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The Investigation of the Induction of Diketocarotenoids Senescence in SHSY-5Y Cells

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Abstract

Introduction: Recently, studies of diketocarotenoids such as astaxanthin (Ax) and canthaxanthin (Cx) with powerful antioxidant have focused on numerous biological mechanisms such as singlet oxygen quenching, radical scavenging, anti-diabetic, anti-carcinogenesis, anti-inflammatory, anti-obesity and anti-melanogenesis activities. There is evidence demonstrating that diketocarotenoid confers neuroprotective effects in experimental models of chronic neurodegenerative disorders and neurological diseases. This study used Ax and Cx to detect its role on senescence of SHSY-5Y Cells.

Methods: In this study, the sample included the cell control group (SH-SY5Y cell line) that did not receive Ax and Cx, , and the experimental group that received Ax and Cx (20 mM). Ax and Cx were treated with SH-SY5Y cell line at 48 hours. To measure the expression of BAX, Bcl-2 and PPARγ different groups were compared by real‑time PCR analysis. The cell senescence effects of Ax and Cx, a β-galactosidase (SA-β-gal) senescence assay was evaluated. The results were analyzed by the one-way analysis of variance (ANOVA) using Prism version 6.0 software.

Results: The results showed that treatment with Ax and Cx (20 mM) for 48h induced apoptosis and senescence. The BAX and Bcl-2 gene expression analysis revealed a significant impact of Ax and Cx in apoptosis induction ($P<0.05$). The measuring of cell senescence also indicated that Ax and Cx exhibited a senescence inductive activity as determined by an increase in β-galactosidase activity and PPARγ gene expression ($P<0.05$).

Conclusion: It appears that Ax and Cx have therapeutic properties in SH-SY5Y cells and can cause the proliferation of these cells to cease. The results suggest that Ax and Cx treatment may be beneficial for therapy of neuroblastoma and neurodegenerative disorders.

Keywords: Astaxanthin, Canthaxanthin, Apoptosis, Senescence, Neuroblastoma, SHSY-5Y Cells

Introduction

Neurodegenerative disorders such as parkinson's disease (PD) and alzheimer's disease (AD) are the most common neurodegenerative diseases and the major causes of dementia in people (1). The causes of neurodegenerative disorders have still been unclear, however, several studies suggest the involvement of mitochondrial dysfunction and oxidative stress (2). Xanthophylls such as astaxanthin (Ax) and canthaxanthin (Cx) part of the broader group of carotenoids, are powerful antioxidants that are produced by several microorganisms (Phaffia rhodozyma, Haematococcus pluvialis), higher plants, and invertebrates. They are derived from a carotene precursor that is modified by a combination of different processes (3). Xanthophylls have been reported to possess anti-inflammatory, anti-oxidant and anti-tumor effects. Recently, xanthophylls have been documented to provide important metabolic functions in animals, protection against diseases such as cancer by scavenging of

oxygen radical, and enhancement of immune response (4- 6). Thus, these compounds are thought to provide health benefits by decreasing the risk of various diseases, particularly certain cancers, cardiovascular, neurodegenerative, eye diseases and aging (7; 8). It has also been shown that Xanthophylls can interfere with senescence and aging by various mechanisms. For example, Ax was shown to stimulate retinoic acid receptors such as retinoic acid, resulting in the production of hyaluronan, an important component of the extracellular matrix (ECM) which can play an important role in the process of differentiation (9). However, the mechanism by which Xanthophylls displays such varied biological activities remains to be completely not clarified. In this study, we used two antioxidants, Ax and Cx to check the senescence status neuroblastoma cell line, SHSY-5Y cells, a human tumoral neuroblastoma cells. Antioxidants and cancer are commonly mentioned together although the exact mechanisms underlying the actions of antioxidants relation to oncogenic events are incompletely known. Among the many aspects that have been investigated to elucidate the effects of antioxidants on cell growth regulation does not appear the cellular senescence state. SHSY-5Ycells, therefore, are ideal for investigating how senescence state affects the cellular regulation activity triggered by antioxidants in neuroblastoma cells.

Methods

Human neuroblastoma SHSY-5Y cells were cultured in Dulbecco's modified eagle medium (DMEM, high glucose) containing 100 IU/ml penicillin, 100 mg/ml streptomycin and 5% (v/v) fetal bovine serum (Invitrogen). Cells were maintained at 37 °C in a humidified environment 5% CO2. Culture media were replaced every three days. This condition was for the SHSY-5Y cell alone, control group. The concentration of Ax and Cx was 20 µM/mL for 48 hours. The cell concentration was adjusted to 1×105 cells and 100 µL aliquots were transferred to 96-well plates. SHSY-5Y cells were treated with various concentrations of Ax and Cx (1.10, 20, 50, 100, 1000 µmol/L) for 48 h in vitro. A total of 20 µL of MTT was added as a concentration of 0.5 mg/mL after media (200 µL) was added in each well. After the incubation, media was removed and 100 µL of DMSO was added to each well and, then, the cells were incubated for further 30 minutes at 37℃ with gentle shaking. Finally, the absorbance intensity was measured by a microplate reader (FLUOstar Omega, BMG LABTECH, Germany) at 570 nm. Cell viability was calculated as the ratio of the absorbance of the test groups to that of the control group. The Total RNAs from the SHSY-5Y cells were isolated using the RNA extraction kit (Cinnagen Inc., Iran). The purity, integrity and concentration of RNA were determined by measuring the optical density 260/280 and agarose gel (1%) electrophoresis and the Complementary DNA (cDNA) was synthesized following the manufacturer's protocols using 1 µg RNA using RevertAid™ First Strand cDNA Synthesis kit (Fermentas Inc.). Real‑time PCR was performed according to the protocol of RealQ Plus 2x Master Mix Green (Ampliqon Inc.) in applied Biosystems StepOne™ Instrument (ABI, Step One, USA). Real-time PCR for expression analysis of the primer pairs for BAX, Bcl-2, PPAR and PGK was designed, as shown in Table 1. The PGK housekeeping gene was also used as the internal control of Real‑time PCR reactions. In this study, we assessed the BAX/Bcl-2 mRNA ratios in cell groups. The Real‑time PCR conditions were set for 10 minutes at 94°C followed by 40 cycles of 15 seconds at 94°C, 60 seconds at 60°C and extension steps. The qRT-PCR reactions, including the no-template controls, were performed in triplicate. After each Real‑time PCR run, gel electrophoresis and melting curve analysis were carried out to confirm specific amplification of targets. The amplification

signals of different samples were normalized to PGK Ct (cycle threshold), and then delta-delta CT (2-□□CT) method was applied for comparing mRNA levels of test versus control which represented as fold change in data analysis. Senescence-associated β-galactosidase (SA-β-gal) activity is now an extensively used biomarker in in vivo and culture studies of cellular senescence (10). SHSY-5Y cells (3×105 cells) were seeded in 6-well plate and after being treated in 80% confluence; SHSY-5Y cells were washed with PBS and then fixed with formaldehyde and glutaraldehyde. After being washed with PBS, the SHSY-5Y cells were incubated at 37◦C overnight with freshly SA-β-gal staining solution [1 mg mL−1 X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), 5 mM K4[Fe(CN)6], 5 mM K3[Fe(CN)6] and 2 mM MgCl2 in PBS, pH 6.0, or in citrate-buffered saline, pH : 4.5]. The next day, the cells were washed again in PBS and the percentage of senescent cells was examined as blue-stained cells under the microscope, and a total of 1000 cells were counted in random fields to determine the percentage of SA-β-gal–positive SHSY-5Y cells. All data were reported as mean ± SEM of three independent experiments. The results were analyzed by one-way analysis of variance (ANOVA) using Prism version 6.0 software (GraphPad Software Inc., San Diego, CA, USA). Each experiment was performed in triplicate. The P value less than 0.05 was considered statistically significant.

Results

Once the SHSY-5Y Cells were treated by different concentrations of Ax and Cx, 48 hours following treatment, their viability were investigated. The results showed no significant difference between Ax and Cx in cell viability. The assay indicated that concentrations above 20 μM/ml induced a significant ($P<0.05$) decrease in cell viability compared to the control group. The results of the cell viability assay are presented in Figure 1. Real-time quantitative PCR analysis was employed to compare the expression level of pro-apoptotic and anti-apoptotic genes including BAX and Bcl-2, respectively, in response to different concentrations of Ax and Cx treatment. As the pro-apoptotic gene, the Ax and Cx have been demonstrated to induce induction of BAX as main pathways for apoptosis induction (11). Therefore, we assumed that Ax and Cx may support apoptosis in SHSY-5Y cells by increasing the expression of BAX and reducing the expression of Bcl-2. The expression analysis results indicated that Ax significantly decrease Bcl-2 gene expression (0.66 fold) expressions, compared to the SHSY-5Y cells alone ($P < 0.05$). In accordance with real-time quantitative PCR results, BAX gene expression were found in Ax treated group followed by Cx (3.50 fold and 2.19 fold change, respectively), compared to the SHSY-5Y cells alone ($P < 0.05$). BAX/ Bcl-2 ratio is a measurable aspect of the apoptosis progression which seems that determine the fate of apoptosis. The BAX/Bcl-2 ratio was found to be increased in SHSY-5Y cells treated by Ax and Cx (5.30 fold and 1.79 fold, respectively). These data suggested that Ax and Cx serve as an effective inducer of cell death in neuroblastoma cell line, SHSY-5Y cells through activation of apoptotic genes as is shown in Figure 2. To find out if Ax and Cx enforces SHSY-5Y cells into senescence, a galactosidase assay was performed on treated cells. Actually, it was an idea that Ax and Cx may subvert neuroblastoma cells into senescence process. This hypothesis was checked by using both β-galactosidase assay and PPARγ gene expression analysis. The data revealed that the cells which were treated with Ax and Cx exhibited dramatically increased senescence phenotype in SHSY-5Y cells. As illustrated in Fig 3, treatment of SHSY-5Y cells with Ax and Cx significantly increased the level of senescence to 16.1% and 12.8% for Ax and Cx, respectively, compared to the control group (5.0%). As a marker gene of senescence evaluation, the PPARγ gene

expression was found to be increased in SHSY-5Y cells treated by Ax and Cx (2.17 fold and 1.81 fold, respectively) significantly; ($P < 0.05$).

Table 1. Primers used in the present study

Genes	Primer Sequences	Sizes (bp)
BAX	Forward: 5'- GCCCTTTTGCTTCAGGGTTTCA -3'	108
	Reverse: 5'- CAGCTTCTTGGTGGACGCAT -3'	
Bcl-2	Forward: 5'- ACGAGTGGGATGCGGGAGATGTG-3'	245
	Reverse: 5'- GCGGTAGCGGCGGGAGAAGTC-3'	
PPAR□	Forward: 5'- CTATGGAGTTCATGCTTGT-3'	177
	Reverse: 5'- CTGATGGCATTATGAGACA-3'	
PGK	Forward: 5'-TAAAGCCGAGCCAGCCAAAA-3'	116
	Reverse: 5'-CTCCTACCATGGAGCTGTGG-3'	

Abbreviation: bp, base pair.

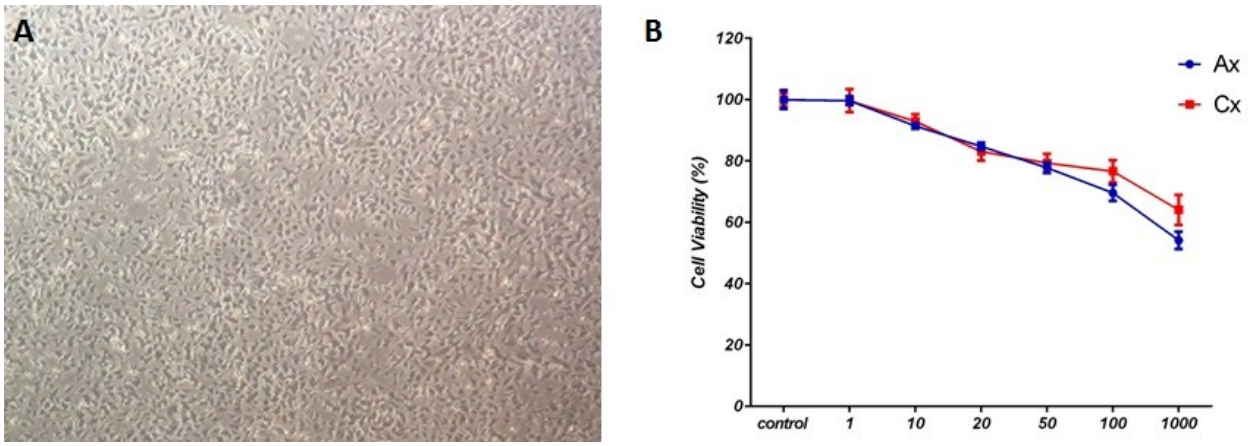

Fig 1. SHSY-5Y cells and the results of cell viability among different cell groups. A) SHSY-5Y cells (magnification X10). B) Cell viability. Cell groups were treated with different doses of Ax and Cx (1, 10, 20, 50, 100 and 1000 µM/ml); then, MTT assay was performed to assess their viability. The results showed no significant difference between Ax and Cx in cell viability. The assay indicated that concentrations above 20 µM/ml induced a significant ($P<0.05$) decrease in cell viability compared to the control group. Data are representative of three independent experiments given as mean ± SEM.

Discussion

These results indicate that Ax and Cx are possible senescence effect for neuroblastoma cell line, SHSY-5Y cells, and such effects may be partly due to suppression of cell proliferation. Recent studies showed that Ax and Cx can induce apoptosis in SHSY-5Y cells. The induction of apoptosis by Ax and Cx may be due to its anti-apoptotic activity and anti-oxidative properties via induction of expression of catalase and superoxide dismutase (SOD) and regulating the expression of Bcl-2 and Bax (2, 12, 13). Ax and Cx have been considered as an apoptotic inducing agents for tumor therapy approaches. Also, such results provide a valuable therapeutic strategy for the treatment of progressive neurodegenerative disease such as PD and AD. In one recent study, an increased level of Ax and Cx in neuroblastoma cells emphasized its potential role for regulation of apoptosis and senescence. Furthermore, existing the Ax and Cx were demonstrated to repress the cell division (9, 13, 14).

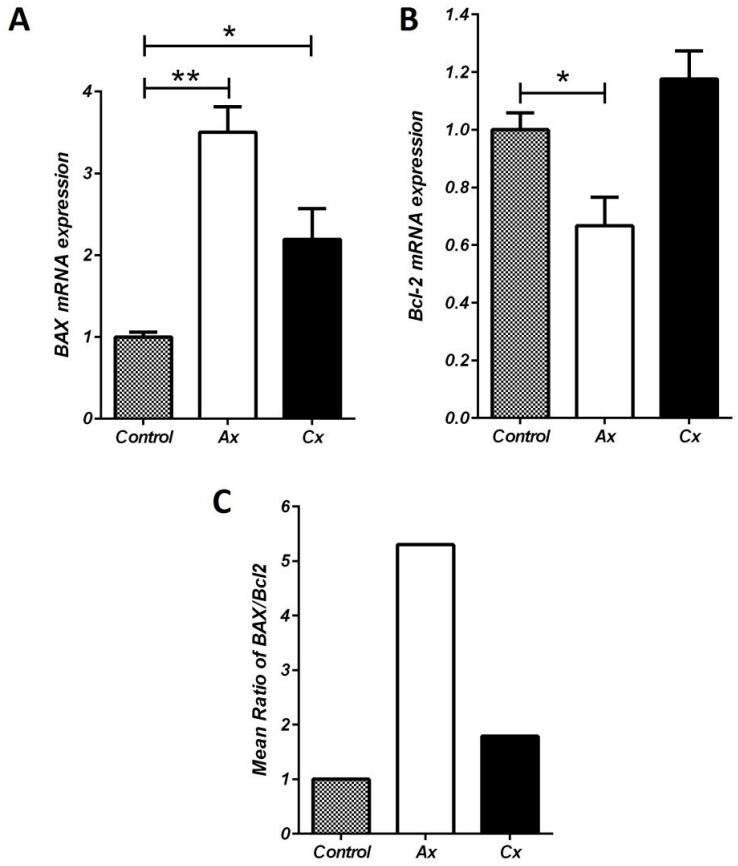

Fig 2. The effect of Ax and Cx on the genes associated with apoptosis BAX, Bcl-2 and BAX/ Bcl-2 ratio in SHSY-5Y cells. BAX and Bcl-2 gene expression levels were estimated by real-time quantitative PCR in SHSY-5Y cells, 48 h after the treatment with Ax and Cx (20 µM/ml). A) The mRNA expression analysis for BAX gene expression level. B) Bcl-2 mRNA expression levels in SHSY-5Y cells. C) Ratios of BAX/Bcl-2 mRNA expression calculated from the mean value of each data. Expression data relative to those of the reference gene from at least three independent assays are given as mean ± SEM. Statistical significance was tested using the one-way ANOVA. * P value < 0.05; **, P value < 0.01.

On the other hand, various studies have suggested that senescence of SHSY-5Y cells may alleviate the proliferation of neuroblastoma cells (15). Herein, the applications of Ax and Cx for proliferation of neuroblastoma cells through the apoptosis and senescence induction in SHSY-5Y cells have been investigated. So, with in vitro study on SHSY-5Y cells our aims were to: (i) evaluate the cell viability of Ax and Cx, (ii) assess the apoptotic role of Ax and Cx and (iii) examine the senescence induction impact of Ax and Cx. As the first finding, viability test demonstrated a similar cytotoxic effect for Ax and Cx on SHSY-5Y cells. The viability test also revealed that concentration higher than 20 µM for Ax and Cx harbor more cytotoxic activity than alone SHSY-5Y cells. Previous study showed that high concentrations of Ax and Cx trigger the apoptosis in cells more efficiently than lower concentration (16, 17). Ax and Cx were shown to stimulate retinoic acid receptors such as retinoic acid, resulting in the production of hyaluronan, an important component of the ECM which can play an important role in the process of differentiation (9). Also, Ax and Cx are known to previously been associated with the inhibition of activator protein (AP)-1 dependent transcription. Retinoic acids are rather known to favor mitochondrial transition and permeability, leading to apoptosis (18).

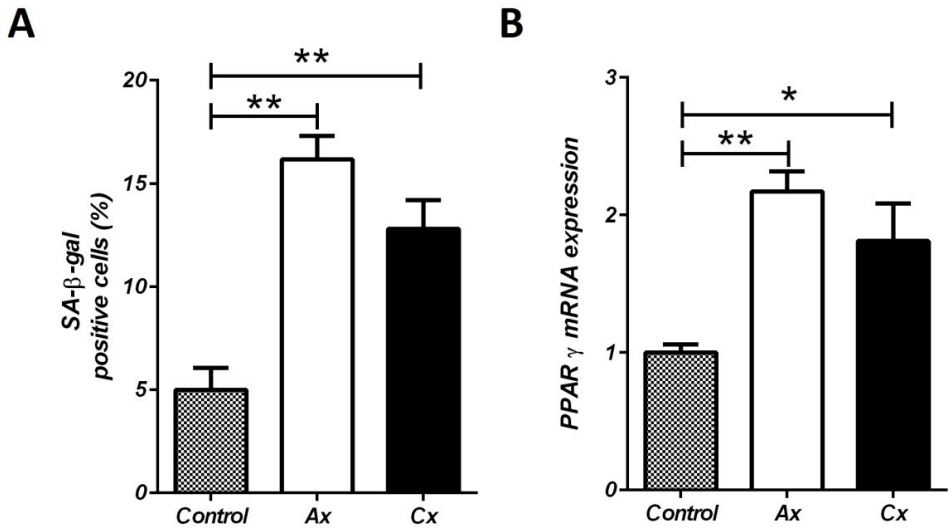

Fig 3. Ax and Cx induced cellular senescence in SHSY-5Y cells. A) The percentage of SA-β-Gal staining positive cells was calculated. B) The mRNA expression analysis for PPAR□ □gene expression level. Data were representative of three or more independent trials and given as mean ± SEM. Statistical significance was tested using the one-way ANOVA. * P value < 0.05; **, P value < 0.01.

Xanthophylls also inhibited phosphorylation of mitogen-activated protein kinase (MAPK) and the transactivation of AP-1 which plays an important role in regulating MMP expression. Diketocarotenoids were found to promoted differentiation via the downregulation protein expression of matrix metalloproteinase (MMP)-1. Based on previous data, the MAPK signaling pathway plays a central role in regulating MMP-1 expression (9; 19). The preliminary study showed that retinoic acid effect on neuroblastoma, and the potential involvement of nuclear retinoid X receptors (RXRs) and retinoic acid receptors (RARs). It has also been shown that retinoic acids involvement in neuroblastoma cells differentiates (20). In addition, the results of previous studies have shown that the relative induced differentiation properties of retinoic acids are similar to their relative effects on gene induction, but with respect to proliferation, these retinoic acids have about the same level of activity at high doses. It poses the question of whether or not retinoic acid-inhibition of proliferation and induced differentiation are controlled by different mechanisms (20, 21). Extra to the role of RARs and RXRs transcription factor, these two factors can be effective transcriptional inhibitors by binding as ligand-dependent monomers to the components of the API complex(c-jun and c-fos), a transcriptional activator involved in controlling cell proliferation. Also, Induction of RARβ is early events in SH-SY-SY cell differentiation induced by retinoic acid (20, 22). Neuronal differentiation is of interest in the prevention of neurodegeneration associated with senescence and neurodegenerative diseases (AD, PD). Also, the α subunit Ca2+/calmodulin (CaM)-dependent protein kinase II (CaMKII) is one of the most important protein kinases involved in brain development and differentiation. For example, α-CaMKII is one of the most abundant protein found in mammalian brain and is highly expressed in the hippocampus indicate that α-CaMKII gene expression is regulated at the level of transcription. A recent study has shown that that the α-CaMKII promoter may

contain sequences that respond directly or indirectly to retinoic acids and these compounds can trigger neuronal differentiation (23, 24).

Conclusions

In conclusion, the findings of our current study revealed that diketocarotenoid and its mechanisms of action propose new methods to approach the differentiation therapy of neuroblastoma. The results showed that the diketocarotenoid increases apoptosis gene expression and cellular senescence in neuroblastoma cells. In other words, enhancing of senescence induction by diketocarotenoid would be a new strategy for controlling the neuroblastoma.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' contributions

All authors equally contributed to the writing and revision of this paper.

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