CHRONOTHERAPY OF JANUS KINASE INHIBITOR AGAINST COMPLETE FREUND'S ADJUVANT-INDUCED RHEUMATOID ARTHRITIS IN *WISTAR* RATS

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MASTER OF PHARMACY
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Submitted by
Mr. P. Subash Raj
Registration No: 261925106

Under the guidance of
Dr. J. Sam Johnson Udaya Chander, M.Sc., M.Pharm., Ph. D
Assistant Professor
Department of Pharmacology

COLLEGE OF PHARMACY
SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES
COIMBATORE- 641044

OCTOBER 2021

Certificate

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Dr. T. K. Ravi, M. Pharm., Ph.D., FAGE.,
Principal,
College of Pharmacy, SRIPMS,
Coimbatore-641 044

Place: Coimbatore

Date:

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Dr. K. Asok Kumar, M. Pharm., Ph. D.
Professor & Head,
Department of Pharmacology,
College of Pharmacy, SRIPMS,
Coimbatore-641 044

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Dr. J. Sam Johnson Udaya Chander, M.Sc., M.Pharm., Ph.D.,
Assistant Professor
Department of Pharmacology
College of Pharmacy, SRIPMS,
Coimbatore-641 044

Place: Coimbatore

Date:

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Acknowledgment

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Abbreviations

5-ASA	- 5-aminosalicylic acid
Aa	- Aggregatibacter actinomycetemcomitans
AAA	- Anti-Ada antibodies
ACPAs	- Anti-citrullinated protein antibodies
Ada	- Adalimumab
ANOVA	- one-way analysis of variance
ATIC	- 5-aminoimidazole-4-carboxamide
Blys	- Anti-B lymphocyte stimulator
BSA	- Bovine serum albumin
CAT	- Catalase
CDAI	- Clinical Disease Assessment Index
CFA	- Complete Freund's adjuvant
CMC	- carboxy methyl cellulose
COX-2	- Cycloxygenase-2
CRP	- C- reactive protein
Cs Dmards	- Conventional Synthetic Disease modified anti-Rheumatic drug
CYP3A4	- Cytochrome P450 3A4
DC's	- Dendritic cells
DMab	- Denosumab
DAS-28	- Disease Activity Score using 28joints
DMARD	- Disease modified anti- rheumatic drug
DNA	- Deoxy ribonucleic acid
EBNA-1	- Epstein-Barr Nuclear Antigen 1

EBV	- Epstein-Barr virus
ECM	- extracellular matrix
FDA	- Food and drug administration
GNS	- N-acetylglucosamine-6-sulfatase
GWAS	- Genome-wide association studies
IC_{50}	- Inhibit concentration 50
IFN-γ	- Interferon γ
IL-17A	- Interleukin -17A
IL-6	- Interleukin -6
JAK	- Janus kinase
JIA	- Juvenile Idiopathic Arthritis
LDAS	- Low disease activity state
M-CSF	- Macrophage colony-stimulating factor
MHC	- Major histocompatibility complex
MSCs	- Mesenchymal stem cells
MTX	- Methotrexate
NET	- Neutrophil extracellullar trap
NFκB	- Nuclear factor κb
NGF	- Nerve growth factor
NSAIDs	- Non-steroidal anti-inflammatory drugs
OA	- Osteoarthritis
PAD	- Peptidylarginine-deiminase
PPARs	- Peroxisome proliferator activated receptors

PUFA	- Polyunsaturated fatty acid
RA	- Rheumatoid arthritis
RANKL	- Receptor activated of nuclear factor kappa-ligand
SCN	- Suprachiasmatic nucleus
SDAI	- Simplified Disease Activity Assessment Index
SEM	- Standard error mean
SLC19A1	- solute carrier family 19 member 1
SNP	- Single nucleotide polymorphisms
SOD	- Superoxide dismutase
SSZ	- Sulfasalazine
TCA-TBA-HCL	- Trichloro acetic acid thiobarbituric acid-hydrochloric acid
TCZ	- Tocilizumab
TIMPs	- Tissue inhibitors of metalloproteinases
TLRs	- Toll-like receptors
TNF	- Tumor necrosis factor
TYK2	- Tyrosine kinase 2
ZT0/ZT12	- Zeitgeber 0/12

Abstract

ABSTRACT

Aim. To explore the circadian rhythm of serum CRP in complete Freund's adjuvant (CFA) rats and effectiveness of tofacitinib administered via chronotherapy.

Methods. CFA rat models were immunized with mycobacterium butyricum. Serum CRP levels in normal and CFA rats were measured at 4,10,16, or 22 h ZT0/ZT12.Tofacitinib was administered to ZT8/ZT20 experimental groups of *Wistar* rats once daily according to the circadian rhythm. The positive control was given with methotrexate, for ZT0 and ZT12 was given with tofacitinib and normal control given (0.5% CMC) once daily simultaneously. Arthritis score, paw volume, body weight was measured on 1^{st}, 5^{th} ,7^{th} ,14^{th} and 21^{st}. Rheumatoid factor and C reactive protein (CRP) levels in the serum were measured by semi-auto-analyser using turbilatex method. Histological changes in the ankle joint were analyzed.

Results. After 3 weeks of treatment, arthritis scores in the experimental group were lower than in the Negative control group. The expression of CRP was lower in the ZT0 treated group than in the negative control or ZT12 treated groups. Histopathology scores in the experimental groups shows less inflammatory cells than in the control group.

Conclusion. The serum CRP levels in CFA rats were higher than in normal rats and showed significant circadian rhythm. Daily dose time dependent administration of tofacitinib is more potent than traditional administration. The therapeutic index of rheumatoid arthritis (RA) may be improved with tofacitinib therapy based on the serum CRP circadian rhythm.

Introduction

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic auto immune disease that arises more frequently in females than males, being predominantly observed in the elderly. The prevalence rate reported in 2002 ranged from 0.5% to 1% of the population and had regional variation [1]. RA primarily affects the lining of the synovial joints and can cause progressive disability, premature death, and socioeconomic burdens. The clinical manifestations of symmetrical joint involvement include arthralgia, swelling, redness, and even limiting the range of motion. Early diagnosis is considered as the key improvement index for the most desirable outcomes (i.e., reduced joint destruction, less radiologic progression, no functional disability, and disease modifying antirheumatic drugs (DMARD)-free remission) as well as cost effectiveness as the *f*irst 12 weeks after early symptoms occur is regarded as the optimal therapeutic window [2][3][4]. However, early diagnosis remains challenging as it relies heavily on the clinical information gathered from the patient's history and physical examination supported by blood tests, and imaging analysis. The reasons for a delayed diagnosis vary markedly between countries with differing healthcare systems [5], while the reasons for a delay in initiating DMARD therapy in RA patients appear to be both patient- and physician-dependent. Noticeably, patient awareness of RA, the willingness of patients to seek medical advice, the time for the patients from symptom onset to receiving appropriate treatment, and the diagnostic capability of the physician all influence the treatment and outcome of RA. With poorly controlled or severe disease, there is risk that extra-articular manifestations such as keratitis, pulmonary granulomas (rheumatoid nodules), pericarditis/pleuritis, small vessel vasculitis, and other non-specific extra-articular symptoms will develop. While there is currently no cure for RA, the treatment strategy aims to expedite diagnosis and rapidly achieve a low disease activity state (LDAS). There are many composite scales measuring the disease activity such as the Disease Activity Score using 28joints (DAS-28), Simplified Disease Activity Assessment Index (SDAI), and Clinical Disease Assessment Index (CDAI) [6]. To achieve full suppression of the activity of the disease (clinical remission), rheumatologists need to monitor disease activity continuously and accurately and to adjust the treatment regimen accordingly.

Universally applied pharmacologic therapy with non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids have proven effective in relieving stiffness and pain, but do not moderate disease progression. Over the last 20 years, the effectiveness of DMARDs has gained much attention as these can efficiently attenuate disease activity and substantially decrease

and/or delay joint deformity [7]. The therapy classification includes the traditional synthetic drugs, biological DMARDs, and novel potential small molecules. Historical DMARDs such as auranofin, minocycline, azathioprine, and cyclosporine are rarely implemented as modern therapies. Several biological DMARDs have recently emerged including TNF-inhibitor (Amjevita, Renflexis, Erelzi, Cyltezo, Imradl), anti-CD20 antibody (Truxima, Rixathon), IL-6 receptor antibody (Kevzara), RANKL antibody (Pralia), and JAK inhibitor (Olumiant). Despite the increasing number of new drugs and treatment regimes, complete long-term disease remission is not achieved for many patients and thus new therapeutic options are required. This review provides a contemporary appraisal of recent literature on the pathogenesis of RA and the potential of new pharmacological interventions for optimizing RA treatment regimes.

EPIDEMIOLOGY

The prevalence rate reported in 2002 ranged from 0.5% to 1% of the population and had regional variation. The objective is to determine the global population prevalence of rheumatoid arthritis (RA) based on population-based studies and assess factors that influence RA prevalence estimates. Four electronic databases were searched (ProQuest Central, MEDLINE, Web of Science, and EMBASE) for peer-reviewed English publications that report prevalence estimates of RA from 1980 and 2019. We included case–control studies, cross-sectional studies, and prospective or retrospective cohort studies in our search strategy. A random-effect meta-analysis model was used to produce the pooled prevalence estimates. The potential between-study heterogeneity was identified using sensitivity analysis, sub-group and meta-regression analyses. A total of 67 studies were included in the meta-analysis, containing 742,246 RA patients and 211,592,925 healthy controls in the study period. The global RA prevalence estimate was 0.46% (95% confidence interval [CI] 0.39–0.54; $I^2 = 99.9\%$) with a 95% prediction interval (0.06–1.27). The RA point-prevalence was 0.45% (95% CI 0.38–0.53%) between 1986 and 2014, while the pooled period-prevalence was 0.46% (95% CI 0.36% and 0.57%) from 1955 to 2015. The highest RA pooled prevalence (0.69%; 95% CI 0.47–0.95) was derived from linked data source studies. Based on meta-regression, the factors that explain the studies' heterogeneity of RA prevalence, including geographical location, the risk bias assessment of studies and sample size. The global prevalence of RA between 1980 and 2019 was 460 per 100,000 population, with variations due to geographical location and study methodology. Linked data are the preferred method to estimate RA population prevalence as they provide the bestcase ascertainment.

SIGNS AND SYMPTOMS

Signs and symptoms of rheumatoid arthritis may include:

- Tender, warm, swollen joints
- Joint stiffness that is usually worse in the mornings and after inactivity
- Fatigue, fever and loss of appetite

Early rheumatoid arthritis tends to affect your smaller joints first — particularly the joints that attach your fingers to your hands and your toes to your feet.

As the disease progresses, symptoms often spread to the wrists, knees, ankles, elbows, hips and shoulders. In most cases, symptoms occur in the same joints on both sides of your body.

About 40% of people who have rheumatoid arthritis also experience signs and symptoms that don't involve the joints. Areas that may be affected include:

- Skin
- Eyes
- Lungs
- Heart
- Kidneys
- Salivary glands
- Nerve tissue
- Bone marrow
- Blood vessels

Rheumatoid arthritis signs and symptoms may vary in severity and may even come and go. Periods of increased disease activity, called flares, alternate with periods of relative remission — when the swelling and pain fade or disappear. Over time, rheumatoid arthritis can cause joints to deform and shift out of place.

TYPES OF ARTHRITIS

- Osteoarthritis

- Rheumatoid arthritis
- Psoriatic arthritis
- Gout
- Lupus

OSTEOARTHRITIS

Osteoarthritis is the most common form of arthritis, affecting millions of people worldwide. It occurs when the protective cartilage that cushions the ends of the bones wears down over time. Although osteoarthritis can damage any joint, the disorder most commonly affects joints in your hands, knees, hips and spine. Osteoarthritis symptoms can usually be managed, although the damage to joints can't be reversed. Staying active, maintaining a healthy weight and receiving certain treatments might slow progression of the disease and help improve pain and joint function [8].

PATHOLOGY

Previously, OA was thought to be a simply a disease of "wear and tear." Chronic overload and impaired biomechanics on the joint were thought to lead to destruction of the joint's articular cartilage and resultant inflammation. This subsequently led to stiffness, swelling, and loss of mobility. OA is most notable for its effect on articular cartilage, which gets severely degraded over the course of the disease.

Articular cartilage is the smooth cartilage at the end of long bones and within the intervertebral discs. It provides a low friction surface for articulation while being able to transmit heavy loads. Although the half-life of the collagen within the cartilage is long, it heals very slowly if at all, even with minor injuries.

Although the cartilage has the most notable changes, the entire joint is affected, including the synovium, joint ligaments, and subchondral bone. Inflammation including active synovitis and systemic inflammation play a key role in the pathogenesis of OA. One potential explanation is that degraded cartilage induces a foreign body reaction within the synovial cells. This may lead to production of metalloproteases, synovial angiogenesis, and production of inflammatory cytokines, which leads to further cartilage destruction.

This suggests other consequences of obesity may be at play beyond body-weight and joint mechanics. This study and others suggest that systemic factors related to obesity, metabolic

syndrome, and atherosclerosis likely play a systemic role in the development of OA, possibly through leptin and other adipokines.

Direct effects of aging on cartilage (due to chondrocyte senescence, DNA damage, aging of the cartilage matrix, oxidative stress, mitochondrial dysfunction, and autophagy) as well as the effect of the endocrine system and estrogen on joint health are also being investigated.

SYMPTOMS

Pain is the most prominent symptom in patients with OA. Pain with OA is also noted to slowly and insidiously progress with time. Early in the course, the pain is predictable and caused by specific (often high-impact) activities. Over time, pain and other joint symptoms become less predictable and more constant, with daily activities beginning to become affected.

In advanced stages, constant dull and aching pain is accompanied by unpredictable, intense, severe pain, which leads to avoidance of certain activities. It is worth noting that the degree of structural pathology noted on imaging and the degree of pain are not always concordant with the symptoms of OA. Some individuals with severe pain have a paucity of findings on imaging and vice versa. Elements such as prior pain experiences, treatment expectations, psychological factors, and sociocultural environment all potentially play a role in the individual's experience of pain.

Other nonpain symptoms of OA include joint swelling, clicking, locking, grating, crepitus, cramping, reduced range of motion, and deformity. Also described are symptoms of instability, buckling, or "giving way." Patients with OA complain of morning stiffness that improves in 30 minutes. This is unlike in rheumatoid arthritis, which typically last longer. The pain of OA also increases throughout the day and with increased activity. Systemic symptoms should be absent. This includes fever, weight loss, or abnormal blood test. The presence of such symptoms would alert the physician to other disease processes such as infection or malignancy.

TREATMENT

There is no current cure for OA. Treatment can be broadly classified into reduction of modifiable risk factors, intraarticular therapy, physical modalities, alternative therapies, and surgical treatments. There is also emerging evidence for several novel treatments. Early on in the course of OA the treatment is focused on the reduction of pain and stiffness. Later, treatment focuses on maintaining physical functioning. Nonpharmacologic Treatment Reduction of modifiable risk factors Obesity may be the strongest modifiable risk factor. Exercise has also

been investigated as a treatment modality for OA. This study also suggested interventions that combined strengthening, flexibility, and aerobic exercise. Aquatic exercise may also be effective.

Other treatments used such as lasers, transcutaneous electrical nerve stimulation, ultrasound, and electromagnetic field therapy are commonly used but evidence is poor on their effectiveness.

Pharmacologic Treatment NSAIDs and acetaminophen are generally considered first-line therapies in the treatment of OA. NSAIDs are effective for overall pain from OA. There is no strong evidence of benefit of any particular NSAID over another. NSAIDS should be used with caution in those with gastrointestinal disease including selective cox-2 inhibitors or nonselective NSAIDs with the addition of a gastroprotective agent.

Acetaminophen has been found to be effective in the treatment of OA, although modestly. They are also less effective than NSAIDs.

Other treatments include serotonin-norepinephrine reuptake inhibitors. Recent evidence has implicated central sensitization as an important factor in pain in OA.

Intraarticular Treatments Intraarticular injection of steroids is an option in the treatment of inflammatory flares of OA, although efficacy is limited and short-lived. Viscosupplements such as hyaluronic acid has uncertain effects when used intraarticulately for the treatment of OA in the knee. Although possibly less effective in the short term, they may provide longer-lasting treatment in OA.

Novel Treatments

Recent improvements in the understanding of the pathophysiology of the disease have led to novel treatments. Strontium ranelate inhibits subchondral bone resorption by regulating the activity of osteoprotegerin, RANK ligand, and matrix metalloproteinases produced by osteoblasts. Strontium may have a direct effect on cartilage; this is supported by the observation that it promotes proteoglycan synthesis, which stimulates cartilage matrix formation in vitro. Nerve growth factor (NGF) is postulated to modulate signals that control expression of peripheral and central pain substances and sensitizes adjacent nociceptive neurons in response to stimulation. Tanezumab is a highly selective immunoglobulin G2 Osteoarthritis antibody against NGF. Several studies have shown that in patients with moderate to severe knee OA tanezumab results in greater improvement in knee pain, stiffness, and increase function compared with placebo. However, side effects include osteonecrosis leading to the medication

being placed on hold by the FDA.

Regenerative therapy has been one the latest rapidly growing strategies to treat OA. Platelet-rich plasma, which is harvested from a patient's blood with the theory that it will provide important growth factors, has been investigated. A systematic review found that platelet-rich plasma resulted in clinical improvement up to 12 months following injection.

Mesenchymal stem cells (MSCs) are a cell source that can be easily obtained from a variety of tissues such as bone marrow, adipose tissue, and synovium. The stem cells are capable of rapid proliferation, chondro-differentiation and immunosuppression. One study showed mild improvements in pain for 5 years after injection of MSCs into the knee joint. Another area of investigation is the use of radiofrequency ablation in the treatment of knee OA. These procedures thermally lesion sensory nerves, which include the superior lateral and medial and inferior medial genicular nerves of the anterior joint capsule of the knee, in order to decrease pain. To date, results are promising for improvement in pain and reducing disability. Other novel approaches being investigated include cryotherapy and geniculate arterial embolization [9].

GOUTY ARTHRITIS

Gouty arthritis is the most common form of arthritis in men. As a consequence of persisting hyperuricemia, uric acid crystals are deposited in the intra-articular and periarticular spaces and activate the innate immune system. The clinically impressive abrupt onset monoarticular arthritis in the lower extremities is highly suggestive of a gout attack. Arthrosonography can be used for early detection of crystal deposition on joint cartilage. In synovial fluid the detection of uric acid crystals in polarization microscopy is proof of gout even without the detection of intracellular uric acid crystals.

Rapidly acting anti-inflammatory drugs are available for acute treatment of attacks; however, the essential therapy is the effective and life-long drug treatment of hyperuricemia from the first attack onwards, typically with allopurinol or febuxostat. This review delineates the clinically relevant knowledge on the pathogenesis, diagnosis and therapy of gout based on the currently available evidence [10].

JUVENILE IDIOPATHIC ARTHRITIS

Juvenile idiopathic arthritis is the most common chronic rheumatic disease of unknown aetiology in childhood and predominantly presents with peripheral arthritis. The disease is divided into several subgroups, according to demographic characteristics, clinical features,

treatment modalities and disease prognosis.

Systemic juvenile idiopathic arthritis, which is one of the most frequent disease subtypes, is characterized by recurrent fever and rash. Oligoarticular juvenile idiopathic arthritis, common among young female patients, is usually accompanied by anti-nuclear antibodie positivity and anterior uveitis. Seropositive polyarticular juvenile idiopathic arthritis, an analogue of adult rheumatoid arthritis, is seen in less than 10% of paediatric patients. Seronegative polyarticular juvenile idiopathic arthritis, an entity more specific for childhood, appears with widespread large- and small-joint involvement. Enthesitis-related arthritis is a separate disease subtype, characterized by enthesitis and asymmetric lower-extremity arthritis. This disease subtype represents the childhood form of adult spondyloarthropathies, with human leukocyte antigen-B27 positivity and uveitis but commonly without axial skeleton involvement. Juvenile psoriatic arthritis is characterized by a psoriatic rash, accompanied by arthritis, nail pitting and dactylitis.

Disease complications can vary from growth retardation and osteoporosis secondary to treatment and disease activity, to life-threatening macrophage activation syndrome with multi-organ insufficiency. With the advent of new therapeutics over the past 15 years, there has been a marked improvement in juvenile idiopathic arthritis treatment and long-term outcome, without any sequelae.

The treatment of juvenile idiopathic arthritis patients involves teamwork, including an experienced paediatric rheumatologist, an ophthalmologist, an orthopaedist, a paediatric psychiatrist and a physiotherapist. The primary goals of treatment are to eliminate active disease, to normalize joint function, to preserve normal growth and to prevent long-term joint damage. Timely and aggressive treatment is important to provide early disease control.

The first-line treatment includes disease-modifying anti-rheumatic drugs (methotrexate, sulphasalazine, leflunomide) in combination with corticosteroids, used in different dosages and routes (oral, intravenous, intra-articular). Intra-articular application of steroids seems to be an effective treatment modality, especially in monoarthritis. Biological agents should be added in the treatment of unresponsive patients.

Anti-tumour necrosis factor agents (etanercept, infliximab, adalimumab), anti-interleukin-1 agents (anakinra, canakinumab), anti- interleukin-6 agents (tocilizumab) and T-cell regulatory agents (abatacept) have been shown to be safe and effective in childhood patients. Recent studies reported sustained reduction in joint damage with even complete

clinical improvement in paediatric patients, compared to previous data [11].

PATHOGENESIS OF RA

There are two major subtypes of RA according to the presence or absence of anti-citrullinated protein antibodies (ACPAs). Citrullination is catalyzed by the calcium-dependent enzyme peptidylarginine-deiminase (PAD), changing a positively charged arginine to a polar but neutral citrulline as the result of a post-translational modification. ACPAs can be detected in approximately 67% of RA patients and serve as a useful diagnostic reference for patients with early, undifferentiated arthritis and provide an indication of likely disease progression through to RA [12][13].The ACPA-positive subset of RA has a more aggressive clinical phenotype compared to ACPA-negative subset of RA [14].It is reported that ACPA-negative RA has different genetic association patterns [15]and differential responses of immune cells to citrullinated antigens [16]from those of ACPA-positive subset. In terms of treatment [17][18],less effective treatment response of methotrexate (MTX) or rituximab was observed in ACPA-negative subset. This suggests a requirement for future study on potential pathophysiology difference between these two subsets. For the purpose of this review, we will focus on the ACPA-positive subset of RA and divide the progression of RA process into several distinct stages. It is noteworthy to mention, however, that these stages may occur sequentially or simultaneously.

TRIGGERING STAGE

The appearance of ACPA is now widely used to diagnose and predict RA due to its high specificity (>97%) in clinical practice. ACPA occurs as a result of an abnormal antibody response to a range of citrullinated proteins, including fibrin, vimentin, fibronectin, Epstein-Barr Nuclear Antigen 1 (EBNA-1), α-enolase, type II collagen, and histones, all of which are distributed throughout the whole body. ACPA production has been associated with genetic and environmental factors. The strongest genetic risk factor associated with ACPA-positive RA is found in genes encoding HLADR, especially HLA-DR1 and HLA-DR4, also known as “shared epitopes” (SEs)[20].It is thought that SE influences RA outcome via the production of ACPA and thus represents a primary risk factor for ACPA production [21].The protein tyrosine phosphatase nonreceptor type 22 (PTPN22), which is a lymphoid specific protein tyrosine phosphatase, has also drawn much attention because of polymorphisms associated with ACPA-positive RA with the contribution of PTPN22 to ACPA-positive RA among various ethnicities[22][23][24] .It may therefore act as a potent inhibitor of T cell activation and in turn

affect in the ACPA production. Genetic variation of α1-antitrypsin has been found to be related to ACPA production in RA [25].

However, whether the production is directly linked to α1-antitrypsin deficiency per se or results from altered autophagy induced by the mutant α1-antitrypsin Z requires further study. The increased response of type I interferon gene associated with Th2 cell induction and B cell proliferation correlates with ACPA production [26] .Some researchers have recently compared the gene expression profiles between ACPA positive RA and ACPA-negative RA patients [11][27].The critical solution to the puzzle is the association between the discovered genes and ACPA production. In addition, the risks of RA increase in individuals with a family history of RA. The risk of developing RA was three times higher in first-degree relatives of RA patients even though familial factors influence RA in men and women equally [28][29][30].It is also reflected in a twin study presenting recurrence risks at 9.5–13.1 in monozygotic co-twins and at6.4–11.7 in dizygotic same sexed co-twins as opposed to a background population risk at only 0.37% [31].Another study of 12,590 twins reveals that environment, lifestyle, and stochastic factors may also play more important roles than genetics in ACPA production while genetic factors are more responsible for the progression from ACPA-positive individuals to arthritis [32].The environment acts as a triggering factor for ACPA production in RA and the epigenetic regulation combines environment with genes. Gene–environment interaction influences the reactivity of autoantibodies to citrullinated antigens in RA [33].

ACPAs can be detected long before the onset of the joint symptoms. This phenomenon suggests that the joints may not be the triggering spot for autoimmunity. Lung exposure to noxious agents ,including smoke, silica dust, nanosized silica, or carbon-derived nanomaterials can trigger mucosal toll-like receptors (TLRs) that activate Ca2+-mediated PADs, but also antigen-presenting cells(APCs), such as classical dendritic cells (DCs) and B cells [34][35][36].The coatomer subunit α gene mutations could disrupt the endoplasmic reticulum (ER)–Golgi transport and cause hereditary autoimmune-mediated lung disease and arthritis, thereby providing a connection between the lung and the joint diseases [37].Moreover, smoking in the context of the HLA-DR SE gene may trigger RA-specific immune reactions to citrullinated proteins [38].DNA methylation mediates smoking and genotype interaction in ANPA-positive RA [39].There is ample evidence for three infectious agents regarded as autoimmunity triggers in RA, namely Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans (Aa), and

Epstein-Barr virus (EBV). The periodontal space can also be a triggering site. In a clinic setting, 47% of the patients with RA showed evidence of previous Aa infection compared with11% in the control group. The pathogen Aa can secret leukotoxin A and form pores in the neutrophil membranes that lead to neutrophil hyper citrullination, which results in the release of citrullinated autoantigens in the gums [40].P. gingivalis infection leads to citrullinated autoantigens and the ACPA production in two reported ways: one way is about PAD and arginine ginpains(Rgps) of P. gingivalis, which can cleave proteins at arginine residues and citrullinate proteins producing more neoantigens [41];another is about neutrophil extracellullar trap (NET) formation induced by the P. gingivalis during the process of NETosis. ACPAs induce NETosis and in turn NETosis provides citrullinated autoantigens [42].EBV can affect ACPA-producing B cells and impaired EBV control can be observed in RA [43].

The intestinal tract is another mucosal organ implicated in the pathogenesis of RA because dysbiosis in RA patients can result from the abundance of certain rare bacterial lineages. It is well documented that gut microbiota may contribute to the pathogenesis of RA via multiple molecular mechanisms [44][45].Several studies have established the role of dietary factors in RA. The omega-3 fatty acids might not only lower the risk of ACPA production but also prevent the onset of arthritis after detecting ACPAs [46].A healthier diet can also make a contribution to reducing the risk of ACPA-positive RA occurring at 55 years of age or younger [47][48].In addition, hormonal levels have been implicated in the pathology of RA [49][50],but the association with ACPA has not been firmly established. Alterations in gene expression regulation proposed to contribute to the pathogenesis of RA. The contribution of other epigenetic modifications (e.g., sumoylation, histone methylation, histone acetylation, and deacetylation) and their functional role in RA currently remain unclear. Translation of above observations to effective treatment and exploring their interaction with the genome is challenging but would be meaningful. It is of significance to clarify the detailed knowledge of each risk factor in the triggering of RA so that tools can be developed to provide susceptibility scores and early diagnosis, as well as to identify new molecular targets for personalized medicine.

MATURATION STAGE

This stage is initiated at the site of secondary lymphoid tissues or bone marrow. Epitope spreading refers to the development of immune responses to endogenous epitopes resulting

from the release of self-antigens. The immune response to autoantigens may exist many years before disease onset and lay outside the joints. In this stage, epitope spreading and a gradually increased titer of ACPA can last several years before the onset of joint symptoms [51]. Initial ACCP levels appear to be of great importance in predicting the interval time to disease onset [9]. The production of ACPA reflects break of immunological tolerance. As a result, many citrullination neoantigens would activate MHC class II-dependent T cells that in turn would help B cells produce more ACPA. ACPA can induce pain, bone loss, and inflammation in RA [52][53]. One study has identified that two RA-specific autoantigens N-acetylglucosamine-6-sulfatase (GNS) and filamin A (FLNA) correlate microbial immunity with autoimmune responses in the joint [54]. What is more, it has been proposed that citrullination plays a unique role during osteoclast differentiation and ACPA-induced osteoclast activation which might explain important features of the gradual development of RA including why the joints are targeted. [55]. The involvement of RA in joints usually has a characteristic presentation with synovitis occurring in symmetrical small joints. Joint swelling is the external reflection of synovial membrane inflammation following immune activation. The normal synovial compartment is infiltrated by leukocytes and the synovial fluid is inundated with pro-inflammatory mediators that interact to produce an inflammatory cascade, which is characterized by the interactions of fibroblast-like synoviocytes (FLSs) with the cells of the innate immune system, including monocytes, macrophages, mast cells, DCs, and so on, as well as cells of adaptive immune system such as T lymphocytes (cell-mediated immunity) and B cells (humoral immunity). The two immune systems and their interactions are intimately involved in the development of ACPA positive RA, which results in the failed resolution of inflammation (chronic synovitis). Monocytes/macrophages have been found to massively infiltrate synovial membranes [56] and be central to the pathophysiology of inflammation. ACPA can enhance NF-kB activity and TNF-α production in monocyte/macrophages via binding to surface-expressed citrullinated Grp78 [57].α-Enolase on the surfaces of monocytes and macrophages induces production of pro-inflammatory mediators [58].The imbalances between proinflammatory M1 macrophage and anti-inflammatory M2 macrophage must also be considered in the context of inflammatory RA [59].Indeed, a recent study reported that an imbalance in M1/M2 monocytes contributes to osteoclastogenesis in RA patients, especially in ACPA-positive RA [60].Further, the pro-inflammatory cytokine interleukin (IL)-17A in RA joint samples is localized primarily to mast cells based on one study [61]and mast cells can be

activated by ACPA and TLRs ligand [62].The accumulation of DCs in the articular cavity has also been reported [63].As an APC, especially myeloid DCs have been shown to induce T cell differentiation. A detailed understanding of how myeloid DCs function in RA may provide more effective RA treatment strategies. Other possible innate immune pathways comprise neutrophil NETosis, nature killer cell activation, etc. On the other hand, many researchers place the adaptive immune system at the center of RA disease pathogenesis. Most interest in the contribution of T cells has focused on their antigen-driven role and cytokine release of specific T cell subsets. CD4 effector T cells are major drivers abnormal immunity in RA by sustaining chronic synovitis and supporting autoantibody production and a lack of reactive oxygen species could boost pro-inflammatory T cells, which shed light on the importance of energy metabolism in RA [64]. As for B cells, the research focuses on their antigen presentation, antibody formation and release, and cytokine release into the milieu. Therefore, better understanding of the mechanisms of disordered innate immunity, including immune complex-mediated complement activation, adaptive immune responses against self-antigens, and abnormal cytokine networks may open up new avenues to restore immunologic homeostasis.

FULMINANT STAGE

Hyperplastic synovium

Synovium is characterized by a mixture of bone marrow-derived macrophages and specialized FLSs [65]. Synovial cells maintain the steady state of the joint by secreting hyaluronic acid and lubricin for joint lubrication and function, as well as processing waste products. In RA, the dysfunction of FLS leads to hyperplastic synovium. The abnormal proliferation of FLS results from a loss of contact inhibition that plays a critical role in RA by producing inflammatory cytokines and proteinases, such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) that perpetuate joint destruction. They create a microenvironment that allows for the survival of T cell and B cell and neutrophil accumulation [66]. Another hypothesis regarding the cause of hyperplastic synovium is likely due to the resistance to apoptosis associated with distinctive pathways. Such pathways include abnormalities of tumor protein p53 function, which contributes to synovial lining expansion and joint destruction in RA; over expression of heat shock protein and enhanced activation of heat shock factor 1 in RA synovial tissues that foster the survival of FLS.The pathogenetic mouse model synoviolin/ Hrd1 triggers synovial cell outgrowth through its anti-apoptotic effects. It appears that synovial hyperplasia contains the proliferation of resident slow-cycling cells, such as mesenchymal

stromal/stem cells and the infiltration of bone marrow-derived cells in lethally irradiated mice after bone marrow transplantation. Although animal models of RA have been useful, they do not always reliably replicate the human disease phenotype, even less the ACPA-positive RA.

Cartilage damage

Cartilage acts as a key component of synovial joints, consisting of chondrocytes and a dense and highly organized extracellular matrix (ECM) synthesized by these chondrocytes and contains type II collagen and glycosaminoglycans (GAGs). The hyperplastic synovium causes major damage to the cartilage in RA through directed adhesion and invasion. Conversely, inflammatory signals, including those released from the ECM, can further stimulate FLS activity. The mediators of cartilage damage include MMPs, a disintegrin-like metalloprotease with thrombospondin type 1 motifs 4 and 5 and cathepsins. MMPs are synthesized by FLS and can promote disassembly of the type II collagen network causing biomechanical dysfunction. Membrane-type I MMP is envisaged to be the predominant proteinase that degrades the collagenous cartilage matrix [67]. However, articular cartilage does not have enough regenerative potential by itself. Consequently, under the influence of synovial cytokines, particularly IL-1 and 17A, and reactive nitrogen intermediates, the cartilage is progressively deprived of chondrocytes that undergo apoptosis. This results in cartilage degradation demonstrable as joint-space narrowing on radiography. These observations may help explain why RA is a site-specific manifestation of a systemic autoimmune disease, in which early cartilage damage in the context of altered immune activation leads to a specific cellular activation of FLS within the articular joints. Nevertheless, a better understanding of the mechanisms underlying cartilage damage is required.

Bone erosion

Bone loss is a pathological hallmark of RA and manifests as localized, periarticular and systemic bone loss. Bone loss is the result of the induction of osteoclasts and the suppression of osteoblasts. “Periarticular” bone loss most likely refers to cellular changes of the subchondral bone marrow, such as osteoclast differentiation and the formation of inflammatory infiltrates. It remains controversial whether inflammation or autoimmunity is the key driver for bone damage. Evidence for the traditional inflammatory theory is as follows: tumor necrosis factor alpha (TNF-α), IL-6, IL-1β, IL-17, and other inflammatory cytokines involved in RA could exert pro-osteoclastogenic effects and suppress bone formation in the appropriate environment via adequate signals, such as the receptor activator of nuclear factor kappa-B ligand (RANKL)

and macrophage colony-stimulating factor (M-CSF).These promote the influx and differentiation of the monocytes into osteoclasts in the context of inflammation, while anti-inflammation therapies for RA arrest the progression of bone damage and vice versa. The second possible pathway for bone loss in RA involves two mechanisms for autoimmunity that act as a trigger for structural bone damage. The first mechanism pertains to the formation of immune complex and Fc-receptor-mediated osteoclast differentiation. The second is the formation of anti-citrullinated vimentin antibodies against the most citrullinated protein, making osteoclasts the ideal antigenic targets for anti-citrullinated protein antibodies (ACPA). It is reported that ACPA binding to osteoclast precursors induces osteoclastogenesis, bone resorption, and bone loss [68]. Bone resorption virtually creates a hole, which is usually found at spots where the synovial membrane inserts into the periosteum, which is known as a bare area according to certain anatomical features. Subchondral bone plays a vital role in maintaining the homeostasis of weight-bearing joints, and the destruction of the subchondral bone can eventually result in the degeneration of the articular cartilage.

Systemic consequences

Multiple studies have documented an elevated risk of cardiovascular events in RA patients. The mechanisms responsible for this risk may be related to cytokines that increase endothelial activation and potentially make atheromatous plaques unstable. Patients with active untreated RA have reduced total cholesterol, low-density and high-density cholesterol [69].RA also influences the brain by causing fatigue and reduced cognitive function; the lungs by causing inflammatory and fibrotic disease; the exocrine glands by causing secondary Sjogren's syndrome; the skeletal muscles by causing sarcopenia; and the bones by causing osteoporosis. Finally, RA patients may be at greater risk of cancer, especially hematologic and kidney cancers [66].

MODERN RA PHARMACOLOGIC THERAPIES

The identification of a preclinical stage and a growing understanding of the natural history and mechanisms of RA development, alongside new potential therapeutic interventions, shapes the prospect that RA might be prevented in future [70].The current treatment principles for established RA involve symptomatic management and disease modification. A meta-analysis of 12 published studies confirmed that patients receiving delayed DMARDs therapy were at higher risk of developing radiographic joint space narrowing and bony erosions. In poorly controlled RA patients, bony erosions become evident on radiographs within 2 years of

onset and these erosive changes are predictive of poorer functional outcome. Oral corticosteroids are potent and effective anti-inflammatory drugs that may contribute to disease modification. However, this needs to be weighed up against its well-known adverse effects. Symptomatic management remains important throughout the course of the disease and consists of everyday practical measures to deal with the primary symptoms of joint stiffness, such as pain and fatigue. Exercise is important to support joint flexibility and function, while abstaining from smoking is a universal advice to all RA patients given its impact on antibody formation.

CONVENTIONAL SYNTHETIC DMARDS (CS DMARDS)

Methotrexate

MTX is a modified form of folate designed to have an increased binding affinity for dihydrofolate reductase (DHFR) compared with its parent molecule. MTX is the cornerstone in the treatment of RA either as a single agent or in combination with other DMARDs [71].In a recent meta-analysis, MTX showed a substantial clinical and statistically significant benefit compared to a placebo in the short term treatment of people with RA, although its use was associated with a 16% discontinuation rate due to adverse side effects. Also, radiographic progression rates measured by an increase in erosion scores of more than 3 units were statistically significantly lower for patients in the MTX group.MTX has been proposed to participate in the process of folate antagonism, adenosine signaling, the blocking of methyl-donor production involved in reactive oxygen species, downregulation of the adhesion molecule expression, modification of cytokine profiles, and the downregulation of eicosanoids and MMPs.Single nucleotide polymorphisms (SNP) analysis and genome-wide association studies (GWAS) have found some SNPs related to MTX responsiveness. For example, those located in the gamma-glutamyl hydrolase (GGH), 5-aminoimidazole-4-carboxamide (ATIC), and solute carrier family 19 member 1 (SLC19A1) genes. Nevertheless, the results from the studies are conflicting, and sufficiently large genomic studies are needed to further develop the understanding.

MTX for RA is administered as a low-dose (5–25 mg) weekly regimen with dosing conditional to the disease state and side effects. Oral MTX has a more variable uptake than subcutaneous administration, which also leads to fewer significant side effects. Subcutaneous MTX administration also demonstrated a greater bioavailability compared with oral MTX.MTX requires regular monitoring to optimize dosing and assess its immunosuppressive and hepatotoxic effects through frequent blood tests (monthly, initially). There are a few well-

established drug interactions for MTX, including cotrimoxazole, which causes pancytopenia, combined with azathioprine or leflunomide, which causes liver and lung complications. NSAIDs can be safely used in conjunction with MTX for symptom control after over 30 years of routine use of the two agents. It is inconclusive that MTX enhances the risk of malignancy beyond the increased relative risk of neoplasia associated with RA. Despite this, the absolute risk is low. Adverse effects associated with the use of MTX additionally include the development of accelerated nodulosis, also known as MTX-induced accelerated nodulosis (MIAN), which occurs in (1–10) % of patients on MTX.However, most adverse effects can be reversed by supplementation with calcium or sodium folinate.

Leflunomide

Leflunomide reduces inflammation in the joints of RA patients by inhibiting dihydroorotate enzymes essential for producing DNA and RNA, particularly in activated proliferation lymphocytes. At higher doses, the active metabolite teriflunomide also inhibits tyrosine kinases responsible for early T-cell and B-cell signaling [72]. Due to its different mechanism of action, Leflunomide is a valuable addition to the armamentarium of drug treatment for RA and is prescribed at a routine starting dose of 10 mg daily for the initial 3 days followed by 20 mg daily. Leflunomide has shown clinical, functional, and structural efficacy similar to MTX and has also been used effectively in combination with biological agents. Dose reduction to 10 mg daily should be considered if side effects occur, with the most common reported side effects being diarrhea, nausea, headache, rash, itching, loss of hair and body weight, hypertension, chest pain, palpitation, infection, and liver failure. It is thus important to monitor gastrointestinal symptoms, allergic reactions, alopecia, and liver function. There are a few well-documented drug interactions, including cholestyramine that impairs the absorption of Leflunomide, rifampin side effects caused by raising Leflunomide levels in the blood, and Leflunomide rarely increasing the anticoagulant effect of warfarin. Leflunomide is deleterious to developing fetuses and breastfeeding infants and therefore should be avoided during pregnancy and lactation. Sulfasalazine (SSZ) Owing to clinical trials, SSZ has been widely available as a therapeutic agent for RA because of its anti-inflammatory and antimicrobial activities. SSZ has significant efficacy in reducing active joint counts and slowing radiographic progression, which is comparable to the effects of Leflunomide. Its metabolites are sulfapyridine and 5-aminosalicylic acid (5-ASA). SSZ has the ability to increase the production of adenosine at the sites of inflammation; inhibit osteoclast formation via modulatory effects

on the receptor activator of nuclear factor κβ (RANK), osteoprotegerin, and RANKL; inhibit TNF-α expression via the apoptosis of macrophages, and suppress B-cell function. Sulfapyridine may reduce IL-8 and monocyte chemotactic protein 1 (MCP-1) secretions in inflammatory cytokines. The common adverse effects of SSZ include gastrointestinal and central nervous system toxicity, rash, liver function abnormalities, leukopenia and agranulocytosis, megaloblastic anemia, oligospermia, and infertility. The way to minimize the side effects is the slow initiation of drug therapy and the serial monitoring of specific laboratory tests. There are no major drug interactions reported but patients should be cautioned about the risk and benefit ratio with pregnancy and breastfeeding. Hydroxychloroquine In RA, hydroxychloroquine is designed to interfere with the interaction between T helper cells and antigen-presenting macrophages that cause joint inflammation and decrease the production of pro-inflammatory cytokines, thus reducing the overall inflammatory response.Whereas, the classical explanation is that, while hydroxychloroquine impaired phago/lysosomal function, it also appears to work in a lysosome-independent manner by impacting on intracellular TLRs, particularly TLR9, by inhibiting the production of TNF, and by interfering with the processing of the conversion of the membrane-bound pro-TNF into soluble mature protein. Hydroxychloroquine has a gradual onset action of 2–6 months, demonstrating improvement of long term functional outcome and retardation of radiographic damage. The common adverse effects are predominantly gastrointestinal, dermatological, and ophthalmologic. High dose and long duration of use of hydroxychloroquine act as risk factors for retinal toxicity which may progress even after cessation of hydroxychloroquine. Therefore, effective screening is important for early detection of retinal toxicity.

BIOLOGICAL DMARDS (BDMARDS)

Although a somewhat vague definition, bDMARDs are a group of drugs that target specific molecules or molecular pathways involved in RA inflammatory processes. A number of bDMARDs have been shown to have clinical and radiological efficacy in the management of RA. TNF-α-inhibiting agents were the initial class of bDMARDs with newer agents targeting B lymphocyte antibodies CD-20, IL6, and CD28 [73].

TNF-α inhibitor (TNFi)

TNF-α triggers inflammatory responses and is produced by activated monocytes, macrophages, and T lymphocytes. TNF-α acts through TNF receptors 1 and 2, which have some species specificity and different affinity with TNF-α. Through the interaction of TNFα

and its receptors, key signaling pathways can be activated, such as the NF-κB pathway, RANKL signaling, the extracellular signal-regulated kinase (ERK) signaling pathway, the tumor progression locus 2 (TPL2) pathway, and proapoptotic signaling. TNF-α has been proposed to mediate local bone destruction in the inflammatory musculoskeletal diseases due to the increased TNF-α levels in these diseases [74].TNF has been involved in the process of endothelial cell activation, the induction of metalloproteinases and adhesion molecules, angiogenesis, and the regulation of fibroblast/keratinocyte/enterocyte chondrocyte/ osteoclast activation, as well as other inflammatory cytokines.

Current evidence implies that TNF-α antagonists may ease arterial stiffness in RA.A substantial proportion of work-disabled patients with RA who start anti-TNF therapy regain work ability. Compared with patients with RA receiving sDMARD therapy, TNFi can decrease the risk of myocardial infarction. In the last 15 years, knowledge on the efficacy and toxicity of the TNFi has been published and was mainly gathered through regional or national registries created after these drugs reach the market. Based on the currently available literature, TNFi has, therefore, become the first choice of bDMARDs therapy in RA patients not responding to, or intolerant of, a conventional sDMARD treatment. Despite differences in biochemical and pharmacological properties of the five currently approved TNFi, there does not seem to be a clinically meaningful difference between them in terms of efficacy and safety. In a large cohort of RA patients, anti-TNF-α therapy does not increase the risk of serious bacterial infections compared with MTX therapy. This leaves the choice of TNFi mainly dependent on practicalities, such as dosing frequency or mode, or on wider economic considerations. In recent years, numerous biosimilar drugs have been developed, and some have already been approved. A biosimilar (bio-originator) refers a biologic medical product almost identical to an original product that is often produced by another company.

Infliximab (IFX) was the first TNFi for RA treatment and consists of a recombinant chimeric monoclonal antibody composed of a human antibody backbone with a mouse idiotype. It can neutralize the biological activity of TNF-α by binding all forms of TNF-α. IFX is administered by intravenous infusion and in overall terms, IFX has an acceptable long-term safety profile. After the treatment with IFX in RA, a decrease of the adhesion molecule, IL-1, IL-6, IL-8, and MCP-1 was observed [75]. Moreover, a reduced thickness of the synovial lining layer could be found. The IFX biosimilars include approved drugs in some countries, such as IFXdyyb, SB2, CT-P13, BOW015, NI-071, PF-06438179/GP1111, STI-002, and ABP

710.IFX has adverse side effects, such as serious infections, the reactivation of hepatitis B or tuberculosis, and the risk of lymphoma and other cancers.

Adalimumab (Ada) is a fully humanized anti-TNF-α monoclonal antibody given by subcutaneous route fortnightly and has a less pronounced toxicity profile.Anti-Ada antibodies (AAA) are detected in more than half of the treated patients with RA. The AAA response is highly restricted and confined to the TNF-α binding region of Ada, thereby neutralizing its therapeutic efficacy and contributing to a loss of clinical efficacy [76]. Ada is proven to be a potent antirheumatic agent to achieve remission and inhibit
radiological progression. Furthermore, combination therapy with MTX is superior to monotherapy. The Ada biosimilars include drugs approved by some countries, such as ABP 501, Adfrar, and ZRC-3197.Ada has the adverse side effects, such as skin reactions, latent infections, and cardiac failure.

Etanercept is a recombinant protein composed of an immunoglobulin backbone and two naturally occurring soluble human 75-kDa TNF receptors. It is given by subcutaneous route twice weekly with toxicity profiles similar to IFX and Ada.Etanercept has shown sustained efficacy and function in rapidly decreasing radiographic progression in elderly and younger patients with RA [77]. The number of patients achieving clinical remission with etanercept varies between 50% and 75% in the literature. Etanercept biosimilars include the approved drugs SB4 and GP2015.

Golimumab is a human IgG1 kappa monoclonal antibody that binds to both the soluble and transmembrane bioactive forms of human TNF-α. It is administered once monthly by subcutaneous injection. While the short-term safety profile is reasonable with no differences in total adverse side effects, including serious infections, cancers, tuberculosis, or deaths. However, long-term surveillance studies are needed for further safety assessment.
One-hundred milligrams of Golimumab showed numerically higher incidences of serious infections, demyelinating events, and lymphoma than 50 mg of Golimubab does. The Golimumab biosimilars include the BOW100 and ONS-3035, which are still in the preclinical phase [78].

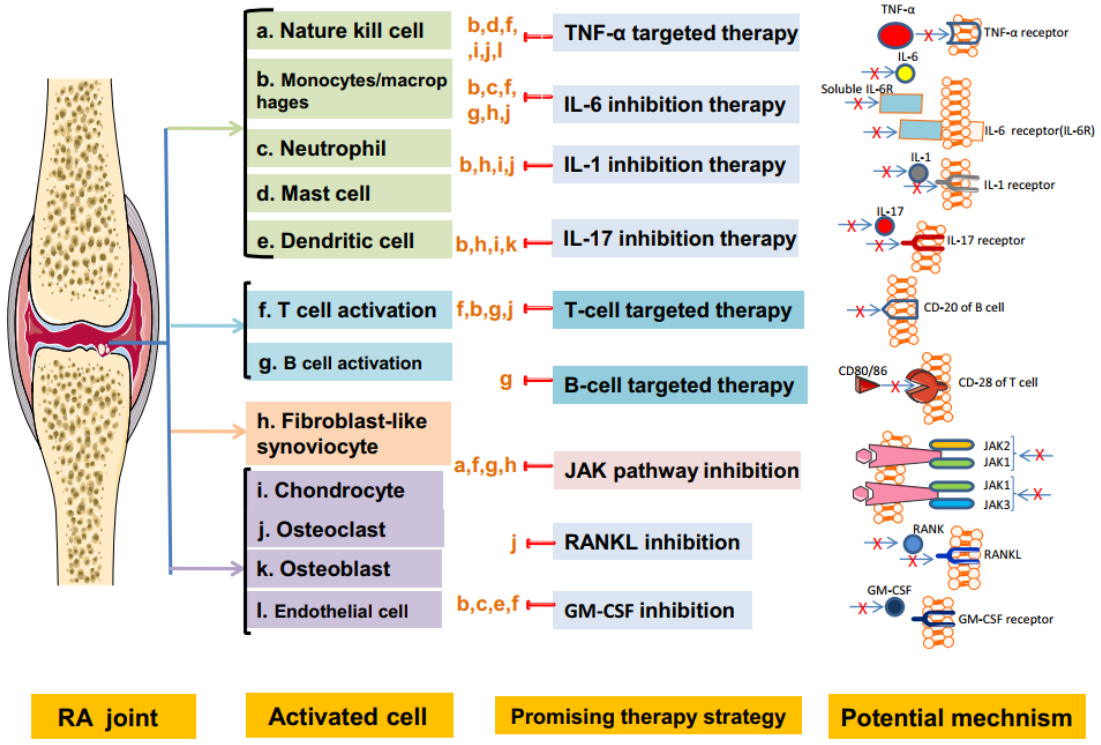


Fig. 1 Cells and key receptors/pathways targeted by current therapy strategies. RANKL receptor activator of nuclear factor-KB ligand, JAK Janus kinase/signal transducers.

Certolizumab pegol is a human anti-TNF-α antibody Fab fragment that is chemically linked to polyethylene glycol and neutralizes membrane-associated and soluble TNF-α. It is administered every 2 weeks by subcutaneous injection and is well tolerated. Certolizumab pegol biosimilars include the PF-688, a drug still in preclinical phase testing. Significant side effects occur in 2% of people who take certolizumab pegol. Incidentally, TNFi (namely onercept and lenercept) failed clinical trials. However, TNF inhibitors have radically altered the approach to treat RA and have become an integral part of disease management. Medical professionals caring for patients should have the basic knowledge of its adverse side effects. Nevertheless, the inactivation of TNF signaling by rationally designed dominant negative TNF variants needs further investigation [79].

B-Cell depletion and inhibition antibodies

Rituximab is a genetically engineered chimeric monoclonal antibody that targets CD20-positive B lymphocytes from early pre-Bcells to later in the differentiation process, but it is absent in terminally differentiated plasma cells. The binding to CD20 enables rituximab to deplete subpopulations of B lymphocytes by way of cell-mediation, complement-dependent

cytotoxicity, and the promotion of apoptosis and growth arrest. B lymphocytes may contribute to the initiation and maintenance of the inflammatory cascade by their action on antigen presentations and through the production of pro-inflammatory cytokines, including IL-1, -4, -6, -8,-10, and -12; TNF-α; vascular endothelial growth factor; MCP; macrophage migration inhibitory factor; and the autoantibodies rheumatoid factor (RF) and ACPA. It has been proposed that Rituximab has an effect on CD4+ cells, inducing substantial T-cell depletion in RA. Rituximab plus MTX demonstrated significant and sustained effects on reducing joint damage progression in RA patients who had a previously inadequate response to TNFi. The Rituximab biosimilars include the drugs BCD-020, Maball, and MabTas, which have been approved by some countries [80].The side effects reported include hypogammaglobulinemia, infection, late onset neutropenia, and mucocutaneous reactions. Rituximab treatment has been linked with rare cases of progressive multifocal leukoencephalopathy (PML).

Belimumab is a monoclonal anti-B lymphocyte stimulator (BLyS) antibody. It binds to soluble human BLyS with high affinity and inhibits its biological activity. BLyS is elevated in the serum and synovial fluid of patients with RA and is associated with increased RF levels. The BLyS mechanism of action of is importance in the survival of B cells, and its inhibition can lead to the apoptosis of autoimmune B-cell clones [81]. However, Belimumab was not effective in phase II clinical trials for RA. Other promising CD-20 targeting antibodies (obinutuzumab, ibritumomab, ocaratuzumab) need more clinical trials. The strategy of depth of depletion of B cell populations may not be the better way compared with the inhibition of B-cell modulatory cytokines.

T-Cell targeted therapies

Abatacept is a T-cell co-stimulation modulator and a fully human soluble fusion protein that consists of the extracellular domain of human CTLA-4, which is linked to the modified Fc part of human IgG1. T-cells infiltrate into the synovial joint and increase the level of pro-inflammatory cytokines such as interferon-γ and IL-17, causing synovial cartilage and bone destruction. Upon antigen recognition, T-cells require a costimulatory signal for full activation. Like the natural CTLA4 molecule, abatacept interferes with CD80/CD86 with higher avidity than CD28. Unlike other biologic drugs, it does not inhibit inflammatory proteins but blocks the communication between these cells by attaching to their surface. It is available in an infusible or injectable form and is administered to patients who have an inadequate response to one or more DMARDs. The data available on abatacept suggests the risk of serious

infections when used together with the TNF-α blocker. Its side effects include headaches, common colds, sore throat, nausea, and infection. By contrast, targeting T cells using ciclosporin, anti-CD4 antibodies, antiCD5 antibodies, or alemtuzumab have not yielded clinically robust responses in patients. The function of T cells and its subsets needs to be further reexamined [82]. Other T-cell medications, such as ALX-0061, Sirukumab, Clazakizumab, Olokizumab, are still in the clinical trial phase.

IL-6 inhibition

Tocilizumab (TCZ) is a humanized monoclonal antibody that targets the IL-6 receptor, which is found on cell surfaces and in circulation. IL-6 is produced by various cell types, including T cells, B cells, monocytes, fibroblasts, and endothelial and synovial cells. It has two receptors: mIL-6R (CD 126) and sIL-6R. In the pathology of RA, IL-6 can stimulate pannus formation through increased vascular endothelial growth factor expression and increase bone resorption as a result of osteoclastogenesis, as well as oxidative stress in leukocytes [83].TCZ is available in subcutaneous and intravenous formulations. Its immunogenicity risk is low. Decreases in neutrophil counts in patients taking TCZ do not appear to be associated with serious infections. Sirukumab, a human monoclonal antibody binding to the IL-6 with high affinity, also shows satisfied outcome with an expected safety profile in clinical phase 3 study. It provides another valuable chance to explore the effect of cytokine inhibition in RA rather than cytokine receptor inhibition. The most common adverse effects observed in clinical trials were upper respiratory tract infections, nasopharyngitis, headaches, and high blood pressure. The candidate IL-6 inhibitors currently undergoing clinical trials include sarilumab, ALX-0061, MEDI5117, clazakizumab, and Olokizumab. Clinical trial data are promising and suggest that anti-IL-6 agents could be a promising therapy.

IL-1 inhibition

IL-1 is a cytokine that has the capability of immune and proinflammatory actions. There are two specific immunoglobulin-like membrane-bound IL-1 receptors, IL-1RI and IL-1RII. At the cell surface, IL-RII, in contrast to IL-1RI, does not transmit signals and acts instead as a decoy receptor that binds and inhibits IL-1. In serum, both IL-1 receptors can bind IL-1, thereby regulating the bioavailability of the cytokine [84].

Anakinra (rHuIL-1ra) is a non-glycosylated recombinant form of the IL-1 receptor antagonist used as a once daily injectable. It is different from the native human protein by having an additional N-terminal methionine. It decreases the activity of IL-1α and IL-1β by

binding to the IL-1 receptor. Its disadvantage includes the requirement of daily injections, and an itchy rash may be observed at the injection site. It can be used as a mono-therapeutic agent or in combination with DMARDs. However, anakinra should not be used in combination with anti-TNF agents. Its side effects include gastrointestinal tract reaction and allergy and infection of the upper respiratory tract; thus, it should be monitored carefully. Interestingly, RA patients receiving anakinra exhibited improved cardiac contractility even within 3 h of a single administration. Therefore, Anakinra should be considered for patients with severe or refractory pericardial disease and(or) heart failure. The benefits of IL-1 inhibition in this population are worth further exploration. Other IL cytokines and their receptors have been studied as the potential target: IL-17 inhibitor (Secukinumab) was finished in a phase III study displaying improvement in patients with active RA who had an inadequate response to TNF inhibitors. However, IL-12/23 blockade, ustekinumab, did not see satisfying outcomes despite being combined with MTX in a randomized phase II study. The drugs targeting IL-7, 15, 18, 21, 32, and 33 are also in a clinical trial.

Osteoclast differentiation factor

Denosumab (DMab) is a human monoclonal IgG2 antibody that inhibits bone resorption by binding and inhibiting the receptor activator of the NF-kB ligand (RANKL), an essential cytokine for osteoclastogenesis and bone resorption. Briefly, RANKL is an essential survival factor for DCs. RANKL-expressing Th17 cells mediate bone resorption. In addition, RANKL secreted by memory B cells promotes bone erosion in RA. Lastly, RANKL was known to induce immune tolerance by promoting the differentiation of Treg cells. It is conceivable that RANKL antagonists may influence immune regulation. The interplay of activated immune cells, synovial cell hyperplasia, and cytokine fosters an osteoclastogenic environment fueled by TNF-α and RANKL. Indeed, the presence of local and systemic bone loss in RA patients raised the possibility that the inhibition of RANKL may be an effective strategy to limit pathologic bone resorption. It has been proved that combining denosumab with DMARDs may be considered for RA patients with progressive bone erosions. Evidence from two phase II trials and one randomized observational trial indicate that DMab inhibits focal and systemic bone loss in RA. Phase III trials are required to discern the magnitude of the inhibitory effect on bone erosions and help to establish an optimal dose. The side effects include low Ca2+ and phosphate levels in the blood, muscle cramps, cellulitis, and numbness. Ultimately, DMab may prove to be a promising drug in the treatment of RA. Besides, the phase IIb study of a novel granulocyte–

macrophage colony-stimulating factor (GM-CSF) receptor alpha monoclonal antibody, mavrilimumab, showed meaningful response by representing a novel mechanism [85].

SMALL-MOLECULE DMARDS

Small-molecule DMARDs revolutionize RA treatment. Many cytokines use the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway to exert their effect in the pathology of RA, rendering them amenable to therapeutic blockade with Jakinibs which have proven effective for the treatment of RA [86].Jakinibs are being developed, and targeting STATs as well as other intracellular signaling pathways may be a future avenue for the treatment of RA, although substantial challenges remain.

Tofacitinib is the first of a new class of oral drugs to have synthetic small molecules that interfere with specific signal transduction pathway and is the third class of DMARD (tsDMARDs) in RA treatment. It created the way to JAK inhibition in RA.

Tofacitinib preferentially inhibits JAK-3 and -1 over JAK-2. With an oral bioavailability of 74% and mean elimination half-life of 3 h, tofacitinib is metabolized via cytochrome P450 3A4 (CYP3A4) with 30% renally excreted; 5 mg bd Tofacitinib has recently been approved by the FDA for moderate to severe RA refractory to DMARDs based on recent efficacy studies, with the onset benefits associated with the treatment occurring earlier. Common adverse side effects were related to infection, hematologic and hepatic disorders, and association of tofacitinib, with carcinogenicity and infections debatable.

Baricitinib is an orally administered molecular that inhibits JAK-1 and -2. It has moderate activity on tyrosine kinase 2 (TYK2) and negligible activity on JAK-3 in both enzymatic and cellular assays.

Baricitinib also proved effective in radiological progression. Peficitinib showed a 14 times higher selectivity for JAK-1/-3 over JAK-2. Filgotinib is a highly selective inhibitor of JAK-1 over JAK-2, JAK-3, and TYK2 in biochemical and cell assays. ABT-494 is also a JAK-1 selective Jakinib. Decernotinib that selectively inhibits JAK3 over the other JAK family members in both enzyme and cellular assays. The new Jakinibs with more restricted JAK isoform selectivity are now between phases 2 and 3 of clinical development. It is advised that Jakinibs will require clinical and laboratory vigilance [87].

FUTURE PERSPECTIVES

With a better understanding of the pathophysiology of RA, new therapeutic approaches are emerging to provide precision medicine for individuals. However, the function

and adverse side effects of these drugs will need to be carefully evaluated and used reasonably. Gene therapy means that treating RA by inserting a gene into a patient's cells instead of using drugs. Targeting gene therapy in RA is a treatment strategy that is still in very early stages of development but could lead to new possibilities because of treating a disease at its root. The availability of Notch1 targeting siRNA delivery nanoparticles and TNF-α gene silencing using polymerized siRNA/Thiolated Glycol Chitosan Nanoparticles has been tested relatively successfully in an animal model. To prevent disease onset or relapses, smoking cessation or avoiding body exposure to environment risk factors is probably the easiest and most cost-effective method. Autoimmunity (tolerance break) develops years before the inflammatory phase of the disease, which can be considered as a golden period for preventing disease progression. Re-establishing immune tolerance and immunological homeostasis are ambitious goals in the way to overcome the disease. T cells and B cells can be targeted by specific drugs in the future to achieve seroconversion or delay the onset of joint destruction. Reduction of the function of APCs and modification of the pro-inflammatory properties of antibodies are being further developed. There is also a great interest in the novel approaches that have the possibility of becoming vital therapeutic targets, such as TLRs; Bruton's tyrosine kinase; phosphoinositide-3-kinase pathway; TGF-β; neuro pathways, and DCs. Bruton's tyrosine kinase is involved in various signaling pathways downstream of the pre-B-cell receptor and FcR, which is a promising therapeutic target for RA. The safety and tolerability of the intravenous infusions of expanded adipose derived stem cells in refractory RA have been reported [88].

Introduction

Symptoms of rheumatoid arthritis (RA) frequently show diurnal variation, with exacerbations in the morning. This variation in disease expression is accompanied by daily oscillations in circulating concentrations of disease-mediating cytokines. In particular, IL-6 shows robust oscillations, and fluctuations in serum IL-6 levels correlate with changes in disease symptoms. This review summarises the evidence for a primary role for the circadian clock in the observed diurnal variations in disease activity, and its role in further aspects of RA disease manifestation. We consider how this information can be utilised, not only to modify existing treatment regimens, but to develop new therapeutic strategies to treat RA.

The circadian clock

The circadian clock drives daily rhythms in physiology necessary to synchronise the function of an organism with the 24-hour environment. Physiological functions under circadian control include the sleep-wake cycle, body temperature, heart rate, blood pressure, hormone regulation and immunity. These daily oscillations are orchestrated by a central pacemaker, which is found within the brain, in a hypothalamic region located above the optic chiasm called the suprachiasmatic nucleus (SCN). The SCN receives light input from the eyes via the retino hypothalamic tract. The central pacemaker synchro nises additional peripheral oscillators found locally within organs, tissues and cells [89]. These secondary clocks are synchronised by the central clock, but are self-sustaining and can be entrained by external cues such as temperature and feeding schedules. The molecular machinery required to enable a cell to oscillate comprises a transcription/translation feedback loop. Central to this loop are the genes *clock* and *bmal*, whose encoded proteins dimerise (CLOCK/ BMAL) and bind to E-box elements on the promoters of the clock genes *period* (*per*), *cryptochrome* (*cry*), *rev-erb* and *ror* to activate their transcription. Translated PER and CRY form a dimeric complex (PER/CRY), enter the nucleus and inhibit CLOCK/BMAL transactivation. Subsequent degradation of PER/CRY allows CLOCK/ BMAL to start a new cycle of transcriptional activation. A second feedback loop is formed by the action of ROR and REV-ERB proteins on *bmal* transcription; these nuclear receptors activate (ROR) and repress (REV-ERB) transcription through their competitive action on response elements (ROREs) on the *bmal* promoter Initially thought of as an auxillary stabilising loop, it is now considered that this circuit is required for circadian oscillation. In addition to these core clock genes, numerous other 'clock-controlled genes' show circadian patterns of expression as a result of action on E boxes, D box enhancers, and RORE sites.

Circadian regulation of the immune system

The biological clock regulates many aspects of the immune system. As a consequence, immune responses often demonstrate measurable circadian variation. Several lines of evidence highlight the contribution of the circadian clock to the function of the immune system. Firstly, multiple immune cells possess the clockwork machinery and show daily variation in their function [90]. Secondly, disruption of the circadian clock has a detrimental effect on the function of the immune system. Finally, deletion of core clock genes can impact on immune responses. These established interactions between the clock and the immune system all have implications for RA.

Oscillating immune cells; a source for rhythms in IL-6 levels

Studies have revealed that the individual cellular components of the immune system that initiate and perpetuate inflammatory pathways are tightly regulated by the circadian clock. These include mast cells, natural killer cells, eosinophils, basophils, T lymphocytes and macrophages. As yet, the dominant cell type driving circadian disease expression in RA remains undetermined. One candidate that may underlie the rhythmic IL-6 secretion is the CD4+ T lymphocyte, which is considered a key mediator of RA with an established role in disease initiation and perpetuation. It has been shown that CD4+ T lymphocytes possess a circadian oscillator that drives rhythmic responses to activating stimuli, as manifest by altered cell proliferation and cytokine secretion [91]. In addition, macrophages possess an intrinsic clock, and over 8% of the macrophage transcriptome is under circadian control. Although macrophages are not thought to be involved in the initiation of RA, their proinflammatory and tissue destructive actions contribute to disease expression and these phagocytes mediate joint inflammation and tissue destruction in erosive disease. A recent study demonstrated rhythmic IL-6 responses to stimulation by both human and mouse macrophages, and established the importance of the clock gene (and nuclear receptor) *rev-erb α* in generating these rhythms.

Finally, fibroblast like synoviocytes, located within the intimal lining of the synovium, also have a circadian oscillator. These cells are a major source of proinflammatory cytokines in RA, although in active disease they may no longer oscillate. To summarise, a number of candidate cells may orchestrate the circadian oscillation in IL-6 secretion. However, the mechanism by which the clock exerts this control is not yet fully established. The production of IL-6 by immune cells may fall under central clock control as a result of circulating mediators (such as glucocorticoids), or alternatively, local clocks may directly drive IL-6 oscillations. Of course, both possibilities may co-exist.

Disruption of the circadian clock and rheumatoid arthritis

Recent studies have identified an intriguing bi-directional interaction between inflammation and the circadian clock. Disruption of the clock has a significant effect on the performance of the immune system, and there is a suggestion that this might impact negatively on the pathogenesis of RA. In converse, inflammation can directly alter cellular expression of core clock genes [92]. Disruption of the clock is exemplified by jet-lag, a consequence of de-synchrony between the internal clock and the environment, resulting in the need to re-set the phase of the clock. Depending on the number of time zones crossed and direction of travel, it

can take a number of days to re-synchronise the body clock. Shift-workers experience the same phenomenon. These constant shifts in the daily schedule are detrimental to health and have been linked with an increased incidence of a number of chronic diseases such as cancer, cardiovascular disease, metabolic syndromes, diabetes and irritable bowel syndrome. Circadian disruption can be modelled by inducing 'chronic jet-lag' in animals through exposure to regular shifts in the lighting schedule. This type of environmental disturbance has negative effects on the function of immune cells, including macrophages and natural killer cells, and has detrimental effects on the survival of mice after immune challenge. Interestingly, a study in 2010 provided a significant link between shift work and an increased risk of RA (in women). This is the first observation of its kind, supporting the concept that the body clock not only impacts on the symptoms of this disease, but is also involved in the pathogenesis.

Core clock genes - links with rheumatoid arthritis

A direct link has been made between core components of the molecular clock and inflammatory pathways known to be relevant to RA. Mice lacking two core clock genes, *cry1* and *cry2*, show disruptions in their circadian clock. Where wild-type mice are able to maintain strong circadian behaviour in the absence of light cues, *cry1*-/- *cry2*-/- mice are behaviourally arrhythmic under constant darkness. These double knockout mice have a more aggressive inflammatory arthritis in response to collagen induction (collagen-induced arthritis). This is attributed to the function of CRY as a transcriptional inhibitor of the *tnfα* gene. It is now established that CRY acts on a broad range of pro-inflammatory target genes, which it modulates through the NFκB pathway [93]. Whilst this is the only established direct regulatory pathway linking the core clock and the pathogenesis of RA, several clock outputs (including hormones and clock-controlled genes) have been attributed roles, and these are expanded on below.

Circadian control of the endocrine system and links with RA

The endocrine system mediates the dissemination of timing signals from the SCN throughout the body. Two hormones in particular act as circadian agents - melatonin and glucocorticoids. Both are important in inflammation and regulation of the immune response, and may contribute to the pathogenesis of RA.

Melatonin

Melatonin is primarily secreted from the pineal gland (other sources include the retina, glands, intestine, skin, leukocytes and bone marrow). The SCN regulates melatonin synthesis

through tonic inhibition of noradrenergic stimulation of the pineal gland. Melatonin affects several aspects of the immune response, and is known to modulate pro-inflammatory cytokines, inhibit proliferation of fibroblast-like synoviocytes and regulate leukocyte function. Interestingly, RA patients have altered levels of circulating melatonin. Sulli and colleagues [94] report higher baseline levels of serum melatonin in RA patients (at the start and end of the dark phase) and an altered temporal profile with a more rapid increase at the start of the night and an earlier peak. These observations suggest that the effects of melatonin may be more pro-inflammatory than anti-inflammatory. In support, *in vivo* studies clearly indicate that melatonin has adverse effects on the development and severity of inflammatory arthritis. Administration of melatonin in a collagen-induced arthritis model resulted in enhanced disease incidence and severity. This is concordant with studies showing that removal of the pineal gland, the main source of melatonin, has a beneficial effect on disease progression in the collagen-induced arthritis model. It is important to consider that melatonin is widely available and often utilised by shift-workers and long-haul travellers, where it is thought to result in faster adaptation to a new time zone. Given that a clinical study of the use of melatonin in RA resulted in poor outcomes, with an increase in some markers of inflammation, this current widespread use of melatonin may need to be addressed.

Glucocorticoids

Glucocorticoids are endogenous anti-inflammatory agents (cortisol in humans and corticosterone in rodents). Circulating levels fluctuate throughout the day in a circadian manner, peaking just before waking in mammals. The diurnal variation in circulating glucocorticoid concentrations is driven by the circadian clock through direct neural connections between the central clock in the SCN, and the paraventricular nucleus in the hypothalamus, the site of central control for the hypothalamic pituitary adrenal axis. Glucocorticoid's act via the ubiquitously expressed glucocorticoid receptor (GR), a ligand-activated transcription factor, belonging to the nuclear receptor superfamily (see below). The activated, hormone bound GR translocates from the cytoplasm to the nucleus, and binds to target gene regulatory regions. Binding to positive glucocorticoid response elements as a homodimer results in transcriptional activation; and binding to negative glucocorticoid response elements results in transcriptional inhibition [95].Additionally, ligand-bound GR can function as a transrepressor by binding (either directly or indirectly) to other transcription factors (such as activator protein-1 and NFκB) in a so-called tethering mechanism, to inhibit their *trans*-activating function and prevent their

association with DNA. Glucocorticoids can also exert rapid effects by coupling the GR to intracellular signalling cascades, including mitogen-activated protein and phosphoinositide-3 kinases. This mechanism of action is less well-characterised, but offers potential cross-talk between growth factors and cytokine signal ling, relevant to inflammatory arthritis, and glucocorticoid action.

In RA patients, the natural morning peak in cortisol levels occurs approximately 40 minutes after the morning rise in IL-6 levels. It is a striking paradox that the stress-responsive hypothalamic pituitary adrenal axis is not constitutively activated in RA patients, and in fact, the dynamic responses to activation have been shown to be blunted, for reasons that remain obscure, but with obvious clinical implications [96]. Recent insights suggest that merely tracking circulating cortisol levels is inadequate as a means to assess glucocorticoid action. Indeed, in certain cell-types, expression of the GR shows daily rhythms; furthermore, the function of the receptor is under clock control, thereby suggesting alternative mechanisms by which the clock regulates glucocorticoid sensitivity.

Nuclear receptors

Nuclear receptors are ligand-dependent transcription factors, which modulate gene expression through direct binding to DNA response elements. Over half of the nuclear receptor family exhibit rhythmic expression in a tissue-specific manner, and many can feedback directly onto the clock itself. Nuclear receptors are recognised as key intermediaries between the molecular clock and a wide array of physiological processes, including immunity. In the context of RA, three nuclear receptors are of particular interest: GR, retinoid-related orphan receptors (RORs) and peroxisome proliferator activated receptors (PPARs).

Glucocorticoid receptor

There are three protein isoforms of the GR (α, β and γ), of which GRα is the dominant form, mediating most of the actions of glucocorticoids. Evidence suggests that receptor levels show circadian variation in some tissues, but not others. In addition, the cellular clock can drive post-translational modification of the GR protein, so affecting its function. This includes GR interaction with NFκB, which consequently affects the anti-inflammatory capacity of glucocorticoids. CLOCK and BMAL proteins can acetylate a cluster of lysine residues in the hinge region of the GR, which selectively attenuates the ability of the GR to bind to glucocorticoid response elements.

The naturally occurring oscillations in CLOCK/BMAL levels consequently translate into circadian fluctuations in the acetylation of the GR; for example, in human peripheral blood mononuclear cells, acetylated GR is almost three-fold higher in the day. An additional insight is provided by the recent discovery that GR and the core clock protein CRY form a dimer, which is critical to normal GR function. As CRY is only present for part of the circadian cycle this imposes indirect clock control upon the GR, with the suggestion that only specific aspects of GR function (including gluconeogenesis and hypo thalamo pituitary axis tone) show a circadian component [97]. This opens up a potential therapeutic opportunity whereby through altering the timing of delivery (chronotherapy) it may be possible to maximise the anti-inflammatory effects of glucocorticoids whilst alleviating the adverse metabolic effects.

Retinoid-related orphan receptors

RORs are a family of nuclear receptors with three members, RORα, RORβ and RORγ (of which there are two isoforms γ and γt). Transcription of *ror* genes is rhythmic, although to varying degrees, and the ROR proteins have a well-established role in the molecular clock via transcriptional control of *bmal* (mice deficient in either RORα or RORβ show aberrant circadian behaviour). The circadian rhythmic RORα has a role in promoting the differentiation of T cells into TH17 cells. These are a subset of T helper cells (distinct from TH1, T H2 and regulatory T cells) characterised by their expression of the pro-inflammatory cytokines IL-17 and IL-17F, which represent a substantial population of infiltrating CD4+ T cells in inflamed synovial tissue. In addition, RORα has a role in regulating inflammation. *Staggerer* (*sg*) mice, which carry a microdeletion in the *rorα* gene preventing translation of the ligand binding homology domain, have immune system deficiencies. Macrophages isolated from *sg* mice are hyper-responsive to lipopolysaccharide, producing elevated levels of cytokines (IL-1β, TNFα and IL-1α). Further studies have identified direct interactions between RORα and inflammatory genes. RORα1 can activate IκBα (the main inhibitory protein in the NFκB pathway) via a response element on the promoter, thereby suppressing inflammatory responses. Conversely, RORα can up-regulate the inflammatory response through binding to a response element on the promoter of the *il6* gene to enhance IL-6 production. Of note, RORγ*t* also plays a key role in the differentiation of T cells into T H17 cells; however, RORγt expression is clock independent.

Peroxisome proliferator activated receptors

PPARs are a further class of nuclear receptors closely interlinked with the circadian clock. They are ligand activated transcription regulators, which act by forming heterodimers

with retinoic acid receptors (RXRs) and binding to response elements on target genes. All three PPARs (α, β/δ and γ) show tissue-specific patterns of circadian expression. Clock regulation of both the expression and function of PPARs is multifaceted. PPARα transcription is directly regulated by CLOCK/BMAL via an Ebox element in the promoter. In comparison, the activity of PPARγ is regulated through the activity of circadian proteins, which include the transcriptional enhancer nocturin, the co-activator protein PGC-1 α, and the core clock protein PER2. Endogenous ligands for PPARs include free fatty acids and eicosanoids and so in response to a high fat diet or fasting (when lipolysis in adipose tissue is active) these receptors become activated. The PPARs play a key role in lipid metabolism, energy partition, and also in regulating macrophage activity. Importantly, PPARγ and δ facilitate 'alternative' activation of macrophages - a cellular state that plays a key role in the resolution of inflammation [98].

PPARs are regulators of T-cell function. Both PPAR α and γ are expressed in T cells, α is down-regulated following T-cell activation, whilst γ is up-regulated. PPARγ is known to mediate the proliferative response of T cells, inhibits their differentiation into T H17 cells, and induces T-cell apoptosis.

Adipokines

The circadian clock is fundamental in the regulation of metabolic processes; controlling expression of genes involved in metabolic pathways but also responding to metabolic cues. Circadian disruption has detrimental effects on metabolism, and is an exacerbating factor in the incidence of metabolic syndrome, which itself is associated with the risk of developing RA. It is proposed that the link between metabolic syndrome and RA is due to the inflammatory milieu associated with metabolic syndrome, much of which is provided by the increased quantities of adipose tissue, which secretes elevated levels of cytokines (TNFα and IL-6) and adipokines. Adipokines are signalling molecules produced primarily by adipose tissue and include adiponectin, leptin, resistin and visfatin. White adipose tissue and its constituent adipocytes are both circadian rhythmic, and many adipokines exhibit 24-hour variation in plasma concentrations. Recent studies have identified clear associations between adipokines and the pathophysiology of rheumatic diseases. For example, visfatin (also known as nicotinamide phosphoribosyltransferase (NAMPT) or pre-B cell colony-enhancing factor (PBEF)) is released by visceral fat, macrophages, liver, skeletal muscle and leukocytes in a rhythmic manner to produce diurnal rhythms in circulating levels. Elevated levels of visfatin are associated with RA; furthermore, levels detected in serum and synovial fluid correlate with

clinical disease severity[99]. Visfatin is pro-inflammatory, and although the underlying mechanisms are not fully understood, it stimulates synovial fibroblasts and mono cytes to release pro-inflammatory cytokines and matrix metalloproteinases. The well-established and complex relationship between the circadian clock and metabolic processes provides another link between the clock and the pathogenesis of RA.

Targeting clock output genes as therapeutics

Synthetic glucocorticoids, such as dexamethasone and prednisone, have long been a cornerstone in the clinical treatment of RA. Yet, prolonged usage is associated with a number of adverse effects, including immune sup pression, metabolic imbalance and diabetes. Although the recent development of biologics (which specifically target components of the immune system that play pivotal roles in driving inflammation) has made great advances in the treatment of RA, these unfortunately are known to increase the risk of infection (that is, tuberculosis and hepatitis B). Consequently, there remains a need to develop new therapies for the treatment of this inflammatory disorder. Given that RORs and PPARs have significant input into key events that contribute to the pathogenesis of RA, both present as potential therapeutic targets in this, and other, inflammatory diseases. Many synthetic PPAR ligands are already in clinical use. The thiazolidinediones are a group of synthetic PPARγ ligands that have anti-hyperglycemic actions and are used in the management of type 2 diabetes.

They include rosiglitazone, troglitazone and pioglitazone. Synthetic PPARγ ligands, and the proposed endogenous ligand 15-deoxy-delta (12,14)-prostaglandin J2 are anti-inflammatory, and improve outcome in animal models of inflammatory arthritis. Recently, a clinical trial has suggested that the concomitant use of pioglitazone and methotrexate may have synergistic effects in RA [100].

However, although these compounds have potent anti-inflammatory properties, off-target effects, possibly through activation of the glucocorticoid receptor , can result in osteoporosis or cardiac failure, which is likely to limit their application. Similarly, there is growing evidence that PPARα agonists may be useful in the treatment of RA. Synthetic PPARα ligands include fibrates (fenofibrate, gemfibrozil and clofibrate), hypolipidemic compounds already utilised in the treatment of metabolic disorders. Promisingly, fenofibrate has been shown to be effective at reducing inflammation in experimental arthritis models.

Novel therapeutic targets

A full understanding of the network through which the circadian clock regulates

inflammatory pathways is likely to provide novel therapeutic targets for treating RA and other inflammatory disorders and autoimmune conditions. Given its role in T-cell function, one such target is ROR. A novel synthetic ligand for ROR (α and γ) has recently been synthesised (SR1001) that inhibits TH17cell differentiation and reduces the severity of murine experimental autoimmune encephalomyelitis, a TH17 cell mediated autoimmune disease. In addition, another key area for therapeutic targeting is the molecular clock. How the clock influences expression of the drug target or elements of the drug's metabolic pathway can be utilised to optimise dosing times to make treatment more effective; this might maximise effectiveness and minimise side effects. Given the strong circadian component to RA, there is obvious potential for the use of chronotherapy in the treatment of this disease. As proof of principle, a study in 1997 demonstrated the advantages of administering prednisone overnight, just prior to the rise in IL-6 levels and joint stiffness. This small study identified a significant improvement in joint stiffness when this glucocorticoid was administered at 2:00 am versus 7:30 am. In response to the impractical nature of dosing during the night-time, modified release prednisone (Lodotra) has been developed. The CAPRA-1 study compared the effectiveness of Lodotra with immediate release prednisone in patients with active RA receiving disease-modifying anti-rheumatic drugs. The group receiving Lodotra demonstrated significant improvements in the duration of their morning joint stiffness after just 2 weeks. Lodotra significantly reduced morning IL-6 levels, which was not seen in the immediate-release prednisone group. These beneficial effects over immediate-release prednisone were sustained over a 12-month period. Furthermore, in a placebo-controlled study (CAPRA-2) the addition of low dose (5 mg) Lodotra to existing treatment regimens proved to be beneficial. These trials highlight the positive effects of chronotherapy on relieving morning joint pain and stiffness, and are the first of their kind to show that modification of pharmacokinetics and the application of timed-dosing can boost the effectiveness of established RA therapies. It is expected that application of chronotherapy to alternative types of RA therapies may prove similarly beneficial.

Induction of arthritis in animal models

1. Complete Freund's Adjuvant Induced (CFA)

Arthritis in Rats

Freund's complete adjuvant induced arthritis in rat model is the best and most widely used experimental model for arthritis. It is a T cell and neutrophil dependent and complement

independent helper (Th) 1 and (Th) 17 inflammatory cytokines are associated CFA induced arthritis. Increased levels of TNFα, interferon γ (INFγ), IL1, IL6 and IL17A mRNA have been detected in this type of model. This model is sensitive to anti-inflammatory and immune inhibiting medicines and best for the study of pathophysiological and pharmacological control of inflammation process as well as for the evaluation of anti-nociceptive potential of drug. AIA is not joint-specific but is associated with granuloma formation in various organs and tissues, such as the spleen, liver, bone marrow, skin and eyes**.**

The main disadvantage of freund's adjuvant is that it can cause granulomas, inflammation at the inoculation site and lesions. CFA can also cause significant side effects such as chronic inflammation, skin ulceration local abscess of tissue sloughing. The mycobacterium in the complete freund's adjuvant attracts macrophages and other cells to the injection site which enhances the immune response. The essential components of this response is an intense inflammatory reaction at the site of antigen deposition resulting from an influx of leukocytes and their interaction with antigen. For this reason, the complete freund's adjuvants is used for the initial injections [101].

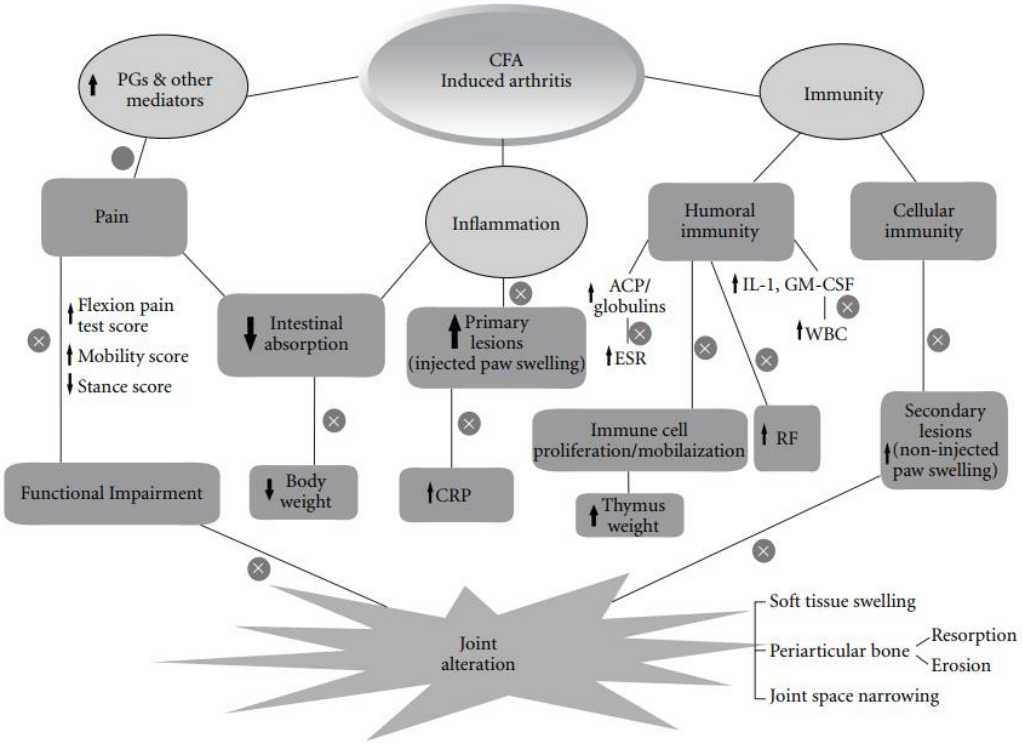
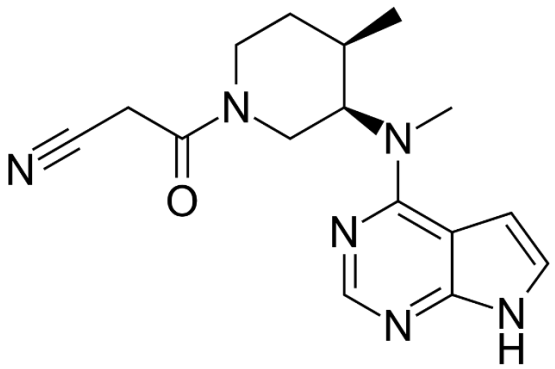


Fig 2: Anti-arthritic activity of tofacitinib. Cross mark indicates inhibition of CFA induced pathological changes in rats by tofacitinib.

Drug profile

DRUG PROFILE

Tofacitinib is an inhibitor of Janus kinases, a group of intracellular enzymes involved in signaling pathways that affect hematopoiesis and immune cell function. It is approved by the FDA for treatment of moderate to severe rheumatoid arthritis that responds inadequately to methotrexate or in those who are intolerant to methotrexate. Besides rheumatoid arthritis, tofacitinib has also been studied in clinical trials for the prevention of organ transplant rejection, and is currently under investigation for the treatment of psoriasis. Known adverse effects include nausea and headache as well as more serious immunologic and hematological adverse effects. Tofacitinib is marketed under the brand name Xeljanz by Pfizer.



Chemical Formula

C16H20N6O

Indication

For the treatment of moderate to severe rheumatoid arthritis which is resistant or intolerant to methotrexate therapy. It may also be used as an adjunct to methotrexate therapy, or other non-biologic disease-modifying antirheumatic drugs (DMARDS), when methotrexate alone is not sufficient.

Tofacitinib has also been investigated as a preventative therapy for kidney transplant rejections, and as a treatment for psoriasis, ulcerative colitis, and ankylosing spondylitis.

It is not to be initiated in patients with a history of chronic or recurrent infections, or in the presence of active infection, even if localized, due to reports of serious and sometimes fatal

infections (commonly pneumonia, herpes zoster and urinary tract infections). Use of tofacitinib is also discouraged in those who have been, or are likely to be, exposed to TB. An increased likelihood of exposure may be encountered by traveling to certain areas. In addition, tofacitinib is not to be used in patients with severe hepatic impairment, or low haemoglobin (less than 9g/dL). Cautioned is advised when using tofacitinib in patients at risk of gastrointestinal perforation, and in the elderly who are more susceptible to infection.

Associated conditions

- Polyarticular-course Juvenile Idiopathic Arthritis (JIA)
- Moderate Rheumatoid arthritis
- Severe Rheumatoid arthritis

Pharmacodynamics

Tofacitinib targets inflammation present in rheumatoid arthritis by inhibiting the Janus kinases involved in the inflammatory response pathway.

In placebo controlled trials of rheumatoid arthritis patients receiving 5mg or 10mg of tofacitinib twice daily, higher ACR20 responses were observed within 2 weeks in some patients (with ACR20 being defined as a minimum 20% reduction in joint pain or tenderness and 20% reduction in arthritis pain, patient disability, inflammatory markers, or global assessments of arthritis by patients or by doctors, according to the American College of Rheumatology (ACR) response criteria list), and improvements in physical functioning greater than placebo were also noted.

Commonly known adverse effects of tofacitinib include headaches, diarrhea, nausea, nasopharyngitis and upper respiratory tract infection. More serious immunologic and hematological adverse effects have also been noted resulting in lymphopenia, neutropenia, anemia, and increased risk of cancer and infection.

Before initiations of tofacitinib patients should be tested for latent infections of tuberculosis, and should be closely monitored for signs and symptoms of infection (fungal, viral, bacterial, or mycobacterial) during therapy. Therapy is not to be started in the presence of active infection, systemic or localized, and is to be interrupted if a serious infection occurs.

Tofacitinib has been associated with an increased risk of lymphomas, such as Epstein-Barr virus associated lymphomas, and other malignancies (including lung, breast, gastric, and colorectal cancers). It is recommended to monitor lymphocytes, neutrophils, haemoglobin, liver enzymes, and lipids.

Tofacitinib use is associated with a rapid decrease in C-reactive protein (CRP), dose dependent decreases in natural killer cells, and dose dependent increases in B cells. Depression in C-reactive protein levels continue after 2 weeks of tofacitinib discontinuation and suggest that pharmacodynamic activity last longer than pharmacokinetic half-life.

Absorption

74% oral absorption (absolute bioavailability), with peak plasma concentrations (T max) achieved in 0.5-1 hour.

Administration with fatty meals does not alter AUC but reduces Cmax by 32%.

Volume of distribution

Vd= 87L after intravenous administration. Distribution is equal between red blood cells and plasma.

Protein binding

40%, mostly bound to albumin.

Metabolism

Metabolized in the liver by CYP3A4 and CYP2C19. Metabolites produced are inactive.

Route of elimination

70% metabolized in the liver by CYP3A4 (major) and CYP2C19 (minor). Metabolites produced are inactive. 30% renally eliminated as unchanged drug.

Half-life

~3 hours

Toxicity

Minimum lethal dose in rat: 500 mg/kg. Maximum asymptomatic dose in non-human primate: 40 mg/kg.

Lymphatic, immune system, bone marrow and erythroid cell toxicity was seen in animal studies involving rate and monkeys. Doses used in these studies ranged from 1mg/kg/day to 10mg/kg/day, over a duration of 6 weeks to 6 months. Lymphopenia, neutropenia, and anemia is seen in human subjects and may call for an interruption or discontinuation of therapy if severe.

Reduced female fertility in rats was seen at exposures 17 times the maximum recommended human dose. Fertility may be impaired in human females and harm may be caused to unborn child. Carcinogenic potential is seen, however evidence for dose dependency is lacking.

Because the Janus kinase pathway plays a role in stimulating the production of red blood cells and is involved in immune cell function, inhibition of this pathway leads to increased risk of anemia, neutropenia, lymphopenia, cancer and infection.

Lymphopenia, neutropenia, and anemia in human subjects may call for an interruption or discontinuation of therapy if severe.

Role of JAK inhibition in the development of gastrointestinal perforation is not known.

Food Interactions

Avoid grapefruit products. Dose adjustments are required when administering CYP3A4 inhibitors (grapefruit) and CYP2C19 inhibitors with tofacitinib.

Avoid St. John's Wort. This herb induces the CYP3A4 metabolism of tofacitinib and may reduce its serum concentration.

Take with or without food [102].

Review of literature

REVIEW OF LITERATURE

In vitro

Alamgeer, (2019) research work was an attempt to appraise the antiarthritic potential of *Ribes alpestre* Decne in rheumatoid arthritis. *In vitro* inhibition of protein (bovine serum albumin and egg albumin) denaturation, Human red blood cell membrane stabilization assays along with formaldehyde induced arthritis in rats were commenced in this study. Findings of present investigation demonstrated significant and dose dependent antiarthritic effect of *Ribes alpestre*. Aqueous ethanolic extract, butanol and aqueous fraction illustrated 95%, 69.233% and 92.840% protection at 6400 ug/mL against bovine serum albumin denaturation respectively. Similarly, plant extract together with butanol and aqueous fractions showed 3653.47%, 1484.03% and 3563.19% inhibition of pathological alteration of egg albumin in that order. Moreover, hydroethanolic extract with butanol and aqueous fraction exhibited 91.29%, 65.73% and 89.62% stabilization against erythrocyte hemolysis at 6400 ug/mL correspondingly. Furthermore, hydroethanolic extract, butanol and aqueous fraction notably 73.49%, 66.42% and 68.87% decreased paw edema at highest dose (200 mg/kg). Similarly aqueous ethanolic extract, butanol and aqueous fraction illustrated 72.38%, 54.90% and 66.33% decrease in paw thickness at 200 mg/kg. Hence results suggested that *Ribes alpestre* possess antiarthritic potential thus supporting its use as natural remedy in rheumatic conditions [(103)].

Arya *et al. (2014)* The present study deals with the *in vitro* anti-inflammatory and anti-arthritic activity in methanolic extracts of *in vivo* (leaf and stem) and *in vitro* (callus) plant parts of *Cocculus hirsutus*. The previous phytochemical analysis of methanolic extract of *Cocculus hirsutus* has indicated the presence of several physiologically active phytochemicals such as phenols, flavonoids, triterpenoids, steroids, alkaloids etc. Since these compounds are of pharmacological interest, coupled with the use of this plant in traditional medicine, prompted us to check all *in vivo* and *in vitro* plant parts of *Cocculus hirsutus* for *in vitro* anti-inflammatory activity by HRBC (Human Red Blood Cell) membrane stabilization method and anti-arthritic activity by the inhibition of protein denaturation method. The methanolic extracts of all plant parts exhibited notable anti-inflammatory activity and remarkable anti-arthritic action. The membrane stabilization was found to be maximum in leaves (88.8% at a dose of 1000µg/ml) and that of protein denaturation was also found to be maximum in leaves (65.85% at a dose of 1000µg/ml) as compare to other *in vivo* (stem) and *in vitro* (callus) plant parts. Therefore, our studies support the isolation and the use of active

constituents from *in vivo* and *in vitro* plant parts of *Cocculus hirsutus* in treating inflammations and rheumatism [(104)].

Nguemnang S *et al.* *(2019)* reviewed the *Dissotis thollonii* Cogn. (Melastomataceae) is a tropical plant widely used in traditional Cameroonian medicine to relieve and treat many pathologies. It is widespread in the western region where it is used to treat typhoid fever, gastrointestinal disorders, and inflammatory diseases. -e purpose of this study is to scientifically demonstrate the anti-inflammatory and antiarthritic properties of the aqueous and ethanolic extracts of the leaves of *Dissotis thollonii*. -e anti-inflammatory properties were evaluated in vitro by inhibition tests for cyclooxygenase, 5-lipoxygenase, protein denaturation, extracellular ROS production, and cell proliferation; while antiarthritic properties were evaluated in vivo in rats using the zymosan A-induced monoarthritis test and the CFA-induced polyarthritis model is study shows that aqueous and ethanolic extracts at a concentration of 1000 μg/ml inhibit the activity of cyclooxygenase (47.07% and 63.36%) and 5-lipoxygenase (66.79% and 77.7%) and protein denaturation (42.51% and 44.44%). Similarly, both extracts inhibited extracellular ROS production (IC50=5.74 μg/ml and 2.96 μg/ml for polymorphonuclear leukocytes, 7.47 μg/ml and 3.28 μg ml for peritoneal macrophages of mouse) and cell proliferation (IC50 =16.89 μg/ml and 3.29 μg/ ml). At a dose of 500 mg/kg, aqueous and ethanolic extracts significantly reduce edema induced by zymosan A (69.30% and 81.80%) and CFA (71.85% and 79.03%). At the same dose, both extracts decreased sensitivity to mechanical hyperalgesia with 69.00% and 70.35% inhibition, respectively. Systemic and histological analyzes show that both extracts maintain the studied parameters very close to normal and greatly restored the normal architecture of the joint in animals. *Dissotis thollonii* would therefore be a very promising source for the treatment of inflammatory diseases[(105)].

In vivo

Singh v s *et al.*, *(2021)* measured the *Calotropis procera* (commonly known as Swallow wort) is described in the Ayurvedic literature for the treatment of inflammation and arthritic disorders. Therefore, in the present work, the antiarthritic activity of potential fractions of Swallow wort leaf was evaluated and compared with standards (indomethacin and ibuprofen). This study was designed in Wistar rats for the investigation of antiarthritic activity and acute toxicity of Swallow wort. Arthritis was induced in Wistar rats by injecting 0.1 mL of Freund's complete adjuvant (FCA) on the 1st and 7th days subcutaneously into the sub plantar region of the left hind paw. Evaluation of our experimental findings suggested that antiarthritic activity of methanol fraction of Swallow wort

(MFCP) was greater than ethyl acetate fraction of Swallow wort (EAFCP), equal to standard ibuprofen, and slightly lower than standard indomethacin. MFCP significantly reduced paw edema on the 17th, 21st, 24th, and 28th days. It also showed significant effect ($p < 0.01$) on arthritic score, paw withdrawal latency, and body weight. The inhibition of serum lysosomal enzymes and proinflammatory cytokines along with improvement of radiographic features of hind legs was also recorded with MFCP. Finally, it was concluded that MFCP can be a feasible therapeutic candidate for the treatment of inflammatory arthritis [(106)].

Triastuti A *et al.*, (2021) reported the anti-arthritic properties of an n-hexane-insoluble fraction of dichloromethane extracts of *P. major* (IPM) were evaluated using Complete Freund's Adjuvant (CFA)-induced arthritis induced in female Wistar rat by CFA. Diclofenac was used as a positive control. The volume of paw oedema, white blood cell count, lymphocytes, neutrophils, expression of TNF-α and Interleukin-6 and the histopathological features of the joint tissues were assessed to characterise IPM activity. The IPM extract at doses of 280 and 420 mg/kg BW and diclofenac inhibited paw oedema by 15.70 %, 15.94 % and 19.71 % respectively. IPM also reduced the incidence of arthritis and arthritic index. Unlike untreated rats, animals treated with IPM showed a significant decrease in the number of neutrophils and decreased expression of TNF-α and Interleukin-6. Histopathological examination showed a reduction in the number of inflammatory cells and hyperplasia of the synovium after IPM treatment[(107)].

Patel R *et al.*, (2021) studied the Calotropis procera treatment significantly lowered paw volume in CFA-induced arthritic rats. Significant improvement was observed in functional, biochemical and hematological parameters in Calotropis procera-treated rats. However, the body weight remained unaffected. Histological and radiographical examination of synovial joints in Calotropis procera-treated animals exhibited less synovial hyperplasia, infiltration and accumulation of inflammatory cell in synovial fluid, cartilage and bone erosion and joint space narrowing. Calotropis procera latex possesses anti-arthritic activity, which is facilitated by modulation in the level of inflammatory mediators and oxidative stress. The improvement in hematological as well as biochemical parameters might be reflected on functional, histopathological, radiological changes and thereby disease progression [(108)].

Khalid M *et al.*, (2021) reported the northeastern part of India, the fruits of Spondias mangifera (S. mangifera) have long been used to treat rheumatism. The current study investigates the ethanolic fraction of S. mangifera fruit extract's anti-arthritis and anti-inflammatory properties

(EtoH-F). To support this research, we used molecular docking on COX-1, COX-2, and TNF- to approach the parameters in silico using the active ingredients of the plant (beta amyrin, beta sitosterol, oleonolic acid, and co-crystallized ligands, i.e., SPD-304). After that, the features of absorption, distribution, metabolism, excretion, and toxicity were evaluated, and then experimental activity was carried out in vitro and in vivo. Parameters including 1,1-Diphenyl-2-picrylhydrazyl (DPPH), free radical-reducing potential, albumin denaturation, and protease inhibitory activity were used to assess the in vitro activities of the plant extract fractions. In Freund Adjuvant (CFA) models, in vivo activity was assessed utilising measures such as COX, TNF-, and IL-6 inhibition assays, as well as arthritis score, at a dosage of 400 mg/kg b.w. per day of various fractions (hexane, chloroform, alcoholic). COX-1, COX-2, and TNF- were used in the molecular docking experiment. At 500 g/mL, in vitro tests revealed a concentration-dependent decrease in albumin denaturation, protease inhibitors, and scavenging activities. In comparison to sick animals, administration of the S. mangifera alcoholic fraction at the above-mentioned dose resulted in a substantial decrease (p 0.01) in arthritis score, paw diameters, TNF-, and IL-6. The docking studies revealed that residues had a high affinity for TNF- and operate as a TNF-antagonist. The alcoholic fraction of S. mangifera extract has anti-inflammatory and anti-rheumatoid arthritis properties, and it might be exploited as a potential agent for innovative target-based therapeutics for arthritis management [(109)].

Arisa *et al.*, (2020) By examine the chronotherapy, differences in drug efficacies according to administration time of Baricitinib, a wide ranged cytokine blocker, were examined in CIA mice. In CIA mice, diurnal variations were observed both in expressions of cytokines and phosphorylation of STAT3. Arthritis scores of ZT0/12 groups decreased from day3 as compared to untreated mice, and those of ZT0 group significantly decreased as compared to ZT12 group from day12[(110)].

Akio (2020) explained the treatment of rheumatoid arthritis has changed dramatically over the last two decades since the development of biological disease-modifying anti-rheumatic drugs (bDMARDs). JAK inhibitors are low-molecular-weight compounds, which exert anti-rheumatic effects by suppressing the action of JAK, an intracellular tyrosine kinase. Of note, biologics bind to extracellular proteins and block their activity. The availability of JAK inhibitors that are as effective as bDMARDs, despite the completely different route of administration and mode of action, has enabled the treatment of rheumatoid arthritis to enter a new stage. Oral administration is convenient for patients. It is necessary to understand the characteristics of JAK inhibitors and use these agents judiciously[(111)].

Xai *et al.*, (2019) reported the nimbolide, a triterpenoid isolated from flower of neem tree possess various therapeutic properties. The objective of the study was to assess the anti-arthritic activity of nimbolide in arthritis induced rats. Nimbolide (20 mg/kg per day) was given orally to arthritic rats induced with Complete Freund's Adjuvant and changes in paw volume, body weight, organ indices (thymus and spleen), arthritic score, biochemical parameters and proinflammatory cytokines levels were determined. Histopathological analysis was also performed. Western blot analysis was also performed. Rats treated with nimbolide displayed marked reduction in arthritic score, organ indices, volume of paw, edema formation, along with substantial enhancement in body weight. Histopathological findings showed significant reduction in destruction of joints and inflammation following nimbolide treatment. The protective action of arthritic rats treated with nimbolide was also substantiated by molecular and biochemical studies. The results of the study show that nimbolide treatment has markedly enhanced health and reduced inflammation via lessening the proinflammatory cytokines expression in arthritic rats. Hence, nimbolide may be used as a potent therapeutic drug in treating rheumatoid arthritis [(112)].

Srinivasa *et al.*, (2019) The present study was undertaken to explore its possible anti-inflammatory and antiarthritic activity. Anti-inflammatory activity of alcoholic and aqueous extracts of the bark was assessed by *in vivo* methods. *In vivo* antiarthritic potential of the extracts was evaluated by Complete Freund's Adjuvant (CFA) induced arthritis in Wistar rats. Our findings showed that the alcoholic and aqueous extracts exhibited anti-inflammatory activity at 500 mg/kg oral dose in carrageenan-induced hind paw edema and carrageenan-induced air pouch inflammation models. We also found alcoholic as well as aqueous extracts of the bark restored the altered blood and serum parameters caused by the Complete Freund's Adjuvant-induced arthritis in Wistar rats. This study shows that the *T. tomentosa* bark extracts possess anti-inflammatory activity and have pronounced effects on adjuvant arthritis also. Future studies are necessary to provide deeper insight into the exact mechanism of the action of anti-inflammatory and antiarthritic activity of *T. tomentosa* [(113).]

Ruckmani L *et al.*, (2018) worked and found the Sesamum indicum, one of the first recorded plants used for its seeds, is reported to have analgesic, antioxidant, anticancer, anti-obesity as well as hepato and nephroprotective activities. The current study evaluated the effects of two doses (400 and 800 mg/kg) of ethanolic extract of S. indicum seeds in Freund's complete adjuvant induced

arthritis in rats in comparison with diclofenac and methotrexate by the changes produced in body weight, body temperature, paw volume and spontaneous activity, hemoglobin, erythrocyte sedimentation rate, total white blood cells, red blood cells, Interleukin-6 and Tumor necrosis factor-a as well as joint changes in X-ray and histological changes in joint tissue. Unlike the untreated group, the groups treated with S. indicum showed significant decrease in paw volume, body weight, white blood cell count, erythrocyte sedimentation rate, Interleukin-6 and Tumor necrosis factor-a and an increase in body weight, spontaneous activity, hemoglobin level, and red blood cell count. Histopathological examination showed gross reduction in synovial inflammation and cartilage damage. X-ray revealed significant improvement in joint space. The effect of ethanolic extract of S. indicum was found to be equivalent to methotrexate and greater than diclofenac [(114)].

Mariam G. F *et al.*, (2016) reported that fibrates were reported to have anti-inflammatory effects while the naturally occurring polyphenol resveratrol was traditionally known as a potent antioxidant agent. The effects of fenofibrate and resveratrol were investigated on complete Freund's adjuvant (CFA)-induced rheumatoid arthritis (RA) in adult female albino rats. Rats were divided into a normal control group, an arthritis control group receiving CFA, two reference treatment groups receiving dexamesathone (1.5 mg/kg/day) and methotrexate (1 mg/kg/day), and two treatment groups receiving fenofibrate (100 mg/kg/day) and resveratrol (10 mg/kg/day) for seven consecutive days. Assessment of RA was performed by measuring serum rheumatoid factor (RF), matrix metalloprotinease-3 (MMP-3) and cartilage oligomeric matrix protein (COMP) as specific rheumatoid biomarkers, immunoglobulin G (IgG) and antinuclear antibody (ANA) as immunological biomarkers, tumour necrosis factor-alpha (TNF-a) and interleukin-10 (IL-10) as immunomodulatory cytokines, myeloperoxidase (MPO) and C-reactive protein (CRP) as inflammatory biomarkers and malondialdehyde (MDA) and glutathione (GSH) as oxidative stress biomarkers, supported by a histopathological study on joints and spleens. Results Serum RF, MMP-3, COMP, IgG, ANA, TNF-a, MPO, CRP and MDA were decreased to about 36, 56, 66, 65, 9, 35, 24, 44 and 31% by fenofibrate, and to about 37, 59, 44, 70, 5, 30, 23, 33 and 28% by resveratrol treatments, respectively. Alternatively, serum IL-10 and GSH were significantly increased to about 215 and 251% by fenofibrate and to about 225 and 273% by resveratrol treatments, respectively. Fenofibrate and resveratrol protect against RA, possibly through their immunomodulatory, anti-inflammatory and antioxidant potential [(115)].

Marius *et al.*, (2016)*:* This study aimed at evaluating immuno modulatory and anti-arthritis

capacity of aqueous and methanol extracts of stem bark of Piptadeniastrum africanum (Mimosaceae). The methods are ROS production from phagocytes, proliferation of T-cells, TNF-α and IL-1β production and cytotoxicity were performed by using chemiluminescence technique, liquid scintillation counter, ELISA and MTT assay, respectively. Anti-arthritic activity was evaluated using a model of adjuvant induced arthritis. These extracts also possess significant ($P < 0.001$) inhibitory activity on T-cell proliferation other than reduced TNF-α and IL-1β production. Piptadeniastrum africanum also significantly exhibited antiarthritic activity in complete Freund's adjuvant induced arthritis in rat associated with a significant anti-inflammatory and anti-hyperalgesia activity. The results show that Immunomodulatory, anti-inflammatory, anti-hyperalgesia and anti-arthritis potential revealed in this study approve that, Piptadeniastrum africanum is a plant rich in compounds with anti-arthritic properties [(116)].

Maurizio *et al.*, (2016) reviewed a clear temporal relationship exists in rheumatoid arthritis (RA) patients between increased nocturnal levels of pro-inflammatory cytokines, such as TNF-α and interleukin (IL)-6, pro-inflammatory hormones (i.e., melatonin, prolactin) and insufficient night production of the anti-inflammatory cortisol (circadian rhythm). Under long-standing chronic stress of disease, insufficient cortisol is available to inhibit an ongoing nocturnal immune/inflammatory reaction. Clinical RA symptoms follow the same circadian rhythm with highest morning severity. Chronotherapy with night-time glucocorticoid (GC) availability optimizes the treatment of RA patients with low-dose GCs through more efficient targeting of mediators of the immune/inflammatory reaction during the night to be available on arising. Circadian use of low-dose, long-term prednisone, by using night-release formulations (ingested at 10 to 11 p.m.) especially in early RA patients, appears characterized by a significantly superior efficacy on decreasing morning stiffness and IL-6 serum levels, compared to conventional daytime immediate-release prednisone. Shift from medium-dose, immediate-release prednisone (over 7.5-10 mg/day) to night-release formulations GC low-dose, long-term chronotherapy requires a gradual passage, since the hypothalamic-pituitary-adrenal axis of the treated RA patients, potentially altered by a negative feedback induced by the medium/high daily exogenous GC administration, needs time to re-synchronize control of endogenous GC production into a circadian and more physiological nocturnal hormone availability/optimized efficacy[(117)].

Elisha I L *et al.*, (2016) examined all extracts inhibited nitric oxide production in a dose-dependent manner in the LPS-stimulated RAW 264.7 macrophages. Extracts of Maesa lanceolata

and Heteromorpha arborescens inhibited NO production by 99.16 % and 89.48 % at a concentration of 30 µg/ml respectively. Elaeodendron croceum and Calpurnia aurea extracts had strong activity against 15-lipoxygenase activity with IC50 values of 26.23 and 34.70 µg/ml respectively. Morus mesozygia and Heteromorpha arborescens extracts had good in vitro anti-arthritic activity with IC50 values of 11.89 and 53.78 µg/ml, the positive control diclofenac sodium had IC50 value of 32.37 µg/ml. The free radical scavenging activity of the extracts in DPPH assays ranged between 7.72 and 154.77 µg/ml. Trolox equivalent antioxidant capacity (TEAC) and FRAP values ranged from 0.06 to 1.32 and 0.06 to 0.99 respectively. Results from this study support the traditional use of the selected medicinal plants in the management of arthritis and other inflammatory conditions. The free radical scavenging capacity of the extracts may be related to an immune boosting potential[(118)].

Patel S S *et al.*, (2013) reported the treatment of formulation to adjuvant induced arthritic animal showed statistically significant ($P < 0.05$) improvement in physical parameters like arthritic index, paw edema, paw thickness as well as reduction of inflammatory markers like C-reactive protein, serum rheumatoid factor, erythrocyte sedimentation rate. The treatment also produced statistically significant ($P < 0.05$) increase in haemoglobin percent and improvement in splenomegaly and thymus index. In the histopathological examination, ameliorative effect of formulation was observed in hyperplasia of synovium, pannus formation, and destruction of the joint space. The results obtained in experiments indicated that the formulation significantly inhibited the adjuvant-induced arthritis which was comparable to dexamethasone and had preferable anti-inflammatory effect without significant side effect. Thus, the formulation may be a potential preventive or therapeutic candidate for the treatment of chronic inflammation and arthritis [(119)].

Tang y *et al.*, (2011) found out the rheumatoid arthritis (RA) is an autoimmune disease associated with advanced joint dysfunction. Madhura indica J. F. Gmel, from the family sapotaceous, is an Indian medicinal plant reported to have an array of pharmacological properties. The aim of present investigation was to determine the anti-arthritic potential of an isolated phytoconstituent from methanolic leaf extract of Madhura indica (MI-ALC) against FCA-induced experimental arthritis. Polyarthritis was induced in female rats (strain: Wistar) via an intradermal injection of FCA (0.1 mL) into the tail. Polyarthritis developed after 32 days of FCA administration. Then rats were treated orally with an isolated phytoconstituent from MI-ALC at doses of 5, 10, and 20 mg/kg. Findings suggested that High-Performance Thin-Layer Chromatography, Fourier-

Transform Infrared Spectroscopy, and Liquid Chromatography-Mass Spectrometry spectral analyses of the phytoconstituent isolated from MI-ALC confirmed the structure as 3,5,7,3′,4′-Pentahydroxy flavone (i.e., QTN). Treatment with QTN (10 and 20 mg/kg) showed significant ($p < 0.05$) inhibition of increased joint diameter, paw volume, paw withdrawal threshold, and latency. The elevated synovial oxidative stress (Superoxide dismutase, reduced glutathione, and malondialdehyde) and protein levels of Tumor necrosis factor-α (TNF-α) and Interleukin (ILs) were markedly ($p < 0.05$) reduced by QTN. It also effectively ($p < 0.05$) ameliorated cyclooxygenase-2 (COX2), Nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-kβ) and its inhibitor-α (Ikβα), and ATP-activated P2 purinergic receptors (P2X7) protein expressions as determined by western blot analysis. In conclusion, QTN ameliorates FCA-induced hyperalgesia through modulation of elevated inflammatory release (NF-kβ, Ikβα, P2X7, and COX-2), oxido-nitrosative stress, and pro-inflammatory cytokines (ILs and TNF-α) in experimental rats[120].

Patil K R *et al.*, (2011) measured the fruits of Barringtonia racemosa are used to cure pain, inflammation, and rheumatic disorders in the ayurvedic literature. The current study reports on the activity-guided isolation of bartogenic acid (BA) and its assessment in rats with Complete Freund's Adjuvant (CFA)-induced arthritis. The ethyl acetate fraction showed strong anti-inflammatory efficacy among the numerous extracts and fractions examined preliminarily for carrageenan-induced acute inflammation in rats. BA was identified as the ingredient responsible for the reported pharmacological effects after large-scale isolation and characterization utilising chromatography and spectrum analysis. The BA was next tested in rats for its ability to prevent CFA-induced arthritis. BA protects rats against the primary and secondary arthritic lesions, body weight alterations, and hematological perturbations caused by CFA at dosages of 2, 5, and 10 mg kg1 day1, p.o., according to the findings. In the BA-treated arthritic rats, blood indicators of inflammation and arthritis, such as C-reactive protein and rheumatoid factor, were also decreased. BA has a significant protective effect against adjuvant-induced arthritis in rats, according to the total severity of arthritis as measured by radiological examination and pain ratings. Finally, the current study supports the ethnomedicinal usage of B. racemosa fruits in the treatment of pain and inflammation. It also establishes BA's powerful anti-arthritic properties. However, further clinical research is needed to show that BA is effective in the treatment of diverse immuno-inflammatory illnesses.

The experimental group's arthritis scores were lower than the control group's after 6 weeks of therapy. TNF-, IL-6, and CRP expression were lower in the 18 HALO group than in the control or 6 HALO groups. The experimental groups had lower histopathology ratings than the control group

(p 0.05)[121].

Ekambaram S *et al.*, (2010) reported the FCA induced arthritic rats, there was significant increase in rat paw volume and decrease in body weight increment, whereas SPP and SPE treated groups, showed significant reduction in paw volume and normal gain in body weight. The altered hematological parameters (Hb, RBC, WBC and ESR) and biochemical parameters (blood urea, serum creatinine, total proteins and acute phase proteins) in the arthritic rats were significantly brought back to near normal by the SPP and SPE treatment at the dose of 200 mg/kg/p.o in both developing and developed phases of arthritis. Further the histopathological and radiological studies revealed the antiarthritic activity of SPP and SPE by indicating fewer abnormalities in these groups when compared to the arthritic control group. Both SPP and SPE at the specified dose level of 200 mg/kg, p.o. showed reduction in rat paw edema volume and it could significantly normalize the hematological and biochemical abnormalities in adjuvant induced arthritic rats in both developing and developed phases of FCA induced arthritis. Further the histopathological and radiological studies confirmed the antiarthritic activity of SPP and SPE.

Otterness (1997) Since 1973, assessment of serum concentrations of C-reactive protein (CRP) has been advocated as an objective measure of disease activity in rheumatoid arthritis (RA). Our review of clinical experience with CRP measurement suggests it has at least two important roles to play in the management of RA. First, persistently elevated CRP levels have prognostic value. In general, such elevated levels are found in those patients who are at greater risk for continuing joint deterioration and therefore may need more aggressive treatment and supportive care. Second, in general, improvement in CRP levels is an objective indication that a drug has produced a beneficial effect and thus may be useful to the physician for monitoring effects of therapy. Since CRP may be elevated in a number of conditions besides RA, a diagnosis of RA must be made before using CRP as a prognostic factor [123].

Aim and objectives

AIM

To study the chronotherapy of Janus kinase inhibitor against Complete Freund's Adjuvant-induced rheumatoid arthritis *wistar* rats.

OBJECTIVES OF THE WORK

The objectives of the study are:

- To evaluate *in vitro* anti-inflammatory activities of tofacitinib and methotrexate.
- To evaluate *in vivo* effect of Chronotherapy of Janus kinase inhibitor against Complete Freund's Adjuvant-induced rheumatoid arthritis rats.
- Analyse 24h variation of cytokine secretion in Freund's adjuvant induced arthritis *Wistar rat* model.
- To study the influence of circadian time of treatment of Janus kinase inhibitor against Complete Freund's Adjuvant-induced rheumatoid arthritis rat

Plan of work

PLAN OF WORK

1. Review of literature
2. Collection of drugs
3. Study of *in vitro* anti-inflammatory activities of tofacitinib and methotrexate.
4. Study of *in vivo* Chronotherapy of Janus kinase inhibitor against Complete Freund's Adjuvant-induced rheumatoid arthritis rats
5. Estimation of biochemical parameters such as,
   - ✓ Rheumatoid factor
   - ✓ C-reactive protein
6. Lipid peroxidation
   - ✓ Malondialdehyde level (MDA)
7. Enzymatic antioxidants
   - ✓ Superoxide dismutase (SOD)
   - ✓ Catalase (CAT)
   - ✓ Glutathione peroxidase (GP_x)
8. Non-enzymatic antioxidant
   - ✓ Reduced glutathione
9. Statistical analysis

Materials and methods

MATERIALS AND METHODS

Chemicals		Manufacture
Tofacitinib	:	Ipca laboratories Ltd, Mumbai
Methotrexate	:	Ipca laboratories Ltd, Mumbai
C-reactive protein	:	Chemleon diagnostics Ltd, Coimbatore
Rheumatoid factor kit	:	Chemleon diagnostics Ltd,
Coimbatore Complete Freund's Adjuvants	:	Sigma Aldrich chemical Ltd,
Bangalore Carboxyl methyl cellulose	:	Loba chemie
Ether anaesthetic	:	Hi-Pure Fine Chemical
Bovine serum albumin	:	Hi media laboratory Ltd,

Mumbai All other chemicals used were obtained commercially and were of analytical grade.

INSTRUMENTS

Instruments	Manufacturer
Centrifuge	Remi
Auto analyzer	Mispa viva
pH meter	ELCO 1/27 pH meter
UV-spectrophotometer	Jasco v530
model Plthesmograph	

EXPERIMENTAL ANIMALS

Male *Wistar* rats weighing between 150 – 250 g was used for the study. The animals were procured from College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala. The animals were maintained under controlled conditions of temperature (23±2˚C), humidity (50±5%) and 12-hrs light-dark cycles. All the animals were acclimatized for 7 days before the study. The animals were randomized to experimental, normal and control groups, housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and drinking water *ad libitum*. Animals were habituated to laboratory conditions for 48 hrs prior to experimental protocol to minimize if any of non-specific stress. The animals used for this study were approved by institutional animal ethical committee (IAEC), College of pharmacy,

SRIPMS, bearing the approval no. 1559/PO/Re/S/11/CPCSEA.

MATERIALS AND METHODS

Animals

Six-week-old, female *Wistar* rats weighing 160 to 170 g each were purchased from the Trissur. They were housed 6 in each cage under standardized light-dark cycle conditions (lights-on and lights-off at 07:00 and 19:00 hours respectively) at a room temperature of 24 ± 1° C and a humidity of 60 ± 10% with free access to food and water.

Induction of rheumatoid arthritis using Complete Freund's adjuvant

Freund's Adjuvant is one of the most commonly used adjuvants in research. It is used as a water-in-oil emulsion. It is prepared from non-metabolizable oils (paraffin oil and mannide monooleate). If it also contains killed Mycobacterium tuberculosis it is known as Complete Freund's Adjuvant. Subcutaneously inject 0.05 ml of CFA containing 10 mg/ml MT into the footpad of a rear paw. Severe and acute inflammation will be observed within 30 minutes of the injection. The inflammation peaks within 3-4 days and lasts for 20-25 days. Secondary arthritis in the non-injected paws typically appears within 12-14 days of the injection. The arthritis peaks within 2-3 days of its onset and lasts for 20 to 25 days [124-128].

IN-VITRO EVALUATION

Inhibition of protein denaturation using Bovine serum albumin

0.5 mL reaction mixture of various concentrations consisting of 0.45 mL bovine serum albumin (5% aqueous solution) and mixed with tofacitinib. pH was calibrated at 6.3 using 1N HCl. After preparation mixtures were incubated at 37 °C for 20 min subsequently heating at 57 °C for 30 min. After cooling the samples, 2.5 mL phosphate buffer saline (pH 6.3) was added to each test tube. Moreover, 0.05 mL distilled water was used in place of plant extract/fractions in control test tube whilst product control did not contain bovine serum albumin. In due course, absorbance was measured spectrophotometrically at 660 nm and percentage inhibition of protein denaturation was calculated [103].

$$\text{Percent inhibition} = \frac{Abs_{control} - Abs_{Treated}}{Abs_{control}} \times 100$$

Inhibition of protein denaturation using fresh hen's egg albumin

5 mL reaction mixture containing 0.2 mL fresh egg albumin, 2.8 mL phosphate buffered saline of 6.4 pH and 2 mL of crude extract/fractions solutions of different concentrations were prepared. Furthermore, similar volume of doubled distilled water was taken as control. Reaction mixture was placed at 37±2 ℃ in incubator for 15 minutes followed by heating at 70 ℃ for 5 min. After cooling, absorbance was taken at 660 nm by using vehicle as a blank. Likewise, diclofenac sodium at same concentrations served as standard control and absorbance was measured afterwards [103].

Percentage inhibition of protein denaturation was calculated by using the following formula:

% of inhibition = 100 X [Vt /Vc-1]

where, Vt = absorbance of test sample

Vc = absorbance of control

***IN-VIVO* EVALUATION**

Thirty female Wistar rats will be divided into 5 groups (n=6 rats in each group).

Groups	**Treatment**
I. Normal control	Vehicle (0.5 % CMC)
II. Negative control	Complete Freund's Adjuvant-0.5mg in 0.1ml
III. Zeitgeber time 0 (5 mg/kg b.w.)	Tofacitinib
IV. Zeitgeber time 12(5mg/kg b.w.)	Tofacitinib
V. Positive control	Methotrexate (1mg/kg b.w.)

End of the study C-reactive protein level was detected using semi auto analyzer machine. During the study rheumatoid factor level was measured whether result became positive or not. On 22nd day, the one animal from each group will be sacrificed for further histopathological evaluation.

Upon conclusion of the experiments, the rats will be sacrificed (one from each group) by diethyl

ether asphyxiation. The hind paws will be radiographed and then histopathologically analyzed. Female *Wistar* rats were divided randomly into five groups (n = 6 per group): the 8 (ZT0) and the 20 (ZT12) experimental groups, the qw positive controls, the negative controls, and the normal control group. Tofacitinib (5mg/kg) was administered at 8 (ZT0) when the serum CRP level started to decrease and at 20(ZT12) when the serum CRP level started to increase, twice daily by *p.o*. separately from day 1 to day 12 after that from day 13 to 21 the animal does not receive any treatment. In the qw (once a week) positive control group, MTX (1 mg/kg) was administered once weekly by *p.o*.

Measurement of body weight and hind paw volume

Hind paw volume of rats was measured on initial day (0^{th} day) before CFA injection and subsequently for different time period till 21^{st} day using Plethysmometer. The hind paw volume can be measured by subtracting final paw volume from the initial volume. Body weight was also measured from animals from 0th day till the end of the experimental period. Body weight can be calculate using difference between initial and final weight of animals.

Measurement of serum CRP level

Blood will be collected and serum will be obtained by centrifugation at 3000 rpm for 10 min and stored at –80 °C prior to analysis. The CRP will be measured by using semi autoanalyzer instrument.

ESTIMATION OF BIOCHEMICAL PARAMETER

Determination of Rheumatoid arthritis measurement [129]:

Reagent composition:

R1 Diluent	Tris buffer mmol/L, pH 8.2
R2 Latex	Latex particles coated with human gamma globulin, pH8.2
RF Calibrator	Human serum RF concentration is stated on vial label, and it is traceable to the "Rheumatoid arthritis serum" 64/002 from WHO(NIBSC)

Working reagent preparation

RF calibrator: Ready to use

Procedure:

1. Bring the reagent and the photometer (curvette holder) to 37ºC
2. Assay conditions: Wavelength :650nm (600-650)
3. Temperature :37 c
4. Cuvette light path :1 cm
5. Adjust the instrucment to zero with distilled water.
6. Pipette into a curvette
7. Mix and read the absorbance immediately Abs(A1) and after 2 minutes Abs (A2) of the sample addition.

Additional sequence	Tests
R1 diluent	400µL
R2 Latex	100 µL
Calibrator or sample	5 µL

Determination of C- Reactive protein measurement [130]:

Reagent composition:

R1 Diluent	Tris buffer 20 mmol/L, pH 8.2
R2 Latex	Latex particles coated with goat anti-human, pH7.3
CRP Calibrator	Human serum CRP concentration is stated on vial label, and it is traceable to the certified Reference material ERM-DA472/IFCC

Working reagent preparation

CRP calibrator: Ready to use

Procedure:

1. Bring the reagent and the photometer (curvette holder) to 37ºC
2. Assay conditions: Wavelength :540nm (530-550)
3. Temperature :37ºc

4. Cuvette light path :1 cm
5. Adjust the instrucment to zero with distilled water.
6. Pipette into a curvette
7. Mix and read the absorbance immediately Abs(A1) and after 2-minute Abs(A2) of the sample addition.

Additional sequence	Tests
R1 diluent	400µL
R2 Latex	100 µL
Calibrator or sample	5 µL

ESTIMATION OF LIPID PEROXIDATION

Estimation of malondialdehyde (MDA):

Chemicals and reagents

- TBA-TCA-HCL reagent

Procedure

The level of lipid peroxidation in serum was measured as malondialdehyde (MDA) according to the method of Niehaus and Samuelson, 1986. About 0.1 ml of the tissue homogenate was combined with 2 ml of TCA-TBA-HCl reagent (1:1:1) (15% trichloro acetic acid (TCA) and 0.375% thiobarbituric acid (TBA) in 0.25 N HCl) and placed in water bath for 15 min, cooled and centrifuged at 100 rpm for 10 min. The precipitate was removed after cooling by centrifugation at 1000 rpm for 10 min. The absorbance of clear supernatant was measured against a reference blank at 535 nm. The levels of MDA were calculated using extinction coefficient calculation. The values are expressed as nmoles of MDA formed/min/mg protein [131].

ESTIMATION OF ENZYMATIC ANTIOXIDANTS

Estimation of superoxide dismutase (SOD):

Chemicals and reagents

- Carbonate buffer
- Epinephrine

Procedure

The activity of SOD was determined according to the method of Kakkar, 1984. To 150 µl of liver homogenate, 1.8 ml of carbonate buffer (30 mM, pH 10.2), 0.7 ml of distilled water and 400 µl of epinephrine (45mM) were added and mixed well. The inhibition of autocatalyzed adrenochrome formation in the presence of liver tissue homogenate was measured at 480 nm using a spectrophotometer. Autooxidation of epinephrine to adrenochrome was performed in a control tube without the homogenate. SOD activity was expressed as nmoles/min/mg protein [132]..

Estimation of catalase (CAT):

Chemicals and reagents

- Hydrogen peroxide
- Dichromate-acetic acid reagent

Procedure

The assay of CAT was done by the method of Sinha, 1972. The reaction mixture contained 1.0 ml of 0.01 M phosphate buffer pH 7 and 0.1 ml of tissue homogenate and was incubated at 37^{O}C for 15 min. The reaction was started by the addition of 0.4 ml of H_2O_2. The reaction is stopped by the addition of 2.0 mL dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid are mixed in 1:3). The absorbance was measured at 620 nm. CAT activity was expressed as the amount of enzyme using the decomposition of µmoles H_2O_2/min/mg protein [133].

ESTIMATION OF NON-ENZYMATIC ANTIOXIDANTS

Assay of reduced glutathione (GSH):

The activity of GSH was determined by Ellman's method. About 1.0 ml of tissue homogenate was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5,5'- Dithiobis -(2-Nitro benzoic acid) [DTNB] in 100 ml of 0.1 % sodium citrate) and 3.0 mL of phosphate buffer (0.2 M, pH-8). The absorbance was read at 412 nm using a spectrophotometer. GSH activity was expressed as nmoles/min/mg protein [134].

STATISTICAL ANALYSIS

Quantitative data were expressed as mean ± SEM and all the comparisons would be made

of one-way analysis of variance (ANOVA) followed by graph pad prism of $P < 0.05$ were considered as significant.

Results

INVITRO ANTI-ARTHRITIC ACTIVITY

Inhibition of protein denaturation:

TABLE 1: Effect of tofacitinib and tofacitib on inhibition of protein (bovine serum albumin) denaturation

Concentration (µg/ml)	**Percentage inhibition**						**Half maximal inhibitory concentration IC_{50} (µg)**
	10	**20**	**40**	**80**	**160**	**320**	
Methotrexate	27.88±0.12	35.07±0.32	46.58±0.34	52.86±0.11	63.22±0.30	75.25±0.19	**56±0.33**
Tofacitinib	33.71±0.1	42.12±0.04	51.37±0.22	64.54±0.8	72.8±0.02	83 ±0.5	**33±0.57**

All the determination were carried out in triplicate manner values are expressed as mean ±SEM

The production of the auto antigen in certain arthritic disease may be due to denaturation of protein. The inhibition of protein denaturation was studied to establish the mechanism of anti-arthritic activity of tofacitinib and methotrexate. The percentage inhibition was calculated for methotrexate and tofacitinib. A dose dependent increase in percentage inhibition was observed for standard drug and also for test drug. The percentage inhibition for methotrexate ranged from 27.88 to 75.25% and the IC_{50} was found to be at 56 µg/ml. For the tofacitinib the percentage inhibition ranged from 33.71 to 83% and its IC_{50} was found to be 33µg/ml. The percentage inhibition of tofacitinib was higher than methotrexate. The IC_{50} for tofacitinib was 33µg/ml. tofacitinib exhibits significant and concentration dependent (10-320 µg/ml) inhibition of protein denaturation with 83% effect at 320 µg/ml regarding bovine serum albumin. Whereas, methotrexate and tofacitinib shows 75.25% and 83% blockade against heat induced structural modification in bovine serum albumin.

Inhibition of protein denaturation:

TABLE 2: Effect of tofacitinib and methotrexate on inhibition of protein (egg albumin) denaturation

Concentration (µg/ml)	**Percentage inhibition**						**Half maximal inhibitory concentration IC_{50} (µg)**
	10	**20**	**40**	**80**	**160**	**320**	
Methotrexate	30.68±0.09	41±0.06	51.1±0.13	61.41±0.19	72.21±0.07	78.51±0.19	**37±0.33**
Tofacitinib	32.67±0.15	45.85±0.13	53.15±0.15	63.16±0.16	79±0.15	81.88±0.09	**28±0.54**

All the determination were carried out in triplicate manner values are expressed as mean ±SEM

The percent inhibition of methotrexate and tofacitinib was measured. Percentage inhibition increased in a dose-dependent manner. The percentage inhibition of methotrexate ranged from 30.68 to 78.51 percent, with an IC50 of 37 µg/ml. Tofacitinib percentage inhibition ranged from 32.67 to 81.88 percent, and its IC50 was found to be 28 µg/ml. Tofacitinib inhibited the denatured protein more effective than methotrexate. The IC50 of tofacitinib is 37 µg/ml. Tofacitinib decreases protein denaturation in a dose-dependent manner (10-320 µg/ml), with an 81.8% effect for bovine serum albumin at 320 µg/ml. Methotrexate and tofacitinib, on the other hand, prevent 78.51 percent and 81.88 percent of heat-induced structural changes in egg albumin, respectively.

INVIVO ANTI-ARTHRITIC ACTIVITY

TABLE 3: The Levels of Rheumatoid factor were Suppressed by tofacitinib treatment

GROUPS	RHEUMATOID FACTOR((IU/ml)
GROUP 1 (0.5%CMC)	14.842±4.6
Group2 (Negative control)	72.730±5.8#
GROUP3 (Zeitgeber time 0)	22.676±4.5*
GROUP4 (Zeitgeber time 12)	30.274±2.71*
GROUP5 (Positive control)	35.042±5.06*

Value are expressed in mean ±SEM, n=6
Data were analysed by one way ANOVA followed by Dunnett's test.

In Negative control group, #p<0.01 with Normal control
In treatment groups, *P<0.01 compared to negative control

Rats treated with complete Freund's adjuvant had statistically significant elevated level of serum rheumatoid factor in disease control group as compared to normal control group. Treatment with tofacitinib and methotrexate to diseased animal showed statistically significant reduction in levels of rheumatoid factor. The group 2 ranged with 72.73 IU/ml, group 3 ranged with 22.67 IU/ml, group 4 ranged with 30.27 IU/ml and group 5 ranged with 35.04 IU/ml.

When group 3 (ZT0) treated showed reduced level of serum RF than the group 4(ZT12).

It depicts that ZT0 treated group shows significantly decreased and had a better activity.

The effects of tofacitinib were dose time dependent had equipotent effects in decreasing the serum RF levels.

TABLE 4: Effect of Tofacitinib Based Chronotherapy Of 24 Hours Variation of CRP Measurement

GROUPS	ZT4	ZT10	ZT16	ZT22
GROUP 1 (0.5% CMC)	2.578±0.31	2.638±0.33	2.376±0.2	2.356±0.2
Group 2 (Negative control)	17.96±1.32#	15.662±1.13#	8.562±0.9 #	12.582±1.2#
GROUP3 (Zeitgeber Time 0)	3.480±0.43*	3.658±0.49*	3.710±0.4*	3.644±0.38*
GROUP4 (Zeitgeber Time 12)	4.830±0.44*	4.590±0.35*	4.712±0.4*	4.590±0.35*
GROUP5 (Positive Control)	5.118±0.55*	5.36±0.45*	5.120±0.3*	5.038±0.6*

Value are expressed in mean ±SEM, n=6
Data were analysed by one way ANOVA followed by Dunnett's test.

In Negative control group,# p<0.01 with Normal control
In treatment groups, *P<0.01 compared to negative control

Circadian variations of serum CRP productions in Complete Freund's Adjuvant models

In untreated Complete Freund's Adjuvant *wistar* rat, the serum CRP was measured that reached maximum levels at ZT4 when compared with the ZT16, ZT22 and ZT10.

The lowest expression of all serum CRP accumulated in ZT16, ZT22 and ZT10 time zone when compared with ZT4. In untreated group, serum CRP was significantly raised from ZT22 ~ 10, especially peaked at ZT4. In ZT0 treated group, serum CRP level were completely suppressed throughout the observation period (table 4).

To evaluate the effects of tofacitinib treatment on inflammatory serum CRP, we first evaluated serum levels of CRP in blood. In all experimental groups, productions of serum CRP showed fluctuations in 24hrs period. Especially in ZT0 treated group, CRP level was significantly decreased as compared to CFA untreated group at ZT4.

Second, in CFA untreated *wistar* rats, CRP in sera was significantly elevated as compared to control group at ZT22 ~ ZT4(table 4). In ZT0 treated group, serum CRP was significantly decreased as compared to CFA untreated group at ZT4, ZT10, ZT16 and ZT22 than ZT12. Low concentration of serum CRP was also determined at ZT16.

The Levels of CRP were Suppressed by tofacitinib treatment

CRP in sera were measured by CRP turbilatex method at four different intervals of time i.e. ZT 4, 10, 14 and 22 on day 21. The serum CRP are markers of systemic inflammation and antibody production against the injected adjuvant. Levels of serum CRP for negative group - 17.96 mg/dL at ZT4,15.66 mg/dL at ZT10, 8.56 mg/dL and 12.58 mg/dL at ZT 10. The serum CRP level raised at ZT22~ ZT4, when compared with 4 different intervals of time. At the same time group 3 (ZT0) treated group suppression the serum CRP level activity than group 4 (ZT12). It depicts that The ZT 0 treatments reduced the increase in the levels of CRP in the serum (table 4). The effects of tofacitinib were dose time dependent, and the 5mg/kg dose of tofacitinib and had equipotent effects in decreasing the serum CRP level.

TABLE 5: Effect of the tofacitinib on body weight in rats

GROUPS	Day 1	Day 7	Day 14	Day 21
GROUP 1 (0.5%CMC)	193±20.4	195±21.6	200.2±22.23	204±23.5
Group2 (Negative control)	207.8±25.4	210±24.6$^{\#}$	208±24.6$^{\#}$	201±23.65$^{\#}$
GROUP3 (Zeitgeber time 0)	194±15.5	196±15.6*	199±15.8*	201±15.5*
GROUP4 (Zeitgeber time 12)	193±19.2	197±19.2*	198±19.7*	200±19.7*
GROUP5 (Positive control)	192±24.9	191±24.7*	187±25.1*	184±25.5*

Value are expressed in mean ±SEM, n=6
Data were analysed by one way ANOVA followed by Dunnett's test.

In Negative control group, $^{\#}$ p<0.01 with Normal control
In treatment groups, *P<0.01 compared to negative control

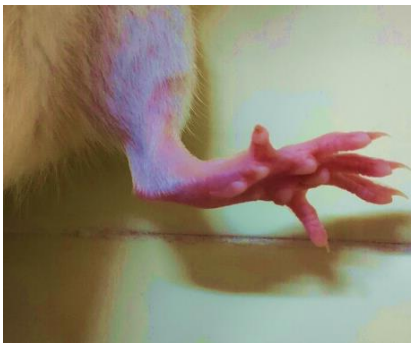
Measurement of body weight

The body weights of all the animals in group-1 to group-5 were recorded on 1st day,7th day,14th day and 21th day (each week up to 3 weeks). There was an increase in body weight in all the treatment groups and the change in body weight for negative control.

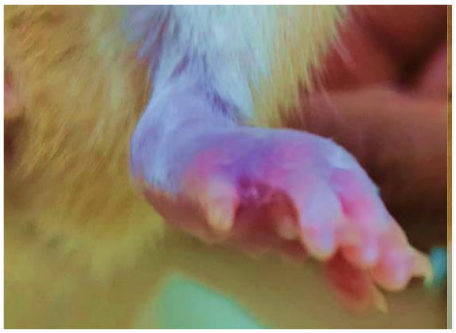
The average gain in the body weight on day 21st as compared with the initial body weight in each treatment group has been given in table 5. The rats in the CFA control group gained less body weight as compared with the group 3 and group 4 treated groups.

There was less body weight gain of FCA control rats as compared to vehicle control rats, due to generation of immune response. In tofacitinib-5mg/kg (ZT0 and ZT12) and methotrexate treated arthritic rats significant weight gain was observed as compared to disease control animals.

This effect on the body weight was clearly evident that ZT0 treated group is better when compared with ZT12. The results shows that the drug treated at ZT0 group produce a better effect.



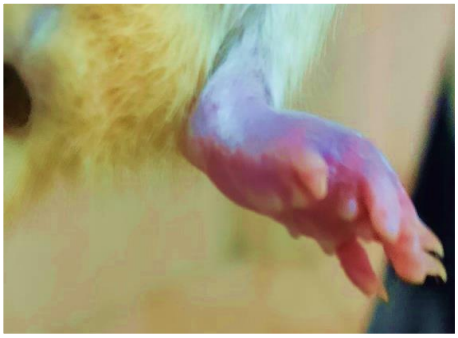
GROUP 1
0.5%CMC



GROUP 2
Negative control-
Complete Freund's adjuvant

GROUP 3
Zeitgeber Time 0-
Tofacitinib(5mg/Kg)

GROUP 4
Zeitgeber Time 12-
Tofacitinib(5mg/Kg)



Group 5
Standard group-
methotrexate(1mg/kg)

Fig 4: left hind paw of the drug treated rats after induction of arthritis

TABLE 6: Effect of tofacitinib on CFA induced arthritis model Primary lesions was measured

GROUPS	PRIMARY LESIONS	PERCENTAGE INHIBITION (%)
GROUP 1 (0.5%CMC)	0.25±0.07	-
GROUP 2 (Negative control)	0.86±0.11$^{\#}$	-
GROUP 3 (Zeitgeber time 0)	0.54±0.05*	37.23±0.14
GROUP 4 (Zeitgeber time 12)	0.64±0.05*	25.55±0.15
GROUP 5 (Positive control)	0.74±0.05*	13.95±0.05

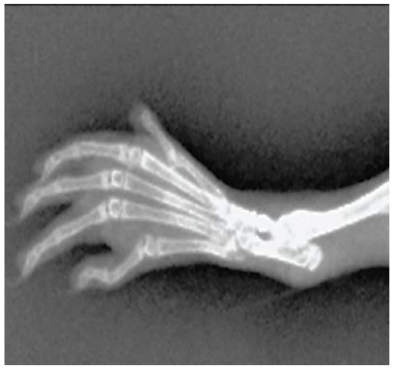
Value are expressed in mean ±SEM, n=6

Data were analysed by one way ANOVA followed by Dunnett's test.

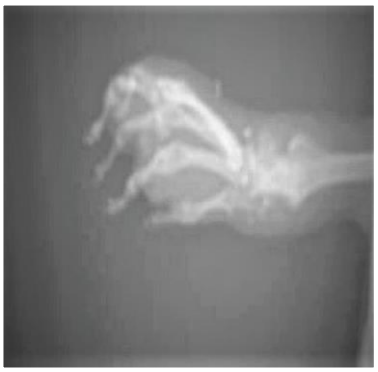
In Negative control group, $^{\#}$ p<0.01 with Normal control

In treatment groups, *P<0.01 compared to negative control

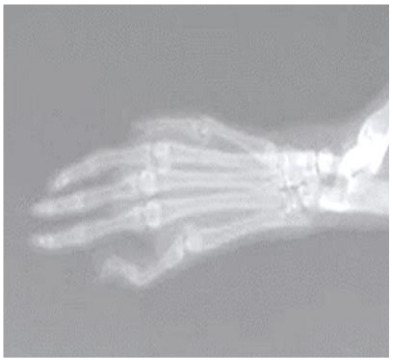
Observations such as the paw volumes were recorded on the 5th days after adjuvant injection. The CFA-induced arthritis control group showed signs of arthritis development, as seen by the increase in the paw volumes in CFA-injected, which indicates primary arthritic lesions. The group 2 shows elevated paw volume. When compared with group 3 and group 4 treated. The group shows reduced paw volume than other groups showed in a table 6.



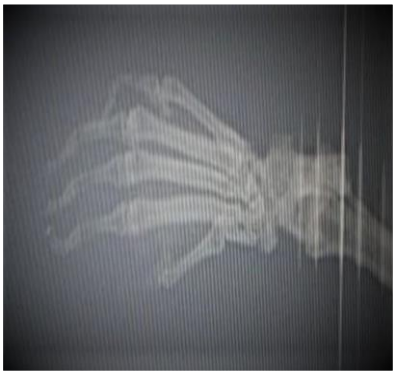
GROUP 1
0.5%CMC



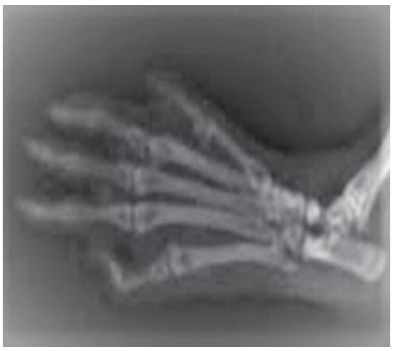
GROUP 2
Negative control-
Complete Freund's adjuvant



GROUP 3
Zeitgeber Time 0-
Tofacitinib(5mg/Kg)



GROUP 4
Zeitgeber Time 12-
Tofacitinib(5mg/Kg)



Group 5
Standard group-
methotrexate(1mg/kg)

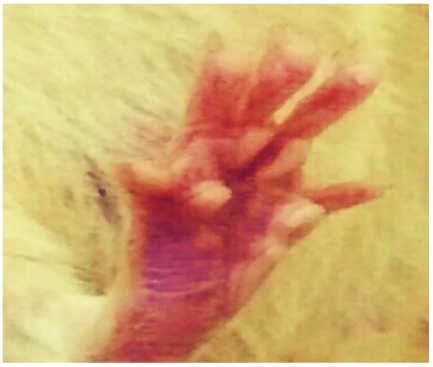
Fig 5: X-Ray of left hind paw of the drug treated rats after induction of arthritis

Tofacitinib Protected the Rats from CFA-Induced Radiographic Changes.

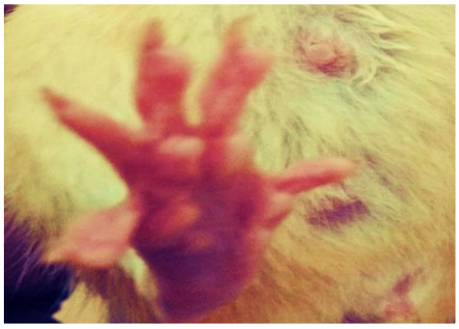
Figure 5 shows representative photographs of the tarsotibial joint swelling of the right hind paws of rats from different groups on the 21st day after CFA injection. It is clearly observed in the X-rays that the soft tissue swelling around the joints, periarticular bone resorption, periarticular bony erosions and joint space narrowing in the rats treated with tofacitinib have been protected from the CFA-induced arthritis-related joint changes.

The x-ray photograph of the rats hind paw depicts that after treatment with ZT0, ZT12 and standard (Methotrexate) a marked reduction joint damage could be observed in rats injected with Freund's adjuvants. The results of this study were compared with negative control group where a marked damage could be observed in hind leg when checked with X-ray photograph against the control which did not receive any treatment and treatment group of rats.

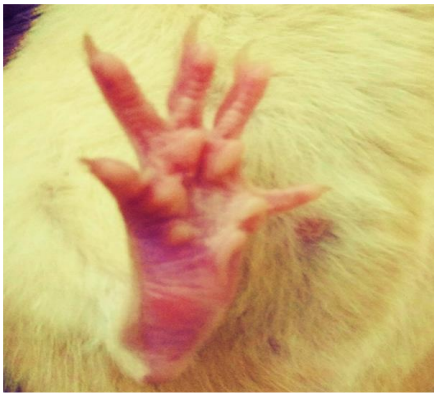
GROUP 1
0.5%CMC



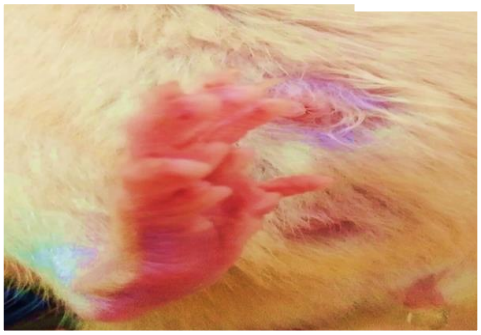
GROUP 2
Negative control-
Complete Freund's adjuvant



GROUP 3
Zeitgeber Time 0-
Tofacitinib(5mg/Kg)



GROUP 4
Zeitgeber Time 12-
Tofacitinib(5mg/Kg)



Group 5
Standard group
methotrexate(1mg/kg)

Fig 6: Secondary lesions of non-injected right hind paw of the drug treated rats after induction of arthritis

TABLE 7: Tofacitinib Reduced Secondary Arthritic Lesions in Rats.

GROUPS	SECONDARY LESIONS	PERCENTAGE INHIBITION (%)
GROUP 1 (0.5%CMC)	-	-
GROUP 2 (Negative control)	0.39±0.03#	-
GROUP 3 (Zeitgeber time 0)	0.29±0.02*	25.64±0.04
GROUP 4 (Zeitgeber time 12)	0.31±0.01*	20.51±0.16
GROUP 5 (Positive control)	0.34±2.4*	12.82±0.35

Value are expressed in mean ±SEM, n=6

Data were analysed by one way ANOVA followed by Dunnett's test.

In Negative control group, # p<0.01 with Normal control

In treatment groups, *P<0.01 compared to negative control

Tofacitinib Reduced Secondary Arthritic Lesions in Rats

Observations such as the paw volumes were recorded on the 14th days after adjuvant injection. The CFA-induced arthritis control group showed signs of arthritis development, as seen by the increase in the paw volumes in CFA-non-injected paws, which indicates secondary arthritic lesions.

The assessment made on the 21st day showed that the group3, group4 and group 5 treatments had significantly reduced the secondary lesions as compared with the CFA control group (Table 7). It is noteworthy that the reduction in the secondary lesions was comparable in the group3, group4 and group5 treated groups.

Table 8: Measurement of paw volume in rats treated with tofacitinib

Group	Day 1	Day 5	Day 7	Day 14	Day 21
GROUP 1 (0.5%CMC)	0.24±0.05	0.25±0.07	0.25±0.13	0.24±0.23	0.24±0.31
GROUP 2 (Negative control)	0.28±0.08$^{\#}$	0.86±0.11$^{\#}$	0.82±0.17$^{\#}$	0.79±0.15$^{\#}$	0.75±0.2$^{\#}$
GROUP 3 (Zeitgeber time 0)	0.28 ±0.08*	0.54±0.05*	0.49±0.06*	0.45±0.05*	0.42 ±0.04*
GROUP 4 (Zeitgeber time 12)	0.28±0.16*	0.64±0.05*	0.58±0.09*	0.51±0*	0.49±0*
GROUP 5 (Positive control)	0.24±0.05*	0.74±0.05*	0.69±0.1*	0.65±0.1*	0.58±0.05*

Value are expressed in mean ±SEM, n=6

Data were analysed by one way ANOVA followed by Dunnett's test

In Negative control group, $^{\#}p < 0.01$ with Normal control

In treatment groups, *P<0.01 compared to negative control

Measurement of paw oedema

Arthritis was induced by complete Freund's adjuvant in female *wistar* rats after anesthetized with diethyl ether by a single intra-dermal injection (0.1 ml) of Complete Freund's Adjuvants (CFA) into the foot pad of the left hind paw. The hind paw volume of both legs was recorded for the experimental animals. Drugs were administered orally and treatment was given to all groups after they were challenged with CFA for 21 days. The drug administration was done every day upto 12 days in the morning (ZT8) and evening (ZT20) and methotrexate was administered once weekly for all the respective groups of rats and the change in paw volume were recorded on 0^{th}, 5^{th} ,7^{th} 14^{th} and 21^{st} day.

Tofacitinib were administered at two different times ZT0 and ZT12. There was a significant change in the mean paw volume in rats treated with ZT0 and ZT12 of tofacitinib. The paw volume increased gradually after intradermal injection with CFA and the paw volume was found to be 0.86ml for negative control group rats and ZT0 treated group rats it was found to be 0.54ml, while rats which received ZT12 treated drugs the value was 0.6ml and for standard drug treatment group paw volume was at 0.74ml respectively on the 5^{th} day when compared with 0^{th} day. The paw volume started to decrease after 7^{th} day of treatment in all the groups significantly and a very good reduction was noticed for ZT0 treated group animals and the mean paw volume was lower for ZT0 and ZT12 treated group when compared to standard methotrexate treatment group. The mean paw volume further reduced to 0.49 and 0.58 ml on 14^{th} day in rats treated with ZT0 and ZT12 and like on 7^{th} day the mean change in paw volume was found to be lower for ZT0 and ZT12 treated group when compared to standard group (0.69ml). A similar remarkable reduction in paw volume was noticed on 21^{st} day in rats which received with ZT0 and ZT12 treated group. The paw volume was found to be 0.42 ml for ZT0 treated group. In all the drug treated group there was a significant reduction in paw volume when compared to negative control group which did not receive drug but were treated with 0.5% CMC. A marked reduction in the paw volume is observed in the rats after treatment with ZT0, ZT12 and standard drug treated group for 21 days. The photograph of the left hind paw of treated rats is shown in the fig 4.

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Table 9: Percentage inhibition of paw volume in rats treated with tofacitinib

Group	Day 1	Day 5	Day 7	Day 14	Day 21
GROUP 1 (0.5%CMC)	-	70.93±0.34	69.91	69.62±0.54	68±0.05
GROUP 2 (Negative control)	-	-	-	-	-
GROUP 3 (Zeitgeber time 0)	-	37.23±0.14*	40.24±0.15*	43.03±0.14*	44±0.22*
GROUP 4 (Zeitgeber time 12)	-	25.55±0.15*	29.26±0.15*	35.44±0.16*	34.66±0.2*
GROUP 5 (Positive control)	-	13.95±0.05*	15.85±0.06*	17.72±0.11*	22.66±1.2*

Value are expressed in mean ±SEM, n=6

Data were analysed by one way ANOVA followed by Dunnett's test.

In Negative control group, #p <0.01 with Normal control

In treatment groups, *P<0.01 compared to negative control

Measurement of percentage inhibition in paw volume

In all the drug treated group (Group1 to Group 5) there was a proportionate increase in paw volume on 5th day and the percentage inhibition in paw edema was found to be 37.23,25.55 and 13.95 in ZT0, ZT12 and standard treated groups respectively. The percentage inhibition in paw edema increased on 7th day to 40.24, 29.26 and 15.85 and values were found to be significant. In 14th day the percentage inhibition paw edema further increased to 43.03, 34.66 and 22.66 respectively. Maximum percentage inhibition in paw edema was observed for ZT0 treated group from 5th day to 21th day. On 21st day the percentage inhibition of paw edema was found to be increase to 44, 34.66 and 22.66 respectively. The percentage inhibition in paw edema was higher in ZT0 group when compared with ZT12 and standard treated group.

TABLE 10: Effect of the tofacitinib on total protein and MDA in liver

GROUPS	TOTAL PROTEIN	LIPID PEROXIDATION MALONDIALDEHYDE (mmole/min/mg)
GROUP 1 (0.5%CMC)	372.9±35.8	14.75±3.8
Group2 (Negative control)	108.5±3.7#	80.25±6.7#
GROUP3 (Zeitgeber time 0)	180.4±38.4*	34.5±2.5*
GROUP4 (Zeitgeber time 12)	184.7±33.8*	36.25±2.5*
GROUP5 (Positive control)	149.1±20.1*	46±3.7*

Value are expressed in mean ±SEM, n=6

Data were analysed by one way ANOVA followed by Dunnett's test.

In Negative control group, # p<0.01 with Normal control

In treatment groups, *P<0.01 compared to negative control

The above table shows the impact of tofacitinib on the levels of MDA in serum of various experimental rats. Assessment of MDA levels in various experimental groups dictate that MDA levels were elevated in arthritis induced animals. On tofacitinib-5mg/kg (ZT0 and ZT12) and methotrexate supplementation, the MDA levels were markedly brought down in group 3, group 4 and group 5 rats in comparison with CFA alone administered rats. When comparing the ZT0treated group with ZT12, ZT0 shows a highly reduced when compared with ZT12. It tells that the ZT0 have a higher anti-arthritic activity and anti-oxidant property in a dose time dependent manner.

TABLE 11: Effect of the tofacitinib on catalase, SOD and GSH in liver

GROUPS	ENZYMATIC ANTIOXIDANTS		NON-ENZYMATIC ANTIOXIDANTS
	SUPEROXIDE DISMUTASE (µmole/min/mg)	CATALASE (mmole/min/mg)	REDUCED GLUTATHIONE (mmole/min/mg)
GROUP 1 (0.5%CMC)	93.75 ±4.9	96.5±2.3	89.25±0.11
Group2 (Negative control)	23.5 ±3.5$^{\#}$	26.5±3.8$^{\#}$	27.26±0.26$^{\#}$
GROUP3 (Zeitgeber time 0)	74.250±3.5*	64±3.5*	62.45±0.25*
GROUP4 (Zeitgeber time 12)	64.75±2.5*	55±2.94*	59.94±0.24*
GROUP5 (Positive control)	56.50±3.1*	54±4.2*	52.45±0.27*

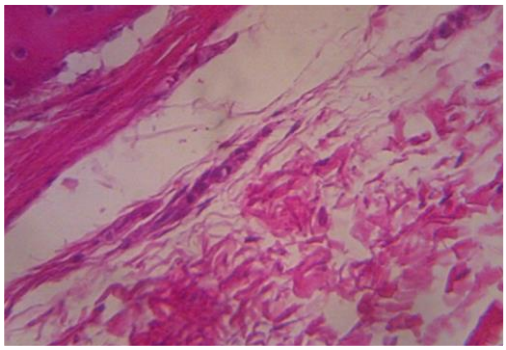
Value are expressed in mean ±SEM, n=6
Data were analysed by one way ANOVA followed by Dunnett's test.

In Negative control group,$^{\#}$ p<0.01 with Normal control
In treatment groups, *P<0.01 compared to negative control

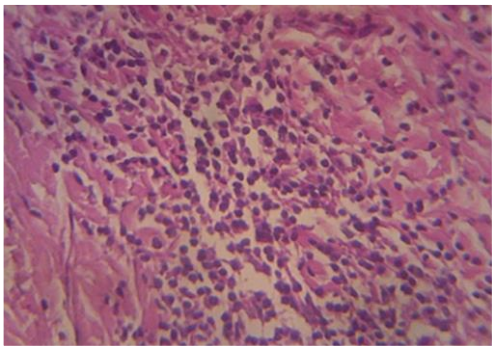
Table 11 shows the activity of antioxidant enzymes SOD, CAT and GST levels are significantly (decreased in Group 2 compared to Group 1, Group 3 and 4 (ZT0 and ZT12, respectively).

Table 11 depicts that the level of GSH is reduced significantly in Group 2, compared to Group 1 and after treatment with tofacitinib, the level is restored significantly near to normally both in Group 3 and Group 4. Elevation of GSH level is more in Group 4 than Group 3 and in case of Group 5 the level is significantly increased compared to Group 2. Comparatively, group 3 showed a higher antioxidant activity when compared with all the treated groups.

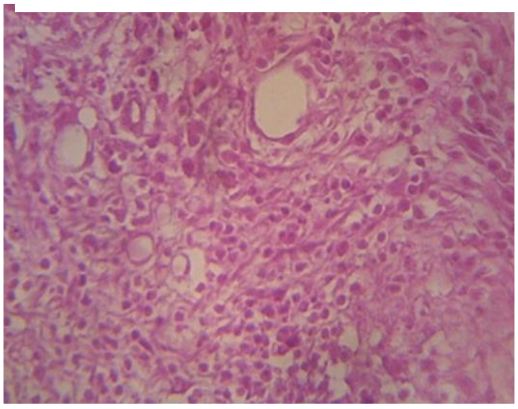
Table 11 portrays that the levels of CAT, were reduced significantly in Group 2 compared to Group 3,4and 5. which show significantly elevated levels seen in group 3. The above results reveal that ZT0 (Group 3) significantly regulated the biochemical alterations compared to ZT12 (Group 4) and hence it could be more effective in affording protection against the arthritic model.



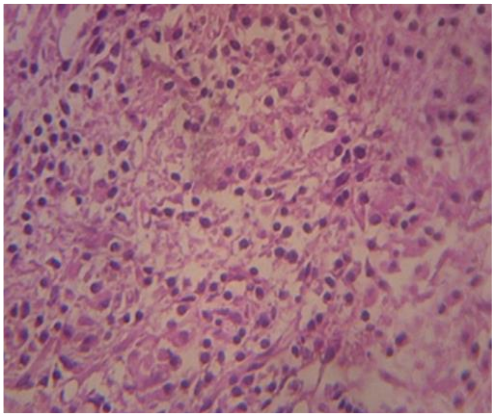
GROUP 1
0.5%CMC



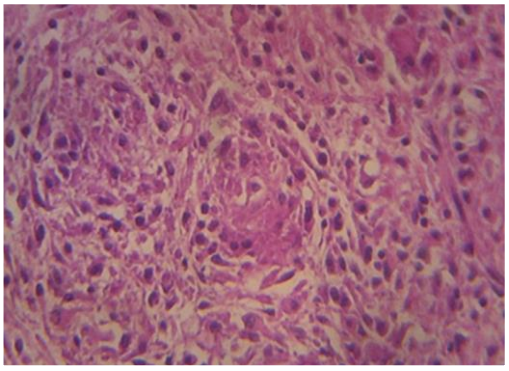
GROUP 2
Negative control-
Complete Freund's adjuvant



GROUP 3
Zeitgeber Time 0-Tofacitinib(5mg/Kg)



GROUP 4
Zeitgeber Time 12-Tofacitinib(5mg/Kg)



Group 5
Standard group methotrexate(1mg/kg)

Figure 7: Histology of the arthritis developing 21 days after immunization with FCA compared with unimmunized complete Freund's adjuvant.

Histopathological data showed CFA-induced arthritis reflected in intense subcutaneous changes associated with granulomatous inflammation. Group1 compared with group 2 it shows significantly increased when compared with group 1. The group 3(ZT0) and group4 (ZT12) reduced these features of inflammation in RA rats, including infiltration of inflammatory cells, and disruption of cartilage. Suppression of disease progression suggests that group 3(ZT0) and group 4(ZT12) might prevent arthritic progression by reducing joint inflammation reactions and paw tissue destruction.

Discussions

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by polyarthritis and joint destruction. Patients with RA demonstrate a characteristic diurnal variation of disease symptoms, including joint stiffness early in the morning, peaked secretion of rheumatoid factor at midnight or sleep disorder. Particularly, this diurnal variation is present in the production of inflammatory cytokines that interleukin (IL)-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ show their peak concentrations during mid night in sera of patients with RA[110].

Thus, in this study, we demonstrated robust 24 h variation of inflammatory mediator secretion in complete Freund's adjuvant model and examined drug efficacies according to administration time of disease modifying anti-rheumatic drugs (DMARDs). Especially about CFA, in comparison with humans, it is reported that rodents exhibit exactly opposite patters in inflammatory mediator secretion since they are nocturnal animals, In which serum CRP levels increase or decrease with a significant 24hr rhythm. Janus kinase family of protein tyrosine kinases (JAKs) are composed of four JAK family members, JAK1, JAK2, JAK3 and Tyk2. Two of four types of JAKs are recruited to bind in various combinations to the intracellular domain of Type I / Type II cytokine receptors.

The anti-denaturation study for investigating anti- arthritis activity was performed using bovine serum albumin (BSA). When BSA is heated, it undergoes denaturation and antigens are expressed which are associated with type-III hypersensitivity reaction, which in turn is related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus. Denaturation of protein is one of the causes of rheumatoid arthritis. Production of autoantigen in certain arthritic diseases may be due to denaturation of protein. The mechanism of denaturation probably involves alteration of electrostatic, hydrogen, hydrophobic and disulphide bonds. In this study, the *in vitro* study tells that when comparing a methotrexate with tofacitinib, tofacitinib shows a better inhibitory effect on the protein denaturation [135].

CFA-induced arthritis is the most widely used chronic test model in which the clinical and pathological changes are comparable with those seen in human rheumatoid arthritis. Chronic inflammation in the CFA model is manifested as a progressive increase in the volume of the injected paw. The volume of the injected paw was comparable with that of ZT0 vs ZT12 (Tofacitinib 5 mg kg-1 day-1). The ZT0 shows a better inhibitory activity, when compared with

ZT12.

Hence this study was undertaken to find out the anti-rheumatoid activity of tofacitinib (dose time dependent) in FCA-induced rheumatoid arthritis in female *wistar* rats. FCA-induced arthritic rat is the most widely used experimental model for RA as it closely mimics the features of human rheumatoid disease. Administration of FCA produced changes in rats similar to what would occur in humans with RA such as joint swelling (paw swelling), restricted joint movement, biochemical and histological changes. Paw swelling, serum CRP and rheumatoid factor are indices of rheumatoid activity. TNF-a, IL-6 and IL-1 are well known inflammatory mediators of RA.

Reduction of oxidative stress markers (SOD, LPO and CAT) in the joint tissue of arthritic rats shows that the antioxidant property of tofacitinib has additionally contributed to its anti-arthritic efficacy. ROS are generated when pathogens (bacteria) or immune complexes are ingested by activated phagocytic cells such as polymorphonuclear leucocytes or macrophages and form an oxidative burst that produce highly toxic ROS to kill those pathogens. More oxygen is consumed during the formation of oxidative burst which is mediated by NADPH oxidases system resulting in the production of superoxide radical. Another strong oxidant, H2O2, is formed spontaneously from superoxide radical, and hydroxyl radical is formed by Fenton's reaction between H2O2 and Fe+2 (ferrous ion).

Apart from these ROS, several other oxidants such as nitric oxide radical, peroxynitrite radical are also similarly responsible for oxidative stress in adjuvant induced arthritis. ROS associated tissue damage can be measured by lipid peroxidation product. It has been well documented that ROS peroxidize the polyunsaturated fatty acid (PUFA) of cell membrane causing lipid peroxidation of which MDA is the end product and it plays an important physiological role in membrane destabilization. Literature data indicate that MDA is increased in CFA-induced oxidative stressed rats and it is due to lipid peroxidation of membrane which is in line with our study. The treatment with tofacitinib (Dose time dependent) decreased the MDA level and protected structural integrity of the cell membrane which might be due to its antiperoxidative effect.CFA-induced arthritis in rats is associated with an increase in the plasma levels of RF and CRP. The treatment with tofacitinib (Dose time dependent) significantly reduced the levels of these biomarkers of inflammation and autoimmune stimulation in the treated rats.

Other miscellaneous information related to the pathology of arthritis that has been obtained during this study includes radiographic examination of the paws, body weight changes, organ weight changes and paw withdrawal latency. The radiographic observations of the rats show that the treatment with tofacitinib inhibited the arthritis-associated joint changes. In the tofacitinib treated groups there was restoration of the body weights of the rats.

Anti-oxidant are the compounds of exogenous or endogenous in nature which either prevent the generation of free radicals or interrupt any that are generated and inactivate them and thereby block the propagation of chain reaction produced by these oxidants. Recently, various studies provided evidences for the role of free radicals in pathological conditions including rheumatoid arthritis. Most of the researcher have reported that inhibition of Freund's adjuvants- induced arthritis in *Wistar* rats in one of the most suitable test models to screen anti-arthritic drugs since it more closely resembled human arthritis. Freund's adjuvant induced arthritis is thought to occur through cell mediated autoimmunity and structural similarity between mycobacteria and cartilage proteoglycan in rats. Freund's adjuvant induced arthritis is used as a model of sub-chronic and chronic inflammation in rats and of considerable relevance for the study of pathophysiology and pharmacological control inflammatory processes. In our study, the arthritis was very stable with inflammatory signs. The inflammatory response was initially developed within few hours, but more critical signs of arthritis were seen on 1st week of post-inoculation and thereafter for three weeks. Body weight was considered as an indirect index of health status and recovery from disease. A dramatic cessation of growth and decline in body weight was indicated in control group of amimal from first week of study. Significant restoration and gain in body weight was evident, when treated with ZT0, ZT12 and standard treated group [(136)].

In our study the ZT0 and ZT12 treated group exhibit a significant anti-arthritic activity in a dose time dependent manner. There was a significant reduction in the paw volume in Freund's adjuvant induced arthritic rats treated with ZT0 and ZT12 group. Reduction of paw swelling in the ZT0 and ZT12 treated rats from the second week onwards may be due to immunological protection rendered by the ZT 0and ZT12 in dose time dependent manner. This finding justifies the usefulness of this product in the management and treatment of inflammation associated disease like arthritis. The pathogenesis or reasons for development of arthritis following injection of FCA were not clear and fully understood. Numerous studies

have contributed to the understanding of various possibilities including reactivity to cartilage proteoglycans, heat shock protein and interaction with intestinal flora. Freund's adjuvant induced arthritis is an experimental model that shares several clinical and pathological features with rheumatoid arthritis. The paw volume was significantly increased in 1st week, and maintained throughout 3rd week of study in negative control group, but decline in paw volume was observed in rats treated with tofacitinib 5mg/kg as benefit at ZT0 treated group when compared with ZT12 treated group was observed on last day (21st day)

The initial reaction of edema and soft-tissue thickening at the depot site in this model is caused by the irritant effect of the adjuvant, whereas the late-phase arthritis and flare in the injected foot are presumed to be immunologic events. The appearance of secondary lesions, that is, non-injected paw swelling is a manifestation of cell-mediated immunity. The suppression of such secondary lesions by a drug shows its immunosuppressive activity. Tofacitinib effectively reduced the secondary lesions in arthritic rats. Moreover, this effect of tofacitinib (dose time dependent) was more potent than that of methotrexate. This reveals potent suppression by tofacitinib of cell-mediated immunity in arthritic rats. Similarly, it reduced the arthritic score and secondary paw swelling. The reduction of the arthritis score by tofacitinib (dose time dependent) as observed in our study indicates a possible immunosuppressant effect.

CFA-induced arthritis in rats is associated with an increase in the plasma levels of RF and CRP. The treatment with tofacitinib significantly reduced the levels of these biomarkers of inflammation and autoimmune stimulation in the treated rats.

Conclusion

CONCLUSION

In this study, we examined characteristic diurnal features of serum CRP in CFA and alsopaw volume measurement, histopathological measurements, RF value measurement, and X-ray imaging and showed that theoptimal administration of tofacitinib based on circadian rhythm of cytokine secretion produced better activity. Chronotherapy further enhances the efficacy of drugs, and also enables the increased drug potencies. Lifetime medical expenses for RA patientswith advanced joint destruction are usually high due to drug costs, surgical treatments, rehabilitations or home renovation costs. In this point of view, our presenting results suggest that night time administration of tofacitinib for patients with RA not only provide a superior drug efficacy but also achieve drug dose reduction. The results indicated that serum CRP levels in CFA rats were higher than in normal rats and showed a significant reduction in paw volume and other biochemical markers or rheumatoid arthritis. The RA therapeutic score may be improved byadministering tofacitinib at night time alone based on the principle of chronotherapy so as to better target time-based excursion of serum CRP.

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Annexures

IAEC Protocol approval certificate

INSTITUTIONAL ANIMAL ETHICS COMMITTEE

(CPSCEA Registration # 1559/PO/Re/S/11/CPCSEA)

College of Pharmacy

Sri Ramakrishna Institute of Paramedical Sciences

(Educational Service of M/s SNR Sons Charitable Trust)

Coimbatore – 641 044.

IAEC PROTOCOL APPROVAL CERTIFICATE

Date: 14/06/2021

Approval: 1559/PO/Re/S/11/CPCSEA dated 30.01.2018

IAEC PROTOCOL: COPSRIPMS/IAEC/PG/Pharmacology/006/2021-2022

IAEC PROTOCOL TITLE: Chronotherapy of Janus kinase inhibitors in Complete *Freund's* adjuvant-induced rheumatoid arthritis using *Wistar* rats

Dear Mr. Subashraj P,

This is to certify that above mentioned animal study protocol has been approved in IAEC meeting held on 14/06/2021 with following conditions:

Principle Investigator	:	Mr. Subashraj P
Duration of Study	:	Months/years (From 14/06/2021 to13/06/2022)
Animal Sanctioned	:	30 *Wistar* Rats
Species	:	Rats
Strain	:	*Wistar*
Sex/Age	:	Male (30 Rats) / 12 Weeks
Total No.	:	30

It is requested to get prior approval of IAEC in case of any deviation/changes in submitted protocol. Please maintain the Form D & provide the photocopy to IAEC along with project report at defined interval.

Member Secretary
IAEC, COP, SRIPMS

Chairman
IAEC, COP, SRIPMS

Main nominee
CPCSEA

K.K. COLLEGE OF PHARMACY

1/161 Sankaralinganar Road, Gerugambakkam(Near Porur), Chennai-128
Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Guindy, Chennai
Approved by the AICTE & PCI, New Delhi & Govt. of Tamil Nadu

CERTIFICATE OF PARTICIPATION

This Certificate is awarded to

Mr. P. Subash Raj

For attending the Webinar Series-5 on the topic: **"DRUG DISCOVERY AND REPURPOSING STRATEGIES"** by **Prof. Dr. S. KAVIMANI** organized by K.K. College of Pharmacy, Chennai on 25th June, 2020. His/Her participation is highly appreciated.

Dr. A. MEENA
Principal

Dr. V. VAIDHYALINGAM
Co-ordinator

Prof. KR. ARUMUGAM
Chairman

Dr. A. SHANTHI
Co-ordinator

Dr. B. PREMKUMAR
Organizing Secretary

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In Association with

Pharmacy Council of India, New Delhi

CERTIFICATE OF PARTICIPATION

This is to certify that **Mr. P. Subash Raj** *has participated in the National Webinar on "Honing Your Job Skills for a Post Pandemic World" organized by SRM College of Pharmacy, SRM Institute of Science and Technology in association with Pharmacy Council of India, New Delhi held on 22nd, June 2020.*

Dr.K.S.Lakshmi	**Dr. A.Ravi Kumar**	**Dr.B.Suresh**
Dean	**Pro Vice Chancellor**	**President PCI, New Delhi &**
SRM College of Pharmacy	**Medical, SRMMCH & RC**	**Pro Chancellor, JSS AHER**