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Research Article
Alpha-linolenic Acid Attenuates Lipopolysaccharide Induced Cystitis

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Abstract

Background and Objectives: Alpha-linolenic acid which is one of polyunsaturated fatty acids is very important for the continuity of human life. Alpha-linolenic acid is a protective food for the heart and cardiovascular system. In addition, this substance is a powerful anti-inflammatory agent. Increases in inflammatory agents are accompanied cystitis induced by lipopolysaccharide (LPS). Therefore, this study aimed to determine whether alpha-linolenic acid, which has anti-inflammatory properties, is protective effect against LPS-induced cystitis. **Materials and Methods:** Cystitis were induced by intravesical LPS application in mice and treated with alpha-linolenic than cyclooxygenase-2, phospholipase A2 and inducible nitric oxide synthase (iNOS) enzymes were analyzed by Enzyme-Linked Immuno Sorbent Assay (ELISA) method in their bladder IL-6 and TNF-α in their serum. Differences in results between tissues were tested by one way analysis of variance (ANOVA) corrected by Bonferroni multiple-comparison. **Results:** While cystitis increased expression of IL-6, TNF-α, NF-κB, cyclooxygenase-2, phospholipase A2 and iNOS enzymes the application of alpha-linolenic acid reversed this increase. **Conclusion:** This study discovered the alpha-linolenic acid that can be beneficial for LPS-induced bladder inflammation. This study will help the researcher to uncover the critical areas of bladder inflammation caused by Gram negative bacteria.

Key words: LPS, cystitis, bladder, alpha-linolenic acid, inflammation, mice

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Polyunsaturated fatty acids are also very important for the continuity of human life. Therefore, they are called essential fatty acids and they are separated into two groups, n-6, n-3 fatty acids. The main source of n-6 is corn and soybean oil with high linoleic acid content. The n-3 is abundant in flaxseed, walnut and especially plankton and fatty fish. These fatty acids must be taken from the outside. Because they are not synthesized by the body, they are called essential fatty acids[1]. Alpha-linolenic acid is a protective agent for the heart and cardiovascular system[2]. It is the precursor of docosahexaenoic acid (DHA) and pentanoic acid (EPA) which are incorporated into the arachidonate structure[3]. Alpha linolenic acid has antioxidant activity. Reducing oxidative stress contributes to the prevention of inflammation[4]. Alpha-linolenic acid reduces inflammation induced by the lipoprotein saccharide (LPS). Alpha-linolenic acid also inhibits nuclear factor kappa-B (NF-κB) translocation and phosphorylation of mitogen-activated protein kinase (MAPK). This inhibition reduces the expression of inflammatory factors such as iNOS, COX-2, TNF-alpha[5].

The LPS is found in the wall structure of Gram negative bacteria and trigger the inflammation to increase the synthesis of inflammatory mediators. Alpha-linolenic acid have anti-inflammatory and antioxidant activity[4-6]. So, it raised curiosity about whether or not it can be effective on LPS induced bladder inflammation. For this purpose this study was designed to demonstrate the protective properties of alpha-linolenic acid on bladder inflammation induced by LPS and to show that it may be used for supplement to treat cystitis.

MATERIALS AND METHODS

Induction of cystitis: Albino female mice (n = 8, weight ≈ 30 g, 8 weeks old balb/c) that are obtained from the Experimental Animal Center in Cukurova University, in Adana are used in study. This study was approved by the Animal Care Committee and Ethics Committee of Cukurova University. The mice were fed *ad libitum* and kept in cages with 8 mice in each cage, with a 12 h/12 h light/dark cycle at a temperature of 20-22°C and 50-55% humidity. Mice divided into three groups as control, cystitis and cystitis together alpha-linolenic acid treated. Alpha-linolenic acid (200 mg kg^{-1}) orally performed to mice twice a day during 10 day before cystitis application to group of cystitis together alpha-linolenic acid treated. Cytitis induced the method proposed by Keay *et al.*[7]. Animals were anesthetized with ketamine HCl (40 mg kg^{-1} im) and xylazine (2.5 mg kg^{-1} im). A polypropylene catheter (24 gauge; 3/4 inch) was introduced transurethrally into the bladder and advanced until the first drop of urine appeared. Urine was drained by applying light pressure to the abdomen and 150 µL of one of the following substances: Pyrogen-free saline (0.9%) or LPS (100 µg mL^{-1}) was instilled into the bladder. Substances were infused at a slow rate (0.1 mL sec^{-1}) to avoid trauma and vesicoureteral reflux. The latter ensured that there was no reflux or leakages at least for 30 min. Twenty four hours after LPS stimulation mice were killed by cervical dislocation and their bloods obtained and bladders were isolated rapidly and stored at -80°C to use in ELISA experiments.

Quantitative analysis

Tissue homogenization: Radio-immunoprecipitation Assay (RIPA) buffer pH 7.4 (3 mL g^{-1}) 30 µL PMSF (phenylmethanesulfonyl fluoride), 30 µlsodyum vanadate, 30 µL protease inhibitor is applied on frozen kidney samples that are stored in eppendorf tubes then homogenates are obtained by using ultrosonication on those tubes on ice. Homogenates are then centrifuged at 10,000 rpm for 10 min and supernatants are taken and pellets are discarded.

Protein quantification: Bradford method is used to quantify the protein in homogenized tissues[6].

Enzyme linked immunosorbent assay (ELISA) test: In bladder expression of NF-κB, cyclooxygenase-2, phospholipase A2 and iNOS were measured by using enzyme-linked immunosorbent assay (ELISA) (Shanghai Sunred Biological Technology Co., Ltd. China) kits according to the manufacturer's instructions.

Blood samples (about 0.5 mL) were obtained from each animal and centrifuged at 3000 rpm for 10 min to obtain the serum. Serum levels of Tumor Necrosis Factor-α (TNF-α), interleukin (IL-6) (Shanghai Sunred Biological Technology Co., Ltd) were measured by using Enzyme-linked Immunosorbent Assay (ELISA) kits according to the manufacturer's instructions.

Statistical analysis: Results were expressed as Mean±SEM and n refers to the number of animals used for each experiments. Differences in results between tissues were tested by one way analysis of variance (ANOVA) corrected for multiple comparisons (Bonferroni corrections). The $p<0.05$ were considered to be significant[8].

RESULTS

ELISA NF-κB enzyme quantification: The LPS application caused an increase in the NF-κB enzyme (7.771±0.6097) in bladder, when compared with control group application of alpha-linolenic acid decreased this increase (5.2±0.4) significantly ($p<0.05$) when compared with gentamicin group (Fig. 1).

ELISA COX-2 enzyme quantification: The LPS application caused an increase in the COX-2 enzyme (13307±1103) in bladder when compared with control group, application of alpha-linolenic acid decreased this increase (9203±635.8) ($p<0.05$) when compared with gentamicin group (Fig. 2).

ELISA cPLA2 enzyme quantification: The LPS application caused an increase in the cPLA2 enzyme (2842±245.7) in bladder when compared with control group, application of alpha-linolenic acid decreased this increase (2048±179.1) ($p<0.05$) when compared with gentamicin group (Fig. 3).

ELISA iNOS enzyme quantification: The LPS application caused an increase in the iNOS enzyme (4565±597.0) in bladder when compared with control group, application of alpha-linolenic acid decreased this increase (2791±429.9) ($p<0.05$) when compared with gentamicin group (Fig. 4).

ELISA IL-6 quantification: The LPS application caused an increase in the IL-6 (158.2±8.9) in bladder when compared

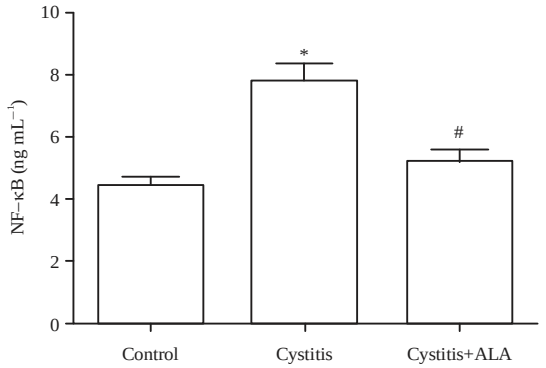

Fig. 1: Effect of alpha-linolenic acid on cystitis induced NF-κB expression (n = 8)

ALA: Alpha-linolenic acid treated group, cystitis: LPS treated group, cystitis+ALA: LPS together alpha-linolenic acid treated group. The amount of NF-κB in tissue homogenates expressed as nanogram per milliliter. *Cystitis $p<0.05$, #Cystitis+ALA $p<0.05$

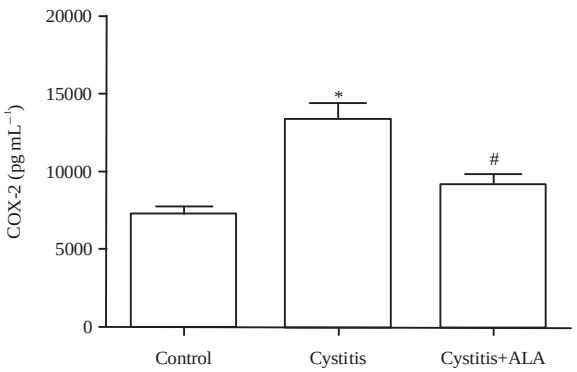

Fig. 2: Effect of alpha-linolenic acid on cystitis induced COX-2 expression (n = 8)

ALA: Alpha-linolenic acid treated group, cystitis: LPS treated group, cystitis+ALA: LPS together alpha-linolenic acid treated group. The amount of COX in tissue homogenates expressed as picogram per milliliter. *Cystitis $p<0.05$, #Cystitis+ALA $p<0.05$

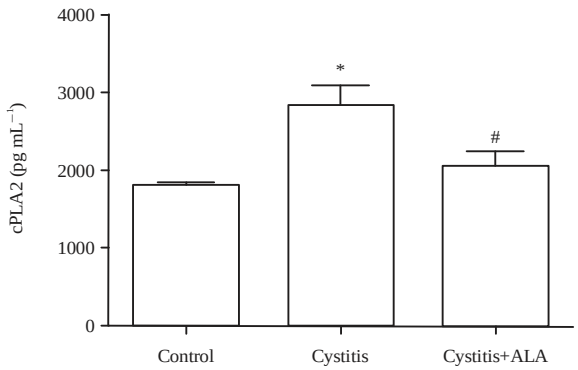

Fig. 3: Effect of alpha-linolenic acid on cystitis induced cPLA2 expression (n = 8)

ALA: Alpha-linolenic acid treated group, cystitis: LPS treated group, cystitis+ALA: LPS together alpha-linolenic acid treated group. The amount of cPLA2 in tissue homogenates expressed as picogram per milliliter. *Cystitis $p<0.05$, #Cystitis+ALA $p<0.05$

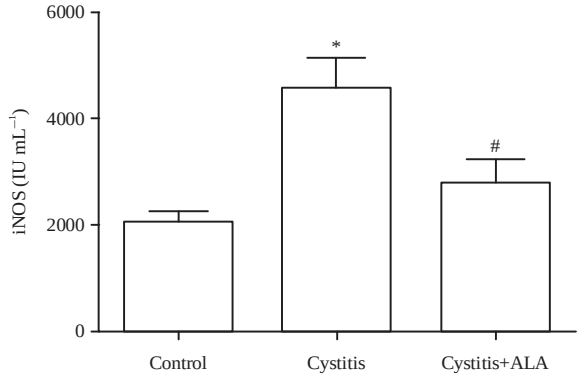

Fig. 4: Effect of alpha-linolenic acid on cystitis induced iNOS expression (n = 8)

ALA: Alpha-linolenic acid treated group, cystitis: LPS treated group, cystitis+ALA: LPS together alpha-linolenic acid treated group. The amount of iNOS in tissue homogenates expressed as international units per milliliter. *Cystitis $p<0.05$, #Cystitis+ALA $p<0.05$

Table 1: Effect of alpha-linolenic acid on cystitis induced IL-6 and TNF-α expression

Groups	IL-6	TNF-α
Control	101.5±2.3	49.520±2.1
Cystitis	158.2±8.9*	92.350±6.2*
Cystitis+alpha-linolenic acid	109.9±5.9#	59.515±5.9#

Results are presented as Mean±SE, *Cystitis p<0.05, #Cystitis+alpha-linolenic acid p<0.05

with control group, application of alpha-linolenic acid decreased this increase (109.9±5.9) (p<0.05) when compared with gentamicin group (Table 1).

ELISA TNF-α quantification: The LPS application caused an increase in the TNF-α (92.35±6.2) in bladder when compared with control group, application of alpha-linolenic acid decreased this increase (59.51±5) (p<0.05) when compared with gentamicin group (Table 1).

DISCUSSION

In this study NF-κB, COX-2, cPLA2, iNOS, TNF-α and IL-6 which are inflammatory mediators were evaluated. While application of LPS induced expression of these mediators alpha-linolenic acid attenuated these mediators. It is known that LPS binds with specific membrane receptors in mononuclear phagocytes and neutrophils, triggering a series of signal transduction events. Inducing this signal transduction system leads to inflammation by the secretion of the inflammation mediators. The LPS react with the CD14 adhesion molecules, the LPS membrane binding protein receptor, the LPS scavenger receptor and the CD18 glycoprotein adhesion receptor complex. In addition, these receptors are also present in macrophages, mononuclear leukocytes and neutrophils. The mediators of inflammation secreted are TNF-α, IL-1, prostaglandins, leukotrienes and the platelet activating factor. These mediators either induce inflammatory reactions directly by infiltrating leukocytes and increasing the vascular permeability or indirectly by interacting with the other molecules[9]. Alfa-linolenic acid is a protective agent for the cardiovascular system and is known to reduce mortality from diseases associated with this system[6]. Studies have shown that this substance has a protective effect with many factors, including anti-inflammatory properties. The IL-6, TNF-α, NF-κB, cyclooxygenase-2, phospholipase A2 and iNOS enzymes, which act as biological mediators in the inflammatory response were evaluated. Previous studies have shown that alpha-linolenic acid reduces iNOS enzyme[4]. The application of alpha-linolenic acid reduced the amount of LPS-induced iNOS enzyme expression.

Nitric Oxide (NO) has a pathogenic role in acute and chronic inflammatory diseases produced by iNOS[8-11]. However, reduction of the increase of COX-2 enzyme caused by alpha-linolenic acid LPS has been shown[5]. In this study alpha-linolenic acid also reduced the increase in LPS-induced COX-2 enzyme[12]. There are studies showing that LPS application increases the enzyme phospholipase A2 that is another enzyme we have evaluated in this study[5]. There is a potential antioxidative property due to double-linkage in the molecular structure of alpha-linolenic acid[4].

Studies have shown that this increase in intracellular calcium level due to oxidative stress leads to an increase in the activity of phospholipase A2 enzyme[13,14]. Superoxide radicals can be produced in the cell as exogenous and endogenously. Endogenous oxidant source is mitochondria. The source of exogenous oxidant is the neutrophil eosinophils and macrophages. In nonpathologic situations, oxidants in the cell take some intracellular roles, but in pathological situations they cause damage to the cells when the amounts increase. Cells have antioxidant defense systems against oxidants, but when exposed to high amounts of oxidative stress, the agents of this system are exhausted than cell damage begins. Alpha-linolenic acid may have reduced the expression of phospholipase A2 by reducing oxidative stress due to its anti-oxidant property. It is known that LPS increases oxidative stress[13]. Phospholipase A2 acts in the cascade at the top of the inflammation pathway. Reduction of phospholipase A2 will result in decreased synthesis of prostaglandins, cytokines and leukotrienes synthesized in the subsequent cascade and may also result in decreased expression of COX-2[9]. This case also helps to inhibition of inflammation by alpha-linolenic acid.

Transcriptional factors such as NF-κB, p53 and AP-1 have been shown to be modulated by oxygen species. Sublethal ROS production, therefore, can interfere with signal transduction pathways[15]. The ROS, in particular H_2O_2 are indeed second messengers for various physiological stimuli, such as, angiotensin inflammatory cytokines and growth factors or transforming factors[16]. In the light of previous studies and our findings suggest that alpha-linolenic acid acts as anti-inflammatory agent cause of its anti-oxidant property[4,5]. In this study, animal cystitis model were performed and used only LPS which found in the wall structure of Gram negative bacteria. However, LPS is only one of Gram negative bacteria's toxins. Furthermore alpha-linolenic acid doesn't have antibiotic effect. It may be used intact bacteria, antibiotics and alpha-linolenic acid together to attenuate severity of cystitis. Therefore, further studies are needed.

CONCLUSION

This study showed that administration of alpha-linolenic acid resulted in a decrease of IL-6, TNF-α, NF-κB, cyclooxygenase-2, phospholipase A2 and iNOS which are mediators of inflammation in mouse bladder and this finding suggested that alpha-linolenic acid may be used to attenuate LPS induced bladder inflammation and for supplement to treat cystitis.

SIGNIFICANCE STATEMENTS

This study showed that anti-inflammatory effect of alpha-linolenic acid resulted in a decrease of IL-6, TNF-α, NF-κB, cyclooxygenase-2, phospholipase A2 and iNOS. Supplementary use of alpha-linolenic acid may be useful to attenuate inflammation in cystitis.

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