

Research Article

Folic Acid Modified Glucosamine/Methotrexate Polymer Targeted Therapy for Rheumatoid Arthritis

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Objective. To develop a targeted drug for the treatment of rheumatoid arthritis. **Methods.** In this study, methotrexate (MTX) was combined with glucosamine (Glu) and modified with folic acid (FA) to prepare folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX). The stability, sustained release, cytotoxicity, and therapeutic effect were evaluated based on in vitro and in vivo experiments. **Results.** FA-Glu/MTX had good stability, sustained release, and low cytotoxicity. In addition, the results of in vivo experiments proved that compared with MTX, FA-Glu/MTX has a stronger therapeutic effect on RA and has certain targeting properties. **Conclusion.** FA-Glu/MTX is expected to become a new option for RA treatment.

1. Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease characterized by synovitis and erosive arthritis [1]. Most of them occur in the knee joint, wrist joint, elbow joint, ankle joint, and so on. The clinical manifestations are joint swelling, morning stiffness, pain, and dyskinesia [2]. If cannot be treated in time, it is easy to cause joint erosion, deformity, and loss of function, in addition, in addition to joint abnormalities, there will be anemia, vasculitis, myocarditis, ischemic neuropathy, scleritis, and other systemic complications, but also the main cause of disability [3–5]. At present, the research results of RA show that it is still a kind of difficult disease and cannot achieve the effect of a radical cure [6]. Therefore, the treatment of RA mainly lies in relieving joint pain, reducing inflammatory reaction, controlling the progression of the disease, protecting joints, and preventing deterioration as far as possible.

At present, the treatment of RA can be divided into six kinds, namely, nonsteroidal anti-inflammatory drugs (NSAIDs), antirheumatic drugs (DMARDs), glucocorticoid drugs (GCs), biological agents, targeted preparations, and plant Chinese herbal medicine [7, 8]. NSAID drugs are the most common in the clinic, mainly by inhibiting the synthe-

sis of COX-2 in inflammatory sites, and it is easy to reduce the occurrence of inflammatory reaction, alleviate the disease, and improve the effect of RA [9]. However, it will lead to cardiovascular, liver, kidney, nervous system, digestive system, and other complications [10]. The main role of DMARDs is to reduce the joint imaging injury of patients, to delay the development of the disease. Because the efficacy of DMARD drugs is slow, long-term treatment is usually needed to improve the clinical symptoms of RA [11, 12]. However, DMARD drugs also have adverse reactions such as dementia, liver and kidney injury, myelosuppression, and digestive tract reactions [13]. GCs are mainly used to treat RA through immunosuppression and are usually used in combination with DMARD drugs. However, the side effects of GCs are serious, which can easily lead to osteonecrosis and atherosclerosis, and have the possibility of recurrence after drug withdrawal [14]. According to the mechanism of action, biological agents can be divided into TNF inhibitors, IL-6 receptor monoclonal antibodies, T cell blockers, B cell depletion agents, and so on. It has a blocking effect on specific cellular inflammatory factors and has a stronger therapeutic effect than other therapeutic drugs. But at the same time, it can also cause serious toxic negative effects, such as tuberculosis, rash, and demyelination

syndrome [15–17]. Targeted inhibitors can treat RA by targeting inhibition of the JAK/STAT signal pathway and controlling the signal transduction of growth factors and cytokines. At present, there are few studies on it, and it is an effective way to treat RA in the future [18, 19]. Sinomenine and other plants Chinese herbal medicine have been found to inhibit RA, which is the main treatment of traditional Chinese medicine at present, which can effectively relieve the pain and swelling caused by RA, but also has the advantages of low price and less adverse reactions, so it is the focus of clinical research [20, 21].

Methotrexate (MTX) is a kind of DMARDs, which can effectively reduce RA-induced inflammatory factors and soft tissue contusion by inhibiting the production of dihydrofolate reductase and promoting the synthesis of thymine synthase to reduce the production of inflammatory factors. As a kind of DMARD drug, MTX has the effects of long-lasting, reliable, low cost, and strong effect, but it has low targeting, short half-life, and strict dosage, so it will produce adverse reactions such as neurotoxicity, hepatorenal toxicity, and myelosuppression when used in large quantities. Therefore, how to improve the targeting and biosafety of MTX is a difficult problem in current research [22, 23].

Glucosamine (Glu) is a natural substance found in human articular cartilage and a kind of amino monosaccharide. At present, it is widely used in the treatment of RA, because it can inhibit Th1 cells and induce Th2 cell response, so it has immunomodulatory and anti-inflammatory effects. At the same time, Glu can effectively relieve the swelling and pain caused by RA to improve the condition [24]. Folic acid (FA) is a vitamin widely found in green vegetables. It is an important coenzyme in human cell metabolism and plays an important role in human metabolism. And because the use of MTX will reduce the synthesis of folic acid in the body, the combined use of FA and MTX can effectively reduce the side effects caused by MTX.

Nanomedicine has the advantages of small particle size and large specific surface area. It can effectively penetrate into the joints and improve the inflammatory symptoms of patients with RA. At the same time, it can target to improve the damaged joint tissue. It is a new type of drug for the treatment of RA [25, 26]. Synthesis of MTX into nanopolymers and modified with folic acid can target MTX to be released at the RA lesions and improve the drug utilization of MTX. At the same time, the addition of Glu can enhance the therapeutic effect of MTX on RA and reduce the occurrence of side effects.

Based on this, this study adopts the method of combined treatment of MTX and Glu and modifies it with FA to obtain FA-Glu/MTX polymer, in order to prepare a targeted therapy for RA that integrates biological safety, high efficiency, and stability. Drugs provide a new way for the treatment of RA.

2. Materials and Methods

2.1. Reagent. FA, MTX, Glu, triethylamine, DMSO, dicyclohexylcarbodiimide, N-hydroxysuccinimide, triphenyl phos-

phate, phenobarbital sodium, dimethyl sulfoxide (MTT), type II collagen, and complete Freund's adjuvant were purchased from Shanghai Tongwei Biotechnology Co., Ltd. (Shanghai, China). Acetone, ether, acetic acid, and sodium acetate are provided by Shandong Xuchen Chemical Co., Ltd. (Shandong, China). Fetal bovine serum, DMEM medium, IL-1 β , and TNF- α ELISA kits were purchased from Sichuan Weikeyi Biotechnology Co., Ltd. (Sichuan, China).

2.2. Instrument. MS-IIIS intelligent digital display magnetic agitator and C1650R-230V microhigh-speed freezing centrifuge were purchased from Beijing Leiputer Scientific instrument Co., Ltd. (Beijing, China). Zetasizer WT potential measuring instrument is purchased from Malvern Panalytical instrument Co., Ltd. (Malvern, UK). Hitachi High-Resolution Cold Field Emission Scanning Electron Microscopy (SU9000), Hitachi Ultraviolet Spectrophotometer (U3900), Hitachi Ultraviolet Spectrophotometer (U3900), and Hitachi Chromaster Series High-performance liquid Chromatography (HPLC) are provided by Hitachi Company (Tokyo, Japan). Rywald gasket carbon dioxide incubator D180Murf was purchased from Shenzhen Ryward Life Technology Co., Ltd. (Guangzhou, China). Ausheng FlexA-200 full-wavelength enzyme label analyzer was purchased from Hangzhou Ausheng instrument Co., Ltd. (Zhejiang, China).

Human bone marrow mesenchymal stem cells (BMSCs cells) were purchased from Shanghai Binsui Biotechnology Co., Ltd. (Shanghai, China). Wistar rats were produced by Beijing Huafukang Biotechnology Co., Ltd. (Beijing, China). All animal-related experiments involved in this study are in line with international ethical standards.

2.3. Preparation and Characterization of FA-Glu/MTX

2.3.1. Preparation of FA Active Ester. 1.5 g of FA was accurately weighed and added to 30 mL DMSO containing 2.0 mL triethylamine. After mixing evenly, excess dicyclohexyl carbodiimide and N-hydroxysuccinimide were added to avoid light in the dark for 24 h, and the side reactants were filtered at the end of the reaction. Under the condition of continuous stirring, the ether solution containing 30% acetone was dripped, and the whole reaction was carried out in an ice bath until yellow precipitation appeared. The product was filtered and washed to obtain FA active ester.

2.3.2. Preparation of FA-Glu. Take an appropriate amount of Glu in the acetic acid-sodium acetate buffer solution of pH = 4.7 (0.5 wt %), add the same amount of FA active ester solution under the condition of magnetic stirring, react at 30°C for 15 h, and adjust the pH value to 9.0. The filtered precipitate was dialyzed with PBS, purified, and lyophilized to obtain FA-Glu.

2.3.3. Preparation and Characterization of FA-Glu/MTX. The prepared FA-Glu powder was dissolved in acetic acid solution (the concentration was 2.0 mg/mL), and the pH value was adjusted to 9.0. The MTX of DMSO solution with a concentration of 10 mg/mL was added to the FA-Glu acetic

acid solution under the condition of magnetic stirring. After dissolving completely, FA-Glu/MTX nanoparticles were obtained by adding triphenyl phosphate solution with a concentration of 4.0 mg/L, stirring for 1.0 h, centrifugation, and drying. The morphology was characterized by Hitachi high-resolution cold field emission scanning electron microscope (SU9000), and the particle size and Zeta potential were measured by Zetasizer WT potential meter.

2.4. Performance Investigation of FA-Glu/MTX

2.4.1. Investigation on the Stability of FA-Glu/MTX. FA-Glu/MTX nanoparticles were resuspended and dispersed in PBS buffer solutions of different pH and stored at room temperature. The absorbance at 500 nm was measured by ultraviolet spectrophotometer at 0.1, 0.5, 1, 2, 4, 6, 8, 10, 12, and 14 days, respectively. According to formula (1), the light transmittance of FA-Glu/MTX nanoparticles at different pH values and different times was calculated. Among them, the light transmittance in pure water is recorded as T_0 , and the light transmittance in buffer solution is recorded as T_1 . The relative light transmittance is calculated according to formula (2).

$$A = -\lg T, \quad (1)$$

$$\text{Relative light transmittance}(\%) = \frac{T_1}{T_0} \times 100\%. \quad (2)$$

2.4.2. Study on Drug Release Ability of FA-Glu/MTX In Vitro. The in vitro drug release ability of FA-Glu/MTX nanoparticles was assayed by dialysis. Equal amounts of MTX and FA-Glu/MTX nanoparticles were weighed in dialysis bags and placed in a PBS buffer solution at pH 6.8. The MTX content in the PBS buffer solution was measured from 0–108 h, respectively.

2.5. Determination of Cytotoxicity of FA-Glu/MTX. BMSCs cells were cultured in a DMEM medium containing 10% fetal bovine serum, the dimension of constant temperature incubator was 37°C, and the gas environment was mixed air containing 5.0% CO₂. FA-Glu/MTX nanoparticles were dissolved in DMSO, as FA-Glu/MTX group. BMSCs cells were treated with DMSO for 24, 48, and 96 h, respectively, and MTT solution of 5.0 mg/mL was added. After 4.0 h of culture, DMSO solution was added. The absorbance was measured at 490 nm (A_1 in FA-Glu/MTX nanoparticles group and A_0 in control group), and the cell proliferation rate was calculated according to the following formula.

$$\text{Cell proliferation rate}(\%) = \frac{A_1}{A_0} \times 100\%. \quad (3)$$

2.6. Therapeutic Effect of FA-Glu/MTX on RA

2.6.1. Establishment of RA Rat Model. The therapeutic effect of FA-Glu/MTX nanoparticles on RA was studied by the collagen-induced RA rat model (CIA). In this study, SPF grade Wistar rats were used to study. 60 rats were randomly divided into four groups. Each rat was anesthetized with 1%

phenobarbital sodium. The hair on the back was removed. Except for the control group, 1.0 mL of normal saline was injected into the control group, and 1.0 mg/mL of type II collagen acetic acid solution and 1.0 mL of complete Freund's adjuvant emulsion were subcutaneously injected into the other groups. One week after the first injection, 0.5 mL was injected intraperitoneally again for strengthening.

2.6.2. Treatment and Observation of RA Rats. After the establishment of the model, the rats in the four groups were injected into the tail vein. Among them, the MTX group was treated with 0.5 mg/mL MTX, FA-Glu/MTX group, while the control group and model group were given PBS buffer of the same concentration. The injection was repeated 7 days later to enhance the therapeutic effect. Its therapeutic mechanism is for RA, such as Figure 1.

The body weight of rats in each group was measured on the 0th, 3rd, 7th, 10th, 14th, 17th, 21st, 24th, 28th, 31st, 35th, 38th, and 42nd day after model administration, and the foot swelling value of each group was measured with Vernier caliper every week.

2.6.3. Comparison of the Release of MTX in Inflammatory Sites. In order to evaluate the targeting effect of FA-Glu/MTX nanoparticles, the drug release of MTX in inflammatory sites of rats in each group was studied on the second day after repeated injection. Five rats in each group were randomly selected. After anesthesia and dissection, the tissue fluid of the inflammatory site was absorbed, and the content of MTX in inflammatory tissue fluid of rats in each group was detected by HPLC.

2.6.4. Effect of FA-Glu/MTX on Immune Organ Index of RA Rats. Spleen and thymus index was used to evaluate the effect of FA-Glu/MTX on immune organs of RA rats. On the 42nd day after administration, after weighing, and measuring the degree of foot swelling, the rats were anesthetized and blood samples were taken from the abdominal aorta. After taking blood, the spleen and thymus of rats in each group were washed with normal saline, dried, and weighed as (W_1), and the weight of the rats was recorded as W . The indexes of spleen and thymus of rats in each group were calculated according to the following formula.

$$\text{Immune organ index} = \frac{W_{\text{Immune organ weight}}}{W_{\text{Rat weight}}}. \quad (4)$$

2.6.5. Effect of FA-Glu/MTX on Inflammatory Cytokines. The blood of rats in each group was centrifuged with low temperature and high-speed centrifuge at 12,000 rpm for 10 min, to detect the effect of FA-Glu/MTX on IL-1 β and TNF- α inflammatory cytokines. The serum of rats in each group was detected according to the ELISA kit of inflammatory factors.

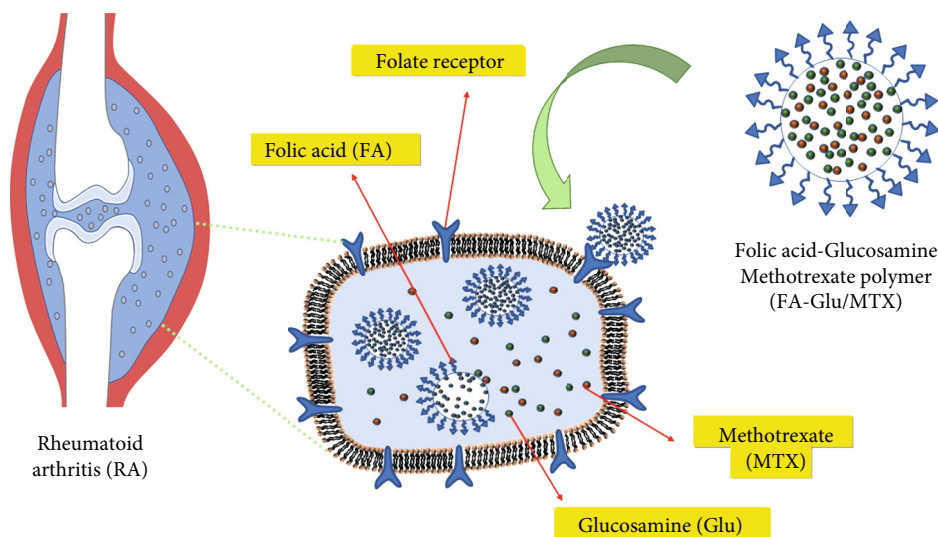


FIGURE 1: Diagram of the mechanism of action of FA-Glu/MTX on RA.

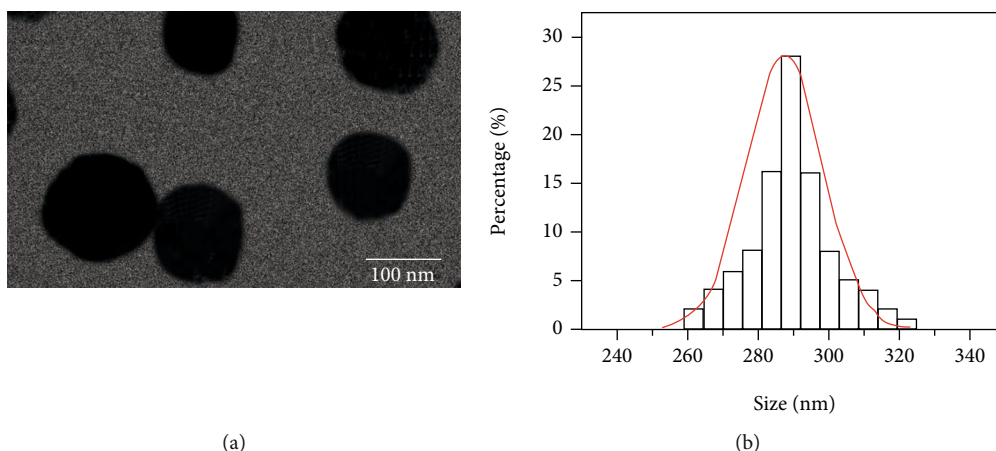


FIGURE 2: Morphology characterization of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX). (a) Electron microscope characterization diagram. (b) Graph of particle size distribution.

3. Results and Discussion

3.1. FA-Glu/MTX Morphology Characterization. The FA-Glu/MTX nanoparticles observed by scanning electron microscope were spherical with a smooth surface, uniform in size, and uniform in distribution (Figure 2). The average diameter of FA-Glu/MTX nanoparticles was 287.36 ± 21.54 nm, and the average Zeta potential was 32.17 ± 4.68 mV by Zetasizer WT potentiometer.

3.2. Investigation on the Stability of FA-Glu/MTX. The relative transmittance of FA-Glu/MTX nanoparticles under different pH conditions was measured to investigate their stability (Figure 3). The results show that the relative transmittance is also different under different pH conditions, and the relative transmittance is the strongest when pH = 6.8. And FA-Glu/MTX nanoparticles have strong stability within 14 days under the same pH conditions.

3.3. In Vitro Release Ability of FA-Glu/MTX. MTX and FA-Glu/MTX nanoparticles were used to simulate the drug release process in vivo in a buffer solution with pH = 6.8, and the cumulative release amount was calculated (Figure 4). The results of drug release showed that the release rate of MTX was faster, and the cumulative release was higher, which was more than 80% at 6.0 h. The cumulative release of FA-Glu/MTX nanoparticles reached 80% at 60 h, indicating that FA-Glu/MTX nanoparticles have a sustained release effect.

3.4. In Vitro Toxicity of FA-Glu/MTX. MTT assay was used to evaluate the cytotoxicity of MTX and FA-Glu/MTX nanoparticles on BMSCs cells (Figure 5). With the increase of action time, the toxic effect of MTX and FA-Glu/MTX nanoparticles on BMSCs cells was greater. And at the same time, MTX had a greater toxic effect on BMSCs cells. When the action time was 96 h, the cell proliferation rate was only

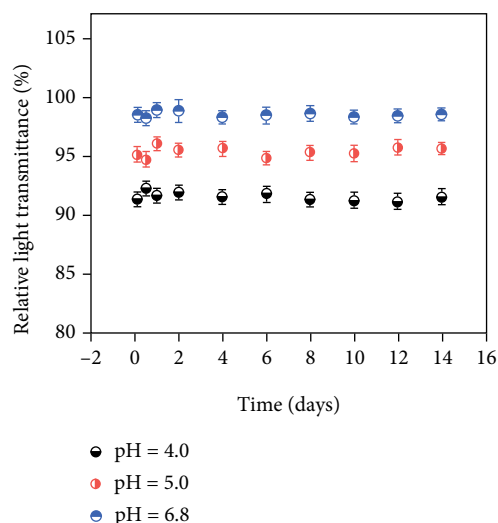


FIGURE 3: Stability analysis of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX).

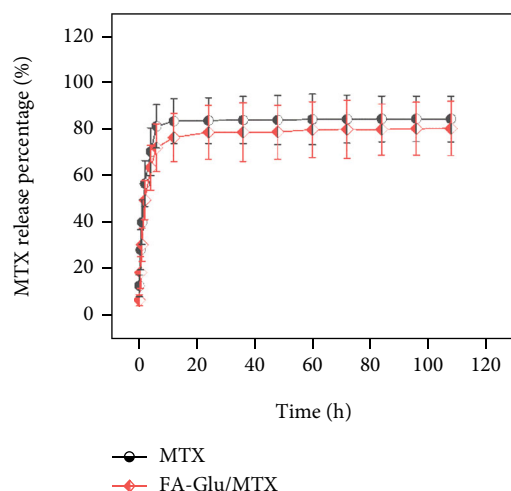


FIGURE 4: Analysis of MTX release rate in folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX).

46.2%, while the cell proliferation rate of FA-Glu/MTX group was 60.7%. These results show that FA-Glu/MTX nanoparticles can significantly reduce the cytotoxicity of MTX.

3.5. The Effect of FA-Glu/MTX on the Swelling Ability and Body Weight of RA Rats. In order to evaluate the therapeutic effect of FA-Glu/MTX nanoparticles on RA rats, the body weight and foot swelling of rats were measured and analyzed (Figure 6). After the establishment of RA model, the diet of rats in each group increased in varying degrees. For this, the body weight of rats in each group was measured twice a week (Figure 6(a)). The results showed that with the increase of time, the body weight of rats in the model group increased the least, while that in the control group increased the most, and the body weight of rats in the FA-Glu/MTX group increased more than that in the MTX group. This shows that FA-Glu/MTX nanoparticles are more effective in the treat-

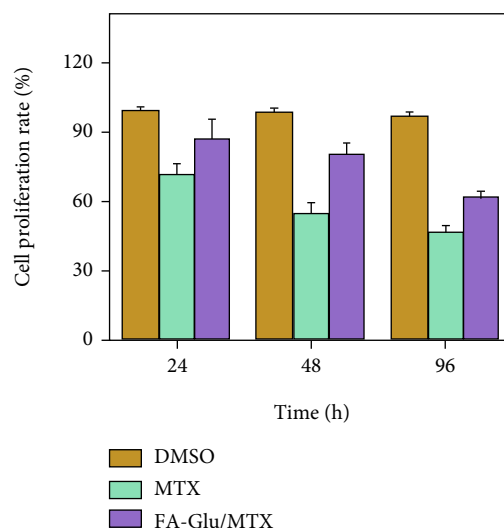


FIGURE 5: Cytotoxicity of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX).

ment of RA rats. Figure 6(b) is the result of the determination of the degree of foot swelling in each group, and the results show that there is no decrease in the degree of foot swelling in the model group, which proves that the RA rat model is established successfully, while the comparison between the MTX group and the FA-Glu/MTX group further proves that FA-Glu/MTX nanoparticles are more effective in the treatment of RA.

3.6. Targeting Effect of FA-Glu/MTX Nanoparticles. The targeting comparison of tissue fluid in inflammatory sites of rats in each group was recorded in Figure 7. As can be seen from the figure, at the same concentration, the release concentration of FA-Glu/MTX nanoparticles in the inflammatory site is higher, which proves that compared with MTX, FA-Glu/MTX nanoparticles have stronger targeting.

3.7. The Effect of FA-Glu/MTX on the Index of Immune Organs in RA Rats. Spleen index and thymus index were used to evaluate the effect of FA-Glu/MTX nanoparticles on immune organs of RA rats (Figure 8). The spleen index of rats in each group was recorded in Figure 8(a), in which the spleen index of the model group was significantly increased, while MTX and FA-Glu/MTX nanoparticles could significantly reduce the spleen index of RA rats, and the effect of FA-Glu/MTX nanoparticles was stronger. Figure 8(b) is the comparison result of the thymus index of rats in each group. Compared with the control group, the thymus index of the model group decreased significantly, while MTX and FA-Glu/MTX could improve the decrease of thymus index caused by RA, and the improvement effect of FA-Glu/MTX nanoparticles was stronger.

3.8. Effect of FA-Glu/MTX on the Content of Inflammatory Cytokines in RA Rats. The most obvious pathological feature of RA is the increase of inflammatory cytokines in vivo. IL-1 β and TNF- α were used as detection indexes to evaluate the therapeutic effect of FA-Glu/MTX nanoparticles on RA

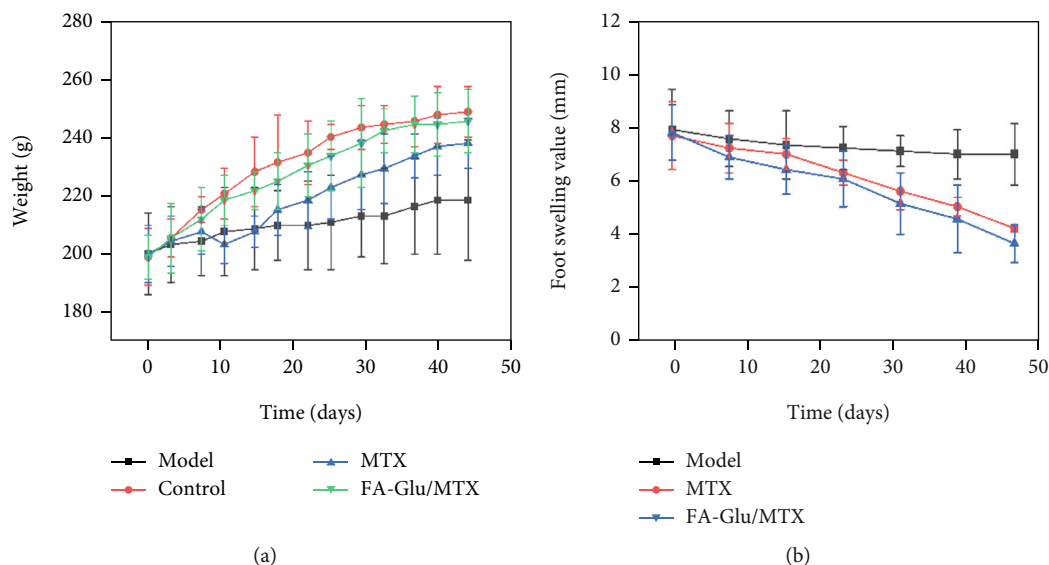


FIGURE 6: Comparative analysis of the effects of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX) and MTX on the macroscopic indicators of rheumatoid arthritis. (a) Comparative analysis of the effects of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX) and MTX on the weight of rats. (b) Comparative analysis of the effects of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX) and MTX on the degree of swelling of the rat's foot.

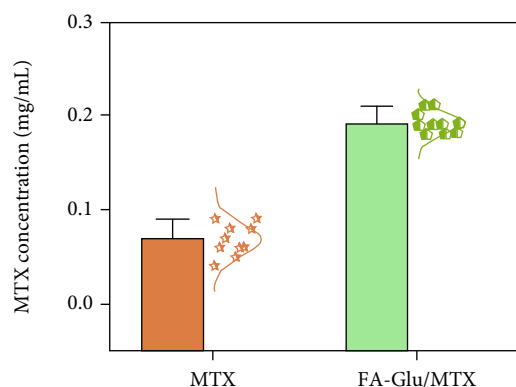


FIGURE 7: Comparative analysis of the effects of MTX and on the concentration of MTX in inflamed tissues.

inflammation (Figure 9). The determination of $IL-1\beta$ is shown in Figure 9(a). FA-Glu/MTX nanoparticles can significantly reduce the increase of $IL-1\beta$ induced by RA, and its inhibitory effect is stronger than that of MTX. The detection results of $TNF-\alpha$ are recorded in Figure 9(b). Both MTX and FA-Glu/MTX nanoparticles can improve the increase of $TNF-\alpha$ caused by RA, and the improvement effect of FA-Glu/MTX nanoparticles is better. In summary, FA-Glu/MTX nanoparticles are more effective in the treatment of RA.

4. Discussion

At present, the pathogenesis of RA is not clear, and therapeutic drugs usually relieve joint inflammation and limit the development of the disease, but its disability rate and fatality rate are still high. At present, conventional therapeutic drugs have low selectivity and high side effects, so there is

an urgent need to find a drug with good targeting and high biological safety for the treatment of RA.

As an important coenzyme involved in human metabolism, FA can be used as a marker to enhance the targeting of drugs. Elkhodiry et al. [27] have proved that FA can be used as a drug delivery carrier to carry chemotherapeutic drugs for the treatment of cancer and can significantly enhance the targeting of drugs. Reactive oxygen species released by activated macrophages can cause a series of inflammatory and pain responses, which is the key cause of RA [28]. The infiltration of macrophages in the synovium of joints was significantly correlated with RA therapy. Chandrupatla et al. [29] proved that folate receptor β can be used as an important target for RA therapy and further proved that FA can improve the targeting of RA drugs. At the same time, Sasaki et al. [30] found that adding a certain amount of FA to patients with RA treated with MTX every day can effectively reduce the side effects caused by MTX without affecting its therapeutic effect on RA.

In this work, MTX and Glu were modified by FA, and their properties were investigated. It is found that FA-Glu/MTX is a uniform spherical particle, which can maintain good stability in different pH buffer solutions. At the same time, the results of drug release experiments in vitro show that FA-Glu/MTX nanoparticles have a sustained release effect compared with MTX. In addition, in the study of the therapeutic effect of FA-Glu/MTX nanoparticles on RA rats, it can be found that FA-Glu/MTX nanoparticles can improve the weight loss and foot swelling caused by RA, and compared with the MTX group, the improvement of FA-Glu/MTX nanoparticles is more significant. Through the comparison of the drug concentration at the inflammatory site, it can be found that FA-Glu/MTX nanoparticles can enhance the drug targeting, which may be one of the reasons why it is more effective in the treatment of RA than

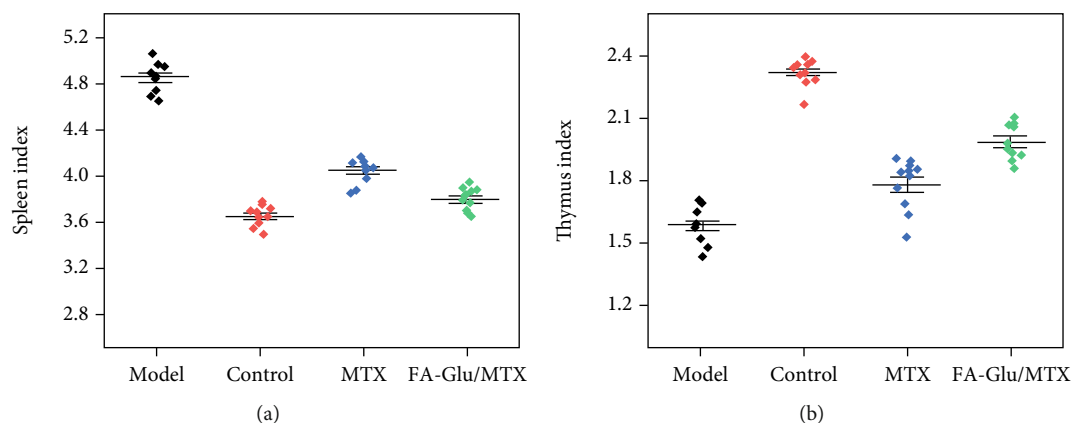


FIGURE 8: Comparative analysis of the effects of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX) and MTX on immune organ index. (a) Spleen index. (b) Thymus index.

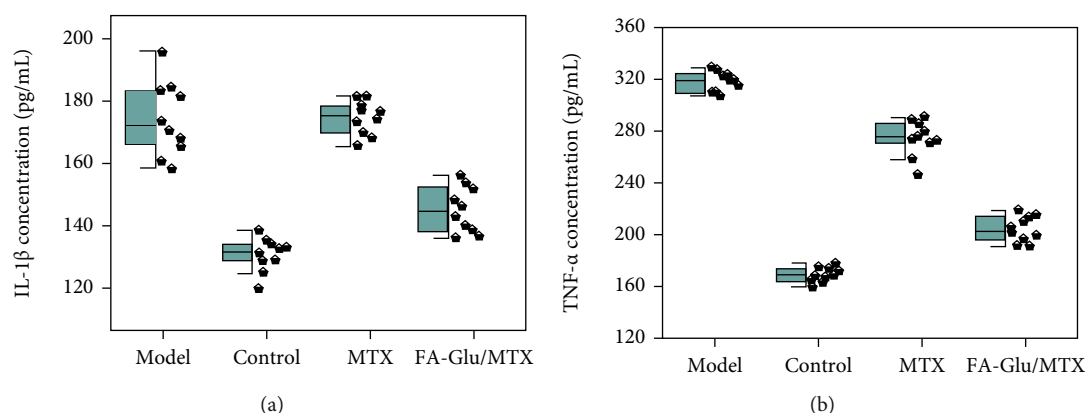


FIGURE 9: Comparative analysis of the effects of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX) and MTX on inflammatory factors. (a) Comparative analysis of the effects of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX) and MTX on IL-1 β . (b) Comparative analysis of the effects of folic acid-glucosamine/methotrexate polymer (FA-Glu/MTX) and MTX on TNF- α .

MTX. The results of its effects on immune organs and inflammatory cytokines further proved that FA-Glu/MTX nanoparticles had a stronger effect on the improvement of RA. This finding provides a new approach for targeted therapy of RA and lays a foundation for follow-up clinical trials.

5. Conclusion

In this study, a novel nanopolymer for the targeted treatment of RA was prepared, which has good stability, slow release, and low cytotoxicity compared to MTX. The in vivo results also demonstrated that FA-Glu/MTX was more potent and targeted for the treatment of RA compared to MTX. This finding makes FA-Glu/MTX a promising new option for the targeted treatment of RA.

Data Availability

The data underlying the results presented in the study are available within the manuscript.

Ethical Approval

Research experiments conducted in this article with animals were approved by the Medical Ethics Committee of Cangzhou Central Hospital following all guidelines, regulations, legal, and ethical standards as required for animals.

Conflicts of Interest

There are no conflicts to declare.

Acknowledgments

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