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Original Article

Improving the anti-inflammatory/anti-angiogenic properties of gold nanoparticles in the treatment of experimental rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease with no apparent cure. There is a continued need for the development of new therapeutic strategies. Nanotechnology has been a proven effective route toward novel and fascinating biomedical applications. The application of gold nanoparticles (AuNPs) in RA treatment is still curious. Thus, this study was designed to evaluate the anti-inflammatory and anti-angiogenic potential of spherical AuNPs with different sizes (5, 25, and 75 nm). Collagen-induced arthritis (CIA) rats were divided into CIA model, AuNP, and methotrexate (MTX)-treated groups. Untreated rats were used as the control group. Pro-inflammatory/anti-inflammatory and angiogenic mediators were measured by enzyme-linked immunosorbent assay (ELISA). A remarkable increase in proinflammatory cytokines [IL-6, IL-17, tumor necrosis factor (TNF-α), and interferon (IFN-γ)] coincides with a remarkable reduction in IL-4, IL-10, and transforming growth factor (TGF-β) anti-inflammatory mediators was observed in the CIA group compared to normal animals. A significant increase in vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) levels was found in the untreated CIA group. Our results confirm the anti-inflammatory properties of AuNPs via downregulating pro-inflammatory (IL-1β, IL-17, IL-23, IL-12, TNF-α, and IFN-γ) and angiogenic mediators (VEGF and PDGF) and increasing anti-inflammatory cytokine (IL-4 and TGF-β) secretion. Our preliminary findings suggest that AuNPs with a diameter of 25 nm could be used as a possible nanotherapeutic agent for RA. In comparison to methotrexate, AuNPs (25 nm) have improved anti-inflammatory/ anti-angiogenic properties in the treatment of experimental rheumatoid arthritis. AuNPs have the potential to be exploited as a novel therapy for RA, bringing a new era in the treatment of autoimmune diseases.

KEYWORDS: rheumatoid arthritis, collagen-induced arthritis, gold nanoparticles, cytokines.

1. INTRODUCTION

RA is a chronic autoimmune disease that mainly affects the joints, leading to joint inflammation and erosive structural damages [1]. It is the most prevailing form of inflammatory arthritis, and globally, approximately 1% of the population is affected by the disease [2]. It is mostly accepted that RA is a multifactorial disease that can betriggered by both environmental and hereditary factors. The interaction between genetic predispositions and

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ecological stimuli would activate both adaptive and innate immune systems [3].

RA has been treated with disease-modifying anti-rheumatic drug (DMARD), especially methotrexate (MTX), but in the last few years, the introduction of biological therapeutics as cytokine antagonists, B cell-depleting agents, and T cell co-stimulation modulator has revolutionized the treatment of RA [4, 5]. Although various drugs have been developed for arthritis treatment, the long-term use of the current therapies will ultimately lead to compromised effectiveness and severe adverse events [6]. Hence, the design of novel therapeutic strategies which inhibit inflammation and reduce joint destruction is still required.

Cytokines are local protein mediators, regulating almost all important biological processes, including cell growth and activation, inflammation, immunity, and differentiation [7]. It is not surprising that they have a role in several autoimmune diseases such as RA. They may participate in chronic inflammation, fibrosis, and the eventual destruction of cartilage and bone associated with these diseases [8, 9]. Angiogenesis plays an essential role in RA pathogenesis. It is a crucial event in the formation and maintenance of the pannus in RA. The newly formed vessels promote synovial inflammation persistence by transporting the inflammatory cells to the site of synovitis and supplying nutrients and oxygen for the hyperplastic synovium [10].

Nanoparticles (NPs) are the fundamental building blocks of nanotechnology and have a broad range of biomedical implementations [11]. To date, many NPs have been synthesized, particularly those made from noble metals such as gold [12]. Gold salts (as Auranofin) have effectively treated inflammatory arthritis and severe RA; however, these pharmaceuticals have undesirable side effects [13]. Lately, research on the medicinal chemistry of gold compounds has focused on developing novel compounds with better performance and/or fewer side effects than existing ones [14].

As an emerging nanomedicine, gold nanoparticles (AuNPs) have received considerable attention because of their unique properties such as biocompatibility, high surface reactivity, and flexibility in functionalization [15]. In addition, it has been shown that AuNPs display anti-inflammatory, immunomodulatory, and anti-angiogenic activities [6].

Our latest findings [16] confirmed the AuNPs' anti-arthritic capabilities and their ability to diminish RA clinical symptoms without causing severe toxicity. AuNPs with a diameter of 25 nm is the most effective treatment among the sizes studied, with a considerable reduction in inflammation, bone and cartilage erosion, and prevention of pannus development. As a result, our former findings suggest that AuNPs with a diameter of 25 nm could be used as a possible nanotherapeutic for the treatment of RA. In continuation, the present study focuses on the ability of polyethylene glycol (PEG)-AuNPs to ameliorate the production of inflammatory/anti-inflammatory and angiogenic mediators in collagen-induced arthritis (CIA) in rats.

2. MATERIALS AND METHODS

2.1. Synthesis of AuNPs

Frens method with modifications was employed to prepare spherical citrate-capped AuNPs with various diameters. The synthesis procedure and characterisation of the PEG-AuNPs produced are described in detail in our prior report [16].

2.2. Animals

Sixty female Wistar rats (6-8 weeks old) weighing 100-120 g were purchased from Modern Veterinary Office, Egypt. They were housed in the Genetic Engineering and Biotechnology Research Institute University of Sadat City (USC), Sadat City, Egypt. Animals were maintained at 25 ^{o}C with an alternating 12-hour light/dark cycle. They were allowed access to food and water *ad libitum* throughout the acclimatization (around one week before the experiment) and experimental period. The experimental procedure was performed on the experimental animals following the approval of Sadat city University Animal Experiments Local Ethics Committee and the Guidelines for the Care and Use of Laboratory Animals. The Sadat city University Ethics Committee granted ethical approval (No. 19 06 2019, Sadat, Egypt).

2.3. Induction, assessment, and treatment of collagen-induced arthritis

Bovine type II collagen (CII) (Chondrex, 2002, Redmond, WA, USA) was dissolved at 2 mg/ml in 0.05 M acetic acid by gently stirring overnight

at 4 ^{o}C. One volume of incomplete Freund's adjuvant (IFA) (Chondrex, 7002, USA) was mixed in a glass tube with an equal volume of CII using a homogenizer until a stiff emulsion resulted. CIA model was initiated as previously described [17]. Wistar Rats were injected intra-dermally at the base of the tail with 200 µl of CII/IFA emulsion on day zero. Eight days after, a booster dose was injected intra-dermally on the other side of the tail with 100 µl emulsion of CII/IFA. On day 11, CIA rats were divided into five groups (10 rats/group): Positive control group (CIA rats without treatment), AuNp groups (CIA rats were injected intra-articularly with 6.5 µg of AuNPs of three different sizes (5 nm, 25 nm, and 75 nm) and MTX-treated group (rats received four intraperitoneal injections of MTX (1 mg/kg) on days 11, 17, 24, and 31). Moreover, normal rats (neither immunized nor treated) were used as the negative control group. Histopathological and radiographic analyses of the anti-arthritic effect of AuNPs are presented in Supplementary Figures 1 and 2, respectively.

2.4. Measurement of pro-inflammatory, anti-inflammatory, and angiogenic mediators

At the end of the experiment (day 35), rats were subjected to light ether anesthesia; blood samples were withdrawn by retro-orbital bleeding and collected in an EDTA-centrifuged tube. The plasma was separated by centrifugation at 2000 rpm for 15 min at 4 °C, aliquotted, and stored at -80 °C. Several pro-inflammatory cytokines (IL-1β, IL-6, IL-17, IL-23, TNF-α, IFN-γ, and IL-12), and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) and angiogenic mediators (VEGF and PDGF) were quantified by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Bostor Biological Co, Pleasanton, CA) according to the manufacturer's instructions. Absorbencies were measured at 450 nm using an ELISA plate reader (Sunrise, Tecan Group Ltd.). The data were analyzed using the Magellan software. Results were expressed as picogram of cytokine per milliliter (pg/ml).

2.5. Statistical analysis

Data were statistically presented in terms of mean ± standard error (SE). Differences between groups were assessed using a one-way analysis of variance (ANOVA) followed by the Tukey test as a post-doc test. The correlation between variables was determined using the Person's correlation test. P-values of <0.05 were considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 19 (LEAD Technology Inc).

3. RESULTS

3.1. AuNP synthesis and conjugation

As previously reported by Abdel-Hakem *et al.* [16], the prepared AuNPs exhibited typical suspension colors: orange-red, red, or violet for the 5, 25, and 75 nm AuNPs, respectively. The UV-vis spectra have a plasm on peak at 508, 525, and 576 nm for the 5, 25, and 75 nm AuNPs, respectively. The size and shape were further confirmed using transmission electron microscopy analysis. PEGylation of the surface of these AuNPs was carried out by displacement chemistry using thiolated PEG, which displaces the physically adsorbed citrate anions from the surface of AuNPs via a thiol-gold solid bond. The Pegylation of AuNPs is a well-known procedure to render these particles hydrophilic with excellent colloidal stability and biocompatibility. The displacement reaction was confirmed by a change in zeta potential for all NPs from a negative origin for the citrate-capped counterparts to a neutral effective surface charge for all NPs.

3.2. Effect of AuNPs on pro-inflammatory cytokines

As shown in Figure 1, a remarkable increase in IL-6, IL-17, TNF-α, and IFN-γ levels ($P<0.01$, $P<0.05$, $P<0.01$, and $P<0.001$; respectively) was observed in the CIA group compared to normal animals. A slight increase in IL-1β and IL-23 secretion levels was observed; however, it is statistically insignificant.

Significant ($P<0.05$) reduction in IL-17 and TNF-α was demonstrated in arthritic rats treated with 5 nm AuNPs. CIA rats treated with 25 nm AuNPs showed a diminution in the plasma levels of IL-23 ($P<0.05$); TNF-α ($P<0.001$); IFN-γ ($P<0.01$), and IL-12 ($P<0.001$) compared to the untreated one. The results in Figure 1 demonstrate that the 75 nm AuNP-treated group significantly reduced IL-1β ($P<0.01$), TNF-α ($P<0.001$), and IL-12 ($P<0.05$) when compared with CIA animals. Treatment with MTX decreased IL-1β ($P<0.001$), TNF-α ($P<0.001$), and IL-12 ($P<0.001$) compared to untreated CIA rats. No significant change was observed in IL-6

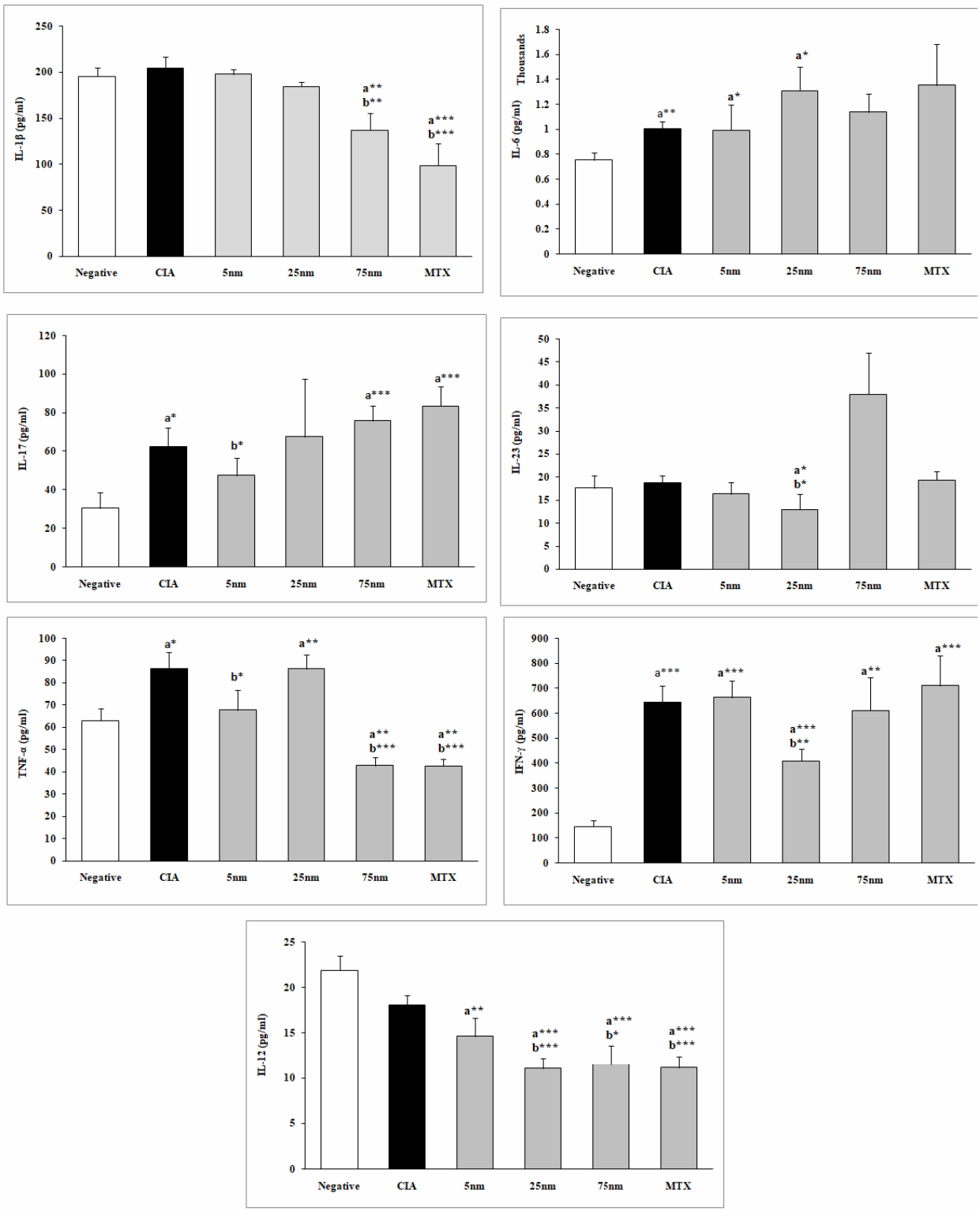

Figure 1. Plasma level of proinflammatory cytokines in CIA-untreated and treated rats. Results are expressed as mean ± standard error. 'a' denotes groups statistically significant from negative controls; 'b' denotes groups statistically significant from CIA (positive control group). *P<0.05, **P<0.01, ***P<0.001.

production in all treatment modalities compared with untreated rats.

3.3. Effect of AuNPs on angiogenic mediators

The results in Figure 2 show a significant increase in VEGF and PDGF levels in the untreated CIA group compared to their normal control counterpart (P<0.05, P<0.001; respectively). CIA rats treated with 5- and 25-nm AuNPs showed a significant reduction in VEGF (P<0.05, P<0.001) and PDGF (P<0.001, P<0.05) in relation to untreated animals. Regarding the CIA group, treatments with MTX decreased both VEGF (P<0.01) and PDGF (P<0.001).

3.4. Effect of AuNPs on anti-inflammatory cytokines

As illustrated in Figure 3, a remarkable reduction in IL-4, IL-10, and TGF-β levels was observed in the CIA group (P<0.001, P<0.01, and P<0.001, respectively). A notable up-regulation of IL-4 (P<0.05) and TGF-β (P<0.001) was found in 25nm-treated rats in relation to the untreated CIA group. Regarding the 75 nm AuNPs group, only TGF-β (P<0.001) was increased compared with CIA rats. Treatment of CIA rats with MTX up-regulated IL-4 (P<0.001) and TGF-β (P<0.001) in relation to untreated CIA group.

3.5. Correlation between pro-and anti-inflammatory cytokines and angiogenic mediators

A positive correlation was observed between IL-1β and IL-17 (r=0.915; P<0.01), and VEGF and IL-6 (r=0.826; p<0.05) in CIA animals. In contrast, a negative correlation was noticed between VEGF

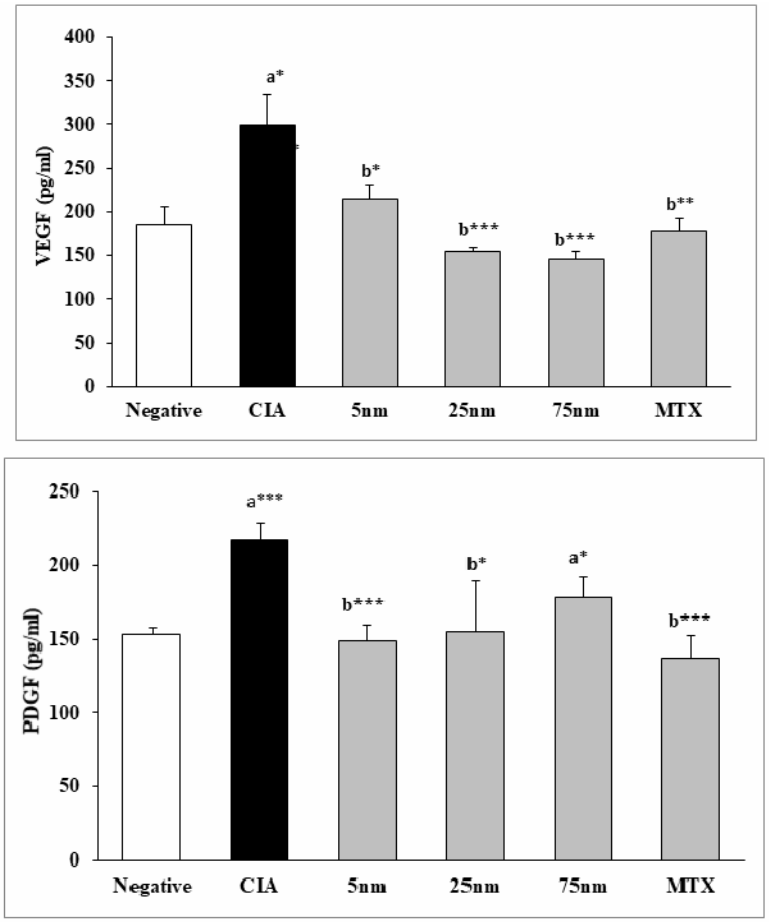

Figure 2. Plasma level of angiogenic mediators in CIA-untreated and treated rats. Results are expressed as mean ± standard error. 'a' denotes groups statistically significant from negative controls; 'b' denotes groups statistically significant from CIA (positive control group). *P<0.05, **P<0.01, ***P<0.001.

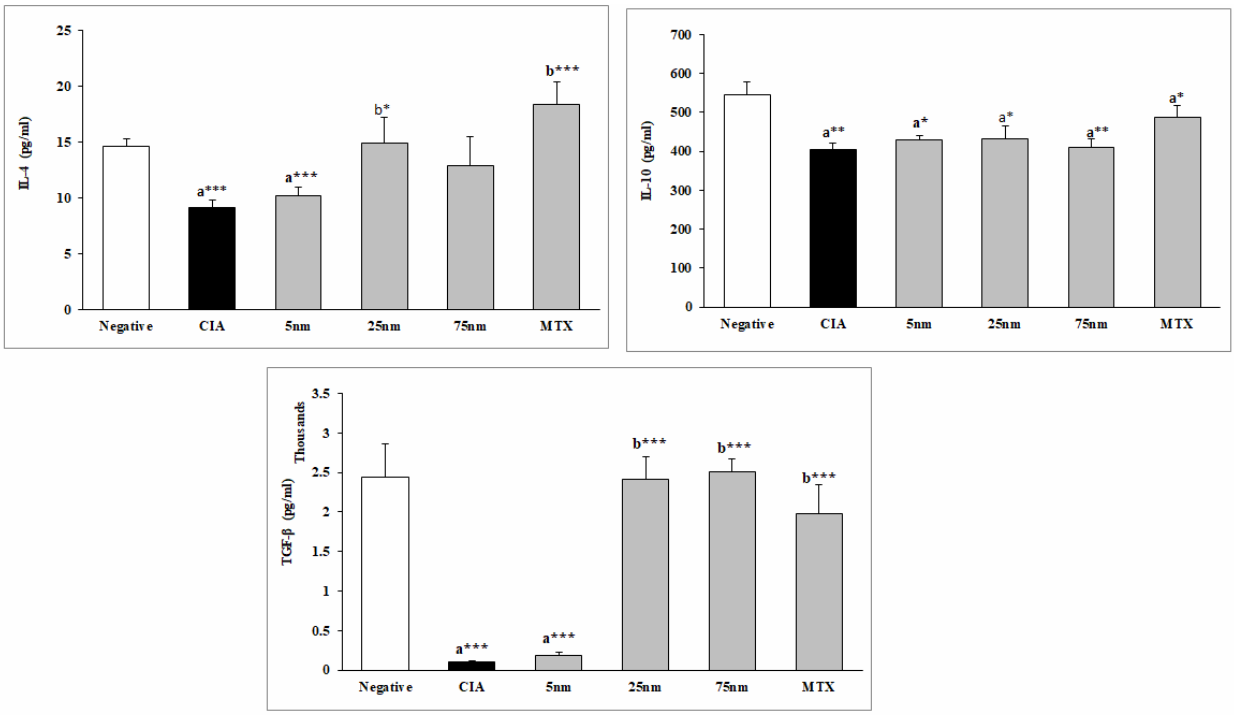

Figure 3. Plasma level of anti-inflammatory cytokines in CIA-untreated and treated rats. Results are expressed as mean ± standard error. 'a' denotes groups statistically significant from negative controls; 'b' denotes groups statistically significant from CIA (positive control group). *P<0.05, **P<0.01, ***P<0.001.

and IFN-γ (r= -0.825; P<0.05) and IL-4 and IL-1β (r= -0.831; P<0.05) in arthritic rats.

4. DISCUSSION

A new prospect to develop effective therapies that specifically target inflamed joints and attenuate damage to healthy tissues in RA patients is urgent. Lately, it has been documented that AuNPs displayed promising RA therapeutic activity without severe toxicity and side effects [16, 18]. The cytokine network in RA is complex, with many cytokines exhibiting pleiotropic actions. They can be divided into two groups, proinflammatory and anti-inflammatory cytokines. Acting on the balance between these two groups of cytokines may lead to new therapeutic approaches to controlling chronic inflammation [19]. Accordingly, the present study was performed to evaluate the effect of AuNP treatment on pro-inflammatory/anti-inflammatory cytokines and angiogenic mediators.

Among the proinflammatory cytokines, TNF-α is a key for cytokine cascade activation as it can boost the production of many other inflammatory cytokines such as IL-1β and IL-6 [20]. During RA pathogenesis, TNF-α and IL-1β play a pivotal role in the inflammatory cell signaling, whereas IL-1β causes cartilage and bone degradation more potently than TNF-α [21, 22]. IL-6 acts on osteoblasts to produce receptor activators of nuclear factor-kappa B ligand (RANKL), which induces osteoclasts and promotes bone destruction [23]. IL-17 is the signature cytokine of Th17 cells. It has pleiotropic effects on many cell types, up-regulating the expression of NF-κB and stimulating the production of several proinflammatory cytokines (TNF-α, IL-1β, and IL-6). Of relevance to the pathogenesis of RA are the effects of IL-17 in driving osteoclastogenesis, leading to bone resorption [24] via increasing the production of IL-17 relative to IFN-γ. IL-23 can enhance osteoclastogenesis presumably in a dose-dependent manner, thereby altering the balance in favor of IL-17 [25, 26].

Reduced TNF-α and IL-1β observed in our results among AuNP-treated rats is in line with many previous studies [27-29]. The immuno-modulatory

and inhibitory effects of AuNPs could be explained based on the interference with the transmission of inflammatory signaling. Sumbayev *et al*. [30] debated that the anti-inflammatory activity of citrate-stabilized AuNPs is mostly attributed to extracellular interactions with IL-1β, which aggregates around AuNPs, thus inhibiting IL-1β binding to its cellular receptors, which in turn reduces the biological activity of IL-1β. Furthermore, spherical AuNPs ameliorated the inflammatory response by decreasing the mRNA expression of IL-1β, TNF-α, and inducible nitric oxide synthase [31].

Our data demonstrated that 5 nm AuNPs decreased IL-17 levels. Additionally, a study by Abdelmegid *et al*. [32] showed that 5 nm AuNPs could ameliorate inflammation progression, as evidenced by the decrease in pro-inflammatory cytokine IL-17 mRNA expression. IL-17 is also a potent inducer for nitric oxide synthase, matrix metalloproteases, and cyclooxygenase expression, and this process is synergized by TNF-α, which mediates tissue infiltration and tissue destruction. Thus, IL-17 downregulation might partially contribute to reduced inflammation.

Interestingly, our results showed that 25nm-treated rats decreased pro-inflammatory cytokine IFN-γ level. Similarly, Bekic′ *et al*. [33] found that spherical AuNPs (20 nm) inhibited the production of Th1-related cytokines such as IFN-γ. Elbagory and his colleagues [34] reported that IFN-γ production was markedly decreased in human natural killer cells 92 (NK92) treated with hypoxis-AuNPs (average size 26 ± 2 nm). Our study revealed that the production of IL-23 was also reduced in 25 nm-treated animals compared with the CIA group. Similarly, a previous study by Aghaie *et al*. [35] reported a decrease in the pro-inflammatory cytokine IL-23 in the 25 nm AuNP-treated group compared with the untreated one. They demonstrated the interference of AuNPs with inflammatory signaling and reduction of the expression of inflammatory cytokines.

IL-12 has a pivotal role in proinflammatory and immuno-regulatory functions [6]. Agarwal *et al*. [36] showed that AuNPs reduce elevated levels of IL-12 production, transposing the cellular immune response from Th1 response (proinflammatory) to Th2 response (anti-inflammatory). This result is in line with the results of this study, where PEG-AuNP-treated rats downregulated the secretion of Th1 cytokine (IL-12) compared to untreated CIA rats.

In RA, uncontrolled neovascularization can induce synovial hyperplasia and progressive bone destruction by fostering the infiltration of inflammatory cells into the joints [37, 38]. In RA, VEGF is a key regulator of angiogenesis secreted by macrophages and synovial tissue fibroblasts. It can promote the proliferation and migration of endothelial cells to support the emergence of new blood vessels. It also plays a direct proinflammatory role in the pathogenesis of RA [39]. The other two growth factors expressed in RA joints are PDGF and TGF-β. They can, together with TNF-α, increase the hypertrophic architecture of the RA synovial tissue-lining layer [40].

Our data showed a remarkable reduction in VEGF concentration after treatment with different AuNPs compared with the CIA group. An early study by Bhattacharya *et al*. [41] showed that ~5 nm spherical AuNPs exhibited potentially anti-angiogenic effects by interacting with the heparin-binding domain of VEGF165. Mukherjee *et al*. [42] reported that spherical AuNPs (~5 nm) bind to VEGF and inhibit the induced proliferation of human umbilical vein endothelial cells. Moreover, Tsai *et al*. [43] found that 13 nm spherical AuNPs bind not only to VEGF165 but also to VEGF121 and inhibit the biological activities of both isoforms. Pan *et al*. [44] noted that spherical AuNP (15 nm) inhibits migration and tube formation in VEGF165-induced human umbilical vein endothelial cells (HUVEC) by preventing protein kinase B (Akt) phosphorylation. Our results indicated a reduction in PDGF levels in rats treated with AuNPs. This was in accordance with a previous study by Abo-Elfadl *et al*. [45]. They documented that ~25 nm width and ~30 nm length PEG-gold nano-semicubes (PEGGNSCs) also produced anti-angiogenic effects in skin cancer animal models by reducing the expression of PDGFR. Similarly, Zhang *et al*. [46] noted that 20 nm spherical AuNPs inhibit the activation of cancer-associated fibroblasts by disrupting communication from tumor and micro-environmental cells. AuNPs exert the effect by downregulating multiple fibroblast inactivation proteins such as PDGF.

Anti-inflammatory cytokines are a panel of immunoregulatory molecules that control the inflammatory response [47]. Regulation of arthritis

can be exerted at the level of these modulatory cytokines, such as TGF-β and IL-4, which can inhibit Th1 cell activity by suppressing inflammatory cytokine (IL-1, IL-2, IL-6, and IFN-γ) production. In addition, they may have a direct inhibitory effect on the macrophage activity in the synovium [48]. Moreover, TGF-β stimulates the production of type I and type XI collagen; hence, it may promote reparative processes in arthritic synovial connective tissue scarring and tissue repair by inhibiting cartilage and bone destruction [49, 50]. In our study, TGF-β concentration was increased in 25 nm and 75 nm AuNP-treated rats, which complied with the *in vitro* study, demonstrating that the AuNPs could increase TGF-β expression and promote the human periodontal ligament cells to form collagen fibers; therefore AuNPs could inhibit the inflammation and promote the periodontal regeneration [51].

In our study, IL-4 concentration was markedly elevated after treatment with 25 nm AuNPs compared with the CIA group. This data is in accordance with Bekic′ *et al*. [33] and Dykman *et al*. [52], who demonstrated the capacity of PEG-AuNPs to up-regulate the production of IL-4 anti-inflammatory cytokine involved in the Th2 polarization of immune response, which is consistent with the predictable immunological effects of PEG-AuNPs. On the other hand, in a polymicrobial sepsis mouse model, spherical AuNP did not show any effects on IL-4 both *in vivo* and *in vitro*, which implied the lesser impact of AuNP against Th2 [53].

Although the efficacy of MTX on proinflammatory cytokines, especially TNF-α and IL-1β, seems to be better than our AuNPs, several drawbacks are present with the therapeutic potential of MTX. One of the major concerns is toxicity [54]. Around 80% of MTX is metabolized in the kidneys, leading to nephrotoxicity, severe neutropenia, sepsis, and, finally, advanced renal failure [55]. Conway and Carey [56] reported several potentially life-threatening adverse events such as neurotoxicity, cytopenias, bone marrow suppression, interstitial lung disease (or MTX pneumonitis), and MTX-related liver disease (fibrosis and cirrhosis). These potential adverse events associated with MTX attract considerable attention as they represent the leading cause of drug withdrawal [54]. Even though the response to MTX is better than to most other csDMARDs, it is not universal. In documented studies, nearly 30% of patients were found to discontinue MTX in the medium term, with half of them stopping due to inefficacy and the other half due to adverse events [57].

5. CONCLUSION

In conclusion, our study reported that spherical AuNPs could be used as a promising therapeutic agent against RA. The underlying mechanisms could be related to the anti-inflammatory/anti-angiogenic potential of AuNPs. Taking our present data together with our former ones [16], it is evident that 25 nm AuNP is the most effective in controlling RA. Besides its anti-arthritic capabilities without causing severe toxicity, it causes a considerable reduction in inflammation and inflammatory mediators such as IFN-γ, IL-23, and IL-12, amelioration in bone and cartilage erosion, prevention of pannus formation, and decline in angiogenic factors (VEGF and PDGF), while increasing the anti-inflammatory cytokines (IL-4 and TGF-β). All over, PEG-AuNPs (25 nm) were more effective than 5- and 75-nm AuNPs. Continuing with this work, a further study combining AuNPs with other RA treatment regimens is in progress.

FUNDING

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ETHICS APPROVAL

All experimental protocols described in this study complied with the rules of Sadat City University Animal Experiments Local Ethics Committee and the Guidelines for the Care and Use of Laboratory Animals. The Sadat city University Ethics Committee granted ethical approval (No. 19 06 2019, Sadat, Egypt).

CONSENT FOR PUBLICATION

All authors approve the publication of this work.

DATA AVAILABILITY

Datasets generated during the current study are

not publicly available but could be obtained from the corresponding author on reasonable request.

ACKNOWLEDGMENT

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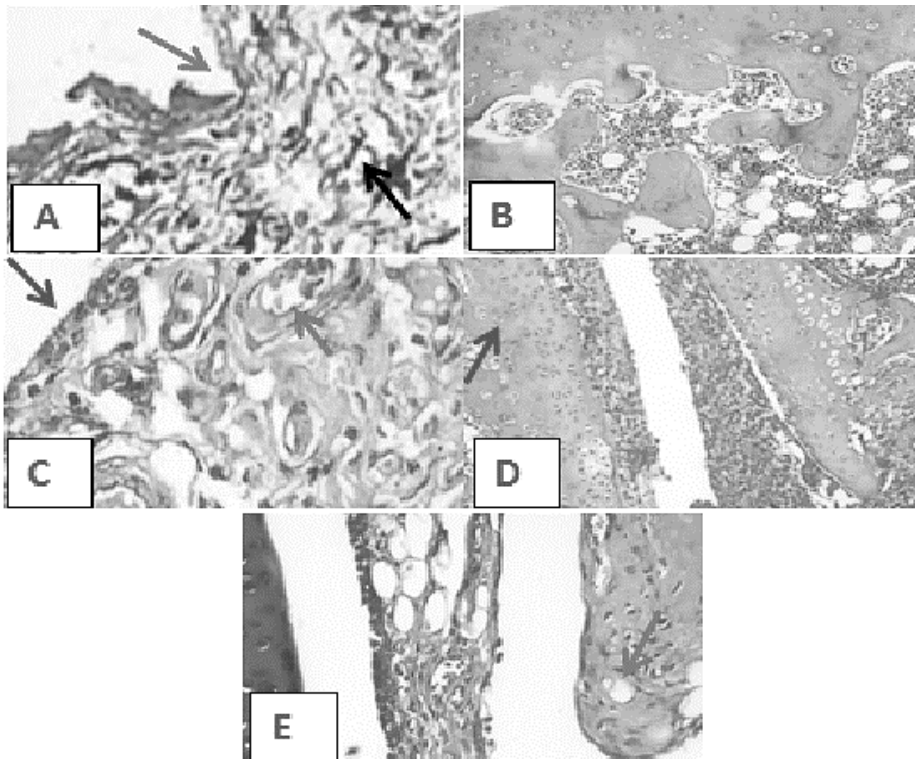
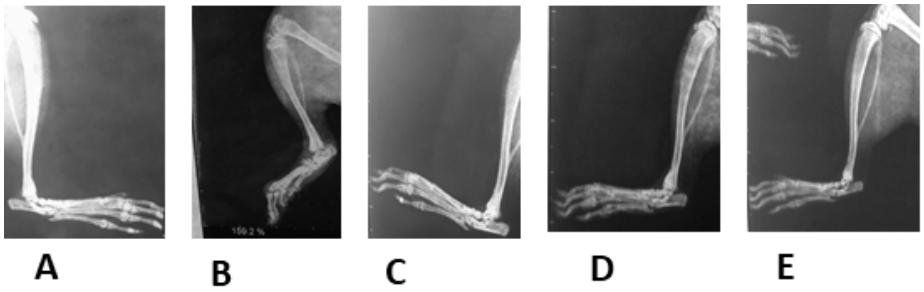
CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.



Supplementary Figure 1. CIA rats. A) Section of the synovial membrane showed destructed and ulcerated synoviocytes (Dark grey). Subsynovium showed edema and haphazardly arranged collagen bundles (Black arrows). AuNP 25 nm-treated rats: B) Normal cartilage and bone. AuNP 5 nm-treated rats: C) Normal synovial membrane (Light grey) and mild congestion (Dark grey). AuNP 75 nm-treated rats: D) Mildly eroded cartilage (Light grey). MTX-treated rats: E) Section shows pannus (Dark grey). (H&E x40 for A, B, &D and x100 for C & E).



Supplementary Figure 2. X-Ray images of female Wistar rats' hind foot joint A: control rat; B: CIA rat, C, D, E: CIA rats treated with AuNPs (5, 25, 75, respectively).

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