MASTER'S THESIS

Inhibitory Effects of *Ganoderma lucidum* Spore Oil on Rheumatoid Arthritis in a Collagen-Induced Arthritis Mouse Model

Yunji Heo

**Department of Animal Biotechnology, Faculty of Biotechnology,
College of Applied Life Sciences**

**GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY**

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Yunji Heo

(Supervised by professor Young-Ok Son)

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This thesis has been examined and approved by

Kyoung-tag Do

Thesis director, Kyoung-Tag Do, Prof. of Animal Biotechnology, Jeju National University

Inhae Kang, Prof. of Food Science and Nutrition, Jeju National University

Young-Ok Son, Prof. of Animal Biotechnology, Jeju National University

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College of Applied Life Sciences
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CONTENTS

국문 초록

몇몇의 의료 종사자들은 RA를 치료하기 위해 특정 약용 버섯에 초점을 맞추기 시작했습니다. 그 중 *Ganoderma lucidum*(GL)은 고대 중국 의학에서 RA와 같은 자가 면역 질환을 포함한 염증성 질환을 치료하는 데 사용된 가장 오래된 버섯 중 하나로 알려져 있습니다. 이 버섯의 포자는 면역 조절, 노화 및 암에 특정한 영향을 미칩니다. 그러나 *G. lucidum* spore(GLS)의 관절염에 대한 효과는 아직 명확하게 밝혀지지 않았습니다. 따라서 콜라겐 유발 류마티스 관절염(CIA) 모델을 활용하여 GLS 오일이 관절염에 주는 효과를 조사하였습니다. GLS오일의 성분 분석에 따르면 GLS 오일에는 10가지 산이 포함되어 있으며 그 중 올레산(52.12%)과 리놀레산(16.77%)이 상당부분을 차지하고 있습니다. GLS 오일을 주입한 CIA 그룹은 일반 CIA 그룹보다 RA에 대해 유의적으로 더 낮은 중증도 점수($p = 0.0384$)를 보였습니다. 또한, GLS 오일은 관절에서 CIA에 의해 유발된 연골 변성과 활막에서의 염증을 감소시켰으며 그리고 CIA에 의해 유발된 판누스 형성도 감소시켰습니다. 이와 더불어 면역세포 활성 측면에서도 GLS 오일을 주입한 그룹은 관절에서의 호산구의 증가정도가 유의하게 감소하였고($p = 0.0056$), 호중구의 침윤을 확인한 결과 호중구의 침윤정도가 무릎($p = 0.0006$)과 발목($p = 0.0023$) 모두 GLS 오일을 주입한 그룹에서 유의하게 감소했습니다. 이러한 결과는 GLS 오일이 RA 약물 개발에 유용할 수 있음을 시사하고 이와 관련된 GLS오일의 더 높은 개선효과를 확인하기 위해서는 추가 임상 연구가 필요합니다.

LIST OF FIGURES

ABSTRACT

Holistic healthcare practitioners have now started to focus on specific traditional medicinal mushrooms to treat rheumatoid arthritis (RA). *Ganoderma lucidum* (GL) is one of the oldest mushrooms that have been used in ancient Chinese medicine to treat inflammatory ailments, including autoimmune diseases such as RA. Spores from this mushroom have specific effects on immunomodulation, aging, and cancer. However, the effect of *G. lucidum* spores (GLS) on arthritis remains unclear. Therefore, we investigated the effects of GLS oil in a collagen-induced rheumatoid arthritis (CIA) model. Metabolomics analysis revealed that GLS oil contains ten acids, of which oleic acid (52.12%) and linoleic acid (16.77%) predominated. The GLS oil-treated CIA mice had a significantly lower clinical score ($p = 0.0384$) for RA than the control CIA mice. Moreover, GLS oil reduced CIA-induced cartilage degeneration and synovial membrane inflammation in the knee. The GLS oil group showed significantly reduced knee eosinophilia ($p = 0.0056$). Immunostaining of neutrophils revealed that neutrophils infiltrated the CIA group; however, infiltrated neutrophils were significantly reduced in the GLS oil group in both the knees ($p = 0.0006$) and ankles ($p = 0.0023$). GLS oil treatment substantially suppressed LPS- or TNF-α–induced IL-6 mRNA expression in primary cultured chondrocytes. IL-6 immunohistochemistry results showed that the protein levels of IL-6 were attenuated in the GLS oil group compared to the CIA group. These findings suggest that GLS oil may be useful for the development of RA drugs. Further clinical research is required to identify significant improvements.

Keywords: Supercritical carbon dioxide, *Ganoderma lucidum* spores, Rheumatoid arthritis, Collagen-induced rheumatoid arthritis

I. INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease that affects 0.5–1.0% of the world population [1]. It is a chronic condition that causes joint swelling, stiffness, and immunosenescence. The latter appears to be a major contributor to the increased risk of disability in elderly patients with RA [2]. Moreover, RA has a high incidence of complications, such as cardiovascular disease and osteoporosis, due to its direct and indirect effects on other systemic symptoms and inflammation [3]. Genetics, infectious diseases, environmental variables, and hormonal influences are all factors that contribute to RA [2]. Additionally, research indicates that the pathogenesis of RA is influenced by interactions and the activation of innate and adaptive immune cells [2, 3]. However, the underlying mechanisms of RA remain unknown.

Disease-modifying antirheumatic drugs (DMARDs) are used to prevent joint damage during the early stages of RA. Anti-rheumatic drugs are sometimes used to bring about remission or slow the progression of RA [3]. These medications include methotrexate, hydroxychloroquine, sulfasalazine, and leflunomide. High doses of these drugs can cause gastrointestinal irritation and other side effects, which can lead to the development of ulcers and perforations in some cases [4]. Liver and kidney damage caused by arthritis medication can also cause skin rashes, urticaria, headache, dizziness, and drowsiness, with some patients experiencing hypertension and edema [5]. Thus, finding effective alternative medicines with fewer side effects is critical.

Ganoderma lucidum (GL) is an annual mushroom that grows on hardwoods in the summer and has a strong bitter taste, and is known as 'Ling zhi' in China and 'Rei shi' in Japan [6]. GL spores (GLS) are discharged from GL in the form of brown ovals. For over 2,000 years, GL has been used as a medicinal mushroom for heart function, memory enhancement, and anti-aging. Organic germanium GL is recommended for cough and asthma relief, dizziness,

insomnia, and breathing difficulties [7]. The physiologically active substances of the GL fruiting body include beta-glucan, sterols, triterpenes, and selenium. The spores of GL have a higher bioactivity than the fruiting body [8] and have been recognized as a promising drug due to their versatile biological activity [9]. The physiologically active substances of GLS include polysaccharides, triterpenoids, peptides, amino acids, fatty acids, and trace elements [10-12]. These biologically active substances have immune regulation, anti-aging, blood lipid reduction, antiviral, and anticancer properties [13]. A plethora of studies have investigated the anti-cancer, anti-pain, and immune-enhancing properties of the fruit and mycelium of GL. However, the efficacy of GLS oil in RA remains to be demonstrated.

The application of natural derivatives (e.g., bioactive compounds) in the pharmaceutical, food, and cosmetic industries, is becoming more commonplace [14-16]. Generally, bioactive compounds are extracted from natural sources using conventional methods, and then fractionated or isolated further. However, many of these methods use organic solvents, which are hazardous to the environment and human health [17, 18]. Moreover, economic systems and government agencies are increasingly demanding safer products for humans and the environment, and that they are produced in a clean, non-polluting manner [19]. CO_2 is non-flammable, non-toxic, inexpensive, and of high purity, with moderate critical pressure and temperature [15]. Although CO_2 lacks the polarity required to extract polar substances, this disadvantage can be overcome using a co-solvent such as a short-chain (sc) alcohol [20]. sc-CO_2 allows for effective and rapid extraction because it can be easily and completely removed by lowering the pressure, at which point it turns into gas [20]. Supercritical fluid technology has received considerable attention as an environmentally friendly and widely applicable method for producing or micronizing pharmaceutical compounds and bioactive compounds [20, 21]. This technology has grown in popularity and is now widely used for extraction, fractionating, and isolation of bioactive compounds from plants and animals.

This study explored the remission effects of GLS in RA. The GLS compounds were

extracted using supercritical CO_2, an extraction method that does not require toxic solvents. Furthermore, we examined the ameliorative effects of GLS oil on RA in the collagen-induced rheumatoid arthritis (CIA) mouse model.

II. MATERIALS AND METHODS

2.1. Reagents and laboratory supplies

Chemicals and Falcon Labware were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Laboratory equipment were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and recombinant human IL-6 (PHC0064) were purchased from Thermo Fisher Scientific (Grand Island, NY, USA).

2.2. Preparation of GLS oil extracts by supercritical CO_2

GLS oil was extracted using the supercritical CO_2 extraction methods [22]. Figure 1a shows a schematic of the extraction apparatus manufactured by Nantong Wisdom Supercritical Science and Technology Development Co., Ltd. (China). The system comprises a 5 L extractor, two separators (separator 1: 3 L, separator 2: 2 L), a chiller, and a high-pressure CO_2 pump, which delivers the CO_2 from the CO_2 cylinder into the system. All the vessels were heated using heating jackets. In a typical experiment, 2 kg of powdered GLS were loaded into the extractor and a certain amount of was CO_2 pumped into the system. Next, valve 9 (V9) was opened to empty the system. Purging with CO_2 was repeated three times to replace the air in the system. After purging, all valves were closed, except for V1 and V2, to allow a certain amount of CO_2 to be pumped into the extractor. After the pressure and temperature of the extractor reached 40 MPa and 55 °C, V5, V8, and V11 were opened for CO_2 circulation and dynamic extraction. The temperature and pressure of separators 1 and 2 were set at 50 °C, 13 MPa, and 40 °C, 5 MPa, respectively. The CO_2 mass flow rate was maintained at 35 kg/h and the extraction process lasted for 2 h. After completing the extraction, the GLS oil was collected via V7 and stored for later testing.

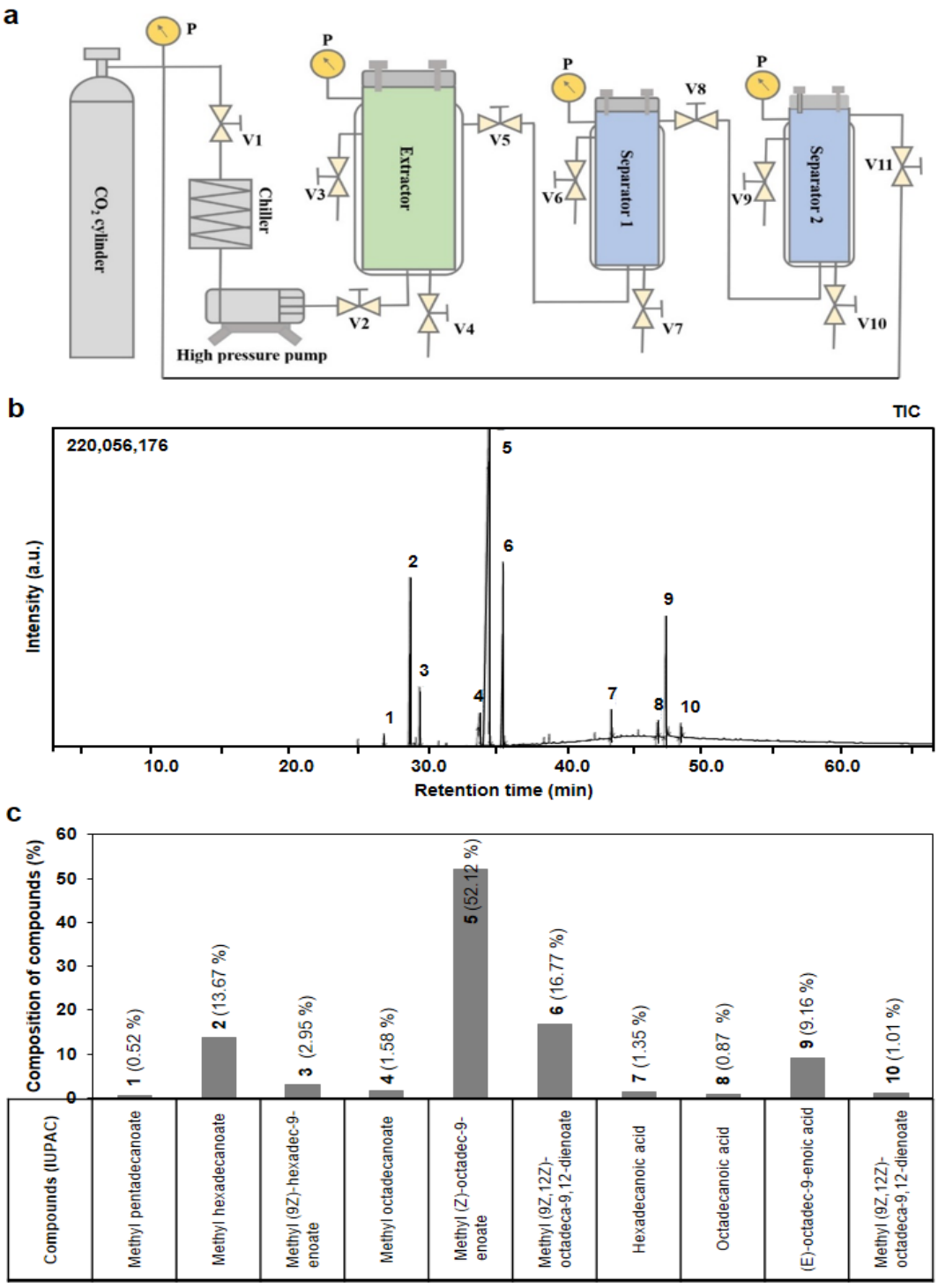

Figure 1. (a) Schematic diagram of the supercritical extraction apparatus. **(b)** Gas chromatography–mass spectrometry chromatogram for the supercritical CO_2 extracted *Ganoderma lucidum* spore (GLS) oil. **(c)** Compound list and corresponding percentages of

chemicals in *Ganoderma lucidum* spore (GLS) oil extracted using supercritical CO_2 fluid.

2.3. Analysis of GLS oil by gas chromatography–mass spectrometry (GC-MS)

The samples were converted to the corresponding fatty acid methyl ester (FAMEs) according to a previously published method [23]. Total fatty acids and fatty acid contents in the oil samples were measured concurrently in two steps: (1) preparation of FAME (2) and chromatographic analysis. One hundred microliters of GLS were added to a test tube with a stopper and 4 mL of potassium hydroxide/methanol solution (0.5 M) was added. The mixture was combined and allowed to evaporate for 15 minutes in a rotary evaporator. Subsequently, 1.6 mL of methanol:hydrochloric acid was added, and the sample was stirred for another 10 minutes, followed by the addition of 6 mL of n-hexane to the sample. This was mixed well until the two layers were separated. The upper layer of n-hexane was extracted to recover the methyl esters in the organic phase into a new test tube. After a certain amount of anhydrous sodium sulfate was added to the n-hexane for dehydration, the mixture was allowed to stand for clarification. The supernatant was analyzed by GC-MS on a Shimadzu QP2010 SE gas chromatography-mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a DB-FFAP (25 m × 0.32 mm × 0.5 μm) column (Agilent, United States). The injector temperature was set to 250 °C and the column temperature from 50–240 °C; the initial column temperature was 50 °C and was held for 2 min, next, the temperature was ramped to 180 °C at a rate of 10 °C/min, and held for 5 min, and then finally increased to 240 °C at a rate of 5 °C/min, and held 25 min. Fatty acid methyl esters were separated and the peaks were identified by comparing the mass spectra with the mass spectral library database.

2.4. Experimental RA model generation and von Frey and hot-plate assays

All experiments were conducted with the permission of the Institutional Animal Care and Committee of Jeju National University (2022-0002). Seven-week-old DBA/1 J mice were used for constructing the experimental RA model. Under isoprene anesthesia, equal volumes of collagen type II and 4 mg/mL of total Freund's adjuvant were prepared and slowly injected into their tails [24, 25].

The von Frey test was conducted once weekly after seven days of arthritis induction. Briefly, the stab of the filament started with a thick size on the sole and was recorded when the mice were licking. Each stab was performed five times and licking was recorded three times. If the mice did not react, the filament was changed to a lower filament and processed for further analysis [26]. The hot-plate assay was performed using the Plantar Test for Thermal Stimulation-Hargreaves Apparatus (ugo basile®, 37570, Gemonio, Italy). The experimental mouse was placed at five different locations, and the patience time (in seconds) when the mouse was removed from the soleplate was recorded. When the mouse licked or popped, the reaction time was recorded for accurate measurements. Each leg was measured five times, and the average values were recorded [27, 28].

2.5. GLS oil injection

GLS oil was injected one week after CIA induction. The GLS oil (6 mg/Kg) was mixed with polyethylene glycol 400 (PEG-400) and intraperitoneally injected (200 μL) twice a week for seven weeks, while 200 μL of PEG-400 was injected in the control group.

2.6. Clinical score & paw thickness

From the first day of intraperitoneal GLS injection, the hind paws of the mice on both sides were examined every 3–4 days, and the severity was determined by the amount of swelling. The CIA scoring index developed by Hooke Laboratories was used to grade the

severity of arthritis (0–4) as follows: an uninflamed, normally functioning foot; one or both toes are inflamed and swollen, while the foot or ankle is not necessarily visibly swollen; two or more toes are inflamed and swollen, but the entire foot may not be swollen or may only have mild swelling; three or all four toes may be severely swollen, preventing the mice from grasping the top of the cage [29]. The most severe swelling in the mouse ankle was measured every 3 or 4 days following the intraperitoneal GLS injection. After seven weeks, the experimental mice were euthanized with CO_2, and knee, ankle, and sole tissues were collected for histological analyses.

2.7. Histological examinations

The collected tissues were fixed in 4% paraformaldehyde for 24 h and then decalcified for two weeks in 0.5 M ethylenediaminetetraacetic acid (pH 7.4). The tissues were sectioned to a thickness of 4 μm for slide preparation after dehydration. A block was created by fixing it in paraffin [30, 31]. Harris hematoxylin, Fast Green, and Safranin O (Sigma-Aldrich, St. Louis, MO, USA) were used for histological analyses. The histological degree of inflammation in RA was classified into three categories: OARSI (Osteoarthritis Research Society International) grade (0–6), synovitis score (0–3), and pannus score (0–4). Five observers evaluated the OARSI, synovitis, and pannus scores. The scoring results were calculated by averaging the scores of the observers for each mouse. A representative image of Safranin-O with the most representative etiology among the sections was chosen.

2.8. Immune cell infiltration analysis

Congo red (Sigma C6277, St. Louis, MO, USA) and Toluidine blue O (Sigma Cat.no, St. Louis, MO, USA) were used for eosinophils and mast cell staining, respectively. The infiltration of eosinophils and mast cells into the synovial membrane around the joint was analyzed. CD15 was used for neutrophil extracellular traps, and the quantification of

neutrophils was analyzed using ImageJ [32].

2.9. Cell culture and treatment

Chondrocytes were isolated and cultured following trypsin and collagenase digestion [33, 34]. Primary chondrocytes (3 x 10^5/30 mm culture dish) were treated with different concentrations of GLS (0-300 g/ml) in the presence or absence of LPS (10 ng/ml) or TNF-α (10 ng/ml). The samples were then subjected to quantitative real-time RT-PCR (qRT-PCR) analysis.

2.10. qRT-PCR-based analysis

Total RNA was extracted from primary cultured chondrocytes using the TRIzol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). The NanoDropTM 2000 spectrophotometer was used to check RNA integrity and concentration (Thermo Scientific, Waltham, MA, USA). Following cDNA synthesis, qRT-PCR analysis for IL-6 expression was conducted on the BIO-RAD Real-Time PCR instrument (CFX96TM Real-Time System, Bio-Health Materials Core-Facility, Jeju National University) using the SYBR premixed Extaq solution (Takara Bio, Mountain View, CA, USA). Relative gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase(GAPDH) levels. Forward: 5'-TCTA TACCACTTCACAAGTCGGA-3', reverse: 5'-GAATTGCCATTGCACAACTCTTT-3' and forward: 5'-TCACTGCCACCC AGAAGAC-3', reverse: 5'-TGTAGGCCATGAGGTCCA C-3' were IL-6 and GAPDH primer sequences, respectively.

2.11. Immunohistochemistry analysis

Hydroperoxide (Dako LSAB2 System-HRP Kit; Dako REAL; Agilent, Santa Clara, CA, USA) was used to inhibit the endogenous peroxidase activity of the cartilage and synovium before antigen retrieval with 0.05% trypsin. The slides were blocked in 1% bovine serum albumin before incubation at 4 ℃ for 12–16 hours with a rabbit monoclonal antibody against IL-6 (1:200 dilution, #12912; Cell signaling, Danvers, MA, USA). The slides were stained with Dako Real EnvisonTM after incubation with a secondary anti-rabbit antibody for 1 hour at 20 ℃ (K5007, Santa Clara, CA, USA).

2.12. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 24 (IBM Corp., Armonk, NY, USA). The data were analyzed by analysis of variance (ANOVA) for determining the relative quantitative gene expressions by real-time qRT-PCR. The results of the histological experiments were analyzed using the Mann-Whitney U test. Significant differences between the means were analyzed at $p < 0.05$ by Student's t-test.

III. RESULTS

3.1. Extraction and identification of GLS components

From the 2 kg of powdered GLS, the GLS oil yield was 600 g. Results of the GC-MS analysis of the GLS oil are shown in Figure 1b. The GLS oil was found to be composed of oleic acid (52.12%), linoleic acid (16.77%), palmitate acid (13.67%), elaidic acid (9.16%), palmitoleate acid (2.95%), stearate acid (1.58%), palmitic acid (1.35%), fatty acid methyl ester (1.01%), stearic acid (0.87%), and pentadecanoic acid (0.52%) (Figure 1c).

3.2. Effects of GLS oil on clinical scores, paw thickness, and pain in a RA model

The GLS oil in combination with PEG-400 (6 mg/Kg) was intraperitoneally administered (200 μL) into the mice twice a week for seven weeks. According to our preliminary animal experiments, 6 mg/kg of GLS oil was an optimal concentration. In the CIA model, administering GLS oil at a dose greater than 6 mg/Kg showed no noticeable significant inhibitory effects. The group treated with GLS oil had a significantly lower severity level than those treated with PEG alone (control). Regarding the clinical RA score, the GLS oil-injected group showed significantly lower severity than the control-injected group ($p = 0.0384$; Figure 2b). Moreover, CIA-induced paw swelling was reduced by GLS oil treatment (Figures 2c and d).

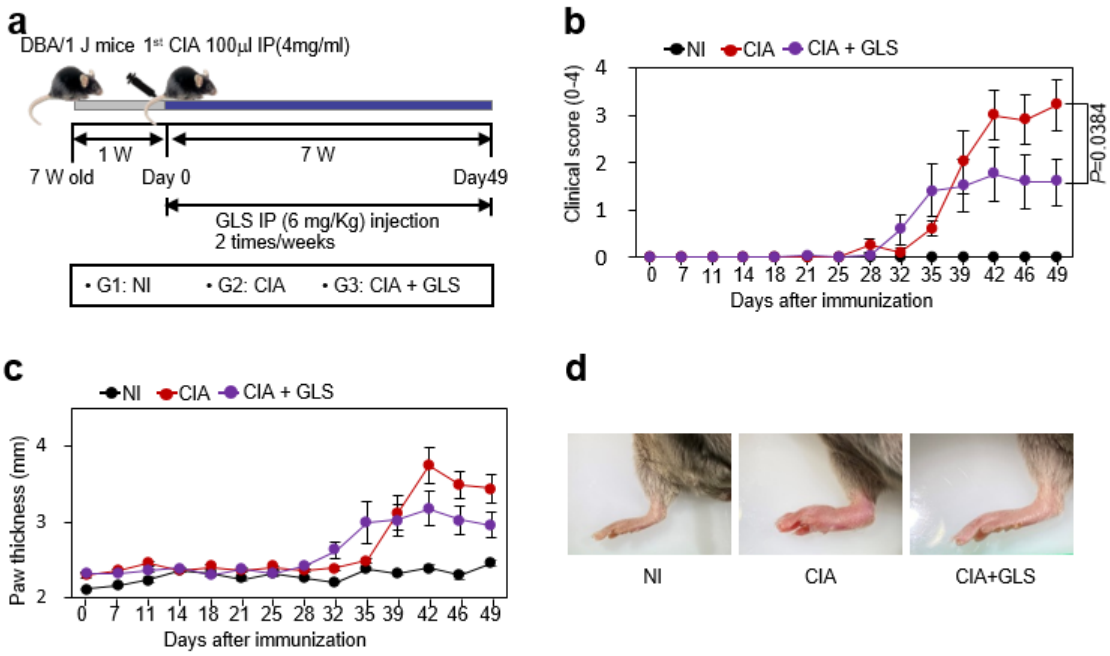

Figure 2. The effects of *Ganoderma lucidum* spore (GLS) oil on the clinical score of collagen-induced arthritis (CIA) model. **(a)** Outline of the experimental plan and animal grouping. Clinical score (0–4) **(b)** and paw thickness **(c)** were measured every three days during the experimental period. **(d)** Differences in paw swelling between the experimental groups.

After 35 days of immunization, the von Frey assay revealed that GLS oil provided pain relief (Figure 3a). Forty-nine days after vaccination, the painkilling effect of GLS oil was found to be significant (p = 0.0248). In addition, a hot plate assay showed that, compared with the control group, the CIA group showed significantly reduced latency (p = 0.03). GLS oil, however, significantly recovered the CIA-induced decrease in latency (p = 0.0309; Figure 3b).

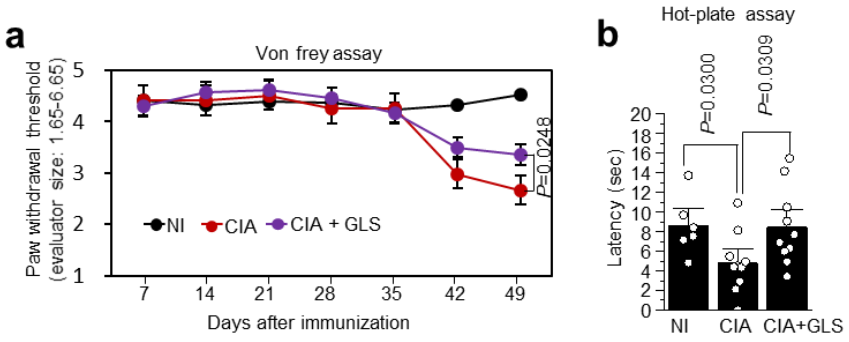

Figure 3. *Ganoderma lucidum* spore (GLS) oil attenuates pain in collagen-induced arthritis (CIA) model. von Frey assays **(a)** and hot plate tests **(b)** for pain responses were performed once a week for seven weeks following immunization.

3.3. Effect of GLS oil on cartilage degeneration in mice knees, ankles, and soles in a CIA-induced RA model

GLS oil treatment reduced CIA-induced synovitis, pannus formation, and cartilage degeneration. Safranin-O staining revealed the degree of cartilage degeneration, synovial membrane inflammation, and inflammation-mediated cell penetration into the bone. GLS oil treatment reduced inflammation of the synovial membrane (Figures 4a and b) and cartilage degeneration in the knee induced by CIA (Figures 4c and d). Moreover, GLS oil treatment lessened the development of panni in the knee induced by CIA (Figures 4e and f).

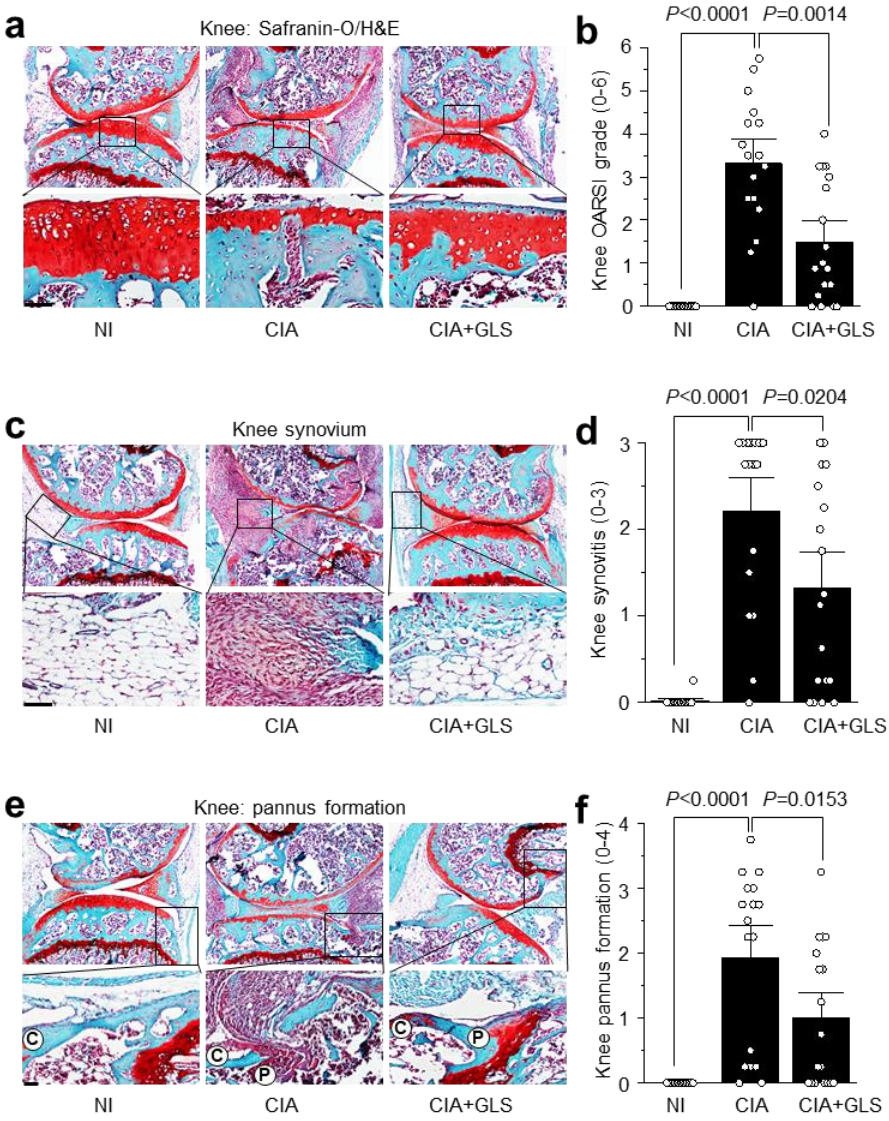

Figure 4. *Ganoderma lucidum* spore (GLS) oil attenuates cartilage destruction, synovitis, and pannus formation of the knee in collagen-induced arthritis (CIA) model. Cartilage degeneration and erosion were analyzed by OARSI staining **(a)**, and the quantification results are shown **(b)**. Knee synovitis for inflammation was analyzed by hematoxylin and eosin staining **(c)**, and the quantification results shown **(d)**. Pannus formation for immune cell penetration to the knee bone is presented in **(e)**, and the quantification results are shown **(f)**. Abbreviations: OARSI, Osteoarthritis Research Society International.

Safranin-O staining of the ankles showed that erosion of ankle cartilage was reversed by GLS oil treatment (Figure 5a). According to the ankle OARSI grade, GLS oil significantly decreased cartilage damage compared to the CIA group (Figure 5b, $p = 0.0187$). Additionally, GLS oil significantly reduced ankle synovitis ($p = 0.0028$) and synovial membrane inflammation (Figures 5c and d). Finally, GLS oil prevented immune cells from penetrating the bone (Figures 5e and f).

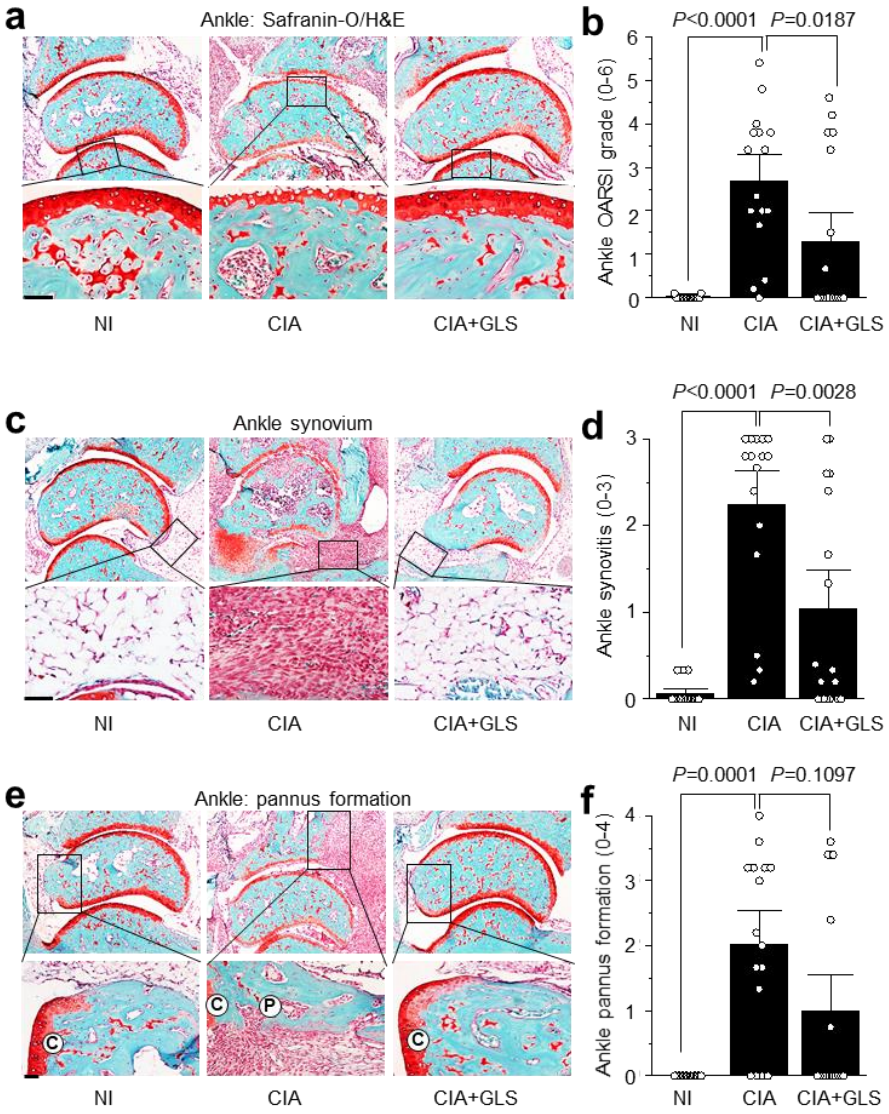

Figure 5. *Ganoderma lucidum* spore (GLS) oil attenuates cartilage destruction, synovitis, and pannus formation of the ankle in collagen-induced arthritis (CIA) model. Cartilage damage to the ankle was visualized by OARSI staining **(a)**, and the quantification results shown **(b)**. The degree of inflammation in the ankle synovial membrane is presented in **(c)**, and the quantification of results are shown **(d)**. The degree of inflammatory penetration into the bone was visualized in **(e)**, and the quantification results are shown **(f)**.

Abbreviations: OARSI, Osteoarthritis Research Society International.

Regarding the sole, GLS oil-treated mice had less cartilage degeneration compared to the PEG-treated mice (Figures 6a and b). The CIA group experienced increased synovial membrane inflammation, whereas the GLS oil-treated group experienced decreased inflammation (Figures 6c and d). Pannus formation was the only variable that showed an increase in the CIA group and was significantly reduced in the GLS oil-treated group (Figures 6e and f).

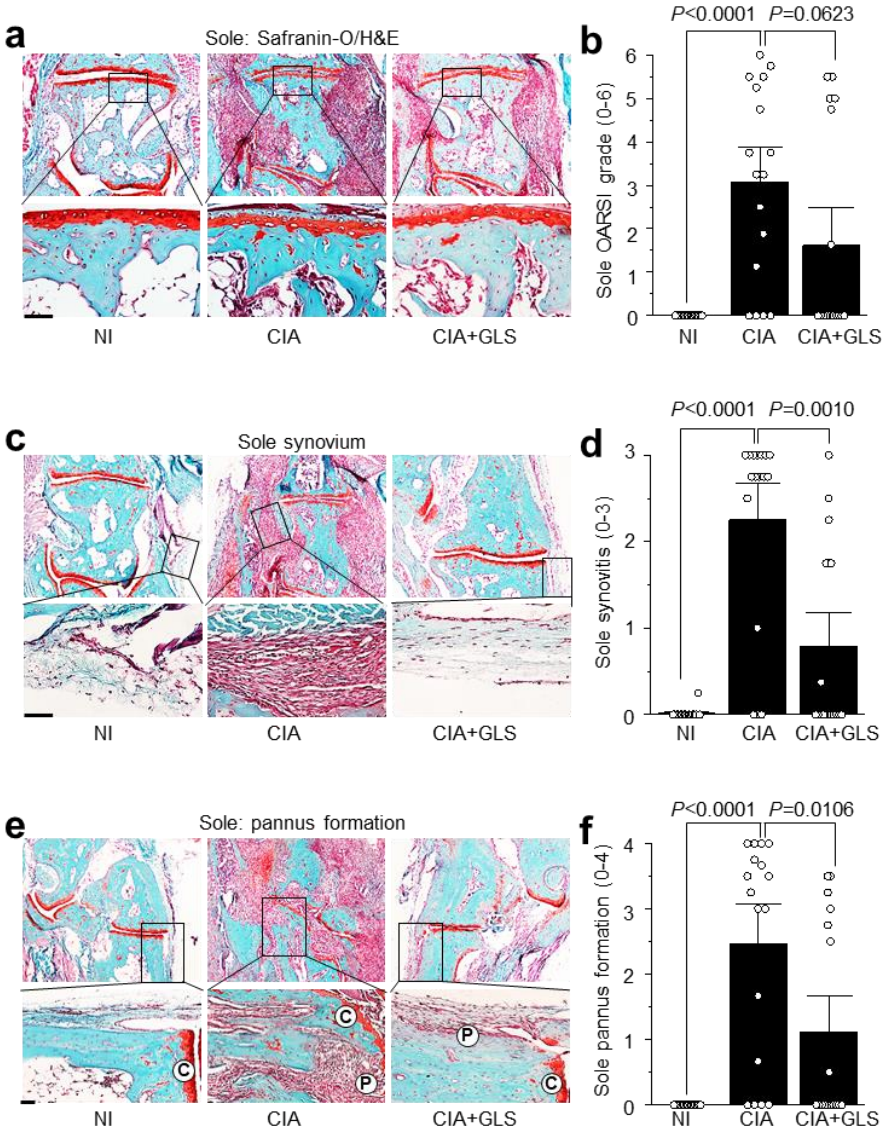

Figure 6. *Ganoderma lucidum* spore (GLS) oil attenuates cartilage destruction, synovitis, and pannus formation of the sole in collagen-induced arthritis (CIA) model. The degree of cartilage degeneration and damage was visualized by OARSI staining **(a)**, and the OARSI grade was quantified **(b)**. The degree of inflammation in the synovial membrane of the sole **(c)** and the synovitis scoring is shown **(d)**. The degree of immune cells penetration into the bone **(e)** and the result of the pannus formation score **(f)**.

3.4. GLS oil attenuates immune cell infiltration in a CIA-induced RA model

Congo red staining revealed eosinophil infiltration in the synovial membranes of the knee and ankle in the CIA group (Figure 7a). The eosinophil count of the knee was significantly reduced in the GLS oil-treated group ($p = 0.0056$). CDr15 immunostaining demonstrated that neutrophils infiltrated the CIA group; however, the infiltrated neutrophils were significantly attenuated in the GLS oil-treated group in both the knee ($p = 0.0006$) and ankle ($p = 0.0023$; Figures 7d-g). The total number of mast cells and degranulated mast cells increased in the CIA group (Figures 7h-j). The number of mast cells decreased in the GLS oil group, but the difference was not statistically significant.

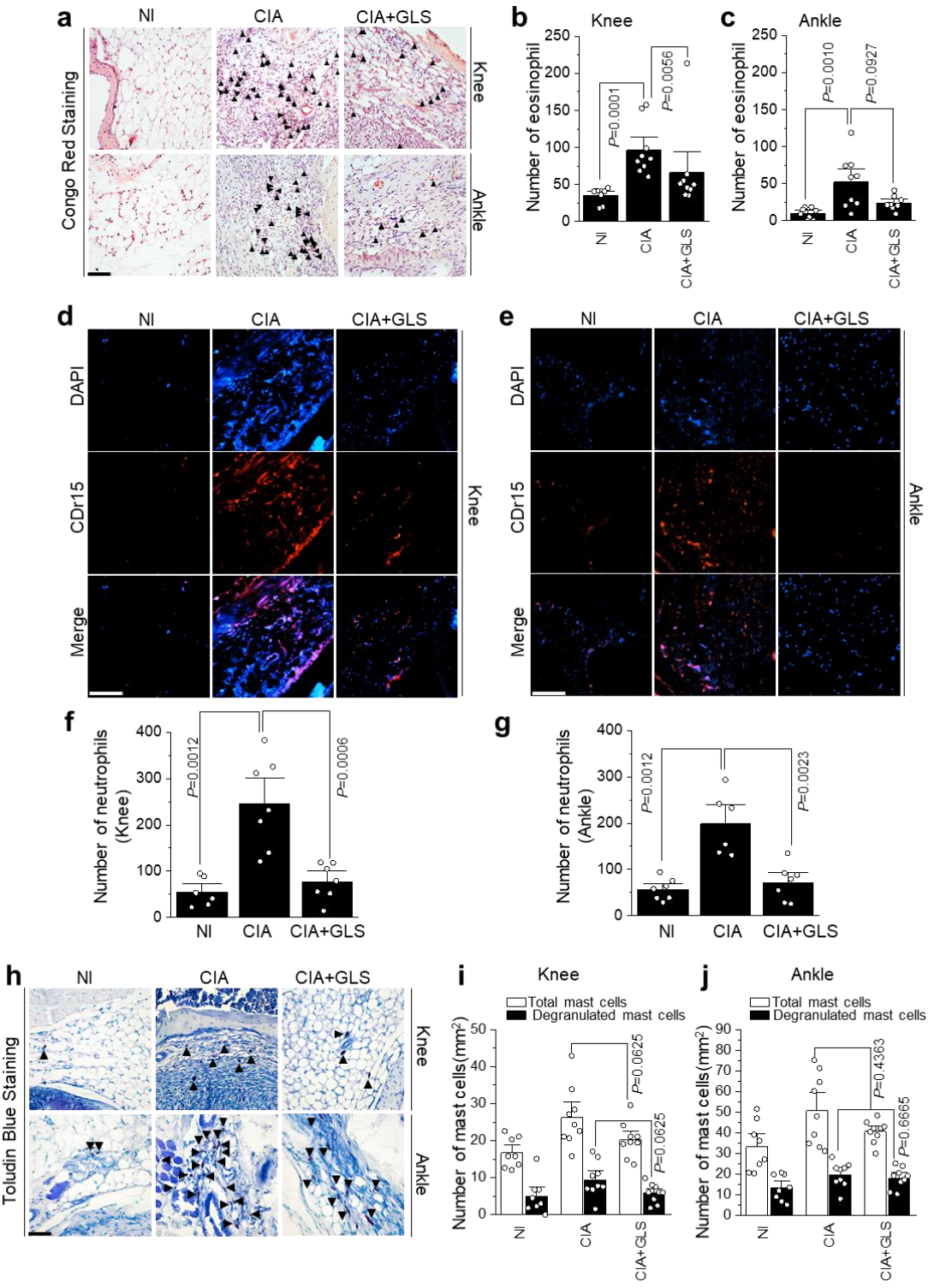

Figure 7. *Ganoderma lucidum* spore (GLS) oil attenuates immune cell activation in collagen-induced arthritis (CIA) model. The eosinophil infiltration in the knee and ankle were analyzed

by Congo red staining **(a)**. The quantification of eosinophil infiltration of the knee and ankle are presented in **(b)** and **(c)**, respectively. In addition, neutrophil penetration of the knee and ankle by CDr15 staining is presented in **(d)** and **(e)**. The quantification of eosinophil infiltration of the knee **(f)** and the ankle **(g)** are presented. Finally, mast cell number and activity in the knee and ankle were analyzed by toluidine blue staining **(h)**. The total mast cell number and degranulated mast cells of the knee and ankle are presented in **(i)** and **(j)**, respectively.

3.5. Effect of GLS oil administration on IL-6 expression in vitro and in vivo

To understand the mechanisms of GLS oil action against RA, we analyzed biomarkers for RA. GLS oil treatment impacts IL-6 expression in both in vitro primary cultured chondrocytes and in vivo CIA-induced RA models. GLS oil reduced IL-6 mRNA expression in LPS- or TNF-treated primary cultured chondrocytes. (Figures 8a and b). In particular, GLS oil administration significantly reduced LPS-induced IL-6 mRNA expression, stimulated at concentrations of 100 ($p = 0.0003$) to 300 ($p = 0.0019$) μg/ml. Moreover, GLS oil significantly inhibited TNF-α–induced IL-6 mRNA expression at 100 ($p = 0.003$) to 300 ($p = 0.0041$) μg/ml concentration. Additionally, immunohistochemistry for IL-6 showed that GLS oil significantly reduced its expression in the cartilage or synovium of the knee and ankle (Figures 8c and d). These findings indicated that in this experiment paradigm, GLS oil specifically downregulated IL-6 expression.

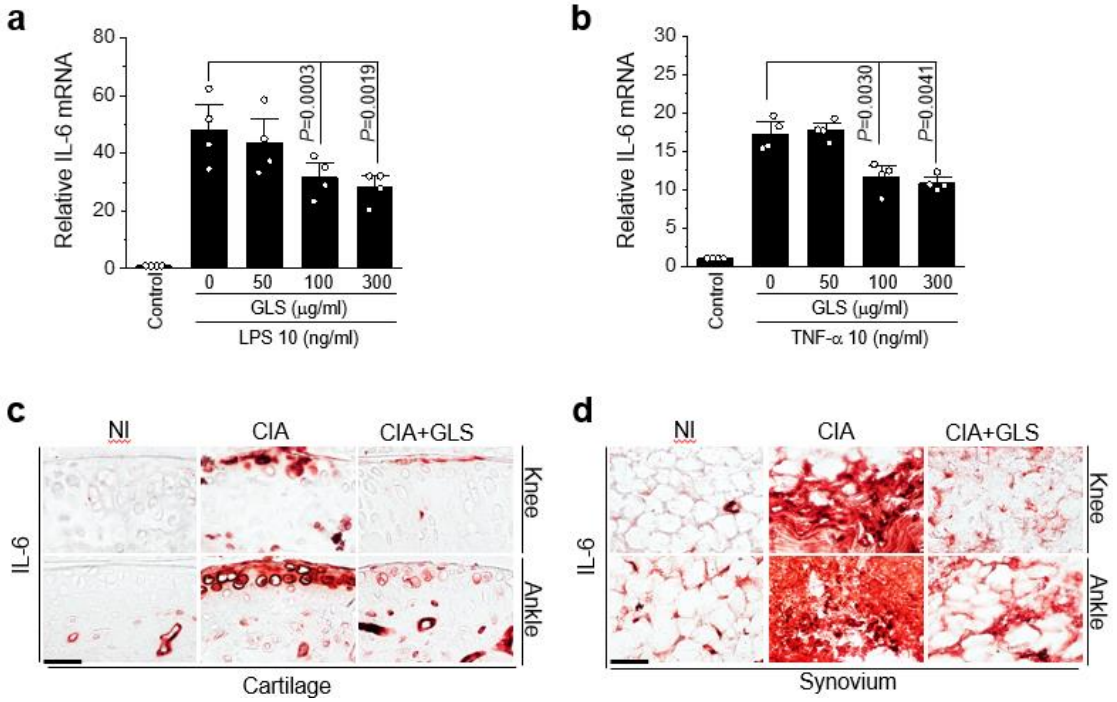

Figure 8. *Ganoderma lucidum* spore (GLS) oil attenuates IL-6 expression in RA. (a)(b) The mRNA expressions of IL-6 in LPS- or TNF-α-exposed chondrocytes after treatment with GLS oil (0 – 300 μg/ml) for 24 h. In this CIA experimental paradigm, the protein levels of IL-6 were analyzed in the cartilage of the knee and ankle (c) and synovium of the knee and ankle (d).

IV. DISCUSSION

RA is a representative autoimmune disease characterized by chronic inflammation and severe damage to the cartilage and bone; however, the underlying mechanisms remain unknown. Indeed, RA is associated with a higher risk of various health conditions, including inflammation, which incurs extra societal and economic consequences [35-37].

GL has been used as a medicinal mushroom to relieve pain and inflammation for hundreds of years [38]. Triterpenoids, polysaccharides, steroids, and fatty acids comprise the core bioactive components of the GLS [23]. Notably, prior studies revealed that 20–30% of lipids are contained in GLS; accordingly, substantial amounts of long-chain fatty acids and unsaturated fatty acids were found to be essential nutrients for normal growth development with various beneficial effects on human health [39].

Previous studies have shown that oleic acid (60.1–68.4%) and palmitate acid (16.3–21.3%) account for most fatty acids in GLS oil obtained from supercritical extraction. Stearic acid, palmitoleic acid, and linoleic acid, however, comprise the minor compounds of GLS oil [39]. Oleic acid has been reported to be effective in immunity, inflammation, cancer, and wound healing [40], while linoleic acid is known to have anti-inflammatory effects [41, 42]. Our findings are in keeping with those from previous studies of GLS [23]. When GLS is extracted using supercritical CO_2, the amount of oleic acid is generally higher [43]. Our GC-MS results showed that oleic acid (52.12%) and palmitate acid (13.67%) were the major compounds. The beneficial outcomes of GLS may be attributed to the synergic effects of fatty acids and the richness of oleic acid.

In this study, we investigated the effect of supercritical CO_2 extracted GLS oil on arthritis pathogenesis *in vitro* and *in vivo*. The clinical indicators, such as clinical score and paw thickness, were attenuated after GLS oil infusion into the CIA-induced RA model. Moreover, GLS oil attenuated the clinical symptoms of pain in the CIA-induced RA model. The GLS oil-

injected group exhibited much lesser damage to the mice's knee cartilage, less pannus development, and less synovial membrane inflammation. Moreover, compared to the CIA group, the GLS oil group had lower eosinophil, neutrophil, and mast cell activities in the knee and ankle. GLS oil causes apoptosis of breast cancer cells in vitro and in vivo by activating caspase-3 and caspase-9 [11]. It also accelerates the process of skin wound healing [6]. In this study, for the first time, we documented the effect of GLS oil on RA modulates. In addition to significantly reducing clinical arthritic scores, the frequency of clinically evident indications for the use of GLS oil suggests an eventual termination of synovial inflammation and erosive joint deterioration. These findings suggested that GLS therapy may prevent CIA from progressing and/or lessen its severity.

Compared to the CIA-only group, the attenuation rate of neutrophils and eosinophils in the GLS oil group was appreciably higher in the knee and ankle. Neutrophils play a crucial role in congenital immune responses such as RA. IL-6 binds to multiple cell types in the preclinical RA state and induces neutrophil migration into the joints, thus contributing to the progression from acute to chronic inflammation and changes in T/B cell differentiation and angiogenesis [44, 45]. Infiltrating neutrophils reduced markedly in the knee and ankle treated with GLS oil. IL-6 activates neutrophils expressing membrane-bound IL-6R. We expected that GLS oil would suppress IL-6 expression, thus regulating the trigger for neutrophils for the expression of membrane-bound IL-6R [46]. The eosinophils tend to increase in other rheumatic conditions, which is thought to be a sign of disease activity and poor prognosis [47]. Comparing the GLS oil group to the CIA group, mast cell infiltration and activation also showed a slight decrease, even though the rate of decrease was insignificant. Mast cells, one of the cells that make up the innate immune system and are found in tissues, are known to play a role in the pathogenesis of RA [48].

RA patients with elevated levels of cytokines, such as IL-6, present with inflammation, which is an established prognostic indicator of treatment response, tiredness, pain, and

depression [49, 50]. In this experiment, GLS oil reduced IL-6 mRNA expression in response to LPS or TNF-α treatment in primary cultured chondrocytes. GLS oil substantially reduced LPS-induced and TNF-α–induced IL-6 mRNA expressions. The IL-6 expression is increased by various transcription factors, including NF-kB, which in turn is triggered by other proinflammatory cytokines and TLR-mediated signals [51, 52]. Nevertheless, as stated previously, anti-IL-6R medications have been used against RA for over a decade. However, the widespread use may raise the health risk of associated illnesses, wherein IL-6 is also directly implicated [53, 54]. The GLS oil may therefore be a promising solution as a natural rehabilitation strategy. According to our findings, GLS oil has anti-rheumatic effects with pain-relieving properties that are mediated by the reduction of immune cells in the tissues of the joints.

V. CONCLUSIONS

Using the CIA-induced RA model, we provide here, convincing evidence that GLS oil reduces pannus formation, inflammation, and inflammation-mediated RA pain and swelling. This result suggested that GLS oil-derived substrates might be used in the treatment of inflammatory illnesses such as arthritis. However, existing research has not assessed the effect of individual substances in GLS oil extracts on RA development as well as its regulation for curing and preventing severity. Consequently, additional research is required to determine the impact of individual active substances on RA.

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감사의 글

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2022년 12월

허 윤 지