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Clinical Evaluation of Collagen-Induced Arthritis in Female Lewis Rats: A Comprehensive Analysis of Disease Progression and Severity

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Abstract

Objective: The collagen-induced arthritis (CIA) model is the most commonly studied autoimmune model of rheumatoid arthritis (RA). In this study, we investigated the usefulness of collagen type II emulsified in Freund's incomplete adjuvant (CII/IFA) as a suitable method for establishing RA in Lewis rats. The aim of the present study was to present a straightforward and effective method for inducing CIA in rats.

Materials and Methods: In this experimental study, animals were divided into two equal groups (n=5); control and CIA. Five rats were injected intradermally at the base of the tail with a 0.2 ml CII/IFA emulsion. On the seventh day, a 0.1 ml CII/IFA emulsion booster was injected. Arthritis symptoms that arose were evaluated at clinical, histological, radiological, and at protein expression levels to find out if the disease had been induced successfully.

Results: Our finding showed a decreasing trend in the body weight during the RA induction period, while the arthritis score and paw thickness were increased during this period. The results of the enzyme-linked immunosorbent assay (ELISA) for serum samples revealed that the levels of proinflammatory cytokines, interleukin (IL)-1β, IL-6, IL-17, and tumor necrosis factor (TNF)-α and anti-CII IgG were significantly increased in CIA rats compared to the control group. After CIA induction, the level of anti-inflammatory protein IL-10 was decreased significantly. Radiographic examination of the hind paws showed soft tissue swelling, bone erosion, and osteophyte formation in CIA rats. Additionally, based on histological evaluations, the hind paws of the CIA group showed pannus formation, synovial hyperplasia, and bone and cartilage destruction.

Conclusion: It seems that CII/IFA treatment can be an appropriate and effective method to induce RA disease in Lewis rats. This well-established and well-characterized CIA model in female Lewis rats could be considered to study aspects of RA and develop novel anti-arthritic agents.

Keywords: Clinical Scoring, Collagen-Induced Arthritis, Freund's Incomplete Adjuvant, Rheumatoid Arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that primarily affects the joints (1). This systemic disorder has an unknown etiology that affects 1% of the adult population worldwide, making RA one of the most common chronic inflammatory diseases (2, 3). In the RA disorder, the immune system attacks the synovial membrane of joints, causes the joints to become swollen and painful due to inflammation, thickening of the joint membrane, and fluid accumulation. Over time, this state leads to cartilage and bone destruction, joint deformity, and eventually severe disability (4, 5). The clinical symptoms of the disease negatively affect the patient's

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quality of life and the ability to work and induce psychological distress. Despite of numerous therapeutic approaches are currently considered to alleviate symptoms, 15-40% of RA patients are resistant to long-term treatments and can become non-responsive to all existing clinical therapies (6). Therefore, suitable *in vivo* models need to assess the safety and efficacy of novel therapies, evaluate the mechanism of action of the drugs, and understand the mechanisms behind the development of the disease.

A Collagen-induced arthritis (CIA) model in mice and rats has been extensively studied because it shares several pathological and immunological features with humans and allows the testing of innovative treatments in preclinical studies (7). Most CIA models are induced by type II collagen (CII), the main protein found in the articular cartilage. Thus, by immunization of some inbreeds or outbred rats and mice, an experimental model of autoimmune arthritis can be developed (8). A CII is provided for immunization from animal sources, including pigs, cattle, and chickens (9). To establish arthritis, adjuvants such as Freund's incomplete adjuvant (IFA) accompanied by CII and Freund's complete adjuvant (CFA) with or without collagen are commonly used. In numerous studies, CFA, employed alongside CII as immunological agents, has been utilized for rat modelling, but this approach has its drawbacks (10). The CFA is composed of an emulsion of water and mineral oil containing heat-killed *Mycobacteria*; in contrast, the IFA contains an emulsion of water and non-metabolizable oils (mineral oil) and does not contain any *Mycobacterium* species (7). In a CII-induced establishment in Wistar rats, it was found that when a CFA adjuvant is used to prepare collagen, the immune response, in addition to collagen, is also produced against other Mycobacterium antigens, leading to a severe inflammatory response (9, 10).

For the reasons stated, the IFA use in preparing CII/IFA emulsions could be preferable to RA model development, which are similar to human RA in different symptoms of haematology, clinical, histological, and radiology (11).

The aim of the present study was to present a straightforward and effective method for inducing CIA in rats. We opted to utilize an Incomplete Freund's adjuvant (IFA), as an alternative to Complete Freund's adjuvant (CFA), to emulsify a bovine Collagen Type II (CII).

Through the implementation of this immunization procedure, diverse mouse strains exhibited distinct responses, which aligned with their sensitivity to the CIA induction. Ultimately, we sought to determine the most effective CIA model. This approach was aimed at ensuring the model's reliability, marked by high consistency and reproducibility, facilitating comparisons across various research studies. Moreover, we intended for this model to be particularly useful in investigations focused on understanding the etiopathogenetic mechanisms and treatment approaches for RA.

Materials and Methods

Ethical statement

The Royan Institutional Review Board and Institutional Ethics Committee, (Tehran, Iran) approved this study (IR.ACECR.ROYAN.REC.1398.05). All experimental procedures were conducted under the standard guidelines of the National Institutes of Health guidelines for the Care and Use of Laboratory Animals (eighth edition) (12).

Animals and establishment of an rheumatoid arthritis model

This experimental study in female Lewis rats, because of more susceptibility of female rodents to develop autoimmune complications than males. Lewis rats were purchased from the Pasteur institute Tehran, Iran.

Ten inbred female Lewis rats (200 ± 50 g in weight; 8-10 weeks old) were randomized into equal two groups (n=5). Animals of each group were housed in pathogen-free cages (two or three rats in each cage) with free access to standard feed and water, while the diameter of each cage was 27×42×17 cm. Also, they were kept in an animal laboratory with 12 h of light/dark cycles at 15-24 ˚C and relative humidity of 30-70%.

In accordance with previous studies (10, 13), the CIA model was induced in the animals. Bovine type II collagen (CII, Chondrex, Redmond, WA, USA, 20021) at a concentration of 2 mg/ml was dissolved in 0.05% acetic acid (Merck, Germany, 137000) and emulsified with an equal volume of Freund's incomplete adjuvant (IFA, Thermo Fisher, USA, 77140) using a homogenizer. This process aimed to generate a stable emulsion that would persist as a solid clump, preventing dispersion in water. Briefly, five rats were injected intradermally with a total of 0.2 ml CII/IFA emulsion (1 mg/ml) at the base of the tail, 1-2 cm away from the tail root, and at two sites on the back of rats, for sufficient and consistent drainage to the inguinal lymph nodes. Seven days after primary immunization, a 0.1 ml CII/IFA emulsion booster injection was administered. These rats were then assigned to the model group. The control group did not receive any compound. After 23 days of primary induction, the rats were euthanized, and their sera and paws were harvested for further analysis.

Clinical evaluation of the collagen-induced arthritis animal model

Induction of arthritis in rats was evaluated by body weight changes, paw swelling (characterized by oedema and erythema), and arthritis score from the 7th day of CII/IFA injection until scarification day, the 23rd day of injection. Body weight changes in the rats were measured every two days. The paw swelling was calculated by the thickness of the ankle joints of both hind paws in millimeters using the digital calipers model (Stoelting, USA, 58750). Arthritis scores on the hind paws of each rat were graded from 0 to 4 according to the extent of oedema and erythema of the specific tissues. The severity scores were defined in Table 1. The maximum total score of every arthritic rat was 8 (4 points×2 hind paws). The arthritic signs were assessed by two independent investigators every two days and were scored individually using a previously described scoring system (15).

Sample collection

All animals were anaesthetized with a mixture of 50 mg/kg ketamine and 10 mg/kg xylazine (1801020-04, 362845 respectively, both from Alfasan, Woerden, Netherland) then the animals were euthanized with carbon dioxide to perform laboratory assessments. Blood samples were collected via cardiac puncture and immediately centrifuged at 3000 rpm for 10 minutes at 4°C, and the harvested sera were kept at -70°C until the enzyme-linked immunosorbent assay (ELISA). The harvested hind paws were fixed with 10% neutral buffered formalin (Merck, Germany, 104002, pH=7.2).

Enzyme-linked immunosorbent assay

The level of inflammatory cytokines (tumor necrosis factor-α [TNF-α], IL-1β, IL-17, and IL-6) and immunoregulatory cytokines (IL-10) were detected using the necessary ELISA kits (R&D Systems, USA). The level of anti-CII immunoglobulin was detected using an ELISA kit (Chondrex, USA, 1021T) according to the manufacturer's instructions.

Radiological assessment

Hind paws were radiographed by a conventional X-ray machine (Toshiba, DC-12M) at 45 kV peak, 15 mA and 5 s exposure time. Radiographs were evaluated for different parameters, including soft tissue swelling, bone erosion, and osteophyte formation. These signs were scored blindly by two independent radiologists who were unaware of the treatment assignments on a scale of 0=regular, 1=mild changes, 2=moderate changes, and 3=severe changes (14).

Histological evaluation

The hind paws were decalcified in 10% EDTA (Gibco, USA, 15040) for 28 days at room temperature after being fixed for 48 h in 10% neutral buffered formalin (pH=7.26). Then the tissue samples were embedded in the paraffin (Biooptica, Italy, 087920) and serially sectioned into 5 µm thick sections. The sections were subjected to hematoxylin, eosin (H&E, Biooptica, Italy, 05-06004, 05-m10003, Biooptica, Italy, respectively) and Masson's trichrome (MT) staining (Sigma, USA, f-7258,). Sections were observed under the light microscope (Olympus BX51; Olympus, Japan) by an expert pathologist trained in joint pathology and blind to our treatment. Histological analysis was carried out based on bone resorption, cartilage damage, pannus formation, and infiltration of inflammatory cells. The severity of the lesions was determined using a graded scale as follows: 0=no signs of change, 1=mild change, 2=moderate change, and 3=increasing degrees of changes (14).

Statistical analysis

All statistical analyses were performed using the GraphPad Prism 8 (GraphPad Software., La Jolla, California, USA) software. The comparisons of radiology and pathology scores, paw thickness and body weight were performed using a 2-way analysis of variance (ANOVA). Unpaired t test were performed for other variables. Data were expressed as mean ± standard deviation of the mean (SD), and $P<0.05$ were considered statistically significant.

Results

Collagen-induced arthritis rats showed clinical signs of rheumatoid arthritis disease

The RA disease progression was assessed based on weight changes, arthritis severity score, and paw thickness. Clinical signs of CIA in the hind paws of rats showed that arthritis established well in animals (Fig.1).

The 23 days after the primary injection, Intense oedema and erythema were observed in the hind paws of the CIA group in comparison with the control group (Fig.1A). Animals in the CIA group showed a trend of mild body weight loss in a time-dependent manner in comparison with the control group (Fig.1B). However, this decreasing trend was insignificant during the model establishment. Erythema and swelling, as disease severity scores, were higher in the CIA group compared to the control group, which was significant at days 15-23 ($P<0.001$, Fig.1C). The paw thickness, indicative of ankle joint swelling, exhibited a notable increase in the CIA group. This increase was statistically significant from day 17 ($P<0.001$) through day 23 following the primary induction ($P<0.001$, Fig.1D).

Table 1: Scoring system for subjective evaluation of arthritis severity

Severity score	Degree of inflammation
0	No evidence of erythema and swelling
1	Erythema and mild swelling confined to the tarsals or ankle joint
2	Erythema and mild swelling extending from the ankle to the tarsals
3	Erythema and moderate swelling extending from the ankle to metatarsal joints
4	Erythema and severe swelling encompassing the ankle, foot, and digits, or ankylosis of the limb

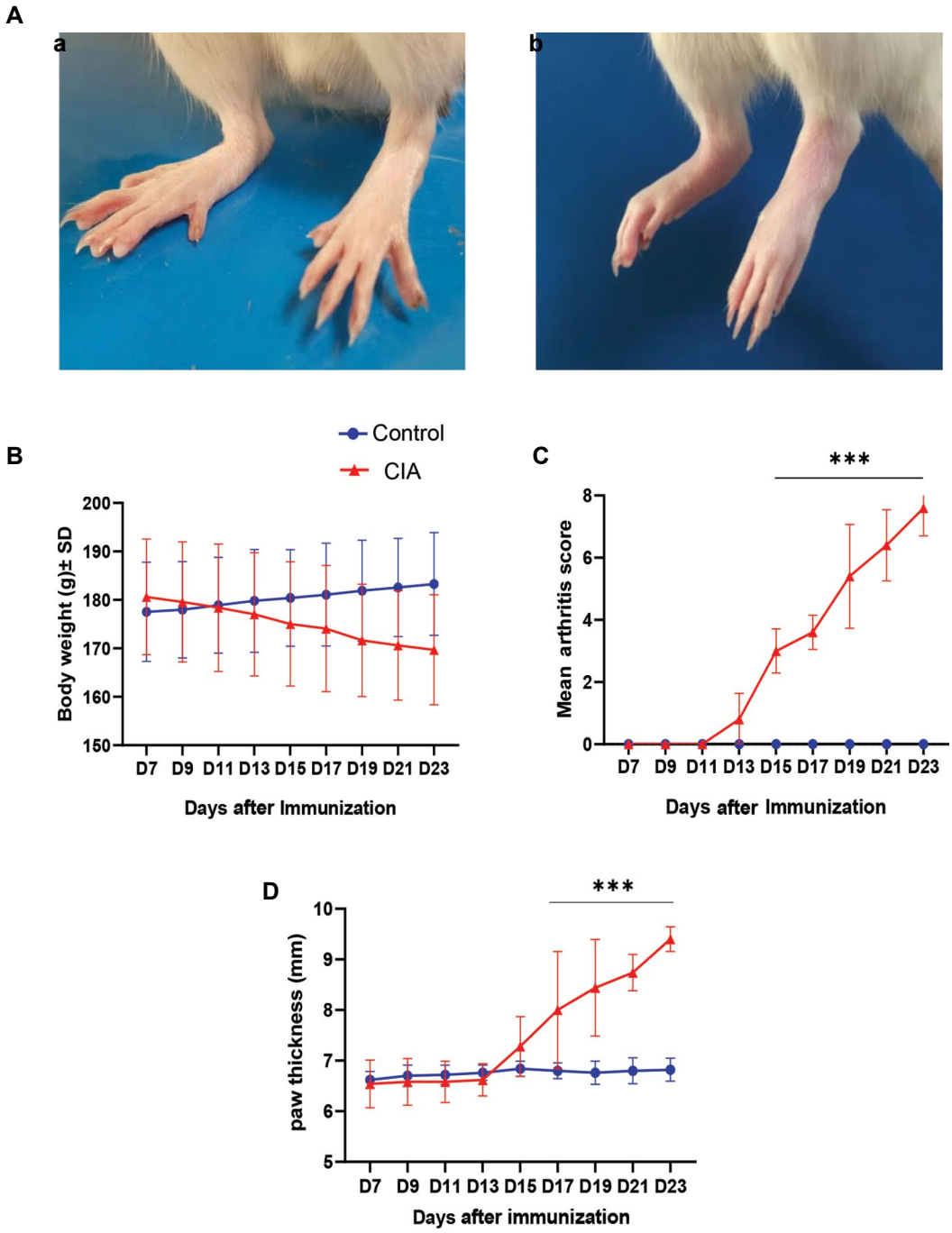

Fig.1: Clinical signs of the CIA induction in rats. **A.** Representative images of the hind paws from the control (a) and CIA (b) groups were taken on the 23rd day after the primary injection of type II collagen emulsified in the incomplete Freund's adjuvant. Intense edema was observed in the hind paws of the CIA group in comparison with the control group. **B.** Body weight changes, **C.** Arthritis scores for hind paws, and **D.** Paw swelling were evaluated in control and CIA groups during the disease induction. All data were presented as the mean ± standard deviation. The comparisons were performed using two -way analysis of variance (ANOVA, n=5). ***; P<0.001 versus control rats and CIA; Collagen-induced arthritis.

Collagen-induced arthritis rats showed inflammation at the protein expression level

To explore the RA induction in rats, the expression of IL-1β, IL-6, IL-17, TNF-α as proinflammatory and IL-10 as an anti-inflammatory cytokine was detected by ELISA assay. The protein level of autoreactive antibodies against CII (CII-specific IgG) was also evaluated.

The anti-CII antibody level in the serum of the CIA group was meaningfully higher than those of the control group (P<0.001, Fig.2A).

The expression levels of TNF-α (P<0.001), IL-1β (P<0.001), IL-6 (P<0.001), and IL-17 (P<0.001) was elevated significantly in the CIA group in comparison with the control group (Fig.2B-E). In addition, the expression

level of IL-10 (P<0.001) in the CIA group was inhibited in comparison with the control group (Fig.2F).

The radiographic features of lateral views of the hind paws in our groups are shown in Figure 3. The radiographic images of the control group revealed normal ankle joints and a higher bone volume than the CIA group (Fig.3A). The severity of RA was radiological measured the shape and volume of bone. The CIA group exhibited bone erosion and osteophyte formation. Specifically, the joint surfaces had a rough and irregular articular appearance with a diffuse lesion pattern on the articular surface. The radiological scoring of soft tissue swelling (P<0.001), bone erosion (osteolysis) (P=0.002), and osteophyte formation (P=0.011) showed significant differences between our groups (Fig.3B).

Collagen-induced arthritis rats showed symptoms of rheumatoid arthritis disease

The H&E staining of the hind paws of the control group showed a normal articular cartilage with the intact joint space, and synovial tissue was without any architectural changes (Fig.4A).

The cartilage surface in the CIA group exhibited damages, while the inflammatory mononuclear cells were infiltrated around and inside the cartilage. Granular hard tissue contains an inflammatory tissue with increased fibroblasts and enlarged blood vessels, with signs of bone destruction (Fig.4A). MT staining photographs of hind ankle joints illustrated a normal synovial tissue with typical collagen fibers in the control group. However, the high rate of articular cartilage degradation and extensive fibrosis completely obliterating the joint cavity was observed on the articular surfaces of the joint in the CIA group. In addition, synovial hyperplasia and inflammatory cell infiltration were observed on the articular surface of the joints from the CIA group (Fig.4A). The ankle histopathological score showed significant increases in different parameters, including inflammation, pannus formation, cartilage damage, and bone resorption in the CIA group (P<0.0001, Fig.4B).

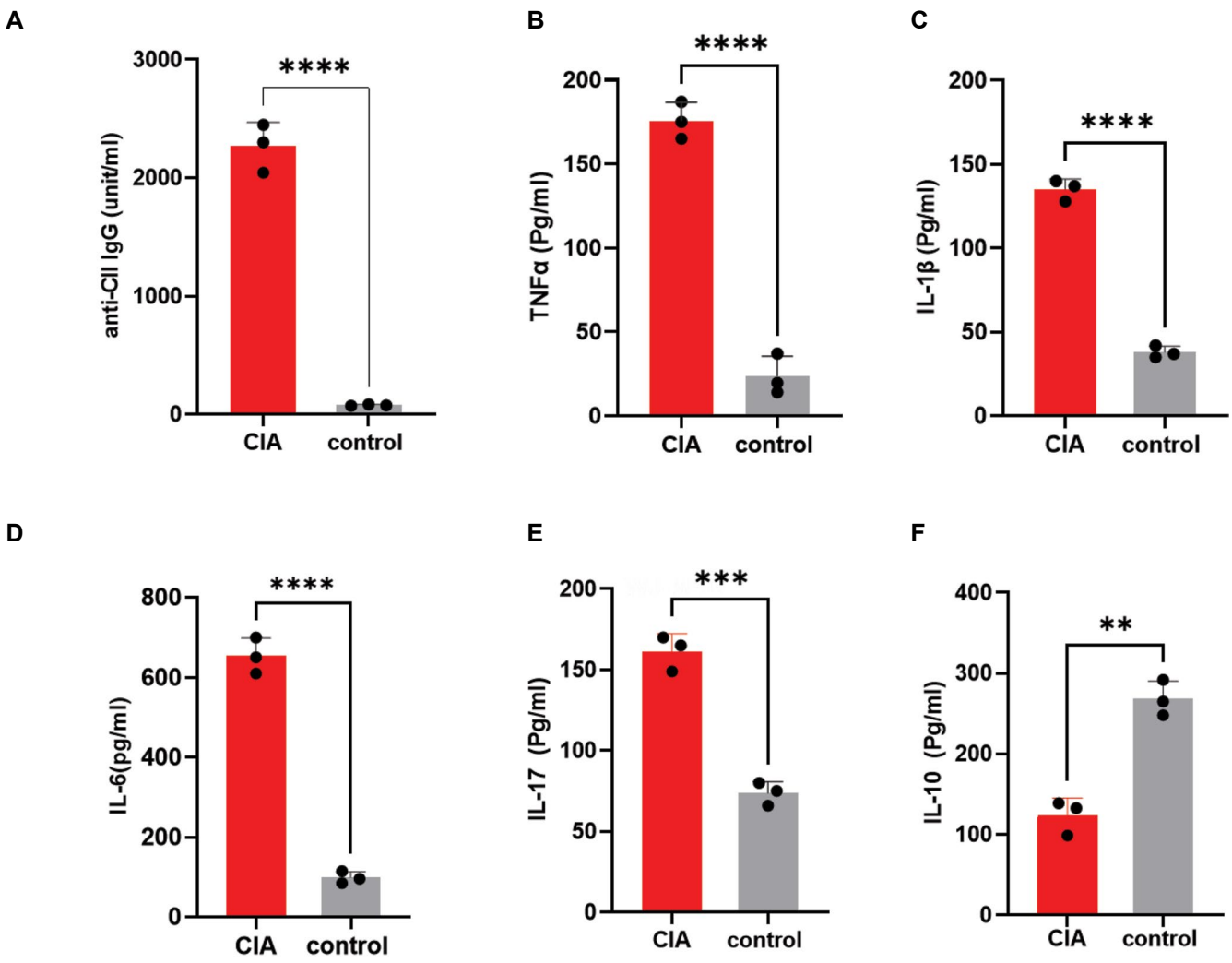

Fig.2: Inflammation produced after CII/IFA injection in rats. **A.** Following CIA induction, the serum levels of anti-CII IgG and **B-E**. Inflammatory cytokines TNF-α, IL-1β, IL-17, and IL-6 were significantly elevated in the CIA group, and **F.** anti-inflammatory factor IL-10 was decreased in comparison with the control group. All data were presented as the mean ± standard deviation (n=3). The comparisons were performed using the unpaired t test. *; P<0.05, **; P<0.01, ***; P<0.001, CII/IFA; Collagen type II/Freund's incomplete adjuvant, CIA; Collagen-induced arthritis, TNF-α; Tumor necrosis factor-alpha, and IL; Interleukin.

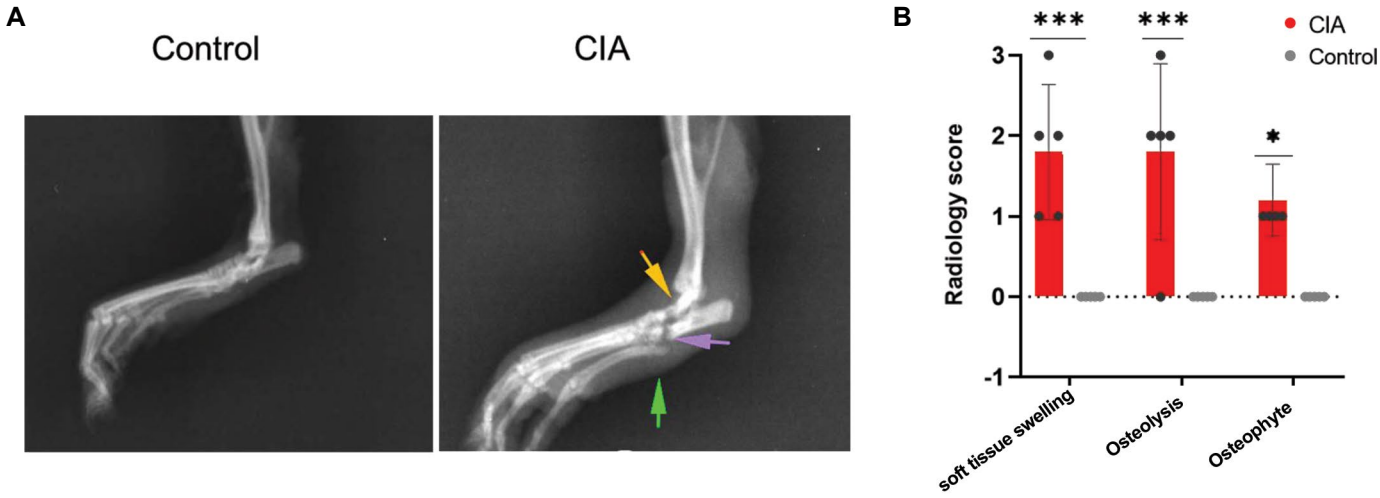

Fig.3: Radiological evaluation of the hind ankle joints. **A.** CIA group had severe soft tissue swelling (green arrow), osteolysis (purple arrow) and periosteal new bone formation (yellow arrow). **B.** Radiological scoring of soft tissue swelling, bone erosion, and osteophyte formation showed significantly increased values in the CIA group in comparison with the control group. All data were presented as the mean ± standard deviation (n=5). The comparisons were performed using two -way analysis of variance (ANOVA). *; P<0.05, ***; P<0.001, and CIA; Collagen-induced arthritis.

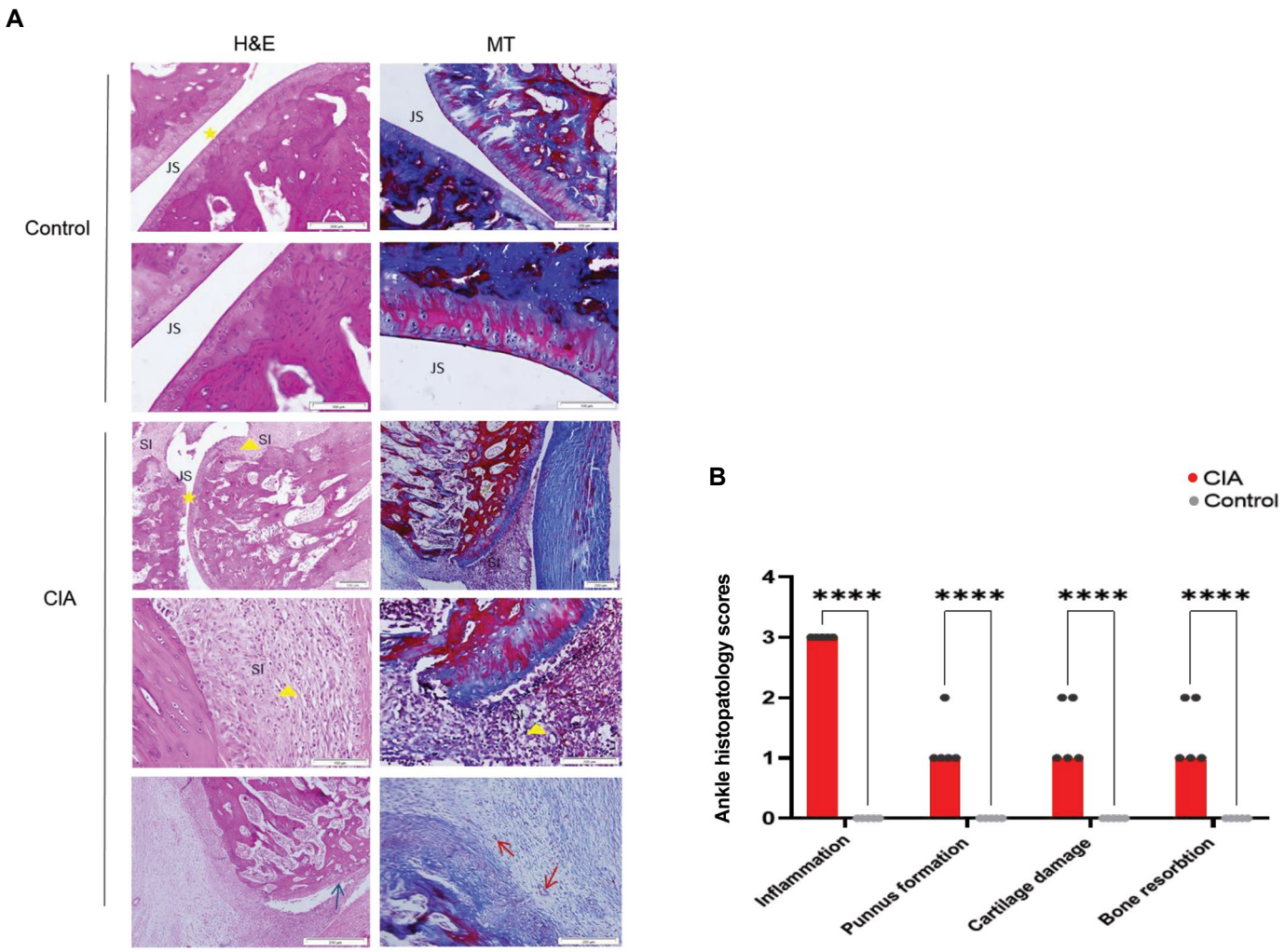

Fig.4: Histological evaluation of the hind ankle joints. **A.** Histological evaluation of the hind ankle joints. H&E and MT staining. The Control group showed an intact articular cartilage, preserved articular space, and typical synovial tissue collagen fibers without any inflammatory infiltration. The CIA group demonstrated synovial hypertrophy with marked mononuclear leukocyte infiltration (inflammation) (triangle), hypervascularity (red arrow) with pannus formation, destruction of the adjacent bone (blue arrow), degenerative changes in articular cartilage with diminished articular space (star). **B.** Histological scores of the rats' ankles in the CIA group showed a significant increase in the inflammation, pannus formation, cartilage damage, and bone resorption changes in comparison with the control group. All data were presented as the mean ± standard deviation (n=5). The comparisons were performed using two-way analysis of variance (ANOVA). ****; P<0.001, CIA; Collagen-induced arthritis, H&E; hematoxylin and eosin, MT; Masson's trichrome, SI; Synovial inflammation, and JS; Joint space.

Discussion

Developing RA animal models may shine the new therapeutic options road. There is a debate regarding the appropriate RA animal models for precise disease characteristics because different models reflect distinct clinical features. These variations could be the result of differences in species, strains, sexes or ages of animals, housing and care refinements, dissimilarity in protocols for arthritis induction, and variation in the materials used for induction such as collagen, lipopolysaccharide, and use of adjuvants such as *Mycobacterium* (16).

Suppose the animal model fails to simulate what is happening in the RA disease. In that case, inaccuracies will occur in finding the underlying biological mechanisms and predicting response to the clinical treatments. One of the most widely used experimental models of autoimmune arthritis CIA which shares several pathological aspects with RA, including synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, and, similar to RA disease in humans, its susceptibility is linked to major histocompatibility complex (MHC) class II molecules (17, 18).

On the other hand, another critical issue is potential harm to animals, i.e., pain, suffering or distress, that should be considered against the possible benefits of each protocol.

Russell and Burch introduced and defined the three Rs (replacement, reduction and refinement) terms, which have become known as ‘alternative methods’ for minimizing the potential pain, distress and fear in animal research (19). Procedures and materials used to induce arthritis in animals can cause pain and suffering, which may be mild, moderate, or severe, depending on the modelling method, animal acceptance, and type of materials used for disease establishment (9). For this reason, researchers need to consider the conduct to minimize this problem in the animal. Although, there is still a challenging issue. Due to the genetic and physiological differences between humans and animals, and the animal findings need to be fully generalized to the human population (20). There are limitations, therefore preclinical studies are critical and helpful regarding efficacy and safety in the living organism (19). In these viewpoints, we induced the CIA model in female Lewis rats in the present study. In the CIA group, symptoms of arthritis appeared in one or both hind paws from 12th to 14th days and showed a significant difference in swelling of hind paws and arthritis scores from the 17th day in comparison with the control group. We used an inbred strain rat for the CIA establishment because using inbred strains can reduce variability and extremes in responses because they have a more uniform genetic background, which minimizes the effects of genetic variation on experimental outcomes (9). Establishing the CIA model in female rats is imperative because, like humans, females are more receptive to this model (21).

In this study, we used 300 μg bovine CII emulsified in the IFA, and the results showed a successful CIA induction. The CII immunity depends on the response of tissue-specific T lymphocytes, followed by the stimulation of B cells and the production of large amounts of antibodies against CII. These antibodies specifically bind to conserve epitopes on the type II collagen (CII), including the C1 epitope. They inhibit the CII self-assembly into fibrils, a process that may play a role in the pathogenesis of CIA. In general, the formation of antibodies that target a CII is a pivotal event in the onset and advancement of the CIA within joint tissues. These antibodies also play a role in the development of RA in both human subjects and CIA models (22).

The IFA induces predominantly T helper 2 (Th2) cells through the formation of a depot at the injection site with the stimulation of B cells; this causes the slow release of an antigen. IFAs, organism contamination free, are less toxic, thus, result in a less painful.

In a review study by Noh et al. (23), different models of RA in rodents were compared. It was concluded that in adjuvant-induced arthritis (AIA) models in comparison with the CIA model, the use of CFA adjuvant causes more severe joint damages and extra-articular involvements, such as inflammation in blood vessels, brain and eyes, and bone loss in the axial skeleton, bone marrow hyperplasia and leukocytosis, and enlargement of spleen and lymph nodes. This systemic response is evoked by ‘danger’ signals available in the CFA. Hence, a CIA could be presumed as an auto-inflammatory rather than an autoimmune situation (24). Therefore, the IFA is preferable to the CFA in creating a CIA model.

Studies revealed that the RA susceptibility in humans and the establishment of a CIA in rodents linked with MHC class II molecules (12), MHC class II regulates B cell activation, proliferation, and differentiation during cognate B cell-T cell interaction (25). In our study, the presence of IgG-specific autoantibodies against CII in the CIA group was significantly increased, confirming B cells’ role in the CIA pathogenesis.

According to previous studies, changes in the balance of the inflammatory cytokines towards disease development detected in the joint tissue and serum of RA patients (26, 27). Therefore, in this study, we assessed the expression of cytokines involved in inflammation markers, including IL-1β, IL-6, IL-17, TNF-α, and IL-10. We observed that the levels of proinflammatory cytokines in the CIA model group increased significantly in comparison with the control group. Proinflammatory cytokines play an essential role in the pathogenesis of RA in patients by causing inflammation, synovial hyperplasia, and destruction of cartilage and bone. During the development and progression of the RA, various cells and many cytokines stimulate the production of fibroblast-like synoviocytes (FLSs) and macrophages. Stimulated macrophages release inflammatory mediators, and FLSs secrete cytokines and enzymes, which all are involved in the cartilage and bone degradation (26, 28). The IL-1β and TNF-α increase the proliferation of

synovial membrane cells in the joint, causing it to thicken and decrease the volume of joint fluid. The TNF-α, as a critical regulator of the inflammatory cascade, is a central player in the pathogenesis of RA and is considered for anti-TNF-α biologic (29).

The IL-1 is a crucial mediator of immune responses and is strongly linked to inflammation and joint damages in the RA (30). Therapeutic effects of IL-1 blockade, either through anti-IL-1 antibody or IL-1 receptor antagonist, have been demonstrated in several models of arthritis, such as CIA (29, 31). Another main cytokine in the development of the RA is IL-6, which has an essential effect on the proliferation and differentiation of macrophages, B and T cells, osteoclasts, chondrocytes, and endothelial cells (32-35). Regarding IL-6, there is some evidence of dysregulated overproduction from various animal models of arthritis, including CIA (36). Several strategies have been used to target IL-6 cytokine/signaling in RA patients.

In the CIA model, similar to the pathogenesis of RA in patients, the protein levels of TNFα, IL1β and IL6 were increased. At the same time, the anti-inflammatory cytokine IL10 was downregulated. The IL-10 is an effector cytokine in mice and humans and is critical in RA pathogenesis (37, 38).

Th17 cells intensify inflammation by the production of IL-17, and elevated serum IL-17 levels are directly related to the severity of RA symptoms in patients (39). The IL-17 can enhance the inflammation and cellular infiltration joint arthritis. Moreover, it mediates bone and cartilage damage, which causes pain and disability in RA patients (40). Thus, the findings in the female Lewis rat CIA model in our study closely resemble the disease characteristics of human RA.

Song et al. (10) induce a simple, specific, and efficient way to induce the CIA model in Wistar rats. They used IFA instead of CFA to emulsify CII, and injected the CII/IFA emulsion into the skin at the base of the tail on the 7th day after the initial immunization. They injected a booster dose of CII/IFA and concluded that this developed CIA model was similar in clinical, hematologic, histopathological, and radiological features to those of humans. In another study, Choudhary et al. (7) used IFA emulsion and autologous collagen to develop severe polyarthritis in Dark Agouti (DA) and Lewis rats. Symptoms of the onset of arthritis were identified two weeks after immunization as paw swelling. As the disease progressed, it eventually led to chronic arthritis, increased inflammatory cytokines, and bone and cartilage destruction (7). Both of these studies confirmed the results of our results.

In our study, in the CIA group, such as RA patients, the IL-10 level declined, which reflects the imbalance between proinflammatory and anti-inflammatory cytokines in the RA pathogenesis. This imbalance might be due to various reasons, such as a specific IL-10 inhibitor, excretion of IL-10, production disruption or increasing degradation.

Alterations in the body weight of animals, paw swelling, and arthritis scores are standard indices used to evaluate arthritis induction and the anti-arthritic effect of drugs in the CIA animal models (37). In the present study, CII/IFAinjected rats exhibited weight loss, paw edema and increasing arthritis scores. The histological and radiological findings also confirmed the establishment of arthritis in the CIA model group. These data demonstrated that increased inflammatory cell infiltration in synovial space and extra-articular tissues in CIA animals could be directly related to cartilage and bone destruction.

Conclusion

This study emphasizes the use of IFA/CII emulsion to establish the CIA model in Lewis rats. This model showed a high disease incidence and low variability in clinical symptoms, closely resembling RA progression in humans. Owing to the remarkable consistency of this model when applied to Lewis rats, combined with the exceptionally high acceptance rate among female Lewis rats (approaching 100%), it boasts a notably reduced mortality rate, aligning it with stringent animal welfare principles. The use of this approach in Lewis rats was associated with lower mortality rates. Emphasizing the ethical priority of adhering to the principles of the three Rs (Replacement, Reduction, and Refinement), it is noteworthy that the chosen method for arthritis induction in animals, involving Freund's incomplete adjuvant without mycobacterial antigens, effectively curbs the risk of severe inflammatory reactions and prevents granuloma formation within the animals' vital organs a problem frequently associated with the use of complete adjuvant.

Additionally, it is prudent to recognize that the Dilute Brown non-Agouti (DBA) 1 mouse strain is not universally accessible in all regions. Consequently, the application of this model, which can be established at a more cost-effective rate, represents a significant advantage.

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Authors' Contributions

M.B., N.M.G.; Performed data collection and assembly, data analysis and interpretation, and wrote the manuscript. B.T.; Performed data collection and assembly. J.P.; Developed the study conception and design. M.B.E., E.H.-S., S-N.H.; Conducted the experiments, developed the study conception and design, performed data analysis

and interpretation, and administrative and manuscript proof. All authors read and approved the manuscript.

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