Squalene suppresses Jam-A, Claudin 5, and Occludin by accelerating cell death and reducing neuronal interaction in the neuroblastoma cell line SH-SY5Y

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ABSTRACT: Although squalene is an isoprenoid compound that plays a role in cholesterol biosynthesis, studies on its molecular mechanism and biological activities on cells have been limited. However, in recent years, it has been found as a functional ingredient in nutrition and taken from various sources due to its anticancer effects. In this study, we evaluated the effect of Squalene on tight junction proteins and cell apoptosis, which are effective on metastasis in neuroblastoma. Squalene was applied at a 1-100 µg/mL concentration to SH-SY5Y human neuroblastoma cell line. Cell viability, colony formation, wound healing, and Annexin-V binding were evaluated with various doses of squalene. In addition, immunofluorescence staining determined junctional adhesion molecule-A (Jam-A), Claudin-5 (Cldn5), and Occludin (Ocln) levels. As a result, squalene in SH-SY5Y cells at 25 µg/mL concentration increases cell death, suppresses colony formation and migration levels, triggers apoptosis, and suppresses Cldn5, Ocln, and Jam-A levels significantly. The mechanism of action of squalene, taken as a supplement, is essential in preserving neurological functions and detecting drug interactions in tumors such as neuroblastoma, which can metastasize and are common in early childhood.

KEYWORDS: Apoptosis, caspases, neuroblastoma, squalene, tight junction

1. INTRODUCTION

Neuroblastoma (NBL) is the most common extracranial solid tumor in children originating from the neural crest cells of the sympathetic nervous system [1]. NBLs are characterized by poor prognosis and account for 15% of pediatric cancer deaths [2]. NBL treatment is based on a combination of several chemotherapeutic drugs. However, these drugs cause cytotoxic effects and the 5-year survival rates are still low [1]. The most important reason limiting the use of chemo-and-radiotherapy combinations in cancer treatment is multi-organ toxicity as a result of tissue damage. Hence, there is a need for alternative approaches with little or no side effects in the treatment of neuroblastoma. Variou s antioxidant supplementations have been shown to protect tissues from toxicity caused by chemotherapy by scavenging free radicals [3].

Squalene (SQ) is a naturally occurring polyunsaturated triterpene [4]. SQ is a precursor for the biosynthesis of secondary metabolites including cholesterol and other sterols and hormones in cells ranging from prokaryotic bacteria to eukaryotic mammalian cells [5]. SQ, which is widely found in nature, is an essential component of shark liver oil and also found in higher concentrations in many vegetable oils, especially extra-virgin olive oil [6]. In humans, it is found in all tissues, especially in the highest concentration in the skin. As a matter of fact, in epidemiological studies, the lower incidence of cancer in the Mediterranean basin suggested that it could be related to daily squalene intake (from olive oil) [7]. It has also been reported that squalene has detoxifying activity against some chemicals such as hexachlorobenzene, arsenic, and theophylline [3]. Studies conducted in both *in vitro* and *in vivo* animal models have shown that squalene has protective effects against chemotherapy-induced toxicity.

It has been shown that dose-dependent administration of SQ is protective against cisplatin-induced bone marrow toxicity, reduces apoptosis, and this protective effect is equivalent to that of reduced glutathione (GSH), a cytoprotective agent. In the same study, it was also reported that SQ significantly inhibited the growth of NBL colonies and increased morphological changes suggestive of differentiation [3]. In another study conducted by the same authors, it was reported that in a dose-dependent manner, SQ reduced tumor growth while protecting healthy cells in the tumor microenvironment against carboplatin and cisplatin toxicity *in vivo* mouse model [3]. In this study, the effect of squalene on SH-SY5Y neuroblastoma cells was examined and its functional role was investigated on tight junction proteins.

2. RESULTS

2.1. Squalene reduced the viability by inhibiting the survival and proliferation of the human neuroblastoma cell line

Initially, we investigated the effect of various concentrations of SQ (1, 10, 25, 50, and 100 µg/mL) on cell viability of the human neuroblastoma cell line. The MTT assay was performed to measure NBL cell viability, as previously described [8]. Cell viability was detected 94±3.11%, 77±5.68%, 61±6.25%, 48±4.10%, and 42.2±4.16% at 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL concentrations of squalene, respectively. MTT analysis showed that the cell viability was reduced in a dose-dependent manner after SQ treatment and cell viability decreased up to 42.2% at the highest squalene concentration ($P<0.05$). In addition, the long-term effects of squalene were investigated by examining the effects of squalene on the proliferative capacity of the colonies formed in SH-SY5Y cell cultures. Squalene treatment was found to cause a significant concentration-dependent reduction in the number and size of colonies (Fig1a), and a SQ concentration of 25 µg/mL and above significantly inhibited the colony forming ability ($P<0.05$; Fig. 1b).

According to the wound healing results, it was determined that even the lowest SQ treatment dose (1µg/mL, Fig2a) decreased the migration ability of SH-SY5Y cells. Furthermore, the wound size was also significantly increased due to the high cell death induced by increasing concentrations of SQ ($P<0.05$; Fig. 2b).
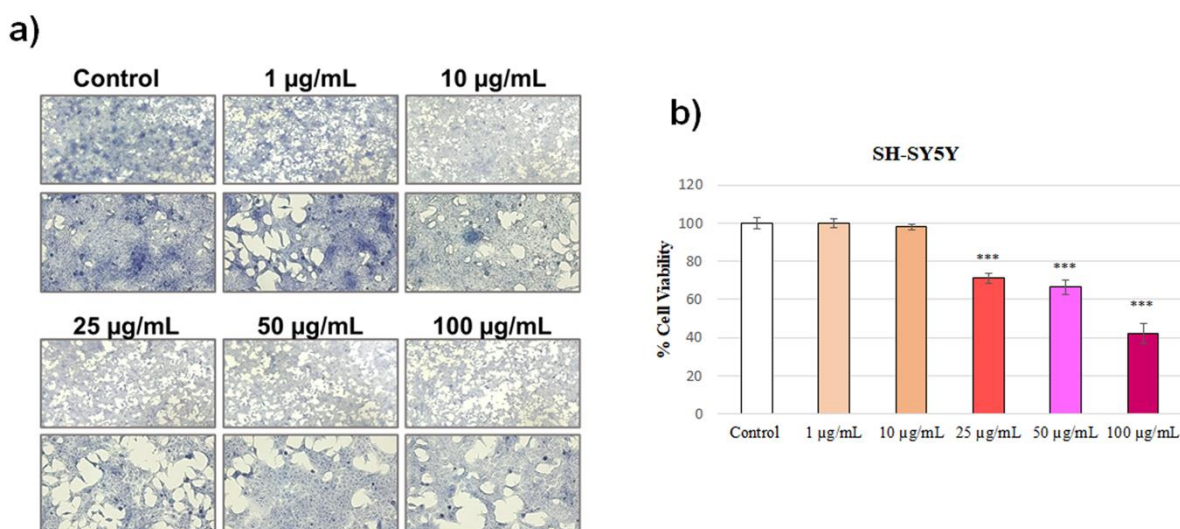

Figure 1. Colony formation of SH-SY5Y cells inhibited with SQ. a) Crystal violet staining for colonies in SH-SY5Y cell cultures treated with the different SQ concentrations. b) The average relative number of crystal violet staining colonies (***p<0.001 compare to control).
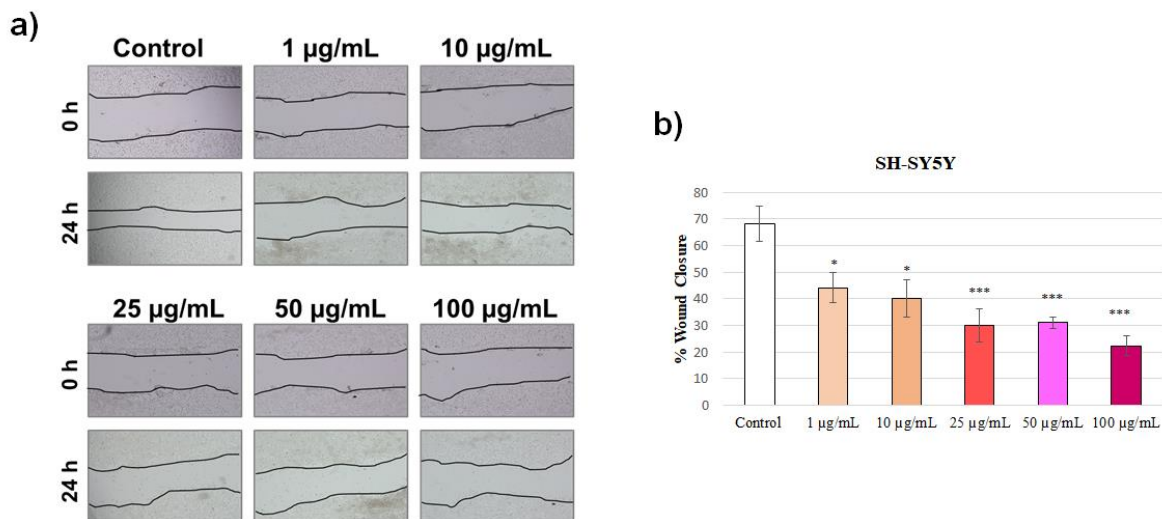
Figure 2. Effects of SQ on cell migration in SH-SY5Y cells. a) Cell migration measurement with scratch wound healing assay SQ (1, 10, 25, 50, and 100 µg/mL) for 24h treated SH-SY5Y cells. b) The rate of wound closure was calculated differences of cells filling the scratched area for the three biological replicates within each group (*p<0.05, ***p<0.001 compared to control).

2.2. Squalene induced apoptosis in the human neuroblastoma cell line

TheAnnexin V assay was performed to investigate the increase in cell death induced by SQ. Annexin V detects the externalization of phosphatidylserine to the cell surface and includes a dead cell marker (7-ADD). Therefore, cells stained negative for Annexin V (lower left quadrant) were healthy cells, cells stained positive for only Annexin V (lower right quadrant) were in early apoptosis, cells stained positive for both Annexin V and 7AAD (upper right quadrant) were in late stage apoptosis, and cells stained positive for only 7AAD (upper left quadrant) were in necrosis. As shown in Figure 3, the total percentages of Annexin V positive cells (undergoing early and late apoptosis) were 15%, 17%, 26%, 27%, and 33% after treatment with SQ at 1, 10, 25, 50, and 100, µg/mL, respectively. According to the Annexin V staining results, treatment with squalene reduced SH-SY5Y cell viability and promoted apoptosis.
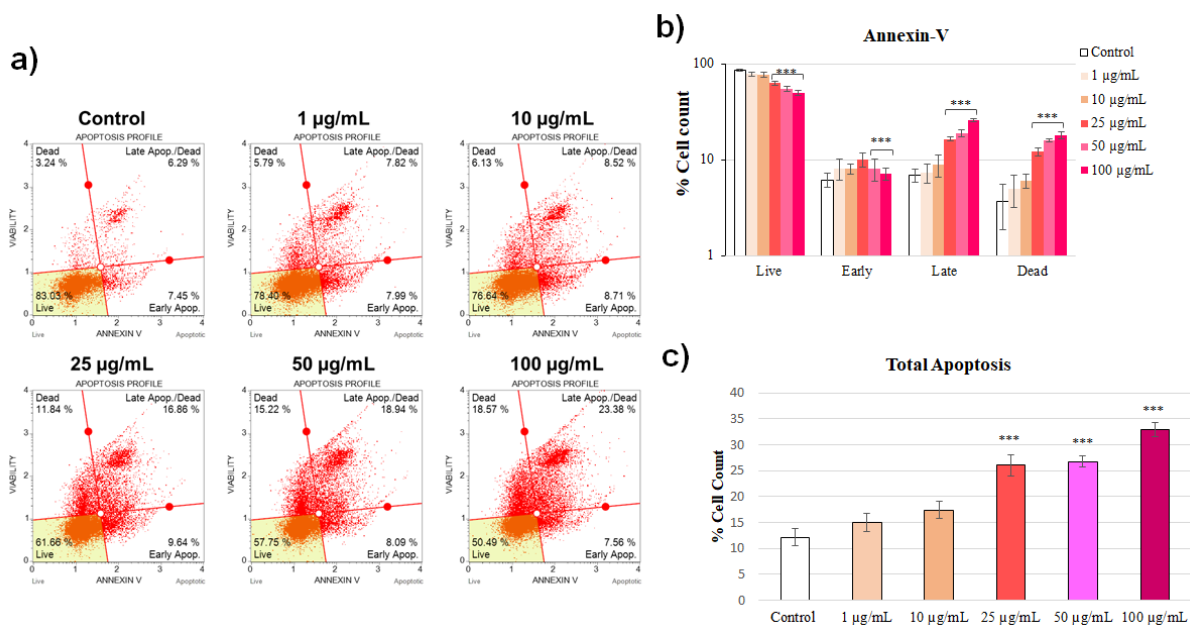

Figure 3. Effect of SQ on SH-SY5Y cells apoptosis. a) Original dot plots of the 7-AAD versus the Annexin V/FITC fluorescence intensities (logarithmic scales). b) Bar diagram shows the percentages of live cells, and early and late apoptotic cells, and dead cells. c) Bar diagram shows the percentages of cells undergoing apoptosis (***p<0.001 compared to control).

2.3. Squalene suppressed Jam-A, Claudin-5 and Occludin

To see the effect of squalene on tight junction proteins, Occludin, Claudin-5, and Jam-A staining were evaluated as immunofluorescence in SH-SY5Y cells (Fig4a, 5a, 6a). Jam-A (Fig 4b), Claudin-5 (Fig 5b) and Occludin (Fig 6b) protein levels in SH-SY5Y cells were significantly decreased after 24 hours of incubation at 25 µg/mL concentration of squalene (P<0.001). This study showed that tight junction proteins were decreased after 25 µg/mL SQ treatment.
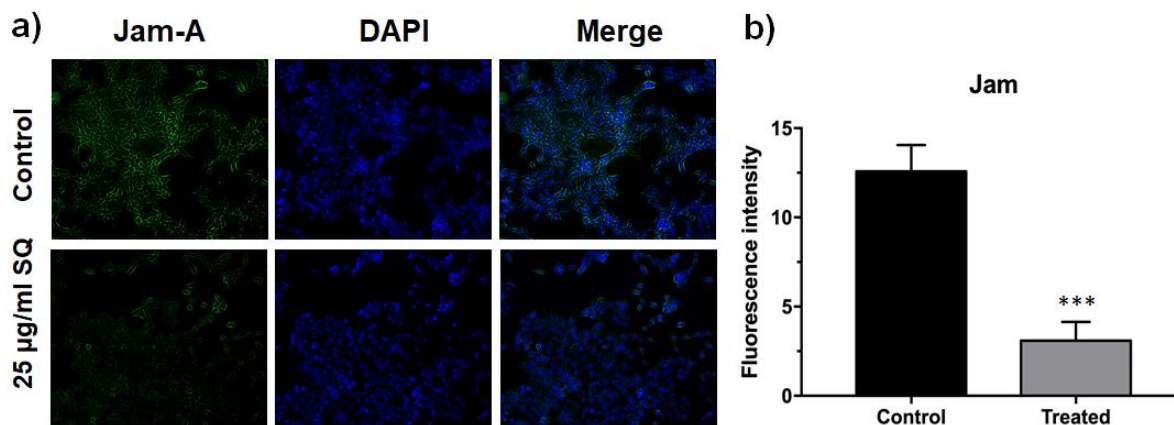

Figure 4. Immunofluorescence staining for Jam-A in SH-SY5Y cell in the control and treatment (25 µg/mL) groups. (a) Immunofluorescence staining. (b) Relative fluorescence intensity. All experiments were performed three times (***p<0.001 compared to control).

Figure 5. Immunofluorescence staining for Claudin-5 (Cldn5) in SH-SY5Y cell in the control and treatment (25 µg/mL) groups. (a) Immunofluorescence staining. (b) Relative fluorescence intensity. All experiments were performed three times (***p<0.001 compared to control).

Figure 6. Immunofluorescence staining for occludin in SH-SY5Y cell in the control and treatment (25 µg/mL) groups. (A) Immunofluorescence staining. (B) Relative fluorescence intensity. All experiments were performed three times (***p<0.001 compared to control).
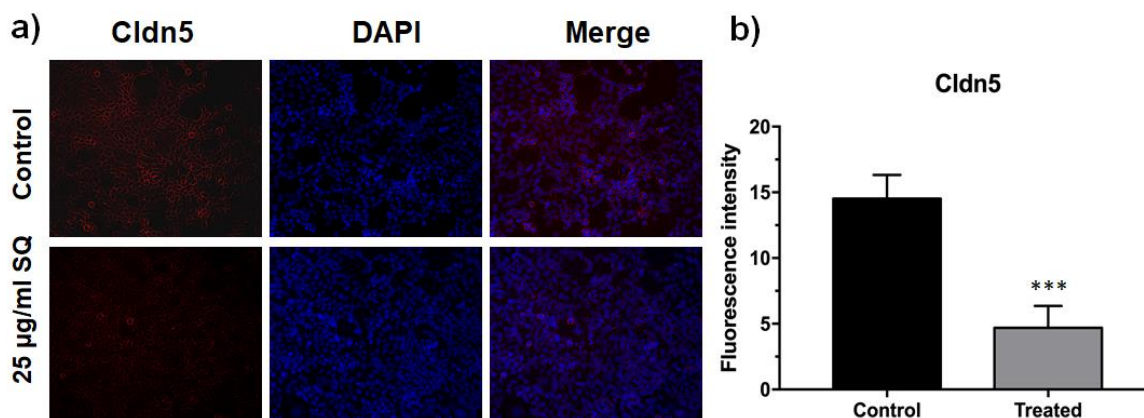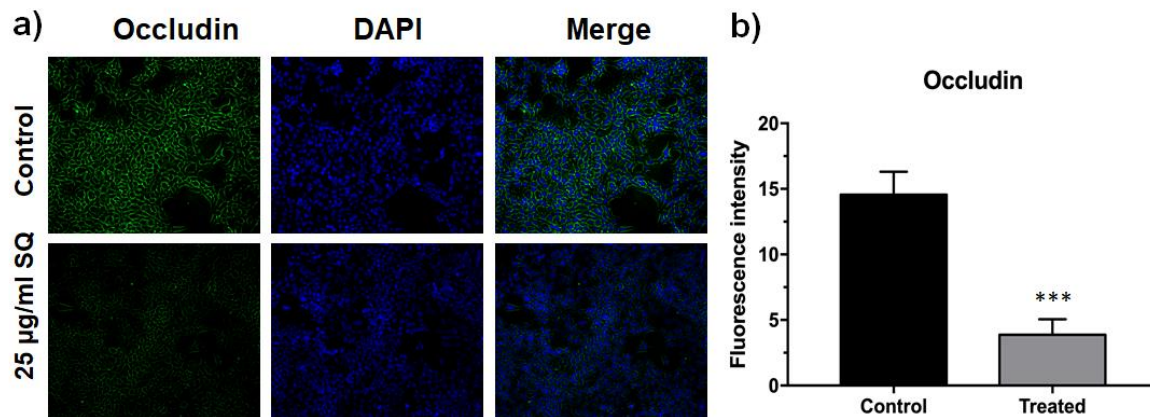
3. DISCUSSION

Cancer treatments are applied according to the type and size, including chemotherapeutic drugs, radiotherapy, phytotherapy, phototherapy, and surgery. Recent studies have reflected that the effects of supplements used in the treatment of cancers and some essential biomolecules taken through food should be investigated in determining the new effects of pharmacological agents on cellular mechanisms.

Squalene is an isoprenoid compound, an intermediate metabolite in cholesterol synthesis, used to potentiate the effects of drugs added to diets or taken as supplements [9]. Squalene is an adjunct therapy in various cancers, but to date, no adequate clinical studies have been conducted to confirm this compound's role in cancer treatment regimens. There seems to be a need for mechanistic studies on its effect on cells. The antitumor activity of squalene purified from shark liver has been demonstrated in sarcoma 180-bearing female ICR mice. Furthermore, when squalene was administered intraperitoneally for ten days at a dose of 0.1 ml/day in mice, the survival time of the mice was significantly prolonged [10]. A similar study showed that syngeneic L-1 sarcoma transplantation suppressed angiogenesis and significantly reduced tumor growth when combined with squalene and conventional drugs against the tumor formed in Balb/c mice [11]. In a mouse lung tumorigenesis model, dietary olive oil and squalene administration for 16 weeks reduced tumor growth and metastasis and inhibited lung tumorigenesis induced by a single dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone for three weeks [12]. A study showed that oral use of 1% squalene for 16 periods in an azoxymethane-induced mouse colon cancer model prevented colon cancer progression and had chemopreventive activity against colon carcinogenesis [13]. It has been reported that the administration of 25 µg/mL of squalene in Hela cancer cells suppresses the phospholipid interaction and causes inhibition of the cells. In the same study, it was reported that local application of squalene in the mouse skin carcinogenesis model induced by 7,12-dimethylbenz[a]anthracene (DMBA) could suppress the tumor, and this effect was achieved by its effect on phospholipids [14].

In this study, the effect of squalene on neuroblastoma cells and its relationship with tight junction proteins were investigated. SH-SY5Y are cells known as human neuroblastoma cell lines and are widely used in experimental studies, including neurodegenerative disorders, neurodegenerative processes, and neurotoxicity. A study using NBL cell lines (SKNBE-2, NUB-7, LAN-5, GOTO, and IMR-32) showed that squalene led cells to apoptosis at a concentration of 25 µM and above and inhibited colony formation. In the same study, it is reported that it triggers the death of neuroblastomas by making a synergistic effect that squalene does not protect from toxicity caused by carboplatin, cyclophosphamide, etoposide, and doxorubicin [3]. In our study, we showed that squalene induces apoptosis and suppresses cell migration in SH-SY5Y cells at a concentration of 25 µg/mL and above. It has been found that squalene (10-20 µg/mL) added at a low concentration in SH-SY5Y cells has a protective effect on cells, and algae's ethanol extracts containing squalene suppress neuroinflammatory effects and can be used as an antidepressant [15].

Tight junction (TJ) proteins play a role in the coexistence of cells, and the adhesion of epithelial cells, which are responsible for forming epithelial layers that act as barriers between organ cavities and body compartments. These proteins mediate the adhesion of epithelial cells and form junctional complexes with different proteins. Tight junction proteins not only act as a barrier to intramembrane diffusion of proteins and macromolecules but are also involved in regulating cell polarity and various other cellular processes, including proliferation and differentiation [16]. In recent years, most of these tight junction-related proteins have come to the fore in the interaction of neurons, neuroplasticity, brain tumors, and neurological diseases. Since cancers such as neuroblastoma metastasize, their cells must overcome the array of barriers, and tight junction proteins have become an essential component in the cell [17,18]. One of the first identified TJ proteins is occludin (Ocln), an integral protein [19]. It has been stated that Ocln is significantly correlated with increased malignancy in brain tumors and may be a prognostic indicator for patient survival [20]. Suppression of Ocln is a common feature of epithelial-mesenchymal transition (EMT) in tumors derived from epithelial cells and is vital in metastasis in cancer [21]. It has been reported that Ocln plays a role in cell-cell adhesion, apoptosis, proliferation, and differentiation in squamous cell carcinoma cells and should be kept under control in tumorigenicity [22]. Claudin (Cldn) is a large family of proteins involved in forming tight junctions in cells and maintaining cell polarity [23]. It is reported that when there are different expressions or inhibitions in these proteins, they affect tumor aggressiveness [24]. Cld5 is a member of the claudin family and is a protein that affects the permeability of blood-brain barrier cells and contributes to brain tumor metastases [25]. If a tumor tries to penetrate the blood-brain barrier, Cldn5 expression increases, and its selective permeability to ions and macromolecules decreases, accelerating the spread of the tumor [26,27]. Regardless of the cellular origin, different expressions of the Cldn5 protein in different tumor tissues have been reported to affect tumor aggressiveness [28]. For example, it has been reported that in high-grade breast cancer, Cldn5 triggers the formation of breast cancer (spheroids) and contributes to neoplastic processes, but the specific molecular mechanism is not yet clear [17,29]. They are type 1 transmembrane proteins of the Junctional adhesion molecule-A (Jam-A) immunoglobulin superfamily from the family of tight junction proteins [30]. When Jam-A protein is suppressed, cell-cell differentiation is facilitated, and it has previously been shown to reduce the spontaneous and random motility of endothelial cells in solid tumors [31,32]. Another study reported a relationship between loss of Jam-A and the acquisition of invasive properties in breast cancer cells [33]. Another study hypothesized that loss of Jam-A could potentially predict poor clinical outcomes in breast cancer. The expression of Jam-A should be controlled because it also affects the microenvironment in tumor cells and tight junction protein expression and function in epithelial cells [34]. In our study, we showed that squalene induces Jam-A, Ocln and Cldn5 in SH-SY5Y cells at a concentration of 25 µg/mL and above. We have shown that squalene in SH-SY5Y cells can be effective on tight junction proteins due to its metabolic effects and cancer protective properties, and it can inhibit the spread, especially in metastatic cancers.

4. CONCLUSION

As a result, the results of this study will be beneficial in determining the effects of dietary squalene and drug combinations to prevent metastases of pediatric tumors such as neuroblastoma and to increase the quality of life of patients.

5. MATERIALS AND METHODS

5.1. Cell culture

SH-SY5Y human neuroblastom acell line was purchased from the American Type Culture Collection cells (ATCC HTB22, Rockville, MD, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mLpenicillin and 100 µg/mL streptomycin. The cultures were incubated in a humidified incubator with 5% CO_2 in air at 37°C. The culture medium was replaced with fresh medium every 2 days until reaching suitable confluency of about 90%. All experiments were repeated three times.

Squalene (SQ) 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane (CAS 111-02-4; purity ≥98%) was dissolved in absolute ethanol and was diluted with ultra pure water to the different concentrations (1, 10, 25, 50 and 100 µg/mL).

5.2. Cell viability assay

The toxic effects of SQ on SH-SY5Y cells were examined using a tetrazolium-based microplate assay with MTT. Briefly, The SH-SY5Y cells were seeded into a 96-well plate at a density of 1×10^4 cells/well with the 100 µL DMEM medium. After 24h incubation to allow cell attachment, different concentrations of SQ (1, 10, 25, 50 and 100 µg/mL) were incubated for 24h. After the incubation, cells were incubated with 10 µL MTT dye (0.5 mg/mL, ODC Inc) each well in the 100 µL medium for 4h at 37°C. After removing all the culture medium, 100 µL DMSO was added in each well. The percentage of cell viability was measured on ELISA reader (BiotekCo., USA) at a wavelength of 570 nm. The % cell viability was calculated using the formula given below in the Eq. (1). Besides, the IC50 values of SQ on the SH-SY5Y cells were calculated using Graphpad Prisim.

$$\text{\% Cell Viability} = (\text{OD test sample}/\text{OD control}) \times 100 \qquad \text{Eq. (1)}$$

5.3. Colony formation assay

SH-SY5Y cells were seeded into 12-well plates at a density of 1000 cells/well in the 800 µL medium and incubated at 37°C. After 24h incubation, cells were incubated with different concentrations of SQ (1, 10, 25, 50 and 100 µg/mL) for 24h. After treatment, the medium containing SQ was removed and replaced with a fresh medium. The medium was changed every 3 days for 10 days until visible colonies were formed. Then, cells were washed with PBS and fixed in cold methanol/acetic acid for 20 min and stained with 0.5% crystal violet solution for 30 min. The stained colonies manually counted under an inverted microscope (ZeissAxio Vert.A1, Zeiss, Germany).

5.4. Wound healing assay

SH-SY5Y cells (1×10^5 cells/well) were seeded in a 12-well culture plate. After the cells were adhered, they were checked under the microscope and drawn with a sterile pipette tip. Different concentrations of SQ (1, 10, 25, 50 and 100 µg/mL) were added to the cells and incubated for 24 hours. After incubation, cells migrated to the plotted area and were photographed under an inverted microscope (ZEISS Axio Vert.A1, Zeiss, Germany). The distance cells migrated to the cell-free space was measured with Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

5.5. Determination of apoptosis by Annexin V staining

The apoptosis was examined using the Annexin V Staining (Luminex Annexin V Dead Cell Kit, MCH 100105). Briefly, SH-SY5Y cells were seeded into 6-well plates with 1×10^6 cells per well and different concentrations of SQ (1, 10, 25, 50 and 100 µg/mL) were incubated for 24h. Then cells were washed with DPBS and removed with 500 µL of trypsin-EDTA solution and transferred to eppendorf tubes. After centrifugation at 1200xg for 5 minutes, the supernatant was discarded. The SH-SY5Y cells were dissolved in 100 µL of DMEM medium and 100 µL of Annexin V binding buffer. After, 10 µL of Annexin-V dye was added to this mixture and incubated in the dark for 20 minutes. SH-SY5Y cells were counted and Annexin V staining of cells was measured with the Muse Cell Analyzer (Merck Millipore).

5.6. Immunofluorescence

SH-SY5Y cells were seeded into cell chamber slides and incubated with SQ (25 µg/mL). After the incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature. It was then washed three times with PBS and incubated with 0.1% Triton X-100 for 30 minutes. After repeat washing, it was blocked with 3% BSA and incubated overnight with primary monoclonal antibodies. SH-SY5Y cells were then treated with goat anti-rabbit IgG secondary antibodies conjugated with Texas Red and FITC at 1:500 dilution, for 1 h at room temperature. Cells were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA), and images were taken using a fluorescence microscope (ZeissAxio Vert.A1, Zeiss, Germany).

5.7. Statistical analysis

The results were expressed as mean ± SD and analyzed using repeated measures ANOVA followed by Tukey's test for post-hoc analysis for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

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