Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Short-term effects of retinoic acid on the proliferation of SH-SY5Y cells via mitophagy and apoptosis

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Article Info

Article history:

Received: March 25, 2024
Accepted: September 05, 2024
Published: November 30, 2024

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Abstract

Neuroblastoma shows the highest lethality in childhood and has poor prognosis at high grade. Our objectives included determining how retinoic acid affected the growth of neuroblastoma cells and the relationship between chemicals unique to neurons and cell death processes like apoptosis and mitophagy. The 50% inhibitory concentration of retinoic acid on SH-SY5Y neuroblastoma cells was determined at the 24th, 48th and 72nd hours. At the optimal concentration of retinoic acid on SH-SY5Y cells, Ki-67, cytochrome C, HIF-1α, Parkin, α-synuclein, DJ-1 and tyrosine β- hydroxylase gene expressions were determined by using RT-PCR. Tyrosine β-hydroxylase protein expression was assessed by ELISA. The optimal time and concentration for retinoic acid in SH-SY5Y cells was 10 µM at the 24th hour. The decreased gene expressions of Ki-67, α-synuclein, DJ-1 and tyrosine β-hydroxylase were observed while Cyt C, HIF-1α and Parkin gene expressions were up-regulated ($p<0.001$). Tyrosine β-hydroxylase protein expression increased at the 24th and 72nd hours although it decreased at the 48th hour ($p<0.001$). Retinoic acid has short-term effect on the proliferation of SH-SY5Y neuroblastoma cells. It was observed that short-term retinoic acid treatment improved neurodegeneration parameters, but it decreased the proliferation by inducing mitophagy and apoptosis of SH-SY5Y neuroblastoma cells.

Keywords: Apoptosis, Mitophagy, Neuroblastoma, SH-SY5Y, Retinoic Acid

1. Introduction

Neuroblastoma is the most common solid extracranial neoplasm in children, accounting for approximately 10% of childhood cancers and 15% of cancer deaths in children. Approximately 90% of patients with early-stage neuroblastoma respond well to treatment, while the survival rate for late-stage neuroblastoma patients is at most 50% [1].

Retinoic acid is one of the intermediates formed because of the metabolism of vitamin A, which is essential for growth and development. Retinoic acid is also a potent compound in stimulating differentiation, inhibiting cell growth, and reducing tumorigenesis. Retinoic acid has been shown to inhibit the growth of breast cancer cells and reduce tumor numbers in animal models [2]. The antiproliferative effect of retinoic acid has been demonstrated in several types of cancer, including lung cancer, cervical cancer, prostate, breast, ovarian, bladder, colorectal cancer, and neuroblastoma [3].

Ki-67 is a nuclear protein associated with cell proliferation, and Ki-67 expression correlates with tumor cell growth, making it a valuable tool for estimating the proliferation index of tumors in routine pathology. It has revealed the importance of Ki-67 as a prognostic marker in several types of cancer, including head and neck squamous cell carcinoma and Merkel cell carcinoma, where its expression correlates with clinical outcomes and tumor aggressiveness [4]. Different forms of Ki-67 reflect baseline proliferation and tumor sensitivity to neoadjuvant systemic therapy in the assessment of response to treatment in breast cancer [5]. Exactly the opposite of the Ki-67; cytochrome C (Cyt C) is a protein found in the intermembrane space of mitochondria and participates in cell death. With the onset of apoptosis, Cyt C moves into the cytosol and activates caspases to cause cell death [6].

Mitophagy is a type of autophagic cell death that occurs in damaged or senescent mitochondria. Mitophagy has two pathways. One is related to Parkin, and the other is related to Hypoxia-inducible factor one alpha (HIF-1α). HIF-1α is a key regulator of cellular responses to hypoxia and plays a significant role in various physiological and pathological processes. HIF-1α has been shown to be involved in

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.11.9

the regulation of proliferation, vascularization, invasion, mitophagy and epithelial-mesenchymal transition (EMT) in different cell types and disease conditions [7]. HIF-1α has been shown to attenuate high glucose-induced renal tubular cell injury by promoting Parkin/PINK1-mediated mitophagy, highlighting its cytoprotective role in reducing cellular stress [8]. HIF-1α has also been shown to affect proliferation, apoptosis, cell cycle, and metastasis in cervical cancer cells [9]. Parkin, like HIF-1α, is a molecule involved in the regulation of mitochondrial function and initiator of autophagic removal of mitochondria from the cell. Parkin participates in the clearance of misfolded proteins through the ubiquitin-proteasome system and regulates mitophagy. A defect in the mitophagy system leads to the accumulation of damaged mitochondria, carcinogenesis, and tumor progression [10].

α-synuclein protein binds to the cell membrane and regulates the release of synaptic vesicles by degrading phospholipase D activity. α-synuclein mutations disrupt the α-helix structure of target proteins and trigger aggregate formation with a β-pleated sheet structure. α-synuclein aggregates are cytotoxic, and mitochondria and organelles have been suggested to be cellular targets of α-synuclein in α-synuclein-induced neurotoxicity [11]. A study reported that α-synuclein has an inhibitory effect on membrane fusion and directly leads to mitochondrial fragmentation when overexpressed in cell cultures [12]. α-synuclein, which induces oxidative stress, also causes cytochrome c release in mitochondria and increases mitochondrial calcium and nitric oxide. Furthermore, Parkin protein regulates α-synuclein metabolism through the ubiquitin-proteasome system, preventing the accumulation of excess α-synuclein in the cytoplasm. DJ-1 serves as a redox-sensitive molecular cytoprotective chaperone involved in preventing α-synuclein accumulation. 11 different mutations have been identified in the DJ-1 gene located at the PARK-7 locus [13]. Overexpression of DJ-1 is induced by many oxidative agents such as cancer cells [14]. Furthermore, DJ-1 has a protective role against α-synuclein-induced toxicity [15].

The rate-limiting enzyme in the production of the neurotransmitter dopamine is tyrosine beta-hydroxylase. Abnormal tyrosine metabolism is known to play a role in cancer development. Reduced blood tyrosine levels have been reported in stage I and stage IV esophageal malignancies [16]. Tyrosine hydroxylase expression is reported to be regulated by epidermal growth factor receptor (EGFR) signaling in esophageal cancer cells [17].

In this study, we determined the short-term impact of retinoic acid on SH-SY5Y cell proliferation for 24 hours. In addition, retinoic acid was found to cause mitophagy and consequent apoptosis, even though it improved neuronal degeneration in this short term. These findings all point to the possibility that retinoic acid is not a beneficial supplement for the treatment of neuroblastoma.

2. Materials and Methods

2.1. Cell Culture, Retinoic Acid Treatment and 50% Inhibitory Concentration (IC50) Determination

SH-SY5Y cells (ATCC, Washington, DC, USA) were kept at 37°C in a humidified environment containing 5% CO_2. Growth media consisted of phenol red-free Dulbecco's Modified Eagle's media (DMEM; Gibco, UK) with 10% fetal bovine serum (FBS; Gibco, UK) and 1% Penicillin/Streptomycin (Capricorn, Germany). After confluency, cells were collected with trypsin. $7x10^3$ SH-SY5Y cells were seeded into 96-well plate (Greiner, Australia) with seven replicates. A sham group was utilized to investigate the effect of retinoic acid's (Cayman, USA) solvent, ethanol. SH-SY5Y cells were treated with different doses of retinoic acid (20 µM, 10 µM, and 5 µM respectively). The 50% Inhibitory Concentration (IC50) of retinoic acid on SH-SY5Y cells was detected by using XTT Kit (Biological Industries, Israel) at the 24th, 48th and 72nd hours after retinoic acid treatments. The SH-SY5Y cells were placed in 96 well plate (Greiner, Germany) and following different concentrations of retinoic acid treatment. The XTT solution was added to all wells and cultivated for 2 hours at 37°C with 5% CO_2. The absorbance at 450 nm was determined for all wells at the 24th, 48th and 72nd hours. IC50 or/and proliferation was detected by using microplate reader (Biotek, Japan).

2.2. Total RNA Isolation and Real Time Polymerase Chain Reaction (RT-PCR)

Total RNA was obtained using a Nucleospin RNA Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Total RNAs were reverse transcribed into cDNA (Bioneer, USA). The cDNA reverse transcription was performed at 42°C for 60 min and 95°C for 5 min. SYBR Green primer sets for the amplification of Ki-67, Cytochrome C (Cyt C), Hypoxia Inducible Factor-1 Alpha (HIF-1α), Parkin, α-synuclein, DJ-1, tyrosine β- hydroxylase, and Glyseraldehide-3-phosphate dehydrogenase (GAPDH) were designed and supplied by Bmlabosis (Ankara, Türkiye). The primer sequences are shown in Table 1. The RT-qPCR was performed under the following conditions: 5 min of pre-denaturation at 95°C, 40 cycles of denaturation at 95°C for 10 s, and 30 s of annealing/extension at 60°C. RT-PCR was performed in Roche Lightcy-

Table 1. Forward and Reverse Primer Sequences used in RT-PCR.

Gene	Forward Sequence	Reverse Sequence
Ki-67	5'-TCCTTTGGTGGGCACCTAAGACCTG-3'	5'-TGATGGTTGAGGTCGTTCCTTGATG-3'
Cyt C	5'-TCGTTGTGCCAGCGACTAAA-3'	5'-TCTTGTGCTTGCCTCCCTTT-3'
HIF-1α	5'-GGCGCGAACGACAAGAAAAAG-3'	5'-CCTTATCAAGATGCGAACTCACA-3'
Parkin	5'-CTGCCGGGAATGTAAAGAAGC-3'	5'-CCACAGTTCCAGCACCACTC-3'
α-synuclein	5'-CTTGAATTTGTTTTTGTAGGCTCC-3'	5'-AATGGAGCTTACCTGTTGCCA-3'
DJ-1	5'-GCGAGCTGGGATTAAGGTCA-3'	5'-CCTTCACAGCAGCAGACTCA-3'
Tyrosine β- hydroxylase	5'-CTGCGACCCCAAGGATTACC -3'	5'-GATGGACCGACACGACCTT-3'
GAPDH	5'-CGAGGGGGGAGCCAAAAGGG-'3	5'-TGCCAGCCCCAGCGTCAAAG-3'

cler96 (Vedbaek, Denmark). ΔΔCT formula was used to determine the gene expressions [18].

2.3. Protein Expression by ELISA

Protein expression level of Tyrosine β- hydroxylase was determined by ELISA based on the kit manufacturer (Elabscience, USA). Samples were prepared and collected at the 24th, 48th and 72nd hours after 10 µM retinoic acid treatment in SH-SY5Y cells to determine protein expression based on the kit manufacturer. The kit protocol started with the incubation of samples (cell mediums) for 90 min at 37°C. Then, biotinylated detection antigen was added to each well and incubated for 60 min at 37°C. The next step was washing each well 3 times with phosphate buffer saline (PBS; Gibco, UK). After washing steps, horseradish peroxidase (HRP) was added and incubated for 30 min at 37°C. At the end of the protocol, stop solution was added to each well and then read at 450 nm by using a microplate reader (Biotek, Japan).

2.4. Statistical Analysis

The Kolmogorov-Smirnov test was used to determine if the continuous variables had a normal distribution. Using One-Way variance analysis, comparisons between groups of normally distributed variables were assessed. The Sidak test assessed non-normally distributed variables' multiple comparisons. The software program IBM SPSS Statistics 26.0 was used for all analyses. The acquired data is presented as the mean ± standard deviation.

3. Results

3.1. The Inhibitory Concentration 50 (IC50) of Retinoic Acid for SH-SY5Y cells

According to the obtained data, 10 µM retinoic acid treatment at the 24th hour was effective in decreasing proliferation of treated group (517.125±154.252) compared to control group (1499± 132.401; p<0.001). Although cell death was seen at 24th, 48th, and 72nd hours, in 50% of all cases, no dose proved to be more efficient than 10 µM at 24 hours (Fig. 1).

3.2. Impact of Retinoic Acid on Cyt C and Ki-67 Gene Expressions

Compared to the control group (2.189±0.25), 10 µM retinoic acid-treated SH-SY5Y cells' (1.526±0.11) Ki-67 gene expression decreased (p<0.001). Cyt C gene expression level of 10 µM retinoic acid treated SH-SY5Y cells (6.364±0.23) upregulated compared to the control group (2.908±0.19; p<0.001; Fig. 2).

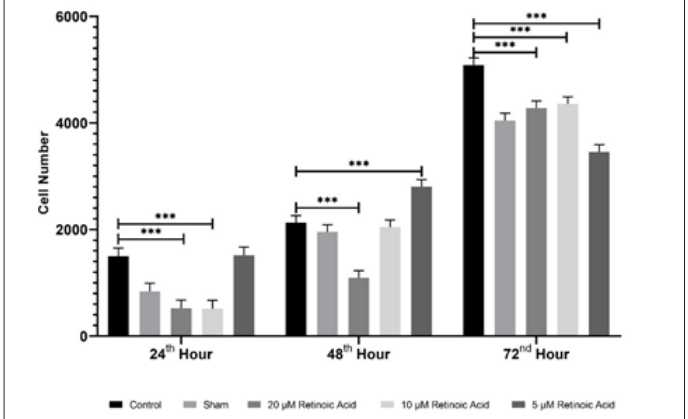

Fig. 1. IC50 values measured after 24, 48, and 72 hours of treatment with control, sham, 20 µM, 10 µM, and 5 µM Retinoic Acid in SH-SY5Y neuroblastoma cells (p<0.001).

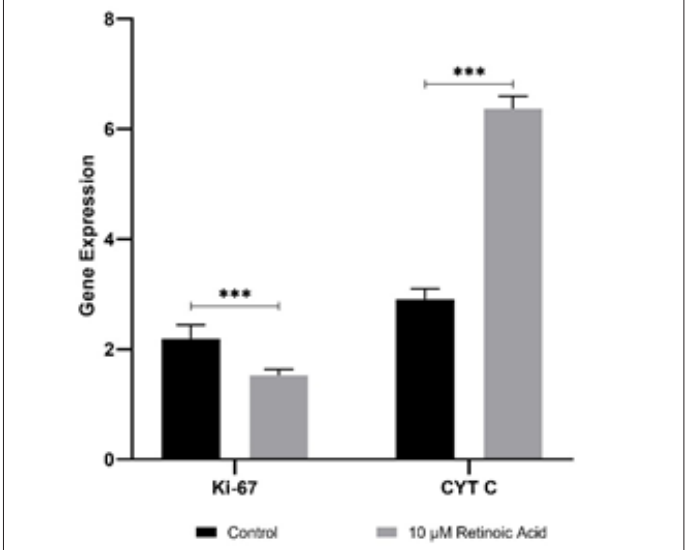

Fig. 2. The effect of 10 µM retinoic acid on the proliferation parameters (Ki-67 and Cyt C) of SH-SY5Y cells (p<0.001).

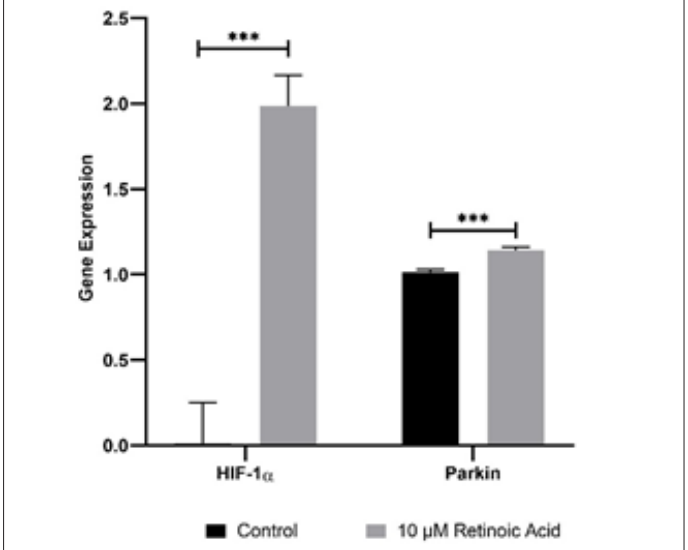

Fig. 3. The effect of 10 µM retinoic acid on the mitophagy pathway starters (HIF-1α and Parkin) of SH-SY5Y cells (p<0.001).

3.3. Impact of Retinoic Acid on HIF-1α and Parkin Gene Expressions

HIF-1α gene expression of 10 µM retinoic acid treated SH-SY5Y cells (1.986±0.18) upregulated compared to the control group (0.011±0.24; p<0.001). Parkin gene expression level of 10 µM retinoic acid treated SH-SY5Y cells (1.141±0.02) upregulated compared to the control group (1.014±0.015; p<0.001; Fig. 3).

3.4. Impact of Retinoic Acid on α-synuclein, DJ-1 and Tyrosine β-Hydroxylase Gene Expressions

α-synuclein gene expression of 10 µM retinoic acid treated SH-SY5Y cells (0.241±0.21) upregulated compared to the control group (0.486±0.29; p<0.001). DJ-1 gene expression level of 10 µM retinoic acid treated SH-SY5Y cells (0.529±0.3) upregulated compared to the con-

trol group (2.129±0.2). The decreased tyrosine beta-hydroxylase gene expression in 10 µM retinoic acid-treated SH-SY5Y cells (0.555±0.2) was observed compared to the control group (1.073±0.09; p<0.001; Fig. 4).

3.5. Tyrosine β-Hydroxylase Protein Expression and Retinoic Acid

No difference was observed between the sham groups and the control groups for all three time periods (p>0.05). In the 10 µM retinoic acid treated SH-SY5Y cells, Tyrosine β-Hydroxylase protein expression increased at the 24th (65.6±0.88) and the 72nd hour (59.4±0.02) and decreased at the 48th hour (51.5±0.83) compared to the control groups (55±0.91; 54.2±0.89 and 47.5±0.012 respectively; p<0.001; Fig. 5).

4. Discussion

Research on the effects of retinoic acid on several types of cancer reveals its potential as a promising tool in cancer therapy. Several mechanisms including inhibition of cell growth, suppression of migration, restoration of sensitization and modulation of cancer cell phenotype, highlight retinoic acid in the prevention and treatment of cancer in the literature. Teppola et al. (2016), it was thought that retinoic acid-induced differentiation in neuroblastoma patients could be used to stop tumor growth, inhibit cell proliferation and form healthy mature neurons [19].

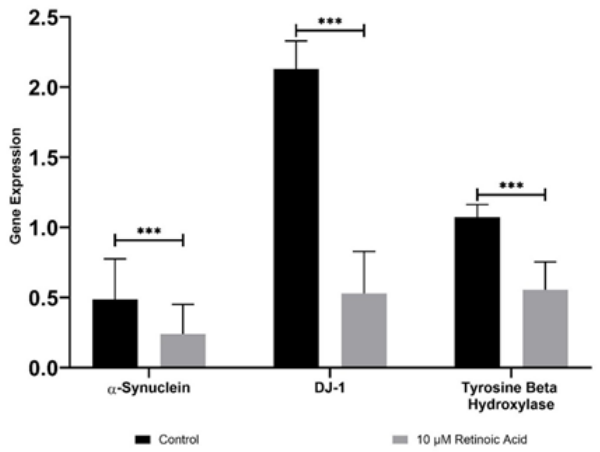

Fig. 4. The effect of 10 µM retinoic acid on the neurodegenerative parameters (α-synuclein, DJ-1 and Tyrosine Beta Hydroxylase) of SH-SY5Y cells (p<0.001).

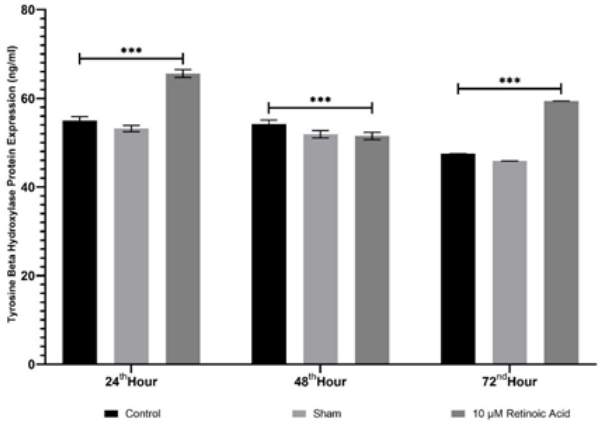

Fig. 5. The effect of 10 µM retinoic acid on the protein expression of Tyrosine Beta Hydroxylase in SH-SY5Y cells at the 24th, 48th and 72nd hours (p<0.001).

The increase of the retinoic acid-degrading enzyme CYP26 is thought to be the cause of resistance to retinoic acid treatment in neuroblastoma and acute promyelocytic leukemia. This phenomenon has been extensively investigated and it has been suggested that increasing the dosage of retinoic acid or inhibiting the CYP26 enzyme may prevent this resistance. This indicates that the metabolism of retinoic acid makes it unsuitable for long-term usage without the need for a supplementary drug [20]. The efficient use of retinoic acid alone is hindered by the fast metabolism of retinoic acid by cytochrome p450 and epigenetic modifications in humans. It has been noted that these factors contribute to the retinoic acid's decreased impact with prolonged therapy [21]. 10 µM retinoic acid was chosen instead of 20 µM because of the increased lethality. In IC50 assay, we should choose the optimal concentration which caused to reduction of 50% proliferation. At the 24th hour, both 20 µM and 10 µM concentrations suppressed proliferation by 50%, however, the greater than 50% enhanced lethality of 20 µM persisted after 48 hours.

This study has some limitations. In this study, only SH-SY5Y neuroblastoma cells could be examined, and the retinoic acid applied was only applied to cancer cells, and its effect on healthy cells could not be examined. Due to the limited project funding, genes and proteins involved in different pathways related to the subject could not be analyzed and advanced methods could not be applied.

The genetic factors used in the study were chosen from three perspectives. Ki-67 and Cyt C levels were measured to assess changes in cell proliferation. Tyrosine β hydroxylase, α-synuclein, and DJ-1 were identified as key indicators of neurodegeneration-induced cell proliferation. The analysis of Parkin and HIF-1α focused on mitophagy, a process linked to mitochondrial dysfunction.

Ki-67 has been studied in the context of many tumor forms, including ameloblastic tumors, and has been determined to be a particular marker for tumor cell proliferation [22]. Ki-67 has been studied in the context of pituitary adenomas, where its expression has been linked to hormone phenotype and invasive behavior, underlining its significance in evaluating tumor proliferation and behavior [4]. The importance of Ki-67 as a proliferation marker was highlighted in SH-SY5Y neuroblastoma cells. The SH-SY5Y neuroblastoma cell line was utilized as a model to evaluate cell proliferation and Ki-67 expression [23]. Cytochrome c is released from mitochondria into the cytosol, where it activates caspases and triggers apoptotic pathways. Exogenous cytochrome c activates apoptosis without additional stimulus [6]. Retinoic acid was found to increase mitochondrial membrane potential and cytochrome C oxidase levels [24]. The treatment with retinoic acid reduced the growth of SH-SY5Y cells. The gene expression of Cyt C further supports these findings. The high expression of Cyt C suggested that the retinoic acid therapy could have triggered apoptosis. According to knowledge, the release of Cyt C from mitochondria into the cytosol is the first step in apoptosis. The decrease in Ki-67 gene expression is dependent on the rise in Cyt C gene expression.

HIF-1α is involved in the maintenance of mitochondrial homeostasis and cell survival [7]. In the context of retinal protection against hypoxia-induced by anti-vascu-

lar endothelial growth factor treatment, HIF-1α induction was associated with upregulation of mitophagy-related proteins, highlighting its role in attenuating hypoxia-induced damage [25]. HIF-1α has been found to enhance migration and invasion in a variety of malignancies, including gastric cancer, hepatocellular carcinoma, and papillary thyroid carcinoma, showing its role in tumor growth and metastasis [26, 27]. Parkin, an E3 ubiquitin ligase, is a neuroprotective protein that also destroys damaged mitochondria via the mitophagy pathway [28]. Deletion or inactivation of the Parkin gene, which has tumor suppressor properties, leads to the development of mutations that disrupt function and cause cells to grow and proliferate uncontrollably [10]. According to research on colorectal and breast cancer, parkin gene suppression reduces breast cancer growth, and may be a predictive factor for individuals with colorectal cancer [29]. The final stage in mitophagy is apoptosis. At the start, there are two pathways that govern mitophagy. HIF-1α and Parkin initiate these pathways. According to our findings, both pathways could be activated by retinoic acid. Increased gene expression of HIF-1α and Parkin stimulated mitophagy, leading to apoptosis via Cyt C. We hypothesized that this indicated mechanism results in decreased proliferation.

Overexpressed α-synuclein accumulates in the mitochondria of neuroblastoma cells and causes mitochondrial dysfunction by increasing oxidative stress [30]. Watanabe et al (2012) suggested that toxic oligomeric α-synuclein may affect impaired mitochondria accumulation or autophagy/mitophagy dysfunction [31]. In SH-SY5Y cells treated with retinoic acid, elevated levels of α-synuclein expression reduced neuronal development and dopaminergic activity. DJ-1 is a protein involved in a variety of processes, including the antioxidative stress reaction and mitochondrial regulation. Increased DJ-1 levels expected to improve cell viability by lowering oxidative stress-induced death. It was investigated whether DJ-1 could be used as a prognostic marker in pancreatic, cervical, and many other cancer types. It was discovered that DJ-1 gene expression levels increased in advanced-stage cancers, leading to the conclusion that DJ-1 can provide information about tumor aggressiveness and prognosis [32]. Tyrosine levels are lower in blood samples from esophageal cancer patients compared to healthy controls [33]. Tyrosine β-hydroxylase levels were raised during the progression and regression of micrometastases in children with stage 4 neuroblastoma [34]. Franco et al. (2009) showed that SH-SY5Y cells transfected with tyrosine beta-hydroxylase were more resistant to cell death caused by oxidative agents [35]. Tyrosine beta-hydroxylase levels were also considered to be useful for determining the stage of neuroblastoma patients [36]. These three genes are the primary markers of neurodegeneration. In this work, we attempted to understand how these genes relate to mitophagy and apoptosis. According to the results, retinoic acid treatment decreased the expression of these three genes. Previous research suggests that these three genes are also associated with mitochondrial function and oxidative stress. Both enhanced gene expression of mitophagy parameters and lower proliferation indicate that retinoic acid treatment influenced these three genes and indirectly altered mitophagy.

5. Conclusion

Unlike the mitophagy and apoptosis studies, this study shows how the α-synuclein, DJ-1 and tyrosine beta-hydroxylase normally used to assess neurodegeneration indirectly affect these mechanisms. However, it was discovered that retinoic acid causes cell death and targets mitochondria. It was discovered that retinoic acid had no long-term impact on an advanced and extremely aggressive cancer type like neuroblastoma, but gradually lost its efficacy after the first 24 hours. Though not recommended to totally eliminate cancer cells, retinoic acid may be used at the beginning of treatment to prepare cancer cells for subsequent therapies. Instead of utilizing retinoic acid alone to treat neuroblastoma, it is expected that retinoic acid combined with another drug will be more effective.

Acknowledgements
Not applicable.

Conflict of interest
All authors have to declare their conflict of interest.

Consent for publications
The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The authors have to declare that they embedded all data in the manuscript.

Authors' contributions
Dilara AYDIN; Project administration, Funding acquisition, Conceptualization, Methodology, Software, Validation, Investigation, Visualization, Supervision, Writing- Reviewing and Editing.
Çağrı ÖNER; Corresponding Author, Project administration, Funding acquisition, Conceptualization, Methodology, Software, Validation, Investigation, Visualization, Supervision, Writing- Reviewing and Editing.
Senem ASLAN ÖZTÜRK; Methodology, Validation, Investigation, Visualization, Writing.
Ertuğrul ÇOLAK: Data curation, Formal analysis, Writing- Original draft preparation.

Funding
This research was funded by Maltepe University Scientific Research Projects Commission.

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