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Original Article

Therapeutic effect of dihydroartemisinin on lupus-prone MRL/lpr mice and its mechanism of action

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Abstract: Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease and female lupus-prone MRL/lpr mice are commonly used models of SLE, in this study, the therapeutic effect of dihydroartemisinin (DHA) on lupus-prone MRL/lpr mice and its mechanisms was investigated by comparing differences between lupus-prone MRL/lpr mice given DHA and control mice in molecular biology and histopathology. The results show that DHA could remarkably relieve the symptoms, decrease the level of urine protein, and alleviate pathological renal lesions. DHA alleviated the symptoms of lupus-prone MRL/lpr mice by reducing production of autoimmune antibodies and inflammatory factors in serum, up-regulating DNMT1 expression and down-regulating NF-κB expression in renal tissue. These results prove that it is reliable and effective to use DHA to treat lupus-prone MRL/lpr mice and its therapeutic mechanisms should closely be related to the fact that DHA can decrease serum levels of autoimmune antibody and inflammatory factors and down-regulate expression of NF-κB p65 protein and up-regulate DNMT1 expression in renal tissues.

Keywords: Dihydroartemisinin, lupus-prone MRL/lpr mice, DNMT1, NF-κB p65

Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by various immunologic abnormalities. SLE is accompanied by excessive inflammatory responses in a wide range of organs [1].

Lupus nephritis (LN) is one of the major complications and causes of death in SLE. Hormones and immunosuppressive drugs are the main treatment options, but long-term use of such agents can cause severe adverse reactions [2].

The traditional Chinese medicine artemisinin has been used for malaria treatment. Recent studies have shown that artemisinin derivatives also have roles in SLE treatment [3, 4]. The artemisinin derivative SM934 can reduce production of auto-antinuclear antibodies, alleviate kidney damage and prolong the life of SLE mice [5, 6].

Dihydroartemisinin (DHA) is another semi-synthetic derivative of artemisinin. DHA can inhibit cell proliferation and induce apoptosis of cancer cells [7, 8], DHA also has been reported to possess an immunoregulatory effect on inflammation and autoimmune diseases [9-11].

Female lupus-prone MRL/lpr mice are commonly used models of SLE because their responses to SLE are similar to those of humans. They develop autoimmune syndromes spontaneously, such as LN, hematologic changes, massive lymphadenopathy, splenomegaly, and autoantibody formation. Among those, LN is the key factor that leads to death [12].

Here, female MRL/lpr mice were used to investigate the immunoregulatory effect of DHA.

Material and methods

Ethical approval of the study protocol

Experimental protocols were approved by the Ethics Committee of Laboratory Animal Care and Welfare within Zhejiang Chinese Medical University (Zhejiang, China). Animal experi-

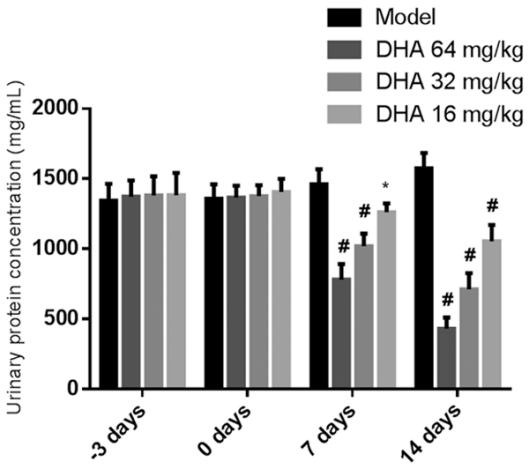

Figure 1. Effects of DHA on urinary protein in MRL/lpr mice. #$p < 0.0001$, *$p < 0.05$.

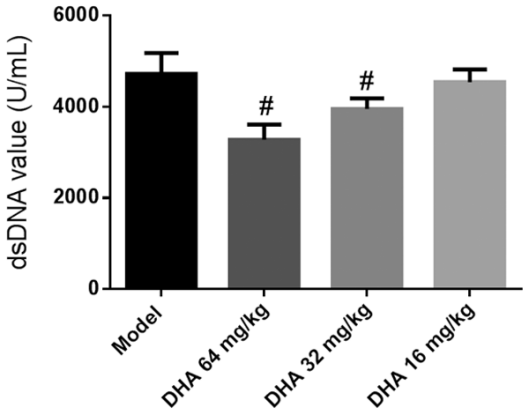

Figure 2. DHA decreased anti-dsDNA production. #$p < 0.0001$.

ments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA).

Animals and DHA treatment

Female lupus-prone MRL/lpr mouse strains were purchased from the Shanghai Laboratory Animal Center within the Chinese Academy of Sciences (Shanghai, China). Mice were maintained under specific pathogen-free conditions in accordance with institutional guidelines. Efforts were exerted to minimize the number of animals used as well as their suffering.

DHA was purchased from MedChem Express (Monmouth Junction, NJ, USA). For *in vitro* experiments, a stock solution of DHA (64 mg/ml) was prepared by reconstitution in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Saint Louis, MO, USA) and stored in aliquots at -20°C. Each day, a fresh stock solution was diluted in a mixture of corn oil and DMSO to a final concentration of 5% DMSO and 95% corn oil. Twenty-eight MRL/lpr mice divided into four groups (7 in DHA 64 mg/kg, 7 in DHA 32 mg/kg, 7 in DHA 16 mg/kg and 7 in the control group) were used for experimentation. MRL/lpr mice were treated for 14 days.

Evaluation of the therapeutic effect

By comparing the changes in urine protein, renal histopathology, and physical conditions among the four groups, the therapeutic effect of DHA on lupus-prone MRL/lpr mice was evaluated. From 3 days before administration to 14 days after administration, urine was collected from mice by bladder compression every morning. Urinary levels of protein were measured using the bicinchoninic acid (BCA) method. At the end of the experiment, part of the kidney cortex were harvested and fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin using a routine histology method.

Anti-double-stranded (ds) DNA antibody and measurement of inflammation factors

MRL/lpr mice were randomized to treatment with different concentration of DHA for 2 weeks to assess the impact of DHA on autoantibodies and inflammation factors. Sera were obtained from mice by retro-orbital bleed at the end of 2-week treatment. The serum concentration of anti-dsDNA antibody was measured using a mouse anti-dsDNA immunoglobulin (Ig) G enzyme-linked immunosorbent assay (ELISA) kit (lot number MDD-416; Shibayagi, Tokyo, Japan). The serum level of inflammation factors (tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, -2, -4, -10 and -17A, monocyte chemoattractant protein-1) were measured using a cytometric bead array (CBA) kit following manufacturer (BD Pharmingen, Franklin Lakes, NJ, USA) protocols. All samples were run in triplicate.

Protein extraction from kidney tissue

Part of the kidney tissue was harvested after mice had been killed, and homogenized with an

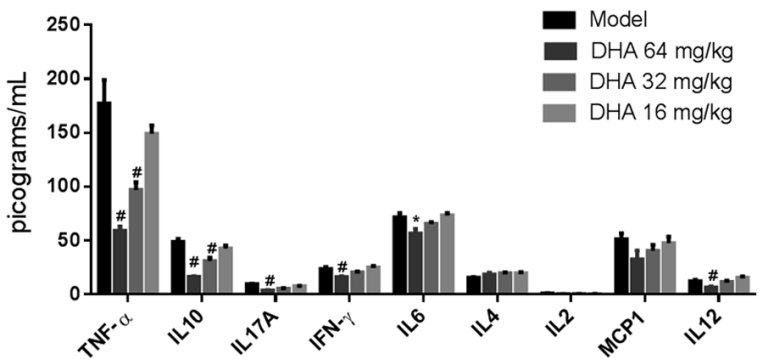

Figure 3. Serum levels of cytokines in MRL/lpr mice. #$p < 0.0001$, *$p < 0.05$.

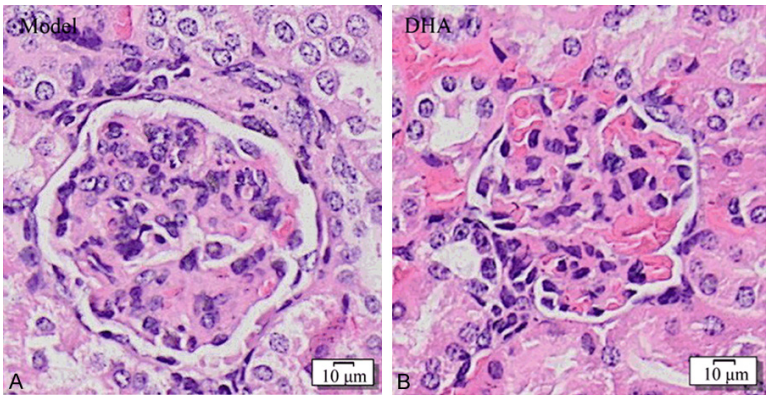

Figure 4. Renal pathologic changes (H&E staining, × 400). A. Model mice. B. Mice treated with DHA 64 mg/kg.

automatic homogenizer (Roche, Basel, Switzerland). Proteins were extracted with M-PER Mammalian Protein Extraction Reagent (78-503; Thermo Fisher Scientific, Waltham, MA, USA) containing protease and a phosphatase inhibitor for 30 minutes at 4°C. Then, samples were centrifuged at 14,000 × *g* for 10 minutes at room temperature. Supernatants were transferred to new tubes for analyses.

Automated western immunoblotting

Before blotting, protein was quantified using the bicinchoninic acid (BCA) method. Western immunoblotting was done on a Peggy Sue system (ProteinSimple, San Jose, CA, USA) using a Size Separation Master kit with Split Buffer (12-230 kDa) according to manufacturer instructions and using anti-nuclear factor-kappa B (NF-κB) p65 (ab32536; Abcam, Cambridge, UK), anti-DNA (cytosine-5)-methyltransferase1 (DNMT1; 5032S; Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin (4970S; Cell Signaling Technology) antibodies. Compass v2.7.1 (ProteinSimple) was used to program the Peggy Sue system as well as for presentation (and quantification) of western immunoblots. Output data were displayed from the software calculated from an average of seven exposures (5-480 s).

Statistical analysis

Data are the mean ± SEM from at least three sets of samples. Differences between groups were evaluated with one-way analysis of variance (ANOVA), followed by the Newman-Keuls test or Student's *t*-test. Statistical analyses were undertaken using Prism 5.0 (GraphPad, San Diego, CA, USA). $p < 0.05$ was considered significant.

Results

DHA reduced urinary protein content in MRL/lpr mice

Urinary protein content of MRL/lpr mice from 3 days before administration to 14 days after administration was determined. The urinary protein content of MRL/lpr mice was very high before DHA administration, but after administration, it began to decrease. After treatment with DHA for 7 days, the urinary protein content in MRL/lpr mice decreased significantly (**Figure 1**).

DHA decreased autoimmunity in MRL/lpr mice

To ascertain the effect of DHA on autoimmunity, MRL/lpr mice were randomized to treatment with DHA or vehicle. Retro-orbital bleeds enabled measurement of mouse anti-dsDNA IgG-specific antibodies in sera by quantitative ELISA. Mice treated with DHA showed a significant decrease in mean antibody levels compared with model mice ($p < 0.0001$; ANOVA) (**Figure 2**). These results demonstrated that DHA treatment reduced anti-dsDNA levels effectively in MRL/lpr mice.

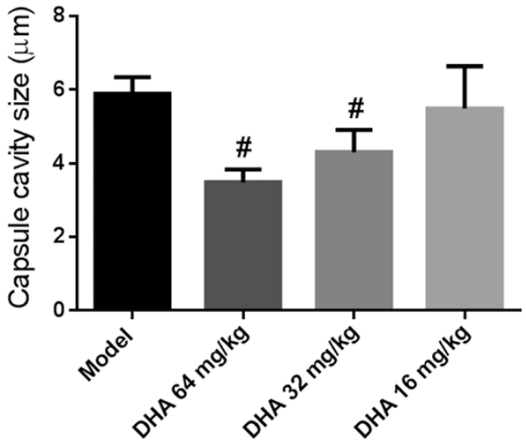

Figure 5. Changes in capsule cavity size. #p < 0.0001.

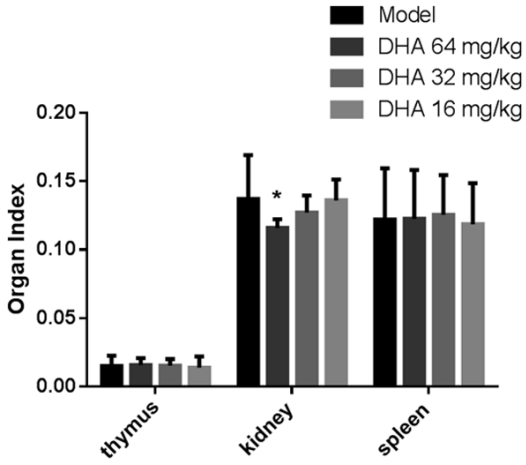

Figure 6. Organ Index. *p < 0.05.

DHA decreased serum levels of cytokines in MRL/lpr mice

Serum levels of cytokines in MRL/lpr mice were measured by the CBA method. Levels of TNF-α, IL-10, IL-17A, IFN-γ, and IL-12 decreased significantly in the high concentration of DHA-treated group (p < 0.0001) compared with those in the model group. TNF-α and IL-10 also decreased significantly in the middle concentration of DHA-treated group (p < 0.0001) compared with those in the model group. IL-6 levels decreased, to a lesser extent, in the high concentration of DHA-treated group compared with the model group (p < 0. 05) (**Figure 3**).

DHA relieved renal pathologic changes

The chief pathologic manifestation of MRL/lpr mice was atrophy in some glomeruli. This was seen mainly as narrowing of the capillary lumen, varying degrees of asymmetric mesangial proliferation, and enlargement of the mesangial region (the width of the glomerular mesangial region was larger than the diameter of the capillary). DHA-treated mice had a smaller width of the glomerular mesangial region and a relatively wider or more open capillary lumen than model mice. The capsule cavity size of model mice was wider than that of DHA-treated mice (**Figures 4** and **5**).

There was no difference between the Thymus Index and Spleen Index (**Figure 6**), but the Kidney Index of the model group was significantly higher than that of the DHA-treatment group. These results suggest that DHA had a greater impact on the kidney than the other organs tested.

DHA treatment affected expression of NF-κB p65 and DNMT1 proteins in the kidney tissue of MRL/lpr mice

To understand the mechanism of action of the therapeutic effect of DHA on MRL/lpr mice, expression of NF-κB and DNMT1 protein were measured by Western blotting. Expression of NF-κB p65 protein was down-regulated and that of DNMT1 protein was up-regulated in renal tissue after DHA treatment (**Figure 7**).

Disscusion

SLE is a chronic autoimmune disease that leads to impairment of multiple systems caused by abnormal immune function. The urinary protein content, anti-dsDNA antibody and cytokines content in MRL/lpr mice were very high, but the indexes of the DHA administration group decreased in varying degrees (**Figures 1**-**3**). DHA has little effect on thymus and spleen of MRL/lpr mice, but it can improve the kidney index (**Figures 4**-**6**).

NF-κB is an important transcription factor that mediates signal transmission between cells. NF-κB protein participates in the information-transmission process of the defense response, tissue injury and stress, cell differentiation and apoptosis, and inhibition of tumor growth. Zheng and colleagues [13, 14] found that that the severity of proteinuria in LN and non-proliferative glomerulopathy was positively correlated with the podocyte-staining scores of NF-κB p65. Dong and co-workers [15, 16] demon-

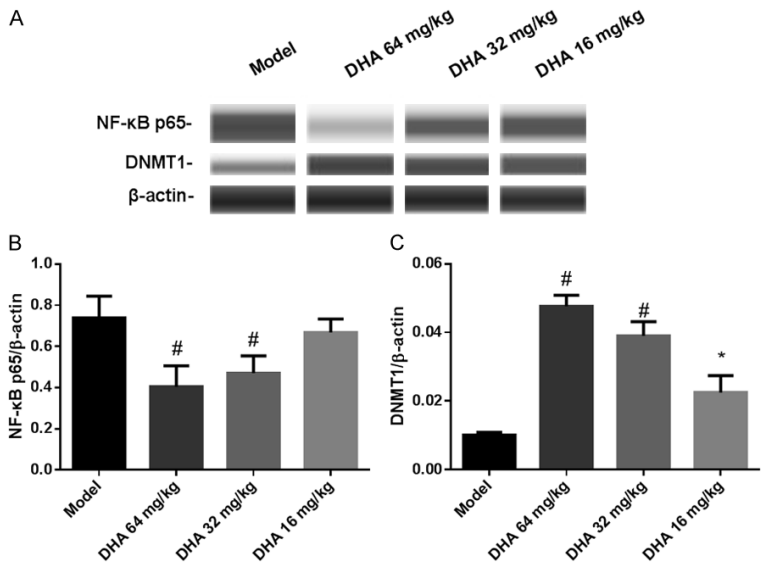

Figure 7. Expression of NF-κB p65 and DNMT1 proteins in kidney tissue. *$p < 0.05$, #$p < 0.0001$.

strated that DHA can inhibit production of anti-dsDNA antibodies, reduce expression of NF-κB p65 protein, and improve the pathologic lesions of LN in BXSB mice, and these are consistent with our results.

Hypomethylation of genomic DNA is common in the cluster of differentiation (CD)4^+ T cells of SLE patients, which is related to abnormal expression of methylation-regulatory genes in SLE patients [17]. DNMT1 is the most important factor in transformation of DNA methylation. This binding rate-limiting enzyme maintains the methylation level of genomic DNA, and its activity and concentration are correlated positively with the methylation level. In SLE patients, the DNMT1 concentration and enzyme activity decrease significantly, resulting in low methylation of genomic DNA [18].

Expression of DNMT1 protein and NF-κB protein in the kidney tissue of mice treated with DHA was analyzed in our study. Expression of DNMT1 in the kidney tissue of mice treated with DHA was significantly higher than that of the model group, whereas expression of NF-κB protein was significantly lower (**Figure 7**). Hence, DHA alleviated the symptoms of lupus-prone MRL/lpr mice by reducing production of autoimmune antibodies and inflammatory factors, up-regulating DNMT1 expression and down-regulating NF-κB expression.

This study proves that the use of DHA in lupus mice is effective and reliable, and its corresponding therapeutic mechanisms should be related to the fact that DHA can alleviate the symptoms by decreasing the serum level of cytokines, such as TNF-α and IL-10, and inhibit expression of NF-κB p65 protein in renal tissue. Furthermore, the use of DHA promotes expression of DNMT1, which shows that it also plays a role in epigenetic regulation. This conclusion offers not only a theoretical basis for the clinical application of DHA but also a new therapeutic method for SLE.

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Disclosure of conflict of interest

None.

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