Article

Discovery Medicine 2024; 36(181): 385–392
https://doi.org/10.24976/Discov.Med.202436181.36

Artesunate Inhibits Lipid Accumulation and Inflammation by Regulating the NLRP3 Inflammasome in Nonalcoholic Fatty Liver Disease

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Published: 20 February 2024

Background: Non-alcoholic fatty liver disease (NAFLD), recognized as a chronic liver condition, has emerged as one of the most prevalent worldwide. This study explores the impact of artesunate (ART) on lipid accumulation and inflammatory factors within NAFLD model cells.

Methods: LO2 cells were subjected to treatment with oleic acid (OA) to establish NAFLD cell model. Subsequently, these cells were categorized into distinct groups: a control group, an OA group, an OA + 2.5 µm ART group, and an OA + 5 µm ART group. The activity of LO2 cells was determined using the Cell Counting Kit-8 (CCK-8) method. The presence of intracellular lipid droplets was examined through oil red O staining. Levels of triglycerides (TG), total cholesterol (TC), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were evaluated using enzyme-linked immunosorbent assay (ELISA). Additionally, the protein expressions of nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR), and pyrin domain-containing protein 3 (NLRP3), Cleaved caspase-1, N-terminus of Gasdermin-D (GSDMD-N), and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) were measured via Western blot assay.

Results: In comparison to the control group, the OA group exhibited a significant increase in the contents of lipid droplets, TC, and TG ($p < 0.01$). Notably, ART effectively reversed the impact of OA ($p < 0.01$). Following OA stimulation, there was a pronounced elevation in the levels of IL-6 ($p < 0.01$), IL-1β ($p < 0.01$), and TNF-α ($p < 0.05$). In comparison to the OA group, the 2.5 µm ART group showed no significant difference in TNF-α content ($p > 0.05$), while the 5 µm ART group significantly reduced TNF-α content ($p < 0.05$). Furthermore, both the 2.5 µm ART ($p < 0.05$) and 5 µm ART ($p < 0.01$) groups notably reduced IL-1β and IL-6 content. When compared to the control group, the expressions of NLRP3, ASC, GSDMD-N, and Cleaved caspase-1 in the OA group significantly increased ($p < 0.01$). ART, however, mitigated this heightened expression trend ($p < 0.05$).

Conclusions: ART demonstrated a reduction in TC and TG content, improvement in the deposit of intracellular lipid droplets, and a decrease in the release of inflammatory factors in LO2 cells. This effect was achieved through the regulation of the NLRP3 inflammasome, presenting a novel approach to the treatment of NAFLD.

Keywords: artesunate; lipid accumulation; inflammation; NLRP3; nonalcoholic fatty liver disease

Introduction

As a metabolic disorder, non-alcoholic fatty liver disease (NAFLD) is characterized by liver cell steatosis and substantial fat accumulation in liver tissues, occurring in the absence of a history of excessive alcohol consumption [1]. The global prevalence of NAFLD is escalating due to the widespread adoption of high-fat diets and sedentary lifestyles, with an increasingly younger age of onset. Notably, the occurrence of NAFLD is closely linked to conditions such as hyperlipidemia, diabetes, and obesity [2]. Key predisposing factors in NAFLD development include hepatic lipid deposition and oxidative stress. Given the intricate nature of its pathogenesis, there is currently no effective and specific pharmacological treatment for NAFLD. The primary focus of NAFLD management revolves around lifestyle modifications, encompassing physical exercise, weight loss, and a healthy diet [3].

Of significant concern is the rising prevalence of NAFLD in children worldwide, driven by the increasing rates of obesity and overweight in this demographic. In obese children, the reported NAFLD prevalence is as high as 34.2% [4]. Consequently, NAFLD has emerged as a prominent contributor to the heightened incidence of chronic liver disease among adolescents and adults. Hence, the quest for safe and effective drugs to treat NAFLD poses a considerable challenge for both general practitioners and pediatricians.

A growing body of research evidence suggests that naturally sourced bioactive substances can effectively improve NAFLD [5]. The representative anthocyanin M3G found in blueberries demonstrates efficacy in en-

hancing NAFLD by regulating Transcription Factor EB-mediated lysosomal function and activating the nuclear factor erythroid 2-related factor 2/antioxidant response element (Nrf2/ARE) signaling pathway [6]. Baicalin has been shown to ameliorate NAFLD in mice by polarizing macrophages into anti-inflammatory M2c subtype macrophages [7].

Artesunate (ART), a water-soluble derivative of artemisinin, exhibits anti-tumor [8], anti-inflammatory [9], and anti-parasitic [10] effects, in addition to its efficacy in treating malaria [11]. Artesunate has been demonstrated to significantly reduce plasma triglyceride and cholesterol levels in rabbits, alleviate liver steatosis, and effectively prevent atherosclerosis [12]. However, the specific impact of ART on NAFLD remains to be studied.

The nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR), and pyrin domain-containing protein 3 (NLRP3) inflammasome constitutes a substantial intracellular multiprotein complex comprising ASC, NLRP3, and caspase-1. Several studies have validated that inhibiting the activation of the NLRP3 inflammasome can alleviate NAFLD [13]. In model cells with a heightened expression of NLRP3, the knockdown of NLRP3 expression notably mitigated urate-induced fat accumulation [14]. Significantly, various Chinese medicines have demonstrated regulatory effects on the NLRP3 inflammasome during the progression of NAFLD [15,16]. For instance, Kinsenoside has been shown to reduce fibrosis, inflammation, and lipid accumulation in the liver of non-alcoholic steatohepatitis mice by restraining the NF-κB/NLRP3 signaling pathway [17]. Recently, NLRP3 has emerged as a key player in anti-oxidative stress, inflammation, and lipid metabolism regulation, making it a focal point in NAFLD research.

In the present study, a lipid accumulation model of LO2 cells was established through the induction of oleic acid (OA). The roles of ART in inflammatory factors and lipid accumulation in LO2 cells were observed, providing valuable insights for the potential use of ART in the treatment of NAFLD.

Materials and Methods

Cell Culture Condition

Human normal liver cells LO2 (TongPai, Shanghai, China) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (CM10041, MACGENE, Beijing, China) supplemented with 10% fetal bovine serum (FBS, FCS500, EXcellBio, Shanghai, China) and 100 U/mL penicillin-streptomycin (P1400, Solarbio, Beijing, China). The cultivation was carried out in a 37 °C cell incubator containing 5% CO_2. To ensure purity, LO2 cells were tested for mycoplasma and confirmed to be uncontaminated. Additionally, they were identified through short tandem repeat (STR) identification.

Upon reaching a cell density of approximately 70%, LO2 cells were subjected to treatment with OA (75090, Merck, Darmstadt, Germany) for 24 hours to establish NAFLD model cells [18]. The successful construction of the NAFLD cell model was verified by the results of oil red O staining.

Oil Red O Staining

To observe the formation of intracellular lipid droplets, oil red O staining was employed [19]. The cells were fixed for 10 minutes using 4% paraformaldehyde (E672002, Sangon Biotech, Shanghai, China) followed by soaking in 0.5% isopropyl alcohol (A600918, Sangon Biotech, Shanghai, China). Subsequently, they were stained with oil red O (G1262, Solarbio, Beijing, China) for 20 minutes and counterstained with hematoxylin (E607317, BBI, Shanghai, China) for 1 minute. The staining results were observed using a microscope (CKX53, OLYMPUS, Tokyo, Japan), and the lipid droplet area was analyzed using ImageJ 8.0 (NIH, Bethesda, MD, USA).

Cell Viability

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay [20]. Following the digestion of LO2 cells from the culture vial with pancreatic enzyme (8049-47-6, Klamar, Shanghai, China), a single-cell suspension (0.8×10^4 cells) was inoculated into a 96-well plate. Various concentrations of ART (0, 2.5, 5, 10, 20 µm, A3731, Merck, Darmstadt, Germany) were added, and the cells were cultured for an additional 6 hours. Upon completion of the culture period, incubation continued for 2 hours with 10 µL CCK-8 (HY-K0301, Merck, Darmstadt, Germany). The absorbance was then detected at 450 nm, and a cell viability diagram was generated. The formula for calculating the cell survival rate is as follows: cell survival rate = (OD value of the experimental group – OD value of the blank group)/(OD value of the control group – OD value of the blank group) × 100%, OD, Optical Density.

Enzyme-Linked Immunosorbent Assay (ELISA)

The cell supernatant from each group was collected, and the detection of triglycerides (TG) and total cholesterol (TC), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) was carried out using enzyme-linked immunosorbent assay (ELISA) according to the relevant kits (TG detection kit: SP12002, Saipei, Wuhan, China; TC detection kit: SP11930, Saipei, Wuhan, China; IL-6 detection kit: KLC006(H).96, KALANG, Shanghai, China; IL-1β detection kit: KL-IL1b-b, KALANG, Shanghai, China; TNF-α detection kit: KL-TNFb-b, KALANG, Shanghai, China) [21]. Absorbance measurements at 450 nm were conducted using a microplate reader (A51119700DPC, Thermo Fisher Scientific, Waltham, MA, USA).

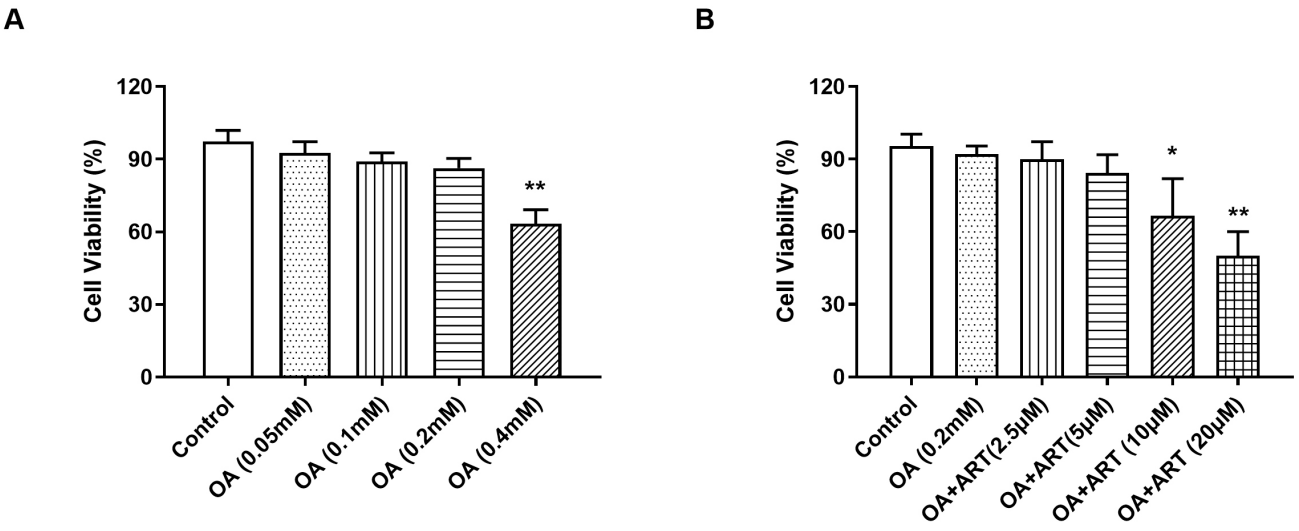

Fig. 1. Effect of artesunate (ART) on cell viability in LO2 cells after oleic acid (OA) treatment. (A) Effect of OA at different concentrations on LO2 cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay (n = 3). (B) Effect of ART at different concentrations on LO2 cell viability was investigated by CCK-8 assay (n = 3). *$p < 0.05$; **$p < 0.01$.

Western Blot Assay

To investigate the impact of ART on the NLRP3 pathway, the protein expressions of NLRP3, N-terminus of Gasdermin-D (GSDMD-N), Cleaved caspase-1, and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) were examined using Western blot assay. Total protein was extracted from the treated cells using radioimmunoprecipitation assay (RIPA) buffer (20101ES60, Yeasen, Shanghai, China). Following separation by 8%–12% polyacrylamide gel electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (IPFL00010, Millipore, Billerica, MA, USA). The PVDF membrane was then blocked with 5% skim milk and co-incubated with primary antibodies (anti-NLRP3, 27458-1-AP, 1:1000, Proteintech, Chicago, IL, USA; anti-Cleaved caspase-1, #89332, 1:1000, Cell Signaling, Danvers, MA, USA; anti-ASC, 1:500, Proteintech, Chicago, IL, USA; anti-GSDMD-N, ab215203, 1:1000, Abcam, Cambridge, UK; anti-Tubulin, 1:10,000, Proteintech, Chicago, IL, USA). Subsequently, the secondary antibody (PR30011, 1:5000, Proteintech, Chicago, IL, USA) was added at room temperature and incubated for an additional 1 hour. Each protein strip was exposed using ECL color development and analyzed using ImageJ software (NIH, Bethesda, MD, USA). Tubulin served as the internal reference, and the relative gray values were utilized to represent the expression of target proteins.

Statistical Analysis

Data analysis was conducted using GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA) and SPSS 20.0 (IBM, Armonk, NY, USA). The results were presented as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's test was employed for the comparison of multiple groups. Statistical significance is considered when the p-value is less than 0.05.

Results

Effect of ART on Cell Viability in LO2 Cells after OA Treatment

The results revealed that lower concentrations of OA (0.05, 0.1, 0.2 mM) did not exhibit obvious toxicity on LO2 cells ($p > 0.05$). However, cell viability decreased significantly after 24 hours of treatment with OA at 0.4 mM ($p < 0.01$, Fig. 1A). Consequently, we selected 0.2 mM OA for a 24-hour treatment to establish NAFLD model cells in LO2 cells.

To assess the toxicity of ART on LO2 cells, a CCK-8 assay was employed. In comparison to the OA group, ART concentrations of 2.5 μM and 5 μM had no significant effect on cell viability ($p > 0.05$). However, at ART concentrations of 10 μM ($p < 0.05$) and 20 μM ($p < 0.01$), evident cytotoxicity to LO2 cells was observed (Fig. 1B). Consequently, we opted for 2.5 and 5 μM ART as the stimulus concentrations in the subsequent experiments.

ART Inhibited Lipid Accumulation in LO2 Cells after OA Treatment

Intracellular lipid accumulation was assessed through oil red O staining. Following OA stimulation, there was a significant increase in intracellular orange-red lipids ($p < 0.01$). Importantly, after treatment with ART, the cytoplasmic fat content exhibited a notable decrease ($p < 0.01$, Fig. 2A). Comparison with the control group revealed a significant elevation in TG and TC in the OA group ($p < 0.01$). Furthermore, in comparison to the OA group, both TG (p

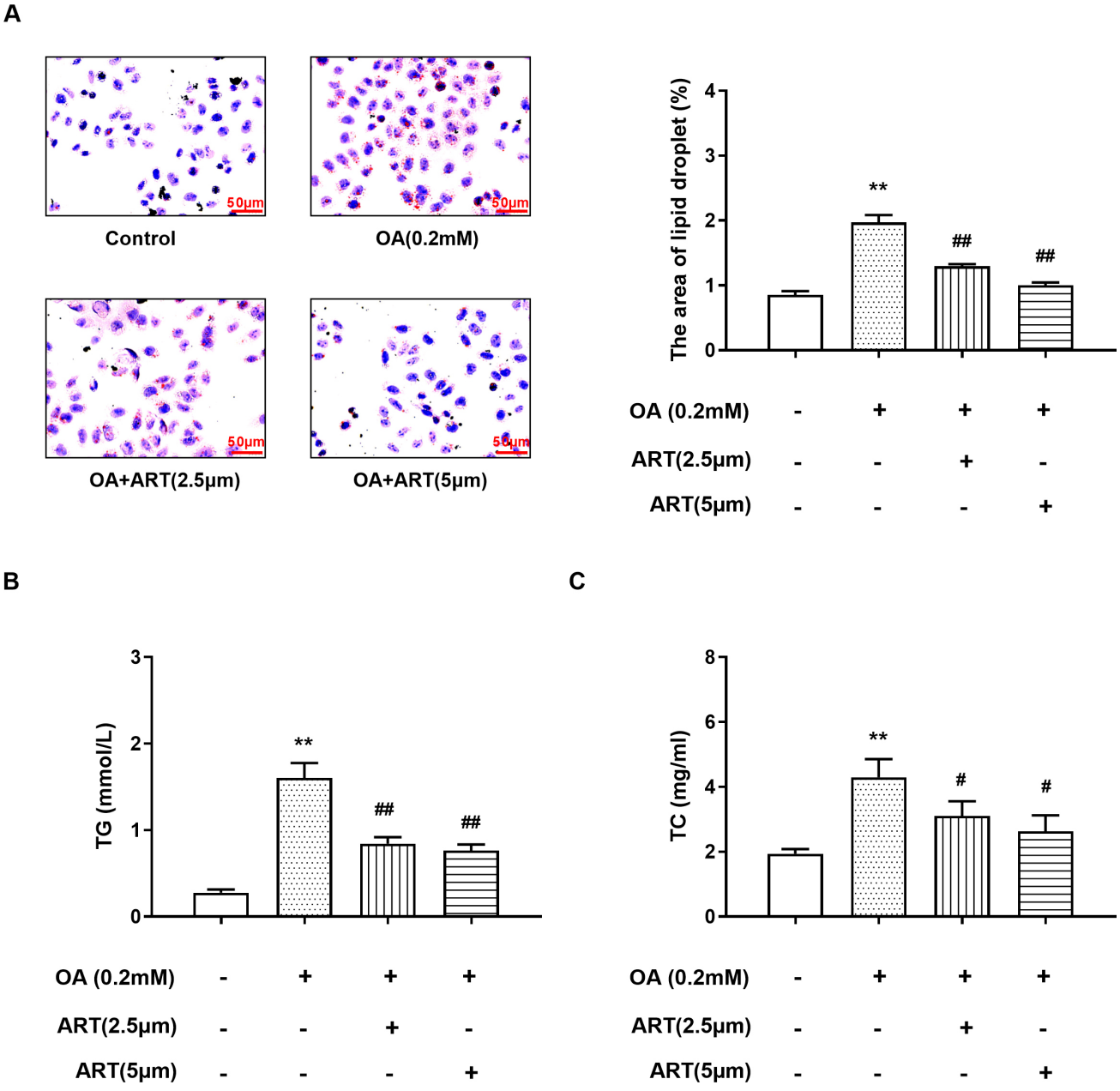

Fig. 2. ART restrained lipid accumulation in LO2 cells after OA treatment. (A) Lipid accumulation was demonstrated by oil red O staining (n = 3) (scale bar: 50 μm). (B) Effect of ART on triglycerides (TG) in LO2 cells after OA treatment (n = 3). (C) Effect of ART on total cholesterol (TC) in LO2 cells after OA treatment (n = 3). #$p < 0.05$, ##$p < 0.01$, vs OA group; **$p < 0.01$, vs control group.

< 0.01) and TC ($p < 0.05$) contents were conspicuously reduced in the ART group (Fig. 2B,C). Collectively, ART treatment effectively restrained lipid accumulation in OA-treated cells.

ART Decreased the Secretion of Inflammatory Cytokines in LO2 Cells after OA Treatment

Subsequently, we investigated the impact of ART on inflammatory cytokines in OA-treated LO2 cells. Following OA stimulation, there was a significant increase in the levels of TNF-α ($p < 0.05$), IL-1β ($p < 0.01$), and IL-6 ($p < 0.01$). In comparison to the OA group, the 2.5 μm ART group showed no significant difference in TNF-α content ($p > 0.05$). However, the 5 μm ART group notably reduced TNF-α content ($p < 0.05$, Fig. 3A). Importantly, in comparison to the OA group, both the 2.5 μm ART ($p < 0.05$) and 5 μm ART ($p < 0.01$) groups exhibited a decrease in the content of IL-6 and IL-1β (Fig. 3B,C). These findings suggest that ART effectively decreased the production of inflammatory factors in OA-treated LO2 cells.

ART Regulated NLRP3 Signaling Axis

The Western blot assay results revealed a substantial increase in NLRP3 expression in the OA group, and

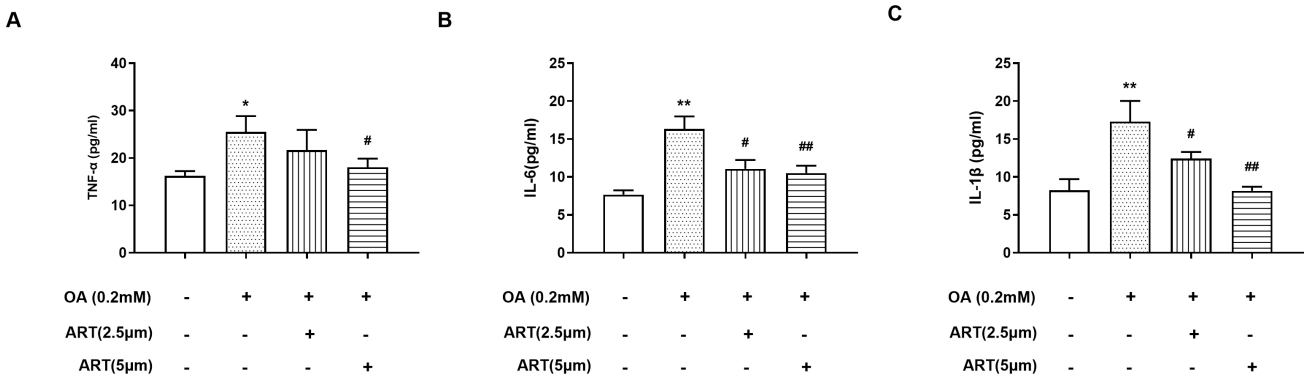

Fig. 3. ART decreased the secretion of pro-inflammatory factors in OA treated LO2 cells. (A) Effect of ART on tumor necrosis factor-α (TNF-α) content in LO2 cells induced by OA (n = 3). (B) Effect of ART on interleukin-6 (IL-6) content in OA treated LO2 cells (n = 3). (C) Effect of ART on interleukin-1β (IL-1β) content in OA treated LO2 cells (n = 3). #$p < 0.05$, ##$p < 0.01$, vs OA group; *$p < 0.05$, **$p < 0.01$, vs control group. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β.

ART effectively mitigated this elevated expression trend ($p < 0.01$, Fig. 4A). The expression of Cleaved caspase-1 significantly increased in the OA group but notably decreased in the ART group compared to the OA group ($p < 0.01$, Fig. 4B). Similarly, when compared to the control group, ASC and GSDMD-N significantly increased in the OA group ($p < 0.05$) and markedly reduced in the ART group ($p < 0.05$, Fig. 4C,D). These observations suggest that ART may ameliorate NAFLD by inhibiting the NLRP3 inflammasome signaling pathway.

Discussion

With the escalating global prevalence of overweight and obesity among children, NAFLD is manifesting as a condition affecting individuals at increasingly younger ages. Currently, NAFLD has emerged as the primary cause of chronic liver disease in children and adolescents, impacting their growth and development. Mounting evidence suggests that traditional Chinese medicine exhibits characteristics of multi-target action in the treatment of NAFLD, accompanied by relatively few side effects [22,23].

ART, as an artemisinin derivative, plays an indispensable role in malaria treatment. Beyond its antimalarial properties, ART has demonstrated various pharmacological activities, including anticancer effects and sepsis prevention. Kong *et al.* [24] made a noteworthy discovery, revealing that ART induces activated iron ptosis in fibrotic livers. Additionally, ART has been shown to restrain bacterial translocation from intestinal flora and reduce inflammation by regulating intestinal microflora in cirrhotic rats [25]. In this study, we successfully utilized LO2 cells cultured with OA to establish a model of hepatic steatosis non-alcoholic fatty liver cells. The findings strongly indicate that ART effectively suppresses the progression of NAFLD.

Excessive accumulation of lipids in the liver is a significant hallmark of NAFLD. The main contributors to hepatic lipid accumulation involve an increase in lipid sources and a decrease in lipid routes. The aberrant buildup of lipids in liver cells serves as the initiating factor in the pathogenesis of NAFLD, and is also recognized as a pivotal element leading to liver oxidative stress, cell damage, inflammatory responses, and fibrosis. In this study, it was observed that ART effectively curtailed the rise in intracellular TG and TC levels induced by OA, countering the abnormal lipid levels provoked by OA. Intriguingly, ART demonstrated a robust anti-lipogenesis effect by modulating peroxisome proliferator-activated receptor-γ (PPAR-γ), CCAAT/enhancer-binding protein-α (C/EBP-α), signal transducer and activator of transcription-3 (STAT-3), fatty acid synthase (FAS), and perilipin A [26]. Furthermore, ART inhibited the adipogenic differentiation of 3T3-L1 preadipocytes [27]. Therefore, our study suggests that ART inhibits lipid accumulation in NAFLD, thereby contributing to the improvement of NAFLD progression.

In our study, ART effectively reduced the levels of IL-6, TNF-α, and IL-1β in OA-treated LO2 cells. The inflammatory response is a crucial factor in the pathological progression of NAFLD. TNF-α, IL-6, and IL-1β are pivotal pro-inflammatory cytokines that promote the onset of liver inflammation [28]. ART exhibits a broad spectrum of anti-inflammatory effects, resisting inflammation in the respiratory system, kidneys, and neuroinflammation by inhibiting inflammatory factors and inducing apoptosis in inflammatory cells. ART has been shown to ameliorate inflammation caused by liver injury by alleviating histopathological changes and regulating the release of cellular inflammatory factors [29]. Notably, ART has been observed to increase the levels of IL-6, interferon-γ (IFN-γ), IL-1β, TNF-α, and interleukin-17 (IL-17) while decreasing interleukin-10 (IL-10) levels in mice with hepatitis [30]. Our study suggests that ART may impede the progression of NAFLD through its anti-inflammatory effects.

In this study, our focus was on investigating whether ART acts on NLRP3 in the progression of NAFLD. Signif-

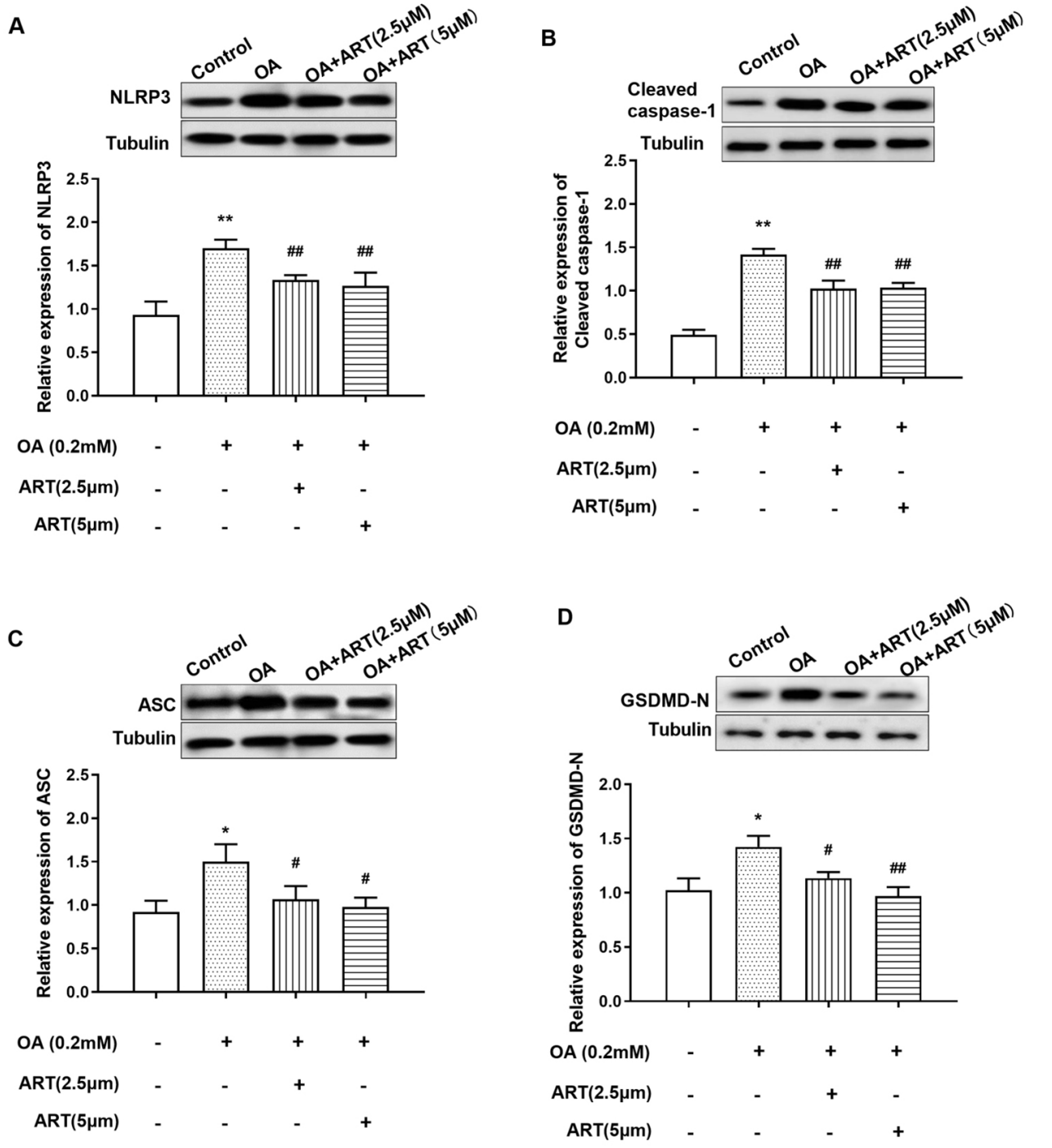

Fig. 4. ART regulated nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR), and pyrin domain-containing protein 3 (NLRP3) signaling axis. (A) Effect of ART on NLRP3 expression in LO2 cells after OA treatment (n = 3). (B) Effect of ART on Cleaved caspase-1 protein expression in LO2 cells after OA treatment (n = 3). (C) Effect of ART on apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) protein expression in LO2 cells after OA treatment (n = 3). (D) Effect of ART on N-terminus of Gasdermin-D (GSDMD-N) protein expression in LO2 cells after OA treatment A (n = 3). #$p < 0.05$, ##$p < 0.01$, vs OA group; *$p < 0.05$, **$p < 0.01$, vs control group.

icantly, ART reduced the expressions of NLRP3, GSDMD-N, Cleaved caspase-1, and ASC in OA-treated cells, indicating that ART improves NAFLD by inhibiting the NLRP3 inflammasome. The NLRP3 inflammasome is a multiprotein complex mainly composed of NLRP3, ASC, and pro-caspase-1 precursor. This inflammasome is a component of the immune system that facilitates the immune response by mediating the release of downstream inflammatory factors

upon activation. Activation of the NLRP3 inflammasome can trigger pyroptosis by activating caspase-1, thereby promoting the progression of NAFLD [31]. In mice with non-alcoholic steatohepatitis, the inhibition of NLRP3, ASC, and Caspase-1 activation significantly reduced liver inflammation [32]. Additionally, down-regulation of NLRP3 suppressed the secretion of IL-18 and IL-1β, alleviating the inflammatory response [15].

Nevertheless, the impact of ART on NAFLD requires validation through animal experiments. Furthermore, it remains unclear whether ART affects lipid synthesis-related proteins, necessitating further studies.

Conclusions

ART regulated lipid accumulation and inflammatory factors in NAFLD by inhibiting the NLRP3 inflammasome. This finding provides a reference for the clinical application and efficacy study of ART treatment in NAFLD.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JW designed the research study; JW, JS and CC performed the research; JS and CC collected and analyzed the data; JS and JW have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors give final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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