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Preparation Of Leflunomide Nanocomposite And Study Of Its Effect On Some Immunological Parameters In Male Rats Induced With Rheumatoid Arthritis

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Abstract: The results of the current study showed a significant increase in the arthritic-induced positive control group compared with the negative control group, and the leflunomide drug group did not record any significant differences compared with the positive control, while the nano-drug group - leflunomide recorded a significant decrease While interleukin-6 .compared with the arthritic induction group and Leflunomide group recorded a significant increase in the positive control group compared to the negative control group, with a slight significant decrease in the leflunomide-treated group compared to the induction group for arthritis, and the drug group nano-loaded with leflunomide recorded a significant and clear decrease compared to the positive control While the rheumatoid factor (RF) .group and the drug-treated group. Leflunomide recorded a significant increase in its levels in the positive control group compared to the negative control, and the group treated with leflunomide recorded a slight decrease in RF levels, while the nano-loaded drug group with leflunomide recorded a significant and clear decrease compared to the two groups inducing arthritis and The group treated with .leflunomide

Keywords/ Leflunomide, ZnO-NPs, Arthritis, TNF-α, RF, IL-6.

1. INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune and inflammatory disease, which means that the immune system mistakenly attacks healthy cells in the body, causing inflammation (painful swelling) in the affected parts of the body, it attacks many joints simultaneously, there are many causes of arthritis, including joint weakness from excessive use due to old age (Organic Agriculture "OA" is more common in adults over the age of 50), injuries, obesity, autoimmune disorders, genetics, family history, or muscle weakness (Junior *et al*., 2021). Rheumatoid arthritis most commonly affects the joints of the hands, knees, and wrists, and in the affected joint occurs rheumatoid arthritis, in which the lining of the joint becomes inflamed, causing damage to joint tissue, and this tissue damage thus causes long-term or chronic pain and unsteadiness (lack of balance), and deformity (misshapenness).

Recent studies have relied on new technologies, including nanotechnology, which has become widely used to improve immune responses directed to the prevention and treatment of infectious and non-communicable diseases (Hamida *et al*., 2019). The field of

nanotechnology is one of the most popular areas of research and development, primarily in all disciplines (Paul & Robeson, 2008). This is due to its high strength, light weight, excellent chemical reactivity, very small volume, high surface area, and high stability , among the most widely used nanomaterial's, zinc oxide has gained great interest in the scientific and medical communities, due to its important use in many biomedical and antibacterial applications, due to its chemical and physical properties (Noor *et al*., 2017).

The use of nano-zinc oxide was not limited to these areas only, but it was an important element in the medical field, especially in the past few years, where it witnessed a remarkable development in the use of nanotechnology in medicine, where nanoparticles can be manufactured in the specific shape and size for the desired purpose, as there is a possibility to provide new methods regarding the treatment of diseases that were difficult to focus on due to size limitations, and the increasing demand for environmentally friendly metal oxide nanoparticles for pharmaceutical applications, there is a growing need to search for alternative methods to formulate new types of safe and cost-effective antibiotics to eliminate on pathogens and their control since most biological processes take place at the nano level, the combined efforts of nanotechnology and biology can solve important biomedical problems. Among the many semiconductors, metal oxides, especially zinc oxide, are considered biologically safe, and effective In terms of cost, non-toxic, and very useful against pathogenic bacteria, zinc oxide has received increasing attention in recent years due to its stability in Due to harsh environmental conditions and because zinc compounds are (GRAS) listed, i.e. generally considered non-toxic and safe by the US food and drug administration, ZnO-NPs are used in many different medical industries such as pharmaceuticals and cosmetics (Wahab *et al.* , 2012 & Mirzaei & Darroudi, 2017), it is also widely used to treat a range of different skin diseases and for its latent ability to absorb ultraviolet rays, it is used as a UV blocker in sunscreens, antimicrobials and many medical products, and studies have shown that particles ZnO-NPs can be highly toxic to cancer cells or bacteria and leukemia cells, and ZnO-NPs nanomaterials have been investigated as compounds for drug delivery, gene delivery and biosensing as well as being studied for cancer treatment (Zhang *et al*., 2013). Nanomedicine of the drug leflunomide for the treatment of rheumatoid arthritis, and the study of the effect of this nanomedicine on some immunological parameters in male rats induced with rheumatoid arthritis by evaluating (IL-6 and general l and Rheumatoid Factor RF and TNF-α).

2. MATERIALS AND WORKING METHODS

3-1: Experimental animals

The study was conducted in the animal house of the College of Veterinary Medicine, Al-Qasim Green University. Adult white male rats were used in this study, their ages ranged between 12-10 weeks and their weights ranged between 180-175 g. The animals were subjected to similar laboratory conditions in terms of ventilation and temperature2. ±22 mº and lighting (12 hours of light to 12 hours of darkness), water and food were served openly *ad libitium*, as the ration was made in the form of fingers (Ward, 1970).

(3-2)Preparation of nano drug using zinc oxide carrier

(3-2-1): Free solution of leflunomide:

Leflunomide was used at a dose of 20 mg/kg. The solution of this drug was prepared by dissolving 1.2 gm of leflunomide in an amount of Di Methyle Sulf Oxide **(DMSO)** and after completing the dissolution process, the volume was completed to 50 ml with deionized water.

3-2-2 : Preparation of the hybrid nano-drug from zinc oxide layers with leflunomide by ion-exchange sol gel method:

The hybrid nano-drug was prepared by adding 50 ml of the drug prepared above dropwise to 50 ml of zinc oxide solution (the result of dissolving 1 g of zinc oxide in 50% ethanol) and stirring the mixture magnetically at room temperature for two hours, then placing the mixture in the vibrating incubator at the temperature is 37°C for 18 hours according to the method described before (Kolekar *et al*.,2013) .

3-2-3: Examination of the hybrid nano-hybrid leflunomide drug:

Several methods were used to examine the hybrid nano-liflunomide drug, including Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), as well as the use of Atomic Force Microscope (AFM) and analysis Flour for elements (C, H, N).

3-3: Induction of arthritis

3-3-1: Induction of arthritis in animals

Induction of arthritis was induced in rats with formaldehyde, where 0.1 ml of formaldehyde at a concentration of 2% was injected into the right hind paw on the first and third days of the experiment according to (Kore *et al*., 2011).

3-4: Immunological criteria

3-4-1:Immunological parameters

3-4-1-1:Tumor necrosis factor and interleukin-6 concentrations were measured

TNF-α was investigated in this study and its levels in the blood serum were measured by ELISA method and according to the kit prepared by the Swiss company ABO. This test uses the immune technology of the Sandwich enzyme according to the method (Abbas *et al*., 2018; Hendy *et al*., 2017).

3-5: Determination of serum Rheumatoid factor

The rheumatic factor test was carried out using the examination kit supplied by the Chinese company CUSABIO. The Latex reagent consists of particles covered with gamma globulin for humans. When the latex reagent is mixed with a serum sample that contains the rheumatic factor, we notice agglutination,according to the method (Mahmoud *et al*., 2021). The results were statistically analyzed to find out the significant differences using one-way analysis of variance ANOVA, and the significant differences between the means were tested using the least significant difference test L.S.D at the level of probability (<0.05).

3. RESULTS

4-1: Immunological parameters

4-1-1: Tumor Necrosis Factor (TNF)

The results of the current study indicated a significant increase ($p \leq 0.05$) in the concentration of TNF in the positive control group G2 that induced arthritis when compared with the negative control group G1, while the group G3 treated with leflunomide in its free form in conjunction with the development of arthritis in it, has It showed a significant decrease in TNF concentration compared to the positive control G2 and a significant increase ($p \leq 0.05$) compared to the negative control, while the G4 group treated with the leflunomide nanocomposite witnessed a significant improvement through the significant decrease ($p \leq 0.05$) in the level of TNF compared to the group G4. The positive control G2 and the free

leflunomide group G3, with the significant difference remaining ($p \leq 0.05$) compared to the negative control group G1, as shown in Table (4- 1).

4-1-2: Interleukin IL-6

As for interleukin IL-6, its concentration showed a significant increase ($p \leq 0.05$) in the positive control group G2 compared to the negative control G1 as shown in the table (4-1). While the concentration of the mentioned criterion witnessed a significant decrease ($p \leq 0.05$) in both groups G3 and G4. Compared with the positive control G2 and a significant increase ($p \leq 0.05$) compared to the negative control G1, also significant differences ($p \leq 0.05$) were observed in the concentration of IL-6 between the groups G3 and G4.

4-1-3: Rheumatic Factor (RF)

The results of the statistical analysis of the concentration of rheumatic factor RF in table (4-1) clearly indicated that there was a significant increase ($p \leq 0.05$) in the concentration of rheumatic factor in the positive control group G2 that induced arthritis compared to the negative control group G1, as it was noted from the results that the synchronization of the creation of Arthritis in the G3 group with the administration of leflunomide in its free form led to a significant decrease in the concentration of rheumatic factor ($p \leq 0.05$) in this group compared with the positive and negative control groups. This decrease in the mentioned criterion in the G4 group, in which the development of arthritis coincided with its dosing with the nanocomposite of the drug leflunomide, increased significantly ($p \leq 0.05$) compared to the groups G2 and G3, and there was no significance with this decrease compared to the normal level of this factor in the negative control group G1 .

Table (4-1) Effect of the anti-arthritis drug free and nano-hybrid leflunomide on immune factors

Groups	**Treatments (Mean±SD)**		
	TNF-α (pg/ml)	**IL-6 (pg/ml)**	**RF (IgM)**
G1	42.675±0.140 **c**	2.264 ±0.010 **d**	7.722 ±0.030 **C**
G2	77.252± 0.062 **A**	8.842±0.042 **a**	12.36 ± 0.037 **a**
G3	69.115 ±0.116 **A**	7.036± 0.023 **b**	10.187±0.014 **b**
G4	55.846 ±0.138 **B**	4.188± 0.016 **c**	8.267 ±0.025 **c**

$LSD_{0.05}$	**8.937**	**1.724**	**1.239**

G1: the negative control group (uninfected rats).
G2: the positive control group (the group of rats with induced arthritis).
G3: A group of rats with arthritis treated 14 days after induction of arthritis with the drug (leflunomide) in its free form at a dose of 20 mg/kg
G4: A group of rats with arthritis treated 14 days after induction of arthritis with the drug in the nano form at a dose of 20 mg/kg.

4. DISCUSSION

We noticed a significant increase in TNF in the positive control group compared to the negative control group, as TNF-α contributes to the inhibition of tissue growth factors by secreting an enzyme called collagenase, which is called the tissue extracellular matrix protein inhibitor of MMP (TIMP) enzyme, which leads to stimulating the erosion of tissues. Bone, cartilage, and synovium degradation in joints in animal models (Robert *et al*., 2016). It also causes dysfunction of B cells, hematopoietic mononuclear cells, T helper cells and increased efficacy of suppressor T cells as they produce several proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α). It is clear that intracellular proinflammatory cytokines play an important role In the pathogenesis of this disease, TNF-abnormally stimulates the differentiation and proliferation of osteoclasts, responsible for bone erosion (McInnes & Schett, 2011), whereby immune cells (lymphocytes) in the inflammatory zone stimulate the production of cytokines, including Tumor necrosis factor (TNF-α), which plays an important role in the occurrence of the disease (Gladman *et al.*, 2005).

We note a significant decrease in the third group G3, which was treated with free leflunomide as a result of the rapid conversion of leflunomide after the first passage to the liver to its active metabolite A77 1726, teriflunomide inhibits dehydrogenase dehydrogenase (DHODH), a mitochondrial enzyme necessary for the de novo synthesis of pyrimidines; Lymphocytes are highly dependent on this pathway for the synthesis of nucleotides, thus inhibiting cell proliferation. Pyrimidines are also essential for lipogenesis and macromolecules essential for many cellular functions (Fox *et al*., 1999).

We show a significant decrease in the G4 group, where the above results are in line with what was shown by the results of the study (Xue *et al*., 2012) that daily treatment with tripterygium-loaded solid lipid nanoparticles reduced the rate of inflammatory responses during the development of rheumatoid arthritis, including alleviation of inflammatory-induced swelling of the feet as well as reduction of other inflammatory markers such as IL-1β, IL-6 and TNF-α in the serum of disease-induced mice.

We also show a significant increase in 1L-6 in the G2 group when compared with the G1 group, where IL-6 is secreted mainly from mononuclear cells, IL-6 stimulates T-cells as well as B-cells for growth and differentiation and induction of antibody production, and in addition, IL-6 activates osteoclasts, and in addition to that IL-6 is a secondary factor in arthritis, studies have shown that giving inhibitors to this cytokine and its receptor will reduce arthritis, and IL-6 It induces A 5acute phase proteins such as **CRP** or may even be considered itself acute phase proteins which are hematological features that are a function of disease activity (Kono *et al*., 1991; Wendling *et al*., 1993).

While we notice a significant decrease in the free leflunomide-treated G3 group compared to the control group where when the inhibition of DHODH by A77-1726 inhibits rUMP

production through the de novo synthesis pathway, the activated lymphocyte undergoes a stop in the G1 phase of the cell cycle. Leflunomide has shown that the observed anti-inflammatory effects of A77 1726 may be related to its ability to selectively suppress interleukin-1 and tumor necrosis factor-1 over their inhibitors in T lymphocyte/monocyte contact activation, and it has also been shown that A77 1726 Inhibits activation of nuclear factor-êB, a potent mediator of inflammation when stimulated by inflammatory factors (Moon *et al*., 2017).

While we observe a significant decrease in G4 attributed to the therapeutic role of the nano-drug and agree with his study showing the effect of stable silver nanoparticles on eliciting anti-inflammatory activity in chronic inflammation through their critical inhibition of the formation of pro-inflammatory cytokines (tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL) -6, developed a gold nanocomplex hyaluronate/tocilizumab (HA-AuNP/TCZ) for the treatment of rheumatoid arthritis., by targeting the interleukin-6 (IL-6) receptor (Zhang *et al*., 2020).

As for RF, we notice a significant increase in the G2 group compared to the control group, and this is consistent with a study where an increase in erythrocyte sedimentation (Mayada *et al*., 2016) is due to the increase in the secretion of interleukin from macrophages, which leads to an increase in the filtration of white blood cells into the bloodstream and an increase in their accumulation and prepared and this result agreed with (Kay & Calabrese, 2004).

It stimulates T cell cells, which is evidence of inflammation, and protein C stimulates tumor necrosis factor and fibrinogen, which increases the rate of sedimentation of blood cells, and cytokines also reduce red blood cell production, thus reducing ferritin, which is the iron depot in the body, and thus the occurrence of anemia. Normal iron in the endothelial reticulum and bone marrow failure to compensate for the response to anemia may be the cause of higher erythrocyte sedimentation rate compared to the control group where excessive production of reactive oxygen species (ROS) damages cellular components leading to apoptosis, in rheumatoid arthritis.

Significant decrease in the G3 group a low dose of this drug has been shown to be effective in controlling the inflammatory manifestations of rheumatoid arthritis. This is attributed to the targeting of reactive oxygen species production centers from inducible inflammatory cells that lead to apoptosis. Thus, inhibiting their interaction with Synovitis, also reduces the production of pro-inflammatory cytokines and mediators of inflammation, especially interleukin-6 and tumor necrosis factor-14. The current study demonstrated that use of DMARDs by rheumatoid arthritis patients is associated with reduced inflammation and changes in lipids.

And a decrease in the G4 group, where nanotherapy works to inhibit pro-inflammatory cytokines and also: protect the therapeutic agent from decomposition, and stay in the circulation for a longer period, and it is designed to take macrophage cells to target specific receptors and permeate through some diseased tissues because the cell vacuoles between the epithelium are Generally 1-2 nm in healthy tissue (Yang *et al*., 2021) but can reach 600 nm in diseased tissue (Zhang *et al*., 2020).

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