

SPC212 Human Mesothelioma Cell Underwent Apoptosis, Oxidative Stress, Morphological Deformation Following Astaxanthin Treatment

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Abstract

Although astaxanthin (ASX) is one type of carotenoid, it is a more bioactive compound than other carotenoids. Despite its utilization against different cancer types, the effect of ASX on mesothelioma has yet to be well-studied. In this study, our goal is to ascertain how ASX will affect SPC212 human mesothelioma cells. First, the effective doses of ASX against SPC212 cells were uncovered by the MTT test. Thereafter, with flow cytometry analysis, Annexin-V and caspase 3/7 assay were implemented for the evaluation of apoptotic cell death and an oxidative stress test was carried out to determine how the free radicals changed. Ultimately, the cells' morphology was examined under a light microscope. The effective doses of ASX were found as 50, 100 and 200 μM . In Annexin V assay, the total apoptosis increased to around 12%, 30%, and 45% with increasing doses of ASX. In the caspase 3/7 assay, the total apoptosis was around 25% and 38% at 100 and 200 μM . In oxidative stress analysis, ROS-positive cells rose from 4.54 at the lowest dose to 86.95 at the highest dose. In morphological analysis, cellular shrinkage, decrease in cell density, swelling and vacuolations in some cells, membrane blebbing and apoptotic bodies are observed in ASX-treated cells. To conclude, the current study provided insights into the efficacy and effects of ASX against SPC212 mesothelioma cells regarding morphology, proliferation and cell death for future studies.

Introduction

Malignant pleural mesothelioma (MPM) is an uncommon and malignant thoracic disease closely linked to exposure to asbestos and its prevalence has been on an upward trend globally (Patel & Dowell, 2016). Recently, the MPM prevalence is noted to reach a peak due to the difficulty in its early prognosis and unsatisfactory treatment modalities. Although asbestos production and its commercial use is restricted or forbidden, its exploitation is still widespread. Thus, the global incidence of MPM is predicted to rise over the next decade on the grounds that mesothelioma develops long after being exposed by inhalation (Lococo, 2021). While males have a higher incidence of disease, females have a better prognosis. Hereditary background poses a risk for the predisposition to mesothelioma.

To date, there is no reliable or approved serum biomarker in cases where the risk of developing pleural mesothelioma is high. The diagnosis of mesothelioma pathologically is difficult. The poor prognosis of this disease is on the account of its being diagnosed in the advanced stage. In addition, the response rates and survival of the MPM patients are very low. Therefore, other treatment modalities are under investigation (Catino et al., 2019).

Astaxanthin (ASX), a ketocarotenoid, was discovered by Kuhn and Sorensen who initially extracted it from lobster (Davinelli et al., 2018). ASX is a red-orange pigment found in marine organisms, especially salmonids, shrimps and crayfish. It is known for its commercial applications in a variety of industries. ASX can be produced by some organisms including plants, bacteria and microalgae (Davinelli, Nielsen, & Scapagnini, 2018). ASX has various biological functions; immune response enhancing, protection against oxidative stress and ultraviolet, providing reproductive ability and stress tolerance (Donoso,

González-Durán, Muñoz, González, & Agurto-Muñoz, 2021). ASX, akin to other carotenoids including zeaxanthin, lycopene and β -carotene, has many metabolisms and physiology-associated roles. In addition, ASX is a more bioactive compound than other carotenoids. Owing to the molecular arrangement, ASX has many characteristics that enable its utilization to improve human well-being. For example, it possesses a relatively stronger antioxidant capacity than its correspondences (Feng et al., 2018). Recently ASX is of great interest due to its possible versatile effects against cancer, diabetics, inflammation and oxidative stress (Faraone et al., 2020). In the last thirty years of research, it was pointed out that oxidative stress causes chronic inflammation, which leads to chronic diseases such as cancer and skin damage (Simioni et al., 2018). ASX was shown to improve oxidative stress metabolism and prevent inflammation by downregulating and inhibiting inflammatory mediators. ASX, in the esophageal cancer model, was reported to boost antioxidant and anti-inflammation capacity by curbing the NF κ B and COX2 expressions (Cui et al., 2019). Kim et al. showed that AST performs anti-migration and anti-invasion activity in the treatment of colorectal cancer by increasing the miR-29a-3p and miR-200a levels and thus suppressing the MMP2 and ZEB1 protein levels (Cui et al., 2019). In SCC131 and SCC4 oral cancer cells and cancer models, ASX was documented to inhibit PI3K / Akt and the associated NF-B and STAT-3 signaling pathways; thereby, hampering the cancer-related symptoms. (Kowshik et al., 2019). Utilization of ASX in experimental animals with sepsis and acute lung injury was suggested to hinder MAPK / NF- κ B signaling pathway and inflammatory factors, and significantly enhance survival (Cai et al., 2019). In another study, whether ASX initiates apoptosis and boosts antioxidant activity in the colorectal carcinoma cells was evaluated. Eventually, ASX was demonstrated to enhance the upregulation of Bax and Caspase3 and to downregulate BCL2; thus, triggering apoptosis and preventing the colorectal cancer cells' growth. Similarly, in another study, ASX was documented to be capable of initiating apoptosis by augmenting the expression of apoptosis-related genes and the antioxidant enzyme levels (Hormozi, Ghoreishi, & Baharvand, 2019). Furthermore, ASX is also reported to significantly reduced in vitro proliferation and migration rates of breast cancer cells (McCall, McPartland, Moore, Frank-Kamenetskii, & Booth, 2018).

ASX is also documented to have some synergic effect when co-used with other proteins. For example, ASX was shown to increase its anti-cancer activity when used with human serum albumin (HSA), the most abundant blood protein in SKOV3 ovarian cancer SKOV3 cells. In this study, SKOV3 cells were given ASX + HSA to explore the effects of cell proliferation, cell cycle, and drug resistance. ASX + HSA treatment was shown to increase the anticancer effects of AST, reduce the G1 phase cell cycle and induce apoptosis in SKOV3 cells. (Su et al., 2019). Another example of the synergic effect of ASX, Chen et al. evaluated ASX and erlotinib for Xeroderma pigmentosum C (XPC) expression-mediated cytotoxicity in non-small cell lung cancer (NSCLC) cells. Activation of p38 MAPK with ASX reduced XPC expression in lung adenocarcinoma cells. Inhibition of p38 MAPK activity decreases cytotoxicity and cell growth inhibition and destruction of XPC using siRNA increased the cytotoxic effects of ASX (Chen et al., 2018). In another study, co-treatment of ASX and anti-cancer drug carbendazim (Carb) to MCF-7 breast cancer cells increased the anti-proliferative effect of Carb treatment alone and reduced G2/M phase cell cycle and decreased intracellular ROS levels (Atalay, Kuku, & Tuna, 2019).

SPC212 human mesothelioma cells are suitable cell lines being a model cell line for MPM, which are derived from malignant mesothelioma a 47-year old female patient, exposed to asbestos and with a biphasic tumor of the pleural cavity. To the best of our knowledge, there are no studies investigating the effect of ASX on these particular cell line and exploring its effective concentration. To fill this lacuna, we proposed carrying out research to unravel how different doses of ASX will affect SPC212 human mesothelioma cells.

Materials And Methods

2.1 Cell Culture

Human mesothelioma cells (SPC212) were grown in Dulbecco's modified Eagle medium containing 1% penicillin-streptomycin, 10% fetal bovine serum at 5% CO₂ and 95% relative humidity at 37°C in culture medium. When the culture flask has a cell density of 70% (about 2-3 days) it is separated into subcultures. Before doing this, the old medium of SPC212 cells was removed by washing the adhered cells' surface 2 times with 1 mL of PBS. Then the cells were dislodged by 1 min- treatment with Trypsin-EDTA, and then appropriately subcultured.

2.2 Cytotoxicity Analysis

The effect of ASX on the cell viability of SPC212 cells was evaluated by MTT colorimetric assay. The cells were always incubated for 24 hours at 37°C in 95% relative humidity in a 5% CO₂ incubator. The cells were seeded at a density of 5×10³ per well in the 96-well plates. ASX was dissolved in the cell medium (Gibco, Germany) at various concentrations and applied to the cells. Plates were incubated for 24, 48, 72 hours at 37°C in 95% relative humidity in a 5% CO₂ incubator. At the end of the incubation periods, MTT stain was added to each well. The plates with MTT dye were kept in the incubator for 2-4 hours. At the end of 2-4 hours, the medium was removed from the plates and DMSO was added to dissolve the formazan crystals and placed on an orbital shaker in the dark. Microplate reader (BIOTEK ELx808IU, USA) was spectrophotometrically read at 540 nm wavelength and the effective doses of ASX are determined statistically by comparing the treated groups with the control group.

2.3 Annexin V test

Annexin V assay was accomplished in compliance with the manufacturer's instructions of the kit. The SPC212 cell line was cultivated in six-well plates and The cells were applied the different doses of ASX including 50, 100 and 200 µM. After 24 h SPC212 cells were dislodged from plates with trypsin-EDTA and taken into 1.5 ml tubes. The SPC212 cells were gained in the bottom of the tubes by 5-min centrifugation at 300×g. Next, the pellet was re-suspended by ready-to-use medium (100 µL) and blended with annexin V and left to a 20 min incubation at dark at 24 °C. In the end, the results are evaluated by Muse™ Cell Analyser.

2.3 Caspase 3/7 Assay Results

Caspase 3 and 7 dual activity was evaluated by a commercial caspase 3/7 kit (Muse™ Caspase-3/7 kit, Merck Millipore, Hayward, CA, USA) based on flow cytometry. Concisely, SPC212 cells were cultivated in six-well plates and allotted into 3 groups as untreated and two ASX -treated groups (100 and 200 µM). After treatment and cell detachment, the caspase working solution provided by the kit was prepared by mixing caspase 3/7 reagent and PBS (1:8). Thereafter, the caspase 7-AAD working solution provided by the kit was prepared by taking 2 µL of 7-AAD, a cell death marker, and mixing it with 148 µL buffer solution. Next, 5 µL of caspase working solution was added to 50 µL of cell suspension and incubated for 30 minutes at 37 °C. Finally, 150 µL of the 7-AAD working solution was added to the suspension and the caspase activity in cell suspension was evaluated via Muse™ Cell Analyzer.

2.4 Oxidative stress

Oxidative stress was gauged according to the manufacturer's instructions. The SPC212 cell line was cultivated in six-well plates and administered with 50, 100 and 200 µM of ASX. After 24 h, SPC212 cells were dislodged from plates with trypsin-EDTA and taken into 1.5 ml tubes. Once the SPC212 cells were gained in the bottom of the tubes by 5-min centrifugation at 300×g, they were diluted with provided buffer in the kit at a cells density of $1 \times 10^6 - 1 \times 10^7$ /mL. Next, this mixture was blended with the working solution procured by the kit with a ratio of 1:19 (10 µl + 190 µl, respectively). Followingly, SPC212 cells were incubated at 37 °C for 30 min and analyzed by Muse™ Cell Analyser (Merck, Millipore, Hayward, CA, USA).

2.5 Hematoxylin - Eosin Staining

Hematoxylin-eosin stain is regularly exploited for investigating morphological alterations of cells regarding cytoplasmic and nuclear as well as extracellular matrix-related modifications. The morphological alterations of ASX-treated cells were observed by hematoxylin and eosin stain. Initially, SPC212 cells were grown in 6-well plates at a density of 3×10^5 and treated with 50, 100, 200 and 300 µM of ASX for 24 h. After that, the cells were exposed to a 10 min-fixation with pure ice-cold methanol, followed by incubation in hematoxylin and eosin stains, respectively for 5 min each. Then the cells were dipped into 1% ammonia solution for 1 min. Finally, the cells were rinsed in PBS and examined under a light microscope.

2.6 Statistical Analysis

First, we checked the data whether it is normally distributed or not. Shapiro-Wilk test showed that all the data follows the normal distribution. Therefore, a one-way analysis of variance (ANOVA) was exploited to unravel the significant difference between the groups. Levene's test confirmed the equality of variance of the data; therefore, as a posthoc multiple comparison test, we utilized Tukey's test. The p-values less than 0.05 were deemed as statistically significant. All statistical analyses were implemented employing the GraphPad Prism 7.0 statistical software (San Diego, CA). All statistical analyses were employed by one-way analysis of variance (ANOVA) followed by using $p < 0.05$ was considered significant. All the findings were displayed as mean \pm SD (standard deviation). All the results are displayed as mean \pm S.D.

Results

3.1. MTT Assay Results

The anti-proliferative effect of ASX on SPC212 cells was ascertained through the MTT test, as displayed in **Figure 1 and Table 1**. 7 different doses of ASX were applied at four-time intervals including 24, 48, 72 and 96 hours. As a result of the cytotoxicity test, as for 24-hour treatment, at the concentrations of ASX under 50 μM , no significant decline in cell viability was detected. On the other hand, at the doses of 50 μM , 100 μM and 200 μM (all $p < 0.001$ vs control), significant inhibition on the proliferative activity of SPC212 cells was detected. At the maximal applied dose, 200 μM cell viability dropped to 54.3 %. As for the 48-hour treatment, no significant change in cell viability was identified until the dose of 25 μM , where the cell viability was 83.7 % ($p < 0.05$ vs control). At the doses of 50, 100 and 200 μM , cell viabilities decreased to 69.3, 57.9 and 48.7%, respectively (all $p < 0.001$ vs control). As for 72-hour treatment, no significant decrease in cell viability was identified until the dose of 12.5 μM , where the cell viability was 86.7% ($p < 0.05$ vs control). At the doses of 25, 50 and 100 μM , cell viabilities decreased to 81.2, 59.5 and 51.7%, respectively (all $p < 0.001$ vs control). At the maximal applied dose, 200 μM cell viability dropped to almost 37.9% ($p < 0.001$ vs control). In the 96-hour treatment, similarly, no significant decrease in cell viability was observed until the dose of 12.5 μM . At the doses of 25, 50 and 100 μM , cell viabilities decreased to 77.6, 49.6 and 49.8%, respectively (all $p < 0.001$ vs control). At the highest applied dose, 200 μM cell viability dropped to almost 27.7%. %50 inhibitory concentrations of ASX against SPC212 mesothelioma cells were found as 241.7 μM , 185.6 μM , 112.3 μM and 73.3 μM for 24, 48, 72 and 96-hour long experiments, respectively.

Table 1. The cell viability percentages of SPC212 mesothelioma cells after ASX application for 24, 48, 72 and 96 hours (0-200 μM).

Astaxanthin Doses (μM)	Cell Viability (%) (mean \pm sd)			
	24 h	48 h	72 h	96 h
Control	100.0 \pm 5.4	100.0 \pm 4.8	100.0 \pm 4.2	100.0 \pm 4.0
3.125	100.2 \pm 3.6	100.9 \pm 3.6	98.9 \pm 4.0	91.8 \pm 6.4
6.250	100.3 \pm 2.1	93.0 \pm 3.7	89.8 \pm 6.7	89.1 \pm 5.2
12.50	99.5 \pm 6.2	92.2 \pm 4.6	86.7 \pm 7.4 ^a	84.4 \pm 3.5 ^a
25.00	90.6 \pm 4.0	83.7 \pm 6.1 ^a	81.2 \pm 1.1 ^b	77.6 \pm 3.6 ^b
50.00	76.6 \pm 1.8 ^b	69.3 \pm 4.9 ^b	59.5 \pm 3.6 ^b	49.6 \pm 9.0 ^b
100.00	64.5 \pm 4.9 ^b	57.9 \pm 5.4 ^b	51.7 \pm 1.7 ^b	49.8 \pm 1.7 ^b
200.00	54.3 \pm 4.0 ^b	48.7 \pm 5.2 ^b	37.9 \pm 4.9 ^b	27.7 \pm 6.4 ^b

a: differs with a significance of $p < 0.05$ and b: differs with a significance of $p < 0.001$ when compared to the correspondent control.

3.2. Annexin V Assay Results

According to the Annexin V findings (**Figure 2 and Table 2**), the cell viability was detected as 97.26% in untreated SPC212 cells, while the total apoptotic cell rate was 1.58% (early apoptosis rate 0.95% and late apoptosis rate 0.63%). At 50 μM of ASX, the lowest applied dose, the viability of SPC212 cells dropped to 79.42%, while the ratio of total apoptotic cells was revealed to be 13.25% (early apoptosis rate 0.92% and late apoptosis rate 12.33%). At 100 μM of ASX, the viability of SPC212 cells decreased to 54.92%, the ratio of total apoptotic cells was found to be 37.86% (early apoptosis rate 8.21% and late apoptosis rate 29.65%). Eventually, at 200 μM of ASX, the highest applied dose, the viability of SPC212 cells was 38.43%, the total apoptosis ratio was 51.24% (early apoptosis rate 6.65% and late apoptosis rate 44.59%).

Table 2. The percentage of cell viability and cell death in SPC212 mesothelioma cells according to Annexin V assay (0-200 μM).

Astaxanthin Doses (μM)	Annexin V Assay (%)				
	L	EA	LA	TA	D
Control	97.26	0.95	0.63	1.58	1.16
50.0	79.42	0.92	12.33	13.25	7.33
100.0	54.92	8.21	29.65	37.86	7.22
200.0	38.43	6.65	44.59	51.24	10.33

L: live cells; EA: early apoptosis; LA: late apoptosis; TA: total apoptotic cells and D: dead cells.

3.3. Caspase 3/7 Assay Results

According to the Caspase 3/7 assay findings by flow cytometry (**Figure 3 and Table 3**), the viability of SPC212 cells was disclosed as 98.30%, and the rate of total apoptotic cells (early apoptosis ratio: 0.07% and late apoptosis ratio: 1.36%) was 1.42%. The rate of dead cells was 0.27%. At 100 μM of ASX, cell viability decreased to 63.25%, and the ratio of total apoptotic cells (early apoptosis rate: 0.36% and late apoptosis rate: 24.84%) was 25.20%. The rate of dead cells was 11.55%. At 200 μM of ASX, cell viability further decreased to 45.31%, and the ratio of total apoptotic cells (early apoptosis rate: 4.69% and late apoptosis rate: 37.81%) was 42.50%. The rate of dead cells was 12.19%.

Table 3. The percentage of cell viability and cell death in SPC212 mesothelioma cells according to Caspase 3/7 assay.

Astaxanthin Doses (μM)	Caspase 3/7 Assay (%)				
	L	EA	LA	TA	D
Control	98.30	1.36	0.07	1.42	0.27
100.0	63.25	0.36	24.84	25.2	11.55
200.0	45.31	4.69	37.81	42.5	12.19

L: live cells; EA: early apoptosis; LA: late apoptosis; TA: total apoptotic cells and D: dead cells.

3.4. Oxidative Stress Results

As regards the oxidative stress results as shown in **Figure 4 and Table 4**, the percentages of ROS negative and positive cells were 95.46% and 4.54%, respectively in control cells. In 50 μM of ASX-treated cells, the

percentage of ROS negative cells decreased to 86.55%, while the percentage of ROS positive cells rose to 13.45%. In 100 μM of ASX-treated cells, ROS negative cells lowered to 64.40% while ROS positive cells further increased to 35.60%. On the other hand, ultimately, in 200 μM of ASX-treated cells, the ROS negative cells fell to their lowest level by decreasing to 13.05% and the ROS positive cells topped among all groups with the percentage of 86.95%.

Table 4. The percentage of reactive oxygen species positive and negative cells after astaxanthin treatments (0-200 μM).

Astaxanthin Doses (μM)	ROS - (%)	ROS + (%)
Control	95.46	4.54
50.0	86.55	13.45
100.0	64.40	35.60
200.0	13.05	86.95

ROS: Reactive oxygen species.

3.5. Light Microscope Analysis

Light microscope analysis was carried out with hematoxylin-eosin staining. The cells were divided into 5 different groups: control group, 50, 100, 200 and 300 μM of ASX-treated cells. In the untreated group, the density of cells was more than in ASX-treated groups. The density of cells diminished with the ascending ASX concentrations. Furthermore, the cells and cellular cytoplasm got shrunk as the dose increased. In some ASX-treated cells, vacuole-like structures and swelling were observed. In some other cells, membrane blebbing and kidney-shaped nuclei were apparent (**Figure 10 A-E**).

Discussion

According to the results of our literature research; this is the first study to investigate the effect of ASX on human mesothelioma cells, SPC212.

Previous reports deduced that ASX has reduced the viability of cancer cells among different types of cancer cells studied so far. In the study of Kim et al, the MTT assay was performed for ASX-treated (50 and 100 μM) colon cancer cells to measure cell proliferation (H.-Y. Kim, Kim, & Hong, 2019). In another study, the cytotoxic activity of ASX on A549 and H1703 cells was investigated. ASX reduced cell viability in a concentration and time-dependent manner. In the colony-forming test, ASX was suggested to significantly suppress the ability of A549 and H1703 cells to colonize (Ko et al., 2016). Furthermore, ASX

was shown to suppress the proliferation of LS-180 colon cancer cells and decreased the cell viability in a dose-dependent manner (Hormozi et al., 2019). ASX exerted an inhibitory effect in non-small cell lung cancer (NSCLC) cell proliferation and viability (Chen et al., 2018). ASX blocked the cell proliferation in the breast cancer cells investigated (McCall et al., 2018). An appropriate concentration of ASX is effective for preventing the proliferative activity of Caco-2 cells (S. H. Kim, Lim, & Kim, 2019). The common point in ASX toxicity studies is that ASX causes dose and time-dependent cytotoxic effects on cell lines. ASX decreased SPC212 cell viability dose- and time-dependently and that IC₅₀ value for 24 h ASX treatment against SPC212 cells was detected as 241.7 μM.

Innate oxidative stress is considered to the pathophysiology of cancers because the potent factor of angiogenesis. ROS is generated by aerobic cellular metabolism and scavenged by antioxidant mechanisms. ROS production and antioxidant defense capability cause oxidative stress. Oxidative stress results in cell damage (Meng, Ni, Yu, Wang, & Shen, 2017). ASX has antioxidant, anti-inflammatory and immunomodulatory properties. Antioxidants are the focus of researches due to their anticancer properties. Previous studies of ASX reported reducing oxidative stress and preserve metabolism (Yan et al., 2017). ASX has much more potent antioxidants than other carotenoids such as zeaxanthin, lutein, tunaxanthin, cantaxanthin and b-carotene and α-tocopherol. ASX inhibited stress in the liver by reducing of lipid peroxidation and NK cell activity. Also, ASX showed an inhibitory effect on hepatic metastases by its antioxidative property (Kurihara, Koda, Asami, Kiso, & Tanaka, 2002). ASX inhibited cytotoxic effects of glutamate in mouse hippocampal HT22 cells. ASX reduced cell viability, glutamate-induced caspase activation and ROS nuclear accumulation (Wen et al., 2015). Oxidative stress is an important factor in diseases such as cancer and causes mitochondrial dysfunction. Wolf et al. analyzed basal oxidative stress, superoxide measurement and mitochondrial membrane potential. They reported that ASX decreased oxidative stress and protected HeLa cells against oxidative stress (Wolf et al., 2010).

ASX provided decreases in SOD2 level and SOD activity and reduced mitochondrial ROS in gastric epithelial AGS cells (S. H. Kim et al., 2019). In rat glioma cells, ASX showed reactive oxygen species (ROS) and free radical salvaging properties as a quencher of singlet reactive oxygen, nitrogen and two-electron oxidants (Afzal et al., 2019). ASX is a potent antioxidant and has a terminal carbonyl group which is conjugated to a polyene. It scavenges free radicals. So ASX protects cells from cancer. ASX may result variously from its antioxidant and anti-cancer properties interactions with altering gene expression and cellular signaling cascades (Ambati, Phang, Ravi, & Aswathanarayana, 2014). In our oxidative stress analysis, consistent with the literature, ASX also decreased oxidative stress in a dose-dependent way in SPC212 cells

Apoptosis is an organized programmed cell death that occurs in physiological and pathological conditions. Apoptosis can be triggered either by the extrinsic pathway and the intrinsic pathway. During apoptosis caspase proteins including caspase-3, caspase-8 and -9 play critical roles. Caspase-3 is important in this process. Wen et al. examined the activities of caspase proteins in HT22 cells. They demonstrated that glutamate generated the activation of caspase 8/9 in HT22 cells by co-treatment with ASX and ASX prevented activation of caspase-3 (Wen et al., 2015). In vitro studies using cancer cell lines

and in vivo tumor models have indicated the anti-proliferative and pro-apoptotic effects of ASX (Liu et al., 2016; Sowmya, Arathi, Vijay, Baskaran, & Lakshminarayana, 2017; Wu et al., 2016). ASX reduced oxidative stress and cell death in HT22 cells. ASX decreased ROS and thereby aborted intrinsic apoptosis by upregulating anti-apoptotic Bcl-2 expression and downregulating pro-apoptotic Bax expression (Wen et al., 2015). Zhang et al. examined the effect of ASX and other carotenoids at the different dose ranges on K562 cells. They reported that carotenoids (b-carotene, ASX, capsanthin and bixin) decreased the viability of K562 cells and induced cell apoptosis (Zhang, Zhao, Hu, Zhao, & Huang, 2011). Kavitha et al. evaluated the effect of ASX in the hamster buccal pouch (HBP) carcinogenesis model by caspase 3/9 test by enzymatic assay. ASX significantly increased the expression of caspase-9, caspase -3 and PARP (Kavitha, Kowshik, Kishore, Baba, & Nagini, 2013). Meng et al. reported ASX treatment is not a clear indicator of protective effects on apoptosis for RWPE-1 or PC-3 cells by Annexin V (Meng et al., 2017).

We analyzed ASX's apoptotic effect on SPC212 cells after the cytotoxic and antiproliferative effect analyses. We recognized this finding by Annexin V and caspase 3/7 analysis by flow cytometry. Annexin V test and caspase 3/7 assays exhibited that SPC212 cells, to which ASX was applied, were noticed to be driven to apoptosis different than control cells, which showed relatively higher viability and almost underwent no apoptosis.

In conclusion, we indicated that ASX induced growth inhibition, morphological deformation, oxidative stress and apoptosis in human mesothelioma cells and its IC50 value for SPC212 cells was found to be 241.7 μ M for 24 hours. We think that the present results will be an incentive for and provide new insights into future studies since there are no studies about ASX's effect on lung cancer cells.

Declarations

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Conflict of Interest

The authors declare that there is no conflict of interests.

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Figures

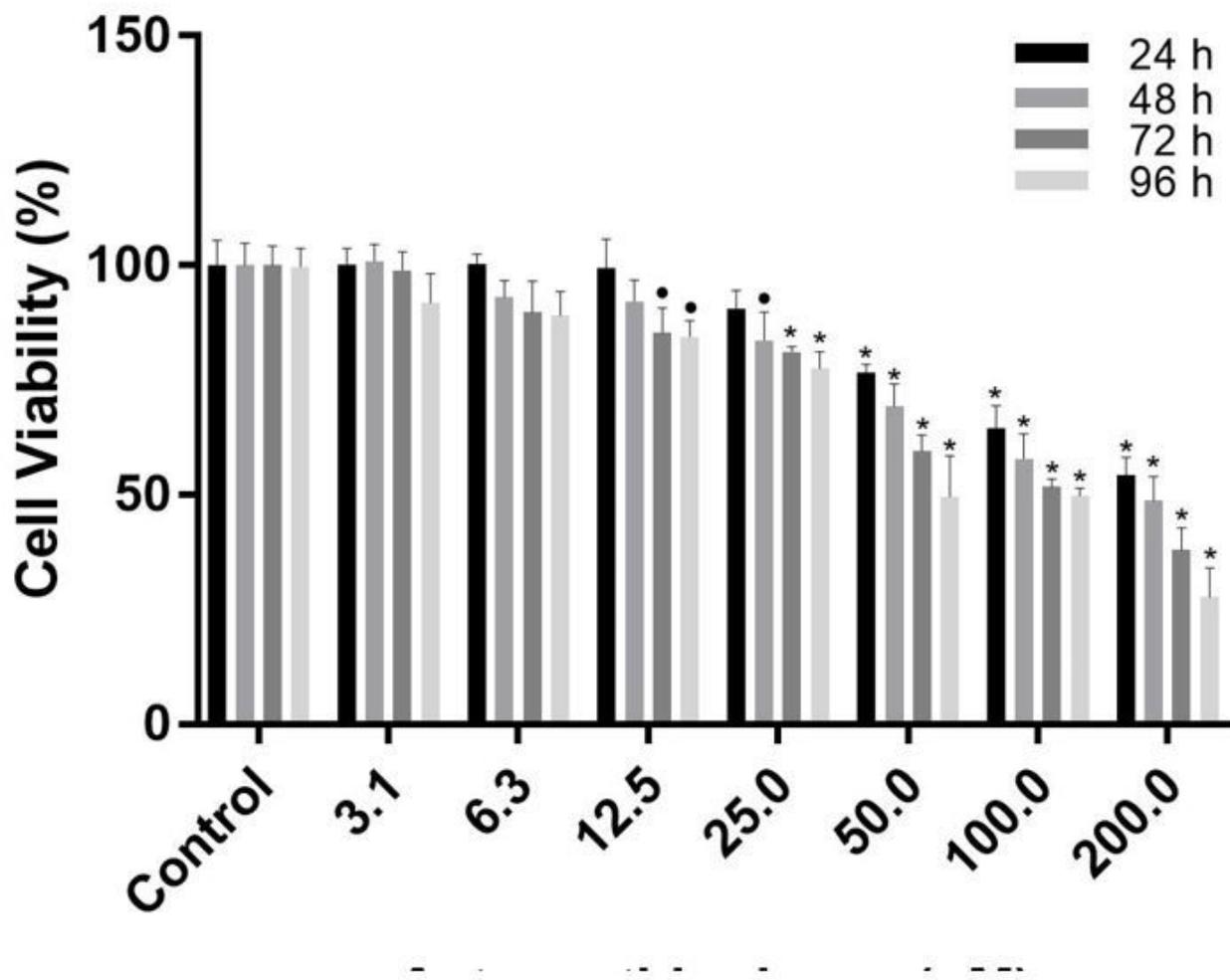


Figure 1

MTT Assay. The changes in cell viability (%) of SPC212 cells with varying ASX doses (24, 48, 72 and 96 h-long assays). “•” and “*” designate significant difference of $p < 0.05$ and $p < 0.001$, respectively when compared to the correspondent control

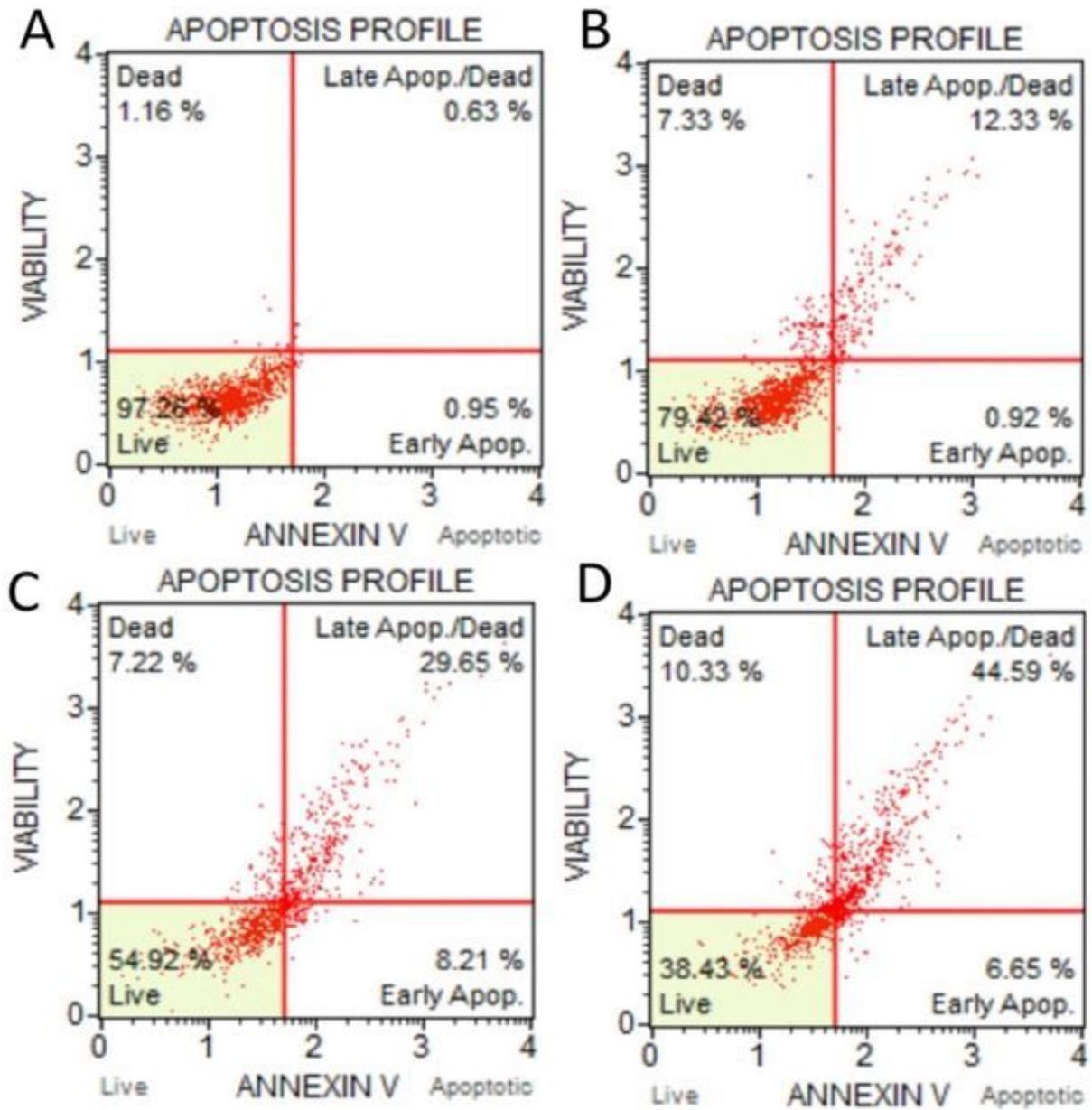


Figure 2

Annexin V results of the ASX-treated cells. A: untreated cells, B: 50 μ M of ASX-treated cells, C: 100 μ M of ASX-treated cells, D: 200 μ M of ASX-treated cells.

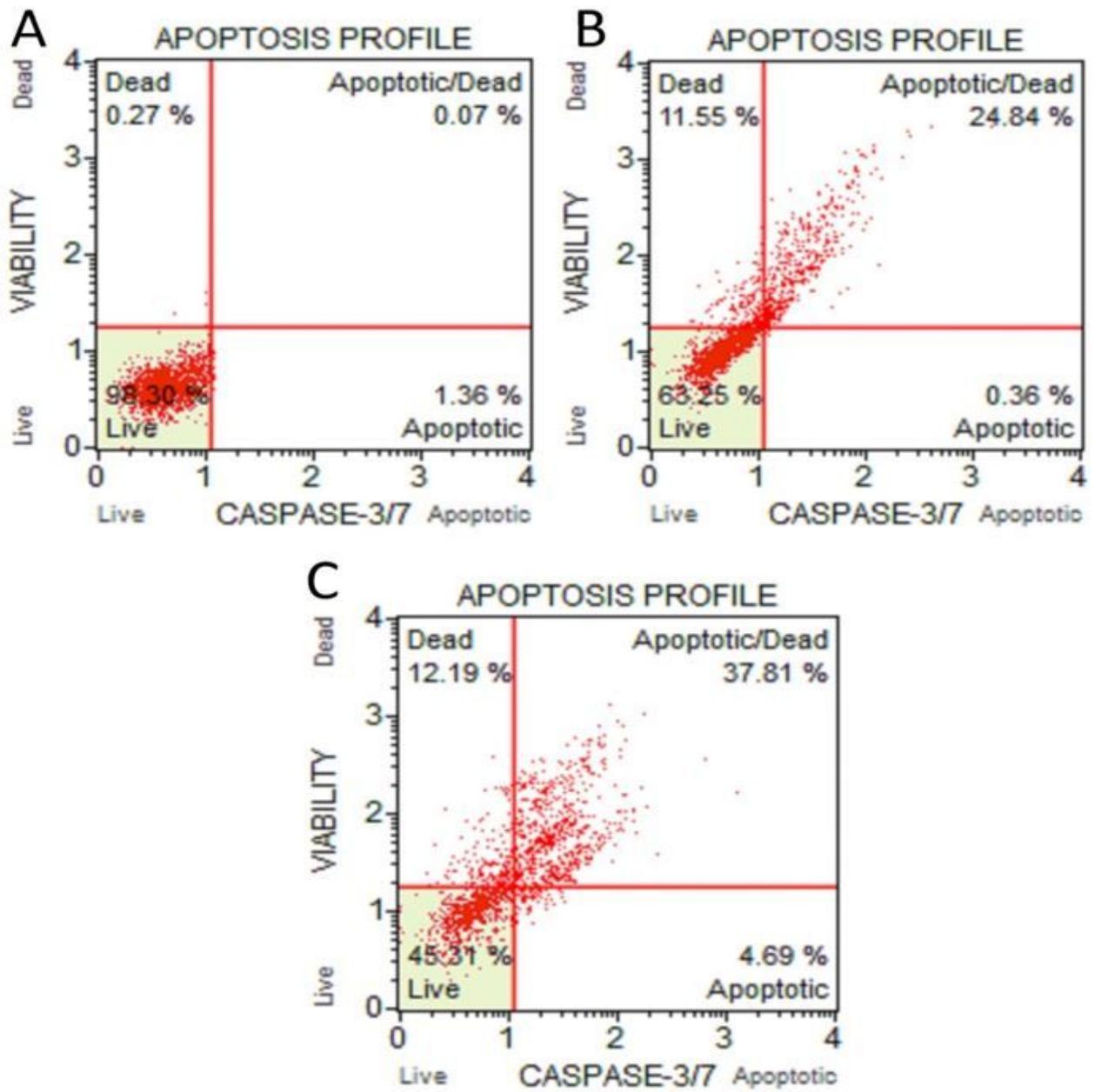


Figure 3

Caspase 3/7 results of the ASX-treated cells. A: untreated cells, B: 100 μM of ASX-treated cells, C: 200 μM of ASX-treated cells.

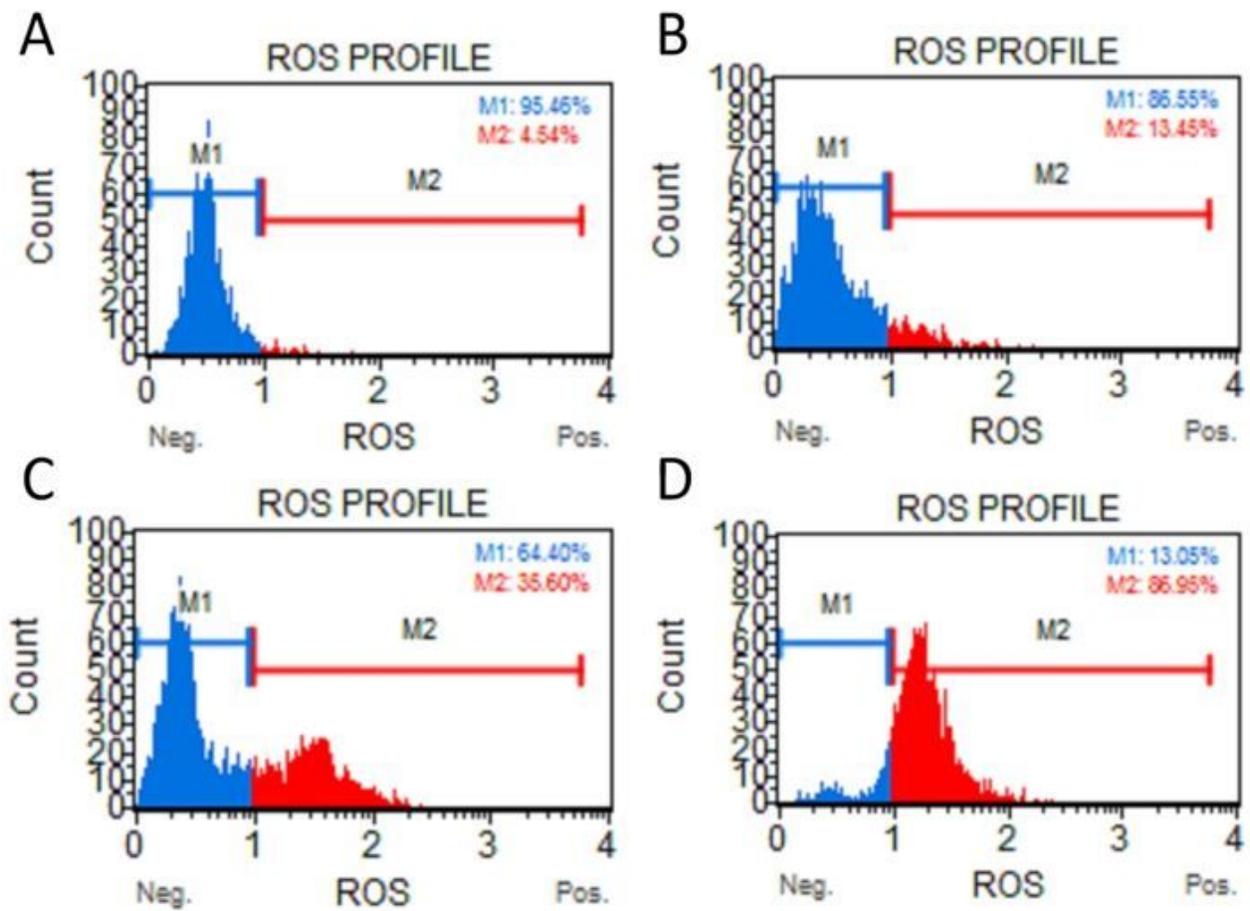


Figure 4

Oxidative stress results. A: untreated cells, B: 50 μ M of ASX-treated cells, C: 100 μ M of ASX-treated cells, D: 200 μ M of ASX-treated cells.

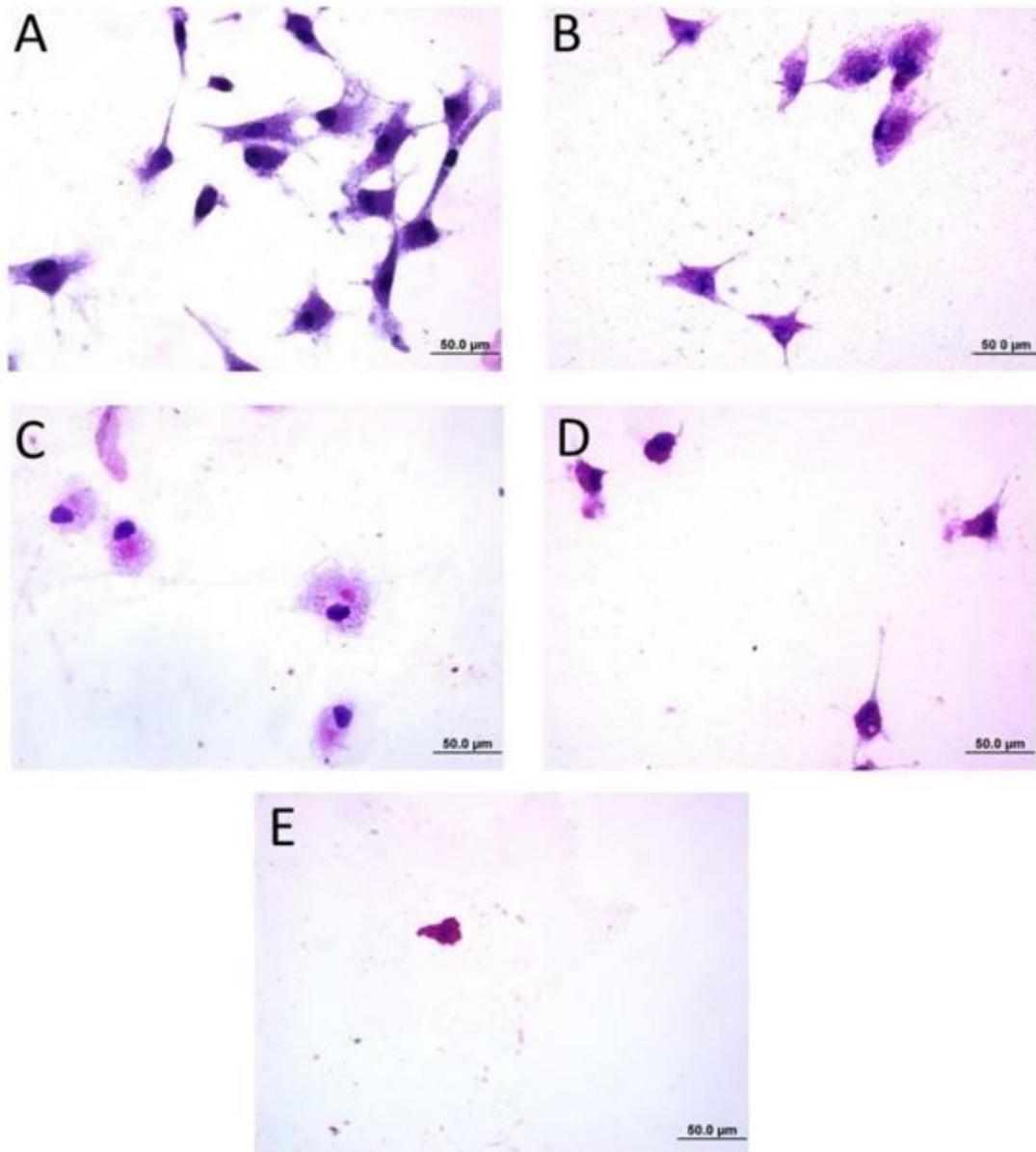


Figure 5

The hematoxylin-eosin micrographs of SPC212 cells. A: untreated cells, B: 50 μ M of ASX -treated cells. C: 100 μ M of ASX-treated cells. Note of vacuole-like structures and cell swelling. Shrunken cells with eccentric nuclei. D: 200 μ M of ASX-treated cells. E: 300 μ M of ASX-treated cells. Note of membrane blebbing and evidently acidophilic cytoplasm. Bar scales indicate 50.0 μ m.