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Ellagic acid alleviates adjuvant induced arthritis by modulation of pro- and anti-inflammatory cytokines

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown aetiology, but it is now clear that pro-inflammatory cytokines play a central role in its pathogenesis. Ellagic acid (EA) has a variety of biological activities including anti-oxidant, anti-inflammatory, and anti-cancer properties. The aim of the present study was to evaluate the potential effect of ellagic acid on the prevention and/or treatment of adjuvant induced arthritis (AIA) model in mice. Ellagic acid treatment was started one week before AIA induction and continued for three weeks after induction of AIA. Ellagic acid treatment significantly ($p < 0.01$) inhibited foot paw oedematous swelling and attenuated AIA-associated pathology. Ellagic acid significantly ($p < 0.01$) reduced serum levels of pro-inflammatory cytokines: interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and interleukin 17 (IL-17). However, serum levels of IL-10 and interferon γ (IFN-γ) significantly increased ($p < 0.01$ and $p < 0.05$, respectively), while serum level of transforming growth factor β (TGF-β) did not significantly alter with EA treatment. In conclusion, these results suggest that EA attenuated AIA-associated pathology in the mouse model by downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines.

Key words: *adjuvant induced arthritis, ellagic acid, pro-inflammatory cytokine, anti-inflammatory cytokine.*

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by inflammation of the synovia, synovial hyperplasia with increased cell density, and infiltration of inflammatory cells leading to pannus formation and irreversible cartilage and bone destruction [1]. It affects about 0.5-1% of the world population, with more women being affected than men, and its incidence increases with increasing age [2-4]. The exact cause of RA is unknown, but genetic and environmental factors are contributory [4]. However, it is now clear that inflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), and IL-6 are overexpressed in RA joints and play an important role in its pathogenesis [5, 6]. In addition, the current view of the cytokine network in RA joints supports the notion that TNF-α activates a cytokine cascade characterised by simultaneous production of proinflammatory cytokines such as IL-1β and IL-6, whereas anti-inflammatory cytokines such as IL-10 and soluble TNF receptor are suppressed [2, 7]. Long-term use of biological agents targeting TNF-α gives rise to sustained improvement in symptoms and signs of RA. In addition, TNF-α blockade protects joints from structural damage [8]. Although TNF-α has been hypothesised to be the "master cytokine" driving joint inflammation, about 50% of patients with RA do not respond to anti-TNF therapy [9].

The pathogenesis of RA can involve either the inappropriate activation of T cells or the lack of appropriate suppression of T cells [10]. It was found that $CD4^+$ T cells from patients with RA express some striking abnormalities in their differentiation into effector cells, in their functional capabilities, and in their responsiveness to regulatory forces. A shift in the Th1/Th2 balance toward Th1 has been described in patients with RA, and

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this also has prognostic value for the course of the disease [11, 12]. The intrinsic bias to Th17 cell differentiation as observed defines a novel aspect of abnormal $CD4^+$ T cell biology in RA and provides additional evidence of an important role of altered $CD4^+$ T cell biology in the pathogenesis of rheumatoid inflammation [13]. Th17 is the pivotal driving force of autoimmune inflammation in several animal models of human autoimmune diseases, including collagen-induced arthritis (CIA) [14] and adjuvant-induced arthritis (AIA) [15]. The inhibition of Th17 cells occurs via the neutralisation of IL-6, a critical Th17 cell–inducing cytokine in mice, leading to suppression CIA [16] and prevention of the onset of AIA [15]. Interleukin 17 exerts potent proinflammatory and joint-destructive activities. The mechanisms orchestrating the cellular pathogenesis and the impact of Th17 cells on the pathogenesis of autoimmune arthritides is therefore unknown but would be important to understand, not least because of the novel treatment strategies being utilised to inhibit Th17 cell effector functions via the neutralisation of IL-17 [13].

Ellagic acid (EA) is a phenolic compound found in a wide variety of nuts and fruits [17-19]. Interest in EA has increased recently due to its potential anti-inflammatory properties. Ellagic acid mediates its anti-inflammatory effects via the modulation of NF-κB activity and by inhibiting IL-1β-induced nuclear translocation of p65 and p50 [20]. Several studies have shown that EA modulates both pro-inflammatory and anti-inflammatory cytokine production. EA decreases production of IL-13 and TNF-α from stimulated human peripheral blood mononuclear cells (PBMC), whereas no change was observed in IL-4 production [21]. Ellagic acid significantly down-regulates mRNA expression and lowers cardiac levels of IL-1β, IL-6, TNF-α, and monocyte chemoattractant protein (MCP)-1 [22]. Moreover, EA lowers renal levels of IL-6, IL-1β, TNF-α, and MCP-1, and down-regulates TNF-α and MCP-1 mRNA expression in the kidney. In addition, intake of EA substantially decreases renal IL-1β, IL-6, and TNF-α levels in diabetic mice [23]. Topical application of EA diminishes production of pro-inflammatory cytokines IL-1β and IL-6, and blocks infiltration of inflammatory macrophages in the integuments of SKH-1 hairless mice exposed to UV-B [24]. Treatment of gastric ulcerated mice with EA significantly reduces pro-inflammatory cytokine (TNF-α, IL-1β, and IL-6) levels and induces anti-inflammatory cytokines (IL-4 and IL-10) [25]. EA shows strong inhibition of neutrophilic infiltration as well as suppression of TNF-α and IL-1β in AIA [26]. Furthermore, EA treatment leads to reduced bronchoalveolar lavage fluid levels of IL-6 and increased levels of IL-10 [27].

Taken together, EA has been shown to down-modulate pro-inflammatory mediators and stimulate the production of anti-inflammatory cytokines. Therefore, EA may have a potential role in the prevention and/or treatment of RA. The aim of the present study was to investigate the potential effect of EA in prevention and/or treatment of AIA.

Material and methods

Animals

Eight-week-old MF1 male mice were purchased from the King Fahd Centre for Medical Research, College of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia. The mice were maintained at 22 ±2°C with a 12-hour (h) light/dark cycle, housed in a specific pathogen-free environment, and fed standard rodent chow and given water *ad libitum*. All procedures were performed with the approval of the Institutional Animal Care and Research Ethics Committee.

Drugs and chemicals

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. All the chemicals used were of analytical grade.

Induction of arthritis and treatment schedule

MF1 mice were induced with Complete Freund's Adjuvant (CFA) containing *Mycobacterium* (*M.*) *tuberculosis*, a subcutaneous injection of 0.02 ml CFA containing 5 mg/ml of heat-killed *M. tuberculosis* into a foot pad of the right hind limb [28]. This arthritis model, called adjuvant-induced arthritis (AIA), has been widely used as a model for rheumatoid arthritis [29]. Ellagic acid was dissolved in pure dimethyl sulfoxide (DMSO), and then diluted with phosphate buffer saline (PBS, pH 7.2) to a final DMSO concentration equal to 2% (v/v). EA suspension was administered intraperitoneally (i.p.) at a total dose of 700 mg/kg body weight divided equally into 12 injections with three injections/week for four consecutive weeks, commencing one week before subplantar injection with CFA containing *M. tuberculosis*. The arthritic control (AC) and normal control (NC) mice groups were given the same amount of the vehicle (2% DMSO) at the same time as the arthritic treated (AT) and normally treated (NT) mice. The dose of EA and route of administration were selected according to Soh *et al.* [30], who reported that mice treated with up to 1000 mg/kg/day by intraperitoneal route for four consecutive days did not show any mortality until day 30 post treatment.

Blood sampling

At the end of the third week post arthritis induction (the fourth week post treatment) mice were anesthetised by ether inhalation and blood was collected. Sera were collected from the clotted blood samples after centrifugation at 400 g for 15 minutes (min), then divided into aliquots and stored at –80°C until use.

Measurement of arthritis severity

The thickness of the right hind paws was measured with Vernier callipers every third day commencing on the first day of arthritis induction (0 day), and the values obtained were used as an index of foot paw oedematous swelling. The percentage reduction of paw swelling in the test drug-treated group was calculated by using the following formula according to Shruthi *et al*. [31].

% Reduction in paw swelling (RPS) = 1-(Vt/Vc) × 100,

where Vt = paw swelling in the test drug-treated animals; Vc = paw swelling in the control group animals.

Histopathological investigations

After sacrifice (21 days after arthritis induction), the right ankles and hind paws were removed and placed in 10% buffered formalin for 48 hours. The skin of paws dorsi were incised longitudinally, and their nails were removed to accelerate the decalcification process. Decalcification was performed with 10% EDTA (pH 7.4) at 18°C. EDTA solution was replaced twice weekly for seven weeks, and the end point of decalcification was assessed physically with a surgical blade. After complete decalcification, the samples were washed with PBS, dehydrated in a graded ethanol series, and embedded in paraffin wax. Sagittal sections measuring 5 µm in thickness were prepared and stained with haematoxylin and eosin (H&E) [32-34].

Histological examinations of synovial inflammation, cartilage, and bone damages were performed by a pathologist blindly. Sections were graded according to the system described by Sancho *et al*. [35] for synovial hypertrophy (pannus formation), inflammation (mononuclear cell infiltration), cartilage destruction, and bone erosion. A 0-3-point scale used for each parameter (0 = normal, 1 = mild inflammation, 2 = moderate inflammation, 3 = severe inflammation). The maximum possible score was 12.

Cytokine measurements

Serum levels of IL-1β, IL-10, IL-17, TNF-α, IFN-γ, and transforming growth factor β (TGF-β) were estimated by using high-sensitivity sandwich ELISA kit (abcam®, Cambridge, UK) according to the manufacturer's instructions. Briefly, a monoclonal antibody specific for each cytokine (IL-1β, IL-10, IL-17, TNF-α, IFN-γ, and TGF-β) was coated onto wells of the microtiter plates provided. Samples and standards of known concentrations were applied into plates. After the incubation period, the biotinylated monoclonal antibodies specific for IL-1β, IL-10, IL-17, TNF-α, IFN-γ, and TGF-β were added and incubated for one hour with gentle shaking. After washing, the enzyme Streptavidin-HRP that binds biotinylated antibody was added and incubated for 45 minutes at room temperature with gentle shaking. TMB substrate solution was added and the plates were incubated in the dark for 30 minutes at room temperature. The reaction was stopped by using 2N HCl and the absorbance was measured at 450 nm using a microplate reader (BioTech, CA, USA). Cytokine concentrations were determined by reference to standard curve construction.

Data analysis

Statistical tests were performed with SPSS (version 16) software. Significant differences were determined by two-tailed *t*-test for comparisons between two groups and one-way analysis of variance (ANOVA) with application of Tukey's test for multiple comparisons. Results were expressed as mean ± standard deviation (SD) and values of $p > 0.05$ were considered statistically insignificant, while those of $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively.

Results

Ellagic acid attenuates pathological manifestations in an adjuvant-induced arthritis mice model

To evaluate the potential anti-arthritic activity of EA, arthritis was induced by sub-plantar administration with CFA containing *M. tuberculosis* into the foot pad of the right hind limb. EA-treated mice received 12 doses of EA, three injections per week, each of 58.33 mg/kg body weight. Treatment with EA started one week before immunisation with CFA and continued for three weeks after arthritis induction. Arthritis severity was assessed by measuring foot paw swelling every three days. Immediately before injection of CFA, on day 0, there was no significant difference ($p > 0.05$) in foot paw thickness of the right hind limb of normal control (NC, $n = 15$), normal treated (NT, $n = 15$), arthritic control (AC, $n = 15$), and arthritic treated (AT, $n = 15$) mice groups (Table 1). Individuals of both NC and NT groups showed no gross pathological lesions in the right hind paws, digits, and ankles. At each time interval throughout the experiment, foot paw thickness of NT and NC mice did not significantly change ($p > 0.05$, Table 1). On the other hand, clinically apparent arthritis developed at day 3 in all AIA mice and reached the peak at day 6 (Table 1), with marked swelling and redness of hind paws. AC mice showed rapid and marked progress. The right hind paws of AC mice revealed marked congestion and swelling (extending to involve digits and ankles of some individuals). However, the right hind paws of AT mice revealed a similar gross pathological picture but of moderate degree. Throughout the time of the experiment, EA treatment significantly ($p < 0.01$) reduced foot paw swelling of AT as compared to AC mice, and the highest reduction percentage was 17.79% at day 21 (Table 1).

Table 1. Effect of ellagic acid administration on foot paw oedematous swelling (mm)

Time/day	NC	NT	AC	AT	% RPS	*p* value
0	2.31 ±0.20[a]	2.31 ±0.20[a]	2.31 ±0.20[a]	2.31 ±0.20[a]	0	> 0.05
3	2.31 ±0.20[a]	2.32 ±0.12[a]	3.81 ±0.16[b]	3.38 ±0.21[c]	11.29	< 0.01
6	2.53 ±0.37[a]	2.40 ±0.17[a]	3.91 ±0.25[b]	3.43 ±0.21[c]	12.28	< 0.01
9	2.53 ±0.37[a]	2.40 ±0.17[a]	3.91 ±0.15[b]	3.40 ±0.22[c]	13.04	< 0.01
12	2.60 ±0.13[a]	2.59 ±0.21[a]	3.85 ±0.46[b]	3.30 ±0.18[c]	14.29	< 0.01
15	2.68 ±0.13[a]	2.70 ±0.28[a]	3.85 ±0.53[b]	3.27 ±0.18[c]	15.07	< 0.01
18	2.76 ±0.17[a]	2.72 ±0.11[a]	3.87 ±0.33[b]	3.19 ±0.14[c]	17.57	< 0.01
21	2.85 ±0.16[a]	2.85 ±0.39[a]	3.71 ±0.44[b]	3.05 ±0.21[c]	17.79	< 0.01

Mice were monitored on daily basis, and thickness of the right hind paws were measured every three days during the experiment. Percentage reduction in paw swelling (RPS) was calculated using the following formula: % RPS = 1-(Vt/Vc) × 100, where Vt = paw swelling in the test drug treated animals, Vc = paw swelling in the arthritic control group animals. Data are presented as mean ± SD of 15 mice per group. For each time interval, values not sharing common superscripts denote significant differences.
NC – normal control mice, NT – normal treated mice, AC – arthritic control mice, AT – arthritic treated mice.

Ellagic acid ameliorates histopathological alterations in AIA mice

In order to investigate the effect of EA treatment on arthritic histopathology, the right ankles and hind paws were removed after sacrifice on the 21st day post arthritis induction and processed for histopathological examinations. As shown in Fig. 1, H&E stained sections of paws and digits tissues from both normal control (NC) and normal treated (NT) mice revealed no inflammation. However, stained sections of arthritic control (AC) mice revealed marked histopathological changes in the form of synovial hyperplasia, with a large number of inflammatory cells (lymphocytes, macrophages, and sometimes plasma cells), extensive pannus formation, and severe cartilage destruction. On the other hand, arthritic-treated (AT) mice showed less severe arthritis pathology with moderate inflammation (Fig. 1).

Concerning AC mice, microscopic examination showed synovitis characterised by proliferating synovial lining cells, in 2-3 layers, as well as proliferation of the underlying blood vessels, which was associated with perivascular oedema and diffuse cellular infiltrates composed of mononuclear cells (Fig. 2A). In many specimens, the inflammatory cellular exudates extended to involve whole periarticular soft tissues of the connective tissue and muscles. There was synovial sloughing in some areas of synovial membrane and mild proliferative lesion of fibroblast-like cells. Pannus formation was in the form of single or multiple proliferating granulation tissues containing hyperplastic synoviocytes and inflammatory cells at the articular cartilage margin, and at the cartilage-bone level. The articular cartilages of some arthritic mice had uneven articular surface and demonstrated superficial fibrillation accompanied by cell death or proliferation and in some cases extended to the mid-zone portion of the articular cartilage. Moreover, the articular bone destruction was visualised by osteoclast activity and fibroplasia (Fig. 2). However, AT mice showed the previously mentioned histopathological lesions of arthritis, but with mild to moderate degree (Fig. 2, AT).

Ellagic acid reduces histopathological arthritis score in AIA mice

Histologic analysis of synovitis, pannus formation, bone erosion, and cartilage destruction were scored on a 0-3 scale in H&E-stained sections by a blinded observer. The present data revealed a high significant ($p < 0.01$) reduction in the total histopathological score of AT mice compared to AC ones (Fig. 3). In detail, EA treatment significantly ($p < 0.01$) attenuated the synovitis, reduced pannus formation, and decreased bone resorption in treated mice, compared to control mice. However, cartilage destruction in AT mice did not change significantly ($p > 0.05$) as compared to AC mice (Fig. 3).

Ellagic acid downregulates pro-inflammatory cytokines and upregulates anti-inflammatory cytokines in AIA mice

To investigate the anti-inflammatory effect of EA on the AIA model, IL-1β, IL-10, IL-17, TNF-α, IFN-γ, and TGF-β were quantified in the serum on the 21st day post-arthritic induction, using standard sandwich ELISA procedures. As depicted in Table 2, circulating levels of IL-1β and TNF-α did not change significantly ($p > 0.05$) in normal mice with EA treatment, compared to NC mice. However, AC mice showed high significant ($p < 0.01$) serum levels of both IL-1β and TNF-α, compared to NC mice. Nevertheless, AT mice showed a high significant ($p < 0.01$) reduction in the circulating levels of both IL-1β and TNF-α, compared to AC mice.

Ellagic acid treatment did not significantly ($p > 0.05$) affect circulating levels of IL-17 in normal (NT) mice, compared to NC mice. At the same time, IL-17 levels of

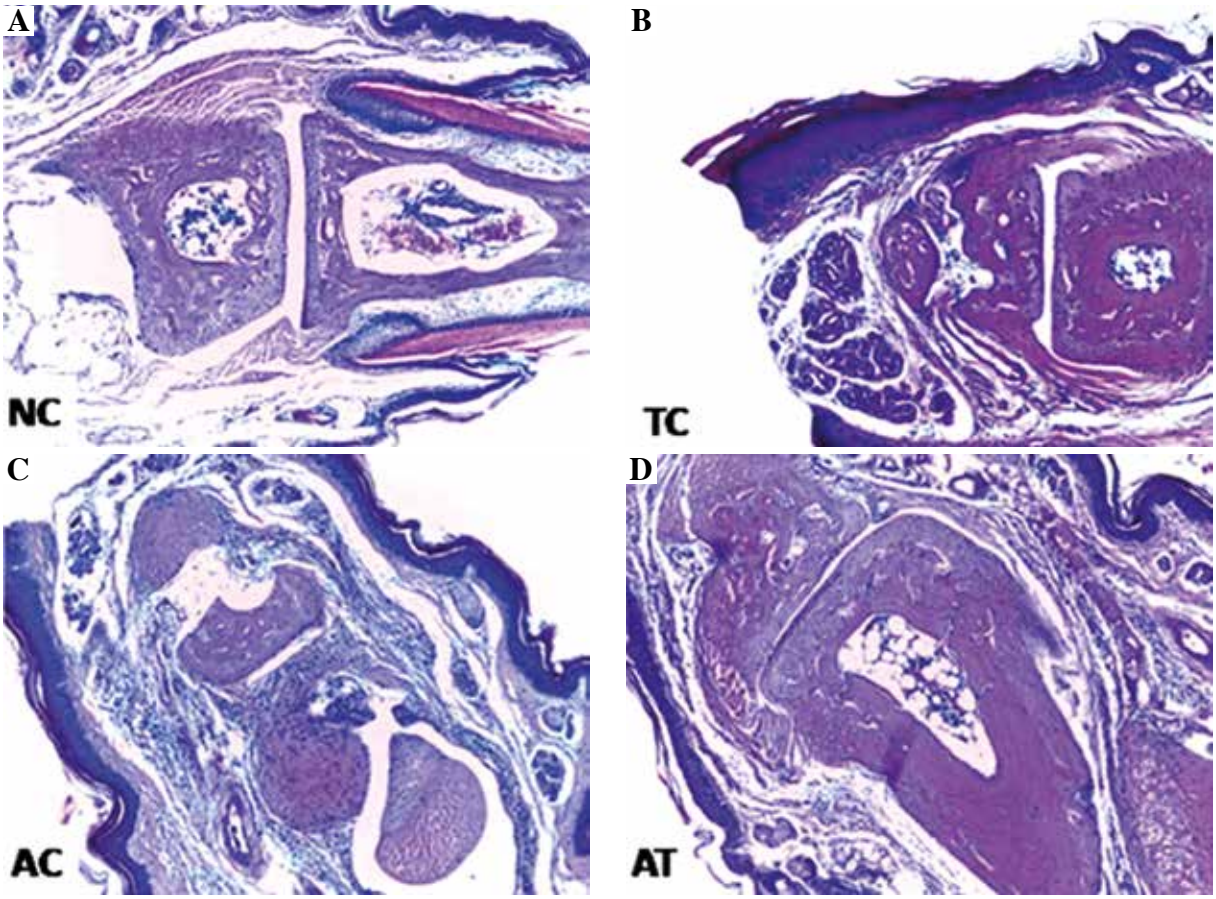

Fig. 1. Histopathological evaluation of ellagic acid treatment on adjuvant induced arthritis in mice. Representative results showing the histopathological picture of hind paws and digits of normal control (NC), normal treated (NT), arthritic control (AC) and arthritic treated (AT) mice in H&E (100×) stained sections. The normal histological picture of hind paws joints was presents in both NC and NT mice. AC mice showed synovial hyperplasia, inflammatory cells infiltration, pannus formation, and cartilage destruction; whereas AT mice revealed less severity of arthritis pathology

AC mice were significantly ($p < 0.01$) increased compared to NC ones. However, the serum levels of IL-17 significantly ($p < 0.01$) decreased in AT mice, compared to the AC group. Interestingly, there was no significant difference ($p > 0.05$) in IL-17 levels of NC, NT, and AT mice (Table 2).

The serum levels of IFN-γ did not significantly ($p > 0.05$) change in AC mice, compared to the NC group. However, circulating levels of IFN-γ significantly ($p < 0.05$) increased in NT and AT mice compared to NC and AC groups, respectively. Meanwhile, there was no significant difference ($p > 0.05$) in circulating concentrations of IFN-γ between NT and AT mice (Table 2).

Transforming growth factor β serum levels of normal (NT) mice did not significantly ($p > 0.05$) change with EA treatment, compared to NC animals. Circulating concentrations of TGF-β decreased in AC mice, but not significantly ($p > 0.05$) differently than those of NC mice. On the other hand, EA treatment significantly ($p < 0.01$) decreased TGF-β levels of arthritic (AT) mice, compared to NC mice. Transforming growth factor β levels of AT mice decreased more that of AC animals; however, such a decrease was not significantly ($p > 0.05$) different (Table 2).

Interleukin 10 serum levels of NT mice did not differ significantly ($p > 0.05$) from that of NC animals, but significantly ($p < 0.01$) increased compared to those of AC and AT mice groups. AC mice showed significantly ($p < 0.01$) decreased circulating levels of IL-10, compared to NC mice. However, IL-10 levels of AT mice significantly ($p < 0.01$) increased, compared to AC mice. Meanwhile, there was no significant difference ($p > 0.05$) in IL-10 levels between AT and NC mice (Table 2).

Discussion

Even though several studies have been performed, an efficient medicine for RA treatment still has not been found [31]. Due to the lack of efficacy and major side ef-

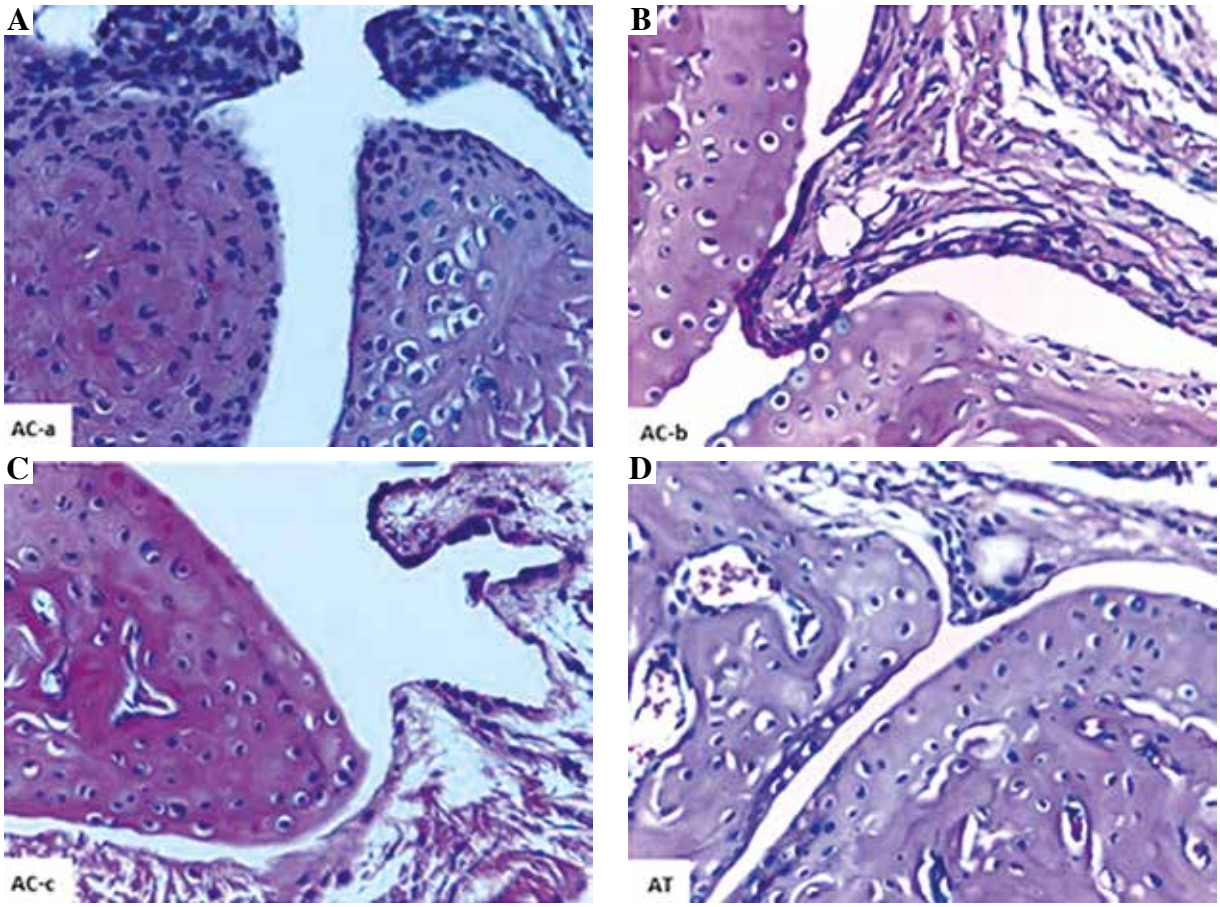

Fig. 2. Representative photomicrographs showing the histopathological picture of hind paws and digits of arthritic control (AC) and arthritic treated (AT) mice in H&E stained sections (× 400). AC mice showed hyperplastic synovial membrane composed of multiple layers of synoviocytes (AC-a), marked pannus formation and fibroplasia of the underlying connective tissues associated with cartilage erosion and bone resoprption (AC-b), and multiple panni were formed from synovial membrane (AC-c). However, AT mice revealed mild pannus formation, fibroplasias, cartilage erosion and bone resoprption (AT)

fects related to many medical treatments, a wide variety of botanicals are used to treat RA [36, 37]. The current study was conducted to evaluate the preventive and treatment potential of EA on the AIA model in mice. This arthritis model is a convenient rodent model of RA [38]. In this study, EA was administered intraperitoneally because absorption seems to be higher by this route than by oral administration [30]. Previous studies on oral administration of EA have found that 10% of the dose given to rats was absorbed and excreted as a metabolite in urine and faeces [39], whereas mice given higher doses showed absorption rates of 28% [40].

Bioavailability and metabolism of EA in mice following i.p. administration are not fully understood and need further investigation. However, studies with oral intake of EA or its natural sources have contributed to shedding light on EA metabolism. Urolithins are considered as biomarkers of human exposure to dietary EA derivatives [41]. Several studies have shown that urolithins appear in human systemic circulation within a few hours after pomegranate product consumption, reaching maximum concentrations at between 24 and 48 hours. They are present in plasma and urine for up to 72 hours, in free and conjugated forms [42-44]. In the current study, mice were injected with EA every 48 hours. Therefore, we assumed that the concentration of EA in plasma was not decreased and could reach biological targets at relevant concentration.

Data from the present study showed that sub-planter injection of CFA significantly increased foot paw oedematous swelling and arthritis score. The pathogenesis for development of AIA following injection of atherogenic preparations is not fully understood. However, the influx and/or local activation of mononuclear cells (including T cells, B cells, plasma cells, dendritic cells, macrophages, and mast cells), and by angiogenesis, is the cause of synovitis. Leukocytes and other cells in the synovial tissue

produce several inflammatory mediators, including chemokines and chemotactic cytokines that recruit more leukocytes and inflammatory cells to the inflamed joint and enhance angiogenesis [45, 46]. The synovial membrane becomes hyperplastic and extends to form villi. The osteoclast-rich portion of the synovial membrane, or pannus, destroys bone whereas enzymes secreted by neutrophils, synoviocytes, and chondrocytes degrade cartilage [47-49]. Histopathological investigations of this study showed slight cartilage destruction, bone resorption, periosteal proliferation, and marked synovitis, as well as periarticular inflammation in arthritic untreated mice. Treatment with EA largely retrieved such pathological manifestations. Our data also showed that EA treatment significantly reduced foot paw oedematous swelling, arthritis score, and disease-associated hyperalgesia. These results are in accord with previous studies that have demonstrated that treatment with EA reduced paw oedema in the AIA rat model [31]. Bulani *et al*. [50] reported that ellagic acid hydroxypropyl-β-cyclodextrin treatment attenuates disease progression and associated hyperalgesia in CFA-induced arthritic rats. Moreover, red raspberry fruit extract containing EA reduced the severity of arthritis by inhibition of inflammation, pannus formation, cartilage damage, and bone resorption in both CIA [51] and AIA [52] models in rats. Pomegranate fruit extract, which is rich in EA [53], was shown to delay the onset and reduce the severity of arthritis in the CIA model in mice [54]. Therefore, the therapeutic effects of EA on AIA probably lie in its inhibition of leukocyte recruitment, down regulation of pro-inflammatory cytokines, and NO neutralising property.

RA pathogenesis is regulated by proinflammatory cytokines such as IL-1β and TNF-α that activate a broad array of intracellular signal transduction mechanisms that contribute to the progression of inflammatory arthritis, viz. leukocyte infiltration, cytokine networks formation, cartilage catabolism elevation, and anabolism suppression [7, 55-58]. Our results showed that arthritic control mice had higher levels of serum IL-1β and TNF-α. We also found that EA treatment significantly reduced the serum levels of these pro-inflammatory cytokines, in agreement with previous studies on different experimental models [21-26, 59-61]. The precise mechanism by which EA decreased levels of IL-1β and TNF-α is unclear and needs further investigation. However, Ahad *et al*. [61] suggest that EA suppressed the synthesis of IL-1β and TNF-α by inhibiting the NF-κB pathway. Furthermore, a growing body of evidence exists for the inhibitory activity of pomegranate extract containing EA on the activation of mitogen-activated protein kinases and NK-kB, which regulates the transcriptional activation of many inflammatory biomarkers [62-64]. Inflammatory genes that have been reported to be downregulated by pomegranate extract include TNF-α, IL-1β, MCP1, iNOS, COX-2, PGE2, and matrix metalloproteinases (MMPs) [36, 62, 63]. All these findings suggest that EA ameliorates AIA pathogenesis by inhibiting pro-inflammatory cytokines.

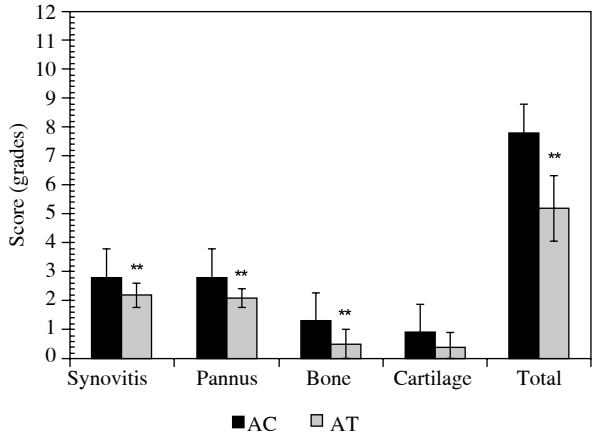

Fig. 3. The effect of ellagic acid treatment on the histopathological arthritis score of arthritic control (AC) and arthritic treated (AT) mice. The histopathological score of synovitis, pannus formation, bone resorption and cartilage destruction were examined per grades out of total score 12. Data reported as mean ± SD of 10 mice per each group. ** $p < 0.01$

Table 2. Effect of ellagic acid treatment on serum cytokine profile

Cytokine (pg/ml)	Normal control (NC)	Normal treated (NT)	Arthritic control (AC)	Arthritic treated (AT)	*p* value
IL-1β	58.47 ±9.62[a]	55.86 ±11.93[a]	94.37 ±15.72[b]	71.13 ±13.41[c]	< 0.01
TNF-α	45.02 ±7.11[a]	42.98 ±8.52[a]	100.79 ±14.16[b]	75.23 ±12.84[c]	< 0.01
IL-17	108.67 ±14.59[a]	104.19 ±16.02[a]	140.19 ±17.66[b]	118.55 ±15.64[a]	< 0.01
IFN-γ	101.545 ±15.67[a]	128.263 ±15.24[b]	97.354 ±18.95[a]	121.091 ±16.18[b]	< 0.05
TGF-β	147.56 ±17.27[a]	146.55 ±19.22[a]	128.41 ±18.19[ab]	114.50 ±17.62[b]	< 0.01
IL-10	124.35 ±15.52[ad]	140.09 ±18.90[a]	96.75 ±11.31[c]	118.91 ±17.48[d]	< 0.01

Blood was collected by orbital puncture on the 21st day after arthritis induction, and serum cytokine levels were estimated using sandwich ELISA. Data are expressed as mean ± SD of 10 mice per group. For each cytokine, values not sharing common superscripts denote significant differences.

Interleukin 17 plays a crucial role in the development of CIA by activating autoantigen specific cellular and humoral immune responses [14, 65]. Interleukin 17 has been shown to drive neutrophil differentiation, maturation, activation, and cytokine release; monocyte activation and cytokine release; and synovial fibroblast activation, cytokine and chemokine release, prostaglandin production, and MMP synthesis [66]. Inhibition or overexpression of IL-17 in the joints suppresses or worsens joint inflammation and damage, respectively [67]. Exogenous IL-17 strongly enhances cartilage damage and leads to erosion and chondrocyte death in the CIA mice model [68]. Antibodies against IL-17 are effective in the treatment of RA and psoriasis in clinical trials [69, 70]. The present study clearly demonstrated that circulating levels of IL-17 significantly increased in arthritic control mice, whereas EA treatment reduced serum levels of IL-17 in arthritic mice, in harmony with a previous study that demonstrated that pomegranate juice rich in EA inhibited the synthesis of IL-17 from PBMC [71]. Consistent with our data, Zhu *et al.* [72] reported that the level of IL-17 in the serum increased in the CIA mice model. These results suggested that EA alleviates RA pathogenesis by inhibited the synthesis of IL-17.

It has been shown that mice with a deficiency in IFN-γ [73, 74] or IFN-γ receptor [75-78] are more susceptible to CIA. These data indicate that deletion of the IFN-γ response somehow disrupts an endogenous protective mechanism against CIA [79]. Therefore, IFN-γ has been suggested to play a beneficial role in the CIA model in mice. The data of the current study showed that the serum levels of IFN-γ significantly increased with EA treatment in both normal and arthritic mice. In an accord with our results, EA has been shown to stimulate IFN-γ production in lymphocytes isolated from hepatitis B virus-e antigen transgenic mice [80]. Furthermore, macrophages treated with EA showed a 26.9% increased secretion of IFN-γ [81]. Irmler *et al*. [82] reported that IFN-γ has anti-inflammatory properties during the initial phase of the AIA model in mice, and concluded that IFN-γ deficiency exerts disease-promoting effects, preferentially via IL-17-modulated pathways. These findings suggest that EA reduced RA-associated pathology by enhancing IFN-γ secretion.

The role of TGF-β in the development and pathogenesis of RA is conflicting. Kuruvilla *et al*. [83] and Thorbecke *et al*. [84] reported that TGF-β protected against CIA in the mouse model because anti-TGF-β increased CIA incidence and/or severity. However, Xu *et al.* [85] demonstrated that systemic or local blockade of TGF-β activity in the subchondral bone attenuated articular cartilage degeneration in RA mouse/rat models. In addition, conditional deletion of TGF-β receptor II (Tgfbr2) in nestin-positive cells also effectively halted progression of RA joint destruction. Our data demonstrated that EA treatment decreased circulating levels of TGF-β in arthritic mice, but not in normal mice; such a decrease in TGF-β level was not significantly different from that of arthritic control, but significantly decreased compared to that of normal control mice. Consistent with our results, Vanella *et al.* [86] demonstrated that EA reduced TGF-β levels from LNCaP human prostatic cancer cell line. Furthermore, Ahad *et al*. [61] reported that treatment of diabetic rats with EA significantly lowered renal pathology and suppressed TGF-β expression in renal tissues, and suggested that EA exerts its renal protective effect by suppressing the NK-κB activation, which in turn leads to decreased expression of TGF-β.

On the other hand, IL-10 protein treatment has been shown to suppress established CIA model in mice [87, 88] and rats [89]. Interleukin 10-deficient mice had exacerbated CIA development, which was associated with increased production of IL-17 [90]. Our data clearly demonstrated that arthritic mice showed significantly decreased IL-10 levels as compared to the other tested groups. However, EA treatment significantly increased system levels of IL-10 in arthritic mice to normal levels. Such a finding is in agreement with the data obtained by Chatterjee *et al*. [25], who demonstrated that treatment of gastric ulcerated mice with EA increase IL-10 level, and Cornélio Favarin *et al*. [27], who reported that EA treatment led to an increase level in IL-10 in bronchoalveolar lavage fluid of acute lung injury murine model. In addition, EA acid exhibited a potent anti-inflammatory effect against carrageenan-induced inflammation in rats with an increased IL-10, and a decrease in TNF-α and IL-1β [91]. Recently, different approaches have been used to prevent and/or treat the CIA model in mice by enhancing IL-10 production and suppressing inflammatory cytokines through either oral administration of type II collagen [92], IL-10 gene therapy [93], treatment with ES-62, a molecule secreted by the parasitic filarial nematodes [94], or *Salmonella*-colonisation factor antigen I (CFA/I) to stimulate Treg cells [95]. Therefore, we suggest that EA alleviates AIA-associated pathology in mice by inhibiting inflammatory cytokines (TNF-α, IL-1β, IL-17) and by stimulating anti-inflammatory cytokine (IL-10) production.

Conclusions

Taking together, the data from this study demonstrate that EA treatment ameliorated AIA associated pathology in mice model, as shown by the reduction of foot paw oedematous swelling, synovitis, pannus formation, and bone resorption. EA mediated its anti-arthritic effect through downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines. Therefore, EA could provide an additional therapeutic strategy for RA,

particularly in situations in which the patient has elevated levels of pro-inflammatory cytokines.

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