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Ameliorating effect of S-Allyl cysteine (Black Garlic) on 6-OHDA mediated neurotoxicity in SH-SY5Y cell line

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ABSTRACT

Therapeutic approaches based on isolated compounds derived from natural products are more common in preventing diseases involving inflammation and oxidative stress at present. S-allyl cysteine (SAC) is a promising garlic-derived organosulfur compound with many positive effects in cell models and living systems. SAC has biological activity in various fields, enclosing healing in learning and memory disorders, neurotrophic effects, and antioxidant activity. In this study, we purposed to identify the neuroprotective activity of SAC toward 6-OHDA-induced cell demise in the SH-SY5Ycell line. For this purpose, 6-OHDA-induced cytotoxicity, and biochemical, and gene expression changes were evaluated in SH-SY5Y cells. SH-SY5Y cells grown in cell culture were treated with SAC 24 h before and after 6-OHDA application. Then, cell viability, antioxidant parameters, and gene expressions were measured. Finally, immunofluorescence staining analysis was performed. Our results showed that SAC increased cell viability by 144 % at 80 μg/mL with pre-incubation (2 h). It was observed that antioxidant levels were significantly increased and oxidative stress marker levels were decreased in cells exposed to 6-OHDA after pre-treatment with SAC ($p<0.05$). SAC supplementation also suppressed the increase in pro-inflammation levels (TNF-α/IL1/IL8) caused by 6-OHDA ($p < 0.05$). While 8-OHdG and Nop10 expressions were observed at a mild level in SAC pretreatment depending on the dose, 8-OHdG, and Nop10 expressions were observed at a moderate level in SAC treatment after 6-OHDA application ($p<0.05$). Our findings demonstrate the positive effect of pretreatment with SAC on SH-SY5Y cells injured by 6-OHDA, suggesting that SAC may be beneficial for neuroprotection in regulating oxidative stress and neuronal survival in an in vitro model of Parkinson's disease.

1. Introduction

The incidence of neurodegenerative illness is increasing globally. Parkinson's disease (PD) is one of the most widespread neurodegenerative illnesses [1,2]. Pathological features of PD include neuronal cell loss in the substantia nigra, reduced dopamine secretion or loss of dopamine neurons in the midbrain, and Lewy body accumulation in other brain tissues, thus aggravating motor functions [3–7]. Although the pathogenesis of PD is still unclear, it may cover genetic and environmental hazards, including exposure to metals and pesticides [8]. Among these factors, abnormal protein homeostasis, neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress play important roles in the development of PD [9,10]. There is currently no pharmaceutical treatment that can significantly reverse the motor and non-motor symptoms of PD [11]. For example, levodopa treatment can only manage the motor symptoms of PD, while other clinical problems such as dyskinesia and neurodegeneration remain untreated [12]. In recent years, dietary bioactive compounds and nutraceuticals have gained great interest as an alternative treatment to combat PD.

The SH-SY5Y cell line is a cell-based model used to study PD's pathogenesis and appraise the therapeutics of novel supposed anti-PD medicines [13]. Neurotoxin compounds such as 6-hydroxydopamine (6-OHDA) are commonly used to make up PD models in vitro and in

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vivo. 6-OHDA is a toxical oxidative metabolite with a chemical construction akin to catecholamines and weakly crosses the blood-brain barrier. 6-OHDA has a loud affinity against dopaminergic neurons of the substantia nigra pars compacta. So, it stimulates oxidative stress, neurodegeneration, apoptosis processes, and neuroinflammation in the nigrostriatal system and lets the thriving of PD models [14,15].

In the last few decades, various phytochemicals such as organosulfur compounds, polyphenols, and isothiocyanates have been found to reduce neurotoxicity in cellular and animal PD models [16–18]. S-allyl cysteine (SAC) is one of the main characteristic compounds in Black Garlic. SAC, which is extremely important for human health, is a biologically active compound used in medical applications due to its antioxidant, neuroprotective, anticarcinogenic activity, antihepatotoxic, and neurotrophic effects reported previously [19–22]. The pharmacological impact of SAC is cell conservation through its antioxidant effect, and it is efficient in treating nervous system illnesses like Alzheimer's by preventing oxidative injury [23]. SAC has been able to exhibit retinal protection against kainate neurotoxicity [24], ameliorate β-amyloid-induced brain injury [25], preservation of dopaminergic neurons against the toxic effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridinium [26] and a neuroprotective effect in ischemic neuronal injury via a nuclear factor-like 2-dependent antioxidant response element [27]. In parallel, in our study, we purposed to determine the neuroprotective activity of SAC towards 6-OHDA-induced cell demise in the SH-SY5Y cell line. For this purpose, 6-OHDA-induced cytotoxicity (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)), biochemical (malondialdehyde (MDO), myeloperoxidase (MPO), glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD)), gene expression (interleukin-1 (IL-1), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α)) and immunofluorescence (8-hydroxy-2′-deoxyguanosine (8-OHdG) and nucleolar protein 10 (Nop 10)) changes were evaluated in SH-SY5Y cells.

2. Materials and methods

2.1. Cell culture and in vitro treatment

The SH-SY5Y cell line was received from the Pharmacology Department at Ataturk University (Turkey). Cells were cultured in dulbecco's modified eagle medium high glucose (Sigma Aldrich) medium containing 15 % fetal bovine serum, and 1 % antibiotic at 37°C under 5 % CO_2 in a humidified atmosphere.

SAC and 6-OHDA were obtained from Sigma Aldrich (USA). They were exposed to a 6-OHDA dose for 24 and 48 h to evaluate neurotoxicity and thrive in the in vitro PD model. In the first stage, 200 μM 6-OHDA [28] was applied to the cells (except control and treatment groups) for 24 h. The cell culture dose of SAC was determined by reference to previous studies [29]. SH-SY5Y cells (except control and 6-OHDA group) were pretreated with SAC (5–10–20–40 and 80 μg/mL) 2 h before 6-OHDA application. In the second stage, 200 μM 6-OHDA was applied to the cells (except the control group) in another 96-well microtiter plate for 24 h. After 24 h, SAC (A-SAC) doses were administered (except for the control and 6-OHDA groups). Cells were incubated for 24 h.

2.2. Cell viability assay

Cell viability was identified by MTT assay. MTT (Sigma Aldrich) assay evaluated cellular metabolic activity to indicate cell viability and cytotoxicity. After incubation, 10 μL of MTT solution was supplemented to every well and incubated at 37°C in 5 % CO_2 for 4 h. Then, 50 μL dimethyl sulfoxide (Sigma Aldrich) was supplemented to solve the consisted formazan salts. Absorbance was measured at 570 nm with a spectrophotometer [30].

2.3. ELISA

MDA (Cat.No.:E- EL-0060), MPO (Cat.No.:E-BC-K074-S), GSH (Cat. No.:E-EL-0026), CAT (Cat.No.:E-BC-K031-M), and SOD (Cat.No.:E-BC-K020-M) were measured with specific ELISA kits (Elabscience, USA). The supernatant was collected after 24 h and processed for the presence of selected antioxidants by ELISA according to protocols provided by the manufacturer. The resulting absorbance was recorded at 450 nm using a microplate reader.

2.4. Determination of TNF-α, IL-1, and IL-8 expression levels

To measure the expression levels of IL-1 (HP200544), IL-8 (HP200551), and TNF- α (HP200561) (Origene, USA) in cultured SY-SH5Y, we first extracted total RNA with the High Pure RNA Isolation kit (Roche, USA). Switzerland), then the Transcriptor First Strand cDNA Synthesis kit (Roche, Switzerland) was used for reverse transcription from the resulting RNA to cDNA. Real-time PCR analysis was also done as formerly explained [31]. GAPDH (HP205798) (Origene, USA) mRNA expression levels were used as an endogenous control, and relative mRNA expression levels were calculated by the ΔΔCt method.

2.5. Double immunofluorescence staining method

Cells cultured in cell culture were incubated in paraformaldehyde solution for 30 min. Then, the cells were kept in 3 % H_2O_2 for 5 min. 0.1 % Triton-X solution was added to the cells, which were washed with phosphate-buffered saline and waited for 15 min. After incubation, protein blocks were dripped onto the cells and kept in a dark environment for 5 min. Then, the primary antibody (8-OHdG) cat no: sc-66036, dilution rate: 1/100 US) was dropped and incubated according to the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (Fluorescein isothiocyanate Cat No: ab6785 diluent ratio: 1/500, UK) and kept in the dark for 45 min. Then, the primary antibody (Nop10) Cat no: ab134902, dilution rate: 1/100 UK) was dropped onto the sections and incubated according to the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (Texas Red Cat No: b6719 diluent ratio: 1/500, UK) and kept in the dark for 45 min. Then, 4′,6-diamidino-2-phenylindole with mounting medium (Cat no: D1306 dilution rate: 1/200 UK) was supplemented to the preparations and left in a dark environment for 5 min, then the sections were covered with a coverslip. The stained sections were analyzed under a microscope with a fluorescence attachment (Zeıss AXIO GERMANY).

2.6. Statistical analyzes

One-way analysis of variance (ANOVA) was performed for post-hoc comparisons with LSD using IBM SPSS 23.0 software for data analysis. Values less than $p < 0.05$ were admitted as statistically meaningful.

To identify the density of positive staining from the images acquired as a conclusion of staining; 5 randomly different fields were chosen from every image and appraised in the ZEISS Zen Imaging Software program. Data were statistically described as mean± standard deviation (SD) for % field. One-way ANOVA after the Tukey test was done to compare positive immunoreactive cells with controls. As a conclusion of the test, $p < 0.05$ was admitted meaningful and the data were offered as mean ± SD.

3. Results

3.1. Effects of SAC against 6-OHDA mediated neurotoxicity in SH-SY5Y cells

To evaluate whether SAC protects SH-SY5Y cells from 6-OHDA-mediated cytotoxicity, we investigated metabolic activity in SH-SY5Y

cells pretreated with distinct concentrations of SAC for 2 h before application of 6-OHDA neurotoxin for 24 h. We also observed the effect of different concentrations of SAC on cell viability for 24 h after applying 6-OHDA neurotoxin to SH-SY5Y cells. According to our findings, SAC-80 (144 %) showed the highest cell viability compared to 6-OHDA (60 %) in pretreatment, followed by SAC-40 (130 %), SAC-20 (125 %), and SAC-10 (112 %). concentrations ($p < 0.05$) (Fig. 1A). According to the findings obtained at different SAC concentrations after applying 6-OHDA, the survival rate was 39 % in the 6-OHDA group, while it increased dose-dependently in the treatment groups. SAC-80 (57 %) showed the highest cell viability, followed by SAC-40 (47 %) ($p < 0.05$) (Fig. 1B).

3.2. Effects of SAC on 6-OHDA mediated MPO, SOD, CAT, GSH, and MDA level in SH-SY5Y cells

While the levels of oxidative stress markers MDA and MPO in the 6-OHDA group were 3.09±0.45 and 61.53±2, respectively, their levels reduced meaningfully in the treatment groups in a dosage-dependent manner compared to the 6-OHDA group ($p < 0.05$). As for antioxidant parameters, CAT, GSH, and SOD values were found to be 524.54±4.3, 25.58±1.8, and 130.76±3.85, respectively, in the 6-OHDA group, while their levels increased significantly in the treatment groups in a dosage-dependent manner compared to the 6-OHDA group ($p < 0.05$) (Fig. 2).

3.3. Effects of SAC on IL-1, IL-8 and TNF-α expressions

The effects of SAC on IL-1, IL-8, and TNF-α expressions in 6-OHDA-induced neurotoxicity were determined by RT-PCR analysis. We found an important rise in IL-1, IL-8, and TNF-α expression levels following exposure to 6-OHDA. The expression of SAC in pretreated cells was lower than that in 6-OHDA-treated cells in a dosage-dependent manner (Fig. 3). A meaningful difference was found in IL-1, IL-8, and TNF-α expression levels in the SAC 20–40 and 80 groups ($p < 0.05$).

3.4. Results of immunofluorescence staining

As a result of immunofluorescent examinations performed in cell culture; 8-OHdG and Nop10 expressions were appraised as negative in the control group. Very intense levels of 8-OHdG and Nop10 expressions were identified in the cells in the 6-OHDA group. In the pre-treated groups, severe intracytoplasmic 8-OHdG and Nop10 expressions were identified in SAC-10 cells while at severe levels in SAC-5 cells. In addition, intracytoplasmic 8-OHdG and Nop10 expressions were observed at a moderate level in the SAC-20 group, a mild level in SAC-40, and a mild level in SAC-80. A meaningful distinct was identified when compared to the 6-OHDA group ($p < 0.05$) (Fig. 4).

As a conclusion of immunofluorescence examinations of groups treated with SAC after 6-OHDA application; 8-OHdG and Nop10 expressions were observed at very severe levels in A-SAC-5 cells, at very severe levels in A-SAC-10 cells, and severe levels in S-A5–20 cells. Additionally, while moderate levels of 8-OHdG and Nop10 expressions were observed in A-SAC-40 cells, they were observed at mild levels in S-A5–80 cells. A meaningful distinct was identified when compared to the 6-OHDA group ($p < 0.05$) (Fig. 4). Data of immunofluorescence staining findings are offered in Table 1.

4. Discussion

In neuroblastoma cells, 6-OHDA is a neurotoxin commonly used in the thrive of in vitro PD models and leads to neurotoxicity by inducing oxidative stress through the manufacture of reactive oxygen species (ROS) [32]. Various studies have also stressed that 6-OHDA induces excessive autophagy, causing the release of cytochrome C from mitochondria [33]. So, cytotoxicity happens in dopaminergic neurons and neuron injury occurs. This study used 6-OHDA, a neurotoxic composite, to thrive in vitro PD model. It was determined that after cells were exposed to distinct doses of SAC, there was a decrease in the levels of pro-inflammatory cytokines and an increase in antioxidant levels.

Neuroinflammation plays a significant role in the pathogenesis of PD [34]. Cytokine aggregation and microglia activation are substantial factors that stimulate neuroinflammation [35]. Additionally, excitotoxicity, oxidative stress, and rising ROS manufacture lead to neuronal injury by raising the accumulation of oxidative produce in neurons [34, 36]. Phytochemicals are suggested to have many helpful properties like anticancer, antioxidant, and anti-inflammatory features. SAC, the most plentiful organic garlic compound, has long been used as a dietary supplement and conventional medicine. In various studies, SAC has been known to have strong antioxidant and anti-inflammatory activities. [16–18]. Aged garlic extract has been reported to raise lifetime and learning in mice [22]. Previous studies have shown that SAC protects nerve growth factor-differentiated PC12 cells [37] and for amyloid beta and tunicamycin-induced toxicity [38]. Another study showed that SAC

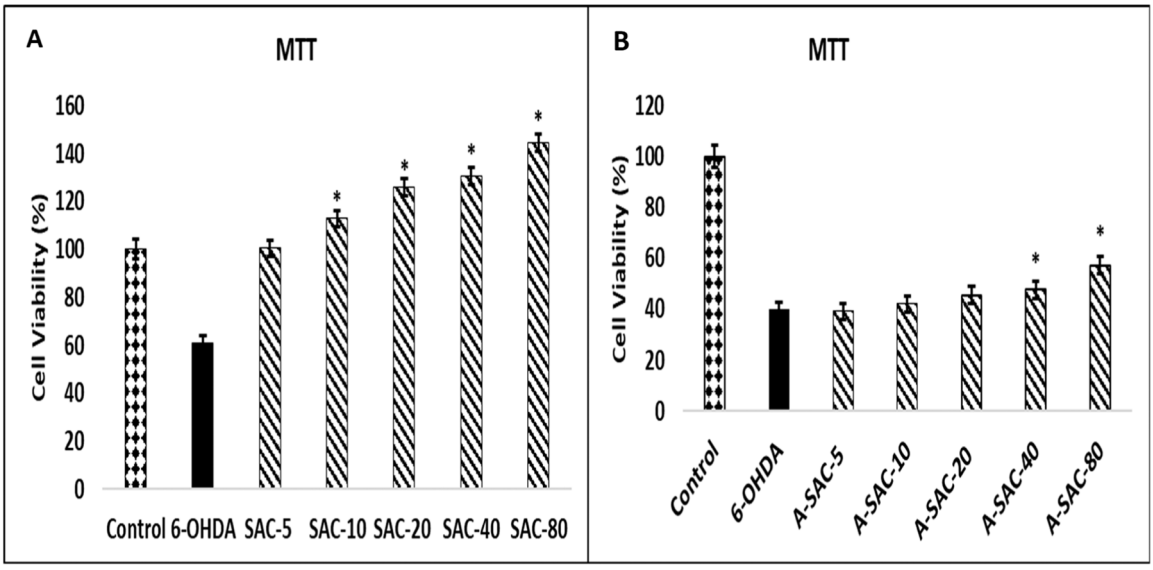

Fig. 1. Metabolic activities of SH-SY5Y cells pretreated with SAC for 2 h (A) and treated with SAC for 24 h after 6-OHDA application (B). Data are expressed as the means ± SD. *$p < 0.05$ vs. 6-OHDA group.

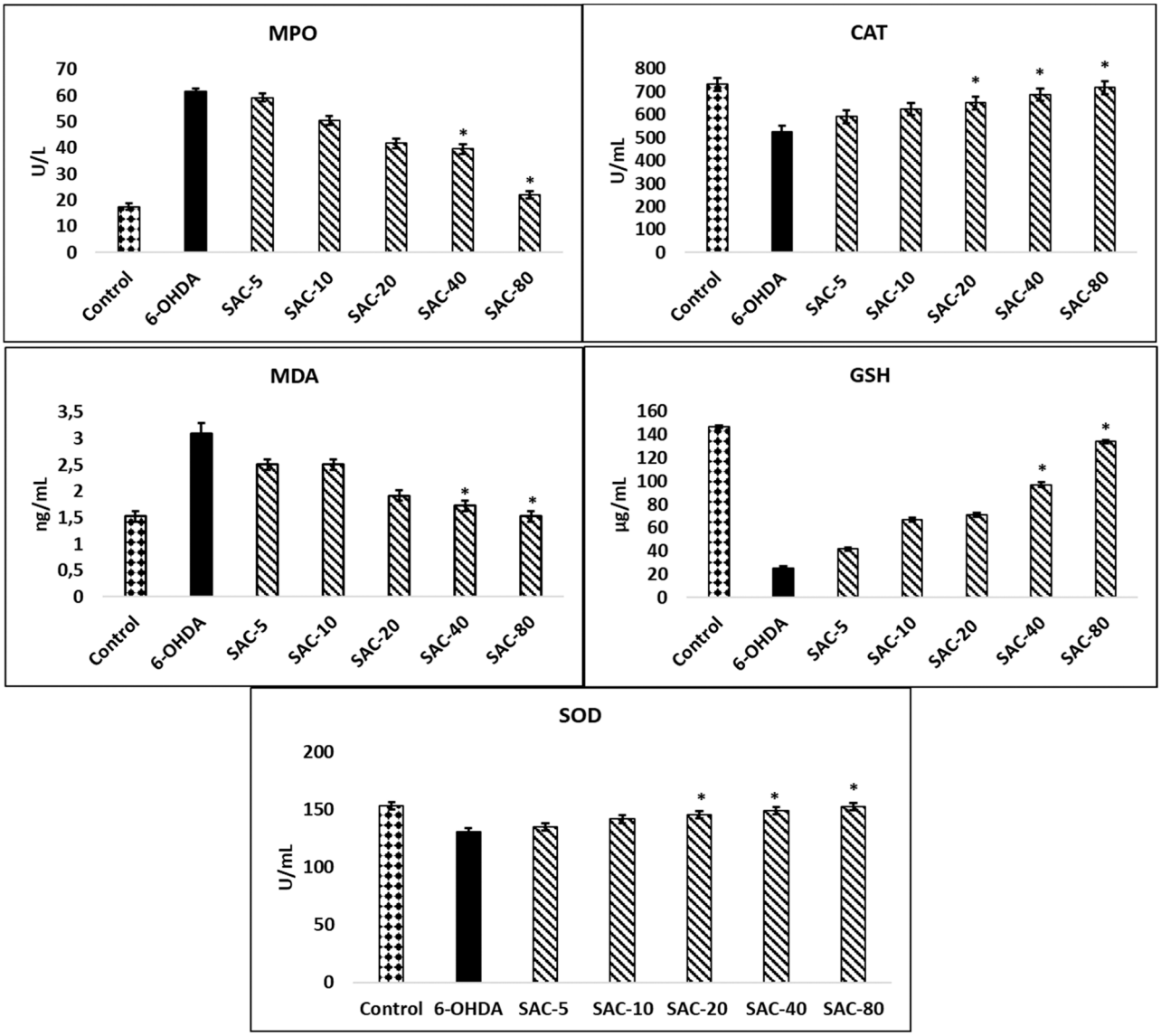

Fig. 2. Effects of SAC on the oxidative stress parameters (MDA, MPO, CAT, GSH, and SOD) of SH-SY5Y cells. Cells were treated with different doses of SAC two hours before exposure to 6-OHDA. Data are expressed as the means ± SD. *$p < 0.05$ vs. 6-OHDA group.

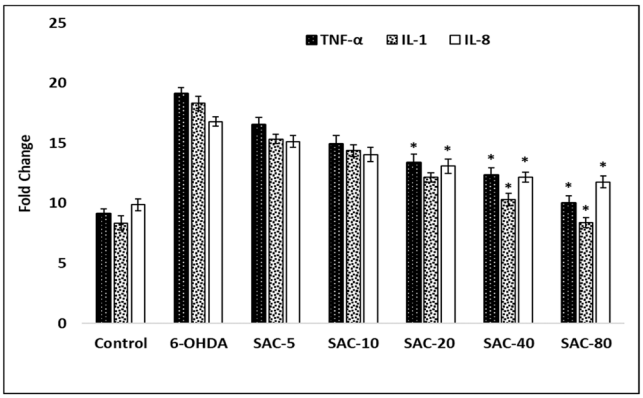

Fig. 3. Effects of SAC on IL-1, IL-8, and TNF-α expression. Cells were treated with distinct doses of SAC two hours before exposure to 6-OHDA. Data are expressed as the means ± SD. *$p < 0.05$ vs. 6-OHDA group.

provided neuroprotection in the cornu ammonis 3 areas and dentat girus, but not in the cornu ammonis 1 area, on neuronal demise induced by amyloid beta + ibotenic acid in certain hippocampus regions. This shows that the neuroprotective impact of SAC is cell-type and area-specific. SAC also has an anti-inflammatory effect by reducing the release of inflammatory cytokines like IL-6, IL-1β, IL-1, and TNF-α and suppressing inflammatory pathways like cyclooxygenase-2 and nuclear factor kappa B. Because of these effects, they protect for inflammation-connected neurodegeneration and neuroinflammation [39–41]. Our in vitro PD model study found that the expression levels of TNF-α, IL-8, and IL-1 (19.15, 16.8, and 18.32 fold chance, respectively) were increased in the 6-OHDA group. However, a significant decrease was found in the elevated levels of TNF-α, IL-8, and IL-1 (10.04, 11.79, and 8.39 fold chance, respectively) after pretreatment of SAC, especially at the dose of 80 µg/mL. Reduced levels of neuroinflammation indicate that SAC has anti-mitochondrial dysfunction, anti-neuroinflammatory, and antioxidative effects.

8-OHdG, the oxidized product of DNA, is the most frequently measured biomarker of oxidative stress [42]. Both in vitro and in vivo studies of the PD model demonstrated 6-OHDA mediated 8-OHdG accumulation in MPTP and SH-SY5Y cells in the mouse striatum [43].

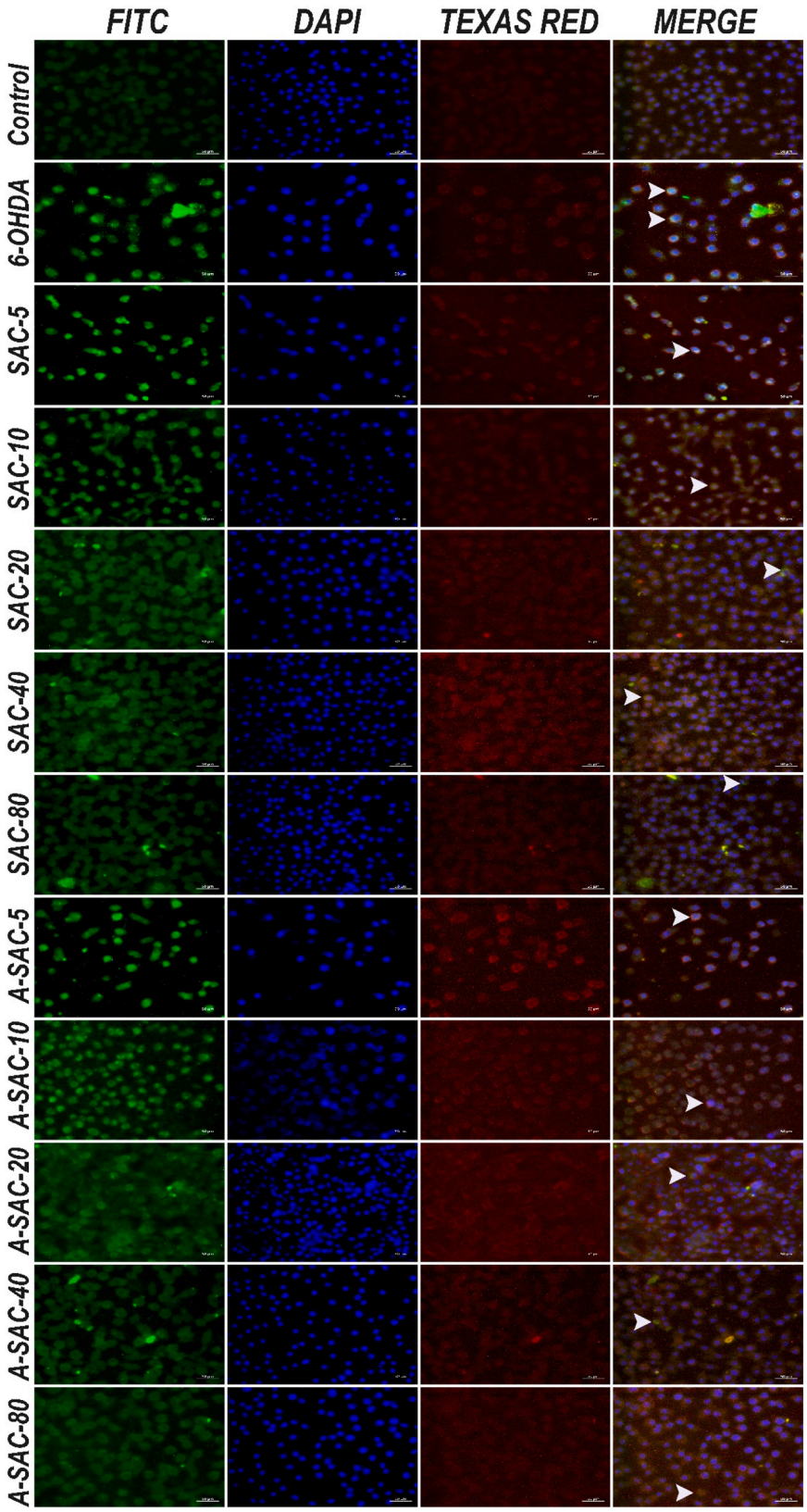

Fig. 4. Cell culture, 8-OHdG (FITC) and Nop10 (Texas Red) images, IF, Bar:50 μm.

Consistent with this study, in our research, 6-OHDA induced the accumulation of 8-OHdG, and SAC partially suppressed this accumulation. Nop10 is needed for telomere maintenance and ribosome biogenesis and plays a major role in carcinogenesis [44]. An invasive breast cancer study showed that there was a significant relationship between high expression of Nop10 at both mRNA and protein levels and bad prognostic clinicopathological parameters and poor survival, and its significance in the progress of invasive breast cancer [45]. Moreover, these

Table 1
Results and statistical analysis of immunofluorescence findings in cell culture.

	8-OHdG	Nop10
Control	20.16±0.57[a]	18.45±0.37[a]
6-OHDA	104.83±2.49[b]	98.59±2.48[b]
SAC−5	82.6±1.49[c]	71.59±1.29[c]
SAC−10	80.45±2.06[c]	69.5±2.12[c]
SAC−20	62.94±2[d]	53.44±1.89[d]
SAC−40	41.37±1.42[e]	37.92±1.66[e]
SAC−80	29.15±1.68 [f]	25.81±1.66 [f]
A-SAC−5	103.29±1.68[b]	98.26±2.15[b]
A-SAC−10	100.57±2.33[b]	95.81±2.6[b]
A-SAC−20	79.46±1.75[c]	68.9±1.43[c]
A-SAC−40	60.84±1.03[d]	51.83±1.35[d]
A-SAC−80	41.18±1.51[e]	35.59±1.49[e]

Distinct letters on the identical line show a statistically meaningful distinct ($p < 0.05$).

data are consistent with a study showing that changes in Nop10 mRNA are related to bad prognosis in endometrial cancer [46]. Nop is a critical player in cellular stress. According to our findings, Nop10 protein expression in the 6-OHDA group was observed mostly in the nucleolus and nucleus of the cells. Dose-dependent a mild expression level was observed in the treatment groups.

5. Conclusion

We found that SAC, a potential compound for the repair of PD in vitro, provides neuroprotection. This study demonstrated the positive effect of pretreatment with SAC (5–10–20–40 and 80 μg/mL) on SH-SY5Y cells injured by 6-OHDA, revealing the in vitro efficacy of SAC in regulating oxidative stress and neuronal survival in the in vitro model of PD. Nevertheless, further preclinical and clinical studies are required to demonstrate the effectiveness of SAC drugs in PD patients.

Research involving human and animal rights

This article includes no studies that have been done with human participants or animals.

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CRediT authorship contribution statement

Yesim Yeni: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization, Writing – review & editing. **Betül Cicek:** Investigation, Formal analysis, Data curation, Conceptualization. **Serkan Yildirim:** Writing – review & editing, Investigation, Formal analysis, Data curation. **İsmail Bolat:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Ahmet Hacimuftuoglu:** Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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