Effect of Folic Acid Conjugated Silver Nanoparticles in Treatment of RA like Adjuvant Arthritis in Rats

Hajar K. Ahmed

Al-Azhar University Faculty of Medicine for Girls, Cairo, Egypt.

Adel A. Elbeialy (✉ aelbeialy@azhar.edu.eg)

Al-Azhar University Faculty of Medicine for Girls, Cairo, Egypt.

Ahmad M. Mansour

Al-Azhar University Faculty of Medicine for Girls, Cairo, Egypt.

Hala Elzomor

Al-Azhar University Faculty of Medicine for Girls, Cairo, Egypt.

Amany I. Abosaif

Al-Azhar University Faculty of Medicine for Girls, Cairo, Egypt.

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Abstract

Objective: Nanomedicine has become one of the promising research areas, opening new horizons in disease diagnosis and treatment. Recent years have witnessed a surge in the development of nanomedicine for combating rheumatoid arthritis (RA), the most common autoimmune arthritis. RA is characterized by progressive inflammation and persistent synovitis, leading to joint destruction, functional incapability, and ultimately disability. Although there has been a tremendous evolution in disease assessment and treatment, many patients still fail to attain remission. Therefore, developing new drugs that specifically target inflamed joints and simultaneously attenuate other possible damages to healthy tissues is indispensable. This study was done to evaluate the potential of folic acid conjugated silver nanoparticles (FA-AgNPs) as RA therapy.

Methods: In the CFA-arthritic rat model, FA-AgNPs & methotrexate were administered for 8 consecutive weeks. Therapeutic efficacy was evaluated by measuring paw volume, ESR, CRP, TNF- α , and IL-6 levels. For safety concerns, CBC, liver, and renal function tests were evaluated. Joints histological assessment was also carried out.

Results: FA-AgNPs significantly reduced paw volume, paw weight, ESR, CRP, RF, TNF- α , and IL-6 levels compared with arthritic non-treated rats, demonstrating good anti-inflammatory activity. Likewise, histology of tarsal joints depicted comparatively lesser inflammatory cellular infiltration and diminished cartilage erosions. Methotrexate displayed comparable results. In contrast to methotrexate, FA-AgNPs showed normal CBC & significantly improved liver and renal function tests.

Conclusion: FA-AgNPs exhibited substantial anti-arthritic activity. This notable anti-arthritic potential of FA-AgNPs was as good as the current standard treatment of MTX with higher biosafety.

Introduction

Rheumatoid arthritis (RA) represents most common inflammatory arthritis [1]. It is characterized by poly-articular joint involvement with subsequent bone and cartilage inflammation and erosions [2]. It has multifactorial pathogenesis [3]. Several studies have identified that activated macrophages play a crucial role in the occurrence and perpetuation of RA [4], therefore, they may be considered as the primary targets in RA management [5].

While the available treatment options can alleviate the symptoms and slow arthritis progression, high frequent doses and long-term treatments are often required which inevitably cause undesirable systemic side-effects due to inability to selectively target the inflammatory areas [6]. Limitations of the current therapies have driven the development of nanomaterial-based devices in RA treatment.

Recently, AgNPs have been used as vehicles to deliver anti-inflammatory drugs for RA treatment [7] and interestingly it was indicated that AgNPs on its own showed anti-inflammatory activity [8,9]. Therefore, it is suggested that AgNPs can be used as effective nontoxic therapeutic agent for RA.

Moreover, as cells involved in RA pathogenesis undergo series of alterations such as overexpression of specific surface receptors or transformation of phenotype, nanocarriers conjugation with targeted ligand would promote targeted drug delivery to inflamed tissues [10]. Many reports have shown that folate receptors (FR β) are overexpressed on activated macrophages that present in abundance at sites of inflammation [11,12]. These FRs of activated macrophages have been utilized as a target for nanomedicine diagnostic and therapeutic agents [13].

Aim Of Work

This work was done to Evaluate the potential of FA conjugated AgNPs as a DMARD therapy for RA.

Materials And Methods

3.1. Drugs and Chemical

Silver nanoparticles (<100 nm particle size), folic acid powder and Freund's adjuvant (Each mL contains 1 mg Mycobacterium tuberculosis, heat killed and dried, 0.85 mL paraffin oil and 0.15 mL mannide monooleate) purchased from Sigma-Aldrich. Methotrexate purchased from Mylan SAS. Serum TNF- α and IL-6 ELISA kit purchased from Bioassay technology laboratory of Jiaying Korain Biotech Company.

3.2. Preparation of folic acid conjugated silver nanoparticle

176.5 mg of folic acid and 76.6 mg ethylene dichloride (EDC) were dissolved in 40 ml DMSO (dimethyl sulfoxide) -water medium (1 : 1) at room temperature by 3 h. Then 130 mg Ag nanoparticles were added to that mixture solution with continuous stirring at room temperature for 12 h. The nanoparticles were collected by centrifugation at 5000 rpm. The product was dried in vacuum at room temperature. The functionalized material was yellowish in color.

3.3. Doses determination

Animal equivalent dose calculated using the following formula:

Animal dose (mg/kg) = Human effective dose (HED) (mg/kg) conversion factor

The HED of MTX in arthritis (7.5 mg/60 kg body weight/week) was used for calculation of rat dose and the conversion factor was taken as 6.17. The animal dose of MTX was calculated to be 0.7 mg/kg/week. Rat dose of FA-AgNPs was calculated to be 84.8 μ g/kg/day.

3.4. Animals

Thirty-eight female Sprague Dawley Albino rats weighing of 200 $\pm$ 10 gm purchased from the animal house of El-Nile Company for Pharmaceuticals. The animals were housed under standard laboratory

conditions of temperature (24-28°C), average humidity (55 ± 5%), a 12/12h light/dark cycles. The animals were allowed free access to water and were fed a standard rodent pellet diet. All the animals were acclimatized for 7 days prior to commencement of the procedure.

All animal procedures adopted in this study were consistent with the ethical guidelines of international association for study of pain in conscious animals (Zimmermann, 1983)[14], and guidelines of the local ethics committee, which comply with international laws for the use and care of laboratory animals, which complies with ARRIVE guidelines (<https://arriveguidelines.org>).

(Ethics Committee of Faculty of Medicine for Girls, Al-Azhar University, Nasr City, Cairo, Egypt, Registered at Central Administration of Research & Development; Egyptian Ministry of Health: Reg No. RHBIRB2018122001).

All rats were apparently healthy at the time of starting the experiment with no evident joint problem (no swollen joints, limping, deformities or aggressive behavior denoting pain).

3.5. Induction of arthritis

Systemic arthritis was induced in 32 rats by subcutaneous injection of 0.4 ml divided in three doses [one dose every four days] of Complete Freund's Adjuvant (CFA) into footpad of right hind paw [15].

3.6. Experimental design

Rats were randomly allocated into four groups and drugs were administrated on day 12 with the onset of arthritis. At first, they were divided into 2 groups:

Group I: Normal healthy control group of six rats.

Group II: Arthritic 32 rats, in which adjuvant arthritis was induced via CFA injection; then they were subdivided randomly into 3 groups:

Group IIa: Arthritic control group of twelve rats received only saline (as *Placebo*).

Group IIb: methotrexate RA-treated group of ten rats. Received methotrexate in a dose of 0.7 mg/kg/week; IP, for 2 months.

Group IIc: FA-AgNPs RA-treated group of ten rats. Received FA-AgNPs treatment in a dose of 84.8 µg/kg/day; P.O., for 2 months.

3.7. Measurement of paw volume

Hind paw edema volume was measured using a digital vernier caliper (the difference between the final volume minus the initial volume results in paw total volume).

3.9. Measurement of biochemical parameters

At the end of the study, blood samples were collected for estimation of CBC, ESR, CRP, serum ALT, AST, urea and creatinine.

3.10. Measurement of proinflammatory cytokines

Serum TNF- α and IL-6 were estimated using ELISA kit according to the manufacturer's instructions.

3.11. Histopathological analysis

All the 38 rats have been sacrificed after ether inhalation anesthesia. The hind paw was amputated proximal to ankle joint and fixed in formalin 10% followed by decalcification in 5% nitric acid. Then we intersected the hind paw in a mid-sagittal plane and transverse plane. After intersection the tissues were processed and embedded in paraffin blocks to cut into 4-5 μ m thick and stained with hematoxylin and eosin (H&E) for microscopic evaluation.

3.12. Statistical analysis

All data are expressed as mean $\pm$ SEM. The data were subjected for statistical analysis using GraphPad Prism 7.0 (CA, USA). The statistical significance of difference between various groups was tested by one-factor analysis of variance (ANOVA) followed by Tukey's multiple comparison test. P values ≤ 0.05 were considered statistically significant.

Results

4.1. Physical Parameters

Paw volume

Compared to the control group, the CFA injection was accompanied with obvious swelling and significant increase in paw volume ($P \leq 0.001$). Compared to arthritic non-treated group, the deleterious effect of paw swelling associated with CFA was inhibited by treatment with either FA-AgNPs or MTX ($P \leq 0.001$), while by comparing effect of FA-AgNPs with effect of MTX on paw volume there was non-significant difference as p value is > 0.05 (**Table 1**).

Paw weight

CFA injection resulted in a significant increase ($P \leq 0.001$) in paw weight when compared to healthy control group. Compared to arthritic non-treated group, treatment with FA-AgNPs or MTX showed a significant decrease ($P \leq 0.01$) in the paw weight (**Table 1**).

Body weight

The arthritic non-treated group recorded a significant reduction ($P \leq 0.01$) in the percentage change of body weight compared with healthy control group. However, FA-AgNPs or MTX treatment showed a significant improvement in weight gain when compared to arthritic non-treated rats ($P \leq 0.05$) (**Table 1**).

4.2. Acute phase reactants

CFA injection resulted in significant increase ($P \leq 0.001$) in ESR and CRP levels when compared to healthy control group. Treatment with FA-AgNPs or MTX showed significant decrease ($P \leq 0.001$) in both ESR and CRP levels when compared to arthritic non-treated group (**Figure 1**).

4.3. Serum TNF- α and IL-6

Serum levels of TNF- α and IL-6 exhibited significant elevation in CFA rats compared to healthy controls ($P \leq 0.001$), suggesting the model was successfully established. MTX treatment induced significant decrease ($P \leq 0.01$) in serum TNF- α and IL-6 as well as FA-AgNPs treatment ($P \leq 0.001$) when compared to the arthritic non-treated group. Results of FA-AgNPs regarding serum TNF- α and IL-6 levels were insignificantly better than MTX as P value is > 0.05 . (**Figure 2**)

4.4. Hematological parameters

CFA injection resulted in non-significant decrease ($P > 0.05$) in the RBCs count, Hb concentration and platelet count, by contrast, WBCs count exhibited significant increase ($P \leq 0.05$) when compared to healthy control group. MTX treatment showed significant decrease ($P \leq 0.001$) in all the hematological parameters when compared to arthritic non-treated group. FA-AgNPs resulted in non-significant increase ($P > 0.05$) in RBCs count, Hb concentration and platelet count while it causes significant decline ($P \leq 0.01$) in WBCs compared to arthritic non-treated group. By comparing the effect of MTX and FA-AgNPs on hematological parameters, the deleterious effect of MTX was significantly abolished ($P \leq 0.01$) in FA-AgNPs treated group (**Table 2**).

4.5. Liver function tests

Compared to normal control group, serum ALT and AST levels of arthritic rats were significantly elevated ($P \leq 0.001$). MTX group showed non-significant increase in the serum ALT and AST levels more than non-treated arthritic group ($P > 0.05$). However, the FA-AgNPs group showed a significant decrease in these enzymes than that in non-treated arthritic and MTX groups. ($P \leq 0.01$) (**Table 3**).

4.6. Renal function tests

Compared to normal control group, serum urea ($P \leq 0.001$) and creatinine ($P \leq 0.01$) levels of arthritic rats were significantly increased. Compared with non-treated arthritic group, MTX group showed non-significant increase in serum urea and creatinine ($P > 0.05$). However, FA-AgNPs treatment resulted in a significant decrease in the serum urea ($P \leq 0.001$) and creatinine ($P \leq 0.05$) levels when compared to non-treated arthritic and MTX groups. ($P \leq 0.01$) (**Table 3**).

4.7. Histopathological analysis

Histopathological assessment of tarsal joints revealed that CFA induced signs of inflammatory

infiltration, synovial hyperplasia, partial cartilage and bone destruction compared to normal control group. Furthermore, histopathological scoring of the finding revealed that, FA-AgNPs and MTX treatment produced tarsal joints protection by reducing the levels of cellular infiltration, hyperplasia and cartilage destruction compared to the arthritic non-treated rats (**Figure 3**).

Discussion

Over last decades, the interest in silver as a therapeutic agent has been rekindled because of the accessibility of silver nanoparticles [16]. The current study elucidated the efficacy and safety of the FA-AgNPs in treating CFA RA model in rats.

The study revealed that CFA injection induced redness and swelling in the paws, as evidenced by significant elevation in paw volume ($P \leq 0.001$). This was in agreement with Li et al. [17] who reported significant elevation of paw volume ($P < 0.05$) following CFA injection.

The therapeutic efficacy was measured by treating the CFA arthritic rats with FA-AgNPs daily for two months and MTX was chosen as a comparative clinical standard treatment. Compared to the non-treated arthritic rats, the rats subjected to FA-AgNPs and MTX showed obvious improvement in the degree of swelling as well as much less redness ($P \leq 0.001$). This is supported by Kedi et al., [18] who reported that oral administration of AgNPs induced significant ($P < 0.001$) inhibition of paw edema when compared to arthritic group.

Pro-inflammatory cytokines and acute phase reactants were measured as another index to evaluate the therapeutic efficacy of both compounds. Our results showed significant elevation ($P \leq 0.001$) of serum TNF- α and IL-6 as well as high ESR and CRP levels in the arthritic group of rats. This is in agreement with Ahsan et al. [19] who reported significant rise ($P < 0.001$) in the mRNA expression of TNF- α and IL-6 following CFA injection in rat model. Furthermore, arthritic rats showed conspicuous rise in the levels of ESR and CRP. FA-AgNPs resulted in remarkable decline ($P \leq 0.001$) in the cytokines demonstrating a good anti-inflammatory activity. This was in agreement with Yang et al. [20] who studied the efficacy of FA-AgNPs as targeted treatment for RA on collagen-induced arthritis mice model and reported significant reduction in levels of TNF- α and IL-6 as well as in ESR and CRP titer with FA-AgNPs and MTX demonstrating good anti-inflammatory activity ($P \leq 0.001$).

Moreover, histopathological analysis of hind limb revealed that non-treated arthritic rats showed cellular infiltration, synovial hyperplasia, partial cartilage and bone destruction compared to normal control. Treatment with FA-AgNPs and MTX showed an obvious improvement with less cellular infiltration, hyperplasia and milder cartilage destruction compared to the non-treated arthritic rats. This is in agreement with Mani et al., [21] who reported amelioration of histopathological features of CFA with AgNPs and methotrexate treatment.

Generally, body weight usually reflects the health condition of the treated rats. The arthritic group of rats showed slow gradual weight gain that is significantly lower than weight gain in the normal control group. FA-AgNPs significantly ameliorated disease effect on body weight gain with significant increase ($P \leq 0.05$) in percentage change of body weight almost similar to the normal control group, suggesting a well tolerance of the rats toward FA-AgNPs at the therapeutically efficacious dose. Similarly, MTX treatment significantly improves ($P \leq 0.05$) weight gain compared with arthritic non-treated group.

For the safety concerns, the hepatotoxicity was evaluated by measuring serum ALT and AST levels and the nephrotoxicity was tested by measuring serum urea and creatinine. Compared to normal rats, all these parameters were not changed after FA-AgNPs treatment ($P > 0.05$), in contrast with MTX group, in which a significant increase of ALT, AST, urea and creatinine levels were noticed ($P \leq 0.01$).

As for the hematological parameters, such as RBC, Hb, WBC and platelets, they were at normal level when compared to normal rats ($P > 0.05$), indicating no significant risk associated with FA-AgNPs treatment. Counterwise, MTX group showed a significant decrease in these parameters compared to normal control ($P \leq 0.01$).

We can explain what happens in that folic acid receptors (FR) are overexpressed on activated M1 macrophages, which are abundant at areas of inflammation (22). These receptors have been employed as a therapeutic target for folic acid linked nanosilver. Because normal macrophages and neutrophils rarely express folate receptors, FA-AgNP targets active macrophages other than healthy macrophages (23). In reaction to intracellular glutathione (GSH), FA-AgNPs dissolved and released Ag⁺, causing M1 macrophage death and reactive oxygen species (ROS) scavenging, allowing M2 macrophages to repolarize. FA-AgNPs were gradually removed from the body, primarily by faeces, with no evidence of tissue buildup, and no evidence of long-term harm. Furthermore, AgNP were found to inhibit folate reductase enzymes (24)

Conclusion And Recommendations

FA-AgNPs effectively suppressed the pathological condition in CFA-induced arthritic rats. The notable anti-arthritic potential of FA-AgNPs was as good as the current standard treatment of MTX with higher biosafety. Therefore, we can envisage that FA-AgNPs would be a promising clinical therapeutic modality for RA therapy. We recommend further studies on larger groups using different doses for longer durations to establish the efficacy of FA-AgNP as a treatment of RA and other autoimmune diseases.

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Tables

Table (1): The effects of FA-AgNPs on physical parameters

Parameters	Paw volume (mm)	Paw weight (gm)	Percentage change in body weight (%)
Groups	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
Control	1.22 $\pm$ 0.09	1.14 $\pm$ 0.02	18.11 $\pm$ 1.49
RA	7.85 $\pm$ 0.38 ^a	2.39 $\pm$ 0.13 ^a	8.54 $\pm$ 1.17 ^a
RA + MTX	2.77 $\pm$ 0.14 ^{a,b}	1.76 $\pm$ 0.11 ^{a,b}	15.52 $\pm$ 1.95 ^b
RA + FA-AgNPs	2.89 $\pm$ 0.11 ^{a,b}	1.79 $\pm$ 0.11 ^{a,b}	16.19 $\pm$ 2.00 ^b

Data are presented as means $\pm$ SEM, n = 6. ^aSignificantly different from healthy control group; ^bSignificantly different from RA group.

Table (2): The effects of FA-AgNPs on hematological parameters.

Parameters	RBCs (10 ⁶ / mm ³)	Hb (g/dl)	PLT (10 ³ /mm ³)	WBCs (10 ³ /mm ³)
Groups	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
Control	7.81 $\pm$ 0.12	14.8 $\pm$ 0.42	557.7 $\pm$ 14.97	7.67 $\pm$ 0.25
RA	7.19 $\pm$ 0.34	14.16 $\pm$ 0.49	555.8 $\pm$ 21.04	10.14 $\pm$ 0.94 ^a
RA + MTX	5.31 $\pm$ 0.31 ^{a,b}	10.72 $\pm$ 1 ^{a,b}	415.2 $\pm$ 37.75 ^{a,b}	5.12 $\pm$ 0.87 ^{a,b}
RA + FA-AgNPs	7.48 $\pm$ 0.2 ^c	14.7 $\pm$ 0.18 ^c	573.5 $\pm$ 23.91 ^c	7.34 $\pm$ 0.33 ^{b,c}

Data are presented as means $\pm$ SEM, n = 6. ^aSignificantly different from healthy control group; ^bSignificantly different from RA group; ^cSignificantly different from MTX group.

Table (3): The effects of FA-AgNPs on biochemical parameters.

Parameters	ALT (U/L)	AST (U/L)	Urea (mg/dl)	Creatinine (mg/dl)
Groups	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
Control	58.89 $\pm$ 4.23	107.1 $\pm$ 8.07	18.10 $\pm$ 0.98	0.84 $\pm$ 0.17
RA	91.65 $\pm$ 4.27 ^a	160.3 $\pm$ 6.83 ^a	28.05 $\pm$ 0.59 ^a	1.93 $\pm$ 0.17 ^a
RA + MTX	90.73 $\pm$ 6.94 ^a	169 $\pm$ 5.45 ^a	28.77 $\pm$ 1.92 ^a	1.95 $\pm$ 0.25 ^a
RA + FA-AgNPs	61.56 $\pm$ 2.97 ^{b,c}	116.9 $\pm$ 7.97 ^{b,c}	18.81 $\pm$ 1.04 ^{b,c}	0.93 $\pm$ 0.23 ^{b,c}

Data are presented as means $\pm$ SEM, n = 6. ^aSignificantly different from healthy control group at ($P \leq 0.001$); ^bSignificantly different from RA group at ($P \leq 0.01$); ^cSignificantly different from MTX group at ($P \leq 0.01$)

Figures

Figure 1

Effect on acute phase reactants

Figure 2

Effect on serum cytokines.

Figure 3

Histopathological analysis of hind limb showing Microscopic pictures of H&E stained sections of tarsal joints at magnification power $\times 40$. A) normal rats demonstrating one layer of normal synovial membrane and normal cartilage. B) arthritic non-treated rats demonstrating perisynovial inflammation, pannus formation, massive inflammatory cells infiltration into the synovial cavity and cartilage destruction and slight bone erosion. C) MTX RA-treated rats demonstrating mild synovial hyperplasia and decreased inflammatory cells infiltration into the synovial cavity and slight cartilage erosion. D) FA-AgNPs RA-treated rats demonstrating mild synovial hyperplasia and decreased inflammatory cells infiltration into the synovial cavity and slight cartilage erosion.

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