Amelioration of Autoimmunity and Inflammation by Zinc Oxide Nanoparticles in Experimental Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects the lining of the synovial joints and approximately affects 0.5-1% of the total population imposing a socioeconomic burden. Currently, there is no cure for RA, but receiving proper medical care at early stages of the disease is of high importance, to prevent the progressive disability and premature death. Using rat animal model injected with Complete Freund's adjuvant proved to be successful in induction of a state highly resembling RA in human. Zinc oxide nanoparticles (ZnO NPs) are considered as one of the most important metal oxide nanoparticles due to their exclusive properties, and they are currently merged in several biological applications due to their biocompatibility, low cost, and high safety profile. In this study, we demonstrated the novel possible beneficial effects of using zinc oxide nanoparticles, on such devastating severe disease. Zinc oxide nanoparticles (ZnO NPs) proved to reduce the adjuvant-induced increased productions of IL-1β, TNF-α, IL-10, total leukocyte count, rheumatoid factor, anti-CCP levels in rats, suggesting an interesting option to be available either alone or in combinations to better control RA.

In conclusion we recommend the expansion of more in vivo studies to highlight the benefits which could be obtained of nanoparticles either alone or in combination with the known anti-arthritic and/or anti-inflammatory agents; giving rise to new protocols to maximize the control of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that approximately affects 0.5-1% of the total population (Silman and Pearson 2002; Moreland 2005; Alivernini et al. 2019). Clinically RA is characterized by the presence of persistent symmetrical joint affection including arthralgia, redness, swelling, stiffness, and decreased range of motion (Guo et al. 2018). It is not only characterized by its disabling progressive articular damage, but also by the presence of extra-articular manifestations in some cases including hematological, skin, cardiac, ocular, and pulmonary manifestations (Dai et al. 2018; Muravyev 2018). Currently, there is no cure for RA, but receiving proper medical care at early stages of the disease are of high importance, and can greatly improve the patient's life by either stopping or retarding the progression of the joint's erosion, and therefore reducing the severity of the disease, or even reaching a state of remission (Heidari 2011). The occurrence of RA seems to be influenced by both genetic and environmental factors; where individuals with genetic predisposition are at higher risk of having RA if exposed to certain environmental agents e.g. smoking, infection, and obesity (Croia et al. 2019).

Pathogenesis of RA is very complicated. Immune system activation and the presence of some pathological manifestations depend on both the adaptive and innate immune pathways, besides the presence of cytokines, growth factors, and intracellular signaling molecules. Certain molecules of the innate immune system such as macrophages, mast cells, and natural killer cells, are important in this process. Macrophages secrete TNF-α, IL-1, IL-6, which are involved in the release of matrix degradation enzymes, phagocytosis, and antigen presentation (Gibofsky 2012). T-cells, B-cells and macrophages are highly involved in the pathogenesis of RA, for example B-lymphocytes have critical roles to play in this

process, as they are the source of the Rheumatoid Factors (RF) and Anti-citrullinated protein auto-antibodies, which contribute to immune complex formation and complement activation (Silverman and Carson 2003; Kay and Calabrese 2004). Fibroblast like synoviocytes (FLS) in the synovial lining of the joints also has a key role in the pathogenesis, as activated FLS has the ability to produce several mediators, and activate several molecules e.g. matrix metalloproteinases (MMPs), and other proteolytic enzymes that have a role in this destructive process (Kay and Calabrese 2004; Bartok and Firestein 2010; Dai et al. 2018). Interleukin-1 (IL-1) & tumor necrosis factor (TNF) are two of the most important pro-inflammatory cytokines, that have several roles to play in the progression of RA (Kay and Calabrese 2004).They have the ability to activate each other, triggering the release of matrix metalloproteinase (MMP) and other proteinases leading to several devastating articular injuries. They can also stimulate the production of chemokines, enhance the differentiation and the activation of osteoclasts, up-regulate the expression of pro-inflammatory genes like cyclooxygenase 2, nitric oxide synthase (Schiff 2000; Kay and Calabrese 2004). Currently RA management strategies and options are expanding; due to the discovery of several new treatment options, starting from the classical methods of treatment, passing by the conventional synthetic disease modifying anti-rheumatic drugs (DMARDs), and ending up to the biological response modifiers (BRMs) (National Institute for Health and Clinical Excellence 2018). Many novel molecules are now designed to target specific cytokines and/or their receptors especially TNF- α, IL-1β based on their pivotal role in the inflammatory response and as a consequence on the disease progression (Cascão et al. 2012).

Nanoparticles (NPs) have been extensively studied for their beneficial effects in several fields for example: food, cosmetics, and agriculture industries. Concerning the medical research field, NPs have been used based on its astonishing properties, as they have a high penetration power, superb ligand-binding properties, high safety margins, and some of them can also have distinctive powerful antimicrobial, anti-inflammatory, anti-oxidant, and anti-angiogenic properties (Agarwal et al. 2019). Zinc oxide nanoparticles (ZnO NPs), are considered as one of the most important metal oxide nanoparticles due to their exclusive properties, and they are currently merged in several biological applications due to their biocompatibility, low cost, and high safety profile (Jiang et al. 2018). It has been identified that they work against inflammation by several ways. As reported, zinc itself can strengthen the antioxidant defense mechanisms; through the activation of some antioxidant proteins and enzymes e.g. catalase (Jarosz et al. 2017). Their anti-inflammatory properties have been attributed to their ability to suppress the production of nitric oxide, nuclear factor kappa B (NF-κB), caspase-1 which is responsible for the activation of the pro-IL-1β, and as a consequence, they have the ability to suppress the production of Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) (Lawrence 2009; Jiang et al. 2018; Agarwal et al. 2019). In this study, we tried to shed the light on the possible beneficial effects of using zinc oxide nanoparticles on the rate of RA progression, and trying to determine the probable mechanisms behind these effects. We successfully set a model of RA by using 0.1 ml complete Freund's adjuvant (CFA) in the left hind paw followed by 0.1 ml in the root of the tail. We found that ZnO NPs significantly decreased the production of TNF-alpha, IL-1β, Rheumatoid Factor, and Anticyclic citrullinated peptide (Anti-CCP) in the serum of the rats.

2. Materials And Methods

2.1. Materials and Animals:

2.1.1. Chemicals

ZnO NPs were supplied by the Egyptian Atomic Energy Authority; Diclofenac ampoules were obtained from Novartis. Complete Freund's adjuvant (CFA), Cell suspension [F5881] from Sigma-Aldrich, each 1 ml contains 1 mg of heat killed and dried Mycobacterium tuberculosis [H37Ra, ATCC25177], 0.15 ml mannide monooleate and 0.85 mL paraffin oil. Equipment including centrifuge, refrigerator, test tubes, ELISA kit and a caliper were used.

2.1.2. Animals

Twenty-four male wistar albino rats were used, weighing from 150 to 200 g. They were housed six rats in each cage and they had free access to commercial diet and tap water. Handling of the rats was done according to the local ethics committee guidelines (PH1/EC1/2020PD) that comply with the international laws for the care and use of laboratory animals.

2.2. Methodology

2.2.1. Zinc oxide nanoparticles preparation

Gallic acid solution of concentration 1mg/1ml was prepared. Zinc oxide (24 mg) was dissolved in 15 ml bi distilled water. Gallic acid solution was heated at 40^{o}C then Zn solution was added with continuous stirring for 2 h. The reaction parameters, such as pH, reaction time, reaction temperature and molar ratios, were optimized till the pale-yellow color of ZnO NPs solution was observed. ZnO NPs was characterized and confirmed by TEM: ZnO NPs was prepared with average size 22 nm **(figure s1).**

2.2.2. Rheumatoid arthritis induction:

The induction of rheumatoid arthritis in rats was done using complete Freund's adjuvant. Rats were injected with 0.1 ml S.C. CFA in the left hind paw sub-planter region, followed by 0.1 ml in the root of the tail. On the second day, they were injected with another dose of 0.1 ml CFA in the root of the tail (A Ali et al. 2016).

2.2.3. Study design:

The rats were allowed to acclimate for one week. After that, they were divided randomly into 4 groups; each group containing 6 rats. Group 1 was kept as a normal group, while group 2 was injected with 0.1 ml CFA in the left hind paw followed by two doses of 0.1ml in the root of the tail, one on the same day and the other on the second day for the induction of rheumatoid arthritis. Groups 3, 4 were injected with CFA and then the treatment started the day after the induction as follow: Group 3 treated by oral dose of Zn

oxide nanoparticles 2 mg/kg/day daily for 14 days; group 4 treated by i.p. dose of diclofenac Na, 1mg/kg/day the day after CFA daily for 14 days.

2.2.4. Collection and processing of samples

The collection of blood samples was done under light anesthesia, from the tail vein. They were collected in non-heparinized tubes, and then centrifuged for 15 minutes at 5000 rpm to separate the serum. The obtained serum was then collected in eppendorff tubes and stored at -20º C for biochemical estimation (Ahmed WMS 2015).

2.2.5. Biochemical parameters:

For the assessment of the inflammation that accompanies the disease, we determined the levels of tumor necrosis factor α [TNF α] using ELISA Kit (Cat. No. CSB-E1218r); interleukin 10 [IL-10] using ELISA kit (Sandwich Cloud clone Corp.; Cat. No. SEA-0169), interleukin-1beta [IL-1β] using ELISA kit cohesion biosciences (Cat. No. # CEK1976), rheumatoid factor [RF] (Sandwich ELISA; Cat. No. MBS720877), total leucocyte count and Anti- CCP using ELISA kit (E-EL-R1521) in the serum (Viana et al. 2012).

2.2.6. Statistical analysis:

Data are presented as the mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons. Significant difference was considered at $P \leq 0.05$. Data analysis and graphs presentation were carried out using Graph Pad Prism software version 6.

3. Results

3.1 Biochemical Parameters:

3.1.1 Effect of ZnO NPs on TNF-α, IL-1 β and IL-10 in CFA-induced rheumatoid arthritis in rats

The levels of the inflammatory mediators (TNF-α, IL-1 β and IL-10) in the control group showed a significant elevation (150.4 ± 15.92, 374.1 ± 79.60, 234.5 ± 42.66) respectively in comparison to the normal group (85.13 ± 10.34, 111.1 ± 12.09, 40.91 ± 5.470) respectively, while in the treated groups with ZnO NPs (129.8 ± 13.26, 118.6 ± 15.23, 69.76 ± 9.756) respectively and the standard treatment group with diclofenac (106.3 ± 9.32, 118.9 ± 26.63, 39.78 ± 5.814) respectively were significantly lowered as shown in Fig. 1.

3.1.2. Effect of ZnO NPs on Anti CCP, RF and Total leucocytes in CFA-induced rheumatoid arthritis in rats

Evaluation of Anti-cyclic Citrullinated Peptide in the control group (50.55 ± 6.819) showed a significant elevation in comparison to the normal group (17.77 ± 2.237), while in the treated groups with ZnO NPs (35.71 ± 3.785) and the standard treatment group with diclofenac (28.11 ± 3.991) were significantly lowered as shown in Fig. 2.

Rheumatoid factor levels in the control group showed a significant elevation (33.30 ± 4.309) in comparison to the normal group (18.72 ± 2.632), while in the treated groups with ZnO NPs (30.17 ± 0.6524) and the standard treatment group with diclofenac (22.96 ± 1.842) were significantly lowered as shown in Fig. 3.

Total Leucocytes counting in the control group (9628 ± 864.2) showed a significant elevation in comparison to the normal group (3154 ± 438.1), while in the treated groups with ZnO NPs (4714 ± 382.0) and the standard treatment group with diclofenac (3920 ± 785.1) were significantly lowered as shown in Fig. 4.

4. Discussion

Adjuvant- induced arthritis in rats has been approved as an experimental model to study the pathogenesis of human rheumatoid arthritis (RA) (Halloran et al. 1996; Pan et al. 2017). In the present study, complete Freud's adjuvant (CFA) induced arthritis in male Wistar albino rats since elevated levels of total leukocyte count, rheumatoid factor, anti-CCP antibody and inflammatory cytokines were observed in sera of studied rats. Rheumatoid arthritis is characterized by three stages, autoimmunity, chronic inflammation and joint destruction. The autoimmunity phase involves autoantibodies (rheumatoid factor and anti-CCP) that are produced specific for IgG and is the earliest phase which is often referred to as the pre-articular phase because it precedes the inflammatory and articular destruction phase (McInnes and Schett 2007).

Zinc supplements have been proven to produce beneficial effects in elder population due to reduction of inflammation and oxidative stress (Prasad 2014). Similarly zinc oxide nanoparticles have been recently reported to exhibit various anti-inflammatory effects like reversing LPS-induced liver cell injury and human mononuclear cells inflammation (Prasad 2014; Kim and Jeong 2015). In the present study, zinc oxide nanoparticles (ZnO NPs) reduced the adjuvant-induced increased productions of IL-1β, TNF-α, IL-10, total leukocyte count, rheumatoid factor, anti-CCP levels in rats.

Tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) are central inflammatory cytokines involved in the pathogenesis of rheumatoid arthritis (RA), since they are involved in bone resorption and cartilage remodeling through maturation of osteoclast cells and inhibition of collagen synthesis (Goldring 2003). In addition, they augment several inflammatory mechanisms such as angiogenesis, fibrosis and cell adhesion (Szekanecz et al. 2000). In the current study ZnO nanoparticles suppressed the serum levels of these cytokines to a level comparable to that of diclofenac Na, the standard drug. This effect might be due to the ability of ZnO NPS to suppress NF-κB nuclear translocation and increasing A20 levels which down regulates NF-κB and eventually inhibiting TNF-α and IL-1β production (Agarwal et al. 2019).

Interleukin 10 is an immunoregulatory cytokine that plays a key role in prevention of tissue damage due to inflammation. Autoimmune diseases show high level of this cytokine during the early stages of the disease, however, immune equilibrium is reached during later stages (Mingomataj and Bakiri 2016). In the present study, IL-10 was elevated after two weeks post injection of CFA, however its level might not have been sufficient to counteract the elevated levels of proinflammatory cytokines namely TNF-α and IL-1β.

ZnO NPs were able to reduce the level of IL-10 and this effect was comparable to that of the standard drug.

Rheumatoid factor (RF) is an autoantibody which targets the Fc region of IgG and is the first antibody discovered in RA. It was reported to contribute to the pathogenicity of the disease by enhancing immune complexes formation therefore increasing the arthritogenicity of itself and other autoantibodies like anti-CCP (Song and Kang 2010). In the current study, treatment with ZnO NPs was able to reduce the level of RF, however unlike the standard drug diclofenac Na, its effect was non-significant.

Anti-cyclic citrullinated peptide (anti-CCP) has a higher specificity to RA than RF and is detected before the clinical development of RA. Induction of RA involves the formation of citrullinated proteins within the joint like fibrin, vimentin and collagen followed by a local chronic immune response that provokes the erosive disease (Kinloch et al. 2006; Niewold et al. 2007). In the present study, ZnO NPs strongly reduced the level of anti CCP and its effect was more potent than diclofenac Na.

Taken together these results suggest the anti-arthritic and anti-inflammatory activity of ZnO NPs and their potential usefulness as a promising tool for the treatment of RA.

Declarations

Ethics declaration

Conflict of interest: The authors declare that there is no conflict.

Ethics approval and consent to participate:

All experimental procedures adopted for in vivo studies were in accordance with to the local ethics committee guidelines at Modern Sciences and Arts University (PH1/EC1/2020PD) that comply with the international laws for the care and use of laboratory animals.

Contribution:

AM Fayez and S Shabaan conceived and designed research. AM Fayez and M Abdelaziz conducted the experiment. D Abou El-ezz, S Shabaan and M Abdelaziz analyzed the data and wrote the manuscript. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Figures

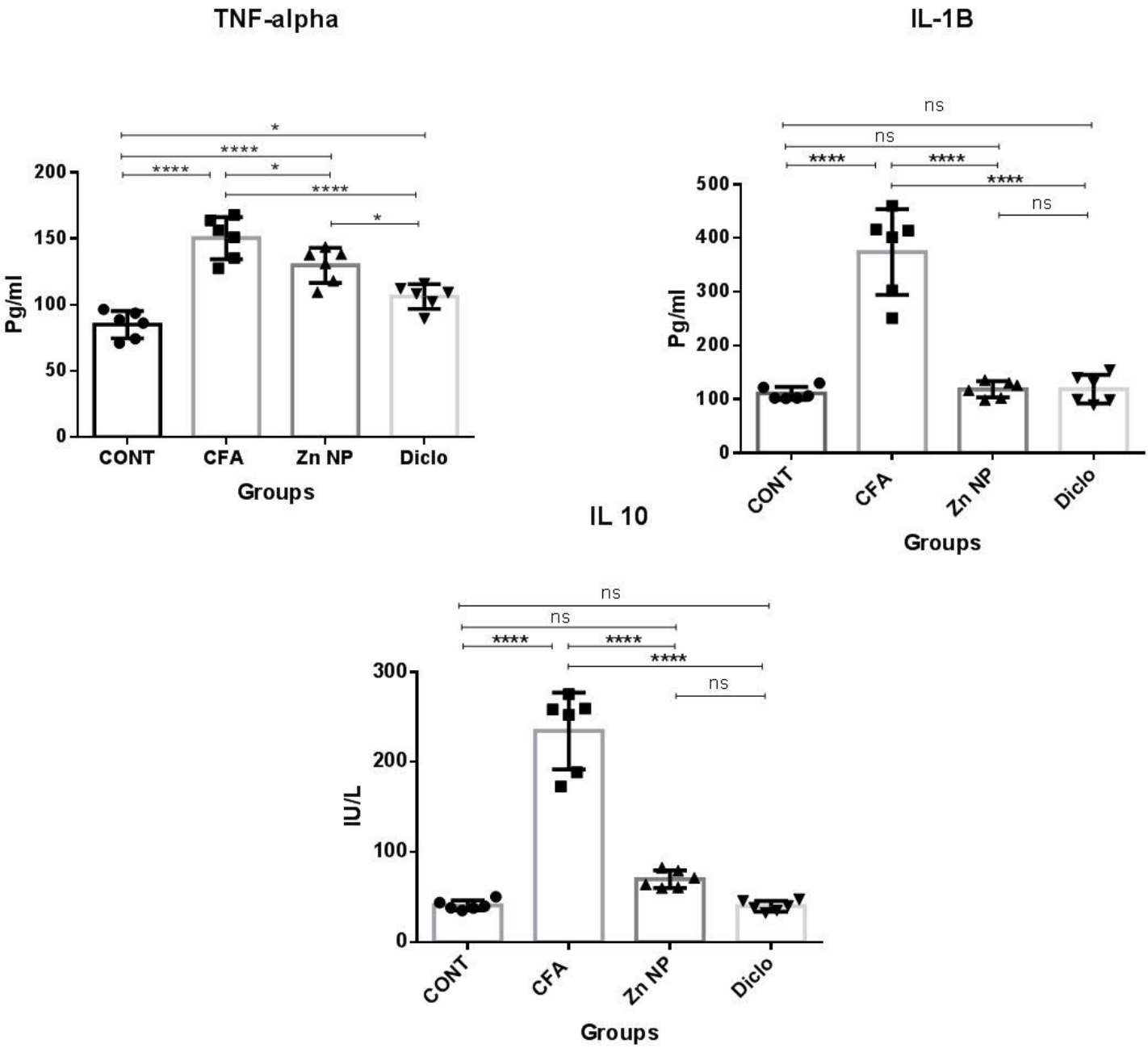

Figure 1

: Effect of ZnO NPs on TNF-α, IL-1 β and IL-10 in CFA-induced rheumatoid arthritis in rats. - Data are presented as the mean ± SD. - Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons.

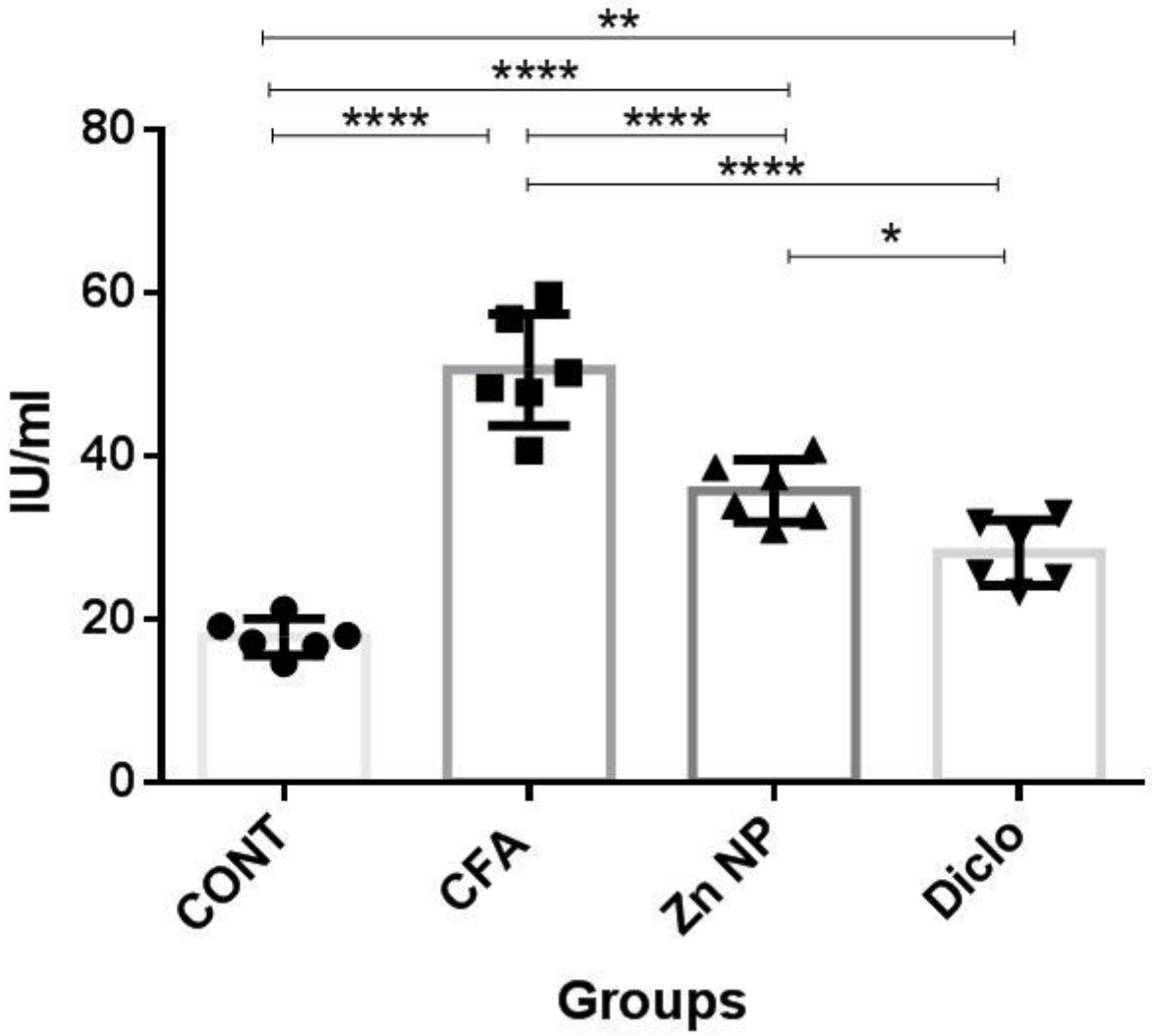

Figure 2

Effect of ZnO NPs on Anti CCP in CFA-induced rheumatoid arthritis in rats - Data are presented as the mean ± SD. - Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

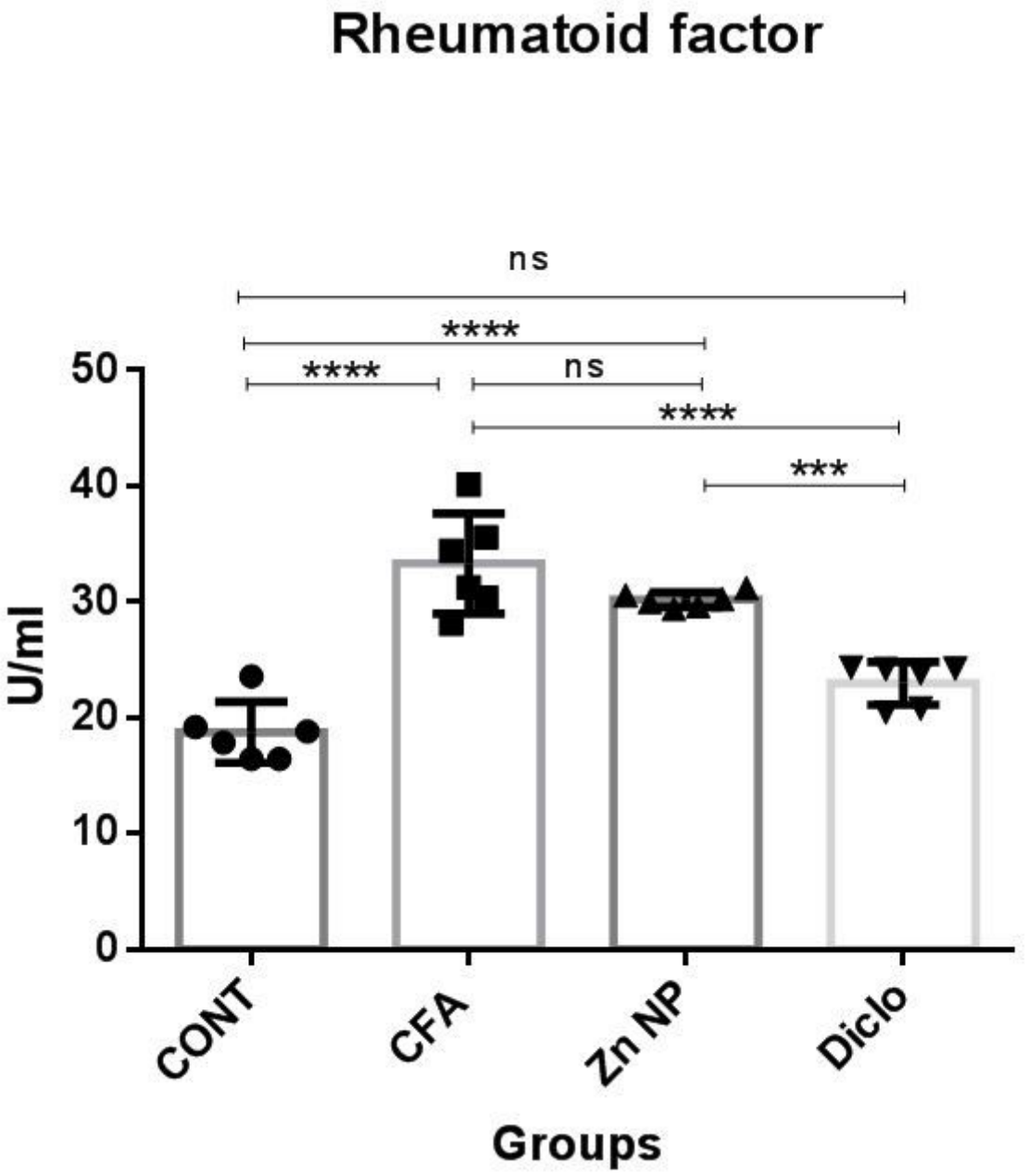

Figure 3

Effect of ZnO NPs on RF in CFA-induced rheumatoid arthritis in rats - Data are presented as the mean ± SD. - Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

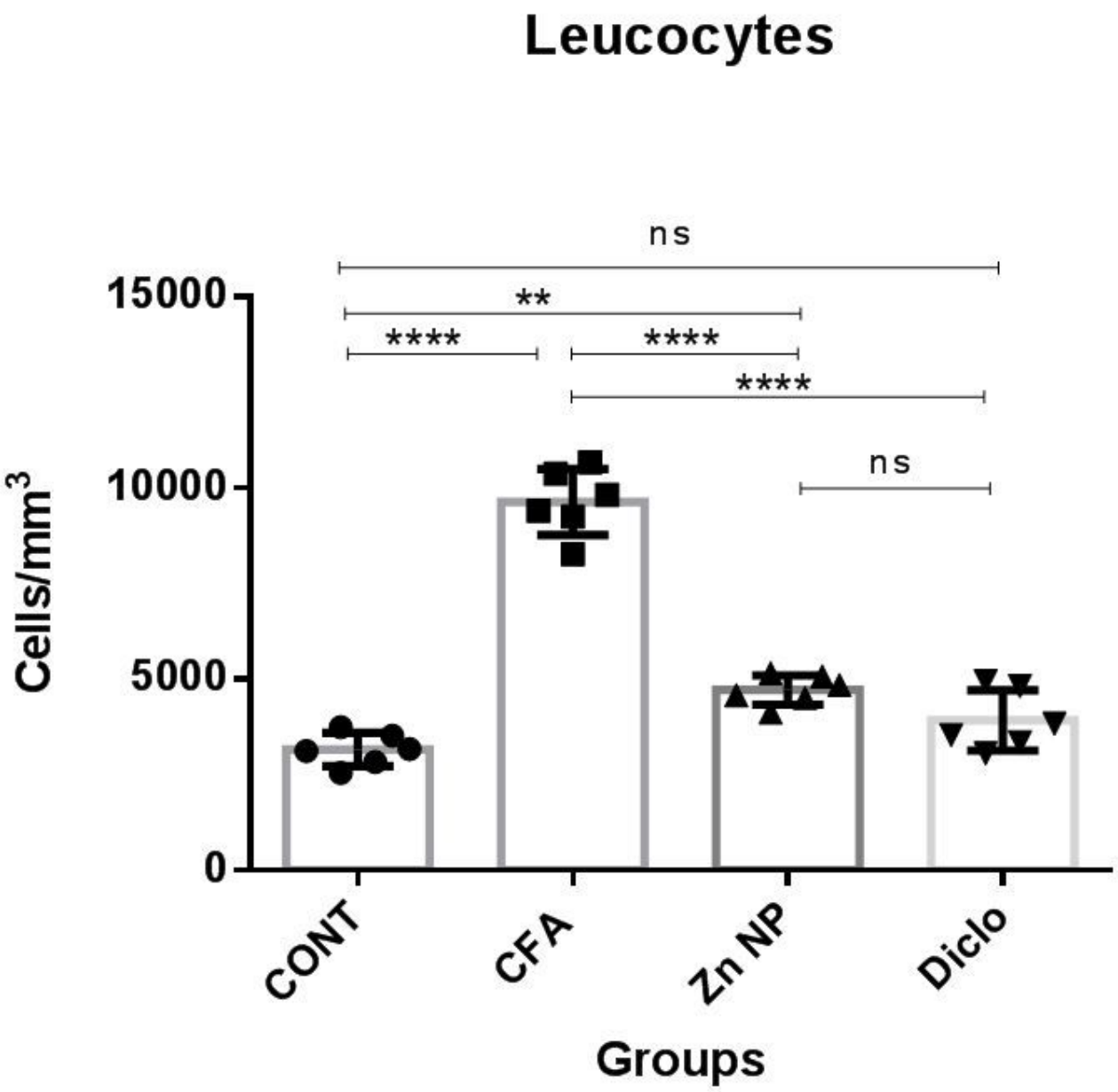

Figure 4

Effect of ZnO NPs on Total leucocytes in CFA-induced rheumatoid arthritis in rats - Data are presented as the mean ± SD. - Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

Supplementary Files

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- sfig1.jpg