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ORIGINAL ARTICLE

Protective Effect of Niclosamide on Lipopolysaccharide-induced Sepsis in Mice by Modulating STAT3 Pathway

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니클로사마이드를 이용한 STAT3 신호전달 조절을 통해 LPS로 유발된 패혈증 동물모델 보호 효과 검증 연구

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ABSTRACT

Sepsis is a systemic inflammatory response, with manifestations in multiple organs by pathogenic infection. Currently, there are no promising therapeutic strategies. Signal transducer and activator of transcription 3 (STAT3) is a cell signaling transcription factor. Niclosamide is an anti-helminthic drug approved by the Food and Drug Administration (FDA) as a potential STAT3 inhibitor. C57BL/6 mice were treated with an intraperitoneal injection of lipopolysaccharide (LPS). Niclosamide was administered orally 2 hours after the LPS injection. This study found that Niclosamide improved the survival and lung injury of LPS-induced mice. Niclosamide decreased the levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) in serum. The effects of Niclosamide on phosphoinositide 3-kinase (PI3K), AKT, nuclear factor-κB (NF-κB), and STAT3 signaling pathways were determined in the lung tissue by immunoblot analysis. Niclosamide reduced phosphorylation of PI3K, AKT, NF-κB, and STAT3 significantly. Furthermore, it reduced the phosphorylation of STAT3 by LPS stimulation in RAW 264.7 macrophages. Niclosamide also reduced the LPS-stimulated expression of proinflammatory mediators, including IL-6, TNF-α, and IL-1β. Niclosamide provides a new therapeutic strategy for murine sepsis models by suppressing the inflammatory response through STAT3 inhibition.

INTRODUCTION

Sepsis is a systemic inflammatory response caused by pathogenic infection [1]. A "cytokine storm" that occurs during sepsis makes these inflammatory reactions worse, which can result in multiple organ failure and even death [2]. Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and is a potent activator of the immune system [3]. LPS can cause pathological reactions such as septic shock [4]. The pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 are secreted excessively or inappropriately in response to LPS [5].

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Various therapeutic drug candidates for treating sepsis are being studied preclinically and clinically. Although monoclonal anti-TNF antibodies, IL-1 receptor inhibitors, Toll-like receptor 4 (TLR4) antagonists, and monoclonal anti-LPS antibodies have demonstrated encouraging preclinical results, candidates with satisfactory efficacy have not yet been shown in clinical trials [6]. The only drug approved by the United States Food and Drug Administration (U.S. FDA) to treat some of the effects of sepsis is Drotrecogin alfa (Xigris) [7].

An anti-helminthic drug with FDA approval, Niclosamide is generally wide safety and few side effects [8]. Furthermore, several groups have reported that it is effective in various solid cancers, and it is also known to be effective in autoimmune diseases due to its potential anti-inflammatory effect [9, 10]. Niclosamide is reported as potent inhibitor of signal transducer and activator of transcription 3 (STAT3) phosphorylation [11]. STAT3 acts a pivotal molecule in regulating innate/adaptive immunity and inflammation pathway [12]. As an acute-phase response factor that can specifically bind to the IL-6 responsive region in the promoter of genes encoding various acute-phase proteins, it was originally identified in hepatocytes in 1994 [13]. Furthermore, previous studies have demonstrated that STAT3 is critical roles in the sepsis pathophysiology [14, 15]. The effects of Niclosamide on LPS-induced mouse models have not yet been investigated.

In this study, we examined whether in vivo treatment with Niclosamide had a therapeutic effect in LPS-induced mice by examining sepsis-like phenotype characterized by increase of pro-inflammatory cytokines, lung inflammatory infiltrate, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and blood urea nitrogen (BUN). We additionally investigated the impact of Niclosamide on RAW 264.7 cells in vitro. The purpose of this study was to demonstrate whether inhibition of STAT3 through oral administration of Niclosamide could suppress the inflammatory response in LPS-induced mice.

MATERIALS AND METHODS

1. Animals and Treatment

C57BL/6 mice were purchased from Orient Bio. Niclosamide (Sigma-Aldrich) was resuspended in 0.5% methyl cellulose (Sigma-Aldrich) for in vivo studies or in 5% dimethyl sulfoxide (DMSO) for in vitro use. Male 8-week-old C57BL/6 mice were treated via intraperitoneal (i.p.) injection of LPS (N=7; 20 mg/kg) dissolved in PBS, and Niclosamide (N=7; 100 mg/kg) was administered orally 2 hours after injection of LPS. The control group (N=7) was administered with PBS. All procedure of animal research were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent experiment provided by the Institutional Animal Care and Use Committee (IACUC) in school of medicine, The Catholic University of Korea (Approval numbers: CUMC-2021-0225-01).

2. Histologic Assessment of Lung

To observe the pathological changes of lung tissues, lung tissues were fixed with formalin and embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin & eosin (H&E) stain. The degree of lung injury (alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, alveolar and interstitial edema, necrosis, and overdistension) was determined using the following criteria: 0, no injury; 1, injury to 25% of the field; 2, injury to 50% of the field; 3, injury to 75% of the field; 4, diffuse injury [16].

3. Enzyme-linked Immunosorbent Assay

Serum obtained after euthanasia was separated by centrifugation at 3,000 rpm for 10 minutes at 4℃. Collect the culture supernatant to carry out the assay. Cytokines in sera or culture supernatants were assayed by using mouse IL-6, TNF-α, IL-1β Duoset enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions.

4. Western Blot

Total protein was extracted using RIPA buffer containing Halt protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific). For immunoblotting, 30 μg of protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinylidene fluoride membrane (Bio-Rad) and probed with the following antibodies: anti-p-phosphoinositide 3-kinase (PI3K), anti-PI3K, anti-p-AKT, anti-AKT, anti-p-nuclear factor-κB (NF-κB), anti-NF-κB, anti-p-STAT3, anti-STAT3 (Cell Signaling Technology), and anti-β-actin (Sigma-Aldrich). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Thermo Fisher Scientific). Reactive proteins on the membrane were visualized by SuperSignal® West Pico Chemiluminescent substrate (Thermo Fisher Scientific), and the membrane was then exposed to an Amersham Imager 600 (GE HealthCare).

5. Analysis of AST, ALT, LDH, and BUN in Mouse Serum

Serum obtained after euthanasia was separated by centrifugation at 3,000 rpm for 10 minutes at 4℃. Serum parameters including AST, ALT, LDH, and BUN were measured using an automated clinical chemistry analyzer, FUJI DRI-CHEM NX500 (FUJIFILM) according to the manufacturer's protocol.

6. Cell Culture

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco) in a humidified atmosphere of 95% air and 5% CO_2 confluent at 37℃. Before the experiments, the RAW 264.7 cells were plated at ~10^5 viable cells per well in 6-well plates. Twenty-four hours later, the cells were pretreated with LPS (0.1 μg/mL) for 30 min, followed by Niclosamide (200, 400 nM) for an additional 24 hours.

7. Cytotoxicity

We investigated whether Niclosamide demonstrates cytotoxicity in the concentration range of 100, 200, 400 nM. Cytotoxicity was analyzed using a Cell Counting Kit-8 (CCK-8, Dojindo) 24 hours after stimulation. Optical density at 450 nm was calculated as the percentage of viable Niclosamide-treated cells relative to the number of viable unstimulated cells.

8. Statistics

Statistical analyses were performed in GraphPad Prism version 7.0 Software (GraphPad). Statistical significance was determined by T tests for two groups, and by one-way ANOVA with Tukey's multiple comparisons tests for three or more groups. Kaplan-Meier survival curves were analyzed using log rank tests. $P<0.05$ was considered statistically significant.

RESULTS

1. Effects of Niclosamide on Inflammatory Response in LPS-induced Sepsis Mice

To assess whether Niclosamide ameliorates inflammatory response in LPS-induced sepsis mice, we orally administered 8-week-old C67BL/6 mice with Niclosamide or vehicle 2 hours before LPS injection. We showed that Niclosamide pretreatment increased the survival rate to approximately 60% (Figure 1A). Niclosamide ameliorated lung pathological features, such as mononuclear cell infiltration, as judged by changes in the histological scores (Figure 1B). We next measured pro-inflammatory cytokine in these mice. Niclosamide treatment significantly decreased the serum levels of IL-6, TNF-α, and IL-1β (Figure 1C). These data suggest that Niclosamide treatment significantly prevented inflammatory response in LPS-induced sepsis mice.

2. Niclosamide Regulates Multiple Signaling Pathways

We analyzed the activity of the PI3K, AKT, NF-κB, and STAT3 pathways in lung tissue of each group to

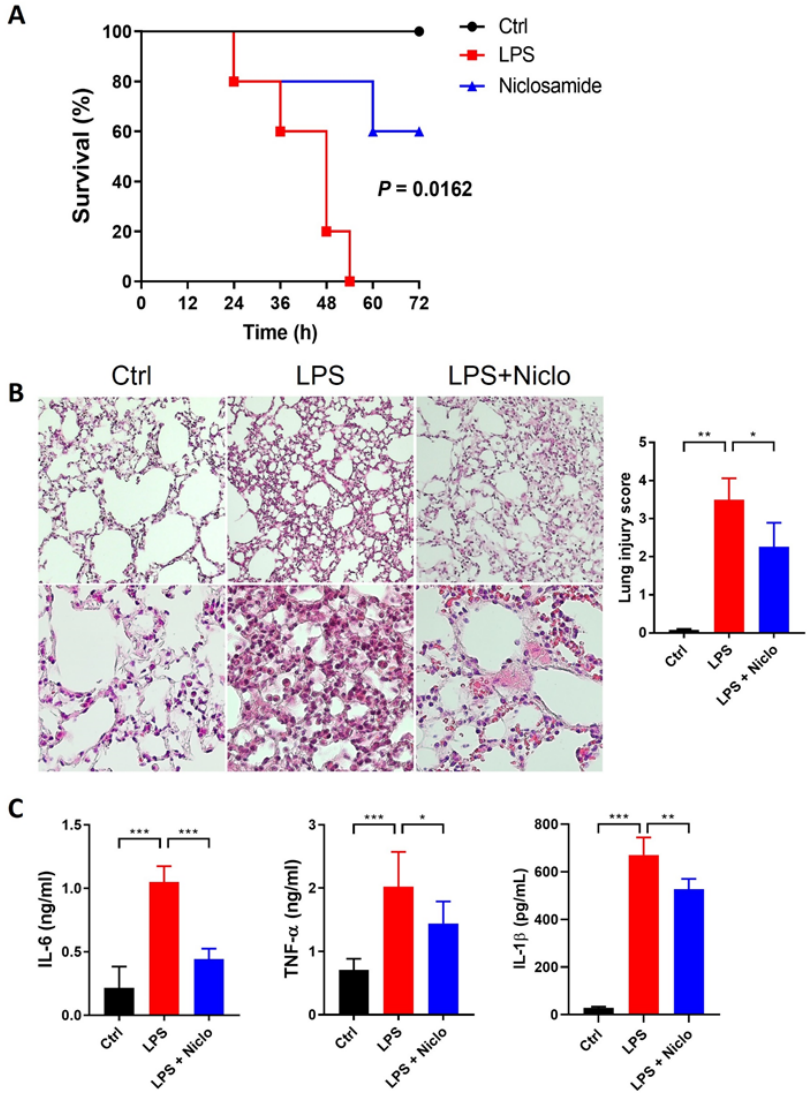

Figure 1. Protective effects of Niclosamide on an LPS-induced sepsis mice. Mice were administered orally with Niclosamide (100 mg/kg) 2 hours after intraperitoneally injection of LPS (20 mg/kg). (A) The survival rate was observed over the next 72 hours. (B) Left, representative photomicrographs of H&E stained sections of lung. Original magnification ×100 (upper), ×200 (bottom). Right, lung injury score. (C) Serum cytokine levels of IL-6, TNF-α, and IL-1β were measured by ELISA. Data shown as mean±SD. One-way ANOVA was performed. *P<0.05, **P<0.01, ***P<0.001.

elucidate the molecular mechanisms by Niclosamide treatment. Previous studies have shown that these signaling are important intracellular signal transduction cascades, modulating cell growth and proliferation, and survival and invasion [17]. Western blot analysis revealed that constitutive activation of these signaling pathways was shown by LPS treatment. Expression of p-PI3K, p-AKT, p-NF-κB, and p-STAT3 was significantly decreased by Niclosamide-treated mice as compared to the vehicle-treated mice (Figure 2). Our data indicated that Niclosamide significantly reduced phosphorylation of PI3K, AKT, NF-κB, and STAT3.

3. Niclosamide Alleviate Physiological Parameters in LPS-induced Sepsis Mice

To confirm whether Niclosamide regulates physiological parameters in LPS-induced sepsis mice. Serum AST, ALT, and LDH levels were measured to monitor liver function, and BUN levels were measured to monitor kidney function. Serum levels of AST, ALT, LDH, and BUN were significantly increased in the LPS-induced mice as compared to the control mice. The levels of AST, ALT, and LDH were significantly decreased by Niclosamide, while BUN levels were not affected (Figure 3). These results confirmed that AST, ALT, and LDH, well-known markers of liver damage,

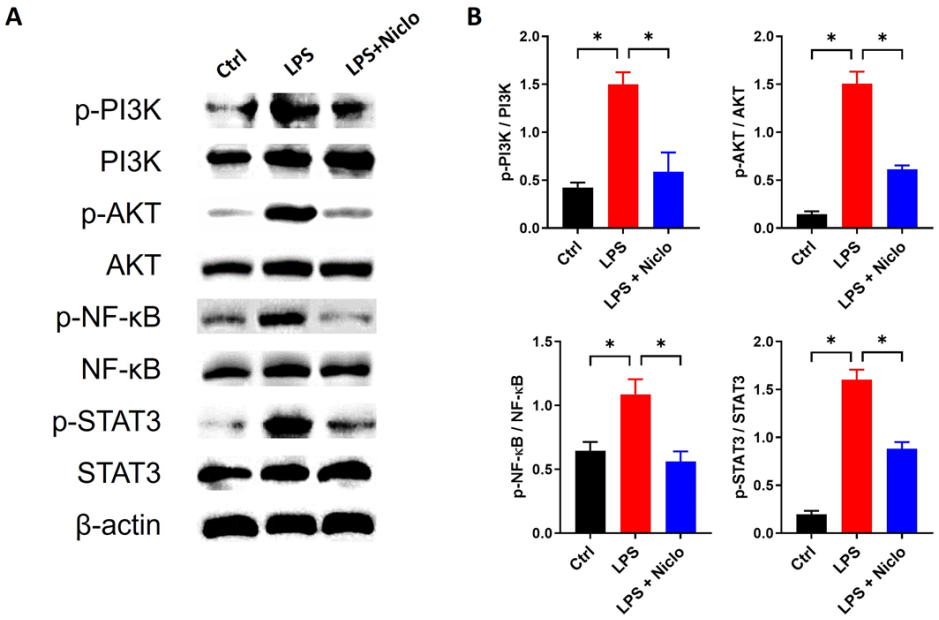

Figure 2. Niclosamide regulates PI3K/AKT/NF-κB/STAT3 signaling in lung tissue of LPS-induced sepsis mice. (A) p-PI3K, p-AKT, p-NF-κB, and p-STAT3 expression in the lung were analyzed by western blot. (B) Graphs show the relative density of the target proteins. Data shown as mean±SD. One-way ANOVA was performed. *P<0.05. Abbreviations: PI3K, phosphoinositide 3-kinase; NF-κB, nuclear factor-κB; STAT3, signal transducer and activator of transcription 3; LPS, lipopolysaccharide; Ctrl, control; Niclo, Niclosamide.

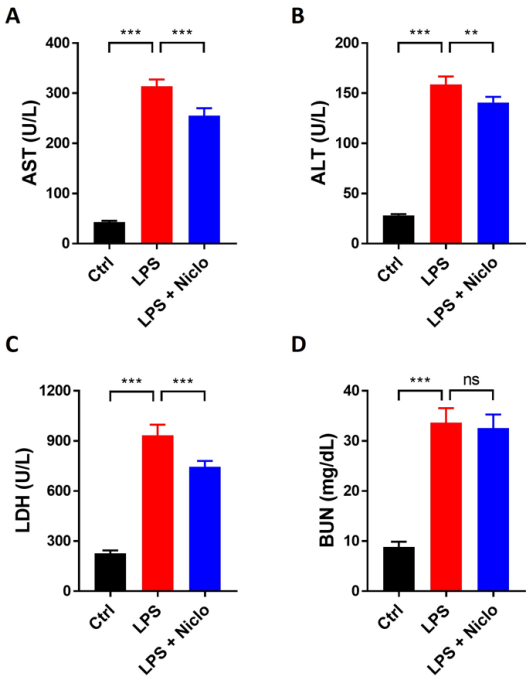

Figure 3. Niclosamide inhibits organ damage in LPS-induced sepsis mice. The serum levels of (A) AST, (B) ALT, (C) LDH, and (D) BUN were measured using a veterinary clinical chemistry analyzer. Data shown as mean±SD. One-way ANOVA was performed. **P<0.01, ***P<0.001. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; ns, not significant; LPS, lipopolysaccharide; Ctrl, control; Niclo, Niclosamide.

were elevated by LPS and significantly reduced by Niclosamide. There was no effect of Niclosamide on renal function marker.

4. Niclosamide Attenuates LPS-induced Immune Response in RAW 264.7 Cells

We further investigated the effect of Niclosamide in RAW 264.7 murine macrophage cells. To determine the effects of Niclosamide on cell viability, cell survival was assessed by CCK-8 assay. The CCK-8 assay was performed in RAW 264.7 cells following treatment with Niclosamide (100, 200, 400 nM) for 24 hours. Niclosamide had no effect on RAW 264.7 cell viability (Figure 4A). STAT3 signaling has been identified as a major contributor in the induction and maintenance of inflammation. Niclosamide reduced protein levels of p-STAT3 in RAW 264.7 cells (Figure 4B). In addition to, Niclosamide inhibited the levels of pro-inflammatory cytokine IL-6, TNF-α, IL-1β in culture supernatants from RAW 264.7 cells (Figure 4C). Overall, these results indicate that Niclosamide has a potential inhibitory effect on LPS-stimulated inflammatory responses in RAW 264.7 macrophages.

DISCUSSION

Sepsis, a serious problem on public health, incurring high hospital charges and causes most deaths in intensive care units [18]. In the early stages of sepsis,

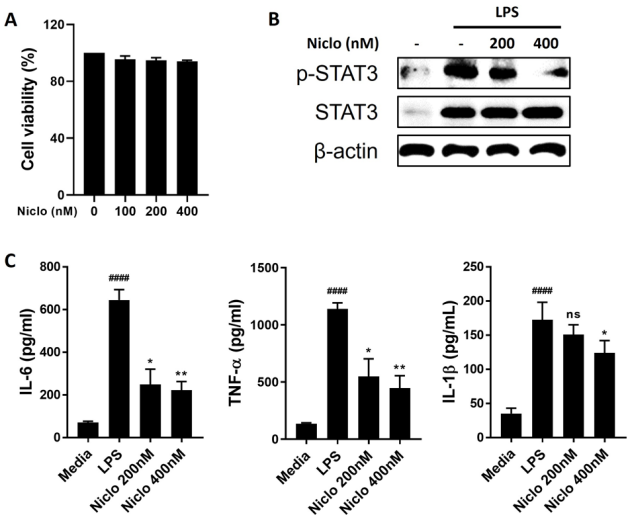

Figure 4. Effects of Niclosamide on cell viability, STAT3 expression and pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. (A) The cytotoxicity assessed in RAW 264.7 cells treated with different doses of Niclosamide for 24 hours. (B) p-STAT3 expressions in RAW 264.7 cells were analyzed by western blot. (C) Levels of IL-6, TNF-α, and IL-1β in the cell culture supernatants were detected by ELISA. Data shown as mean±SD. One-way ANOVA was performed. *$P<0.05$, **$P<0.01$ versus LPS. $^{\#\#\#\#}P<0.0001$ versus Media. Abbreviations: STAT3, signal transducer and activator of transcription 3; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; Niclo, Niclosamide; ns, not significant.

the treatment strategy is administration of appropriate antibiotics in a very short time. To identify the causative pathogens of sepsis, it is necessary to culture the bacteria in the patient's blood, but this requires at least 3 to 5 days. Thus, waiting for appropriate antibiotics is not reasonable because sepsis progresses too rapidly [19]. Antibiotics treatment may increase the amount of pathogen-associated molecular patterns (PAMPs) released as bacteria die, exacerbating the patient's health and inflammation, which is another problem with treating sepsis. Still another crucial challenge is the potential for treatment failure by antibiotic-resistant bacteria [20].

PAMPs, a host defense system that uses specialized receptors known as pattern recognition receptors (PRRs), can recognize molecular components of invading pathogens. LPS, commonly known as a bacterial endotoxin, is a well-known PAMP. The canonical cytokine storm and consequent end-organ failure are caused by a PAMP-mediated and TLR-driven mechanism, which are often associated to the systemic pathogenesis of sepsis [21].

The predominant cause of morbidity and mortality in sepsis is the development of multiple organ failure. The liver plays a major role in the defense response during sepsis by eliminating bacteria and producing inflammatory mediators. However, the liver becomes a target organ for abnormal inflammatory responses [22]. Sepsis-associated liver dysfunction is considered a late feature of severe disease with jaundice and hyperbilirubinemia, but recent studies reported that liver dysfunction has been shown to be an early feature of sepsis. Liver dysfunction is not the most common feature of organ damage that occurs in patients with sepsis. When liver dysfunction leads to liver failure, it becomes a serious complication [23]. Therefore, it is important to understand the pathophysiological changes involved in liver dysfunction caused by sepsis. In further studies, we will explore the mechanisms of liver dysfunction by sepsis.

Acute lung injury (ALI) is one of the leading causes of death in sepsis. Alveolar macrophages are considered a therapeutic target because they are poised to rapidly release large amounts of inflammatory cytokines in response to LPS [24]. Several studies have shown that suppressing alveolar macrophage STAT3 phosphorylation, thereby reducing the production of pro-inflammatory cytokine, such as IL-6, TNF-α, IL-1β, a cytokine known to be involved in the pathogenesis of inflammatory lung disease [25]. In our study, we showed that lung injury score was significantly reduced by Niclosamide administration in LPS-induced mice. Further, the expression of PI3K, AKT, NF-κB, and STAT3 were significantly decreased in lung tissue by Niclosamide treatment. In further studies, we will explore the effects of Niclosamide on macrophage-mediated inflammatory responses.

Our data showed LPS induce PI3K, AKT signaling, and NF-κB as well as increased STAT3. The PI3K, AKT signaling pathway serves a crucial role in inflammation and discovered to be important mediators that drive the production of inflammatory cytokines in sepsis. LPS

stimulates macrophages to produce IL-6, TNF-α, and IL-1β, whereas inhibition of STAT3 exerts significant suppressive effects [26]. Matsukawa et al [27] found that macrophages and neutrophils specific STAT3 deficiency mice were highly susceptible to sepsis associated with impaired bacterial clearance, higher systemic inflammation, more serious organs damage and increased lethality. Our results showed that sepsis was alleviated by systemic pharmacological inhibition of STAT3.

This is the first study to provide a therapeutic strategy for LPS-induced sepsis treatment by Niclosamide. We demonstrated that Niclosamide was significantly alleviates sepsis phenotype in LPS-induced mice. This study is also that it showed the regulatory function of Niclosamide on RAW 264.7 cells in vitro. Niclosamide may aid in developing better therapeutic strategies for the treatment of proinflammatory disorders.

요 약

패혈증은 병원성 감염에 의해 여러 장기에 나타나는 전신성 염증 반응으로, 현재로서는 유망한 치료제가 없다. Signal transducer and activator of transcription 3 (STAT3)은 세포 신호전달 전사 인자로서 항염증 및 염증 반응과 관련된 다양한 세포의 생물학적 과정에서 중요한 역할을 한다. Niclosamide는 FDA에서 승인된 구충제로 STAT3 조절에 관여한다고 알려져 있다. C57BL/6 마우스에 복강 주사로 지질 다당체(lipopolysaccharide, LPS)를 투여해 패혈증을 유발하였고, Niclosamide를 LPS 주사 2시간 후에 경구 투여하였다. 본 연구에서 Niclosamide가 LPS로 유발된 패혈증 모델의 생존률과 폐 손상을 완화시켰고, 혈청 내 interleukin (IL)-6, 종양괴사인자(tumor necrosis factor-α, TNF-α), IL-1β, AST, ALT, LDH 수치를 유의하게 감소시켰다. 또한 폐 조직 면역 블롯을 통해 PI3K, AKT, NF-κB, STAT3 신호 전달 경로가 Niclosamide에 의해 조절되는 것을 확인하였다. Niclosamide는 LPS를 자극한 RAW 264.7 세포주에서 IL-6, TNF-α, IL-1β와 같은 염증성 사이토카인의 발현을 감소시켰으며, 또한 STAT3의 인산화를 감소시켰다. 본 연구를 통해 Niclosamide에 의한 STAT3 조절이 염증 반응을 억제함으로써 패혈증 모델에 대한 새로운 치료 전략을 제시하였다.

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Ethics approval

The study provided by the Institutional Animal Care and Use Committee (IACUC) in school of medicine, The Catholic University of Korea (Approval numbers: CUMC-2021-0225-01).

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