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Comparative Study on Induction of Apoptosis in A549 Human Lung Adenocarcinoma Cells by C2 Ceramide or Ceranib-2

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Authors' Contributions

GK and HMK conceived and designed the study. All authors were involved in experimental studies. DV and EC analyzed the data. GK, HMK and DV wrote the article.

Key words

C2 ceramide, Ceranib-2, Human lung adenocarcinoma cells, A549, Apoptosis.

ABSTRACT

Investigation of ceramide and ceramidases on cancer therapy had become popular in recent years. Ceramide is a metabolite in sphingolipid pathway of the cells that has an ability to promote cell death in tumor cells when produced endogenously in the cells or applied exogenously. Ceramidases, which are main component of ceramide pathway catalyze metabolism of proapoptotic ceramide to anti-apoptotic sphingosine and other fatty acids. In this study we have evaluated the pro-apoptotic effect of ceranib-2, a human ceramidase inhibitor and of a short-chain C2 ceramide on human lung adenocarcinoma cells, A549 by determining cytotoxicity of these agents by trypan blue assay, apoptosis inducing ability by annexin V and JC-1 staining and ultrastructural alterations by transmission electron microscopy. Our data clearly shows that ceranib-2 is more effective than C2 ceramide in promoting apoptosis. The cytotoxic effects of these components cause ultrastructural changes in A549 cells.

INTRODUCTION

Lung cancer is one of the widespread diseases, which is difficult to cure and relapses easily, often causing death (Li *et al*., 2004; Molina *et al*., 2006). It is therefore, important to develop new diagnostic methods and search for new ways of treatment.

Ceramides as bioactive sphingolipids are described as molecules with a capability to modulate the balance between the proliferation of the cell and the programmed cell death (Radin, 2001; Senchenkov *et al*., 2001). Ceramides exactly induce apoptosis and repress the growth of cells (Mitra *et al*., 2007). Ceramide as a main unit in the structure of sphingolipids has the ability to promote the signals for cell death under stressful conditions such as chemotherapeutics, radiation and cytokines (Hannun and Luberto, 2000).

Modulation of a ceramide pathway has revealed the sensibility of cancer cells to antineoplastic agents. Moreover, it has been reported that exogenously applied low doses of ceramide enhance the sensitivity of pancreatic cancer cells to gemcitabine, leading to early senescence (Modark *et al*., 2009). Zhang *et al*. (2007) have demonstrated a need for detailed understanding of the ceramide-mediated apoptotic pathway.

Ceramidases play a vital role in ceramide pathway that metabolize ceramide into sphingosine, and in turn, generate an anti-apoptotic metabolite sphingosine-1-phosphate (Vejselova *et al*., 2014). Thus ceramidases are reported to be mediators of cell growth in tumor cells (Strelow *et al*., 2000; Vejselova *et al*., 2014). Treatment of cells with inhibitors of ceramidases or exogenous ceramides promote programmed death of cells (apoptosis) causing a rise in the ceramide concentration in cells (Vethakanraj *et al*., 2015).

Recent studies have revealed that exogenously administered ceramide induced apoptosis in A549 (Zhang *et al*., 2007; Kurinna *et al*., 2004). In addition, inhibition of acid ceramidase with ceranib-2 has been reported to lead to apoptotic cell death in 5RP7 cells (Vejselova *et al*., 2014), LNCaP prostate cancer cells (Kus *et al*., 2015), melanoma (Proksch *et al*., 2011) and hepatomas (Osawa *et al*., 2005). Recently promoting the apoptosis targeting the ceramide pathway has been one of the most commonly used methods in the treatment of cancer (Ravid *et al*., 2003).

Histologically, lung cancers are divided into two groups: non-small cell lung cancer (NSCLC, 85%) and small-cell lung cancers (SCLC, 15%) (carcinomic human

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alveolar basal epithelial cell A549) is one of NSCLC (Samarghandian *et al*., 2013).

The present study examines and compares the cytotoxic and apoptotic effects of exogenous short-carbon chain phospholipid, C2 ceramide and a novel ceramidase inhibitor, ceranib-2 in human lung adenocarcinoma cells, A549.

MATERIALS AND METHODS

Materials

Ceranib-2 and C2 Ceramide were obtained from (Cayman, USA), Roswell Park Memorial Institute Medium (RPMI), fetal bovine serum (FBS), penicillin-streptomycin, Trypan blue, dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Seelze, Germany). The Annexin V-FITC [propidium iodide (PI)] apoptosis detection kit and MitoScreen JC-1 kit were purchased from BD Pharmingen (Franklin Lakes, USA). Human lung adenocarcinoma cell line (A549) was purchased form ATCC (Rockville, MD, USA).

Trypan blue assay

Cellular survival in the presence or absence of the drugs was determined by trypan blue assay. Trypan blue assay is based on that viable cells do not take up trypan blue dye, whereas non-viable cells do (Hajighasemi anf Mirshafiey, 2009). Stock solutions of C2 ceramide and ceranib-2 (10 mM) were prepared in dimethyl sulphoxide and further dilutions were prepared with fresh medium. The cells ($1x10^6$ / mL) were seeded in 25 cm^2 flasks and incubated for 24 h. After treatment with the drugs (C2 ceramide and ceranib-2 at concentrations ranging from 5 µM to 100 µM) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air, cells were trypsinized and diluted with complete medium. A cell suspension was prepared in Hank's balanced salt solution (HBSS, Sigma) and then each sample were mixed with trypan blue solution, stained and dye-excluding cells were counted with a cell counter (CEDEX, Roche, Switzerland). Percentage of the number of viable cells according to the total number of cells is accepted as viability percentage (n=3).

Flow cytometric analyses of apoptosis

Annexin V staining

The externalization of phosphatydilserine as early apoptotic indicator was measured by Annexin V-PI (BD Pharmingen) on a flow cytometer (BD FACSCaliburTM) and by Muse® Annexin V & Dead Cell Assay Kit on a Muse® Cell Analyser (Merck Millipore, Germany). In this manner, cells were incubated with IC_{50} concentrations of C2 ceramide and ceranib-2 for 24 h were washed in PBS and suspended in a binding buffer. Then, 100 µL of cells were transferred to a FACS tube containing 5 µL of Annexin-V and PI and then they were incubated for 15 min at room temperature in the dark. Then 400 µL of binding buffer was added to each tube and analyzed on a Becton–Dickinson FACS Aria using FACSDIVA Version 6.1.1. Software and Muse® Cell Analyser . Annexin V staining was applied according to the manufacturer's instructions (Merck Millipore, Germany) and (BD Pharmingen).

JC-1 staining

The change in mitochondrial potential is considered as a hallmark of apoptosis or as a permeabilization of plasma membrane. This change in mitochondrial potential was measured by using Muse® Cell Analyser (Merck Millipore, Germany). The staining protocol was applied according to the manufacturer's instructions (The Muse® MitoPotential Assay kit, Merck Millipore, Germany). The results of the analysis, as percentages, were shown as four populations of cells: live, depolarized, depolarized/dead and dead.

TEM analysis of ultrastructural changes in A549 cells

The ultrastructural changes in A549 cells, treated with IC_{50} concentrations of C2 ceramide and ceranib-2 for 24 h, were examined by transmission electron microscope (TEM). Both the treated and untreated cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. Fixed cells were post-fixed in osmium tetroxide (2% in distilled water) and dehydrated in graded alcohol (70%, 90%, 96%, and absolute alcohol). Dehydrated samples were embedded in Epon 812 epoxy and epoxy blocs were sectioned on ultramicrotome (Leica EMUC6) then thin sectioned (maximum 100 nm). The thin sections were stained in lead citrate and uranyl acetate and observed under a TEM (FEI Tecnai BioTWIN).

Statistical analysis

SPSS 11.5 for Windows was used to conduct one-way variance analysis for multiple comparisons.

RESULTS AND DISCUSSION

Viability of cells

Eradicating the cancer cells by using cytotoxic drugs is considered to cause cell death by inducing apoptotic mechanisms (Kabadere et al., 2014). The first step of metabolic pathway of sphingosine1-phospate is hydrolysis in sphingomyeline by sphingomyelinases in turn generating ceramide, a proapoptotic sphingolipid in the cells (Hofmann

et al., 2000). The hydrolysis of ceramide to sphingosine phosphate is performed by ceramidases (Koch et al., 1996) in turn converting them to sphingosine-1-phosphate, an anti-apoptotic sphingolipid by phosphorylation (Liu et al., 2000). Current study elucidates the effects exogenous C2 ceramide and a ceramidase inhibitor- ceranib-2 on cell death in carcinomic human alveolar basal epithelial cells, A549. Our cytotoxicity assays showed that IC_{50} concentration of ceranib-2 on A549 cells for 24 h is 14 µM and 22 µM for C2 ceramide. The viability of A549 cells in both groups decreased as the concentration of the treated agents increased (Table I).

Table I.- Viability percentages of A549 cells exposed to IC_{50} concentrations of ceranib-2 and C2 ceramide for 24 h.

Applied doses (µM)	Viability (%) (Mean±S.D)	
	C2 Ceramide	Ceranib-2
0	100.0±0.0	100.0±0.0
5	87.1±4.1	87.5±1.5
10	76.2±2.2	69.1±1.2
15	55.8±4.4	44.1±1.4
20	52.7±2.7	45.6±4.7
25	40.6±0.3	39.8±2.9
30	16.8±1.1	40.8±0.6
35	9.0±1.4	32.0±0.7
40	7.7±1.6	21.8±0.3
45	4.4±1.1	19.2±1.3
50	3.2±1.4	9.9±1.6
55	5.3±0.5	9.7±2.2
60	4.8±0.5	8.9±0.9
65	5.8±0.3	8.1±1.1
70	4.3±0.5	9.7±0.9
75	2.3±0.8	5.6±1.6
80	1.4±0.5	3.2±3.4
85	0.8±0.3	2.1±0.3
90	0.5±0.5	2.8±0.8
95	0.5±0.5	5.2±0.8
100	0.3±0.3	5.0±0.5

On the base of these findings ceranib-2 can be interpreted as more cytotoxic on A549 cells than C2 ceramide. It is reported that 100 µmol/L of C2 ceramide induced apoptosis in A549 cells causes cell death in half of the cells in 24 h (Zhang et al., 2001). Similarly, ceranib-2 was reported to be cytotoxic in high level (3µM-IC_{50} concentration) on H-Ras transformed 5RP7 cells (Vejselova et al., 2014). Furthermore, Kurinna et al. (2004) have reported that 50 µmol/L C6 ceramide application has led to the death of half of the A549 cells.

The treatment using only 20 µM of C2 ceramide H1299 cells has been reported to be less effective when compared to the C2 ceramide application in combination with paclitaxel (Chen et al., 2010). It is well documented that short carbon chain ceramides, C2-C8 may be distributed in the cell compartments and recycled when applied exogenously (Sot et al., 2005; Ogretmen et al., 2002). This may trigger the lower cytotoxicity on A549 cells than that of ceranib-2 in our study.

Apoptosis quantification

Inhibition of ceramidases might lead to the use of ceramides such an anti-tumor agent. Thus it is reported that it is necessary to cause an augmentation in ceramide levels in tumor cells to promote apoptosis (Henry et al., 2013). In our results obtained from annexin V-PI staining, the percentage of early apoptotic cells in C2 ceramide applied A549 cells was detected to be 1.19% and 25.49 % for late apoptotic cells (Fig. 1).

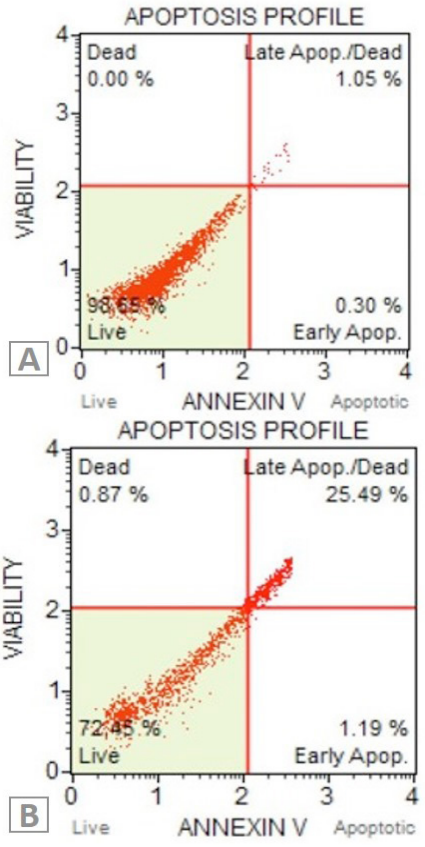


Fig. 1. Apoptosis quantification of A549 cells treated with C2 ceramide for 24 h. **A,** Untreated A549 cells; **B,** C2 Ceramide treated A549 cells.

In the cells exposed to ceranib-2, the percentage of early apoptotic cells was 29.75 and the percentage of late

apoptotic cells was 6.70 when compared to untreated A549 cells (Fig. 2). From these findings it can be concluded that ceranib-2 induced apoptosis in A549 cells has high percentages than that of C2 ceramide.

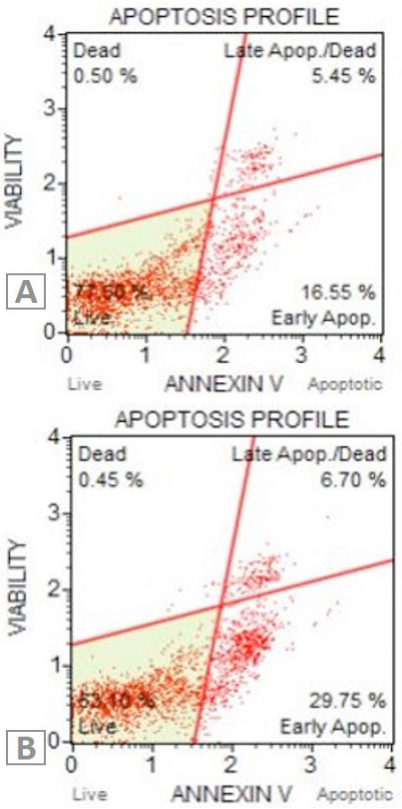


Fig. 2. Apoptosis quantification of A549 cells treated with ceranib-2 for 24 h. **A,** Untreated A549 cells; **B,** Ceranib-2 treated A549 cells.

Pinto *et al*. (2011) have reported that B13 or N-oleoyl-ethanolamine, as inhibitors of acid ceramidase have caused apoptosis in prostate cancer cells and hepatoma cells by reducing ceramide clearance (Pinto *et al*., 2011). In current study we examined the effects of C2 ceramide and ceranib-2 on the potential of mitochondrial membrane of A549 cells. We found that 30.60 % of cells in C2 ceramide treated group (Fig. 3) had reduced mitochondrial membrane potential while 60.10 % of the cells treated with ceranib-2 had reduced potential compared to the control group (Fig. 4). Reduced membrane potential of mitochondria exerts a big clue of apoptosis. Our data present that ceranib-2 is more effective in reducing mitochondrial membrane potential than C2 ceramide.

Ultrastructural changes

Our TEM results showed that A549 cells exposed to C2 ceramide for 24 h exert autophagic vacuols, loss of cristae, chromatin condensation and cells are found to have circular shape compared to the control cells (Fig. 5).

Cells exposed to IC_{50} concentration of ceranib-2 for 24 showed ultrastructural changes as fragmented nucleus, circular shape, membrane blebbings, lipid droplets in the cytoplasm, DNA disintegration and holes in cytoskeleton in comparison with the control cells ultrastructure (Fig. 6). The function of ceranib-2 can be considered to be more effective in the alteration of cells ultrastructure when compared to the sparks of apoptosis in C2 ceramide treated cells.

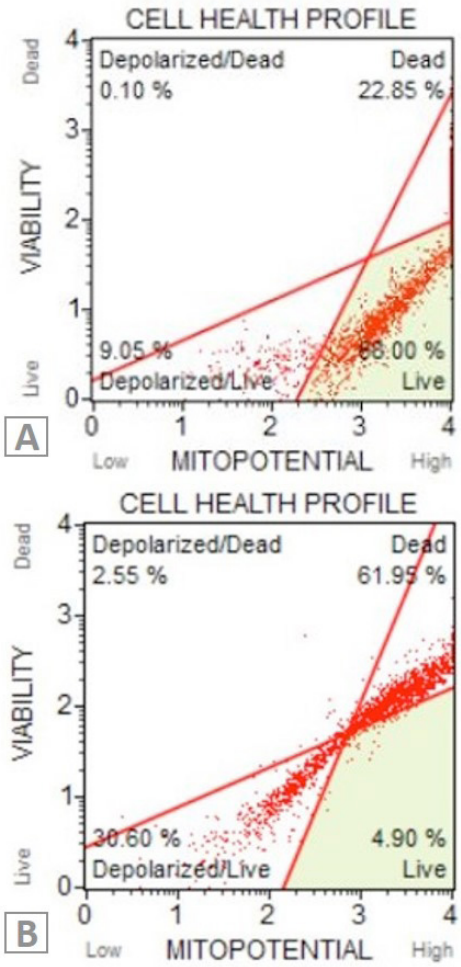
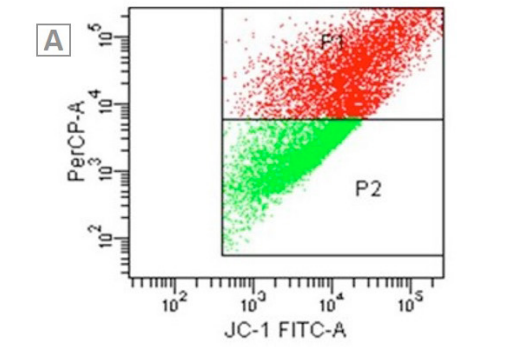
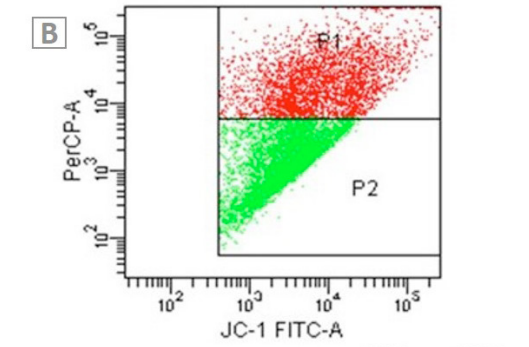


Fig. 3. Analysing the mitochondrial membrane potential changes of A549 cells. **A,** Untreated A549 cells; **B,** A549 cells exposed to C2 ceramide.

Ceramide levels in cells may be augmented by induced autophagy using drug application (Grösch et al., 2012). This induce/induction of autophagic cell death may be mimicked by exogenous application of short chain



Population	#Events	%Parent	FSC-A Mean	SSC-A Mean
All Events	10,000	####	102,011	51,010
P1	5,299	53.0	142,339	77,724
P2	4,547	45.5	59,600	21,860



Population	#Events	%Parent	FSC-A Mean	SSC-A Mean
All Events	10,000	####	61,500	32,928
P1	3,635	36.4	98,130	53,847
P2	6,009	60.1	43,036	22,201

Fig. 4. Mitochondrial membrane potential changes of A549 cells. **A,** Untreated A549 cells: **B,** A549 cells exposed to ceranib-2.

ceramides (Scarlatti et al., 2004). In this study, we evaluated the cell death mechanism of ceranib-2 and C2 ceramide exposed to A549 cells and showed for both of these agents to promote apoptosis in A549 cells. But autophagic vacuoles were also detected in both groups of cells in addition to clear apoptotic cell death sparks in our TEM micrographs. Recently it is indicated to the cell death caused by exogenous and endogenous ceramide through accumulation in the mitochondria (Hou et al., 2011; Beckham et al., 2013). The damage detected in the mitochondria of ceranib-2 and C2 ceramide treated A549 cells might have occurred after the accumulation of ceramide in the mitochondria of the treated cells.

Apoptosis is defined as a genetically controlled and regulated process of removal of unhealthy and potentially dangerous cells failure or absence of which lead to cancer (Kus et al., 2013). There is no detailed examination regarding the function of ceramide mitochondrial pathway of apoptosis (Carpinteiro et al., 2008). Recently, Carpinteiro et al. (2008) have reported that C16 ceramide affects mitochondria via forming large channels in the membranes of mitochondria leading to cytochrome c release from the mitochondria resulting in death of the cells (Carpinteiro et al., 2008). Recent study indicates therapy via increasing ceramide levels in tumors. This increase in ceramide level in the cells has contributed to the promotion of the cell death. Thus exogenous application of ceramides, drug induced ceramide production or blocking hydrolysis of ceramides were indicated as potential treatment methods for cancer therapy (Henry et al., 2013). According to the findings of this study, inhibiting the consumption of ceramides by applied ceramidase inhibitor, ceranib-2 is a better/more efficient way of treatment when compared with exogenous C2 ceramide application on human lung

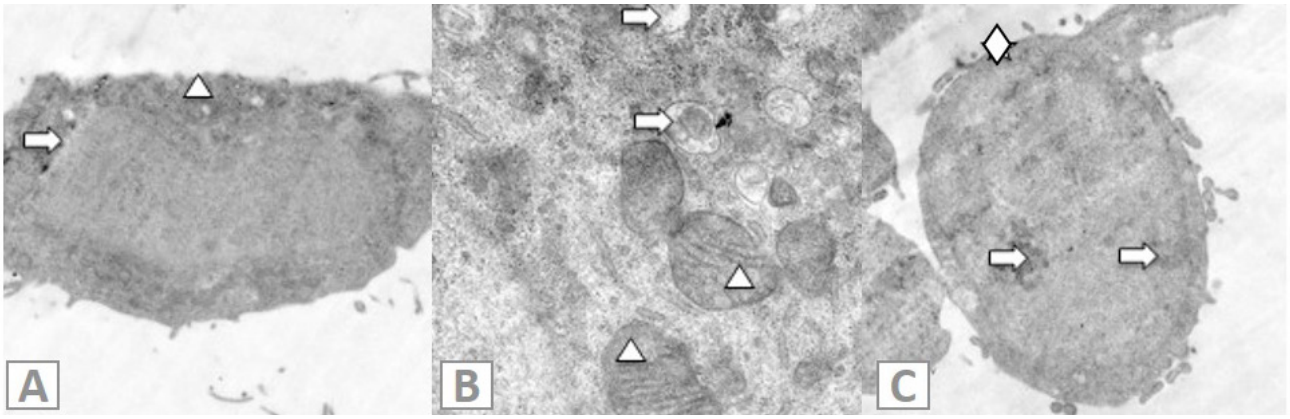


Fig. 5. Ultrastructural changes of A549 cells. **A,** Untreated A549 cells: Δ**,** normal cell membrane; →**,** normal nuclear membrane. **B,** A549 cells exposed to C2 ceramide for 24 h: →**,** autophagic vacuols; Δ**,** loss of cristae. **C,** chromatin condensation and ◊**,** is circular shaped cell.

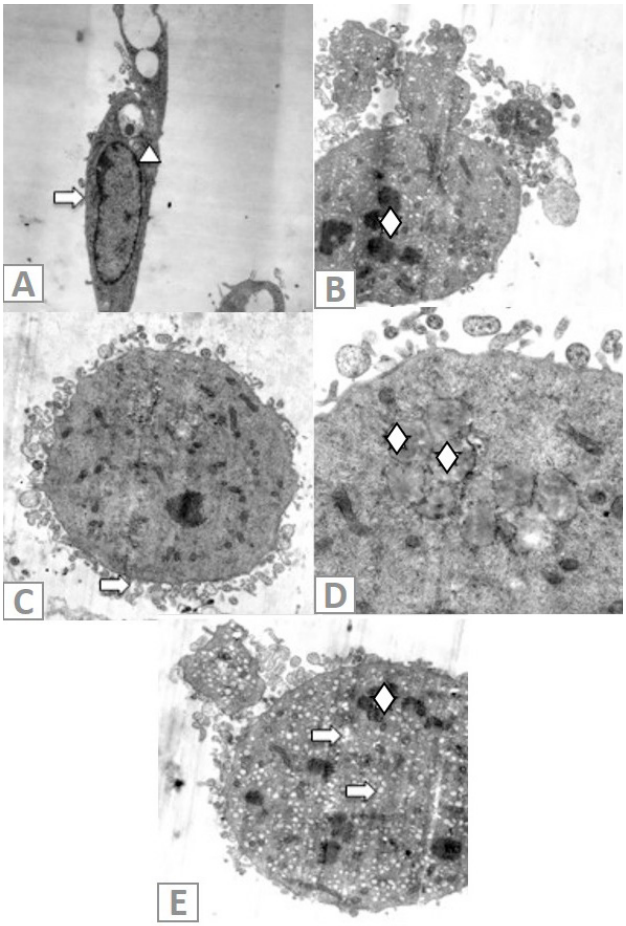


Fig. 6. Ultrastructural changes of A549 cells. **A,** Untreated A549 cells: →, undamaged cell membrane; Δ, normal nuclear membrane. **B,** A549 cells exposed to ceranib-2 for 24 h. ◊, fragmented nucleus. **C,** →, Circular shaped cell and membrane blebbing. **D,** ◊, Lipid droplets in cells. **E,** ◊, DNA disintegration, →, holes in cell.

lung adenocarcinoma cells, A549. In conclusion, this may serve as an inspiration in designing novel drugs for clinical use in cancer treatment as there are not enough kind and number of agents that manipulate ceramide metabolism for clinical use are not adequately, and more of them are need to be further examined *in vitro* and *in vivo* and need to be appropriately defined for cancer treatment.

CONCLUSION

The present study indicates a path to therapy via increasing ceramide levels in tumors. This increase in ceramide level in the cells has indicated promotion of the cell death. Thus exogenous application of ceramides, drug induced ceramide production or blocking hydrolysis of ceramides were indicated as potential treatment methods for cancer therapy. According the findings of this study inhibiting the consumption of ceramides by applied ceramidase inhibitor, ceranib-2 is better way for treatment when compared with exogenous C2 ceramide application on human lung adenocarcinoma cells, A549. This study may serve as an inspiration in designing novel drugs for clinical use in cancer treatment, because there are not enough agents available presently that manipulate ceramide metabolism for clinical use. More studies are required to further examine those drugs under *in vitro* and *in vivo.*

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Statement of conflicts of interest

Authors claim no conflicts of interest for this research.

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