WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 10, Issue 4, 52-61. Research Article ISSN 2277– 7105

THE APOPTOTIC EFFECT OF ALPHA-LINOLENIC ACID THROUGH ANTI-INFLAMMATORY IN BLADDER CANCER CELLS

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Article Received on
25 Jan. 2021,

Revised on 17 Feb. 2021,
Accepted on 09 March 2021

DOI: 10.20959/wjpr20214-20076

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ABSTRACT

Introduction: Alpha-linolenic acid (ALA) is one of the omega-3 fatty acids and has anti-oxidant and anti-inflammatory effects. Previous studies have shown that ALA affects apoptosis, known as cell death, in some cancer cells and reduces inflammation. Apoptotic pathways are important for cancers, chemotherapeutic resistance and cancer development. Furthermore, some studies have reported that the use of anti-inflammatory agents may be a potential strategy for the inhibition of cancer. Therefore, we planned to investigate the activities of apoptotic mediators such as caspase-3, B-cell lymphoma 2 (bcl-2), BCL2 Associated X (bax), wee1 (a nuclear protein), growth arrest and DNA damage 153 (gadd153), Glucose Regulated Protein 78 (grp78), allograft inflammatory factor-1 (AIF) and inflammatory mediators Inducible Nitric Oxide Synthase (iNOS), Siklooksijenaz-2 (COX-2), Cytosolic phospholipases A2 (cPLA2) and Nuclear factor-κB (NFkB) in bladder cancer cells treated with ALA. **Material and methods:** Bladder cancer cell lines were treated with ALA and their activity of caspase-3 and expression of bax, bcl-2, wee1, gadd153, grp78, AIF, and iNOS, COX-2, cPLA2 and NFkB were analyzed using ELISA assay. **Results:** While ALA treatment increased caspase-3 activity and the expression of bax, wee1, gadD153, grp78 and AIF, reduced the expression of bcl-2, an anti-apoptotic protein. ALA also decreased the expression of iNOS, COX-2, cPLA2 and NFkB in bladder cancer cells. **Conclusion:** Based on our findings, we conclude that ALA may prevent the development of bladder cancer and its anti-inflammatory effect can contribute to this effect.

KEYWORDS: Alpha-linolenic acid, bladder cancer, apoptosis, inflammation, cell line

1. INTRODUCTION

Polyunsaturated fatty acids are liquid at room temperature and at the same time they are very important for the continuity of human life. Therefore, they are called basic fatty acids and are separated into two groups, n-6, n-3 fatty acids. While the main source of n-6 is corn and soybean oil with high linoleic acid content, n-3 is abundant in flaxseed, walnuts and especially planktones and fatty fish. Alpha-linolenic acid (ALA) in flaxseed and walnuts, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in fish oils are the most important fatty acids. EPA and DHA must be taken absolutely externally. Because they cannot be synthesized by the human body, they are called essential fatty acids.[1]

ALA is the precursor of docosahexaenoic acid (DHA) and pentanoic acid (EPA) which are incorporated into the arachidonate structure.[2] In a previous study, it has been reported that 12-week ALA diet reduces systolic and diastolic blood pressures of Japanese subjects with high-normal blood pressure and mild hypertension.[3] ALA possesses antioxidant activity and the subsequent reduction of oxidative stress contributes to the prevention of inflammation.[4] ALA also reduces inflammation caused by lipoprotein saccharide (LPS) as well as nuclear factor kappa-B (NF-kB) translation and phosphorylation of mitogen-activated protein kinase (MAPK). The latter inhibition reduces the expression of inflammatory factors such as iNOS, COX-2, TNF-alpha.[5] It has been reported that ALA decreased the ratio of bcl-2/bax and subsequently increased the release of proapoptotic mediator cytochrome c and caspase-3 expression in human breast cancer cells.[6] Furthermore, previous studies showed that using anti-inflammatory agents may be a potential strategy for the inhibition of cancer.[7] (Rahman et al. 2012). For this purpose, we planned to investigate the apoptotic effect of ALA on bladder cancer cells.

2. MATERIALS AND METHODS

2.1. Cell Culture

Human bladder cancer cells HTB-9 were obtained from ATCC. Bladder cancer cells were grown on RPMI 1640 (ATCC) medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate. Cells were incubated at 37°C in a 5% CO_2 air.

2.2. Cell Homogenization

Cells (5×10^4 cells/cm^2) were exposed to 100 µmol/L ALA for 48 h. They were then washed in PBS and lysed in RIPA buffer (150 mmol/L NaCl 0.5%, TritonX-100, 20 mmol/L EGTA, 1 mmol/L dithiothreitol, 25 mmol/L NaF, 50 mmol/L Tris–HCl [pH 7.4], 1 mmol/L Na_3VO_4) for 15 min on ice followed by centrifugation at 15000 rpm for 20 min, then supernatants are taken and pellets are discarded.

2.3. Protein Quantification

Bradford method is used to quantify the total protein in homogenized tissues. By using Bovine serum albumin (1µg/ml), 1, 2, 3, 5, 7, 8, 10 (µg/ml) standarts are prepared. 10 µl is taken from every sample and completed to 100 µl by adding distilled water. 1 ml Bradford solution is added to standards and samples, vortexed and absorbances at 595 nanometer are measured manually. Protein quantification (µg/µl) was performed according to the standart curve drawn in Prism software.

2.4. ELISA (Enzyme Linked Immunosorbent Assay) Test

ELISA test is used to examine the expression of caspase-3, bax, bcl-2, wee1, gadD153, grp78, AIF, iNOS, COX-2, cPLA2 and NFkB according to the manufacturer's instructions and ELISA kits were purchased from Shenzhen Genesis Technology (Shanghai Sunred Biological Technology, China).

2.5. Statistical Analyzes

For the comparison of parameters from control and ALA treated group unpaired Student's t test was used. Data is presented as means ± SEM. Statistical analysis of differences with $p < 0.05$ was taken as the indicator of significance.

3. RESULTS

ALA treatment increased the activity of caspase-3 (fig 1) and expression of bax (fig 2), wee1, gadd153, grp78 and AIF (tab I) and decreased the expression of the anti-apoptotic bcl-2 (fig 3) protein. ALA also decreased iNOS (fig 4), COX-2 (fig 5), cPLA2 (fig 6) and NFkB (fig 7) in bladder cancer cells.

FIGURE LEGENDS

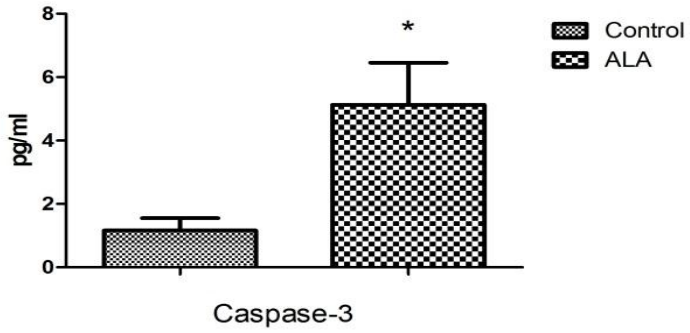

Figure 1: Effect of ALA treatment on caspase-3 activity. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

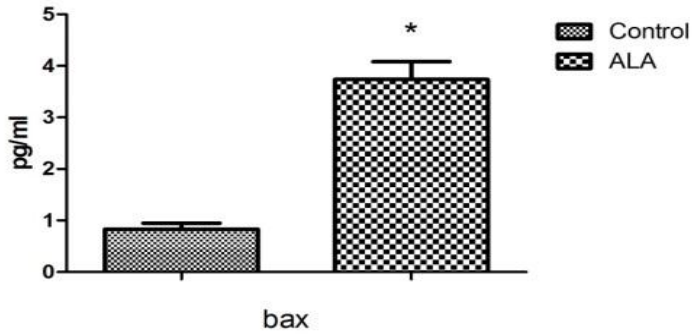

Figure 2: Effect of ALA treatment on bax expression. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

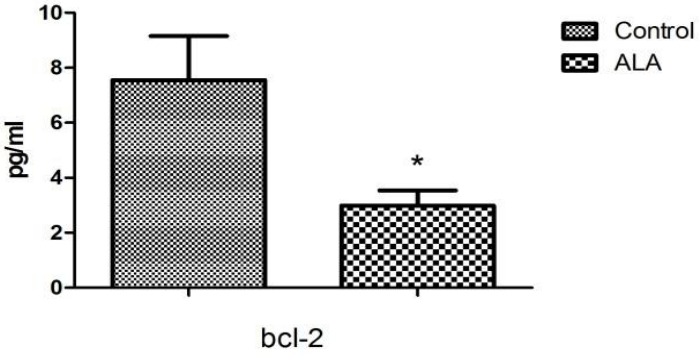

Figure 3: Effect of ALA treatment on bcl-2 expression. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were

analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

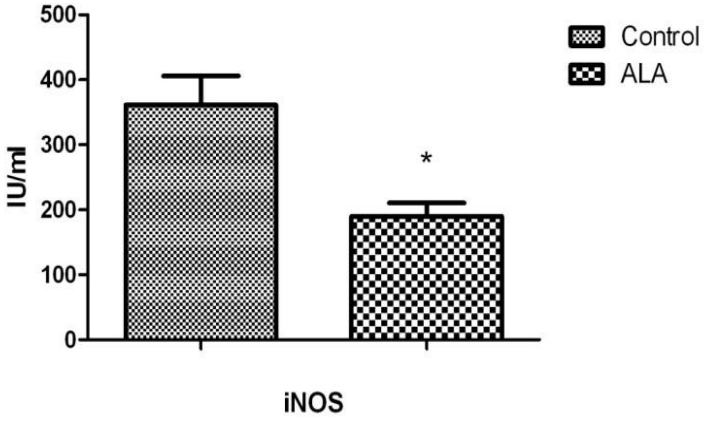

Figure 4: Effect of ALA treatment on iNOS expression. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

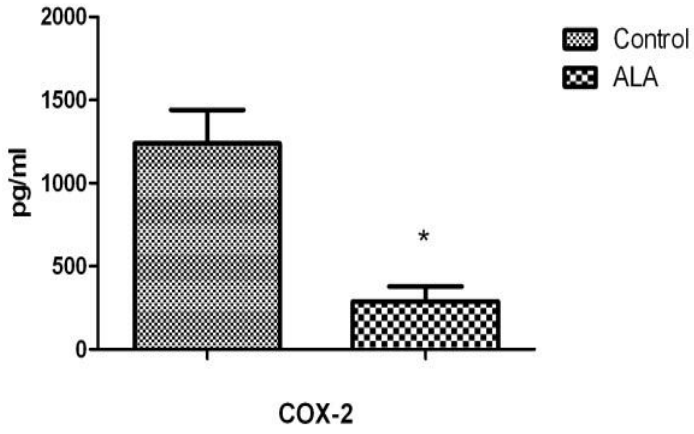

Figure 5: Effect of ALA treatment on COX-2 expression. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

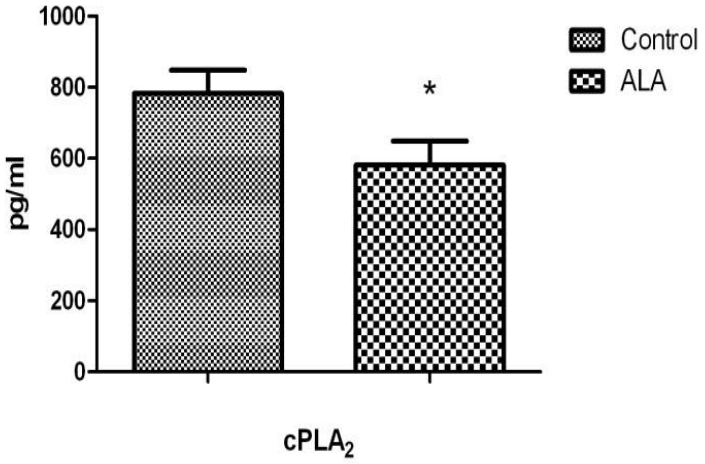

Figure 6. Effect of ALA treatment on cPLA2 expression. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

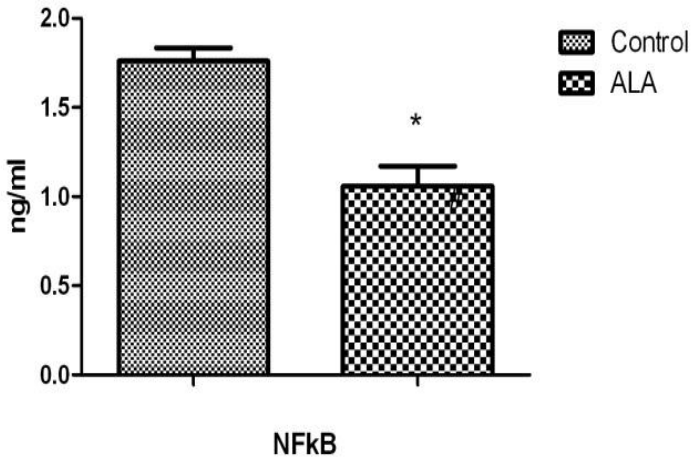

Figure 7. Effect of ALA treatment on NFkB expression. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

Table I. Effect of ALA treatment on wee 1, gadd153, grp78 and AIF expression. The data are presented as mean ± SEM. Differences between parameters of control and ALA treated group were analyzed by applying unpaired Student's *t* test. *difference between control and ALA treated group is significant with $p < 0.05$.

Table I	Control	ALA Treated
wee 1	0.18±0.015 pg/ml	0.83±0.012 pg/ml*
AIF	0.88±0.013 pg/ml	1.4±0.08 pg/ml*
gadd153	0.22±0.014 pg/ml	1.63±0.25 pg/ml*
grp78	0.38±0.01 pg/ml	1.03±0.15 pg/ml*

4. DISCUSSION

ALA is one of the essential omega-3 fatty acids. Increased dietary intake of omega-3 fatty acid is associated with the reduced risk of cardiovascular disease. Likewise, it has been suggested that ALA may retard the growth of breast and colon cancer cells.[6,8] In this study, we have shown that ALA inhibits cell proliferation and induces apoptosis in bladder cancer cells. Also, our study showed that ALA induced expression of proapoptotic proteins, however, reduced the expression of anti-apoptotic proteins and inflammatory mediators. Previous studies have reported that ALA induced apoptosis in some cancers.[8] It has been also reported that the use of anti-inflammatory agents is a potential strategy in fighting against cancer.[7] We have demonstrated the anti-inflammatory effect of ALA in our previous studies.[9,10] In this study, we showed the overexpression of cPLA2, iNOS, COX-2 and NFκB in bladder cancer cells as well as the decreased expression of these mediators by ALA. Anti-proliferative and proapoptotic effect of ALA on bladder cancer cells may be due to its anti-inflammatory effect. Inhibition of cPLA2, COX-2 and it's products prostanoids, prostaglandins (PGs), and thromboxane A2 (TxA2) plays an important role in carcinogenesis.[11] It has also been reported that ALA has an antioxidant effect.[9,10] Meanwhile, COX-2 contributes to immune evasion and resistance to cancer immunotherapy. Increasing activity of COX-2-PGs suppresses Dendritic cells, natural killer, T cells, type-1 immunity, but promote type-2 immunity, which promote tumor immune evasion.[11]

Oxidative stress is known to play a role in inflammation and apoptosis and reactive oxygen species (ROS) have both beneficial and negative effects in this process. At a very low concentration, oxidative stress acts as a second messenger on signal transduction pathways.[12] However, when overproduced, it can cause damage to many vital components of the cell. There has been a relationship between ROS production and antioxidant capacity. Some oxidation processes are involved in some processes within the cell. Transcription

factors such as NF-κB, p53 and AP-1 have been shown to be modulated by oxygen species.[13] Therefore, non-lethal ROS production can affect signal transduction pathways. ROS is the second messenger for angiotensin inflammatory cytokines and various physiological stimuli such as growth factors or transforming factors.[12, 13] Apoptosis is known as programmed cell death and is essential for the normal functioning and survival of most multicellular organisms. However, the morphological and biochemical characteristics of apoptosis are highly conserved during evolution. Recent studies have shown that ROS and oxidative stress play an important role in apoptosis.[14, 15] Antioxidants may block or delay apoptosis. It has been shown that Bcl-2, an endogenous anti-apoptotic protein, is inhibited by an antioxidant mechanism in cells escaping from apoptosis.[16] Taken together, the ROS and the resulting cellular redox exchange may be part of the signal transduction pathway during apoptosis.[16] Moreover, the antioxidant effect of ALA may have roles on its anti-cancer effect as well. Furthermore inhibition of COX-2 and inflammatory mediators may have off-target effects on immune cells and can counterbalance their activity as enhancers of susceptibility to immune elimination. So, COX-2 may serve as predictive biomarker and as therapeutic target for modulation of immune resistance in cancer.[11]

In conclusion, ALA inhibits bladder cancer development and its anti-inflammatory effect may contribute to this outcome.

ACKNOWLEDGMENT

This research was supported by a grant from Mustafa Kemal University Research Fund (16543/2017).

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