotic death due to its self-regulatory mechanism [49]. Low dopamine level as a result of extensive loss of dopaminergic neurons, may also be a contributing factor to the dysregulated microglial function [50]. With accumulating evidence suggesting the central role of SAL in PD aetiology, it can potentially be employed as a disease biomarker for PD staging. Together with the present study findings, numerous studies have demonstrated that its intracellular level is closely linked to increased cell damage and ROS production in PD condition. Therapeutic strategies that specifically target SAL or its mechanisms could ameliorate its toxicity and cell death-related events. For example, memantine and pituitary adenylate cyclase-activating polypeptide (PACAP) have been demonstrated to interfere with SAL-mediated apoptotic pathways and reverse cell death [32, 51]. Inhibition of apoptosis alone however, may not be sufficient to reverse PD pathology as the affected cells that do not undergo apoptosis may still contain degenerated mitochondria which impede physiologic cell functions. Some cells may still be subjected to death *via* necrotic pathway [39]. Therefore, the targeting of underlying mechanisms that could induce cell apoptosis, such as oxidative stress is a more practical approach in preventing cell death.

CONCLUSION

It can be concluded from the present study that SAL exerts anti-proliferative effects on BV2 microglial cells and induce their apoptosis *via* caspase-dependent mechanism. Both intrinsic and extrinsic apoptotic pathways are activated upon SAL treatment. Furthermore, SAL also demonstrated oxidative stress-induced apoptosis when BV2 cells were treated at high concentration. These results provide further insights into the pathogenic mechanisms of PD. Owing to the neuroprotective effects of microglia in CNS, depletion of microglial cells by SAL can significantly impact the survival of dopaminergic neurons and CNS homeostasis. As SAL is a well-known mitochondrial toxin as reported in previous studies, mitochondrial impairment in microglia can give rise to abnormal phenotype, which further contributes to disease progression. The present findings again demonstrated the dose-dependent toxicity of SAL as reported in existing literature, which further supports the progressive nature of PD. This signifies that the slow accumulation of SAL as a result of perturbation in its normal metabolism or other unknown factors could eventually induce various pathogenic consequences in the diseased patient.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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