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Original Article

Artesunate ameliorates non-alcoholic fatty liver disease cells by regulating the inflammatory cytokines and oxidative stress *in vitro*

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Abstract: Recently, nonalcoholic fatty liver disease has become the most common cause of chronic liver disease with the increasing prevalence of obesity, diabetes, and the metabolic syndrome in the general population. Nevertheless, it still has no obvious treatment mechanism so far. Artesunate is the water soluble derivatives of artemisinin which shows high activity against both drug-resistant and drug-sensitive of malaria. In the present study, we employed artesunate to assess whether it could improve nonalcoholic fatty liver disease. The 20 μg/mL oleic acid was employed as a treating reagent to induce steatosis, this was used for building a hepatocyte LO-2 nonalcoholic fatty liver disease cell model, which could mimic the histological features and pathological symptom. Therefore, this model was treated with different concentrations of artesunate. The total content of triglyceride (TG), alanine aminotransferase, aspartate amino transferase, alkaline phosphatase and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-18 (IL-18) were determined by Elisa, and immunoflurescence and Western blot were employed for further confirmation. Optical microscopy, Western blot, Elisa technique, immunoflurescence and dichlorofluorescin diacetate (DCFH-DA) assays suggested that artesunate was able to improve nonalcoholic fatty liver disease by regulating the downstream inflammatory cytokines (TNF-α, IL-6, IL-8 and IL-18) and oxidative stress which were associated with TLR4/PI3K/Akt pathway. These results demonstrated that artesunate could be a potential therapeutic strategy for preventing the progression and development of nonalcoholic fatty liver disease cells. This report is conductive to deepening the understanding of nonalcoholic fatty liver disease mechanism and provides theoretical basis for clinical application of artesunate in treating nonalcoholic fatty liver disease.

Keywords: Artesunate, nonalcoholic fatty liver disease, TLR4/PI3K/Akt signal pathway, inflammation, oxidative stress

Introduction

Non-alcoholic fatty liver disease (NAFLD) is becoming an important public health concern and the incidence of which is rising rapidly due to the increasing epidemic of obesity in both adults and children [1]. Fatty liver includes a serious of histological alterations, characterized by inflammation and fibrosis [2]. Therefore, NAFLD has been traditionally defined as a condition which may progress to liver-related complications such as cirrhosis, liver cancer and liver mortality [3]. In the recent years, NAFLD has been considered as cryptogenic cirrhosis, an important early stage of liver cancer and a possible cause of liver failure [4]. With the rapid development of social economy, the prevalence of NAFLD is rapidly increasing. It has become one of the three major liver diseases endangering human health and one of the most important public health problems in the 21st century. Nevertheless, the pathogenesis of NAFLD is not entirely clear and has no special therapeutic methods. Therefore, exploring its pathogenesis and finding a positive and effective prevention methods have far-reaching significance.

At present, the most mature explanation of NAFLD pathogenesis is the two “hits” theory [5]. The first is insulin resistance. The oxidative stress and lipid per oxidation are the second

hit to liver cells and it also lead to liver inflammation, necrosis and fibrosis. In the first hit, the excess secretion of tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and other inflammatory cytokines may induce insulin resistance by affecting insulin signal transduction directly. These inflammatory cytokines also promote the oxidative stress on liver in the second hit. Thus, abnormal secretion of inflammatory cytokines is one of the key factors in the pathogenesis of NAFLD. In addition, the unbalanced secretion of IL-10, IL-18 and the resistance in inflammatory cytokines also could be found in NAFLD [6]. Therefore, the regulatory mechanism of inflammatory cytokines is critical for researching the mechanism of NAFLD.

Toll-like receptors family (TLRs), which recognize pathogen-associated molecular patterns, are widely distributed and expressed in a variety of immune cells. The most prominent biological function of TLRs is to promote the synthesis and release of cytokines, which cause inflammation effectors [7]. TLRs can activate nuclear transcription factor-κB p65 (NF-κB p65) and transcribe it into the nucleus. It also activates target genes transcription and cause a series of cytokine synthesis and release, such as TNF-α, IL-6 and IL-8, which ultimately result in a series of immune and inflammatory responses [8]. Therefore, the up-regulation signaling pathway of inflammatory cytokines is mainly TLRs/NF-κB pathway. The recent studies have indicated that TLRs, in particular TLR4, represented a major mechanism linked to saturated fatty acid activation [9]. The TLR4 which is the receptor for lipopolysaccharide (LPS) and components of the TLR4 signaling pathway are widely distributed in the liver [10]. In addition, activation of the TLR4 signaling pathway may be perturbed at multiple steps during the initiation and progression of NAFLD [11]. Hence, the role of TLR4 in NAFLD has attracted increasingly attention by researchers. Phosphoinositide-3 kinase (PI3K) /serine/threonine kinase (Akt) is one of the main signal pathways of the downstream TLR4 [12]. With this in mind, it is speculated that TLR4/PI3K/Akt pathway played an important role in the regulation of inflammatory cytokines in NAFLD.

Significant research endeavors are being directed toward understanding the pathogenesis of NAFLD. Designing therapeutic strategies and exploring suitable drugs to treat NAFLD is necessary. Artesunate, a water-soluble artemisinin derivative, has been the most widely used derivative for more than 15 years [13], and is an essential component of the combination treatment of uncomplicated falciparum malaria, which is now accepted as the treatment of choice [14]. Many clinicians think that parenteral administration of artesunate is the most effective treatment for severe malaria. Additionally, artesunate was also demonstrated that it could be a potential drug against Babesia infection [13]. However, to the best of our knowledge, there are few reports regarding the artesunate to be a drug for treating NAFLD. Our preliminary research revealed that artesunate could down-regulate TLR4, NF-κB p65 and some other inflammatory cytokines, and had anti-inflammatory effects on asthmatic rats. Moreover, artesunate also has the antilipemic effect and reduces TNF-α and IL-6 expression, these inflammatory cytokines are closely related to the occurrence of NAFLD. With this in mind, we propose that artesunate may have the potential for improving NAFLD by regulating the expression of inflammatory cytokines which are associated with TLR4/PI3K /Akt signaling pathway.

In the present study, artesunate was first utilized as a drug for improving NAFLD, and we have verified that artesunate could improve NAFLD by reducing the level of liver enzymes in hepatocyte LO-2 NAFLD cell model, affecting the TLR4/PI3K/Akt signaling pathway and decreasing the expression of inflammatory cytokines. The reactive oxygen species (ROS) formation when oxidative stress occurred also supported these conclusions. This study suggests that artesunate could be a potential drug for treating NAFLD, and it is expected to provide new ideas for the therapy of NAFLD.

Materials and methods

Cell culture

The human normal hepatocyte LO-2 cells were obtained from the Shanghai institute of cell research, Chinese Academy of sciences. These cells were maintained in a humidified incubator containing an atmosphere of 5% CO_2 and 1640 medium (Gibco, USA) supplemented with 1% L-glutamine (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), and 10% fetal bovine serum (Gibco, USA); the temperature of this medium was maintained at 37°C before used.

The establishment of hepatocyte LO-2 NAFLD cell model and Oil red-O staining

After human hepatocyte LO-2 cells were treated with oleic acid, the degree of steatosis of cells was measured using Oil Red-O staining. In this assay, cells were fixed with formaldehyde, while lipids were stained using 0.5% Oil Red-O in isopropyl alcohol for 20 minutes, and nuclei were counterstained with hematoxylin for 1 minute. Cell images were then observed under a bright field microscope (Olympus, Japan) at 400 × magnification.

Enzyme-linked immunosorbent assay

The Enzyme-Linked Immunosorbent Assay (ELISA) Kits (R&D Systems, USA) were used to determine the concentrations of total triglyceride (TG), alanine aminotransferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP), TNF-α, IL-10, IL-18 and IL-6 in NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. The process of measuring these concentrations was performed according to the manufacturer's instructions. These enzymes and inflammatory cytokines concentrations in the culture supernatants were calculated using their standards. All ELISA assays were repeated three times (n = 5 for each experiment).

Western blot

After the cells were treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hour, they were lysed in RIPA buffer (Beyotime, China) containing a complete protease inhibitor cocktail and 2 mM phenylmethylsulfonyl fluoride. The protein concentrations were determined using a BCA Protein Assay Kit (Beyotime, China). Total protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a 0.45 μm nitrocellulose membrane (Millipore, USA). The membranes were incubated with primary antibodies overnight at 4°C and then hybridized with the appropriate HRP conjugated secondary antibody (Abcam, USA) for 2 hours at room temperature. Protein signals were visualized using the ECL detection system. The primary antibodies were anti TRL4 (Abcam, USA), anti-PI3K (Abcam, USA), anti-NF-κB p65 (Abcam, USA), anti-Akt (Abcam, USA), anti-p-Akt (Abcam, USA) and anti-β-actin (Abcam, USA). Each experiment was performed in triplicate.

Immunohistochemical staining

The cells were treated according to the protocols of the Fast Immuno Cyto Chemistry Staining Kit (BPICC30-1KT, Protein Biotechnologies, USA). The primary antibody was anti-NF-κB p65 (Abcam, USA). The working concentration of DAPI (Sigma, USA) was 5 μg/mL in PBS. Each experiment was performed in triplicate.

Dichlorofluorescin diacetate (DCFH-DA) assay

The formation of intracellular ROS was measured using 2', 7'-DCFH-DA stain method by fluorescence microscope analysis. The cell permeable DCFH-DA is non-fluorescent unless oxidized by the intracellular ROS to form a highly fluorescent compound 2', 7'-dichlorofluorescin (DCF). Briefly, LO-2 cells were seeded in 96-well plates for 24 hours and then treated with 20 μg/mL oleic acid for 48 hours. After the cells were rescued by 2, 10 and 40 μg/mL artesunate for another 24 hours, fresh media containing 5 μM DCFH-DA were added for 30 minutes at 37°C, and then washed with PBS three times and the fluorescence was analyzed immediately by using fluorescence microscope and a fluorescence plate reader (λex = 485 nm, λem = 535 nm). Each experiment was performed in triplicate (n = 5 in each experiment).

Statistical analysis

All experiments were performed in triplicate, and the data are shown as the means ± SDs of three separate experiments. Statistical analysis was performed with SPSS software 11.0 and it was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Two-group comparisons were tested by Student's test. The probability values of $^{*}P<0.05$ were considered significant compared to control group, and $^{\#}P<0.05$ was considered significant compared to 0 μg/mL artesunate group.

Results

Artesunate decreases NAFLD-associated lipid accumulation in the LO-2 hepatocytes model

Oleic acid is a fatty acid that occurs naturally in various animals and vegetable fats and oils, oleic acid could induce steatosis of LO-2 hepatocytes cell lines, as described previously [15]. In this study, the cells were treated with

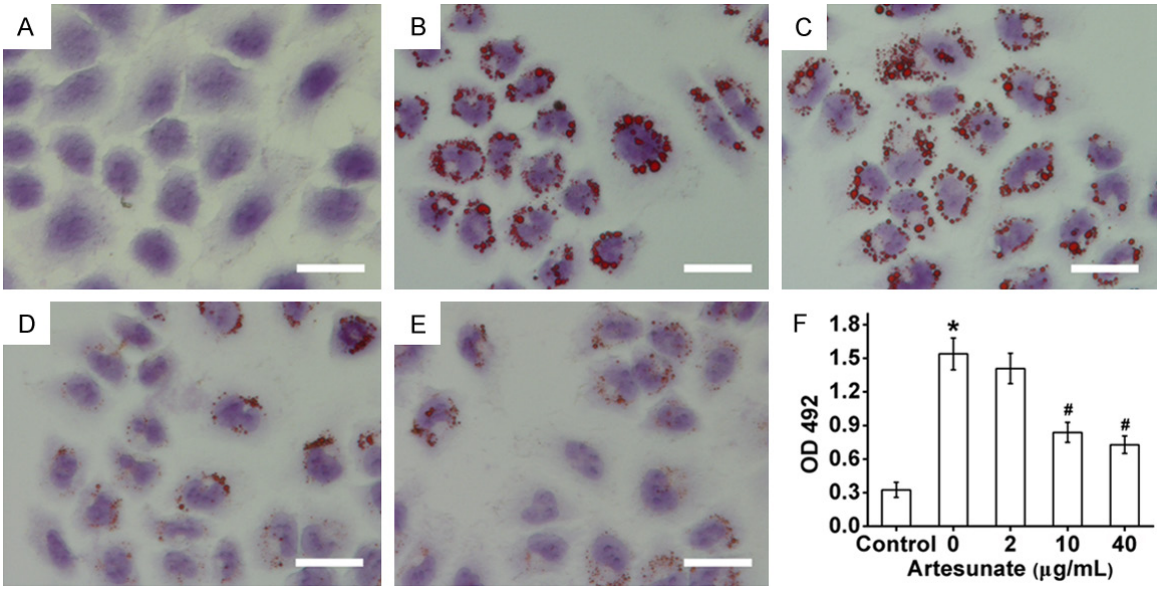

Figure 1. The effect of artesunate on hepatocyte LO-2 NAFLD cell model. A: The optical microscopy images of control cells staining with Oil Red-O. B-E: Oil Red-O staining of NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. F: The absorbance of Oil Red-O of control cells and NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. The data are presented as the means ± standard deviations (*P<0.05 vs. control group; #P<0.05 *vs.* NAFLD cells treated with 0 μg/mL artesunate). The scale bar was 50 μm.

20 μg/mL of oleic acid for 48 hours to induce steatosis. The control cells (**Figure 1A**) and cells treated with various concentrations of artesunate (0, 2, 10, 40 μg/mL) for 24 hours (**Figure 1B-E**) were all stained by Oil Red-O, from **Figure 1A-E** we could observe that the number of positive lipid droplets were significantly reduced with the increase of artesunate concentrations. To further verify the above conclusion, the absorbance of Oil Red-O was detected (**Figure 1F**). When the cells treated with 10 and 40 μg/mL of artesunate for 24 hours, the absorbance of Oil Red-O was significantly decreased. Therefore, these results demonstrated that artesunate treatment could reduce steatosis and displayed a concentration-dependent manner.

Artesunate reduces NAFLD-associated liver TG, ALT, AST and ALP contents

NAFLD is characterized by the accumulation of TG, ALT, AST and ALP in the liver, caused by multiple factors, as the increment of fatty acid uptake as a result of the enhancement of the lipolysis from the adipocytes or the increased intake of dietary fat [16]. After being exposed to 0, 2, 10, 40 μg/mL artesunate for 24 hours, the contents of TG, ALT, AST and ALP accumulation in LO-2 hepatocytes NAFLD cells were reduced in a concentration-dependent manner, and significantly reduced when the cells were treated with 10 and 40 μg/mL of artesunate, compared to the cells treated with 0 μg/mL artesunate group cells (**Figure 2**). These results implied that artesunate could improve NAFLD cells by decreasing the accumulation of these enzymes in the liver.

The changed expression of TLR4/PI3K/Akt pathway, down-regulated NF-κB p65, TNF-α, IL-10, IL-18 and IL-6 in NAFLD cells treated with artesunate

TLR4/PI3K/Akt pathway plays an important role in the pathogenesis of NAFLD [10-12]. In order to investigate the effect of artesunate on this pathway, we assessed the protein expression of TLR4, PI3K, and phosphorylation Akt (p-Akt) by Western blotting analysis when the NAFLD cells treated with 0, 2, 10, 40 μg/mL artesunate for 24 hours (**Figure 3A-C**). As compared to that of control group, the NAFLD group significantly demonstrated higher expression of TLR4, PI3K, and p-Akt. Meanwhile, artesunate was able to attenuate this increase, notably at 10 and 40 μg/mL treatments. NF-κB p65 is a crucial factor in the production of inflammatory mediators, and TLR4 is involved

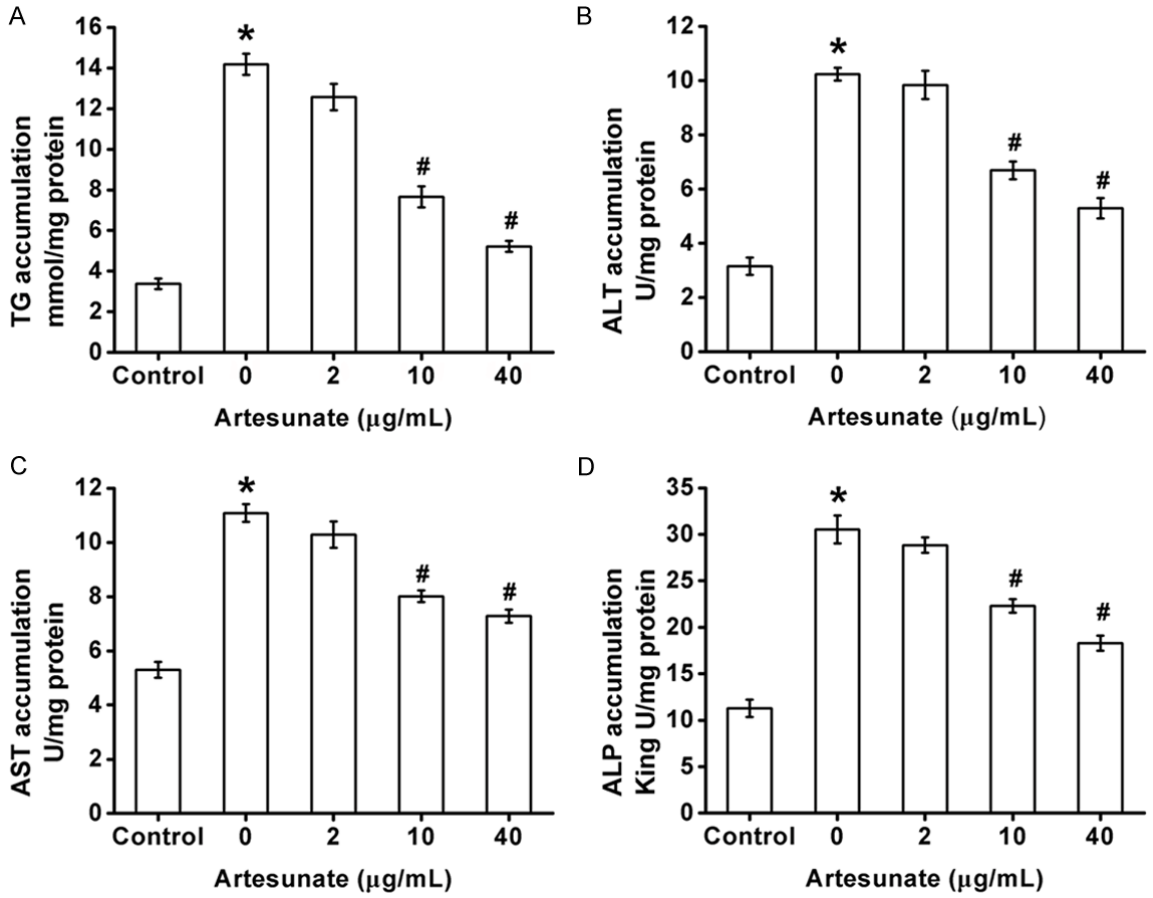

Figure 2. Artesunate could decrease the liver enzymes accumulation of NAFLD cells. TG (A), ALT (B), AST (C) and ALP (D) content measurement in NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. The data are presented as the means ± standard deviations (*P<0.05 *vs.* control group; #P<0.05 *vs.* NAFLD cells treated with 0 μg/mL artesunate).

in triggering the intracellular signal transduction pathway of NF-κB p65 activation [17]. In order to investigate whether the expression of NF-κB p65 was inhibited by artesunate, Western blot analyses were also used. As showed in **Figure 3D**, artesunate also attenuated NF-κB p65 activation in a concentration-dependent manner which was consistent with the above results. The downstream inflammatory cytokines (TNF-α, IL-10, IL-18 and IL-6) of TLR4/PI3K/Akt pathway could promote the progression of NAFLD [18]. The activation of NF-κB p65 was inhibited by artesunate was further confirmed by the immunostaining assay (**Figure 4**). The NAFLD cells were stained with NF-κB p65 antibody (green) and counter-stained with DAPI (blue), and the NF-κB p65 protein activated within NAFLD cells and transferred into the nuclei (indicated by the white arrows in **Figure 4B**). When the cells were treated with artesunate, the NF-κB p65 activation was inhibited and the nuclear translocation was blocked (**Figure 4C-E**). To verify whether artesunate could suppress the inflammatory effect of NAFLD cells, the contents of TNF-α, IL-10, IL-18 and IL-6 cytokines were detected by Elisa when treated with various concentrations of artesunate for 24 hours. As shown in the **Figure 5**, the four kinds of inflammatory cytokines expression levels were significantly higher in NAFLD cells, and artesunate was able to attenuate this increase, especially when the concentration of artesunate were 10 and 40 μg/mL, the cytokines expression levels were significantly reduced. These results demonstrated that artesunate could improve NAFLD cells by regulating the downstream inflammatory cytokines, which might associated with TLR4/PI3K/Akt pathway.

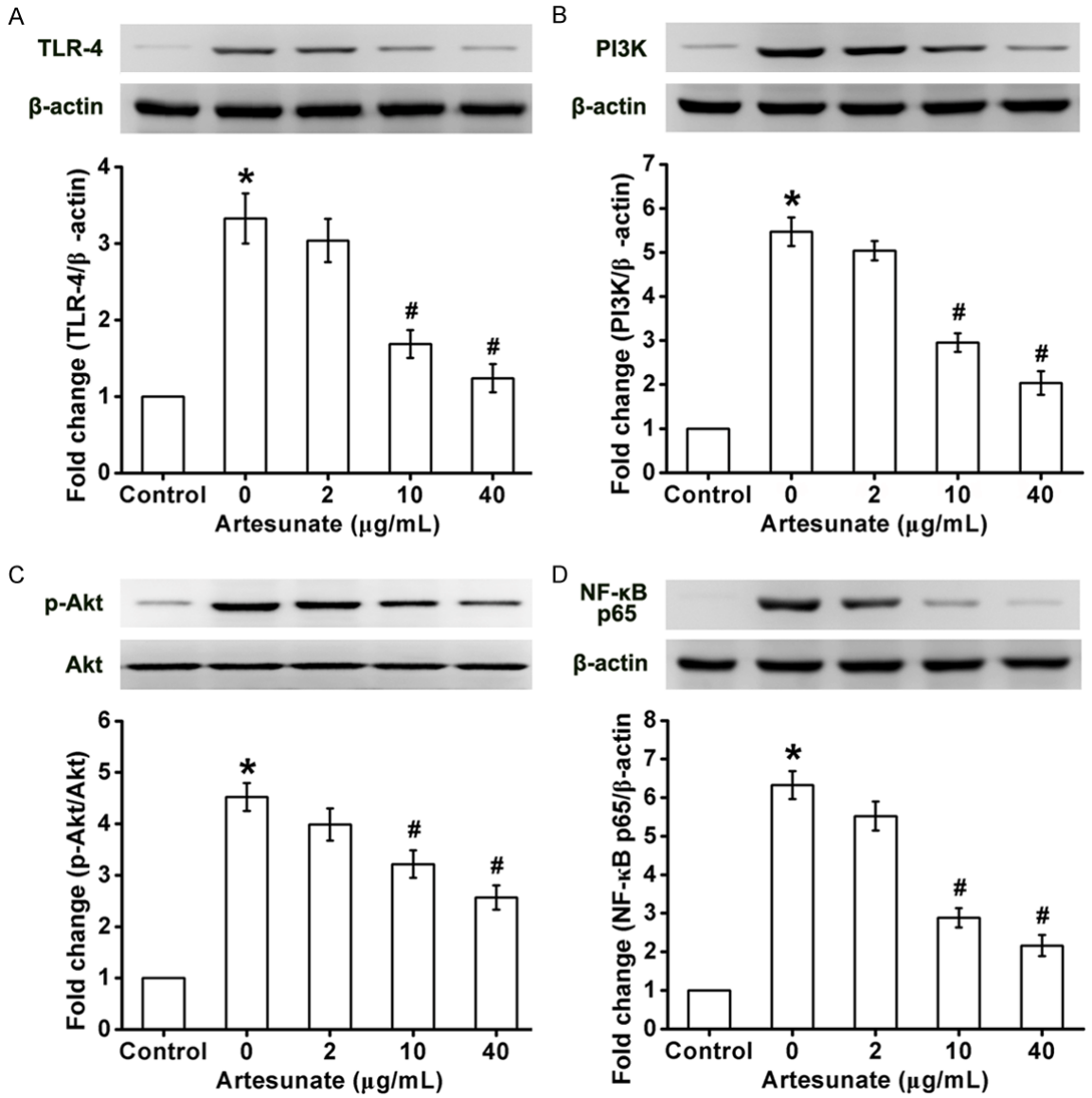

Figure 3. Artesunate could affect the expression of TLR4/PI3K/Akt pathway and reduce the expression NF-κB transcription factor of NAFLD cells. Western blot analysis of TLR-4 (A), PI3K (B), p-Akt (C) and NF-κB p65 (D) protein changed in NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. The data are presented as the means ± standard deviations (**P*<0.05 vs. control group; #*P*<0.05 vs. NAFLD cells treated with 0 μg/mL artesunate).

Artesunate decreases NAFLD-associated oxidative stress

ROS is continuously formed in biological systems, and any increase in radical production or decrease in the defense against ROS induces oxidative stress [19]. Excessive ROS levels trigger inflammatory responses through the activation of transcription factors that increase the synthesis of inflammatory cytokines [20]. In order to investigate the oxidative stress in NAFLD cells after exposure to various concentrations of artesunate for 24 hours, DCFH-DA assay was used. As shown in **Figure 6A**, **6B**, significant ROS formation occurred in NAFLD cells compared to control groups. However, when the cells treated with artesunate, the ROS formation became weekly and significantly reduced when the concentration of artesunate was 10 and 40 μg/mL (**Figure 6C-E**). The ROS formation was further determined by a DCF fluorescence plate reader (**Figure 6F**), and it was

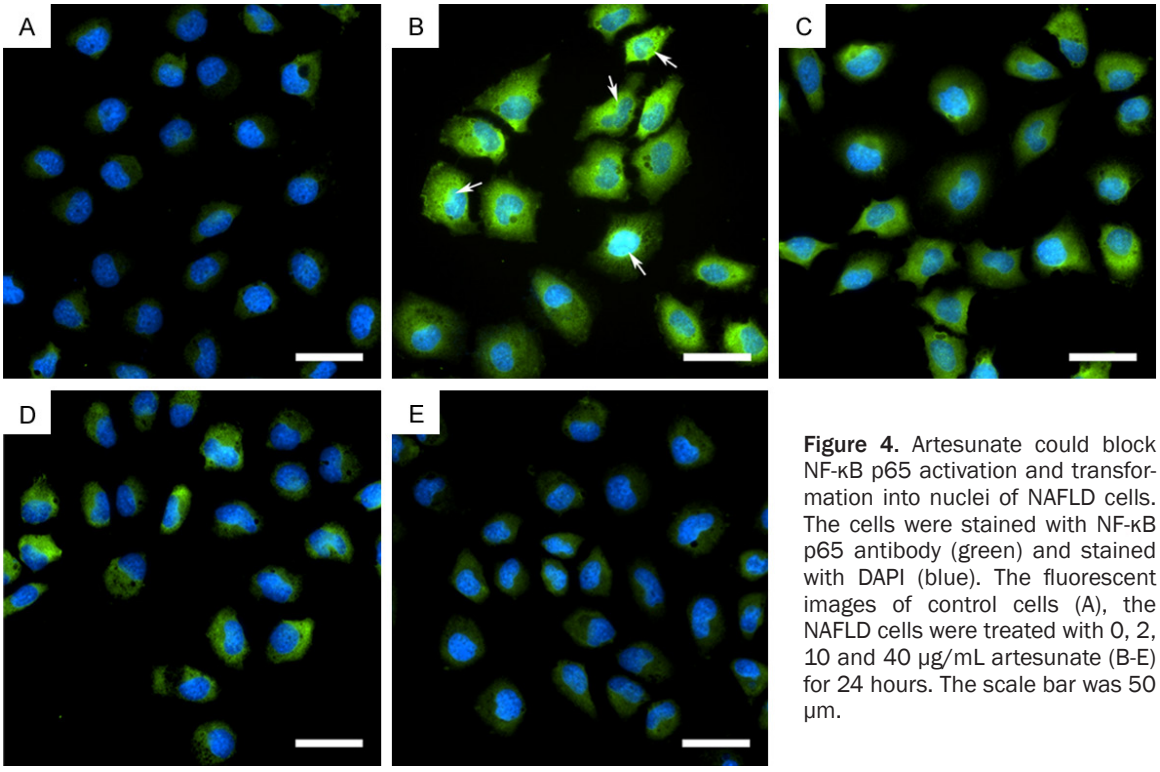

Figure 4. Artesunate could block NF-κB p65 activation and transformation into nuclei of NAFLD cells. The cells were stained with NF-κB p65 antibody (green) and stained with DAPI (blue). The fluorescent images of control cells (A), the NAFLD cells were treated with 0, 2, 10 and 40 μg/mL artesunate (B-E) for 24 hours. The scale bar was 50 μm.

consistent with the fluorescence images of ROS formation results.

Discussion

The increasing prevalence of obesity has a rise and is associated with liver injury namely NAFLD, to date, 20-30% of adult populations and some of the children in developed countries suffer from NAFLD [21]. Currently available drugs proposed for NAFLD treatment have been demonstrated to present poor efficacy and safety [16]. Therefore, it is necessary to explore the molecular mechanisms which are correlated to the establishment and progression of the disease as target for new therapies. In the present study, we show for the first time that artesunate could be a potential strategy in NAFLD development. We have studied systematically the effect of artesunate to lipid droplets formation, liver enzymes level, TLR4/PI3K/Akt pathway, inflammatory cytokines generation and oxidative stress occurred in NAFLD cells. These results suggested that artesunate was able to improve NAFLD by regulating the downstream inflammatory cytokines and oxidative stress which was associated with TLR4/PI3K/Akt pathway.

Normal human LO-2 hepatocytes cells induced by oleic acid are the classical liver model for the study of NAFLD *in vitro*. Oleic acid induces steatohepatitis rapidly and the cells develop the histological features that most closely resemble those seen in human NAFLD compared with other models. This NAFLD model allows the increased surface area of small lipid droplets and liver enzymes, as previously described [17]. In this regard, we found that artesunate was able to reduce the number of lipid droplets and liver enzymes, leading to the changed expression of TLR4/PI3K/Akt pathway. On the other hand, the inflammatory is associated with oxidative stress related ROS formation in the typical NAFLD. As expected, artesunate was able to attenuate the ROS formation because of its well-known anti-inflammatory and antioxidant capacities.

The inhibition of NF-κB p65 activation may play a key role in anti-inflammatory effect of artesunate through its ability to induce transcription of proinflammatory genes [22], furthermore, it has also been demonstrated that artesunate could inhibit TLR4. TLR4, a member of TLRs family, plays a pivotal role in initia-

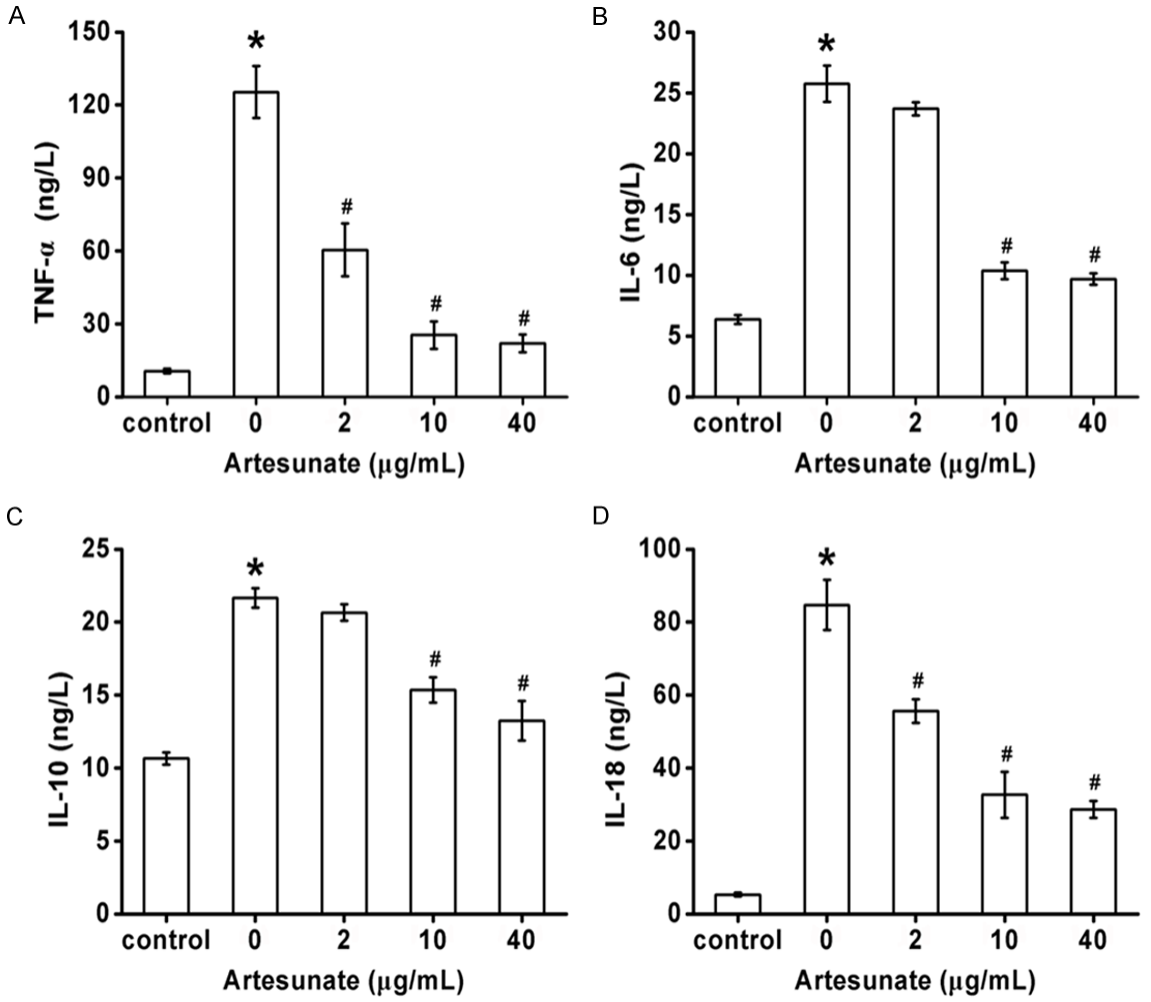

Figure 5. Artesunate could reduce the content of inflammatory cytokines of NAFLD cells. TNF-α (A), IL-6 (B), IL-10 (C) and IL-18 (D) content measurement in NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. The data are presented as the means ± standard deviations (*P<0.05 vs. control group; #P<0.05 vs. NAFLD cells treated with 0 μg/mL artesunate).

tion of innate immune responses and has been shown to trigger the release of inflammatory cytokines, and activate NF-κB p65. There have been several evidences to indicate that TLR4 plays a critical role in the pathogenesis and progression of many chronic liver diseases, such as alcoholic liver disease, liver cancer and NAFLD. PI3K/Akt plays a role in activating downstream signaling pathway of TLR4. LPS, a TLR4 agonist, activates PI3K, resulting in the phosphorylation of Akt, a downstream target of PI3K. Akt, is a serine/threonine kinase that is activated in response to various of cytokines and is activated via phosphorylation by PI3K and further phosphorylates its downstream signaling cytokines. Akt has also been shown to induce p65 phosphorylation, resulting in enhanced NF-κB transactivation. Therefore, PI3K/Akt signaling pathway is closely linked to TLR4 and NF-κB. It has been reported that PI3K/Akt pathway activation was involved in the pathogenesis of NAFLD and promoted the release of inflammatory cytokines [16]. Furthermore, research has shown that artesunate mediated anti-inflammation by inhibiting TNF-α-induced production of IL-1β, IL-6, and IL-8 which are key regulatory molecules in the development and progression of NAFLD via NF-κB signaling pathway [18]. Those results are in agreement with our investigation in this report and our reports proved that artesunate played a pivotal role in inhibiting the TLR4/PI3K/Akt pathway which is activated in the development and progression of NAFLD.

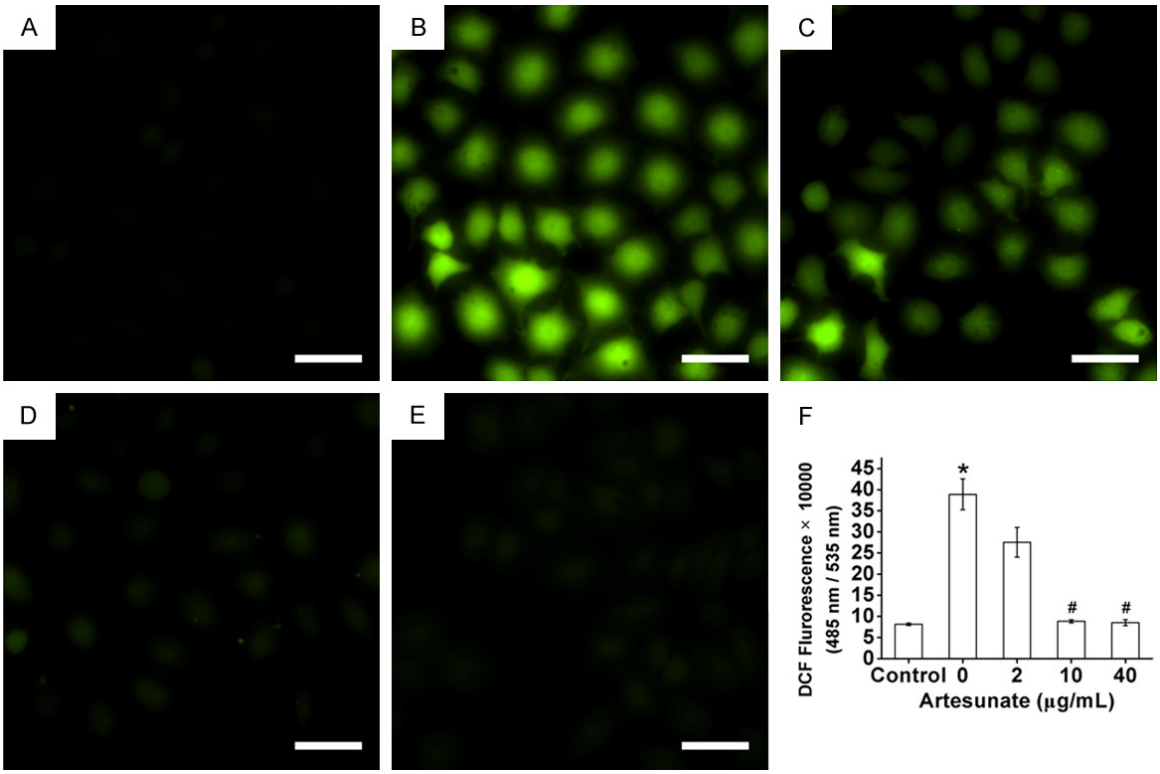

Figure 6. Effect of artesunate on the oxidative response of NAFLD cells measured by DCF-DA staining. A-E: ROS formation fluorescence images of control cells and NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. F: ROS formation was determined by DCF fluorescence absorbance at 485 nm of control cells and NAFLD cells treated with 0, 2, 10 and 40 μg/mL artesunate for 24 hours. The data are presented as the means ± standard deviations (*P<0.05 *vs.* control group; #P<0.05 *vs.* NAFLD cells treated with 0 μg/mL artesunate). The scale bar was 50 μm.

Oxidative stress refers to elevated intracellular levels of ROS that cause damage to lipids, proteins and DNA and has also been incriminated in the pathophysiology of NAFLD [23]. Several lines of evidence suggest that chronic oxidative stress may be important in the progression of NAFLD. ROS production participates in the two 'hits' of NAFLD physiopathology and may indirectly activate redox sensitive transcription factors such as NF-κB. The fluorescence microscopy images showed that expression of ROS was increased significantly in the NAFLD cells of our study. Previous reports have demonstrated ROS expression was markedly attenuated by artesunate [17], and this was in accordance with our study. These observations indicate that artesunate may be developed as the therapeutic agents for NAFLD.

This report has described that the hepatocyte LO-2 NAFLD model could be established by oleic acid treatment. Moreover, when the cells were treated with artesunate, the content of liver enzymes TG, ALT, AST and ALP was reduced in a concentration-dependent manner as well as the production of inflammatory cytokines TNF-α, IL-10, IL-18 and IL-6, which was possibly by inhibiting the TLR4/PI3K/Akt signal pathway, furthermore, NF-κB expression and oxidative stress were also attenuated as we observed in the artesunate-treated groups of our study. These results demonstrated that artesunate could be used as a potential therapeutic strategy for preventing the progression and development of NAFLD.

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Disclosure of conflict of interest

None.

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