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The Effect of Apilarnil on the Autophagia Against Lipolysaccarite-Based Sepsis in Liver

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Abstract: Sepsis, triggered by highly bacterial lipopolysaccharide (LPS) endotoxins, exhibits high morbidity and mortality despite medical advances. Damage to the liver occurs due to the production of highly reactive oxygen species (ROS) and the release of various proinflammatory cytokines. It is suggested that autophagy, which regulates inflammation and selectively destroys damaged mitochondria, suppresses apoptosis and provides a possible protective mechanism in the endotoxic liver. However, apilarnil, a bee product, is known to have high antioxidant activity and positive effects against various diseases thanks to its polyphenols. In this study, it is aimed to reveal the potential protective effect of apilarnil on the autophagy mechanism in the endotoxic liver model LPS-induced. 64 male *Sprague Dawley* rats weighing 200-250 g; control, apilarnil treated groups (0.2, 0.4 and 0.8 g/kg), LPS (30 mg/kg) group and LPS+apilarnil treated groups (LPS+0.2 g/kg, LPS+0.4 g/kg and LPS+0.8 g/kg) are randomly divided into eight groups. Beclin-1, LC3 and P62 proteins were analyzed immunohistochemically in order to determine the activity level of autophagy pathway in the liver tissues taken after the completion of the experiment protocol. The data obtained showed that Beclin-1 immunoreactivity decreased while LC3 and P62 expression increased in the tissues of the LPS group compared to the control group. When apilarnil was applied with LPS, it was determined that there was an increase in Beclin-1 level ($p>0.05$) and a decrease in P62 levels ($p<0.05$) depending on the dose increase. Apilarnil increases the activity of the autophagy pathway and shows potential positive effects by providing a significant decrease on LC3 and P62 protein expression increased by LPS. However, the role of apilarnil in the autophagy pathway, which is a possible protector against LPS-induced sepsis, should be further investigated.
Keywords: Beclin-1, LC3, P62, Apilarnil, LPS, Liver.

1. Introduction

Sepsis; an uncontrolled immune response that is triggered by infection, trauma, or toxins and occurs systemically. In the following process, it can lead to death by causing septic shock and multiple organ failure (1, 2). Lipopolysaccharide (LPS) is the main structural component of the outer cell membrane of gram-negative bacteria (3). Bacterial LPS, an endotoxin, is involved as a powerful microbial agent in

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the pathogenesis of sepsis and septic shock. LPS delivered to the blood initiates a potentially fatal series of inflammation mediators and procoagulant factor release.

Widespread endothelial damage in tissues leads to hypoperfusion and intravascular coagulation (4). During sepsis, pro-inflammatory responses occur with activation of the complement system, coagulation system, vascular endothelium, neutrophils and platelets. However, the immune system is suppressed due to the reprogramming of antigen presenting cells and apoptosis and depletion of lymphocytes (5).

Despite the constant advances in medicine, sepsis continues to be a global problem with high morbidity and mortality rates (6, 7). However, it is known that liver dysfunction accompanying sepsis has a significant effect on mortality rates. However, the pathological mechanism of sepsis-related liver dysfunction is very complex and has not been fully elucidated yet (8).

The liver plays a key role in the regulation of a wide range of metabolic, homeostatic and host defense activities under septic conditions (9). The liver provides both the clearance of circulating pathogenic microorganisms and toxins and the release of components of the liver-induced cytokines, inflammatory mediators, and coagulation cascade (10). The irregular inflammatory response that occurs following excessive LPS stimulation can kill bacteria as well as damage the liver, which has an effective function in defense responses (11).

LPS increases hepatocyte damage and leukocyte infiltration by interacting with TLR-4, a sub-member of the Toll-like receptor (TLR) family, which is highly expressed in Kupffer cells in the liver (12). Following increased TLR-4 stimulation, chemokine from Kupffer cells, various cytokines (tumor necrosis factor-alpha (TNF-α), interleukin (IL) -1β, IL-6, IL-12 and IL-18), reactive oxygen species (ROS) and nitric oxide (NO) is secreted (13, 14). However, activation of the TLR-4 signal can interestingly contribute to the destruction of pathogens by inducing autophagy in immune cells (15, 16).

Autophagy is one of the innate and well-protected defense mechanisms against microbial attack. It controls the destruction of damaged organelles and various macro molecules, such as protein, through the formation of double membrane autophagosomes to provide cellular homeostasis (17, 18). It also plays an active role in the elimination of bacteria and pathogens in the cytoplasm (19).

Current studies in the literature reveal that in the case of sepsis, autophagy suppresses immune reactions and inhibits apoptosis by regulating inflammation and metabolism (20, 21). It also reduces cellular stress by preventing high ROS-induced LPS production and accumulation of damaged mitochondria (22). Autophagy can eliminate damaged mitochondria by selectively (23, 24). It also promotes regeneration of damaged proteins and organelles through lysosomal dependent degradation to deal with oxidative stress damage caused by ROS excess (25). Thus, autophagy has been shown to have a protective role against multiple organ failure, including liver damage (26). Various regulatory proteins such as Beclin-1, LC3 and P62, located in the autophagy pathway, allow examination of the autophagy activity in tissues.

Apilarnil is a poorly studied and little known biologically active bee product (27). It is obtained by freezing and breaking the 3-7-day old bee larvae with the nutrients they contain (28). In the analysis of the chemical composition of drones, it was observed that it contains a high rate of water, carbohydrates and lipids (29). In another analysis study conducted in 2019, similarly high protein content was determined, and 16 kinds of amino acids were detected. It has also been shown to be rich in phenolic compounds. It is suggested that it can perform its biologically beneficial activity through its antioxidant and radical inhibitory activity (30). In addition, current studies reveal that apilarnil has positive effects against gastrointestinal diseases, toothache, muscle fatigue, respiratory problems and male infertility (31).

In this study, the potential protective effect of apilarnil against LPS-induced liver sepsis will be examined. It was aimed to reveal the effect of apilarnil on the autophagy mechanism by performing immunohistochemical staining of Beclin-1, LC3 and P62 proteins in the liver tissue of the rats with sepsis model.

2. Material and Methods

2.1. Chemicals

Lipopolysaccharide was provided from Sigma Aldrich (*Escherichia coli* LPS, serotype 0127: B8) and Lyophilized Apilarnil was provided from Nutral Therapy Co Ltd (Erciyes University Technopark, Kayseri).

2.2. Experiment groups

Ethics committee approval was received from Erciyes University Animal Experiments Local Ethics Committee (HADYEK) for this study (Protokol No: 18/063). Animals were housed under controlled conditioning (on a 12-h light/12-h dark cycle at a temperature of 22-25 °C and air humidity of 55 %). Throughout the study, the animals were provided with ad libitum rat feed and drinking water. Lyophilized apilarnil distilled water was also dissolved and administered to animals by oral gavage. Sixty-four adult male *Sprague Dawley* rats (200-250 g weighing) were randomly divided into 8 groups:

- Control group: 1 ml saline (SF) (0.9% NaCl) i.p.
- LPS group: 30 mg/kg/body weight (bw) LPS single dose i.p. (32).
- 0.2 g/kg API group: 0.2 g/kg/bw oral gavage for 10 days (33),
- 0.4 g/kg API group: 0.4 g/kg/bw oral gavage for 10 days,
- 0.8 g/kg API group: 0.8 g/kg/bw gavage for 10 days,

- 0.2 g/kg API+LPS: 0.2 g/kg API for 10 days, after 60 min LPS (single-dose i.p., 30 mg/kg)
- 0.4 g/kg API+LPS: 0.4 g/kg API for 10 days, after 60 min LPS (single-dose i.p., 30 mg/kg)
- 0.8 g/kg API+LPS: 0.8 g/kg API for 10 days, after 60 min LPS (single-dose i.p., 30 mg/kg)

Experimental animals were anesthetized with Ketamine (70 mg/kg/bw) and Xylazine (10 mg/kg/bw) six hours after administration and liver tissues were removed.

2.3. Immunohistochemical analysis

Immunoreactivity of Beclin-1, LC3A/B and P62/SQSTM1 in liver tissues of the LPS-induced rat sepsis model was determined using the Avidin-Biotin peroxidase method (34). Briefly, after deparaffinization of sections taken at 5μm thickness, citrate buffer was used to open the epitopes (pH: 6.0). The slides were then taken into a 3% hydrogen peroxide solution in methanol to prevent endogenous peroxidase activity. Ultra V block solution was applied to prevent non-specific staining. Sections were then incubated overnight at 4°C with primary antibodies. Biotinylated secondary streptavidin-HRP and DAB chromogens were applied, respectively. And then sections were counterstained with Gill Hematoxylin. It was dehydrated by passing through increasing alcohol series and closed with a concealer called entellan. Sections were examined with Olympus BX53 light microscope. The evaluation of the immunoreactivity levels was done with Image J program. 10 different areas were evaluated for each slide.

2.4. Statistical analysis

Experimental data were statistically analyzed in GraphPad Prism (version 6.0, GraphPad Software Inc., San Diego, California) and presented as Mean±SEM. Data were analyzed using one-way ANOVA with Tukey's post hoc tests for multiple comparisons. $P<0.05$ was considered significant.

3. Results

As shown in Figure 1, it was observed that Beclin-1 decreased while LC3A/B and P62/SQSTM1 were increased in liver samples belonging to LPS group. In groups where LPS and apilarnil were applied together, while Beclin-1 increased with the increase of apilarnil dose ($P>0.05$), LC3A/B and P62/SQSTM1 decreased with the increase of apilarnil dose ($P<0.05$).

4. Discussion

During sepsis, cells are exposed to increased oxidative stress and metabolic demands. All of these processes can lead to cell damage and cell death (35). However, cells and tissues try to protect themselves from such stresses by activating autophagy and autophagy-like mechanisms (36).

Autophagy is critical for maintaining normal human physiology, such as cellular homeostasis, energy balance, development, and cellular defense (37). Autophagy can also play a role in the pathogenesis of cancer, neurodegenerative diseases, aging, muscle diseases, infectious diseases and immune system diseases (38, 39). In addition, the presence of autophagy in liver tissue in the case of sepsis has been reported in studies (35, 40). In this study, we aimed to determine the effects of apilarnil, a natural bee product, on the autophagy mechanism that may occur as a result of endotoxic shock caused by LPS.

In lysosomal activity during autophagy, the P62 protein is considered a biomarker of autophagy and an indicator of autophagic flow. It is also characterized as an indicator of autophagic flow prevention of P62 reduction (41). Immunohistochemical analysis of LC3A or LC3B has also been used to investigate the level of autophagy occurring in the tissue (42). In addition, in clinical and experimental studies, Beclin-1, which is one of the main proteins of autophagy, is examined as a target for the determination of the presence of autophagy (43). Accordingly, in the sepsis model created with LPS, we performed the immunohistochemical analysis of Beclin-1, LC3A/B and P62 parameters as autophagy markers in liver tissue. According to the results obtained, LC3A/B expression was significantly increased only in the groups injected with LPS compared to the control. These results confirmed the presence of autophagy in liver tissue as a result of sepsis caused by LPS endotoxicity (Figure 1C). However, it was determined that Beclin-1 expression decreased significantly and P62 expression increased as a result of LPS injection (Figure 1B and 1D).

In the study performed by Chen et al. (44), LC3A/B, Beclin-1 and P62 proteins were examined to evaluate autophagy in hepatocytes in the sepsis model created by LPS injection in C57BL/6 mice. As a result of the study, significant changes were observed in LC3 and P62, while no increase was observed in Beclin-1 (44). According to these results, it can be said that autophagy associated with LPS in liver tissue is not related to Beclin-1 and P62 molecules. Since LPS provides its effect with the TLR-4 receptor (45), more detailed studies are needed to clarify the mechanism underlying the relationship between the TLR-4 receptor and the LPS-induced autophagic pathways.

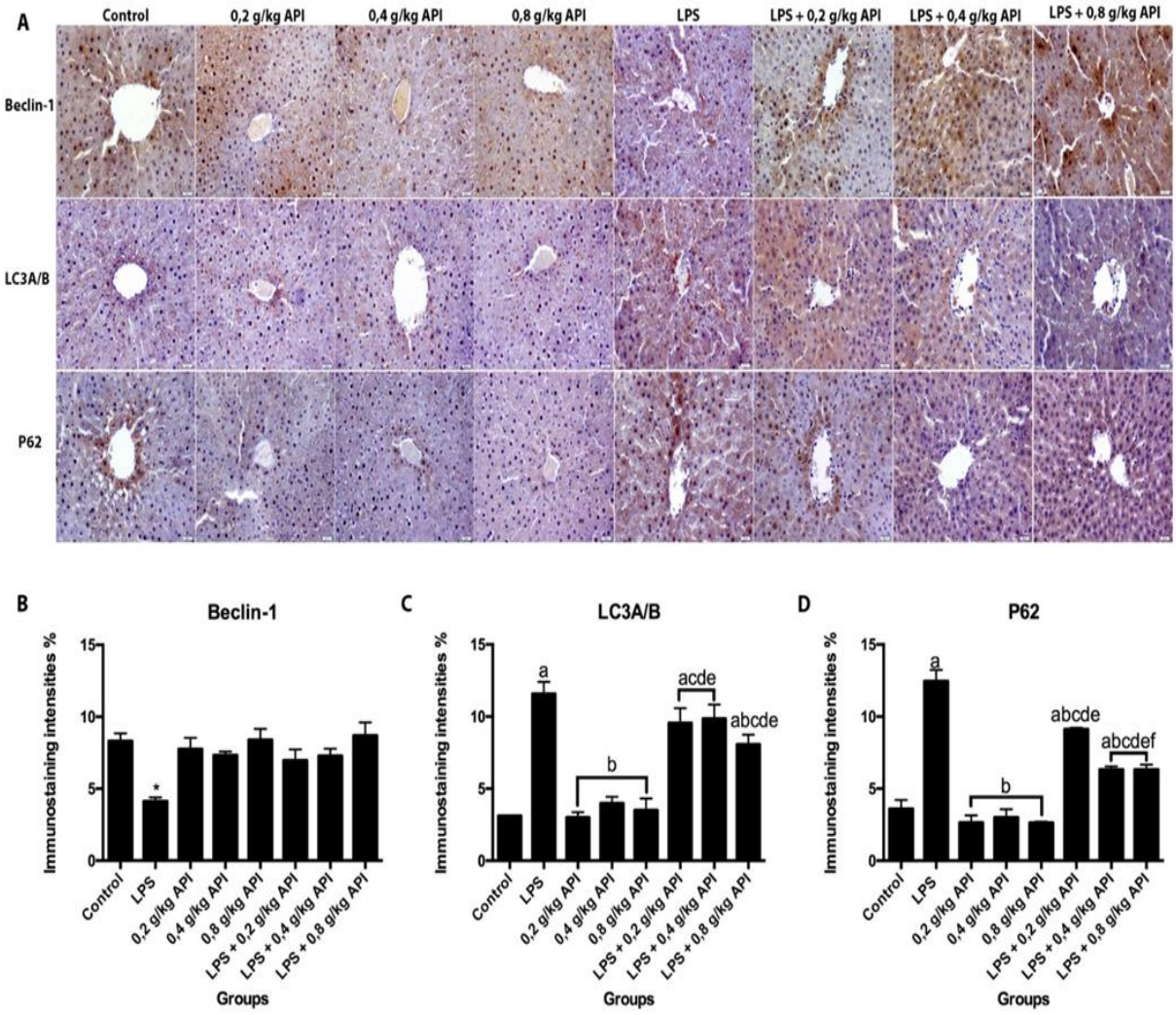

Figure 1. A. Beclin-1, LC3A/B and P62/SQSTM1 images of immunostaining in rat livers belonging to experiment groups. Histogram graphics of Beclin-1 (B), LC3A/B (C) and P62/SQSTM1 (D) represent the intensity values in percent of immunostaining obtained using Image J software. T bar graph data are expressed as mean ± SEM, and compared by one-way ANOVA and TUKEY's multiple comparisons test (a $P<0.05$ vs. control group; b $P<0.05$ vs. LPS group; c $P<0.05$ vs. 0,2 g/kg API group; d $P<0.05$ vs. 0,4 g/kg API group; e $P<0.05$ vs 0,8 g/kg API group; f $P<0.05$ vs. LPS+0,2 g/kg API group; g $P<0.05$ vs LPS+0,4 g/kg API group; * $P<0.05$ statistically different from all other groups).

In sepsis, organ damage initially progresses to dysfunction without cell death and structural damage. Therefore, it is possible to restore organ function (45). Therefore, in another study we conducted, we investigated the protective role of apilarnil in the LPS-induced sepsis model in liver tissue and observed its positive effects on TLR-4 receptor-associated inflammatory response (34). In the results obtained from the current study, P62 expression increased in the LPS group compared to the control and only apilarnil-treated groups. However, in groups in which apilarnil was administered with LPS, it showed decrease compared to the LPS group. Similarly, LC3A/B expression levels increased statistically in the LPS group compared to the control group and apilarnil-only groups (0.2, 0.4, 0.8 g/kg API). The expression amount of LC3A/B decreased in groups where apilarnil was administered with LPS, although it was not significant. No significant change was observed in Beclin-1 activity. With this result, it can be thought that apilarnil plays a role in the adaptation mechanism by acting on autophagy, LC3A/B and P62 pathways, which occur as a metabolic response to sepsis. Further research is needed to clarify the effects of this role on survival and immune response.

In another study conducted by Carchman et al. (46), adaptation metabolic responses of liver tissue to LPS sepsis model were investigated. Mitochondrial homeostasis and mitophagic pathways have been investigated in relation to the TLR-9 receptor. In the results obtained, it has been reported that TLR-9 receptor and mitophagia play an important role in the adaptation of hepatocytes to sepsis (46). Accordingly, it may be important for future studies to investigate the

effects of apilarnil on mitophagia, which is a more featured pathway of TLR-9 and autophagy, and also on mitochondrial homeostasis, one of the key players of oxidative stress.

In the literature review, there was no study evaluating the effect of apilarnil on autophagy in the LPS-induced sepsis model, and the studies evaluating the effects on autophagy in other bee products are quite limited. We believe that this study will contribute to the literature on this subject.

5. Conclusions

In the light of all this information, it is important to develop alternative therapeutic agents targeting the autophagy pathway against sepsis, which results in high mortality. In this study, we investigated the effect of apilarnil on sepsis-induced autophagy in liver tissue through Beclin-1, LC3A/B and P62 molecules. As a result, apilarnil exhibited different effects in terms of different markers on the autophagy pathway. Apilarnil increases the activity of the autophagy pathway and shows potential positive effects by providing a significant decrease on P62 protein. However, an increase in its effect was observed due to the increased dose. In the light of the findings, apilarnil, which is a natural bee product, can be targeted in further studies as an alternative treatment method by playing a which is a natural bee product, can be targeted in further studies as an alternative treatment method by playing a protective role against sepsis, especially through the LC3A/B and P62 pathways.

Conflict of interest statement

There is no conflict of interest.

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