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Effect of inositol -stabilized arginine silicate on arthritis in a rat model

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ABSTRACT

The purpose of this study was to test the effects of arginine-silicate-inositol complex (ASI), compared to a combination of the individual ingredients (A + S + I) of the ASI, on inflammatory markers and joint health in a collagen-induced arthritis (CIA) rat model. A total of 28 Wistar rats were divided into four groups: (i) Control; (ii) Arthritic group, rats subjected to CIA induction by injection of bovine collagen type II (A); (iii) Arthritic group treated with equivalent doses of the separate components of the ASI complex (arginine hydrochloride, silicon, and inositol) (A + S + I); (iv) Arthritic group treated with the ASI complex. The ASI complex treatment showed improved inflammation scores and markers over the arthritic control and the A + S + I group. ASI group had also greater levels of serum and joint-tissue arginine and silicon than the A + S + I group. Joint tissue IL-6, NF-κB, COX-2, TNF-α, p38 MAPK, WISP-1, and β-Catenin levels were lower in the ASI group compared to the other groups ($P < 0.05$ for all). In conclusion, these results demonstrate that the ASI complex may be effective in reducing markers of inflammation associated with joint health and that the ASI complex is more effective than a combination of the individual ingredients.

1. Introduction

In many industrialized countries, rheumatoid arthritis (RA) is the leading cause of major disability. RA primarily affects the joints and causes pain, swelling, stiffness, and synovial hyperplasia (pannus formation), resulting in bone damage and destruction of cartilage (Borchers et al., 2004; Mahajan and Mikuls, 2018). RA is largely known as a chronic systemic inflammatory autoimmune disease associated with nonspecific inflammation of peripheral joints, destruction of articular tissues, and deformities in the joints (Asquith et al., 2009; Mahajan and Mikuls, 2018). Although RA has an uncertain etiology, some multifactorial components such as genetic and environmental factors can together lead to early immune concern and subsequent chronic joint inflammation (Aletaha et al., 2010).

The animal collagen-induced arthritis (CIA) model has been used to test new therapeutic agents as well as to demonstrate the potential pathogenic mechanisms of autoimmunity (Asquith et al., 2009; Choudhary et al., 2018). RA has been shown to be accelerated by some factors including pro-inflammatory cytokines, adhesion molecules, and corticosteroids (Ku et al., 2009). It has been well-defined that pro-inflammatory cytokines and free radicals arising from inflammatory cells create joint dysfunction reflected in the erosion of both bone and cartilage (Schett and Gravallese, 2012).

An arginine complex which consists of arginine, silicate, and inositol (ASI) might be an effective and reliable intervention for the treatment of RA. Amino acids like arginine have been reported to play a pivotal role in the synthesis of growth hormone, insulin-like growth factor-I (IGF-I) and nitric oxide (Colao et al., 1999; Rondón et al., 2017). They have also been seen to hasten events of metabolic disturbance such as in calcium absorption, growth, decalcification, dentition and ossification defects, osteomalacia and rachitism (Fiore et al., 2000; Clementi et al., 2001; Sahin et al., 2018). The ASI complex, a promising agent, may show greater systemic bioavailability than its individual components due to its physicochemical properties (Proctor

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Abbreviations

ALT	alanine aminotransferase
ASI	arginine-silicate-inositol complex
AST	aspartate aminotransferase
CIA	collagen-induced arthritis
COX-2	cyclooxygenase-2
CV	coefficient of variations
DKK-1	dickkopf1
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinases
HDL	high density lipoprotein
HPLC	high-performance liquid chromatography
I-κB	inhibitor of kappa B
IL-6	interleukin 6
IL-7	interleukin 7
LDL	low density lipoprotein
LEF1	lymphoid enhancer-binding factor 1
NF-κB	nuclear factor kappa B
PBS	phosphate buffered saline
p38 MAPK	p38 mitogen-activated protein kinases
RA	rheumatoid arthritis
TC	total cholesterol
TG	total triglyceride
TNF-α	tumor necrosis factor alpha
WISP-1	wnt1-inducible signaling pathway protein 1

et al., 2007). Proctor et al. (2005) exhibited that the ASI complex exerts stimulates the rate of absorption of arginine across the intestinal tract and delivers the highest concentration of available arginine in the blood compared to traditional arginine complexes, such as arginine hydrochloride (HCl). Moreover, it has been found that the ASI complex has no toxic effects on *ex vivo* trial models (Komorowski et al., 2004). An *in vitro* study showed that arginine silicate component of ASI complex dissociates in the acidic environment of the stomach. (Komorowski et al., 2004). Therefore, the safety of ASI complex can be based on the individual safety profiles of its components (arginine, silicon, and inositol). This study was designed to elucidate the effect of the structural presence of the ASI complex and mixture of its ingredients on inflammatory markers and joint health in collagen-induced arthritic rats.

2. Materials and methods

2.1. Animals and experimental design

A total of 28 female Wistar rats (8 weeks old) were obtained from Inonu University Research Center (Malatya, Turkey). Rats were not used previously in other experiments. Rats were maintained in a controlled environment (temperature: 22 ± 2 °C, relative humidity: 55 ± 5% and a 12/12 h light/dark cycle) and were fed a standard diet and water *ad libitum*. The experimental procedures conducted on rats were approved by the Animal Experiments Ethics Committee of Inonu University (Malatya, Turkey) according to the Government of Turkey's accepted principles for the use and care of lab animals.

The animals were randomly divided into four groups (n = 7 per group): (i) Control (no treatment); (ii) Arthritic control group, rats subjected to CIA induction by intradermal injection of bovine collagen type II (A); (iii) Arthritic group treated with equivalent doses of the separate component parts (arginine HCl, silicon, and inositol) of the ASI complex by oral gavage for 29 days (A + S + I); (iv) Arthritic group treated with the ASI complex by oral gavage for 29 days (ASI). The ASI complex consists of 43.8% arginine, 8.0% silicon and 24.0% inositol. Rats in the ASI complex group received 25 mg of the ASI complex per kg body weight (BW) per day. The ASI complex at a dose of 25 mg/kg BW contains approximately 11 mg arginine, 2 mg silicon, and 6 mg inositol. Control and arthritis groups were given to drinking water by oral gavage in the same volume with rats in the treatment group for 29 days. Rats in the A + S + I group received a mixture of 13.3 mg of arginine HCl (approximately 11 mg arginine), 4.3 mg of silicon dioxide (approximately 2 mg silicon) and 6 mg of inositol. Therefore, rats in the ASI complex and mixture of its ingredients (A + S + I) groups consumed the same amount of arginine, silicon, and inositol.

The chicken type II collagen used in this study originated from sternal cartilage (Sigma Aldrich, St. Louis, USA, Product No. C9301) and was dissolved in 0.1 M acetic acid at 1 mg/mL. The collagen solution was emulsified in equal volumes with incomplete Freund's adjuvant (CFA, Difco Laboratories, Detroit, USA). The solution was administered intradermally (total: 200 μg/rat) to induce arthritis in the rats in all groups, excluding the control (no treatment) group. The solution was injected into the dorsal tail (100 μg/rat) and hind paw (50 μg each). Seven days after the first administration, booster injections (100 μg/rat) were given in the dorsal tail (Trentham et al., 1977). Rats in the control groups were injected with an equal volume of saline instead of CFA. After injecting collagen, all rats were evaluated for symptoms of clinical arthritis, scoring between 0 and 4 for the development of arthritis in both hind paws (Larsson et al., 1990).

2.2. Sample collection

At the end of the study, rats were decapitated, blood samples were collected, and the hind paws were amputated from the knee down for further analyses. The blood samples were centrifuged at 3000 rpm for 10 min, and the obtained serum was kept at −20 °C until the day of analysis. Also, the hind paws were cut off for further histopathological analysis. One hind paw was fixed with 10% formalin solution and was established in paraffin for histopathological examination, and the other hind paw was frozen at −80 °C for further western blot analysis.

2.3. Laboratory analyses

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and serum creatinine, total cholesterol (TC), total triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) levels were analyzed by using an automatic analyzer (Samsung LABGEO PT10, Samsung Electronics Co, Suwon, Korea). Serum tumor necrosis factor alpha (TNF-α), interleukin (IL)-7 and IL-6 levels were measured by using enzyme-linked immunosorbent assay (ELISA) rodent-specific kits (R&D Systems, Minneapolis, MN) according to the procedure recommended by the manufacturer's instructions. The intra- and inter-assay coefficient of variations (CV) were 5 and 6.3% for TNF-α, 5.7 and 6.9% for IL-17 and 5.5 and 7.4% for IL-6. Serum ghrelin concentration was detected by ELISA using Rat Ghrelin ELISA kits (Millipore, USA). The intra- and inter-assay CVs for this kit were 5.6 and 7.2%. Serum obestatin, sclerostin and Dickkopf1 (DKK-1) levels were also measured using a commercially available rodent-specific ELISA kit (R&D Systems, Minneapolis, MN). The intra- and inter-assay CVs were 5.3 and 7.6% for obestatin, 6.1 and 8.7% for sclerostin, and 5.3 and 6.8% for DKK-1.

Serum and tissue arginine levels were analyzed by high-performance liquid chromatography (HPLC, Shimadzu, Japan) as described by Pieper and Dondlinger (1997). Sera samples were extracted 1:1 in 35% (wt/vol) sulfosalicylic acid dihydrate. After mixing and centrifugation, the supernatant was mixed 1:1 with lithium-D buffer before analysis. Tissue samples were homogenized in ground-glass microhomogenizing tubes in 800 μl of 0.5 N perchloric acid and centrifuged. An aliquot of supernatant (700 μl) was mixed with 196 mL 2M potassium carbonate, centrifuged and frozen at −20 °C for amino acid

analysis. Amino acid standards and other chemicals were obtained from Sigma-Aldrich Chemicals (St. Louis, USA).

The obtained joint samples (0.1–0.6 g) were placed into Teflon tubes containing concentrated (69%) nitric acid and 40% hydrogen peroxide for microwave digestion in a Berghof Speedwave 4 system (Berghof Products and Instruments, Germany) for 20 min at 180 °C. After cooling, distilled deionized water was added to every tube to obtain a total volume of 15 ml. These solutions were used for further analysis. Serum and joint tissue silicon levels were analyzed by atomic absorption spectrometry (Perkin-Elmer, Analyst 800, Kleve, Germany) with a hydride generator system (HG-GF-AAS) in combination with flow injection analysis (FIAS-400, Waltham, MA, USA) with EDL lamb. Details of the entire analytical procedure for the determination of silicon have been described previously (Bissé et al., 2005; Jugdaohsingh et al., 2015; Díaz-Gómez et al., 2017). The silicon standard of 1000 mg/L in water (N9303799) from PerkinElmer was used by the standard addition technique. The detection limit was 0.72 mmol/L and the limit of quantification was set at 1.54 mmol/L.

2.4. Western blot analysis

Tissue samples were analyzed for the expression of IL-6, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), inhibitor of kappa B (I-κB), cyclooxygenase-2 (COX-2), TNF-α, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinases (p38 MAPK), lymphoid enhancer-binding factor 1 (LEF1), Wnt1-inducible signaling pathway protein 1 (WISP-1), and β-catenin using the western blot technique as described previously (Sahin et al., 2018). The hind paws were excised rapidly from decapitated rats and then quickly frozen at −80 °C. Tissue samples were homogenized in ice-cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). Total protein (20 μg) samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Schleicher and Schuell Inc., Keene, NH, USA). The membranes were blocked with 1% bovine serum albumin in PBS (phosphate buffered saline) for 1 hour prior to the application of a primary antibody. The antibodies specific to IL-6, NF-κB, I-κB, COX-2, TNF-α, ERK1/2, p38 MAPK, LEF1, WISP-1, and β-catenin (Abcam, Cambridge, UK) were diluted (1:1000) in the same buffer containing 0.05% Tween-20. In each analysis, the nitrocellulose membrane was incubated overnight at 4 °C with a protein antibody. The blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Abcam, Cambridge, UK). Specific binding was detected using diaminobenzidine and hydrogen peroxide as substrates. Protein loading was controlled using a monoclonal mouse antibody against a β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). Protein levels were determined with respect to the density of the signals observed by using an image analysis system (ImageJ; National Institute of Health, Bethesda, USA).

2.5. Histopathological evaluations

Samples of the joint tissue fixed in formalin solution to prepare paraffin blocks were decalcified with 10% nitric acid (one mount). Cross sections taken from the blocks were stained with hematoxylin and eosin (H&E). Subsequently, they were examined by an expert pathologist under a light microscope at × 40, × 100, × 200, and × 400 magnifications to evaluate inflammatory cell infiltration, pannus formation, and bone destruction around the joint. Samples were scored with a score ranging from 0 to 4 for histopathological scoring as previously defined (Barsante et al., 2005, Table 1).

2.6. Statistical analysis

All results are shown as mean ± SEM. Data were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Statistical significance of the differences among the groups was determined by ANOVA, followed by Tukey's post hoc test; $P < 0.05$ was considered to indicate a statistically significant result. Differences in continuous values (clinical scoring of arthritis on 14th and 29th days) were assessed using the Wilcoxon ranksum test.

3. Results

3.1. Biochemical parameters

Arthritis induction significantly increased serum ALT, AST, creatinine, TC, TG, and LDL levels compared to the control group (no treatment) ($P < 0.001$ for all; Table 2), but significantly reduced serum HDL levels ($P < 0.01$). Administration of ASI complex significantly lowered the activates of ALT ($P < 0.001$), AST ($P < 0.001$), and levels of creatinine ($P < 0.01$), TC ($P < 0.05$), TG ($P < 0.001$) and LDL ($P < 0.01$) when compared to arthritic controls (Table 2). A + S + I treatment decreased the activities of ALT ($P < 0.001$), AST ($P < 0.05$), and levels of TG ($P < 0.05$) and LDL ($P < 0.05$), and increased HDL levels ($P < 0.05$) compared to arthritis group. However, in the A + S + I group, creatinine and TC levels were not changed when compared to the arthritis group ($P > 0.05$). On the other hand, except for HDL levels ($P < 0.05$), no difference was found in these parameters when compared to ASI complex and A + S + I groups ($P > 0.05$).

3.2. Arthritis score

As shown in Table 3, there was no difference in arthritis score at day 14 ($P > 0.05$). By the 29th day, arthritis scores had significantly decreased in the ASI complex and A + S + I groups compared to the arthritic control group ($P < 0.001$ for both), with ASI complex treatment being the most effective. Moreover, on the 29th day, the mean arthritis score was lower in the ASI complex group versus the A + S + I group ($P < 0.001$) (Table 3).

3.3. Inflammatory markers

Significant increases in serum proinflammatory cytokine levels were detected after 29 days in rats in the arthritic control group when injected with CIA to induce arthritis (Table 3). However, serum TNF-α levels were significantly lower in ASI complex and A + S + I treated groups compared to the arthritic control group ($P < 0.01$ for all). Moreover, the ASI complex dramatically reduced serum levels of TNF-α compared to equivalent doses of ASI's components individually. Similar results were found for IL-17, IL-6 and DKK-1 ($P < 0.01$). Rats subjected to CIA presented a significant decrease in serum ghrelin, obestatin, and sclerostin levels when compared with normal rats ($P < 0.001$).

Table 1
Histopathologic assessment of inflammation severity and arthritis severity.

Perisynovial tissue (PT) inflammation severity	Score
Normal PT	0
PT inflammation, no aggregates	1
PT inflammation, occasional, small, focal aggregates	2
Moderate PT inflammation, many small aggregates	3
Diffuse PT inflammation and large aggregates	4
Arthritis severity	**Score**
Normal cartilage and bone tissue	0
Synovial hyperplasia or hypertrophy	1
Pannus or superficial cartilage erosion	2
Subchondral erosion, mild bone erosion	3
Marked bone erosion	4

Table 2
Effect of inositol-stabilized arginine silicate complex (ASI) or the combination of the individual ingredients (A + S + I) on biochemical parameters in rats with collagen-induced arthritis.

Variable	Groups[a]			
	Control	Arthritis	A + S + I	ASI complex
ALT, U/L	51.29 ± 3.93^{c}	96.57 ± 4.42^{a}	74.14 ± 4.30^{b}	66.71 ± 5.91^{bc}
AST, U/L	138.71 ± 4.45^{c}	242.86 ± 12.51^{a}	175.00 ± 10.19^{bc}	178.14 ± 10.65^{b}
Creatinine, mg/dl	0.42 ± 0.03^{b}	0.65 ± 0.02^{a}	0.54 ± 0.03^{ab}	0.49 ± 0.04^{b}
T-C, mg/dl	109.41 ± 5.72^{c}	169.29 ± 6.16^{a}	161.86 ± 4.97^{ab}	144.43 ± 7.09^{b}
Triglyceride, mg/dl	135.86 ± 5.76^{c}	226,00 ± 15.56^{a}	185.86 ± 5.88^{b}	154.43 ± 9.31^{bc}
HDL-c, mg/dl	62.86 ± 3.45^{a}	30.14 ± 2.10^{c}	42.57 ± 2.59^{b}	33.43 ± 2.11^{c}
LDL-c, mg/dl	19.14 ± 1.60^{c}	94.00 ± 2.05^{a}	82.00 ± 1.53^{b}	80.00 ± 4.40^{b}

[a] Control: untreated rats; Arthritis: rats received a single administration of collagen; A + S + I: rats received a single administration of collagen and arginine HCl + silicon dioxide + inositol; ASI Complex: rats received a single administration of collagen and ASI complex. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; T-C: Total cholesterol; HDL-c High-density lipoprotein-cholesterol; LDL-c: Low-density lipoprotein-cholesterol. Means within the same row without a common superscript differ ($P < 0.05$).

Table 3
Effect of inositol-stabilized arginine silicate complex (ASI) and the combination of the individual ingredients (A + S + I) on the arthritis score and inflammatory markers in rats with collagen-induced arthritis.

Variable	Groups[a]			
	Control	Arthritis	A + S + I	ASI complex
14th day arthritis score	–	1.32 ± 0.16	1.34 ± 0.31	1.35 ± 0.20
29th day arthritis score	–	2.51 ± 0.10^{a}	1.12 ± 0.06^{b}	0.52 ± 0.03^{c}
Inflammation score	–	4.56 ± 0.24^{a}	3.39 ± 0.09^{b}	2.31 ± 0.09^{c}
TNF-α, pg/ml	23.71 ± 1.22^{c}	50.43 ± 2.09^{a}	36.70 ± 1.59^{b}	30.71 ± 1.46^{bc}
IL-17, pg/ml	21.29 ± 1.22^{c}	58.00 ± 2.59^{a}	37.14 ± 1.82^{b}	28.7 ± 0.90^{c}
LL-6, pg/ml	11.40 ± 1.02^{c}	37.57 ± 2.17^{a}	23.43 ± 25.00^{b}	18.14 ± 0.73^{bc}
Ghrelin, pg/ml	337.71 ± 13.03^{a}	144.29 ± 10.07^{c}	222.71 ± 15.22^{b}	249.57 ± 10.38^{b}
Obestatin, pg/ml	259.0 ± 11.17^{a}	115.86 ± 4.06^{c}	135.57 ± 4.94^{bc}	154.57 ± 3.71^{b}
Sclerostin, ng/ml	0.47 ± 0.04^{a}	0.26 ± 0.03^{c}	0.32 ± 0.02^{bc}	0.40 ± 0.03^{ab}
DKK-1, pg/ml	654.86 ± 41.66^{d}	1795.0 ± 34.22^{a}	1357 ± 43.09^{c}	991.87 ± 34.76^{b}

[a] Control: untreated rats; Arthritis: rats received a single administration of collagen; A + S + I: rats received a single administration of collagen and Arginine HCl + silicon dioxide + inositol; ASI Complex: rats received a single administration of collagen and ASI complex. TNF-α: Tumor necrosis factor alpha; IL-6 and 17: Interleukin 6 and 17; DKK-1: Dickkopf-related protein 1. Means within the same row without a common superscript differ ($P < 0.05$).

Table 4
Effect of inositol-stabilized arginine silicate complex (ASI) and the combination of the individual these ingredients (A + S + I) on levels of serum and joint tissue arginine and silicon in rats with collagen-induced arthritis.

Variable	Groups[a]			
	Control	Arthritis	A + S + I	ASI complex
Serum				
Arginine, nmol/L	1.10 ± 0.07^{c}	0.77 ± 0.06^{d}	1.33 ± 0.03^{b}	1.56 ± 0.01^{a}
Silicon, μg/L	131.86 ± 6.85^{c}	109.00 ± 2.14^{c}	580.71 ± 20.94^{b}	712.86 ± 18.14^{a}
Joint tissue				
Arginine, pmol/mg	537.29 ± 24.58^{c}	463.71 ± 28.50^{c}	682.57 ± 23.77^{b}	793.14 ± 15.99^{a}
Silicon, μg/g	0.56 ± 0.03^{bc}	0.44 ± 0.03^{c}	0.64 ± 0.03^{b}	0.79 ± 0.04^{a}

[a] Control: untreated rats; Arthritis: rats received a single administration of collagen; A + S + I: rats received a single administration of collagen and arginine HCl + silicon dioxide + inositol; ASI Complex: rats received a single administration of collagen and ASI complex. Means within the same row without a common superscript differ ($P < 0.05$).

Treatment with ASI complex and A + S + I increased the levels of those markers compared to the arthritic control group ($P < 0.05$). The highest levels of obestatin and sclerostin were observed in the ASI complex group compared to the arthritic control and A + S + I groups. However, the increase in serum ghrelin levels was similar in the ASI complex and A + S + I groups (Table 3).

3.4. Arginine and silicon concentrations

As Table 4 clearly defines, in collagen-induced arthritic rats, the serum and joint tissue arginine and silicon levels were significantly reduced when compared to other treatment groups ($P < 0.001$). Treatment either with the ASI complex or A + S + I significantly increased the concentrations of arginine and silicon in serum and joint tissue ($P < 0.05$ for both). Interestingly, when animals were treated with the ASI complex, a statistically significant increase in serum concentrations of arginine and silicon was found relative to the effects of A + S + I on these parameters. These results show that the ASI complex significantly increased blood serum levels of arginine and silicon compared to equivalent doses of ASI's components individually.

3.5. Western blot analysis

Arthritic induction significantly upregulated the levels of TNF-α, IL-

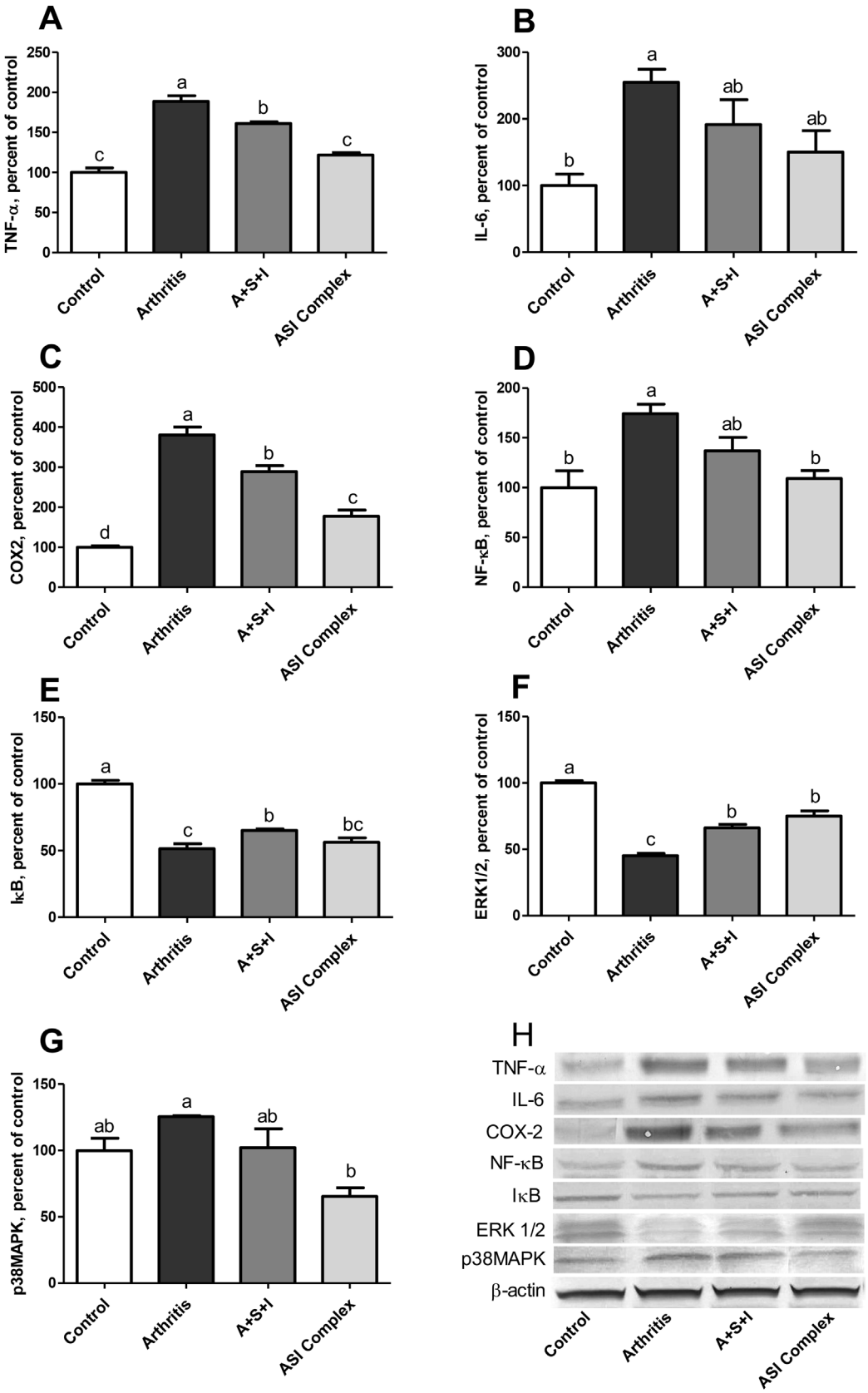

Fig. 1. Effect of inositol-stabilized arginine silicate complex (ASI) or a combination of the individual ingredients (A + S + I) on protein levels of tumor necrosis factor alpha (TNF-α; Panel A), interleukin 6 (IL-6; Panel B), cyclooxygenase-2 (COX-2; Panel C), nuclear factor-kappa B (NF-κB; Panel D), inhibitor of kappa B (IκB; Panel E), extracellular signal-regulated kinase 1 and 2 (ERK1/2; Panel F) and p38 mitogen-activated protein kinases (p38MAPK; Panel G) in rats with collagen-induced arthritis. The intensity of the bands (Panel H) was quantified by densitometric analysis and β-actin was included to ensure equal protein loading. Data are expressed as percent of control value. Each bar represents the mean and standard error. Blots were repeated at least 3 times. Different superscripts (a–c) indicate group mean differences ($P < 0.05$). Control: untreated rats; Arthritis: rats received a single administration of collagen; A + S + I: rats received single an administration of collagen and Arginine HCl + silicon dioxide + inositol; ASI Complex: rats received a single administration of collagen and ASI complex.

6, COX-2, NF-κB, p38 MAPK, LEF1, WISP-1 and β-catenin in the joint tissue when compared with normal controls (Figs. 1 and 2). However, treatment with ASI complex and A + S + I significantly downregulated TNF-α, IL-6, COX-2, NF-κB, p38 MAPK, LEF1, WISP-1 and β-catenin levels in the joint tissue of arthritic rats compared with the arthritic control group (Figs. 1 and 2). Marked down-regulation was observed for the ASI complex. Although β-catenin expression was similar in the collagen-induced arthritic rats and A + S + I groups, it was lower in the

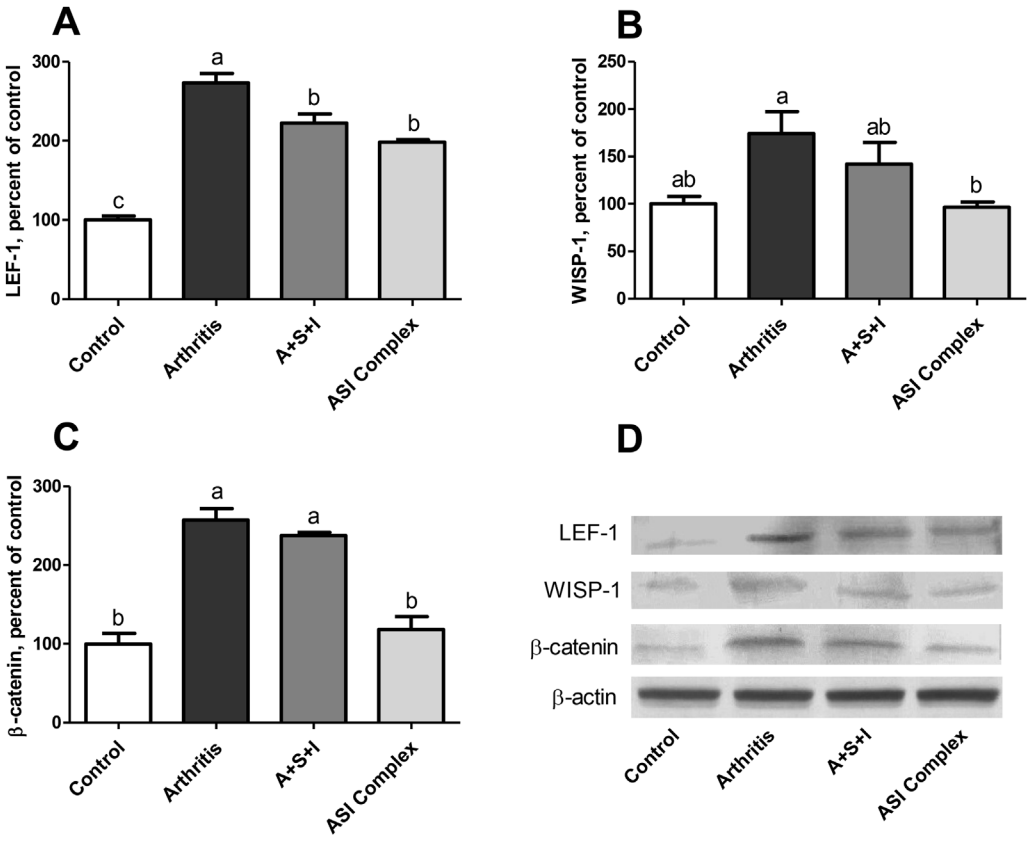

Fig. 2. Effect of inositol-stabilized arginine silicate complex (ASI) or the combination of the individual these ingredients (A + S + I) on protein levels of lymphoid enhancer-binding factor-1 (LEF-1; Panel A), extracellular signal-regulated kinase 1 and 2 (ERK1/2; Panel B) and p38 mitogen-activated protein kinases (p38MAPK; Panel C) in rats with collagen-induced arthritis. The intensity of the bands (Panel D) was quantified by densitometric analysis and β-actin was included to ensure equal protein loading. Data are expressed as percent of the control value. Each bar represents the mean and standard error. Blots were repeated at least 3 times. Different superscripts (a–c) indicate group mean differences ($P < 0.05$). Control: untreated rats; Arthritis: rats received a single administration of collagen; A + S + I: rats received a single administration of collagen and arginine HCl + silicon dioxide + inositol; ASI Complex: rats received a single administration of collagen and ASI complex.

ASI complex group ($P < 0.05$, Fig. 2). Joint tissue LEF1 levels were similar in the ASI complex and A + S + I treatment groups ($P < 0.05$, Fig. 2). The I-κB and ERK1/2 levels were lower in the collagen-induced arthritic control group than in the control group (Figs. 1 and 2). In the treatment groups, joint tissue I-κB and ERK1/2 expression were significantly higher compared to the collagen-induced arthritic control group ($P < 0.05$, Figs. 1 and 2).

3.6. Histopathological assessments

Control rats had the normal appearance of cartilage lining, joint space, and underlying bones with the normal architecture of the joint without inflammatory infiltrates and edema (Fig. 3). However, in CIA rats, joints were highly abnormal, with pronounced synovial hyperplasia, inflammatory cell infiltration, pannus formation, and extensive erosion changes in cartilage. CIA-treated rats with ASI or A + S + I treatment was spared the chronic inflammation of synovial tissue. In these groups, there were significant reductions in inflammatory cell infiltration, pannus formation, and cartilage destruction compared with CIA treated rats. However, ASI complex supplementation was more effective than A + S + I (Fig. 3).

4. Discussion

This study was designed to evaluate the effect of an ASI complex or a combination of the individual ingredients (arginine HCl, silicon, and inositol) of the ASI complex on inflammation score, inflammation markers, serum and joint tissue arginine and silicon levels, and the molecular mechanism by which these effects are expressed in collagen-induced arthritic rats. The present study shows, to our knowledge for the first time, that it is the ASI complex, rather than a combination of the individual ingredients of the ASI complex, that can potentially cause anti-inflammation effects and improve joint health in experimental animal models with collagen-induced arthritis. In addition, with the evidence of normal AST, ALT, urea and creatinine level in blood for all treatment groups, it is suggested that there is no toxic effect on liver and kidney function of ASI complex treatment. Arginine silicate inositol complex has been shown to be more effective in increasing serum arginine levels than commonly used arginine hydrochloride in animals (Proctor et al., 2007) as well as in humans (Komorowski and Perez Ojalvo, 2016). Similar to our results, it was reported that plasma arginine levels increased from baseline at 1, 1.5, 2, 3 and 6 h in humans by ASI complex treatment and plasma arginine levels in the arginine hydrochloride group only increased at 1 hour. The area under the curve (AUC0-6 h) for plasma arginine levels was 12.68 ± 10.83 for ASI complex and 7.40 ± 35.08 for arginine hydrochloride (Komorowski and Perez Ojalvo, 2016). In addition, in a pharmacokinetics (PKs) and pharmacodynamics (PDs) of ASI complex study, the results indicated that the ASI complex was well absorbed (Kalman et al., 2015). The authors reported that the peak concentration of plasma arginine (CMax) was 30.06 ± 7.80 μg/mL, ~1 hour (tMax) after an oral dose of 1500 mg of the arginine silicate dietary supplement and $t_{1/2}$ 15.93 ± 9.55 h. In the same study, the peak concentration (CMax) for silicon was 2.99 ± 0.63 μg/mL approximately 2 h and 44 min after the 1.500 mg dose of the product and $t_{1/2}$ 34.56 ± 16.56 h (Kalman et al., 2015).

The study recorded arthritis score, inflammation score, inflammatory markers, and serum and joint arginine and silicon levels upon administration of ASI complex in rats with CIA (Tables 3 and 4). Consistent with our results, Manicourt et al. (1993) and Rosillo et al. (2015) found that TNF-α, IL-17, and IL-6 levels were high in the serum, synovial fluid, and tissues of RA patients. Further, previous findings reported that dietary ASI supplementation decreased serum concentrations of TNF-α and C-reactive protein in stressed animals (Sahin et al., 2006). Similarly, Proctor et al. (2005) reported that the ASI complex has physicochemical properties that increase the rate of absorption of arginine across the intestinal tract and significantly raise the concentration of available arginine in the circulation, compared with

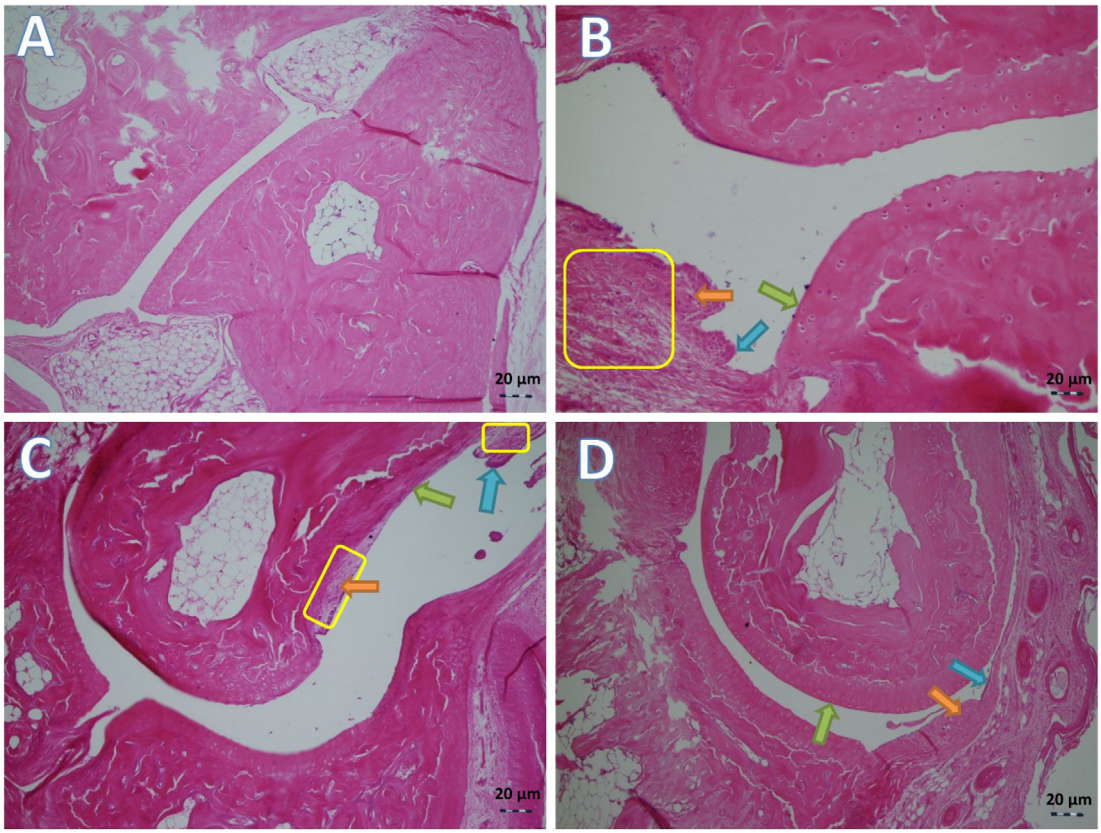

Fig. 3. The effects of inositol-stabilized arginine silicate complex (ASI) or the combination of the individual ingredients (A + S + I) on histological changes (H& E × 200) in joint tissue in rats with collagen-induced arthritis. The normal joint structure is observed in the control group (Panel A). Marked synovial hyperplasia (blue arrow), inflammatory cell infiltration (orange arrow), pannus formation (yellow frame) and extensive erosion changes (green arrow) in the arthritis group (Panel B). Decreased synovial hyperplasia (blue arrow), inflammatory cell infiltration (orange arrow), pannus formation (yellow frame) and extensive erosion changes (green arrow) in the A + S + I group (Panel C) and ASI complex group (Panel D). Control: untreated rats; Arthritis: rats received a single administration of collagen; A + S + I: rats received a single administration of collagen and arginine HCl + silicon dioxide + inositol; ASI Complex: rats received a single administration of collagen and ASI complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conventional preparations. Moreover, arginine is involved in both the synthesis of substrates (*e.g.* polyamine and L-proline) associated with collagen synthesis and the production of growth hormone, insulin-like growth factor-I, and nitric oxide (Fiore et al., 2000). In contrast to our results, Proctor et al. (2007) stated that arginine-HCl decreased the serum TG and TC levels compared to the ASI complex in female obese insulin-resistant JCR:LA-cp rats.

In the present study, administration of the ASI complex and A + S + I significantly increased serum levels of ghrelin, obestatin, and sclerostin in arthritic rats. However, the largest increase was seen in the ASI complex group (Table 3). There are no earlier studies that examined the effects of ASI complex or the combination of the individual arginine, silicon and inositol ingredients on serum ghrelin, obestatin and sclerostin levels in arthritic rats to compare with this study. However, Koca et al. (2008) stated that sulphasalazine and leflunomide, known as anti-arthritic agents, increased ghrelin levels and decreased obestatin levels in patients with RA. In addition, Granado et al. (2005) and Li et al. (2004) demonstrated that ghrelin exerts anti-inflammatory effects and inhibits NF-κB activation and pro-inflammatory responses in human endothelial cells.

TNF-α is a pro-inflammatory cytokine (Waksman, 2002) that is that is essential for the overexpression of inflammatory genes (IL-1β and IL-6) and bone and cartilage destruction (McInnes and Schett, 2007). NF-κB is a transcription factor in macrophages that control gene expression for numerous cytokines and plays an important role in arthritis development, differentiation and provocation of osteoclasts, which lead to bone erosion, and proliferation of RA synovial cells (Kim et al., 2009). When stimulated by proinflammatory cytokines including IL-1β, signal pathways are induced that lead to IKK-β activation, which then phosphorylates IκBα and transports NF-κB from the cytoplasm to the nucleus, where it triggers the expression of inflammation-related genes, including TNF-α, IL-6, COX-2, and MMPs (Marcu et al., 2010).

In this study, IL-6, NF-κB, COX-2, TNF-α, and p38 MAPK levels were downregulated while I-κB and ERK1/2 levels were upregulated in synovial cells after treatment with the ASI complex and A + S + I (Figs. 1 and 2). This change was detected at the highest level in the ASI complex group. This may be because arginine and silicon are more absorbed and active in the ASI complex group (Proctor et al., 2007), which is supported by the fact that, in this study, we found that serum and tissue arginine and silicon levels were higher in the ASI complex group compared to the A + S + I group. It is thought that ASI complex and A + S + I can alleviate the damage of joint tissue by inhibiting the formation of pro-inflammatory cytokines. These changes can be mitigated after the treatment with ASI complex and A + S + I, which indicates that ASI complex and A + S + I protected the synovial tissue from edema and angiogenesis by regulating the activity of p38/NF-κB signaling pathway. ASI complex and A + S + I may also suppress the activation of NF-κB, possibly by reducing the phosphorylation of IκBα and expression of p65. In addition, previous studies stated that inhibition of MAPK phosphorylation and NF-κB p65 subunit translocation to the nucleus pathways caused in the inhibition of IL-1β-induced MMPs, COX-2, and pro-inflammation cytokines in chondrocytes (Akhtar et al., 2017). Moreover, arthritis-linked inflammation can be driven by the NF-κB pathway, which is inhibited by nitric oxide (NO). The effects of ASI

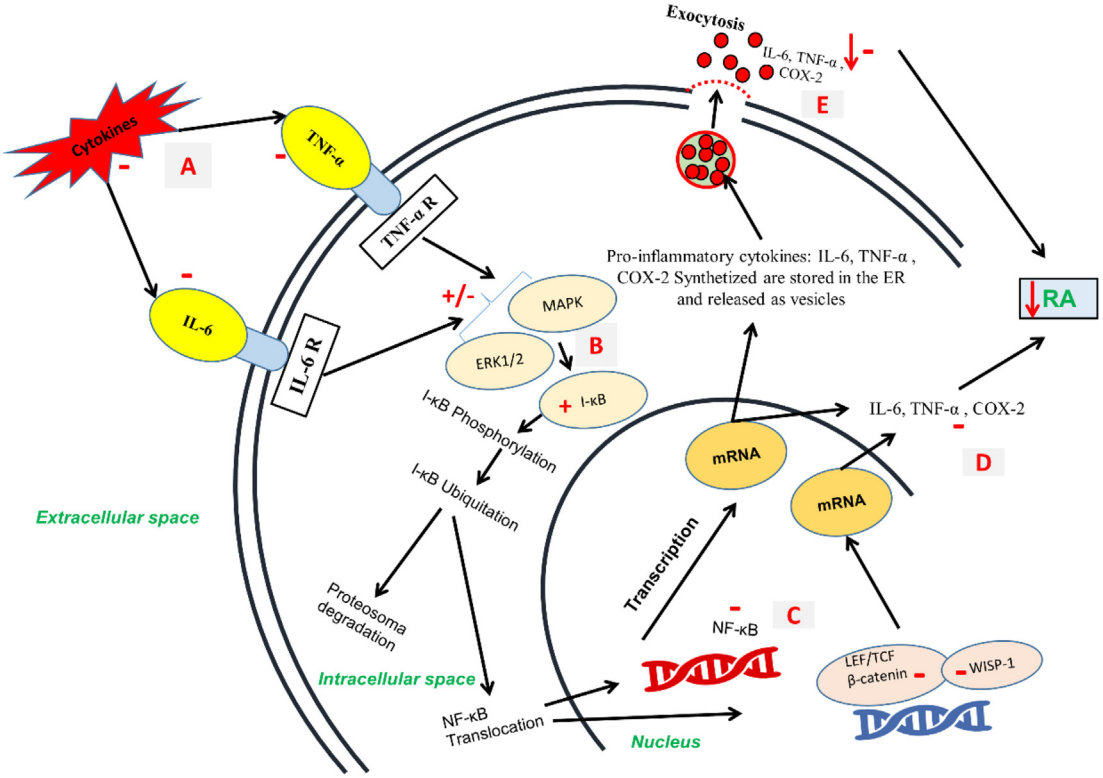

Fig. 4. Proposed mechanism of action by which ASI complex inhibited an inflammatory effect and reduced rheumatoid arthritis. RA: rheumatoid arthritis; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; IL-6 R: interleukin 6 receptor; TNF-α R: tumor necrosis factor alpha receptor; I-κB: inhibitor of kappa B; ERK1/2 extracellular signal-regulated kinase 1 and 2; COX-2 cyclooxygenase-2; NF-κB: nuclear factor-kappa B; p38MAPK: p38 mitogen-activated protein kinases; mRNA: Messenger RNA. -: Inhibition; +: Stimulation.

complex in arthritis could be mediated by NO, as well. For instance, arginine is a common precursor for NO by a NADPH-dependent NO synthase and regulates cellular signalization (Lee et al., 2013). ASI complex was found to be more effective than A + S + I for the protein levels. The reason is that ASI complex rises the arginine bioavailability and the arginine levels in the circulation compared to A+S+I (Proctor et al., 2005). There are several studies that discuss the effects of the ASI complex on the inhibition of inflammation markers in tissues in different models not specific to arthritis. However, there are no studies on how the separate ingredients of the ASI complex affect these parameters. For instance, administration of ASI complex significantly reduced the levels of bone-related molecules (RANK and RANKL), neutrophil infiltration, and cytokine production in gingival tissue (IL-1β), as well as collagenolytic enzymes (MMP8), in experimental periodontitis-induced rats (Dundar et al., 2016). In a previous study, we also reported that topical application of ASI 4% and 10% ointment exerts its wound-healing potential through modulating the nitric oxide synthases, matrix metalloproteinase, and inflammatory mediators (IL-6, TNF-α, and IL-1β, NF-κB) in the full-thickness excisional model in Wistar rats in a dose-independent manner (Durmus et al., 2017). It has been stated that pro-inflammatory cytokines arising from RA are involved in the erosion of articular cartilage by playing a key role in multiple signal transduction pathways including mitogen-activated protein kinases (MAPKs, in particular, p38MAPK) which comprises extracellular signal-regulated kinases (ERK1/2) in RA patients (Criado et al., 2014). Similar to our results, in a CIA mice model, both p38MAPK and ERK1/2 expression was decreased. However, when given dietary hydroxytyrosol acetate, ERK1/2 expression differed from our results (Rosillo et al., 2015). In the present study, ERK1/2 levels were lower in the arthritic group and ASI complex treatment significantly increased ERK1/2 in the joint tissue of CIA rats.

β-catenin is a signal transduction protein responsible for key gene expression in RA (Xu et al., 2000; MacDonald et al., 2009). Wnt1-inducible signaling pathway protein 1 (WISP-1), which is described as a downstream target of Wnt-1 and β-catenin, has been shown to contribute to β-catenin-mediated tumorigenesis (Xu et al., 2000). Bone formation, which is reliant on proliferation, function, and apoptosis of osteoblasts, is coordinated by the Wnt/β-catenin pathway (Garnero et al., 2008). Dkk-1 and sclerostin are two natural inhibitors of the Wnt/β-catenin pathway of bone formation in RA (Garnero et al., 2008; Goldring et al., 2013). In the present study, we found for the first time that ASI complex and A+S+I could suppress collagen-induced activation of the Wnt/β-catenin signaling pathway in the joint tissue (Fig. 2). The accumulation of β-catenin in the joint tissue was significantly reduced and the expression of LEF-1 was significantly diminished by ASI or A+S+I treatment. In addition, protein expression of WISP-1, which was transcribed from two primary Wnt signaling target genes, was decreased as well. These results suggest that the ASI complex, which is more active than A+S+I, might attenuate collagen-induced arthritis via Wnt/β-catenin signaling suppression. Therefore, the potential mechanism of ASI complex may be the down-regulation of β-catenin and WISP-1 expression via inhibition of inflammatory cytokines such as TNFα, IL- 6, and IL-17.

Overall, a single mechanism of action of ASI complex could not explain its therapeutic effect on RA because natural compounds have different mechanisms depending on their chemical structures. They probably have multiple mechanisms acting on multiple sites of cellular machinery. However, based on the results obtained in the current investigation, we can suggest that the ASI complex may act at 5 levels. ASI complex may regulate extracellular cytokines (Fig. 4A) and stimulate downstream adapter proteins and activate specific protein kinases such as ERK 1/2 and MAPK, leading to the activation of IκB kinases (Fig. 4B). ASI complex may also inhibit NF-κB and Wnt/β-catenin genes in the nucleus (Fig. 4C), leading to the reduction of pro-

inflammatory cytokines (Fig. 4D). Moreover, cytokines synthesized are stored in the ER and released as vesicles (via exocytosis) (Fig. 4E). The reduction of inflammatory cytokines finally reduces RA (Fig. 4). A similar study conducted by Armutcu et al. (2015) also showed that caffeic acid phenethyl ester exhibits anti-inflammatory property by inhibiting the NF-κB pathway and reducing pro-inflammatory cytokines.

In conclusion, inositol-stabilized arginine silicate (ASI) complex administration significantly decreased markers of inflammation and overall arthritis and inflammation scores over the arthritic group and the A + S + I group. These results demonstrate that ASI complex may be effective in reducing inflammation and that the ASI complex is more effective than a combination of the individual ingredients of ASI. These results also showed that ASI exerted its anti-inflammatory effects through inhibition of inflammatory and Wnt/β-catenin pathways, suggesting a vital role for the ASI complex in regulating these pathways.

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