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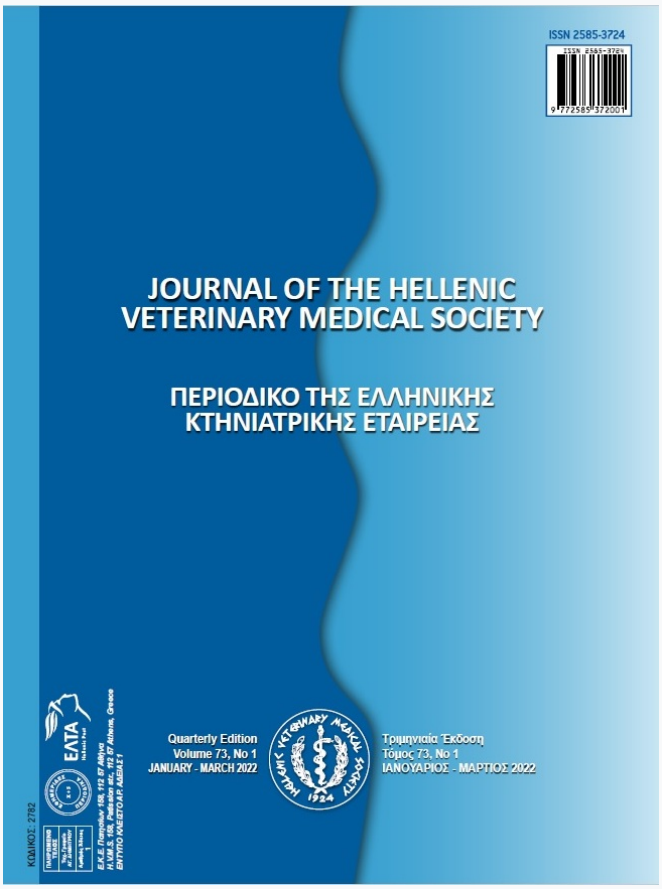

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Research article
Ερευνητικό άρθρο

Pharmacological effect of Lidan Tang against adjuvant-induced rheumatoid arthritis in rats

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ABSTRACT: Rheumatoid arthritis (RA) is a great concern across the globe. It is characterized as an autoimmune disease, where the body's immune system mistakenly attacks bone joints causing them to erode which leads to deformity in structure. Thus, the present study was conducted to elucidate the effect of Lidan Tang (LDT) decoction, a traditional Chinese medicine against rheumatoid arthritis. The rheumatoid arthritis was induced in Sprague-Dawley albino rats by intradermal injection of bovine collagen II type at the tail. Results suggested that LDT reduces paw swelling and arthritic scores in rats. The protective effect of LDT against RA was further substantiated by histopathological analysis of synovial tissue of rats, where LDT improves the architecture of the tissues as compared to CIA rats. It also reduces oxidative stress and inflammation in CIA rats as compared to the disease model group. The serum level of anti-collagen II-specific immunoglobulins (IgG_1 and IgG_{2a}) was reduced significantly in LDT treated group. In a western blot analysis, LDT treated group showed a significant reduction in the expression of NF-κB and COX-2 as compared to CIA rats. Collectively, our study for the first time demonstrated the protective effect of Lidan Tang decoction against rheumatoid arthritis.

Keywords: TCM, CIA, rheumatoid arthritis, NF-κB, COX-2.

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INTRODUCTION

The dramatic change in lifestyle in past decades has predisposed humans to many chronic diseases that affect their overall survivability (Carrera-Bastos et al. 2011). Among such diseases, Rheumatoid arthritis (RA) is a great concern for many health care workers across the globe. It is characterized as an autoimmune disease, where the body's immune system mistakenly attacks bone joints causing them to erode which leads to deformity in structure. It is also associated with inflammation, redness, swelling at the site due to the destruction of ligaments and tendons (Firestein and McInnes 2017; Smolen et al. 2018). The current clinical options to treat RA have been greatly dependent on the use of disease-modifying anti-rheumatic drugs (DMARDs) in combination with physical exercise or even surgery (Aletaha and Smolen 2018). However, these options are not sufficient to manage the disease; thus, it is an urgent need to discover newer agents that can provide relief against RA.

Various studies have shown a close connection between inflammation and oxidative stress (Sies 1986; Reuter et al. 2010; Fernández-Sánchez et al. 2011; Mittal et al. 2014; Hussain et al. 2016). It has been found that the inflammatory condition of RA has been greatly provoked by the generation of reactive oxygen species (ROS). It undermines the oxidative defense mechanism of the body by inducing lipid peroxidation, mitochondrial and DNA damage (Birben et al. 2012; Schieber and Chandel 2014). Moreover, accumulating shreds of evidence suggested that inflammation in RA provoked the release of various cytokines (IL-1β, IL-6, IL-10, IL-17, and TNF-α) from the immune cells (Feldmann 2002; McInnes and Schett 2007; Feldmann and Maini 2008). Studies have also shown that RA patients have a serum level of IgG in comparison to the normal population (Nandakumar et al. 2007; Khosroshahi et al. 2010). Thus, agents that inhibit inflammation and oxidative stress have shown a beneficial effect against RA.

Traditional Chinese medicine (TCM) has been used for centuries in China for treating various diseases and ailments. It's growing interest across the world as an alternative therapy to the modern-day conventional therapies (Yuan et al. 2016). Lidan Tang (LDT) decoction is a highly prescribed medication by the Beijing Integrated traditional Chinese and Western Medicine Hospital, China for the treatment of cholelithiasis. Recently, it was reported to exhibit a protective effect against high-fat-diet-induced gallstone in mice via reducing hepatic oxidative stress by promoting Keap1/Nrf2 pathway (Gao et al. 2020). However, till now, no study has reported the effect of LDT against RA. Thus, in the present manuscript, we intend to study the effect of LDT in adjuvant-induced arthritis in rats.

MATERIALS AND METHODS

Animals

Healthy male Sprague-Dawley albino rats were obtained from the institutional animal house and kept in a strict hygienic environment. They had free access to food and water in controlled humidity. The animal study was approved by the Xi'an Jiaotong University, China.

Establishment of the disease model

The chicken type II collagen (CII) after mixing with acetic acid (0.05 M) was suspended in complete Freund's adjuvant (CFA) (heat killed *Mycobacterium tuberculosis* H37Ra). The CFA mixture was admisntered into the dermis oftail of rats on the day 0. It was further admistered with CII and IFA on day 16. The control rats received no immunization.

Animal groups

Total five groups of rats were formed containg 12 rats in each group. The LDT decoction for administration to rats was prepared as per the given procedure elsewhere. (Gao et al. 2020) Th group treatment are as follows. The whole experiment period is 28 days and LDT decoction was admistered intra-peritonellly to the rats.

Group 1: Control (Saline for 28 days)
Group 2: CIA rats (Arthritis disease group)
Group 3: CIA + LDT (25 mg/kg)
Group 4: CIA + LDT (50 mg/kg)
Group 5: CIA + LDT (75 mg/kg)

The paw swelling of rats was examined on days 0, 14, and 28 of the experimental period using plethysmometer, . Moreover, the arthritic score of RA rats was considered as per the earlier reported method (Zhang et al. 2009)

Sample preparation

At the end of the experiment, i.e. 29th day, the rats were sacrificed, and serum was isolated from the blood of the rats for further biochemical estimation. The synovial joint tissue was cut out from the rats and

set in 10% formalin for histological assessment. On the other hand, an additional set of joint tissue was homogenized in phosphate buffer saline (10%) for further biochemical and molecular estimation.

Histopathological evaluation

The rat's synovial joint tissue was fixed for 24 hr after soaking in 10% buffered formalin. The tissues were processed and implanted in paraffin. A 5 μm thickness sections were cut, deparaffinized, dehydrated, and stained with hematoxylin and eosin (H&E) for the histological assessment estimation under a light microscope. Swelling and erythema of the paws (hind and fore) were graded using a 5-point scale: 0, no sign of swelling or erythema; 1, signs of swelling or erythema in the ankle or wrist; 2, signs of swelling or erythema in the ankle and tarsal or wrist and carpal; 3, signs of swelling or erythema extending to the metatarsals or metacarpals; and 4, signs of swelling or erythema involving the entire hind or fore paw. Hence, the maximum score was 8 (4 × 2 hind/fore paws).

Antioxidant enzymes and lipid peroxidation products

The joint-tissue homogenates were used to estimate the level of Glutathione (GSH) content, catalase (CAT) malondialdehyde (MDA) and superoxide dismutase (SOD) activities using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Estimation of Anti-collagen (C) II Ig and pro-inflammatory cytokines

Anti-collagen (C) II Ig and inflammatory marker levels Serum anti-CII IgG1 and G2a, IL-1β, IL-6, IL-10, IL-17, and TNF-α levels were determined using ELISA kits (Thermo Fisher Scientific).

Western blot analysis of NF-κB and COX-2

The isolated protein from the synovial joint tissues was probed to 10% SDS-PAGE for 50 min and transferred to PVDF membrane. The membrane was blocked with 5% defatted milk in tris-buffered saline with 0.1% Tween 20 for 1 h at 4°C and then incubated with rabbit anti-mouse NF-κB, COX-2 and β-actin primary antibodies at 4°C overnight. Afterward, the membrane was incubated with anti-mouse IgG HRP-conjugated secondary antibodies. The protein expression was perceived with an ECL detection kit.

Statistical analysis

Results are shown as means±standard deviation (SD). Statistical analysis was performed using ANOVA using Graphpad Prism (version 5). $P<0.05$ was taken as statistically significant.

RESULTS

LDT reduces paw swelling and arthritic score

Initially, the effect of LDT was investigated on swelling of the paw and arthritic score after induction

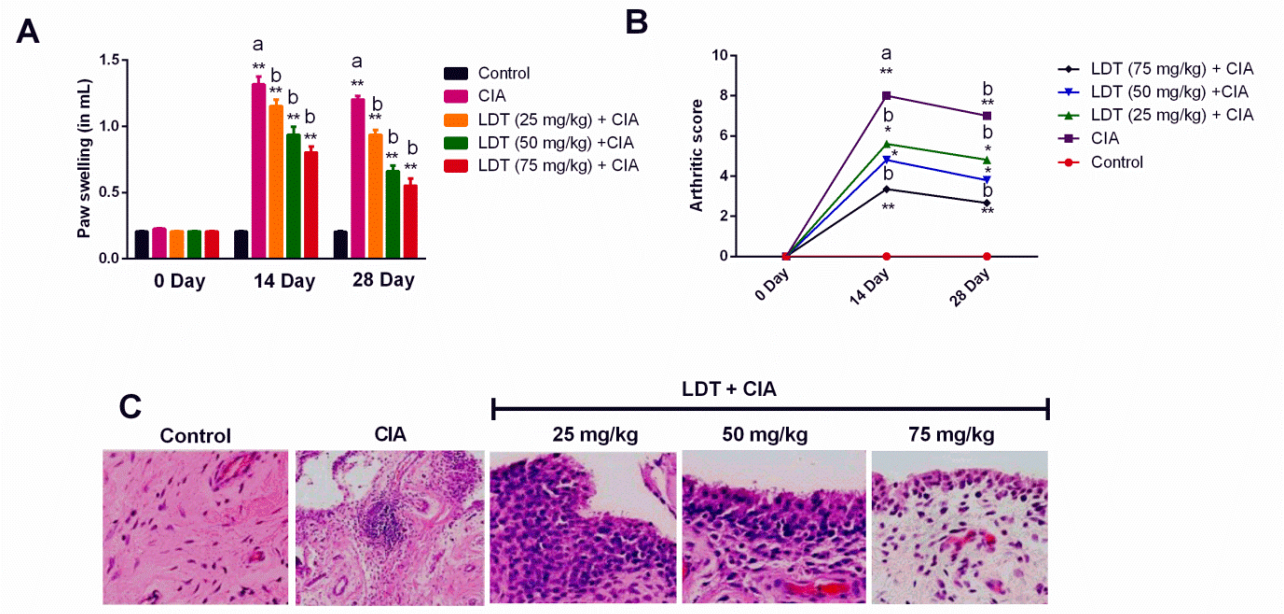

Figure 1. Effect of LDT on A) paw swelling, B) arthritic score and C) histopathological analysis of synovial joints of CIA and control rats. Data are represented as means ± SD. ** $p<0.01$, * $p<0.05$. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

of arthritis by CFA in rats. The rats from the CIA disease model group showed significantly increased swelling along and arthritic score as compared to control. However, upon administration of LDT, the studied indices were found significantly reduced as compared to the CIA model group in the treatment period, Fig. 1. The benefits of LDT were confirmed by the histological analysis of joint tissues, where it prevents tissue erosion and decreased permeability inflammatory infiltrates.

LDT reduces oxidative stress

The effect of LDT was further investigated on various indices of oxidative stress, such as MDA, GSH, CAT, and SOD in the joint tissues of rats. The level of GSH, SOD, and CAT was found significantly reduced in the CIA disease model together with an increase in MDA as compared to control. On the contrary, the levels of these biomarkers were found significantly improved in LDT treated group as compared to control ($p<0.05$), Fig. 2.

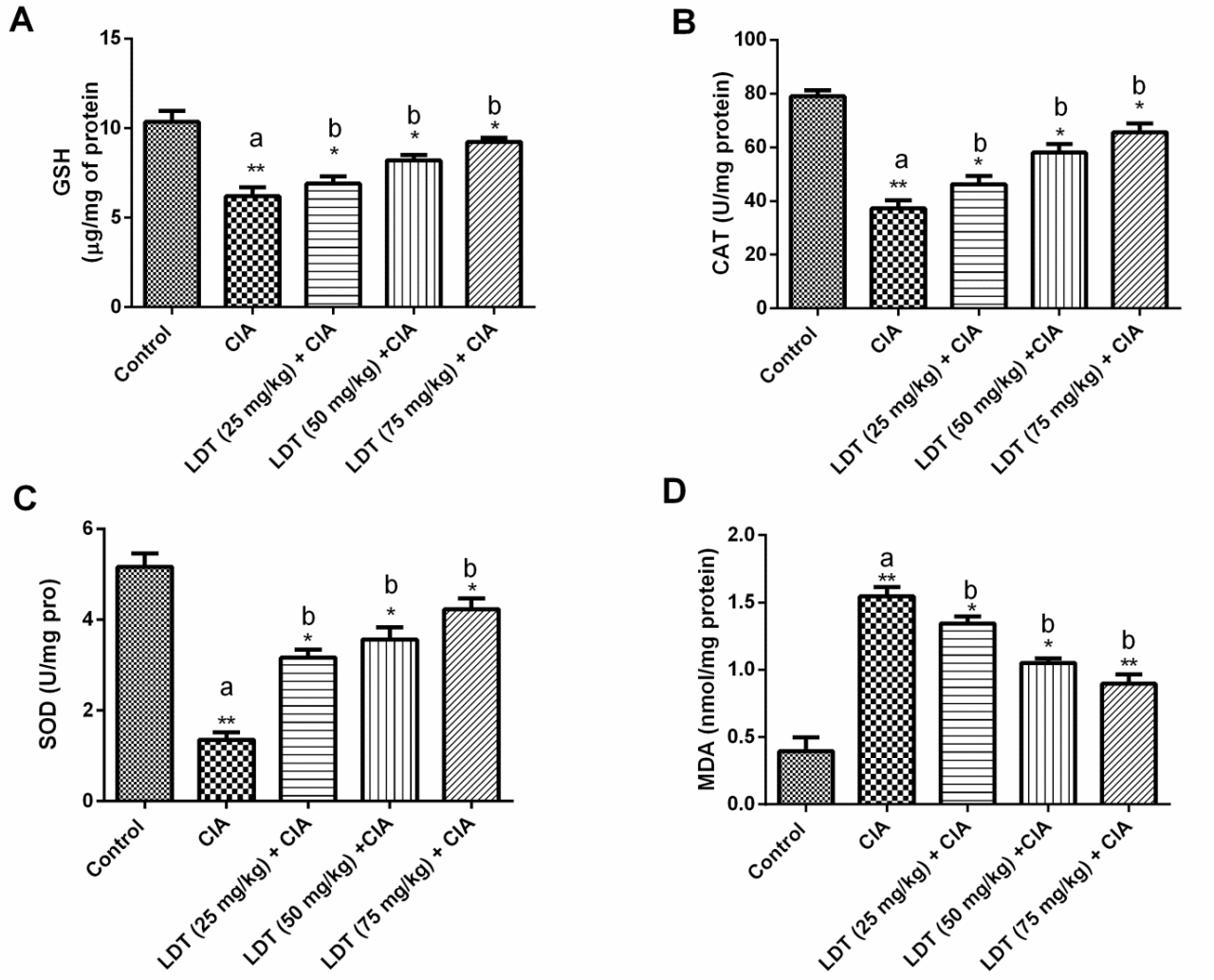

Figure 2. Effect of LDT on various indices of oxidative stress in CIA rats and control. A) GSH, (B) CAT, (C) SOD and (D) MDA. Data are represented as means ± SD. ** $p<0.01$, * $p<0.05$. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

LDT reduces serum Immunoglobins

The effect of LDT was investigated on the serum anti-CII IgG1 and G2a levels using ELISA analysis. The serum level of these tested immunoglobulins was found significantly elevated in the CIA disease model as compared to the control. However, in LDT treated rats, the level of serum anti-CII IgG1 and G2a was reduced significantly in a dose-dependent manner, Fig. 3 ($p<0.01$).

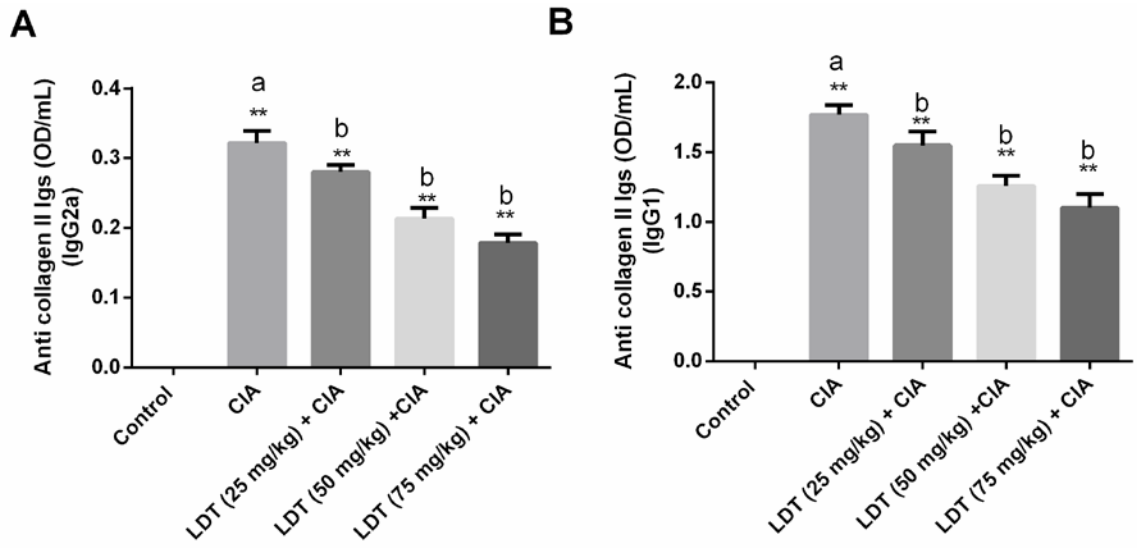

Figure 3. Effect of LDT on various serum anti-collagen II Ig levels in CIA rats and control. Data are represented as means ± SD. ** p<0.01, * p<0.05. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

LDT reduces serum pro-inflammatory cytokines

Standard ELISA was used to determine the effect of LDT on the serum level of various pro-inflammatory cytokines. As shown in Fig. 4, in comparison to control, the serum level of IL-1β, IL-6, IL-17, MCP-1, TNF-α was found significantly increased with low-level IL-10 in the CIA disease model group. On the contrary, LDT restored the level of these cytokines in comparison with the CIA disease group.

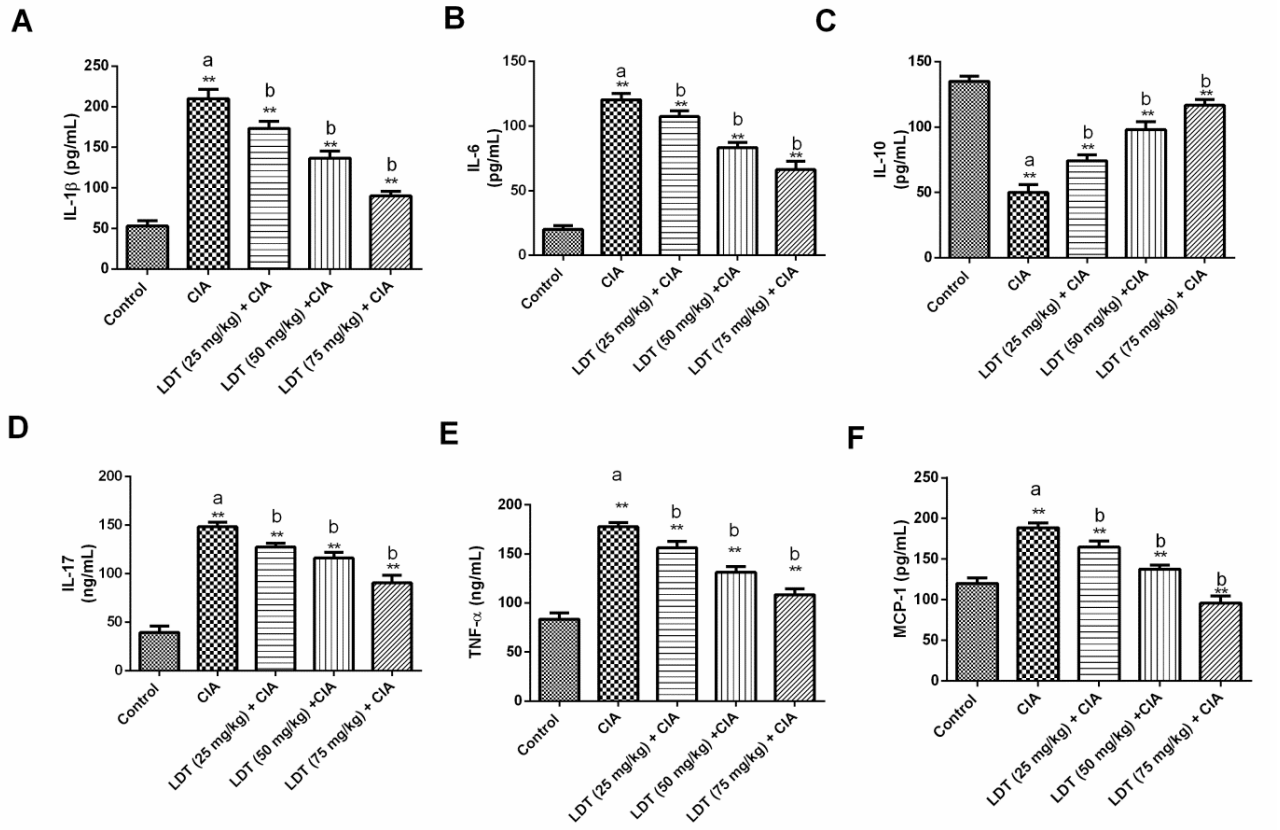

Figure 4. Effect of LDT on serum level of various pro-inflammatory cytokines in CIA and control rats. (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-17, (E) TNF-α and (F) MCP-1. Data are represented as means ± SD. ** p<0.01, * p<0.05. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

LDT reduces COX-2 and NF-κB in western blot analysis

Western blot analysis was conducted to determine the effect of LDT on the expression of COX-2 and NF-κB, an important inflammatory bio-marker. As shown in Fig. 5, LDT causes a significant reduction in the expression of COX-2 and NF-κB in rats as compared with the CIA disease model group.

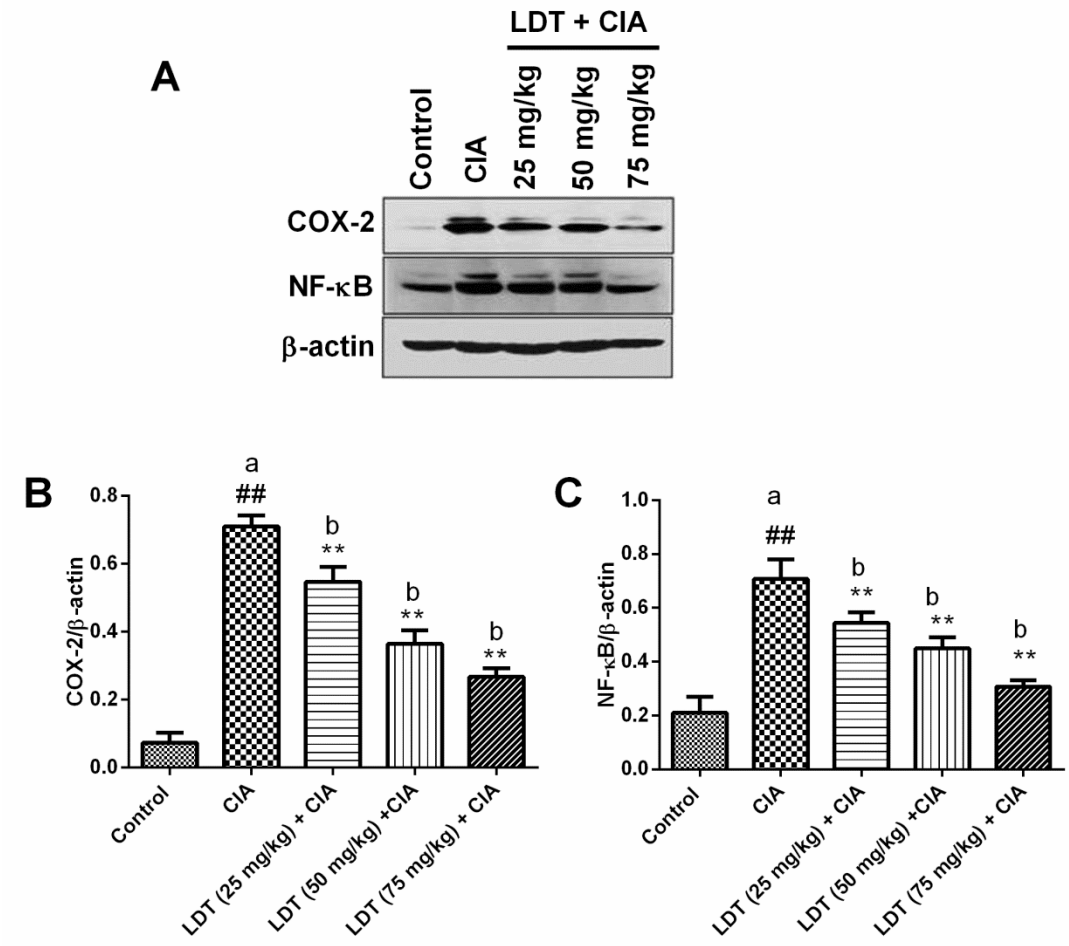

Figure 5. Effect of LDT on the (A) expression of COX-2 and NF-κB in western blot analysis, and representative bar-graph of (B) COX-2 and (D) NF-κB. Data are represented as means ± SD. ** p<0.01, * p<0.05. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

DISCUSSION

Since immemorial time, plants and their products have been used by mankind for treating their ailments and disease. These products have found significance in various traditional forms of medicines across the world, such as traditional Chinese medicine (TCM), Ayurveda, traditional Korean medicine (TKM), and other forms (Yuan et al. 2016). The holistic approach by these systems has garnered attention and as a result they have now blossomed into regulated systems of medicine (Efferth et al. 2007; Xu et al. 2013). In the past few decades, we have witnessed a significant rise in the research activities on the component of these medicines, which provides valid scientific evidence (Xue et al. 2013). Particularly, the medications used in TCM are often derived from hydro-alcoholic extracts of plants or combined extracts of the plant. Lidan Tang (LDT) decoction is a frequently used medical preparation used in TCM for the treatment of cholelithiasis (Gao et al. 2020). In the present manuscript, we have successfully shown the beneficial effect of LDT against adjuvant-induced (CIA) RA in rats. It has been found that LDT improved the pathological state of RA by reducing inflammation and oxidative stress in rats. Initially, the effect of LDT was investigated on the macroscopic features of RA in rats, such as paw swelling. Swelling is the common symptom of RA due to inflammation of the synovi-

al membrane and subsequent fluid build-up causing erosion and degradation of joint (Khurana and Berney 2005; Khanna et al. 2017). It has been found that LDT causes significant dose-dependent inhibition of paw swelling and associated arthritic score. This observation was further supported by histopathological examination of synovial joint tissues. Various studies have shown a close connection between inflammation and oxidative stress (Sies 1986; Reuter et al. 2010; Fernández-Sánchez et al. 2011; Mittal et al. 2014; Hussain et al. 2016). It has been found that the inflammatory condition of RA has been greatly provoked by the generation of reactive oxygen species (ROS). It undermines the oxidative defense mechanism of the body by inducing lipid peroxidation, mitochondrial and DNA damage (Birben et al. 2012; Schieber and Chandel 2014). Thus, agents reducing oxidative stress have shown a beneficial effect against RA. The effect of LDT was further investigated on various indices of enzymatic and non-enzymatic biomarkers of oxidative stress. It has been found that LDT reduces oxidative stress by the significant restoration of examined biomarkers near to normal. It has been suggested that reduction in oxidative stress by LDT prevents ligament damage and tissue erosion. The effect of LDT was further investigated on various pro-inflammatory cytokines. Accumulating shreds of evidence suggested that inflammation in RA provoked the release of various cytokines (IL-1β, IL-6, IL-10, IL-17, and TNF-α) from the immune cells (Feldmann 2002; McInnes and Schett 2007; Feldmann and Maini 2008). Therefore, inhibition of these overexpressed cytokines provides symptomatic relief against RA. In the present study, LDT significantly inhibited the release of tested cytokines in a dose-dependent manner. Moreover, LDT also causes a significant reduction of the serum level of anti-CII IgG, e.g. IgG1 and IgG2A, which is deemed as a characteristic hallmark of RA. Studies have shown that RA patients have a serum level of IgG in comparison to the normal population (Nandakumar et al. 2007; Khosroshahi et al. 2010). To understand the detailed mechanism of LDT, its effect was further quantified against pivotal mediators of inflammation cascade, NF-κB, and COX-2. Accumulating pieces of evidence suggested that NF-κB and COX-2 both mediates inflammation, hyperplasia, and tissue destruction in RA. It has been found that NF-κB activation promotes development of T helper 1 responses, activation, abnormal apoptosis and proliferation of RA fibroblast-like synovial cells, and differentiation and activation of bone resorbing activity of osteoclasts (van Loo and Beyaert 2011; Giridharan and Srinivasan 2018). Activated NF-κB has been detected in human synovial tissue on the early stage of joint inflammation, as well as in specimens obtained at the late stages of the disease. Analyses of nuclear extracts from synovial explants revealed the presence of increased NF-κB DNA binding activity in RA patients, but not in osteoarthritis patients. Immunohistochemical studies detected nuclear RelA (p65) and NF-κB1 (p50) mostly in RA endothelium and synovial lining, particularly in CD14-positive cells, and no staining in the normal synovium (Blom et al. 2014a, a, b; Ahmed et al. 2018). It is well established that NF-κB is involved in the regulation of multiple pro-inflammatory mechanisms. Activation of NF-κB is necessary and sufficient for the transcriptional activation of IL-1β, IL-6, IL-17, MCP-1, and TNF-α receptor activator of NF-κB ligand (RANKL), and cyclooxygenase 2 (COX-2), all of which are required for the initiation, amplification, and maintenance of chronic inflammations, including that seen in RA (Ghosh and Hayden 2008; Skaug et al. 2009; Sun 2012; Napetschnig and Wu 2013). Especially, TNF-α is critical in the pathogenesis of RA. In an inflammatory disorder, TNF-α is believed to play the role of early and crucial cytokine, which triggers various positive and negative feedback loops leading to exacerbated inflammation (Feldmann 2002; Palladino et al. 2003; Popa et al. 2007). Therefore, inhibition of NF-κB, cytokines and TNF-α expression is believed to inhibit RA. The activation of NF-κB also promotes upstream activation of COX-2 and responsible for controlling the transcription of this gene (Liang et al. 1999; Lee et al. 2004; Nishikori 2005; Ke et al. 2007; Ulivi et al. 2008). The results of the present study suggest that LDT causes significant inhibition of NF-κB and COX-2 and exhibit an ameliorative effect on inflammation in RA affected rats.

CONCLUSION

Collectively, our present study successfully demonstrated the anti-RA effect of LDT against CIA induced RA in rats. LDT causes a significant reduction in oxidative stress, inflammation which results in a reduction of paw volume and arthritic score and improves the pathological state of RA rats.

CONFLICT OF INTEREST

None

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