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Original Article

Hydrochloroquino sulfate liposomes can significantly improve the treatment of lupus nephritis

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Abstract: To investigate the therapeutic effect of hydroxychloroquine sulfate liposome on lupus nephritis mice model, the lupus nephritis mice model was established by intravenously injecting female parent DBA/2 lymphocytes to female offspring F1 hybrid mice for four times. The mice were randomly divided into the control group (physiological saline) and treatment group. The mice in treatment group were divided into hydroxychloroquine sulfate liposomes group and hydroxychloroquine sulfate Active Pharmaceutical Ingredients (APIs) treatment group. The urine protein and TNF-like apoptosis weak inducer (TWEAK) were determined after treated for 2 w, 4 w, 8 w and 10 w. The kidneys from 5 mice were randomly selected and made into slices after treatment. The renal pathological changes were observed by HE staining. The levels of urinary protein and TWEAK in the control group were significantly higher than those of treatment group. The level of urine protein in hydroxychloroquine sulfate APIs treatment group was significantly higher than that of hydroxychloroquine sulfate liposome treatment group. The level of TWEAK showed no significant difference between sulfate hydroxychloroquine APIs treatment group and hydroxychloroquine sulfate lipidosome treatment group. Hydroxychloroquine sulfate can effectively alleviate renal pathological changes. Hydroxychloroquine sulfate liposome can reduce the levels of urine protein and TWEAK in lupus nephritis mice and reduce the degree of renal damage.

Keywords: Lupus nephritis, hydroxychloroquine sulfate liposome, urinary protein

Introduction

Lupus nephritis (LN) is an immune complex nephritis in which the kidney is involved and is caused by systemic lupus erythematosus. Its incidence is mainly related to immune complex formation, immune cells and cytokines abnormalities [1, 2]. The main clinical manifestations include hematuria, proteinuria and renal insufficiency in addition to systemic lupus erythematosus (SLE) manifestations. The pathological classification of lupus nephritis is of great value for judging the disease activity, prognosis and formulating the treatment plan. Hydroxychloroquine is an antimalarial drug composed of a 4-aminoquinoline compound [3]. Hydroxychloroquine sulfate was synthesized by Surrey and Hammer in 1946 [4]. Hydroxychloroquine could prevent the damage of SLE and had a protective effect on the clinical manifestations and drug complications that are caused by SLE [5]. Belizna studied [6] the function of hydroxychloroquine, advising that hydroxychloroquine could be used as a basic drug to treat SLE, which made hydroxychloroquine increasingly receive attention. Hydroxychloroquine sulfate is widely used in treating SLE, rheumatoid arthritis and other diseases. Liposomes, composed of phospholipids and cholesterol, as a good drug carrier, can not only enclose water-soluble drugs, but also contain liposoluble drugs.

The liposome drug has many advantages, including a targeting ability, a slow release feature, cell affinity and histocompatibility. Since its advent, it has been a research hot topic for medical workers. The commercially available hydroxychloroquine sulfate is a solid tablet [7, 8]. Hydroxychloroquine sulfate was prepared into a liposome encapsulated liquid phase drug

in the study. The therapeutic effects were compared between the hydroxychloroquine sulfate liposome and hydroxychloroquine sulfate APIs.

Materials and methods

Animals

The research subjects, including 10 two-month-old female DBA/2 mice, and 30 1~1.5-month-old male and female F1 hybrid mice (C57BL6/DBA2), were purchased from the Shanghai experimental animal center.

Reagent and instrument

Hydroxychloroquine sulfate (Shanghai Center for Pharmaceutical Co., LTD), DOPC (Avanti), cholesterol and dichloromethane (Tianjin Bodi Chemical Co., LTD), a urine protein ELISA Kit and a TWEAK ELISA Kit (Enzyme-linked Biological Technology Co., LTD., Shanghai), and HE staining Kit (Shanghai Mr Biotechnology Co., LTD), rotary evaporators (Shanghai Rong Biochemical Instrument Factory), and a cell ultrasonic grinding device (Ningbo New Art Ultrasonic Equipment Co., LTD) were used.

Establishment of animal model

According to female progeny F1 hybrid mice, female parent DBA/2 lymphocytes were intravenously injected into female progeny F1 hybrid mice. The concentration of lymphocytes was 1×10^7 cells/mL. The injection dose was 100 μL. The injection time was 1, 3, 5 and 7 d. After the model was established, the mice were randomly divided into 3 groups: 10 mice in the control group (normal saline) and 20 mice in the treatment groups (the hydroxychloroquine sulfate liposome treatment group and the hydroxychloroquine sulfate APIs treatment group). There were a total of 30 mice.

Preparation of hydroxychloroquine sulfate liposome

The oil phase was formed by weighing and dissolving 4 mL of DOPC and cholesterol in dichloromethane. Hydroxychloroquine sulfate tablets were ground into a powder and dissolved in PBS water. The aqueous phase was poured into the oil phase under ultrasound conditions. The sample was moved into a pear-shaped bottle for rotary evaporation (42°C) after complete emulsification, to remove the organic solvent dichloromethane. Hydroxychloroquine sulfate liposome was successfully prepared. The size, electric potential and distribution of long-circulating nano-liposomes was detected using transmission electron microscopy (TEM) and a laser particle analyzer/Zet.

Drug treatment

The drug was administered by intraperitoneal injection after the animal model was stable. The dosage of hydroxychloroquine sulfate was 0.4 g according to the adult oral dosage given 1-2 times qd. The drug was injected according to the body weight proportion. Hydroxychloroquine sulfate and hydroxychloroquine sulfate liposomes were injected into mice in the experimental group. The concentration of hydroxychloroquine sulfate was 10 mg/ml; 0.1 mL was administered every time. In the control group mice, 0.1 mL of physiological saline was injected. The treatment lasted for 10 w. The therapeutic effects of hydroxychloroquine sulfate tablets APIs and hydroxychloroquine sulfate liposome were compared.

Samples collection and detection

The urine of mice in all groups was collected after treatment for 2 w, 4 w, 8 w and 10 w. The urine protein was detected using the Coomassie brilliant blue method; 0.5 mL of peripheral blood was collected. The serum was centrifuged and separated. TWEAK was detected using ELISA. The kidneys from 5 randomly selected rats were removed, fixed in formalin, embedded in paraffin and cut into sections. The renal pathological changes were observed using HE staining.

Statistical methods

The single factor analysis of variance among multiple groups was performed using SPSS 19 statistical software. Measurement data was presented as mean ± standard deviation and compared by t test. $P<0.05$ was depicted as significant difference.

Results

Physical characterization of hydroxychloroquine sulfate lipidosome

The particle size of hydroxychloroquine sulfate liposome was observed using TEM. The particle

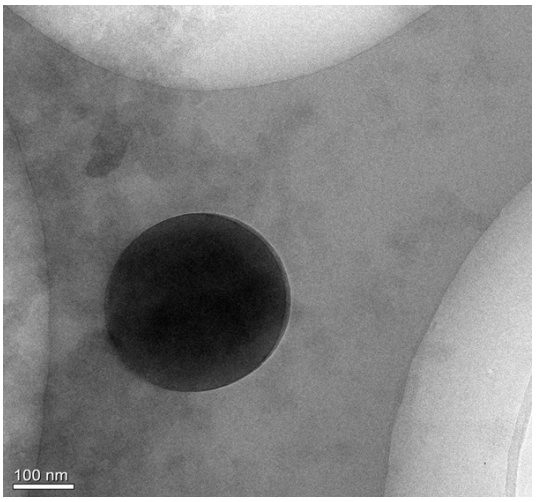

Figure 1. Particle size of hydroxychloroquine sulfate liposomes.

size was approximately 200 nm. The shape was basically spherical (**Figure 1**). The potential of hydroxychloroquine sulfate liposome was detected by the potential instrument; its potential was -68.3 mV (**Figure 2**).

Detection of urine protein

The urine protein content showed less difference between the treatment group and the control group after drug treatment for 2 weeks. The urine protein content in the drug treatment groups (the hydroxychloroquine sulfate APIs treatment group and the hydroxychloroquine sulfate liposome treatment group) were significantly lower than that of the control group after treatment for 4 weeks ($P<0.05$). The urinary protein contents also showed a significant difference between the two drug treatment groups ($P<0.05$). The urinary protein in the hydroxychloroquine sulfate liposome treatment group was significantly lower than that of the hydroxychloroquine sulfate APIs treatment group (**Table 1**). This suggests that to inject liposome encapsulated hydroxychloroquine sulfate could improve the therapeutic effect of hydroxychloroquine sulfate.

Detection of TWEAK

The ELISA result showed that the level of TWEAK showed no difference between the treatment group and control group after treatment for 2 w~8 w. The levels of TWEAK in the drug treatment groups (the hydroxychloroquine sulfate APIs treatment group and the hydroxychloroquine sulfate liposome treatment group) were significantly lower than those of the control group after treatment for 10 w ($P<0.05$), but there was no significant difference between the two drug treatment groups ($P>0.05$) (**Table 2**). This fact suggests that hydroxychloroquine sulfate can effectively reduce the level of TWEAK in LN rats and alleviate the inflammatory reaction of rats. Meanwhile, TWEAK might have a correlation with the pathogenesis of LN.

HE staining results

The HE staining results of kidney tissue were observed using a biological microscope (**Figure 3**). The results showed that moderate hyperplasia of mesangial cells in mice in the control group had significantly increased stroma with neutrophil infiltration, vascular stenosis, occlusions and a subtly thickened basement membrane. Part of the glomeruli was lobulated. The interstitial inflammatory cell infiltration was obvious. The fibrosis formed, and part of the renal tubular was atrophic (**Figure 3A**). The lesion of the hydroxychloroquine sulfate liposome treatment group was the mildest. The mesangial cells were mildly proliferated. The matrix was increased slightly. There were a few inflammatory cells that infiltrated the mesenchyma. The renal tubular cells showed no obvious fibrosis (**Figure 3C**). The lesion of the hydroxychloroquine sulfate drug treatment group was between the control group and the hydroxychloroquine sulfate liposome treatment group (**Figure 3B**).

Discussion

Hydroxychloroquine sulfate is a widely used drug to treat SLE, rheumatoid disease, antiphospholipid antibody syndrome and other diseases, and it has a certain curative effect [9-12]. Although hydroxychloroquine sulfate has a definite effect in the treatment of SLE, rheumatoid arthritis and other diseases, it also has many side effects. For example, it can cause central nervous system excitement, muscle response, oculoreaction and cutaneous reaction. Liposome is a good drug carrier; it can slowly release the drug with a high concentration and reduce the side effects of the drug.

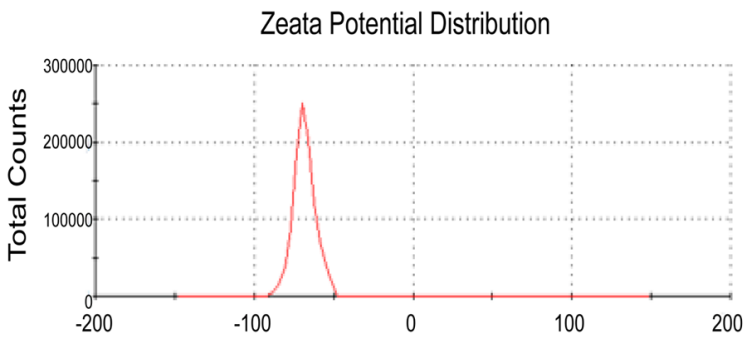

Figure 2. Potential of hydroxychloroquine sulfate liposomes.

Additionally, it can control the distribution of the drug by changing the size and charge of the liposome. Based on this, hydroxychloroquine sulfate was selected as the drug in the experiment. Hydroxychloroquine sulfate tablets were prepared into liposome encapsulated microspheres using a new method. The therapeutic effects of different forms of hydroxychloroquine sulfate drug on LN were compared in order to seek the most effective drug treatment form.

Urinary protein is one of the clinical characteristics of LN, and it is an important index for the occurrence and development of renal lesions [13, 14]. A persistent large amount of proteinuria is a marker of poor renal prognosis and has a correlation with the pathological changes in glomerular mesangial proliferation and renal tubular cell fibrosis [15-17]. The urine protein concentrations were detected at various times in this experiment. The result showed that the level of urinary protein continued to increase in the control group and reached 723.7±26.88 μg/mL by the end of the treatment. However, the level of urinary protein in the treatment group was significantly reduced compared to that of the control group; the hydroxychloroquine sulfate APIs treatment group's concentration was 325.17±31.78 μg/mL, whereas the hydroxychloroquine sulfate liposome treatment group's concentration was 292.7±30.58 μg/mL. This indicated that hydroxychloroquine sulfate could effectively treat LN, which was in accordance with the literature. Moreover, the therapeutic effect of the hydroxychloroquine sulfate preparing liposome encapsulated drug was significantly improved, which may provide the basis to develop a treatment drug for LN.

TWEAK is a new member of the tumor necrosis factor family [18, 19]. It plays a role in inducing cell apoptosis, increasing cell invasion and inducing the expression of inflammatory factors after combining with receptor fibroblast growth factor 14 (Fn14). Recent reports showed that TWEAK was correlated with LN. For example, Schwartz et al. reported that the level of TWEAK in LN patients was significantly higher than those of non-LN patients and normal people; Mnoharan [20] reported that the level of TWEAK had a great significance in clinical changes of LN patients. However, our study showed that the level of TWEAK in LN patients was lower than that of the normal group. The reason may be related to the differences in the experimental sample phenotypes, the sizes and the research methods. The levels of TWEAK in the control group and the treatment group were detected at various times in this experiment. The results showed that there was no significant difference in the level of TWEAK between the control group and control group. By the end of treatment (10 w), the level of TWEAK in the control group (2.96±0.18) was significantly higher than that of treatment group (2.05±0.21, 2.01±0.19). This further indicated that the level of TWEAK was correlated with the clinical symptoms of LN.

LN is the most commonly severe visceral damage of SLE and is the main cause of death. Studies have shown that renal tissue biopsies have shown renal involvement can be increased by up to 90% after SLD has lasted for 2 years [21]. Therefore, the prompt diagnosis of LN has important significance for improving the survival of SLE patients, improving the prognosis and so on. In this experiment, the renal involvement of LN rats was assessed by renal pathological observation. The results showed that the kidney of LN rats in the control group was significantly involved. The glomerular mesenterium was proliferated. The renal tubular was atrophic or occlusive. The interstitial inflammatory cells were obvious. Compared with the control group, renal involvement was reduced significantly in the treatment group. The lesion of the hydroxychloroquine sulfate liposome treatment group was the most extreme. The mesenterium was mildly hyperplastic. There was no obvious fibrosis in the renal tubular cells. There were a few

Table 1. Quantitative determination of urine protein in each group ($\bar{x}\pm s$)

Group	Content of urinary protein (μg/mL)			
	2 w	4 w	8 w	10 w
Control group (normal saline)	195.75±36.78	280.15±36.18	710.75±31.74	723.7±26.88
Treatment group (Hydroxychloroquine sulfate)	189.55±39.21	241.75±35.53	330.55±34.72	325.17±31.78
Treatment group (Hydroxychloroquine sulfate liposomes)	186.23±42.11	210.75±36.78	299.24±36.01	292.7±30.58

Table 2. Quantitative determination of TWEAK in each group ($\bar{x}\pm s$)

Group	OD value of TWEAK			
	2 w	4 w	8 w	10 w
Control group (normal saline)	1.66±0.17	1.95±0.19	2.36±0.23	2.96±0.18
Treatment group (Hydroxychloroquine sulfate)	1.53±0.21	1.89±0.30	1.95±0.24	2.15±0.21
Treatment group (Hydroxychloroquine sulfate liposomes)	1.49±0.11	1.90±0.22	1.80±0.23	2.0 9±0.19

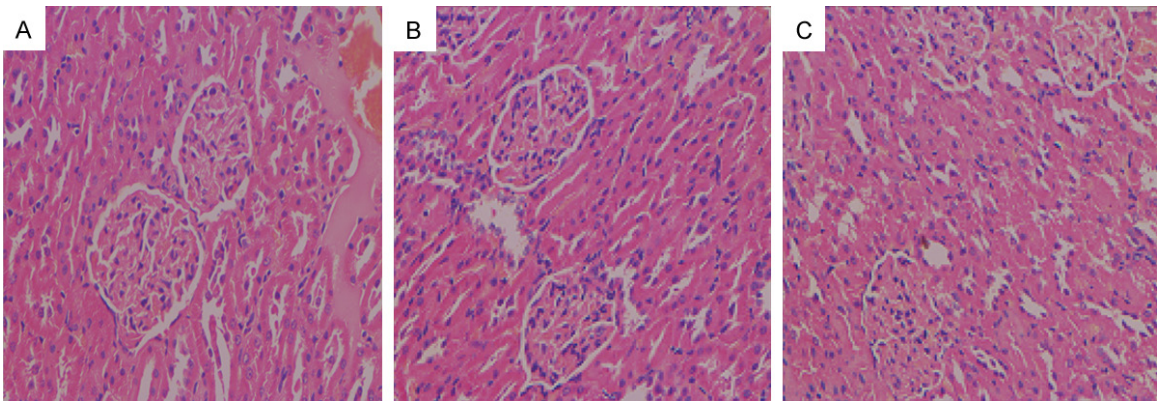

Figure 3. HE staining results of kidney tissue. A. Control group. B. Teatment group of Hydroxychloroquine sulfate. C. Teatment group of Hydroxychloroquine sulfate liposomes.

interstitial inflammatory cell infiltrations. In summary, the hydroxychloroquine sulfate liposome can effectively alleviate LN clinical symptoms, reduce urine protein and TWEAK in LN animals, inhibit the inflammatory response in the body and reduce the damage to the kidney, which may provide a basis for the research and development of new drug carriers.

Disclosure of conflict of interest

None.

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