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Effects of ceramide C2 application on human laryngeal carcinoma cells: a cell culture study

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Abstract. – OBJECTIVE: In the present study, we investigated the effects of Ceramide C2 application on human laryngeal carcinoma cells.

MATERIALS AND METHODS: Human larynx epidermoid carcinoma HEp-2 (ATCC® CCL-23™) cells were purchased from the American Type Culture Collection (ATCC, USA). Human larynx epidermoid carcinoma HEp-2 cells were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS) (10%) and penicillin/streptomycin (1%) in a CO_2 (5%) incubator under standard cell culture conditions. Ceramide C2 was prepared, and further dilutions ranging from 3.13 to 100 µM were prepared in a fresh culture medium. Cells on 96 well plates were exposed to the prepared concentrations of ceramide C2 for 24 and 48 hours. Cytotoxicity evaluation was performed by MTT. Apoptosis profiles of HEp-2 cells were detected by annexin-V analysis. The activated caspases 3/7 on HEp-2 cells after ceramide C2 exposure were evaluated with flow cytometric analysis. The morphological changes on HEp-2 cells caused by ceramide C2 were evaluated by staining with phalloidine and acridine orange *via* confocal microscopy. For the Wound Healing Assay, HEp-2 cells were cultured in 6 well-plates until they became confluent.

RESULTS: MTT cytotoxicity test findings revealed that the viability of human laryngeal carcinoma cells decreased with the increased application of ceramide C2 for 24 hours compared to untreated (control) cells. The highest growth inhibition by ceramide C2 for short-term application for 24 hours was detected at the highest concentration of ceramide C2 (100 µM). Annexin-V findings showed that 98.97 of HEp-2 cells were alive, and 1.63% were detected as early apoptosis for the control group. The results showed that ceramide C2 triggered apoptosis on HEp-2 cells with a percentage of total apoptotic cells of 61,40 compared to untreated HEp-2 cells. Cysteine proteases (caspases) 3/7 activation percentages of HEp-2 cells exposed to ceramide C2 for 24 hours were compared to control cells, and the morphology of HEp-2 cells was changed with clear apoptotic signs that underlined the cytotoxicity and pro-apoptotic activity of ceramide C2. Scratch Assay assessed the migration capability of HEp-2 cells before and after the exposure to ceramide C2. It showed that ceramide C2 reduced human laryngeal carcinoma cells' migration capability and proliferation for 24 hours.

CONCLUSIONS: Based on all study findings, it can be considered that short-chain ceramide C2 exerted cytotoxicity on human laryngeal carcinoma cells in a dose and time-dependent manner and reduced the viability *via* inducing caspase-dependent apoptosis. The overall effect might be derived from the elevated intracellular ceramide levels by the exogenous application of ceramide C2. Consequently, it was concluded that ceramide C2 has good potential to cause cytotoxicity and apoptosis in human laryngeal carcinoma cells and, after deeper *in vitro* and *in vivo* investigations, can be a good candidate for designing anti-cancer drugs with high efficiency.

Key Words:
Human larynx epidermoid carcinoma HEp-2 (ATCC® CCL-23™) cells, Ceramide C2, MTT cytotoxicity test, Annexin-V, Caspase 3/7 activation, Confocal microscopy, Scratch assay, Apoptosis, Cytotoxicity.

Introduction

Sphingolipids are lipids found in cell membranes essential in the signal transduction pathways that control cell death, proliferation, and

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migration. Sphingolipids regulate critical cancer therapeutic pathways in cancer cells, including invasion, metastasis, apoptosis, and death by mitophagy. Among the several sphingolipids, ceramide stands out because it triggers cell death and fatal mitophagy, while sphingosine-1 phosphate is notable because it triggers cell survival and resistance to chemotherapy. These sphingolipids control mitophagy, in which cells dispose of dysfunctional mitochondria by enclosing them in double-membrane vesicles (autophagosomes). Cancer cells can be killed without undergoing apoptosis by a process called lethal mitophagy, which is mediated by ceramide[1].

Head and neck squamous cell carcinomas (HNSCCs) are challenging to treat. HNSCC is one of the top five primary causes of mortality in the United States from solid tumors[2], despite advances in both surgical and chemoradiation treatment. Patients diagnosed with HNSCC at an advanced stage continue to have a 5-year survival rate of roughly 50%, with a slight improvement over the past several decades. Several tissue biomarkers have been linked to HNSCC, including "p16, p53, cyclin D1, cyclo-oxygenase 2 (COX-2), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and matrix metalloproteinases"; however, there is still a need for new diagnostic and/or prognostic markers[3].

Sphingolipids have been linked to controlling HNSCC development and progression in the past. In epidermoid cell A431 carcinoma cells[4,5], ceramide and sphingosine have been demonstrated to block EGF receptor kinase. Further, an inhibitor of glucosylceramide synthase causes fast epithelial cell shape loss, slows cell development, and blocks cell-substrate interaction in human A431 cells by affecting the amounts of membrane glycolipids[6].

Studies[7-15] confirming ceramide's active regulatory role in HNSCC cells have focused on its ability to induce apoptosis[7], modulate EGF receptors[8,9], reverse drug and radiation resistance[10,11], inhibit neo-vascularization[12], and boost the anti-cancer effects of chemotherapy agents[13,14], or photodynamic therapy[15].

Cancer rates continue to rise despite the progress in many areas, especially for kidney, pancreatic, liver, melanoma, and head and neck cancers[16,17]. Cancers of the esophagus, pancreas, liver, and lung all have low 5-year survival rates[18]. A more profound comprehension of the principles behind tumor genesis, metastasis, and medication resistance is required to promote innovations to treat the rising cancer incidence and decrease mortality. Recent research[19,20] has shown that sphingolipids play a role in tumor development and metastasis. Sphingolipids are a type of membrane lipid involved in the signal transduction of many different cellular pathways and the development and progression of many diseases. Particularly intriguing in terms of the involvement of sphingolipids in cancer is the apparent contrast between sphingosine-1-phosphate and ceramide. "Sphingosine-1-phosphate" levels are typically elevated in cancer cells, which play a role in cell survival and resistance to chemotherapy. As an inducer of apoptosis, mitophagy, and necroptosis, endogenous ceramide is often depleted in cancer cells[19,20].

In this study, we tested what happens when ceramide C2 is applied to HEp-2 (ATCC® CCL-23™) cells taken from human laryngeal epidermoid carcinomas.

Materials and Methods

This study was undertaken at the ENT Department of Eskisehir Osmangazi University, working alongside the Department of Biology within the Faculty of Science at Eskisehir Technical University.

Materials

Human larynx epidermoid carcinoma HEp-2 (ATCC® CCL-23™) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Ceramide C2, Fetal bovine serum (FBS), Penicillin/Streptomycin, Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT), Trypsin/ETDA and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3/7, Annexin-V Kits were from Merck Millipore (Hayward, CA, USA).

Culture of HEp-2 cells

Human larynx epidermoid carcinoma HEp-2 cells were cultured in complete DMEM supplemented with FBS (10%) and penicillin/streptomycin (1%) in a CO_2 (5%) incubator[21] under standard cell culture conditions. Cells were passaged each third day and were utilized for investigations at a confluency level of 85% and passage number 15.

Cytotoxicity Evaluation by MTT

HEp-2 cells were seeded in flat bottom 96 well culture plates (Corning, New York, NY, USA) ($2x10^5$ cells/well) and were cultured for 24 hours under standard cell culture conditions. A stock solution (100 mM in DMSO) of ceramide C2

was prepared, and further dilutions ranging from 3.13 to 100 µM were prepared in a fresh culture medium. Cells on 96 well plates were exposed to the prepared concentrations of ceramide C2 for 24 and 48 hours. At the end of the incubation, 20 µL/well MTT dye (5 mg/mL in PBS) was added and incubated again for 3 hours. Liquid parts were changed with DMSO (200 µL/well), and plates were read at 560 nm[22] on an ELISA reader (Bio-Tek HTX Synergy, Winooski, VT, USA). Viabilities (%) were calculated as mean SD values from the absorbances on an Excel program. IC_{50} (inhibition concentration 50 value) was calculated from the calculated viabilities.

Annexin-V Staining

Apoptosis profiles of HEp-2 cells were detected by Annexin-V analysis. In this test, the translocation of phosphatidylserine to the outer membrane was measured as a sign of apoptosis. Briefly, HEp-2 cells were cultured in six-well plates at a density of $5x10^5$ cells per well with IC_{50} concentration of ceramide C2 for 24 hours at standard cell culture incubator conditions. Untreated HEp-2 cells were used as controls. All cell groups were trypsinized and washed in phosphate-buffered saline (PBS). Cell samples were resuspended in PBS and added (100 µL) to the Eppendorf tube (Eppendorf, Hamburg, Germany). Annexin-V reagent (100 µL) was transferred into the tube, and samples were further incubated for 20 minutes in the dark at room temperature. All samples were analyzed with Muse™ Cell Analyzer (Merck, Millipore, Hayward, CA, USA) according to the user guide of the Annexin-V kit (Merck, Millipore, Hayward, CA, USA)[22].

Caspase 3/7 Activation Analysis

The activated caspases 3/7 on HEp-2 cells after ceramide C2 exposure was evaluated with flow cytometric analysis. For this manner, HEp-2 cells ($5x10^5$/well) were treated with IC_{50} concentration of ceramide C2 for 24 hours in six-well plates (Corning, New York, NY, USA). Untreated HEp-2 cells were utilized as controls. All groups were collected *via* trypsinization and washed in PBS at 1,200 rpm for 5 minutes. Samples were incubated with Caspase 3/7 and 7-ADD solutions and were read on a cell analyzer (Muse TM Cell Analyzer, Merck, Millipore, Hayward, CA, USA). The test settings and sample preparation for the Caspase 3/7 kit (Merck, Millipore, Hayward, CA, USA) were prepared according to the manufacturer's instructions.

Confocal Microscopic Analysis for Morphological Changes on HEp-2 Cells

The morphological changes on HEp-2 cells caused by ceramide C2 were evaluated by staining with phalloidine and acridine orange *via* confocal microscopy. Briefly, HEp-2 cells were ($3x10^5$/well) cultured on a coverslip in 6-well plates and treated with IC_{50} concentrations of ceramide C2 for 24 hours. Untreated HEp-2 cells were used as controls. All samples were washed in PBS and fixed in glutaraldehyde (2%) at room temperature for 15 minutes. Cells were washed once in PBS and stained with Alexa Fluor-488 phalloidin and acridine orange. Stained samples were photographed under a confocal microscope (Leica TCS-SP5 II, The Nederlands) supplemented with Leica Confocal Software Version 2.00[23] (Germany).

Wound Healing Assay

For this assay, HEp-2 cells were cultured in 6 well plates until they became confluent. A scratch was made using the tip of a sterile pipette (20-200 µL), and to discard the unattached cells, the plates were washed in PBS. Test wells were labeled and added IC_{50} value of ceramide C2, whereas untreated cells were kept in a fresh complete culture medium. Immediately after adding the solutions, all cells were photographed under a light microscope (DM 6000B, Leica, Germany). Then, all plates were incubated for 24 hours under standard cell culture conditions in an incubator. After incubation, all groups were photographed again under the same microscope to compare the wound width or closure[24].

Statistical Analysis

GraphPad Prism 8 program (GraphPad Software, Boston, MA, USA) was utilized for statistical analysis of the significance of the data. One Way ANOVA analysis and Tukey post-test were used in this manner, and values of $p<0.05$ were considered statistically significant.

Results

MTT Assay Results

MTT cytotoxicity test findings revealed that the viability of human laryngeal carcinoma cells decreased with increased application of ceramide C2 for 24 hours compared to untreated (control) cells. The highest growth inhibition by ceramide C2 for short-term application for 24 hours was detected at the highest concentration of ceramide

C2 (100 µM). Additionally, a statistically significant (p<0.05) decrease in cell viability was recorded also at doses of 50, 25, and 12.5 µM, and no significant decrease was recorded for the lower doses of 6.25 and 3.125 µM. The IC_{50} value for the short-term exposure of ceramide C2 for 24 hours to HEp-2 cells was calculated to be 39 µM (Figure 1). The results underline the dose dependency of the cytotoxic effects of ceramide C2 when applied exogenously to the human laryngeal carcinoma cells for 24 hours.

Cytotoxicity results for an application time of 48 hours uncovered the dose-dependent cytotoxicity of ceramide C2 on HEp-2 cells. Compared to untreated cells, a statistically significant (p<0.05) reduction in cell viability was detected for the doses of 6.25, 12.5, 25, 50, and 100 µM, of which the highest was at 100 µM. The highest growth inhibition by ceramide C2 was detected for short-term application for 24 hours. At the lowest concentration of ceramide C2, no significant decrease was recorded for this incubation period. IC_{50} concentration of ceramide C2 for 48 hours in HEp-2 cells was 62 µM (Figure 2). The results underline that the dose and time depended on the cytotoxicity of exogenous applied ceramide C2 on human laryngeal carcinoma cells for 48 hours (Figure 2). As can be seen, the required concentration of ceramide C2

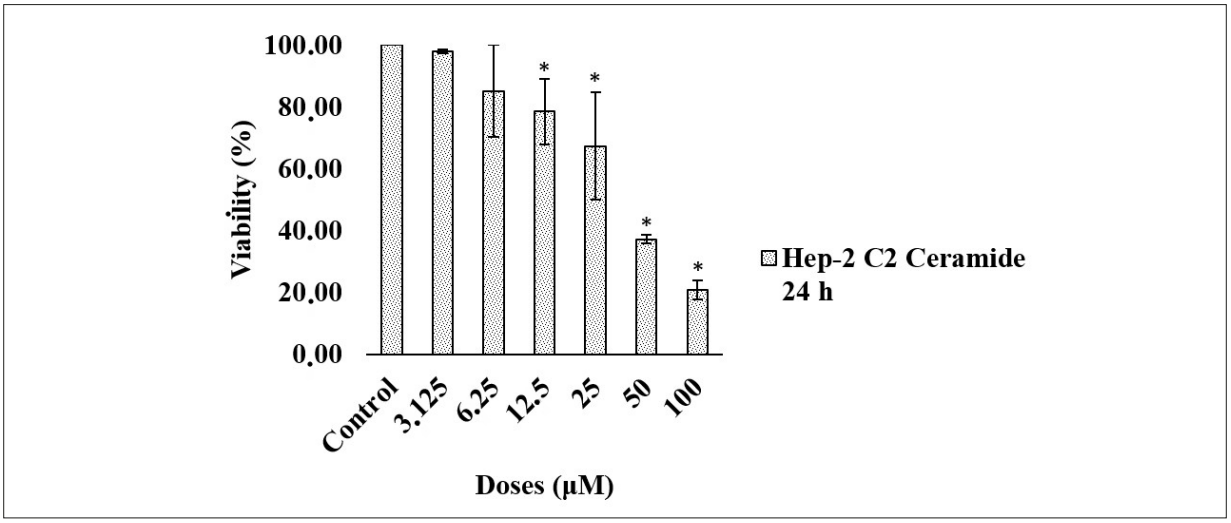

Figure 1. Viability percentages of HEp-2 cells exposed to various ceramide C2 concentrations for 24 hours. (*p<0.05) (IC_{50} value: 39 µM).

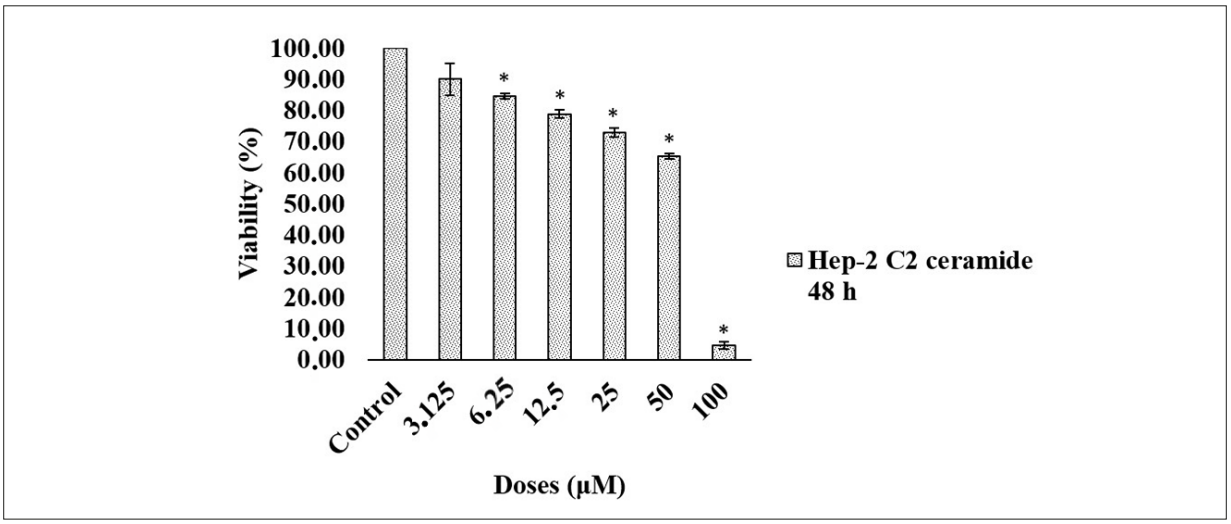

Figure 2. Viability percentages of HEp-2 cells exposed to various ceramide C2 concentrations for 48 hours. (IC_{50} value was detected to be 62 µM) (*p<0.05).

to reduce the cell viability to 50% has been nearly two-fold augmented. This was considered a more relevant application that might be short-time and requires 39 μM of the agent as IC_{50} value.

Annexin-V Findings

A percentage of 98.97 HEp-2 cells were alive, and 1.63% was detected to be in early apoptosis for the control group. After applying the IC_{50} value of ceramide C2 for 24 hours, HEp-2 cells underwent early apoptotic stages (56.25%) and late apoptotic (5.15%), whereas 38.55% of the treated cells were alive. Only 0.05% of ceramide C2-treated cells were dead (necrotic) (Figure 3). The results showed that ceramide C2 triggered apoptosis on HEp-2 cells with a percentage of total apoptotic cells of 61.40 (Figure 3) compared to untreated HEp-2 cells. This may be *via* increased intracellular ceramide levels with exogenously applied ceramide C2.

Caspase 3/7 Activation Results

Cysteine proteases (caspases) 3/7 activation percentages of HEp-2 cells exposed to ceramide C2 for 24 hours were detected to determine the caspase dependency of the triggered apoptosis because this might occur *via* various apoptotic pathways. As it is shown in Figure 4A, only 3.40% of control HEp-2 cells were with activated caspase 3/7, and 96.60% were live cells. In the experimental group of human laryngeal carcinoma cells applied

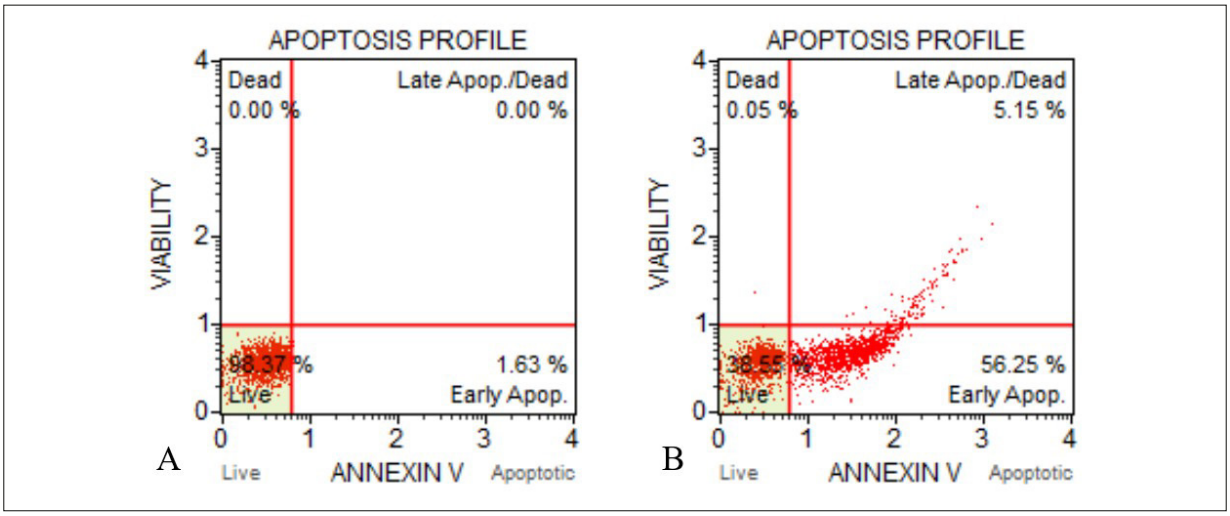

Figure 3. Apoptosis percentages of HEp-2 cells. **A**, HEp-2 control cells, (**B**) Ceramide C2, treated HEp-2 cells with IC_{50} value of ceramide C2 for 24 hours.

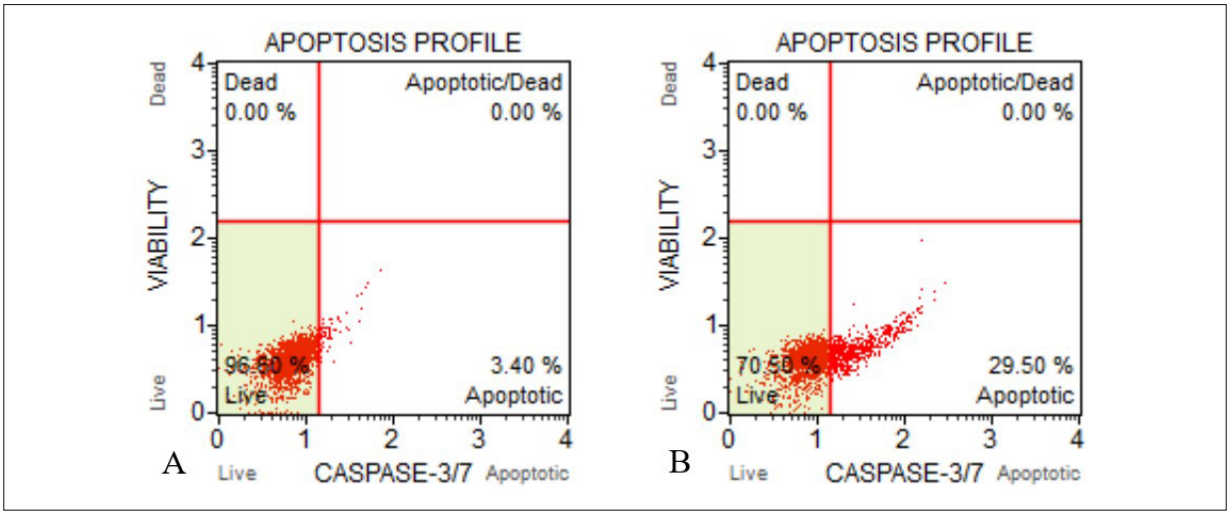

Figure 4. The percentages of programmed cell death *via* activation of caspase 3/7 on HEp-2 cells. **A**, HEp-2 control cells, (**B**) Ceramide C2, treated HEp-2 cells with IC_{50} value of ceramide C2 for 24 hours.

with IC_{50} concentration of ceramide C2 (Figure 4B) compared to untreated HEp-2 cells, 29.50% were with activated caspases 3/7. From here, it can be seen that the exogenous application of short-chain ceramide C2 to human laryngeal carcinoma cells triggered apoptosis *via* activating a cascade of caspases 3/7.

Confocal Microscopy Results

The confocal microscopic findings align with MTT, annexin-V, and caspase 3/7 activation results. The HEp-2 cells not exposed to ceramide C2 (control HEp-2 cells) were detected to have a compact morphology. Control cells were fusiform and with an undamaged cytoskeleton and nuclei (Figure 5A). Human laryngeal carcinoma cells exposed to ceramide C2 for 24 hours were shrunk, with holes in the cytoskeleton. There were fragmented nuclei with condensed chromatin and fragmented cytoskeleton. Compared to control cells, the morphology of HEp-2 cells was changed with clear apoptotic signs that underlined the cytotoxicity and pro-apoptotic activity of ceramide C2 (Figure 5B).

Scratch Assay Results

Figure 6A indicates the initial stage of the artificial injury on plates with untreated HEp-2 cells. Figure 6C shows the first stage of injury for HEp-2 cells exposed to the IC_{50} value of ceramide C2. It can be seen that the wound area is broad for both groups of cells for the initial stages (Figure 6A and Figure 6C). After the incubation period of 24 hours, the final wound areas were observed, and as shown in Figure 6B, cell proliferation and migration continued. The wound opening was near to close without any application of ceramide C2. On the other hand, Figure 6D indicated that migration and proliferation of HEp-2 cells were inhibited; thus, the wound area continued to be opened by applying ceramide C2 for 24 hours.

This assessed the migration capability of HEp-2 cells before and after exposure to ceramide C2 and unraveled that ceramide C2 has reduced the migration capability and proliferation of human laryngeal carcinoma cells for 24 hours.

Discussion

Ceramide synthase 1's (CERS1) generation of pro-apoptotic C18-ceramide is often downregulated, and sphingosine 1-phosphate (S1P)'s generation of pro-survival ceramide is often increased in malignancies. CERS1/4 inhibition or a rise in ceramide metabolizing enzyme levels may diminish C18-ceramide levels in cells. Therefore, chemotherapeutic medicines and radiation therapy sensitize cancer cells and trigger death by reestablishing ceramide levels. In phase II clinical trials[25,26], some

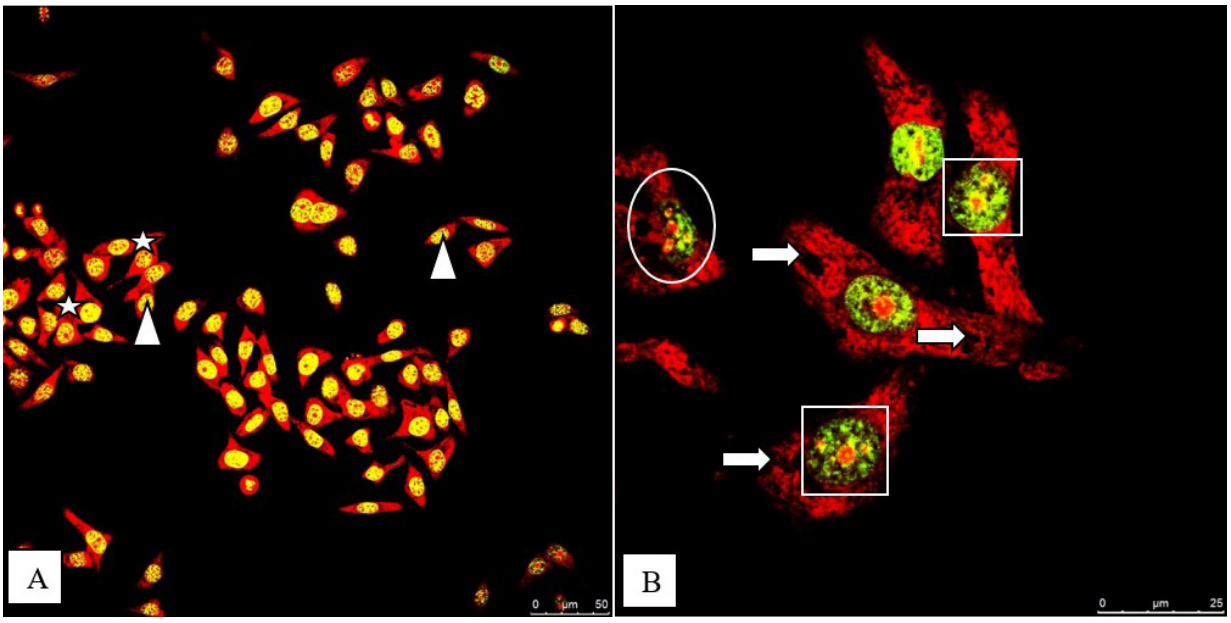

Figure 5. Confocal microscopy micrographs of HEp-2 cells. **A**, Untreated HEp-2 cells (arrowhead-compact nucleus, asterisk-undamaged cytoskeleton) (scale bar; 0-50 micrometer). **B**, HEp-2 cells were exposed to IC_{50} concentration of ceramide C2 for 24 hours (circle-apoptotic nucleus, rectangle-condensed chromatin, arrow-holes on cytoskeleton) (scale bar; 0-25 micrometer).

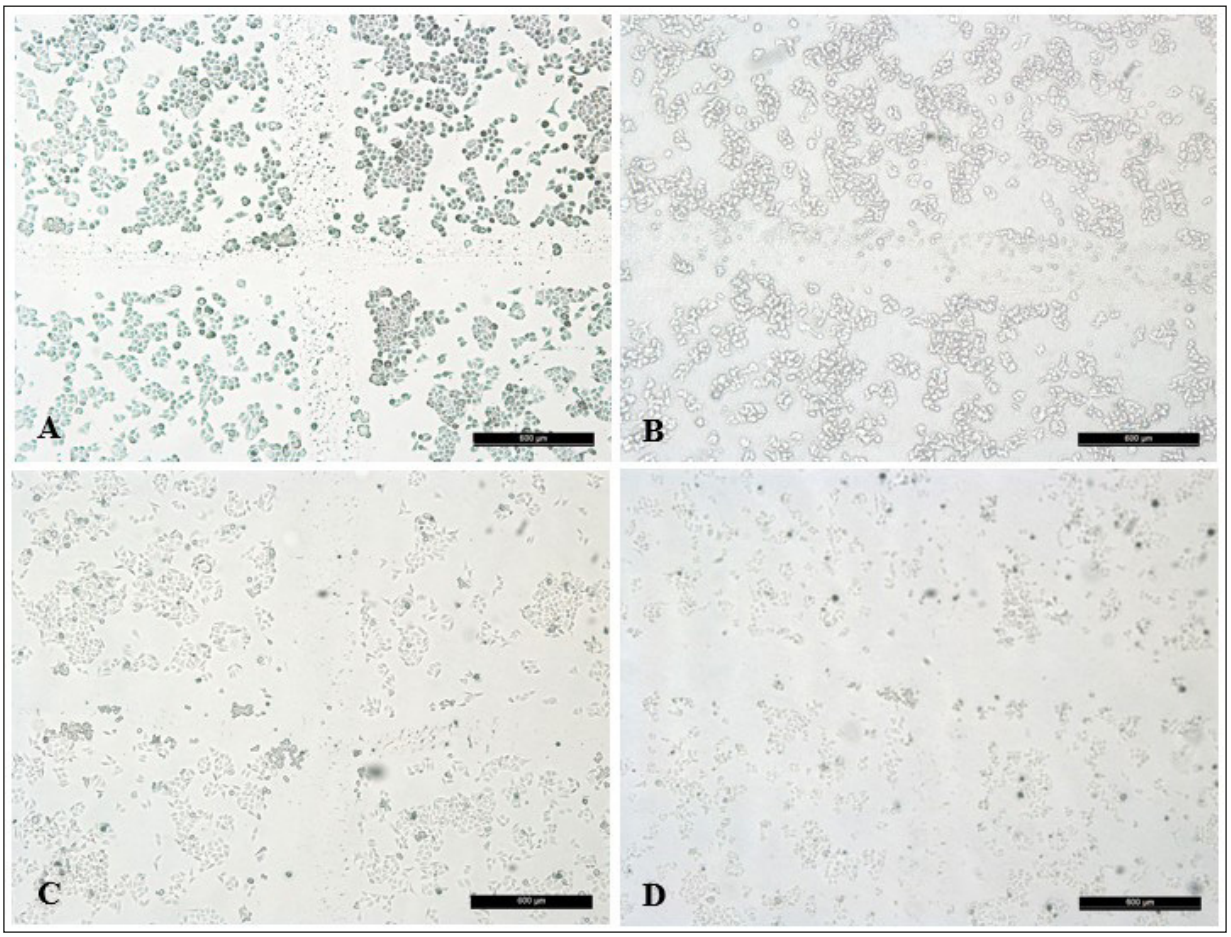

Figure 6. Light microscopy images of the scratch assay for HEp-2 cells. **A**, Control cells at 0. hour. **B**, Control cells at 24 hours. **C**, Ceramide C2 exposed cells at 0 hours. **D**, Ceramide C2 exposed cells at 24 hours (600-micrometer scale bar).

patients treated with gemcitabine and doxorubicin for head and neck malignancies experienced higher serum C18-ceramide. Clinical results (partial response, complete response, or stable disease state) were better in these individuals than those with "normal serum C18-ceramide" (progressing illness condition)[26]. After treatment, CERS1-dependent upregulation of C18-ceramide production was further validated in HNSCC cell lines and animal xenograft tumors[27]. Native solenopsin treatment of melanoma cell lines revealed "anti-proliferative effects *via* mitophagy", as did drugs with structural similarities to ceramide[28].

In the lysosome, ceramide synthase (CERS) re-acylates sphingosine to generate ceramide *via* the catabolic pathway[29]. Each CERS generates ceramide with a unique acyl chain length, which confers unique ceramide characteristics on cells and either suppresses or stimulates cell proliferation[30-32]. Significant links between aberrant CERS expression and the development of several types of cancer were found. For instance, lymphatic infiltration and lymph node metastasis[33] are linked to the lower CERS1 expression level in head and neck cancers than in normal tissues. By reducing Matrix metalloproteinase (MMP)-2 and MMP-9 activity and blocking extracellular matrix degradation[34], overexpression of CERS2 impedes the invasion of breast cancer cells. Chen et al[35] also found that CERS4 was overexpressed in liver cancer tissues and stimulated the growth of cancer cells in the liver by activating the nuclear factor kappa B pathway. Colon, breast, and ovarian cancers and other malignant tumors have all been linked to CERS6 as a cancer-promoting factor[36-38].

Sphingolipids are produced in response to cellular stress and regulate multiple anti-transformation processes, including apoptosis, necroptosis, fatal mitophagy, and senescence. These defensive mechanisms can be compromised by cancer cells due to enzyme dysregulation in sphingolipid metabolism. By increasing the production

of pro-survival sphingolipids like "sphingosine-1-phosphate" and decreasing the production of pro-cell death sphingolipids like ceramide, cancer cells might further boost their proliferation, metastasis, and resistance to chemotherapeutics1.

In this study, we looked into how ceramide affected human laryngeal cancer cells in culture. American Type Culture Collection (ATCC, USA) HEp-2 (ATCC® CCL-23™) human larynx epidermoid carcinoma cells were bought.

Results from MTT cytotoxicity tests showed that ceramide C2 treatment for 24 hours reduced the viability of human laryngeal carcinoma cells compared to untreated (control) cells. For short-term treatment of only 24 hours, the greatest concentration of ceramide C2 (100 M) was found to restrict growth the most. When ceramide C2 was given exogenously to human laryngeal cancer cells for 24 hours, the results showed that the cytotoxic effects were dosage-dependent. Ceramide C2 was found to be cytotoxic to HEp-2 cells in a dose- and time-dependent manner after being applied for 48 hours. These findings demonstrate that exogenously administered ceramide C2 to human laryngeal cancer cells is cytotoxic over 48 hours in a dose- and time-dependent manner. As can be observed, the amount of ceramide C2 needed to kill off 50% of the cells has increased by a factor of nearly two. A shorter time frame, with an IC_{50} value of 39 μM for the agent, was more applicable.

The results of the Annexin-V assay indicated that 98.97% of HEp-2 cells were viable, while 1.63% were in early apoptosis in the control group. According to the findings, ceramide C2 induced apoptosis in HEp-2 cells, as evidenced by a 61.4% increase in the proportion of apoptotic cells relative to those in the control group. This happens if ceramide C2 is introduced exogenously and causes an increase in intracellular ceramide levels.

The caspase dependency of the triggered apoptosis was determined by measuring the percentage of activated cysteine proteases (caspases) 3/7 in HEp-2 cells after they had been exposed to ceramide C2 for 24 hours. The data show that short-chain ceramide C2 applied exogenously to human laryngeal carcinoma cells activated a cascade of caspases 3/7, leading to cell death.

The results from the confocal microscope agree with those from the MTT, Annexin-V, and caspase 3/7 assays. Control HEp-2 cells, which were not exposed to ceramide C2, were shown to have a more compact shape. The nuclei and cytoskeletons of the controls were both intact, and the cells were generally fusiform. Twenty-four hours of exposure to ceramide C2 caused shrinkage and cytoskeleton hole formation in human laryngeal cancer cells. It condensed chromatin in broken nuclei with a shattered cytoskeleton. The shape of HEp-2 cells was altered, and obvious apoptotic indications were present compared to control cells, demonstrating the cytotoxicity and pro-apoptotic activity of ceramide C2.

Using a scratch assay, we compared the capacity of HEp-2 cells to migrate before and after exposure to ceramide C2. We found that even after only 24 hours of treatment, ceramide C2 significantly slowed the growth of human laryngeal carcinoma cells.

Reconstitution of the levels of "C18-ceramide" *via* "*human longevity assurance gene 1 (hLASS1)/CERS1 expression*" resulted in apoptosis. It enhanced chemotherapy-induced cell death in HNSCC both in situ and *in vivo*, suggesting that defects in the generation of C18-ceramide in most HNSCC tumors may play a role in the pathogenesis of this disease[39,40].

C18-ceramide levels were considerably lower in HNSCC tumors, particularly in tumor tissues from male patients. As reported here, significantly increased rates of lymphovascular invasion and pathologic nodal metastasis were also observed in HNSCC tumor tissues with lower-than-expected levels of C18-ceramide. Notably, decreased levels of C18-ceramide correlated with more advanced stages of primary HNSCC tumors[33].

It has been studied[41,42] how males and females experience HNSCC differently, and it has been found that males experience a higher rate of laryngeal cancer occurrences, particularly in glottic tumors. There is some debate over whether or not the larynx has receptors for sex hormones[43-45]; however, evidence[46,47] from voice alterations experienced after hormone replacement therapy provides support for such a connection. Consistent with these findings, Karahatay et al[33] found that ceramide levels in female and male patients were significantly different. Ceramide levels were found to be greater in female patients' tissues than in male patients, and C18-ceramide levels were found to be considerably lower exclusively in the HNSCC tumor tissues of the male patients, which is consistent with the "higher incidence rates" of these cancers being seen in males.

DNA ploidy and sphingomyelin ceramide expression were studied by Yuan et al[48] in normal, precancerous, and malignant laryngeal mucosa. Twenty-three of the original eighty individuals diagnosed with leukoplakia larynx developed malignant laryngeal tumors. Normal tissue, precancerous lesions,

and laryngeal carcinoma were examined for DNA content and ceramide expression using flow cytometry and immunohistochemistry. There were 23 completely aneuploid cases and 57 diploid cases among the 80 individuals diagnosed with laryngeal cancer. A decline in ceramide expression was observed from normal tissue to precancerous lesions and finally into laryngeal carcinoma. 400±30 ($p<0.05$), 180±20 ($p<0.05$), and 10±10 cells were stained per high-power field. DNA diploid cells had higher ceramide expression than aneuploid cells ($p<0.05$). They concluded that ceramide, the second messenger in apoptosis, is crucial in developing laryngeal carcinoma from precancerous lesions. A lack of ceramide may cause laryngeal carcinogenesis.

In human head and neck squamous cell carcinoma, CERS1 overexpression was found[39] to promote C18-ceramide synthesis, disrupting telomerase activity and leading to mitochondrial malfunction and cell apoptosis. C18-ceramide, which CERS1 synthesizes, is significantly lower in glioma tissues compared to normal tissues, and CERS1 overexpression in glioma cells can inhibit cell proliferation and promote cell death by activating the endoplasmic reticulum stress response, as confirmed by Wang et al[49].

Activation of the P38 MAPK signaling pathway by CERS1 is thought[50] to increase the cytotoxicity of cisplatin against malignant cells. Xu et al[36] discovered that CERS1 inhibited NSCLC brain metastasis (BM) through decreasing the activity in "the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway" for the first time. Shorter BM-free survival[51] is related to increased PI3K signaling in NSCLC BM. Several PI3K/AKT activation-specific proteins are overrepresented in BM compared to extracranial metastases[52]. Furthermore, blocking "the PI3K/AKT pathway" in melanoma[53] may reduce the prevalence of BM. A recently discovered and crucial addition to the mechanism of CERS1 in malignant tumors is the link between CERS1 and the "PI3K/AKT/mTOR signaling pathway", as investigated by Xu et al[36].

The induction of autophagy and mitophagy is a biological reaction due to ceramide signaling. These cellular mechanisms are crucial for preventing tissue and cellular homeostasis disruption due to the buildup of damaged organelles and reactive oxygen species, which can lead to systemic inflammation and a tumor-promoting environment. Cancers can alter the activity of autophagy and mitophagy to promote tumor development and metastasis despite these processes having tumor-suppressive effects[1].

We considered that short-chain ceramide C2 reduced cytotoxicity on human laryngeal carcinoma cells in a dose and time-dependent manner and reduced the viability *via* inducing caspase-dependent apoptosis. However, no more studies were performed in this regard, which is the limitation of our study.

Conclusions

All of the results suggest that short-chain ceramide C2 reduced the viability of human laryngeal carcinoma cells by causing caspase-dependent apoptosis and that this effect was dose- and time-dependent. Exogenous injection of ceramide C2 may have an overall effect by increasing intracellular ceramide levels. After further *in vitro* and *in vivo* examination, ceramide C2 may be a promising candidate for building a highly effective anti-cancer therapy, causing cytotoxicity[54] and death in human laryngeal carcinoma cells, as previously demonstrated.

Conflict of Interest

The authors declare that they have not conflict of interest.

Ethics Approval

This is a cell-culture study; therefore, ethics approval was not needed.

Informed Consent

This is a cell-culture study and there is no need to take informed consent.

Funding

None.

Authors' Contributions

Oğuzhan Oğuz: Planning, designing, literature survey, active intellectual support. Felicia Manole: Planning, designing, literature survey, active intellectual support. Nuray Bayar Muluk: Planning, designing, literature survey, interpretation of the results, active intellectual support, writing, submission. Canan Vejselova Sezer: Planning, designing, data collection, literature survey, statistical analysis, interpretation of the results, active intellectual support, writing. Hatice Mehtap Kutlu: Planning, designing, data collection, literature survey, interpretation of the results, active intellectual support. Cemal Cingi: Planning, designing, literature survey, data collection, interpretation of the results, active intellectual support, English editing.

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Availability of Data and Materials
All data used for this study are presented in this paper.

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