RESEARCH

PROTECTIVE EFFECT OF ASTAXANTHIN AGAINST LPS-INDUCED ORAL AND NASAL MUCOSAL DAMAGE

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Abstract

Background: Abnormal response to inflammation in sepsis can cause multiorgan failure and even death due to endothelial dysfunction and failure to maintain hemostatic balance. Astaxanthin (AXA), a xanthophyll carotenoid, holds exceptional promise as an antioxidant, anti-inflammatory, and anticancer agent. The study aimed to investigate the potential effects of AXA on lipopolysaccharide (LPS)-induced sepsis and oral and nasal mucosal damage. **Materials and Methods:** Sepsis was induced by LPS in a rat model. Rats were divided into three groups as control, LPS, and LPS + AXA groups. After euthanasia of rats at at the end of the study, oral and nasal tissue samples were collected for histopathological and immunohistochemical analyzes. **Result:** While slight to moderate hyperemia in the nasal mucosa was noted in rats in the LPS group, oral mucosa appeared normal in rats from the two other groups. Immunohistochemical examination revealed markedly increased expressions of Cas-8, IL-1β, IL-3, OPN, and iNOS in the oral and nasal mucosal sections ($p<0.001$ for all markers for both mucosa) obtained from the LPS group. AXA treatment caused a marked decrease in immunohistochemical expressions in the AXA+LPS group. There were no expressions in the control group. **Conclusion:** Apoptosis and inflammation play a role in the pathogenesis of sepsis-related oral and nasal mucosal damage caused by LPS. AXA treatment prevents inflammation and apoptosis in the oral and nasal mucosa.

INTRODUCTION

Sepsis is a complex syndrome with limited treatment options and a high mortality rate. Pathogenesis of sepsis involves a dysregulated inflammatory response with the production of inflammatory mediators and reactive species. Sepsis can lead to an exaggerated response to inflammation, the occurrence of endothelial dysfunction, and disruption in intracellular homeostasis, leading to multiorgan failure and even death.[1,2] The intensive and uncontrolled release of excessive levels of inflammatory cytokines and free radicals into the systemic circulation triggers various cellular and metabolic changes observed during disease development. Hypoxia and apoptosis are implicated in the development of organ dysfunction.[1,3] Bacterial components such as lipopolysaccharide (LPS) activate an inflammatory cascade during the development of sepsis, resulting in the release of several inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, and of inflammatory mediators such as nitric oxide (NO).[4,5] The outermost LPS layer of gram-negative bacteria interacts with CD14 and TLR4 receptors on the cell surface.[2,3] Overproduction of inflammatory mediators induces endothelial damage leading to vascular leakage, edema, and vasodilation, leading to multiple organ dysfunction syndrome. Significant oxidative stress, as a result of sepsis-specific inflammatory responses, initiates changes in mitochondrial function, potentially leading to organ injuries.[6] Therefore, it would be worthwhile to develop new drugs with effective anti-inflammatory and antioxidant profiles so that the incidence and mortality associated with this devastating condition would be reduced.

Astaxanthin (AXA), which is a type of red xanthophyll from a carotenoid pigment class, is particularly found in shellfish and salmon. It is obtained commercially from Haematococcus pluvialis microalgae.[6,7] The favorable effects of AXA on health are associated with its potential antioxidant capacity.[7] Diseases, in which astaxanthin is thought to have favorable effects include cancer, inflammatory diseases, heart

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diseases, Helicobacter pylori-associated ulcer, liver diseases, and neurodegenerative diseases.[6,8]
In the light of all these data, we aimed to investigate the effectiveness of AXA against nasal and oral mucosal damage caused by LPS-induced sepsis in our study.

MATERIALS AND METHODS

Animal Preparation

All experiments were performed in accordance with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines in 2.0, and approved by the Local Committee on Animal Research of Burdur Mehmet Akif Ersoy University, Burdur, Turkey (16.10.2019/567). All animals were included in the experimental protocol before the experiment started. The rats were kept at 22-24 ^{0} C and exposed to 12 hours of light and dark cycles. Euro type 6 cages were used and ad libitum feeding regimen was applied. All animals were provided tap water and standard commercial rat chow (Korkuteli, Yem, Turkey)

Experimental Design

In the study, a total of 28 female Sprague Dawley rats, 8 for the control group and 10 for each of the other groups, were used. The rats were divided into 3 groups, as follows:

Group 1(Control group, n=8)

An astaxanthin (Orzax Pharmaceutical and Chemical Industry Trade Inc., Istanbul, Turkey)-equivalent volume (5mg/kg-1ml) of saline was administered by gavage (7 days). An LPS-equivalent volume of saline was administered a single dose by intraperitoneal injection.

Group 2

(LPS group, n=10) Before the administration of LPS (048 K4126, Sigma Aldrich, Sweden), a volume of olive oil equivalent to that of AXA was administered by gavage for 7 days. On the 8th day, a single dose of 5 mg/kg LPS (9) dissolved with 0.9% NaCl was administered by intraperitoneal injection.

Group 3

(LPS + AXA group, n=10): Before the administration of LPS, 100 mg/kg AXA (10) was dissolved in olive oil and given by gavage for 7 days. On the 8th day, a single dose of 5 mg/kg LPS was given by intraperitoneal injection.

All animals were sacrificed under Xylazine HCl (Alfazin, Alfasan IBV)+Ketalar HCl (Alfamin, Alfasan IBV) anesthesia 6 hours after LPS administration. Tissue samples of all mice were collected and histopathological and immunohistochemical analyzes were performed.

Histopathological Analyses

During necropsy, oral and nasal mucosal samples were collected and fixed in 10% buffered formalin solution for histopathological and immunohistochemical examinations. After 2 days of fixation, tissue samples were routinely processed by automatic tissue processor equipment (Leica ASP300S, Wetzlar, Germany) and embedded in paraffin. Then, samples of 5 µm thickness were obtained using a rotary microtome (Leica RM 2155; Leica Microsystems, Wetzlar, Germany). Tissue specimens were stained with hematoxylin and eosin (HE) and examined under a light microscope (11). Histopathological changes were evaluated in a blinded manner by a specialized pathologist.

Immunohistochemical Analyses

For histopathological examinations, 5 serial sections with thicknesses of 5-µm each were mounted onto poly-L-lysine microscope slides. Streptavidin-biotin immunoenzymatic antigen detection system [EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436), (Abcam, Cambridge, UK)] was used as a second antibody. Sections were immunohistochemically stained to demonstrate Cas-8, IL-1β, IL-3, iNOS, and OPN expressions. Commercial kits (Abcam, Cambridge, UK) were used for immunohistochemical examinations. Tissue samples were immunostained with active Cas-8 [Anti-Caspase-8 (ab25901), 1/100 dilution]; interleukin-1β (IL-1β) [Anti-IL1 beta antibody (ab2105), 1/100 dilution]; interleukin-3 [Anti-IL-3 antibody (ab126852), 1/100 dilution]; anti-iNOS antibody (ab15323), and osteopontin [(anti-OPN antibody (ab8448)], 1/100 dilution] according to manufacturers' instructions. Then, sections were counterstained with Harris hematoxylin, coverslipped, and examined under a microscope. All slides were analyzed for immunopositivity, and a semiquantitative analysis was carried out. Samples were analyzed by examining five different sections for each sample and each section was then scored from 0 to 3 according to the intensity of staining (0, absence of staining; 1, slight; 2, medium; and 3, marked staining). Rats included in this study were evaluated randomly in a blinded fashion by the researcher without knowing which rat was included in which group. For morphometric examinations of section, an Olympus CX41 light microscope was used. Morphometric evaluations were made by using the Database Manual CellSens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

Statistical Analyses

One-way ANOVA tests were used to determine whether there were any significant differences between the groups. SPSS 22.00 software for Windows (SPSS Inc, Chicago, IL, USA) was used for analyzing the data. Variables were presented as frequencies, percentages, mean ± standard deviations. Duncan test was used for the analysis of immunohistochemical scores between the groups. $p<0.05$ was set as the value for significance.

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RESULTS

Nasal Mucosa Findings

At the clinical examination, all rats in all groups were normal. No clinical signs such as coughing or sneezing suggesting nasal mucosal damage were observed in any rats. There were no pathological findings such as erosion and ulceration in any rats from any groups at the gross examination of the nasal mucosa. At histopathological examinations, while slight to moderate hyperemia and very slight inflammatory reactions were noticed in the submucosa of rats from the LPS group, the nasal mucosal epithelial layer was in normal appearance in all groups. Immunohistochemical examinations revealed markedly increased Cas-8, IL-1β, IL-3, OPN, and iNOS expressions in the nasal mucosal sections from the LPS group. AXA treatment caused a marked decrease in immunohistochemical expressions in the AXA+LPS group. There were no or slight expressions of any markers in the control group. Representative microphotographs were presented in [Figure 1]. Statistical analysis results of the immunohistochemical scores of nasal mucosa sections were shown in [Figure 2].

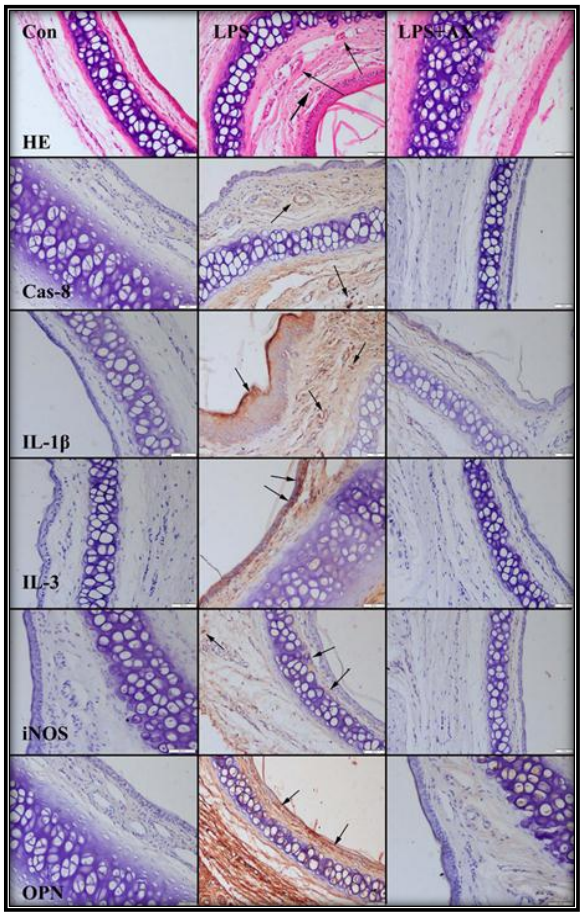

Figure 1: Histopathological appearance of the nasal mucosa; normal tissue histology in the control group, marked hyperemia (thin arrows), and slight inflammatory cell infiltrations (thick arrow) in the LPS group, and decreased pathological findings in the LPS+AXA group; HE (first row). Cas-8 immunohistochemistry findings in the groups; negative expressions in the control group, marked increase in the LPS group (arrows), and no expressions in the LPS+AXA group; Streptavidin biotin peroxidase method (second row). IL-1β immunohistochemical findings in the groups; no expression in the control group, marked increase in epithelial and submucosal cells (arrows) in the LPS group, and marked decreases in the LPS+AXA group; Streptavidin biotin peroxidase method (third row). IL-3 expressions in the groups; negative expression in the control group, increased expression in epithelial cells (arrows) in the LPS group, and no immunoexpression in the LPS+AXA group; Streptavidin biotin peroxidase method (fourth row). iNOS immunoreactions in the groups; no expression in the control group, marked increases in submucosal cells (arrows) in the LPS group, and marked decreases in the LPS+AXA group; Streptavidin biotin peroxidase method (fifth row). OPN expressions in the groups; no expression in the control group, severe expressions in epithelial and submucosal cells (arrows) in the LPS group, and marked decreases in the LPS+AXA group; Streptavidin biotin peroxidase method (sixth row), Bars=50µm.

Oral Mucosa Findings

Clinical examination of rats revealed no clinical signs such as coughing and salivation, which would be potentially related to oral lesions. Gross examination during necropsy revealed normal oral mucosa except slight hyperemia in rats from the LPS group. Histopathological examination of oral mucosa revealed a normal epithelial layer in all groups but marked hyperemia and slight inflammatory cell infiltrations were observed in the oral mucosa of rats from the LPS group. AXA treatment decreased pathological findings. Normal tissue histology was observed in the control group. Immunohistochemical examination revealed marked increases in Cas-8, IL-1β, IL-3, OPN, and iNOS expressions in oral mucosal sections from the LPS group. AXA treatment caused marked decreases in immunohistochemical expressions in the AXA+LPS group. There were no or slight expressions in the control group. Representative microphotographs were presented in [Figure 3]. Statistical analysis results of immunohistochemical scores are shown in [Figure 4].

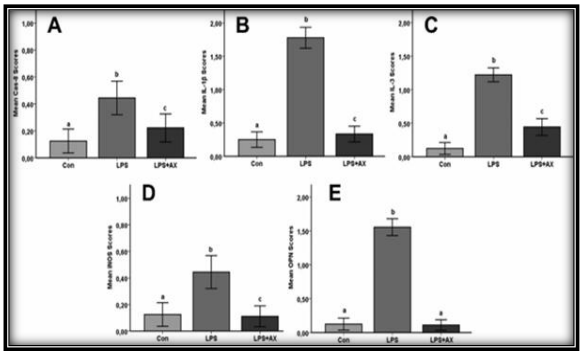

Figure 2: Statistical analysis results of immunohistochemical scores of the nasal mucosa. (A) Cas-8 scores, (B) IL-1β scores, (C) IL-3 scores (D)

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iNOS scores, and (E) OPN scores. Data were assessed by ANOVA and differences between groups were analyzed by Duncan tests. Values represent mean ± standard deviation (SD). Differences between the means of groups marked by different letters indicate statistical significance (P < 0.001).

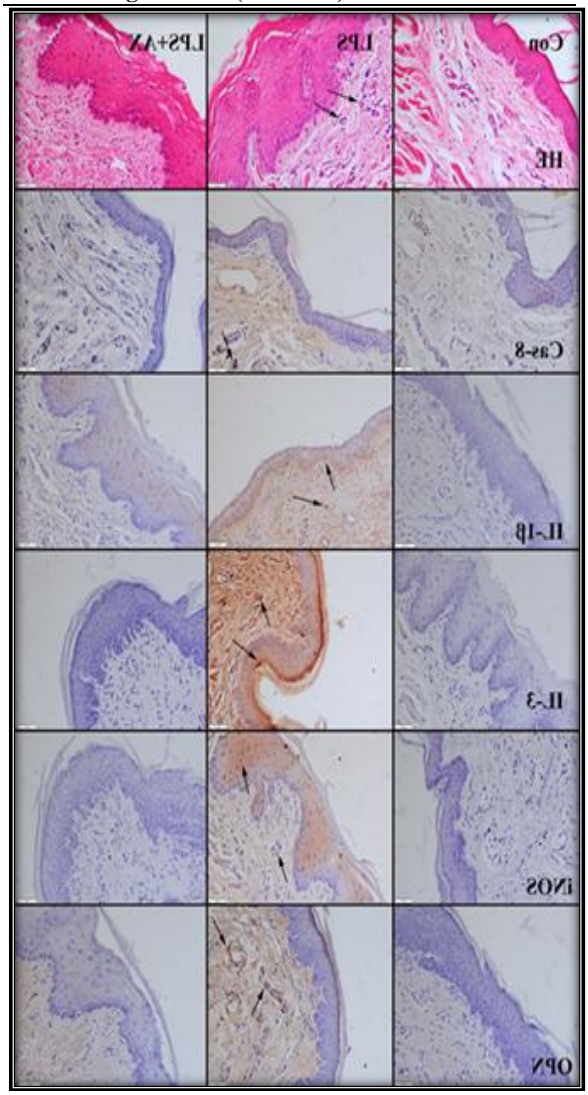

Figure 3: Microscopical findings in oral mucosa; normal tissue architecture in the control group, marked hyperemia (arrows) in the LPS group, and normal tissue histology in the LPS+AXA group; HE (first row). Cas-8 immunohistochemical findings in groups; negative expressions in the control group, slight increases in the LPS group (arrow), and negative expressions in LPS+AXA group; Streptavidin biotin peroxidase method (second row). IL-1β immunohistochemical reaction in groups; negative expression in the control group, marked increases in epithelial and submucosal cells (arrows) in the LPS group, and marked decreases in the LPS+AXA group; Streptavidin biotin peroxidase method (third row). IL-3 expressions in groups; no immunoreaction in the control group, increased expression in epithelial and mesenchymal cells (arrows) in the LPS group, and negative expressions in the LPS+AXA group; Streptavidin biotin peroxidase method (fourth row). iNOS immunoreactions in groups; no expression in the control group, marked increases in both epithelial and submucosal cells (arrows) in the LPS group, and marked decreases in the LPS+AXA group; Streptavidin biotin peroxidase method (fifth row). OPN expressions in groups; negative expression in the control group, slight expression in submucosal cells (arrows) in the LPS group, and no immunoreaction in the LPS+AXA group; Streptavidin biotin peroxidase method (sixth row), Bars=50µm.

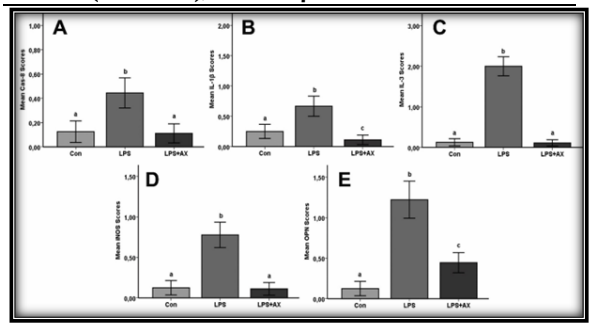

Figure 4: Statistical analysis results of immunohistochemical scores of the oral mucosa. (A) Cas-8 scores, (B) IL-1β scores, (C) IL-3 scores, (D) iNOS scores, and (E) OPN scores. Data were assessed by ANOVA, differences between the groups were analyzed by Duncan's test, and values represent mean ± standard deviation (SD). The differences between the means of groups are statistically significant when different letters were used for indication (P < 0.001).

DISCUSSION

In this study, changes in the oral and nasal mucosa in the sepsis model induced by LPS were examined histopathologically and immunohistochemically. The results showed that LPS produced pathological findings in the oral and nasal mucosa. In addition, AXA has been found to have therapeutic effects in the model of LPS-induced sepsis model.

Sepsis, a life-threatening condition, can cause damage to tissues and organs as a result of the body's overreaction to infection. Sepsis continues to be a leading cause of mortality and morbidity, especially in the elderly population. It is known that widespread inflammation and oxidative stress mechanisms are involved in the pathogenesis of sepsis.[1,2,7] Animal models developed to mimic human sepsis to investigate the pathogenesis and potential of therapeutic agents are important in sepsis research. Endotoxin (LPS), the outer membrane component of gram-negative bacteria, can elicit potent immune responses in animals so it is widely employed as a pathogenic factor in sepsis research.[7,8] LPS administration induces systemic inflammation that mimics many of the initial clinical features of sepsis, including extensive proinflammatory cytokine production leading to multi-organ failure and high mortality.[2,6,9,10,11,12]

In our study, mild hyperemia was noted in nasal and oral mucosa of rats from the LPS group, while the other two groups appeared normal. Immunohistochemical examination revealed significant increases in IL-1β, IL-3, OPN, iNOS, and Cas-8 expressions in oral and nasal mucosal sections from the LPS group. Thus, it has been

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shown that apoptosis and systemic inflammation are involved in the pathogenesis of LPS-induced sepsis.

After exposure to endogenous and exogenous stimuli, iNOS is quantitatively induced in various cells including macrophages, smooth muscle cells, and hepatocytes, resulting in inflammation.[2,3,13] Therefore, the potential anti-inflammatory effects of a drug can be assessed by measuring iNOS levels. In our study, we evaluated iNOS expression in oral and nasal mucosa to elucidate the anti-inflammatory mechanism of AXA. Synthesis of large quantities of NO, induced by bacterial LPS or cytokines, plays an important role in endotoxemia and inflammatory conditions.[13,14] In our study, iNOS expressions in the oral and nasal mucosal sections of the LPS group were higher compared to those of the control group. It was observed that high rates of expression decreased significantly after treatment with AXA. This finding shows that AXA suppresses NO production by directly inhibiting the iNOS enzyme activity. Therefore, we think that AXA, which suppresses NO production by inhibiting the iNOS enzyme activity, would have beneficial therapeutic effects in the treatment of inflammation.

IL-1β, produced by monocytes and macrophages in response to endotoxin, is an inflammatory factor and plays an important role in sepsis.[4,5] Interleukin-3 (IL-3), a hematopoietic growth factor produced by T cells and to a lesser extent by mast cells, eosinophils, and innate response activator B cells, has been described to play a key role during inflammatory diseases.[15] IL-3 can trigger severe sepsis in vivo and, after it recognizes bacterial components, it is involved in the development of a cytokine storm by further inducing cytokine synthesis in increased quantities.[16] For example, a recent study has found that IL-3 potentiates acute inflammation in sepsis by precipitating a cytokine storm and that high levels of IL-3 are associated with mortality.[17] In our study, IL-1β and IL-3 levels in the oral and nasal mucosal sections of the LPS group were higher compared to those of the control group. We observed that high levels of these variables decreased significantly after treatment with AXA.

OPN, an integrin-binding protein involved in the inflammatory response, modulates leukocyte activation, migration, and differentiation, and cytokine secretion in both acute and chronic inflammation.[18] A gradual increase of OPN levels in sepsis has been shown in both animal experimental models and clinical practice. High OPN levels have been associated with increased mortality rates and described as a biomarker for poor prognosis in sepsis.[19,20] In our study, OPN levels were higher in the oral and nasal mucosal sections of the LPS group compared to those of the control group. These high levels of OPN were observed to decrease significantly after the treatment.

It is recognized that apoptosis associated with sepsis induces the death of immune cells with increased ROS production. In addition to ROS, proinflammatory cytokines and LPS can induce apoptosis.[21] Apoptosis is a major mechanism that regulates cell populations for the sustainability of homeostasis, and caspases, a family of cysteine proteases, are key mediators of the apoptosis pathway. Caspase-8 is an initiator caspase of the extrinsic apoptotic pathway induced by extracellular stimuli.[21,22] In our study, it was observed that Cas-8 expression increased in the nasal and oral mucosa tissues studied in immune histochemical analyzes in the LPS group. It was observed that these increased levels were significantly reduced after the treatment. This finding shows that AXA suppresses apoptosis by decreasing Cas-8 expression.

Carotenoids are natural fat-soluble pigments. Based on their antioxidant and anti-inflammatory effects, their protective effects against several diseases have been shown including metabolic, neurodegenerative, cardiovascular, immune, inflammatory, and neurodegenerative disorders, and cancer.[6,15] AXA, a carotenoid of Xanthophyll, exhibits a more potent antioxidant effect compared to other carotenoids because it easily penetrates the cell membrane without interrupting its integrity. The favorable effects of AXA on health conditions are associated with its antioxidant capacity.[2,6,23] Several studies employing LPS-induced models have shown that AXA reduces activities of nitric oxide (NO) and iNOS, decreases levels of TNF-α, IL-1β, and oxidative stress parameters, and decreases apoptosis.[2,14,24] Again, in a study conducted on rats, it was shown that locally administered AXA to the nasal mucosa after injury significantly reduced subepithelial fibrosis and prevented the development of synechia. That study also showed that AXA had no systemic or local toxic effects.[25] Histopathological examinations of oral and nasal mucosal sections from the LPS group in our study revealed higher IL-1β, IL-3, OPN, iNOS, and Cas-8 expressions compared to the control group. It was observed that these high levels decreased significantly after treatment with AXA.

CONCLUSION

This study shows that AXA has a protective effect on oral and nasal mucosa by reducing inflammation and preventing apoptosis. We think that our study contributes to a better understanding of the subject and the evaluation of the effects of AXA on a developing injury since no previous research has been performed on potential histopathological changes in the oral and nasal mucosa in LPS-induced sepsis models. In conjunction with previous studies of AXA in different fields of research, we believe that AXA may be a potential therapeutic candidate for the treatment of oral and nasal mucosal injuries developing due to sepsis. From this point of view, we think that revealing the protective effects of this common commercially available

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agent against sepsis-induced organ injury, which is a critical problem in terms of both mortality/morbidity and costs of treatment, would provide substantial benefits. Therefore, randomized studies including large samples of animals and examining different dosages of AXA are needed to better clarify the role of AXA in sepsis. In addition, significant differences in homology can be observed between the results of animal experiments and studies on humans. Therefore, clinical studies are needed to investigate the protective efficacy of AXA against oral and nasal mucosal damage and to understand its use comprehensively. The results of this study showed that AXA may be one of the important drug options in the treatment of sepsis in the future.

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