Iraqi JMS

Published by Al-Nahrain College of Medicine
ISSN 1681-6579
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq

Effect of *Glycyrrhiza glabra* on Antigen Induced Arthritis in Mice Model

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Abstract

Background Rheumatoid arthritis is a chronic inflammatory autoimmune disease represents the most common form of chronic inflammatory joint diseases. *Glycyrrhiza glabra* (*G. glabra*) was widely known to have several pharmacological activities, which might be beneficial in preventing and treating both acute and chronic inflammatory conditions.

Objective To study the effect of aqueous extract of *G.glabra* on antigen induced arthritis model in mice.

Methods Forty-eight male Swiss albino mice were used in this study. Group 1 arthritic mice without treatment (positive control); group 2 arthritic mice treated with *G. glabra* aqueous extract 750 mg/kg/day; group 3 arthritic mice treated with *G. glabra* aqueous extract 300 mg/kg/day and group 4 negative control (non-immunized, non-treated mice). Antigen induced arthritis was induced by Methylated bovine serum albumin in Imject Alum adjuvant. The mice were given the drug orally and the treatment was started from day 1 of the induction of arthritis until day 20. At day 20 of arthritis all mice were sacrificed and serum TNF-α was measured using ELISA technique. Biopsies of the left knee joint were taken for histopathological evaluation.

Results The results indicate that *G.glabra* caused inhibition of histopathological features of antigen-induced arthritis in dose dependent manner. *G.glabra* also caused reduction of serum TNF-α concentration in antigen-induced arthritis model in dose dependent manner.

Conclusions *Glycyrrhiza glabra* can significantly inhibit antigen-induced arthritis in mice. This effect seems to be in dose dependent manner.

Key words Rheumatoid arthritis, *G. glabra*, TNF-α, antigen induced arthritis.

List of abbreviation: RA = Rheumatoid arthritis, TNF-α = tumor necrosis factor alpha, NSAIDs = non-steroidal anti-inflammatory drugs, DMARD = disease-modifying antirheumatic drugs, ELISA = Enzyme Linked Immunosorbent Assay, H&E = Hematoxyline and Eosin, LE = Liquorice extract, CIA = collagen-induced arthritis.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease that is associated with progressive disability, systemic complications, early death, and socioeconomic costs [(1)]. The epidemiological ratio of arthritis in female: male is 3:1 and the prevalence is 1% of the world population [(2)]. Synovial inflammation underlies the cardinal manifestations of this disease, which include pain, swelling, and tenderness followed by cartilage destruction, bone erosion, and subsequent joint deformities [(3,4)].

Despite intensive research, the precise cause of RA remains elusive. Although a variety of cells play a role in RA disease progression [(5)]. Macrophage-derived cytokines, such as tumor necrosis factor alpha (TNF-α), appear to play a critically important role in the induction and perpetuation of the chronic inflammatory processes in rheumatoid joints as well as in the systemic manifestations of this disease [(6)]. Medications that used to treat rheumatoid arthritis are divided into three main classes: non-

steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease-modifying antirheumatic drug (DMARD) (both synthetic and biologic) [7].

The majority of patients with newly diagnosed RA are started on disease-modifying antirheumatic drug (DMARD) therapy within 3 months of diagnosis such as Methotrexate [6]. Non-steroidal anti-inflammatory drugs (NSAIDS), glucocorticoid joint injections, and/or low-dose prednisone may be considered for control of symptoms [6,8].

Furthermore, the past decade have seen the introduction of seven new DMARDs which include leflunomide, the highly specific and efficacious anticytokine agents, including adalimumab, etanercept, and infliximab, and recently, abatacept, and rituximab, and others [9,10]. These therapies are emerging as important and successful therapeutics for patients with early disease [6,9,10]. Although effective in many patients, they are not without their drawbacks. Methotrexate and Leflunomide require careful monitoring and can cause serious hepatic and pulmonary toxicities [6]. The anti-TNF-α biological agents are costly, require parenteral administration, and have been associated with serious and opportunistic infections and lymphoma [6,9,10]. Furthermore as there are no 'cures', patients will require 30-40 years of ongoing therapy; although most of these agents do not remain effective in an individual for longer than 5 years. Thus, despite major recent advances in the treatment of RA, there is still need for convenient, safe, and effective therapies for many patients [6]. With these difficulties, the field of arthritis research has progressed exponentially towards herbal therapies that have been considered safe and effective in all elevating chronic pain associated with arthritis [2].

Glycyrrhiza glabra (Liquorice) has been used in medicine for more than 4000 years. The plant is distributed in the subtropical and warm temperature region of the world. The root and rhizome of the plant has been used as anti-inflammatory, anti-oxidant, anti-spasmodic, and expectorant [2].

In the present study, aqueous extracts of *G. glabra* was administered to mice with antigen induced arthritis to investigate the suggested benefit on this model as a animal model close to RA in human. Mouse models of antigen arthritis have been used extensively to study efficacy of biologies and the role of specific cytokines in the various aspects of disease pathogenesis [15-17].

The objective of our study is to study the effect of *G.glabra* on antigen induced arthritis model in mice.

Methods

Animals

Forty-eight male Swiss albino mice were used in this study; the animals were obtained from the animal house of the Al-Nahrain University. The animals aged 8 to 10 weeks and weighing 20 to 25 g, housed at a maximum of six per cage on wood shavings with free access to food and water. Before starting study, the animals were left for 48 hours to acclimatize to the animal room conditions and were maintained on an environment of controlled temperature, with a 12 hours light-dark cycle and standard pellet diet and tap water.

Arthritis induction

Mice were immunized subcutaneously with Methylated bovine serum albumin (mBSA) in Imject Alum in a concentration of (100 µg) mBSA/ 0.1 ml Imject Alum. The immunization was done on day zero and booster dose given after 7 days by subcutaneous route. After fourteen days of the second immunization (day 21 of experiment), arthritis was induced by intra-articular injection of 100 µg of mBSA mixed with the Imject Alum adjuvant in 1:1 ratio, in the left Knee joint [18].

Experimental design

The mice were divided into four experimental groups each group consist of 12 mice as follows:

Group 1: Arthritic mice without treatment (positive control).

Group 2: Arthritic mice treated with *G. glabra* aqueous extract 750 mg/kg/day [19].

Group 3: Arthritic mice treated with *G. glabra* aqueous extract 300 mg/kg/day [20].
Group 4: Negative control (non-immunized, non-treated mice).
All mice were weighed, and the drugs were measured according to the weight of each mouse. The mice were given the drug orally and the treatment was started from day 1 of the induction of arthritis (day 21 of experiment) until day 20 (day 41 of experiment) which is the end of the experiment.

Measurement of serum TNF-α

Mice of all groups were sacrificed at days 20 from induction of arthritis (day 41 of experiment). Blood was collected by cardiac puncture; serum was obtained to measure serum TNF-α level using Enzyme Linked Immunosorbent Assay (ELISA) technique.

Histopathological evaluation

Mice of all groups were sacrificed at day 20 from induction of arthritis and hitopathological changes of left knee joint of each mouse were evaluated.

Assessment of histopathological changes

Biopsies of the left knee joint were taken at day 20 of arthritis from the sacrificed mice of all groups to study the histopathological changes in them. The histopathological severity of arthritis was graded on a scale of 0-3 [21], where Zero = normal; One = minimal synovitis, cartilage loss, and bone erosions limited to discrete foci; Two =synovitis and erosions present, but normal joint architecture intact; and three = synovitis, extensive erosions, and disrupted joint architecture.

Biopsies were fixed by formalin 10 % for 4 hours and decalcified by HCL 10% for 6 hours. Dehydrated by using different concentrations of ethanol 70%, 80%, 95% and 100% (2 hours for each concentration), then specimen treated with xylol (2 steps two hours for each) before dipping in liquid paraffin at 55-60 ^{o}C, then tissue was embedded in paraffin (two steps for 2 hours for each), and Paraffin blocks were made. Sections were made with 5-µm thickness by using microtome. Finally, sections were stained by Hematoxyline and Eosin (H&S).

Statistical analysis

Statistical analysis was performed with the SPSS 19.0 statistical package for social sciences and Excel 2010. Descriptive statistics for the numerical data were formulated as mean and standard error (SE). Numerical data were analyzed using independent sample t-test for comparison between two groups. While histological score comparison between each group were done by Wilcoxon Mann whitney test. The level of statistical significant difference (*P*-value) is below (0.05).

Results

Serum TNF-α level

G.glabra seems to reduce serum TNF-α level in dose dependent manner. Mean serum TNF-α level of group 3 was 73.83±1.82 pg/ml which was significantly lower ($P \leq 0.001$) than 173.61±2.88 pg/ml of group 1. Moreover, serum TNF-α level was 53.23±1.82 pg/ml of group 2 which was significantly lower ($P \leq 0.001$) than 73.83±1.82 pg/ml of group 3. However, serum TNF-α level of group 2 and group 3 remain significantly higher ($P \leq 0.001$) than 18.22 ±1.29 pg/ml of group 4 (Table 1).

Table 1. Serum TNF-α level of study groups on day 20 of arthritis induction

Study Group	Serum TNF-α level (Mean± S.EM)
Group 1	173.61±2.88 (B*,C*,D*)
Group 2	53.23±1.82 (C*,D*)
Group 3	73.83±1.82 (D*)
Group 4	18.22±1.29

B: Comparison with group 2, C: Comparison with group 3, D: Comparison with group 4, S.EM: standard error mean, TNF-α: Tumor necrosis factor alpha, *= $P < 0.05$.

Histopathological score evaluation

Glycyrrhiza glabra also seems to reduce the histopathological score in our model of arthritis in dose dependent manner. Histopathological scores of group 3 showed a significant reduction ($P < 0.05$) compared to group 1. Moreover, histopathological scores of group 2 showed a

significant reduction ($P < 0.05$) compared to group 3 (Table 2).

Table 2. Histopathological scores of study groups

Study Group	Histological score Median
Group 1	$2.75^{(B*C*)}$
Group 2	$0.75^{(C*)}$
Group 3	1.00

B: Comparison with group 2, C: Comparison with group 3, *= $P < 0.05$.

Histopathological section taken from group 1 reveals loss of joint architecture with heavy chronic inflammation of the synovium, pannus (an abnormal layer of fibrovascular tissue or granulation tissue), marked erosion of bone and cartilage, hyperplasia and hypertrophy of the synovial lining (Figure 1).

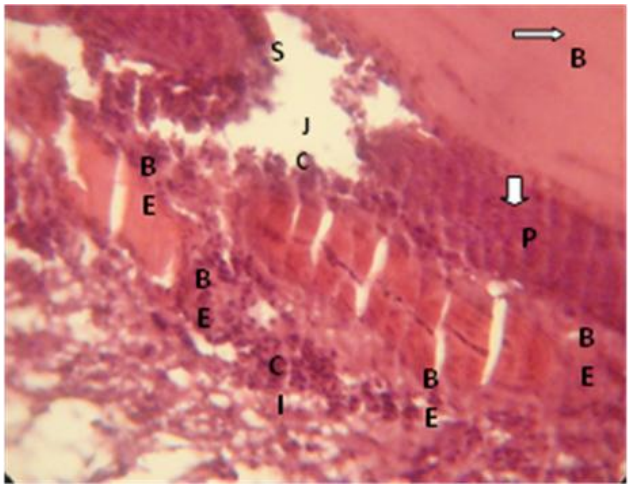

Fig. 1. Arthritic knee joint of group 1 on day 20 of arthritis showing loss of joint architecture with heavy chronic inflammation of the synovium, pannus, marked erosion of bone and cartilage, hyperplasia and hypertrophy of the synovial lining. (S) synovium, (JC) joint cavity , (p) pannus, (B) bone, (BE) Bone erosion, (CI) chronic inflammation. H & E stain (4X).

Histopathological slide examination of group 2 mice shows preservation of the joint architecture, with minimal chronic inflammation and minimal erosion in the bone (Figure 2) and for group 3 showed preservation of the joint architecture, focal synovitis, and pannus caused cartilage and bone erosion limited to discrete foci (Figure 3). Histological section taken from group 4 showed normal joint architecture with normal cartilage, bone and synovium, no inflammatory cell infiltration (Figure 4).

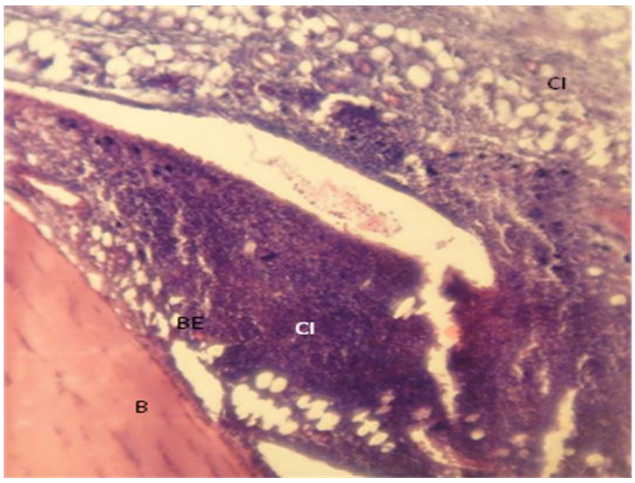

Fig. 2. Arthritic knee joint of group 2 on day 20 of the arthritis showing preservation of the joint architecture, with minimal chronic inflammation and minimal erosion in the bone. (B) Bone, (CI) chronic inflammation, (BE) bone erosion. H & E stain (40X).

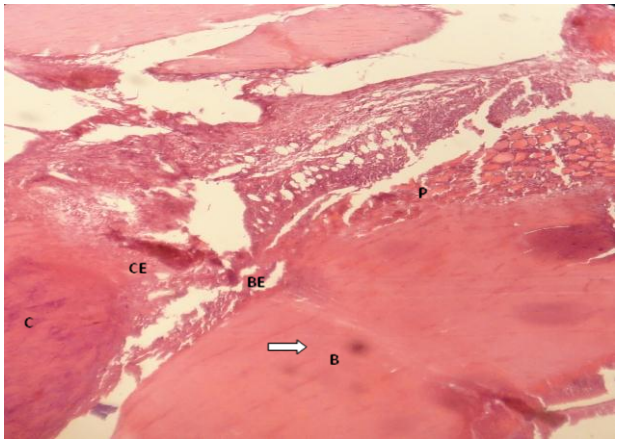

Fig. 3. Arthritic knee joint of group 3 on day 20 of the arthritis showing preservation of the joint architecture, focal synovitis, and pannus caused cartilage and bone erosion limited to discrete foci. (B) Bone, (BE) Bone erosion, (C) cartilage, (CE) cartilage erosion. (P) Pannus. H & E stain (4X).

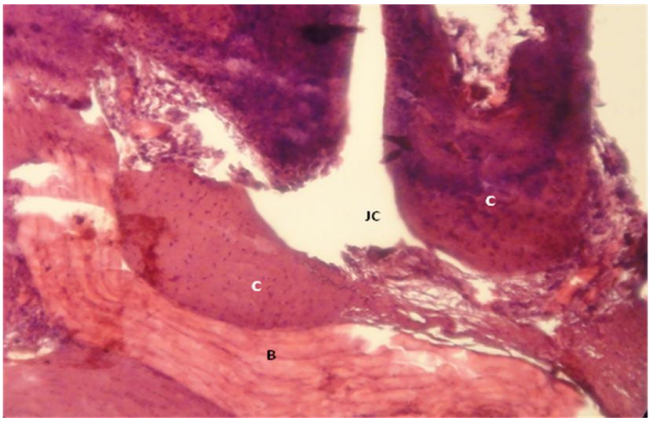

Fig. 4. Left knee joint of group 4 showing normal joint architecture with normal cartilage, bone and synovium, no inflammatory cell infiltration. (C) Cartilage, (JC) Joint cavity, (B) bone. H & E stain (4X).

Discussion

Antiarthritic therapeutic potential of *G. glabra* aqueous extract on mice antigen induced arthritis has been studied in the present study. The results of present study indicate that *G. glabra* was significantly effective in inhibiting serum levels of TNF-*α* in the present model of arthritis in dose dependent manner. The results of present study come in agreement with other study in which Liquorice extract (LE) oral administration to collagen-induced arthritis (CIA) mice result in significant reduction in the serum levels of TNF-*α* compared to CIA mice treated with vehicle.

G. glabra was effective in inhibiting histopathological changes in the present model of arthritis (inflammatory cells infiltration, synovitis, cartilage erosion, bone erosion, loss of joint architecture and pannus formation) in dose dependent manner.

In other study, LE oral administration to CIA mice cause noticeable reduction of the histopathological changes in the joint (including CIA-characteristic synovial hyperplasia, infiltration of inflammatory cells into the joint cavity, and extensive pannus formation) this come in agreement with results of present study [22].

It has been concluded that aqueous extracts of *G. glabra* is the good source of anti-oxidants. In addition, in *G. glabra* anti-oxidant contents were high. Therefore, it is more beneficial for the treatment of various diseases, which are caused due to the oxidative stress [14]. Oxidative stress is involved in the pathogenesis of autoimmune rheumatological diseases, including RA, systemic lupus erythematosus, and systemic sclerosis [23]. Epidemiological studies have demonstrated an inverse correlation between the dietary intake of antioxidants and the incidence of RA [24]. The disease activity correlates inversely with antioxidant levels and positively with the presence of oxidative stress in patients with RA [25,26]. Oxidative damage to proteins, lipids, DNA, cartilage, and extracellular collagen has been demonstrated in patients with RA [28].

Some drugs used to treat RA such as methotrexate; etancercept and infliximab are known to play essential roles as antioxidative agents [29]. Lipid peroxidation markers such as serum malondialdehyde and urine isoprostane are reported to be elevated in CIA compared with those in controls [30,31]. The beneficial effects of antioxidants have been demonstrated in mice with CIA [32-37]. An important mechanism of anti-arthritic activity is the membrane stability modulating effect [38]. *G. glabra* may exert its effects by modifying the lysosomal membrane in such a way that it is capable of fusing with the plasma membrane and there by preventing the discharge of acid hydrolase or by inhibiting the release of lysosomal enzymes [39]. The activity of *G. glabra* may be due to presence of flavonoids i.e. liquiritin and isoliquiritin [2]. Lysosomes are membrane enclosed cytoplasmic organelles, which possess an acidic interior that contain many hydrolytic enzymes. Lysosomal enzymes are widely distributed in tissue and circulating blood cells and are responsible for intracellular breakdown of complex macromolecules. They also degrade endothelial membrane glycol-conjugates. The altered enzyme activities in arthritis can be regarded as an index of lysosomal enzyme activation occurring in response to metabolic need of degrading various

constituents of cells such as mucopolysaccharides and glycoproteins accumulated in tissue due to arthritis associated with vasculopathies [2].

Another mechanism may explain *G. glabra* inhibition of arthritis in the present model can be reducing autoantigen production by inhibition protein denaturation [2]. Denaturation of proteins as one of the causes of rheumatoid arthritis is well documented. Production of autoantigens in certain rheumatic diseases may be due to in vivo denaturation of proteins. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [2]. The results from previous study revealed that *G. glabra* is capable of controlling the production of autoantigens due to in vivo denaturation of proteins in rheumatic diseases [2]. Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinases, which carry in their lysosomal granules many neutral serine proteinases. Leukocyte proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors. *G. glabra* exhibited significant anti-proteinase activity in previous study and this may explain anti-arthritic effect of this herb in the present study [2].

Inhibition of some important proinflammatory cytokine(s) may be additional effect of *G. glabra* on the present model of arthritis. However, there is evidence that antioxidants reduce the activation of NFκB, which is involved in the production of inflammatory cytokines, including TNF-α and IL-17 [40,41]. Taken together, these data indicate that *G. glabra* may inhibit the production of TNF-α and IL-17 by T cells by inhibiting NF-κB [42]. Suppression of osteoclastogenesis also may be another mechanism accounted for *G. glabra* effect in the present experiment, because previous studies, reveal that antioxidants cause suppression of osteoclastogenesis [37,43,45].

Acknowledgement

To the Deanery and Departments of Pharmacology and Pathology of College of Medicine/Al-Nahrain University.

Author contributions

Authors contribute in organizing the idea and protocol of the research, performing the practical aspects and accomplishing writing the final outcome of this work.

Conflict of Interest

No.

Funding

College of Medicine/Al-Nahrain University.

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Received 21st Dec. 2014: Accepted 14th Apr. 2015