Original Research

Evaluation of the antirheumatic effects of isoflavone-free soy protein isolate and etanercept in rats with adjuvant-induced arthritis

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Impact statement

In view of the partial clinical benefit and significant toxicity of traditional rheumatoid arthritis (RA) treatments, there is a growing trend to use complementary therapy. The antiarthritic activity of soy is related to the effect of soy isoflavones. However, little is known about the antiarthritic activity of soy protein itself. This study demonstrates that soy protein isolate (SPI) and etanercept (ETN), a tumor necrosis factor-α (TNF-α) inhibitor, protect rats against the effects of adjuvant-induced arthritis (AIA) by reducing inflammation (TNF-α and matrix metalloproteinase-3), autoantibody production (anticyclic citrullinated peptide), and lipid peroxidation (malondialdehyde). Only SPI improved dyslipidemia accompanied by RA, giving it the advantage of reducing cardiovascular risk. Additionally, the severity of arthritis-induced pathology, including inflammatory infiltrates, synovial hyperplasia, pannus formation, synovial vascularity, and cartilage erosions, was reduced by both SPI and ETN. This research ascertains the possible antiarthritic effect of SPI, making it a recommended alternative therapy for RA.

Abstract

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by persistent synovitis, autoantibodies, and systemic inflammation. This study evaluates the possible antirheumatic effect of isoflavone-free soy protein isolate (SPI) in adjuvant-induced arthritis in rats. Male albino rats were divided into four groups: the control, arthritis, SPI, and etanercept (ETN) groups. Arthritis was induced by a single subcutaneous injection of 0.1 mL of complete Freund's adjuvant. Treatment with 4 g/kg SPI orally every day or 3 mg/kg ETN subcutaneously three times/week started on the day of arthritis induction. After 11, 21, or 31 days, the rats were sacrificed, serum was collected for analysis of different biological markers, and the right ankle of each animal was obtained for histopathological examination. Serum levels of anticyclic citrullinated peptide (anti-CCP) antibodies, tumor necrosis factor-α (TNF-α), matrix metalloproteinase-3 (MMP-3), malondialdehyde (MDA), and hypercholesterolemia gradually increased in the arthritis animals compared to the control animals. Compared to the arthritis group, the ETN group showed significantly reduced anti-CCP levels at each of the three experimental periods, while a significant reduction in TNF-α, MMP-3, and MDA levels was observed after 21 and 31 days. ETN had no significant effect on the lipid profile after the three experimental periods. SPI significantly reduced anti-CCP, TNF-α, and MMP-3 levels after 31 days. The hypocholesterolemic effect of SPI and a significant reduction in MDA levels were observed after 21 and 31 days. Both ETN and SPI reduced the severity of arthritis-induced histopathological changes. These findings indicate that SPI has anti-inflammatory, antirheumatic, and hypocholesterolemic effects, suggesting it as a complementary therapy for RA.

Keywords: Rheumatoid arthritis, soy protein, etanercept, anticyclic citrullinated peptide, tumor necrosis factor-α, matrix metalloproteinase-3

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Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease. Approximately 1% of the world's population suffers from RA.[1] RA is characterized by synovial inflammation and hyperplasia, autoantibody production (anticitrullinated peptide [anti-CCP] antibodies and rheumatoid factor [RF]), cartilage and bone damage and systemic features, including cardiovascular disease

(CVD), and skeletal, psychological, and pulmonary disorders.[2] Although the cause of RA is still unknown, the interplay among genetic and environmental factors is involved in RA development.[3]

The pivotal role of cytokines in the pathogenesis of RA emerges from their direct implication in several immune pathways associated with RA. Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine produced mainly by macrophages and monocytes, is a key molecule in the pathogenesis of RA.[4] TNF-α acts at different steps on B-cell proliferation as well as antibody production and secretion and augments the effects of other cytokines on B-cell function.[5] Furthermore, TNF-α induces endothelial cell and leukocyte activation, synovial fibroblast proliferation and survival, angiogenesis and activation of matrix metalloproteinases (MMPs).[4] Additionally, TNF-α shifts the oxidant/antioxidant balance in favor of lipid peroxidation by increasing the generation of reactive oxygen species, which could lead to tissue damage.[6]

Despite the breakthroughs in the management of RA and the currently available treatment options, such as non-steroidal anti-inflammatory drugs, corticosteroids, and disease modifying antirheumatic drugs, no single agent consistently offers both a high level of tolerability and a maintained degree of efficacy across a wide RA population.[1] Etanercept (ETN), a TNF-α inhibitor, has been shown to reduce disease activity in RA patients; however, an increased risk of infections was a serious side effect.[7,8] Therefore, the use of complementary therapy is a growing trend.

Abundant and relatively inexpensive dietary protein constitutes 36–56% of soybeans and offers functionally significant properties, high nutritional value, and biologically active polypeptides.[9] In most clinical trials and animal studies, the digestion of soy protein releases bioactive peptides that are then absorbed and distributed to target organs or tissues in an intact and active form.[10–12] Soy proteins, soy-derived peptides, and soy amino acids have antihypertensive, hypocholesterolemic, antioxidant, immunomodulatory, antidiabetic, anticancer, and antiobesity activities.[13,14]

The antiarthritic activity of soy beans was related to the effect of soy isoflavones such as genistein.[15] However, little is known about the antiarthritic activity of soy protein itself. This study aimed to ascertain the possible antiarthritic effect of isoflavone-free soy protein isolate (SPI) in adjuvant-induced arthritis (AIA) in rats.

Materials and method

Drugs and chemicals

Complete Freund's adjuvant (CFA) (Sigma-Aldrich, USA) contained 1 mg of heat-inactivated and dried *Mycobacterium tuberculosis* suspended in 0.85 mL of paraffin oil and 0.15 mL of mannide monooleate. ETN (Enbrel®) was obtained from Amgen Inc. (USA). Ethanol-washed SPI powder was purchased from Protein Technologies International (St Louis, MO, USA). The process of SPI preparation nearly depletes the isoflavones.[16,17]

Table 1. Characteristics of SPI.

Crude protein (g%)	91.7(%)
Moisture (g%)	4.45
Fat (g%)	2.02
Ash (g%)	1.83
Total isoflavones (mg/g SPI)	0.05

SPI: soy protein isolate.

The composition of the SPI powder as analyzed by Protein Technologies International is summarized in Table 1. The protein content was measured by the Kjeldahl method and multiplied by a conversion factor of 6.25 to calculate the total protein content. Analyses for isoflavones were performed using liquid chromatography with absorbance detection. Propylene glycol and 0.9% NaCl were purchased from El Nasr Pharmaceutical Chemicals Co. (Egypt).

Animals and experimental design

The protocols for laboratory animal care were approved by the Research Ethics Committee (Faculty of Pharmacy, Tanta University, Egypt). Eighty-four male albino rats weighing between 180 and 200 g each were obtained from VACSERA (Agouza, Giza, Egypt). Animals were housed and acclimatized to laboratory conditions for one week with free access to rat chow and water *ad libitum*. Rats were randomly divided into four groups of 21 animals each:

The control group The rats received vehicle and served as controls.

The arthritis group The rats were injected with a single subcutaneous injection of 0.1 mL of CFA into the subplantar region of the right hind foot paw to induce arthritis.[18]

The SPI group The rats were induced with arthritis as in the arthritis group and given 4 g/kg/day SPI suspended in propylene glycol by oral gavage.

The ETN group The rats were induced with arthritis as in the arthritis group and subcutaneously injected with 3 mg/kg ETN in 0.9% NaCl three times per week.[19]

Treatment with SPI and ETN started the same day as arthritis induction and continued for 11, 21, or 31 days. A pilot study was conducted to evaluate the effect of three different doses (2, 4, and 7 g/kg) of SPI by examining the histopathological changes in the synovial membrane, cartilage, and bone. The selection of SPI doses was based on previous studies.[17,20] The dose of 4 g/kg SPI was superior to 2 g/kg, while 7 g/kg SPI was very high. Therefore, a dose of 4 g/kg SPI was selected for subsequent experiments.

Macroscopic evaluation of arthritis

Hind paw edema was measured before and every 3–4 days after arthritis induction using Vernier callipers (RadioShack Cat. No. 6400192, Egypt). Ankle circumference was

determined by measuring two perpendicular diameters: a: medio-lateral diameter and b: an anterior–posterior diameter[21]

$$\text{Ankle circumference} = 2\pi\sqrt{(a^2 + b^2)/2}$$

Blood collection and serum separation

Twenty-four hours after the end of the three experimental periods, seven rats in each group were subjected to light diethyl ether anesthesia before being sacrificed. Blood samples were collected from the posterior vena cava and centrifuged for 10 min at 1000 rpm to separate serum that was immediately preserved at −80°C for further analysis. The right ankle joints were removed at the fur line (just proximal to the hock) for histopathological examination.

Determination of serum anti-CCP, TNF-α, and MMP-3 levels

ELISA kits for anti-CCP, TNF-α (Mlbio Biotechnology Company, Shanghai, China) and MMP-3 (Glory Science Co., Ltd, China) were used to determine the respective protein concentrations in serum samples according to the manufacturer's protocol.

Determination of the lipid profile

Serum levels of total cholesterol (TC) and triglycerides (TGs) were measured according to Allain *et al.*[22] and Fossati and Prencipe,[23] respectively, using commercial colorimetric kits (Biodiagnostic, Egypt). The high-density lipoprotein-cholesterol (HDL-C) fraction was measured according to Lopes-Virella *et al.*[24] by precipitating very low-density lipoprotein-cholesterol and low-density lipoprotein-cholesterol (LDL-C) by phosphotungstic acid and magnesium ions. LDL-C levels were calculated using Friedewald's formula[25]

$$\text{LDL-C} = \text{total cholesterol} - \text{HDL-C} - \text{TGs}/5$$

Determination of serum malondialdehyde (MDA) levels

The serum level of MDA, an indicator of lipid peroxidation, was determined using a colorimetric kit (Biodiagnostic, Egypt). MDA reacts with thiobarbituric acid in acidic medium producing thiobarbituric acid reactive product, a pink chromogen, which was measured at 534 nm.[26]

Histopathological examination of the right ankle joint

The right ankle joint from each rat was fixed in 10% neutral buffered formalin for 24 h and then decalcified by immersion in 5% formic acid for three days. After decalcification, the ankle joint was longitudinally transected into two equal halves, immersed in ascending alcohol grades, cleared in xylene, embedded in paraffin, sectioned using a microtome (Leica RM2135; Leica Inc., Germany), and stained with hematoxylin–eosin (H&E).[27] Two slides from the same joint were examined using a light microscope (Leica DM500, Switzerland). Histological scoring was performed according to different variables: inflammatory infiltrates, synovial hyperplasia, pannus formation, synovial vascularity, and cartilage erosions. The scoring range was 0–3 (0: absent, 1: mild, 2: moderate, 3: severe).[28]

Statistical analysis

Analysis of data was performed with Statistical Package for Social Science software version 20. All results are presented as the mean ± SEM. Statistical comparisons among groups were performed by one-way analysis of variance using Fisher's least-significant differences method for comparisons between two groups. When $P < 0.05$, the results were statistically significant.[29]

Results

Effects on paw edema

This procedure induced arthritis in 100% of the animals. Hind paw edema, represented as swelling and redness, developed over a 24 h period in the CFA-injected right hind paw and peaked on day 4. After that, swelling declined until the seventh day, and then the paw began to swell again as arthritis spread. The arthritis group showed a significant increase in ankle circumference compared to the control group after 11 (↑1.48-fold), 21 (↑1.59-fold), and 31 days (↑1.72-fold). The ETN-treated group exhibited significantly reduced ankle circumference compared to the arthritis group after 11 (↓7.03%), 21 (↓16.91%), and 31 days (↓25.76%). A non-significant decrease in ankle circumference was observed between the arthritis group and the SPI group after 11 days, while a significant decrease was observed after 21 (↓4.65%) and 31 days (↓14.02%). The ETN group showed a significant reduction in paw edema compared to the SPI group after each of the three experimental periods (Figure 1).

Effect on serum anti-CCP

The serum level of anti-CCP in the arthritis group was markedly elevated after 11 days (↑2.46-fold) in comparison with that in the control group and continued to increase after 21 (↑4.54-fold) and 31 days (↑5.83-fold) after arthritis induction. The ETN-treated group showed significantly reduced anti-CCP levels after 11 (↓16.11%), 21 (↓33.45%), and 31 (↓37.25%) days compared to the arthritis group. However, there was no significant difference in anti-CCP levels between the SPI and arthritis groups after 11 or 21 days of CFA-induced arthritis. The SPI group showed a significant decrease in anti-CCP levels after 31 days compared to the arthritis group (↓15.82%). The ETN group showed a significant reduction in anti-CCP levels compared to the SPI group after the three experimental periods (Figure 2).

Effect on serum TNF-α

During the progression of AIA, the serum levels of TNF-α in the arthritis group were significantly increased after 21

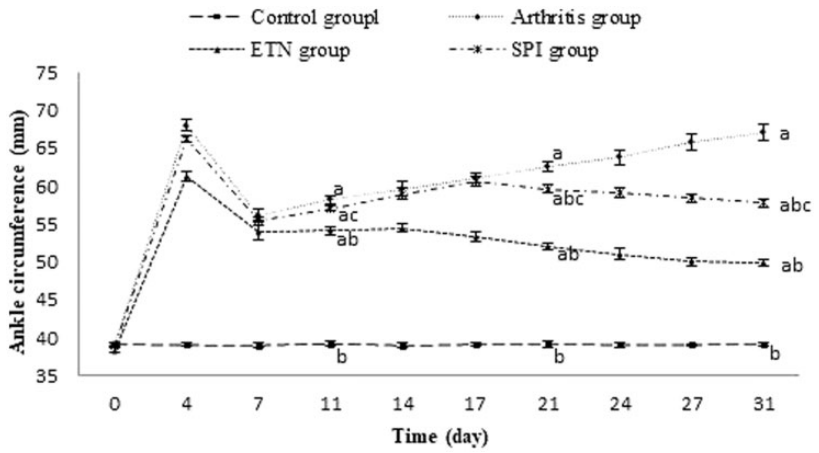

Figure 1. Effect of SPI and ETN on right hind paw edema in AIA rats. Rats were injected with 0.1 mL of CFA into the subplantar region of the right hind foot paw (the arthritis group), and some were treated with 3 mg/kg ETN subcutaneously three times per week (the ETN group) or 4 g/kg/day SPI orally (the SPI group) starting the day of arthritis induction and continued for 11, 21, or 31 days. Healthy control rats were treated with vehicle only. Hind paw edema was measured before and every 3–4 days after arthritis induction. The results are expressed as the mean±SEM; n = 7 rats per group. a: Significant versus the control group ($P < 0.05$). b: Significant versus the arthritis group ($P < 0.05$). c: Significant versus the ETN group ($P < 0.05$). ETN: etanercept; SPI: soy protein isolate.

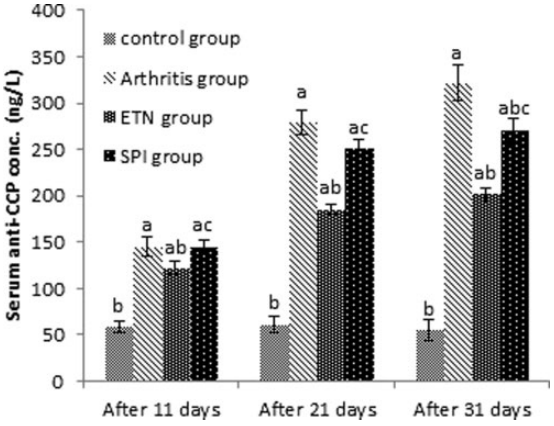

Figure 2. Effect of SPI and ETN on serum anti-CCP levels in AIA rats. Rats were injected with 0.1 mL of CFA into the subplantar region of the right hind foot paw (the arthritis group), and some were treated with 3 mg/kg ETN subcutaneously three times per week (the ETN group) or 4 g/kg/day SPI orally (the SPI group) starting the day of arthritis induction and continued for 11, 21, or 31 days. Healthy control rats were treated with vehicle only. Hind paw edema was measured before and every 3–4 days after arthritis induction. The results are expressed as the mean±SEM; n = 7 rats per group. a: Significant versus the control group ($P < 0.05$). b: Significant versus the arthritis group ($P < 0.05$). c: Significant versus the ETN group ($P < 0.05$). Anti-CCP: anticyclic citrullinated peptide; ETN: etanercept; SPI: soy protein isolate.

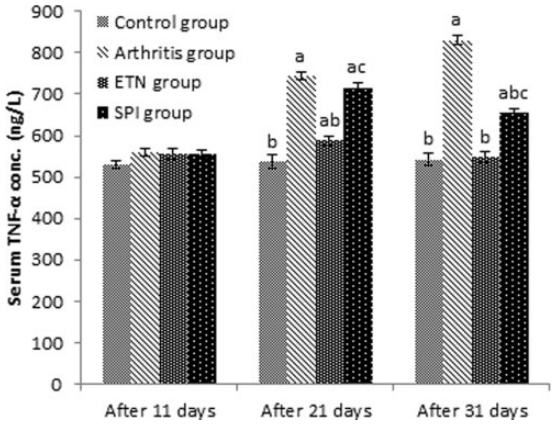

Figure 3. Effect of SPI and ETN on serum TNF-α levels in AIA rats. Rats were injected with 0.1 mL of CFA into the subplantar region of the right hind foot paw (the arthritis group), and some were treated with 3 mg/kg ETN subcutaneously three times per week (the ETN group) or 4 g/kg/day SPI orally (the SPI group) starting the day of arthritis induction and continued for 11, 21, or 31 days. Healthy control rats were treated with vehicle only. Hind paw edema was measured before and every 3–4 days after arthritis induction. The results are expressed as the mean±SEM; n = 7 rats per group. a: Significant versus the control group ($P < 0.05$). b: Significant versus the arthritis group ($P < 0.05$). c: Significant versus the ETN group ($P < 0.05$). ETN: etanercept; SPI: soy protein isolate; TNF-α: tumor necrosis factor-α.

(↑1.39-fold) and 31 days (↑1.53-fold) in comparison with the levels in the control group. The ETN group exhibited significantly reduced TNF-α levels after 21 days (↓21.03%) that normalized after 31 days (↓33.85%) compared to the arthritis group. The SPI group showed a significant attenuation effect on TNF-α levels only after 31 days (↓21%). The ETN group showed a significant reduction in TNF-α levels compared to the SPI group after 21 and 31 days (Figure 3).

Effect on serum MMP-3

The serum level of MMP-3 in the arthritis group was significantly increased in association with AIA progression after 21 (↑1.5-fold) and 31 days (↑1.93-fold). The ETN group showed significantly reduced MMP-3 levels after 21 (↓15.08%) and 31 days (↓28.71%) compared to the arthritis group, while the SPI group showed a significant attenuation effect on MMP-3 levels only after 31 days (↓18.38%). The ETN group showed a significant reduction in MMP-3 levels compared to the SPI group after 21 days (Figure 4).

Effect on serum MDA

Eleven days after arthritis induction, the level of serum MDA in the arthritis, ETN, and SPI groups showed no significant changes. The arthritis group showed a significant increase in MDA levels compared to the control group after 21 (↑2.75-fold) and 31 days (↑3.34-fold). The ETN group

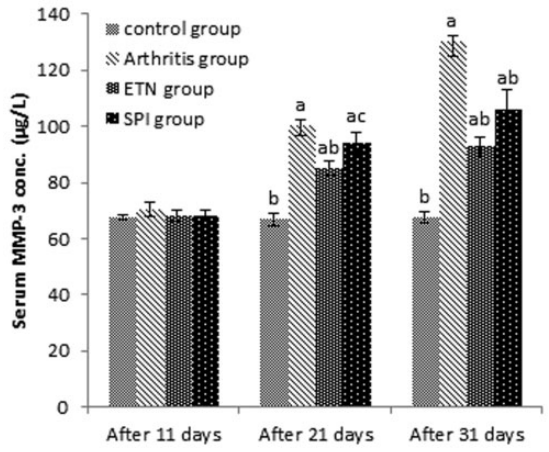

Figure 4. Effect of SPI and ETN on serum MMP-3 levels in AIA rats. Rats were injected with 0.1 mL of CFA into the subplantar region of the right hind foot paw (the arthritis group), and some were treated with 3 mg/kg ETN subcutaneously three times per week (the ETN group) or 4 g/kg/day SPI orally (the SPI group) starting the day of arthritis induction and continued for 11, 21, or 31 days. Healthy control rats were treated with vehicle only. Hind paw edema was measured before and every 3–4 days after arthritis induction. The results are expressed as the mean±SEM; n = 7 rats per group. a: Significant versus the control group ($P < 0.05$). b: Significant versus the arthritis group ($P < 0.05$). c: Significant versus the ETN group ($P < 0.05$). ETN: etanercept; MMP-3: matrix metalloproteinase-3; SPI: soy protein isolate.

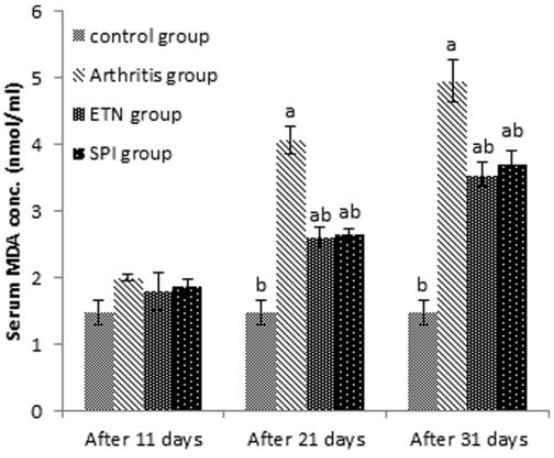

Figure 5. Effect of SPI and ETN on serum MDA levels in AIA rats. Rats were injected with 0.1 mL of CFA into the subplantar region of the right hind foot paw (the arthritis group), and some were treated with 3 mg/kg ETN subcutaneously three times per week (the ETN group) or 4 g/kg/day SPI orally (the SPI group) starting the day of arthritis induction and continued for 11, 21, or 31 days. Healthy control rats were treated with vehicle only. Hind paw edema was measured before and every 3–4 days after arthritis induction. The results are expressed as the mean±SEM; n = 7 rats per group. a: Significant versus the control group ($P < 0.05$). b: Significant versus the arthritis group ($P < 0.05$). ETN: etanercept; MDA: malondialdehyde; SPI: soy protein isolate.

exhibited significantly reduced MDA levels after 21 (↓35.87%) and 31 days (↓28.48%) compared to the arthritis group. Additionally, the SPI group showed a significant reduction in MDA levels after 21 (↓34.8%) and 31 days (↓25.41%) compared to the arthritis group (Figure 5).

Effect on the lipid profile

The arthritis group showed a marked increase in serum TC, TGs, and LDL-C levels and a significant decrease in serum HDL-C levels at 21 (↑1.5-fold, ↑1.5-fold, ↑2.83-fold, and ↓0.79-fold, respectively) and 31 days (↑1.77-fold, ↑1.94-fold, ↑3.77-fold, and ↓0.66-fold, respectively) after AIA induction. The SPI group showed a significant decrease in the levels of TC, TGs, and LDL-C with a significant increase in HDL-C compared to the arthritis group after 21 (↓18.45%, ↓20.05%, ↓35.46%, and ↑12.95%, respectively) and 31 days (↓21.56%, ↓19.35%, ↓42.21%, and ↑27.88%, respectively). On the other hand, ETN showed no significant effect on the lipid profile at any of the three experimental periods (Table 2).

Histopathological evaluation

Histopathological evaluation of the right ankle joints from the control group showed normal synovial lining, synovial vascularity, articular cartilage, and subchondral bone in the control group (Figure 6(a)). On the other hand, the arthritis groups showed heavy aggregates of mononuclear inflammatory infiltration comprising predominantly lymphocytes and histocytes and multiple well-defined granulomas. The synovial layer of the arthritis group showed congested vascular spaces, increased synovial vascularity, and synovial hyperplasia with an adjacent subchondral bone layer (Figure 6(b)). The pannus, highly vascularized granulation tissue formed of T cell macrophages and synoviocytes, crept over the eroding articular cartilage and penetrated into subchondral bone, causing erosion and destruction (Figure 6(c)). As the disease progressed after 21 (Figure 6 (d) and (e) and 31 days (Figure 6(f)), the severity of arthritic histopathological findings increased.

Compared with the arthritis group, treatment with ETN resulted in less inflammatory infiltrates in the synovium after 11 days (Figure 6(g)) and less synovial vascularity and synovial hyperplasia after 21 days (Figure 6(h)). The pannus was almost inhibited, and the destruction of articular cartilage and subchondral bone was alleviated after 31 days (Figure 6(i)). The inflammatory infiltrates in the synovium of arthritic rats treated with SPI for 11 days were minimal (Figure 6(j)). Treatment with SPI for 21 (Figure 6. (k)) and 31 days (Figure 6(l)) moderately ameliorated the histological changes in synovial membrane, cartilage, and bone.

Further histological scoring shown in Table 3 demonstrates that the severity of AIA increased as the disease progressed. Histological scoring of the ETN group and SPI group after 11 days showed no significant decrease except for the inflammatory infiltrates in the ETN group. Treatment with ETN and SPI for 21 days significantly decreased the histological scoring except for the synovial vascularity, cartilage, and bone erosion in the SPI group. The antiarthritic effect of ETN and SPI was significant after 31 days in all histopathological variables.

Discussion

RA is characterized by chronic inflammation of the synovial joints that can progress to joint destruction.[30] In the current study, RA was induced by CFA as evidenced by histopathological features and pannus formation. In agreement with previous studies,[31,32] arthritis onset occurred at

Table 2. Serum lipid profile in the studied groups.

	Groups	Serum TC (mg/dL)	Serum TGs (mg/dL)	Serum HDL (mg/dL)	Serum LDL (mg/dL)
After 11 days	Control group	43.78± 0.89	34.74 ± 0.59	24.33 ± 0.43	12.50 ± 0.86
	Arthritis group	45.92 ± 1.20	35.65 ± 0.84	23.68 ± 0.70	14.39±1.06
	ETN group	44.27 ± 0.93	35.02 ± 0.85	23.78 ± 0.91	13.48 ± 0.73
	SPI group	44.16 ± 0.78	35.24 ± 1.08	23.50 ± 0.44	13.62 ± 0.74
After 21 days	Control group	43.89 ± 1.54b	35.47 ± 1.00b	24.02 ± 1.02b	12.78 ± 0.97b
	Arthritis group	65.89 ± 0.83a	53.26 ± 0.59a	19.02 ± 0.51a	36.21 ± 0.72a
	ETN group	67.17 ± 1.08a	52.55 ± 0.69a	20.54 ± 0.75a	36.11 ± 1.01a
	SPI group	53.73 ± 0.84ab	42.58 ± 1.02ab	21.85 ± 0.50ab	23.37 ± 0.65ab
After 31 days	Control group	44.09 ± 1.37b	35.57 ± 0.84b	24.15 ± 0.79b	12.83 ± 0.73b
	Arthritis group	78.18 ± 0.93a	69.00 ± 0.73a	16.06 ± 0.63a	48.32 ± 0.71a
	ETN group	77.90 ± 1.08a	67.16 ± 0.45a	17.81 ± 0.46a	46.66 ±1.07a
	SPI group	61.32 ± 1.01ab	55.65 ± 1.30ab	22.27 ± 0.57ab	27.92 ± 1.00ab

ETN: etanercept; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; SPI: soy protein isolate; TC: total cholesterol; TGs: triglycerides.
Results are expressed as mean±SEM, n = 7 rats per each group.
a: Significant versus control group ($P < 0.05$).
b: Significant versus arthritic group ($P < 0.05$).

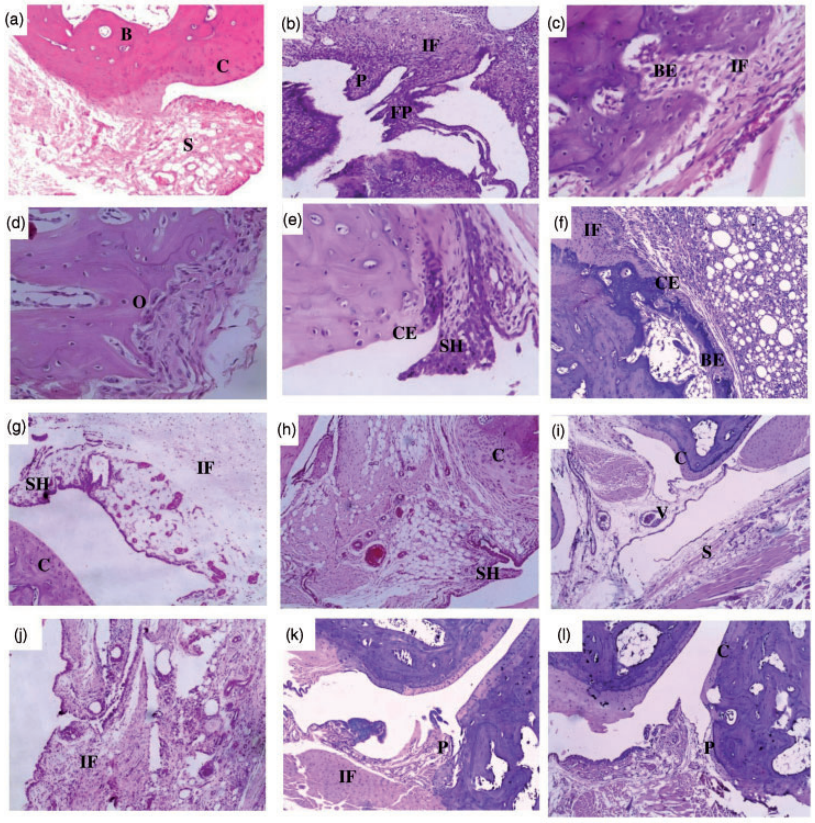

Figure 6. Representative histopathology sections of the right ankle joint of (a): the healthy control group shows normal synovial membrane (S), cartilage (C), and bone (B). (b,c): The arthritis group after 11 days shows severe inflammatory infiltrates (IF), moderate pannus (P), and moderate bone erosion (BE). (d,e): The arthritis group shows increased osteoblastic activity (O), moderate cartilage erosion (CE), and moderate synovial hyperplasia (SH) after 21 days. (f): The arthritis group after 31 days of severe inflammatory infiltrates (IF), severe cartilage erosion (CE), and moderate bone erosion (BE). (g): The ETN group after 11 days shows mild inflammatory infiltrates (IF), mild synovial hyperplasia (SH), and normal cartilage (C). (h): The ETN group after 21 days shows mild inflammatory infiltrates (IF), mild synovial hyperplasia (SH), and normal cartilage. (i): The ETN group after 31 days shows mild congested blood vessels (CV), normal synovial membrane (S), and normal cartilage (C). (j): The SPI group after 11 days shows mild inflammatory infiltrates (IF). (k): The SPI group after 21 days shows mild inflammatory infiltrates and mild pannus (P). (l): The SPI group after 31 days shows mild pannus (P) and normal cartilage (C). H&E, ×40. (A color version of this figure is available in the online journal.)

approximately 9–10 days post-CFA injection, where tissue edema, synovial changes, and inflammatory infiltrates were observed. The longer the disease progressed, the more pannus formed and the more destruction of soft tissue, cartilage, and bone occurred. These histopathological changes were associated with paw edema and swelling, which was represented as increased ankle circumference.

Table 3. Histological scoring of the studied groups.

Groups		Inflammatory infiltrates	Synovial hyperplasia	Pannus formation	Synovial vascularity	Cartilage erosion	Bone erosion
After 11 days	Arthritis group	2.14±0.26	1.57±0.3	1.29±0.36	1.57±0.20	0.71±0.29	0.14±0.14
	ETN group	1.14±0.26b	1.14±0.26	0.86±0.34	0.86±0.34	0.43±0.20	0.00±0.00
	SPI group	2.167±0.40c	1.33±0.21	1.00±0.26	1.50±0.22	0.67±0.21	0.00±0.00
After 21 days	Arthritis group	2.29±0.18	2.00±0.31	1.71±0.28	1.86±0.34	1.29±0.36	0.86±0.26
	ETN group	1.33±0.21b	0.83±0.31b	0.50±0.22b	0.67±0.21b	0.33±0.21b	0.17±0.17b
	SPI group	1.67±0.21b	1.00±0.26b	0.67±0.33b	1.00±0.36	0.67±0.21	0.33±0.21
After 31 days	Arthritis group	2.37±0.26	2.37±0.18	2.00±0.27	2.12±0.23	1.75±0.25	1.37±0.32
	ETN group	0.71±0.28b	1.29±0.29b	0.71±0.28b	0.86±0.26b	0.57±0.20b	0.28±0.18b
	SPI group	1.12±0.29b	1.62±0.26b	1.12±0.29b	1.12±0.23b	0.75±0.31b	0.50±0.19b

ETN: etanercept; SPI: soy protein isolate.
Results are expressed as mean±SEM, n = 7 rats per each group.
b: Significant versus arthritic group ($P < 0.05$).
c: Significant versus ETN group ($P < 0.05$).

In the present study, treatment with ETN or SPI ameliorated the histopathological changes and paw edema, and ETN was slightly better than SPI was. This result coincides with previous studies showing that ETN and SPI treatment significantly decreased paw swelling in an AIA model.[33,34]

In the AIA model, many inflammatory cytokines and oxidative biomarkers, such as TNF-α, are crucially associated with the chronic inflammatory response, as in the joint destruction of RA.[35] Coinciding with previous studies,[36,37] our work showed that the systemic concentration of TNF-α was markedly increased at 20 days after CFA inoculation.

Both ETN and SPI treatment significantly reduced the serum levels of TNF-α in the AIA model, with ETN being more effective. Our results were in accordance with those obtained by Huang *et al.*,[38] who showed that administration of ETN significantly decreased TNF-α serum levels in collagen-induced arthritis, while Yan *et al.*[39] reported that SPI supplementation reduced TNF-α in mice with chronic systemic inflammation. Interestingly, the isoflavone-free soy protein diet strongly inhibited lipopolysaccharide-induced NF-κB activation and the subsequent upregulation of pro-inflammatory cytokines, including TNF-α.[40]

Autoantibodies are a useful tool in understanding the pathogenesis, diagnosis, and prognosis of RA as well as in screening therapeutic strategies.[41,42] In the present study, elevated anti-CCP levels were detectable starting from day 7 after arthritis induction. Our results were in agreement with those of previous studies.[43,44] Treatment with ETN showed a significant decrease in anti-CCP levels compared to treatment with SPI. Nozaki *et al.*[45] reported that the serum anti-CCP levels of the RA patients were significantly decreased after three months of treatment with ETN. Our study showed that SPI slowed the progression of RA, which coincides with a marked decrease in the serum level of anti-CCP. This reduction in anti-CCP levels may account for the suppression of TNF-α.

TNF-α activates the NF-κB pathway, leading to further production, expression, and upregulation of pro-inflammatory cytokines, MMPs and MDA.[46,47] During the early stages of RA, MMP-3 is probably the key enzyme as it degrades non-fibrillar collagen and proteoglycans of the extracellular matrix in cartilage, leading to further joint inflammation, cartilage degradation, and bone erosion.[48] Our study showed a significant reduction in MMP-3 after treatment with both ETN and SPI. Catrina *et al.*[49] reported that ETN markedly reduced MMP-3 serum levels, so it can prevent further progression of joint damage. Additionally, we found in our study that SPI reduced serum MMP-3 levels, which can be explained by its ability to reduce the TNF-α serum level.

An elevated serum level of MDA has been observed in RA.[50] In the AIA model, a gradual but significant elevation in lipid peroxidation was observed during the development of arthritis.[51] Our results showed that treatment with ETN or SPI had almost equal effects on reducing MDA serum levels. Our results were in accordance with those obtained by Yildirim *et al.*[52] and Sehirli *et al.*,[53] who showed that ETN treatment reduced serum MDA levels in the peritonitis model and lung MDA levels in the thermal injury model, respectively. In Takenaka *et al.*'s[54] study, both SPI and soy peptides prohibited the increase in MDA serum levels in paraquat-induced oxidative stress in rats. Moreover, soy protein decreased plasma MDA in postmenopausal women with metabolic syndrome.[55]

Dyslipidemia, a critical CVD predictor, is highly common in RA patients in early and advanced stages.[56] Consistent with previous studies,[57,58] our work showed that elevated levels of plasma TC, TGs, and LDL-C and reduced levels of HDL-C were observed after 28 days of arthritis induction. Treatment with ETN did not improve dyslipidemia, which was also reported by Totoson *et al.*[59] after three weeks of treatment with ETN. In RA patients, ETN treatment did not make any difference in serum lipid levels.[60,61] Therefore, the management of dyslipidemia must be an approach to reduce the cardiovascular risk in patients with RA.[62] Our results confirmed that SPI improved dyslipidemia, which was in agreement with those by Mohammadshahi *et al.*[20] Additionally, Marsh *et al.*[63] reported that soy protein markedly decreased the TC and LDL-C serum levels in a model of systemic inflammation. In a meta-analysis study, the ingestion of soy protein significantly reduced serum levels of TC, LDL-C, and TGs without affecting HDL-C level.[64]

Conclusion

The results from our study suggest that SPI has a potential antirheumatic role against AIA in rats by reducing inflammation and oxidative stress. In addition, the beneficial effect of SPI in improving dyslipidemia may reduce the cardiovascular risk accompanied by RA. Thus, SPI as a natural product could be considered as a complementary alternative therapy to reduce the progression of RA.

Authors' contributions: NE-E, EGK, and MMK participated in the conception and design of this work. Practical work, data analysis and interpretation, and drafting the article were carried out by MMK. MMS conducted and interpreted the histopathological examination. The article was critically revised by EGK and MMS.

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