"INVESTIGATION ON EFFECT OF COMBINATION THERAPY OF FLUOXETINE AND DEXAMETHASONE IN EXPERIMENTAL MODELS OF RHEUMATOID ARTHRITIS"

A THESIS SUBMITTED TO

NIRMA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHARMACY

IN

PHARMACOLOGY

BY

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CERTIFICATE

This is to certify that ***Mr. Shreyans K Patel*** *has prepared his thesis entitled "Investigation On Effect Of Combination Therapy Of Fluoxetine And Dexamethasone In Experimental Models Of Rheumatoid Arthritis" in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under our guidance. He has carried out the work at the Department of Pharmacology, Institute of Pharmacy, Nirma University.*

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DECLARATION

I declare that the thesis entitled "Investigation On Effect Of Combination Therapy Of Fluoxetine And Dexamethasone In Experimental Models Of Rheumatoid Arthritis" has been prepared by me under the guidance of Dr. Shital J. Panchal, Assistant Professor, and Dr. Bhoomika R. Goyal, Assistant Professor, Department of Pharmacology, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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Acknowledgement

Though words are seldom sufficient to express gratitude and feelings, it somehow gives me an opportunity to acknowledge those who helped me during the tenure of my study.

First of all, I want to submit my deep pray to god whose blessings remained with me from beginning of my research work and dissertation.

I would like to thank ***Dr. Shital J Panchal*** *and* ***Dr. Bhoomika Goyal*** *for their keen observations and constructive criticism as well as continues encouragement and everlasting support which helped me to recognize my mistakes and for providing me with the insights and guidance to overcome the obstacles and follow the right path in my research work. I am grateful to* ***Mrs. Shraddha Bhadada*** *and* ***Mr. Shashank Patel*** *for their valuable suggestions which helped this work to acquire the shape what it has today. It is a crucial relief from* ***Mrs. Jignasa Savjani*** *in acquiring Fluoxetine for my work and I am obliged indeed to her.*

With great respect, I express special gratitude ***to Dr. Manjunath Ghate , Dr. Anuradha Gajjar, Dr. Priti Mehta, Dr. Sanjiv Acharya, Dr. Tejal Shah*** *and* ***Dr. Avani F Amin*** *have provided all necessary help and facilities for my work. I would like to thank them for their guidance and helpful comments.*

I thank Mr. ***Dipesh Patel*** *for providing all the requirements for my research on time and for making the laboratory much more than just a laboratory.*

I acknowledge with golden words, my special friends ***Saurabh, Vipul, Preeti, Tej Pratap, Shruti,Kushal, Sagar, Neel, Akshay, Ronak, Ankit, Naishadh, Chintan, Mahek, Saumin, Disha, Janki, Nisarg, Vidip, Dhara, Kinjal and Keshav*** *for their help and support at each and every point of time during dissertation. Appreciatively, its my great fortune to have juniors* ***Vishal, Samir, Pratik, Ujjaval, Sameer****,* ***Devras*** *and* ***Divyansh*** *who have assisted in my work whenever desired.*

Most important, I would like to thank ***my parents and my sister*** *for their blessings, eternal support, love, and encouragement without which I would never been come so far*

Finally, I would like to thank everyone who was directly and indirectly important for the successful completion of the thesis.

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1. ABSTRACT

Objective: The present study is aimed to investigate the effect of combination therapy of fluoxetine and dexamethasone for the treatment of rheumatoid arthritis in experimental models- adjuvant arthritis and methylated bovine serum albumin (antigen) induced arthritis.

Materials and methods: Adjuvant arthritis was induced by injecting complete freund's adjuvant on the subplantar surface in the left hind paw on day 0. Drug treatments of fluoxetine 2 mg/kg and/or dexamethasone (0.3, 0.1 or 0.05 mg/kg) were given from day 1 to day 21 daily. On day 5, arthritic index, paw oedema and C-reactive protein are measured. On day 21, rats were sacrificed and various parameters like Rheumatoid Factor, arthritic index, paw oedema, splenomegaly, erythrocyte sedimentation rate (ESR), oxidative stress markers of synovial tissue and histopathological evaluations of ankle joint were performed. In another set of experiments, in antigen induced arthritis, rats were immunized 14 days prior to induction of arthritis with 2 mg methylated bovine serum albumin suspended in freund's adjuvant: saline (1:1) subcutaneously. Animals were treated with the fluoxetine 2mg /kg and/or dexamethasone 0.1 mg/kg from day 0 to day 7. On day 7, various parameters like joint diameter, WBC count, ESR and oxidative stress markers of synovial tissue were estimated and histopathological evalution of knee joint was performed.

Results: In adjuvant arthritis, combination therapy with 0.3 mg/kg dexamethasone suppressed rheumatoid factor as well as splenomegaly and improved antioxidant assessments and histopathological changes significantly compared to dexamethasone 0.3 mg/kg treatment but showed aggrevation of stomach ulcers and weight loss. Combination group having 0.1 mg/kg dexamethasone also produced similar effect on C-reactive protein, arthritic index, paw oedema, antioxidant assessments, serum rheumatoid factor and histopathological evaluations. Fluoxetine treated group showed reduction only in splenomegaly and primary lesions. In antigen induced arthritis, combination group with 0.1 mg/kg dexamethasone significantly suppressed antioxidant assessments, ESR and

histopathological menifestations compared to dexamethasone 0.1 mg/kg while effects on WBC count and joint diameter were found similar.

Conclusion: Combination therapy of fluoxetine and dexamethasone are more effective in experimental models of adjuvant induced arthritis and antigen induced arthritis.

2. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disorder of unknown etiology that primarily involves the joints but can also cause multiple extra-articular manifestations. RA is the most common autoimmune disease, affecting 1–1.5% of the population worldwide. [Wolf et al, 1968]

However, aetiology of the disease is unknown but claimed to involve genetic factors like HLA-DRB1 class II haplotypes and environmental factors like viral infections and disturbances of neuroendocrine stress responses to antigens. [O'Brien et al,1967;Silman et al,2001] The presentation of the putative antigen by macrophages to the CD4+ helper cells in genetically predisposed individuals is the first event. An inflamed synovium is central to the pathophysiology. It is histologically striking, showing pronounced angiogenesis; cellular hyperplasia; an influx of inflammatory leucocytes; and changes in the expression of cell-surface adhesion molecules, proteinases, proteinase inhibitors, and many cytokines. Immunological response involves both cellular and humoral pathways. [Khuaran et al,2005; McCachren et al,1990]

Current therapies for arthritis involve use of non-steroidal anti-inflammatory agents (NSAIDs), corticosteroids and disease-modifying anti rheumatic drugs (DMARDs). Novel therapies also include recent agents like leflunomide, TNF α and IL-1β receptor antagonist and monoclonal antibodies like rituximab. All these of therapies are proved very effective in controlling the progression of disease and improving quality of life in rheumatoid patients. However, none of the above agents have still cure the disease completely.[Gurmukh et al,1999]

Although corticosteroids are extensively used yet for well developed NSAIDs resistant arthritis due to their high immunosuppressant activity, they still suffer the problem of resistance and adverse effects in patients on long term use. [Gurmukh et al, 1999] Receptor mutations and cell efflux transporter p-glycoprotein are claimed to be responsible for this phenomenon. [Dennison et al, 1998]

P-glycoprotein is membrane transport protein responsible for transport of drug from intracellular compartment to extracellular compartment.[De Rijk et al, 2004; Borst et al,2002] They are present on fibroblasts, lymphocytes, epithelial membrane of intestine, kidney and liver and on endothelial blood brain barrier[Jette et al,1993]. Recent clinical studies stated that p-glycoprotein present on lymphocytes are responsible for refractory rheumatoid arthritis and overcoming drug resistance by p-glycoprotein substrates can be useful for effective steroid treatment[Yvonne et al, 2003; Tsujimura et al,2008]. Various neoplastic agents, opioids, verapamil, quinidine, DMARDs and antidepressants are the substrates for p-glycoproteins. Antidepressants like selective serotonin reuptake inhibitor-fluoxetine have been reported to increase glucocorticoid receptor function by modulating steroid transporters p-glycoprotein in vitro.[Pariente et al,2001] Later, fluoxetine was also found to suppress p-glycoprotein in intestinal caco-2 cell lines also. Thus combining fluoxetine with glucocorticoids can be useful strategy for effective steroid response. [Johanna et al, 2003]

In addition to antidepressant action, fluoxetine has shown anti-inflammatory properties in various animal model of inflammation. [Omar et al, 2004] Fluoxetine is found to have anti-inflammatory action on paw oedema in rats developed separately by carragenan and brewer's yeast suspension. [Omar et al, 2004] Reports suggested contribution of the induction of hypothalamic pituitary axis by serotonin for negative immunoregulatory activity of fluoxetine. It is found to suppress TNF α (tumor necrosis factor) release and thereby effective in suppressing inflammation in acetic acid induced colitis in rats and lipopolysaccharide induced septic shock.[Aida et al, 2008] Fluoxetine has found to repress NF-κB and AP-1 activities in a lung epithelial cell line and found to be effective in asthma.[Kojima et al,2006] Even Fluoxetine have demonstrated a therapeutic benefit in both the murine collagen induced arthritis model and act on toll like receptors in a validated human RA disease tissue model- synovial membrane culture. (Sandra et al, 2010) However, the combined use of SSRIs and NSAIDs strongly increases the risk of gastrointestinal adverse effects and should be avoided [Jeroen et al,2003]. NSAIDs and selective serotonin reuptake inhibitors are reported the potential lethal combinations as increase high the risk of gastrointestinal bleeding and ulcerations. [Loke et al, 2008;

Dalton et al, 2003] However, little known regarding combination effect of fluoxetine and corticosteroids in vivo in effectively treatment of autoimmune disorders.

Systematic review and meta-analysis supported the strength of association between RA and depression, attributed to the level of pain. [Chris et al, 2002]. The report of population based clinical studies in United Kingdom infers that RA is associated with the depression. [Dickens et al, 2001] Out of 2,00,000 RA patients, as many as 40,000 suffered from depression, two third (27000) of whom would quickly respond to the antidepressant treatment. The use of SSRIs- fluoxetine or citalopram is the first line regimen for treatment of depression in RA. [Anderson et al, 2000]

Fluoxetine was found to increase glucocorticoid receptor function by modulating steroid transporters p-glycoprotein in presence of dexamethasone. [Pariante et al, 2003]. P-glycoproteins are responsible for limiting efficacy of dexamethasone treatment by suppression of intestinal absorption in freund's adjuvant arthritis and also delivary to mononuclear cells [Maillefert et al, 2000]. P-glycoprotein expression is also found in synovial tissue. [Jorgensen et al, 1995] Antidepressant – fluoxetine has been found p-glycoprotein suppressing activity in intestinal caco-2 cell lines. [Johanna et al, 2003]. Therefore, concomitant use of dexamethasone and fluoxetine warrants investigation for therapeutic benefit as both the drugs are included in common regimen.

Objective

The present study is aimed to investigate the effect of combination therapy of fluoxetine and dexamethasone for the treatment of Rheumatoid arthritis in experimental models-adjuvant arthritis and methylated bovine serum albumin (antigen) induced arthritis.

3. REVIEW OF LITERATURE

3.1 EPIDEMIOLOGY

RA is the most common autoimmune disease, affecting 1–1.5% of the population worldwide [1–3 Wolfe et al,1968; O'Brien et al,1967; Engel et al,1967]. The disease predominates in female rather than males. The ratio of female to male patients is approximately 2–4:1 [Silman et al,2001]. The basis of the gender differences is not known but presumably is related to effects of the hormonal milieu on immune function. Throughout the world, ethnic groups like the North American Pima Indians and southeast Alaskan Indians have a much higher incidence of RA [silman et al, 2001]. The incidence of RA rises dramatically during adulthood and peaks in individuals aged 40–60 years. [Engel et al, 1967]

3.2 AETIOLOGY

Aetiology of the disease still remains a mystery but suggest the involvement of certain genetic and environmental factors. [Khurana et al, 2005]

Genetic Factors:

Recent reports suggesting presence of genetic factors for RA stated that concordance of RA are 30 % in monozygotic twins, compared to 5% in fraternal twins and first degree relatives [Silman et al,1993]. Environmental factors must be related to RA development otherwise monozygotic twins would have a 100% concordance. A recognized RA genetic risk factor is the presence of the HLA-DR4 or HLA-DRB1 class II MHC haplotypes [Rigby et al,1991; Stastny et al,1978; Gregersen et al,1999; Mu et al,1999]. Associations have also been noted in the humoral system; in particular, the immunoglobulin kappa genotype appears to confer a risk of RA.

Environmental Factors:

Several environmental stimuli such as infections, vaccine inoculations, and emotional trauma have been implicated as inciting factors. Researchers have hypothesized atypical bacteria and viruses such as mycobacteria mycoplasma, Epstein–Barr virus, parvovirus B-19, rubella and retroviruses, may infect an individual with the appropriate genetic background, and through various mechanisms, the inflammatory response becomes focused on self antigens. [Kouri et al,1990; Hajeer et al,1994; Walker et al,1987]

The recent reports stated the disturbances in connections of central and peripheral nervous systems and immune systems can be the potential reason for the development of autoimmune disorders. During an immune attack, the central nervous system (CNS) regulates innate immune responses through hormonal and neuronal routes. The neuroendocrine stress response and the sympathetic and parasympathetic nervous systems generally inhibit innate immune responses at systemic and regional levels, whereas the peripheral nervous system tends to amplify local innate immune responses. These systems work together to first activate and amplify local inflammatory responses that contain or eliminate invading pathogens, and subsequently to terminate inflammation and restore host homeostasis.[Esther et al,2006] (Figure 3.1).

The neuroendocrine stress response comes from the hypothalamic pituitary axis which gives anti-inflammatory signals. The physiological feedback loop through which glucocorticoid release is regulated consists of a set of brain regions (the hypothalamus) and endocrine organs (the pituitary and cortex of the adrenal glands) known as the hypothalamic–pituitary–adrenal (HPA) axis (Figure 3.1). Systemic exposure of the host to pro-inflammatory stimuli (such as bacterial lipopolysaccharide) as well as to physical or psychological stimuli, results in secretion of corticotropin-releasing hormone from cells of the paraventricular nucleus of the hypothalamus into the hypophyseal blood supply around the pituitary gland.[Esther et al,2006]

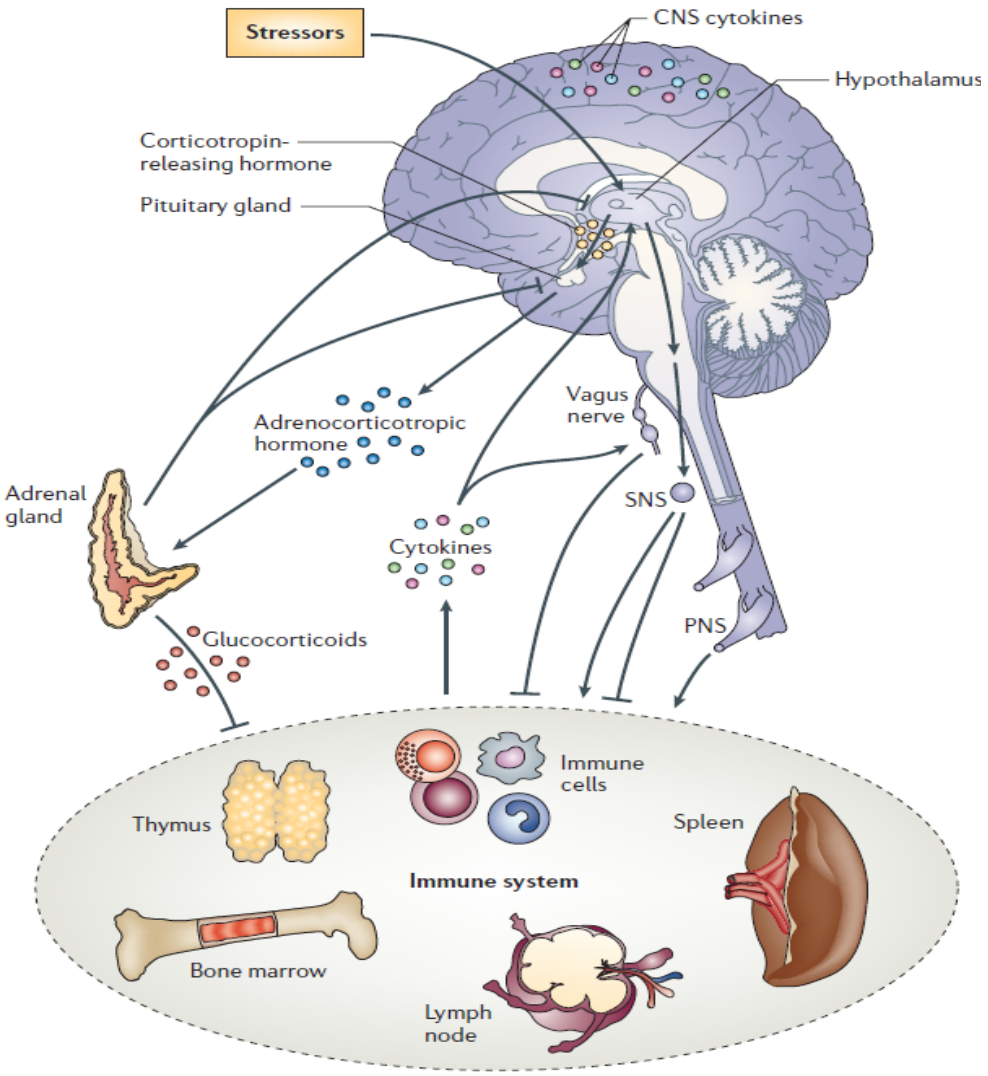

Figure 3.1: Schematic illustration of connections between the nervous and immune systems. Signalling between the immune system and the central nervous system (CNS) through systemic routes, the vagus nerve, the hypothalamic–pituitary–adrenal (HPA) axis, the sympathetic nervous system (SNS) and the peripheral nervous system (PNS) are shown. [Esther et al, 2006]

This stimulates the release of adrenocorticotropic hormone from the anterior pituitary into the blood, which in turn stimulates the synthesis and release of endogenous glucocorticoids from the adrenal cortex (FIG. 3.1). This bi-directional communication between the immune system and the central nervous system (CNS) [15,16] in which

cytokines, including interleukin-1 (IL-1), IL-6 and tumour-necrosis factor (TNF), signal to the brain, and the brain responds by regulating the immune system, in part, through the anti-inflammatory effects of glucocorticoids, constitutes the main hormonal negative feedback loop for CNS regulation of immunity. In addition to their role in regulating the immune system, glucocorticoids also downregulate the HPA axis itself, and are essential for the maintenance of several homeostatic mechanisms in the body, including the CNS and cardiovascular system, as well as for metabolic homeostasis

.

3.2.2 Impaired HPA-axis function and disease:

Impairment of HPA-axis signals are involved in the development of rheumatoid arthritis and other autoimmune disorders.

The roles of cytokines and the hypothalamic–pituitary–adrenal (HPA) axis-immune system feedback loop have been exploited in several novel ways. By evaluating diurnal cortisol secretion patterns in patients with RA, it has been found that there is an abnormal HPA axis response to immune/inflammatory stimuli, which may reside in the hypothalamus [Chikanza et al, 1992], or in a functional insufficiency of adrenal [Cutolo et al,1999]. The defective response of the neuroendocrine system to inflammatory stimuli suggests a non-MHC genetic factor contributing to the pathogenesis of RA [Panayi et al, 1995]. Some experimental models such as adjuvant-induced arthritis (AA) [Sarlis et al, 1992] and inbred Lewis (LEW/N) rats to group A streptococcal cell wall peptidoglycan polysaccharide (SCW) arthritis are also related to defective HPA axis responsiveness to inflammatory mediators.

3.3 PATHOLOGY

Anatomical and Histopathological abnormalities:

An inflamed synovium is central to the pathophysiology of rheumatoid arthritis.[David et al,2001] In the first weeks of the disease, tissue oedema and fibrin deposition are

prominent and can manifest clinically as joint swelling and pain. Within a short period, the synovial lining becomes hyperplastic, commonly becoming ten or more cells deep and consisting of type A (macrophage-like) and type B (fibroblast-like) synoviocytes. The sublining also undergoes striking alterations in cellular number and content, with prominent infiltration of mononuclear cells including T cells, B cells, macrophages, and plasma cells (figure 2). Synovial-vessel endothelial cells transform into high endothelial venules early in the course of the disease.[Girard et al,1995] High endothelial venules facilitate the transit of leucocytes from the bloodstream into tissues (figure 2).

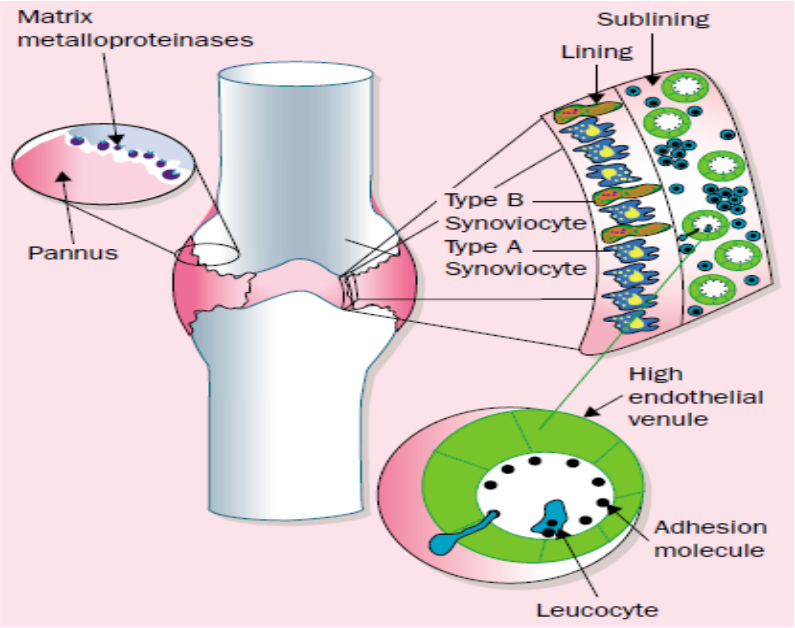

Figure 3.2: Major anatomical features of inflamed joint in rheumatoid arthritis

The formation of locally invasive synovial tissue— pannus—is an another characteristic feature of rheumatoid arthritis. This tissue is involved in the joint erosions seen in rheumatoid arthritis. Pannus is histologically distinct from other regions of the synovium and shows phases of progression. Initially, there is penetration of the cartilage by synovial pannus composed of mononuclear cells and fibroblasts with high-level expression of matrix metalloproteinases by synovial lining cells (figure 3.2) [Mccacheren et al1990; Gravallese et al,1991] In later phases of the disease, cellular pannus can be

replaced by fibrous pannus comprised of a minimally vascularised layer of pannus cells and collagen overlying cartilage. The molecular pathogenic mechanisms driving pannus formation remain poorly understood.

3.4 PROPOSED PATHOGENESIS SEQUENCE

The pathophysiology of the disease is still not completely clear but the proposed mechanism is as follows [Gurmukh et al,1999]

The first event is the presentation of the putative antigen by macrophages to the CD4+ helper cells in genetically predisposed individuals. The antigen could be of bacterial or viral origin.

Activation of helper T cells results in release of interleukin-2 which in turn amplifies the helper T cells by a positive feedback mechanism. Cytokines like IL-4, IL-6, TNF α and gamma-IFN are also released by the activated CD4+ T cells. These cytokines increase the expression of adhesion molecules like ICAM-1, LFA-1 and Mac-1, which help in the localisation of the inflammatory cells. Cytokines stimulate the activation, proliferation and differentiation of B-cells into antibody-producing plasma cells. These plasma cells produce antibodies against the Fc fragment of IgG which is termed as the rheumatoid factor.

Rheumatoid factor forms immune complexes with IgG. These activate the complement cascade. This results in production of C3a, C5a, C3b and C56789. C3a and C5a act as anaphylotoxins causing histamine release and increase in vascular permeability. In addition, C5a acts as a chemotactic factor for neutrophils ; C56789 (membrane attack complex) is capable of damaging cells by drilling pores in their membranes.Infiltration with neutrophils results in further amplification of the inflammatory insult through release of oxygen free radicals, inflammatory metabolites of arachidonic acid pathway like prostaglandins and leukotrienes, metalloproteinases like collagenase, elastase, gelatinase and stromelysin.

These inflammatory mediators lead to damage of articular cartilage, demineralisation of the underlying bone, erosion of the joint margins, laxity of the joint capsule and ligaments and finally total derangement of the affected joint leading to joint deformities.

3.5 CLASSIFICATION CRITERIA

Criterion		Comments
1.	Morning stiffness	For more than 1 hour and for more than 6 weeks
2.	Arthritis of 3 or more of 14 possible joint areas	Should have swelling of soft tissue or fluid for more than 6 weeks. The joint areas are right or left PIP, MCP, wrist, elbow, knee, ankle and MTP joints
3.	Arthritis of hand joints	Wrist, MCP or PIP joints for more than 6 weeks
4.	Symmetrical arthritis	Bilateral involvement of any one of PIP, MCP or MTP joint areas and/or other joints, lasting for more than 6 weeks
5.	Rheumatoid nodules	At bony prominences or extensor surfaces or juxta-articular regions as observed by a physician
6.	Serum rheumatoid factor	As detected by any one of the methods positive in 5% or less of control subjects
7.	Radiographic changes	Erosions and/or juxta-articular osteoporosis of involved joints in the antero-posterior film of hands and wrists

Table 3.1 : 1988 revised ACR criteria for classification of rheumatoid arthritis [Gurmukh et al,1999]

At least 4 of these criteria must be satisfied for classification purposes. These have a sensitivity of 91% to 94% and specificity of 89%.

3.6 CLINICAL FEATURES

• Fever, loss of weight
• Morning stiffness
• Symmetrical involvement of small joints of hands and feet
• May be monoarticular (large joint)
• Deformities of hands (chronic cases)
• Muscle wasting, bursitis, tenosynovitis
• Rheumatoid nodules
• Features of vasculitis (ulcers, nail changes, neuritis, pyoderma)
• Presence of rheumatoid factor(s)
• Eosinophilia, anaemia
• Splenomegaly, lymphadenopathy (Felty's syndrome)
• Episcleritis, scleromalacia, kerato-conjunctivitis sicca (Sjogren's syndrome)
• Pericarditis, endocarditis, myocarditis, aortitis, aortic incompetence, coronary vasculitis, heart block
• Pleural effusion, nodules, bronchiolitis, fibrosing alveolitis
• Mononeuritis multiplex, cervical cord compression
• Amyloidosis
• X-ray Joints - Narrowing, deformities
Chest - Fibrosing alveolitis, bronchiolitis, pleural effusion

Table 3.2 : Important Articular and extraarticular features of rheumatoid arthritis [Gurmukh et al,1999]

3.7 LABORATORY INVESTIGATIONS

Laboratory investigations may help either to establish a clinical diagnosis or to assess the prognosis and the possible course of the disease.

Erythrocyte Sedimentation Rate	Elevates in RA and comes down as disease progress to remission
Rheumatoid Factor	Most important immunological investigation for RA. A cut-off value of 80 IU/ml with latex agglutination test and a differential agglutination titre (DAT) of 16 are more specific
C-reactive protein (CRP)	useful marker of acute phase response and its measurement is valuable in the management and prognosis
Synovial fluid analysis	valuable indicator of the inflammatory status.
leucocyte count	usually in the range of 5,000 to 20,000 cells per mm_3 with neutrophilic predominance. Counts over 50,000 cells per mm3 indicate possible superadded infection
Serum Protein estimation	2.5 g/dL to 3.5 g/Dl
Blood glucose level	Remains relatively low
Plain radiograph of the joint	In early stages detect soft tissue swelling around the affected joint, Erosions and in later stages, juxtaarticular osteoporosis.

Table 3.3: Various laboratory tests and their importance in Rheumatoid Arthritis [Gurmukh et al, 1999]

3.8 TREATMENT

Current treatment of RA frequently includes the use of **nonsteroidal anti-inflammatory drugs** (NSAIDs), such as ibuprofen and diclofenac. As first line drugs, these offer little protection against tissue degeneration. They do, however, reduce the levels of

prostaglandins, bradykinins, and oxygen radicals; thereby contributing to pain relief. [Seitbert et al, 1994]

NSAIDs work by inhibiting cyclooxygenase (COX), decreasing prostanoid production. Common side effects are peptic ulceration, impairment of renal blood flow, renal papillary necrosis, nephrotic syndrome, and hepatic injury. [Jain et al,1997]

Corticosteroids (glucocorticoids) are indicatied for steroids include systemic vasculitis, scleritis, pericardial involvement, pulmonary involvement and severe arthritis not responding to NSAID therapy. Severe vasculitis responds well to pulse intravenous megadose methylprednisolone therapy.

Disease-modifying antirheumatic drugs **(DMARDs)** are drugs which modify the course of the disease. **DMARDs** are often given simultaneously with NSAIDs. DMARDs include gold, hydroxychloroquine, methotrexate, auranofin, sulfasalazine, d-penicillamine, cyclosporin, azathioprine, and cyclophosphamide. These have slow onset of action and thus are also termed as SAARDS (slow-acting antirheumatic drugs).

Immunomodulatory drugs should be used with caution in RA because of potential toxicity. The drugs used are cyclophosphamide, azathioprine and chlorambucil. They are mainly indicated in aggressive RA with extraarticular manifestations like intractable vasculitis.

3.8.2 PHYSIOTHERAPY, SURGERY AND REHABILITATION [Edwards et al, 2004]

Rest during active stages of the disease is absolutely indicated until pain subsides. After disease activity is controlled, muscle-strengthening and joint-strengthening exercises are indicated. It is important to achieve a balance between rest and exercises on an individual basis. Exercise programme can be assisted by giving heat therapy in the form of moist heat (wax baths or hot water fomentation) or dry heat (short-wave diathermy or ultrasonic therapy).

Surgery plays on important role in the comprehensive management of RA and success depends upon selection of appropriate surgery. Soft tissue repair includes repair of the ruptured tendon at the wrist and achilles tendon, release of entrapped nerves in carpal and tarsal tunnel syndromes, and soft tissue release of the trigger fingers. Surgical procedures of the joint include (a) synovectomy of the inflammed joint before joint damage has set in; (b) osteotomy to correct the deformity and relieve pain in the upper end of the tibia, and excision of the lower end of the ulna; (c) arthrodesis of the joint to relieve pain in the atlanto-axial joint, wrists, ankle and subtalar joints; (d) excision arthroplasty and (e) joint replacement.

3.8.3 NOVEL THERAPIES

T cell targeted therapy : leflunamide (LFM): LFM is an anti-inflammatory and immunomodulatory drug that has been used to treat RA and to prevent organ rejection following transplant. In addition to suppressing the effects of IL-2 (and other cytokines) and inhibiting adhesion and migration of inflammatory cells, LFM also retards the proliferation of activated T-cells, by blocking biosynthesis of pyrimidines at the level of dihydroorotic acid dehydrogenase, crucial for DNA synthesis. LFM clearly has been documented to bring about clinical improvement in RA patients.

Monoclonal Antibodies : Rituximab is a chimeric mAb to the B cell-specific antigen CD20. CD20 is expressed on pre-B and mature B cells, and not on stem cells or on plasma cells. When rituximab binds to CD20, B cells may be depleted via mechanisms including complement-mediated lysis, antibody-dependent cytotoxicity, and apotosis

Cytokine targeted therapy :

TNF α antagonist – Etanercept , Infliximab and Adalimumab

Infliximab is a chimeric human/mouse monoclonal anti-TNF alpha monoclonal antibody. Etanercept is a recombinant form of the human p75 receptor that is fused to the Fc fragment of the human immunoglobulin G1. TNF antagonists have several advantages

over traditional DMARDs. They exhibit a rapid onset of action, provide significant clinical response, improve quality of life, and, most importantly, substantially inhibit radiographic progression of the disease.

IL-1R antagonist – Anakinra

Anakinra is a recombinant form of the human IL-1Ra that acts as a competitive receptor antagonist. Subcutaneous injections of Anakinra are found to reduce signs and symptoms of RA. A humanized IL-6R monoclonal antibody called tocilizumab was developed to bind to human IL-6R and be less immunogenic.

3.9 GLUCOCORTICOIDS

For many years, **corticosteroids** have been used extensively for treatment of RA.

Glucocorticoids act on immune cells both directly and indirectly to suppress the induction of proinflammatory responses. They inhibit the production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumour-necrosis factor (TNF), while promoting the production of anti-inflammatory cytokines, such as IL-10, by macrophages and dendritic cells. They also promote apoptosis of macrophages, dendritic cells and T cells, leading to inhibition of immune responses, resulting in inhibition of collagenase and lysosomal enzyme release (as well as reducing prostaglandin and leukotriene synthesis) [Ann et al,2000]

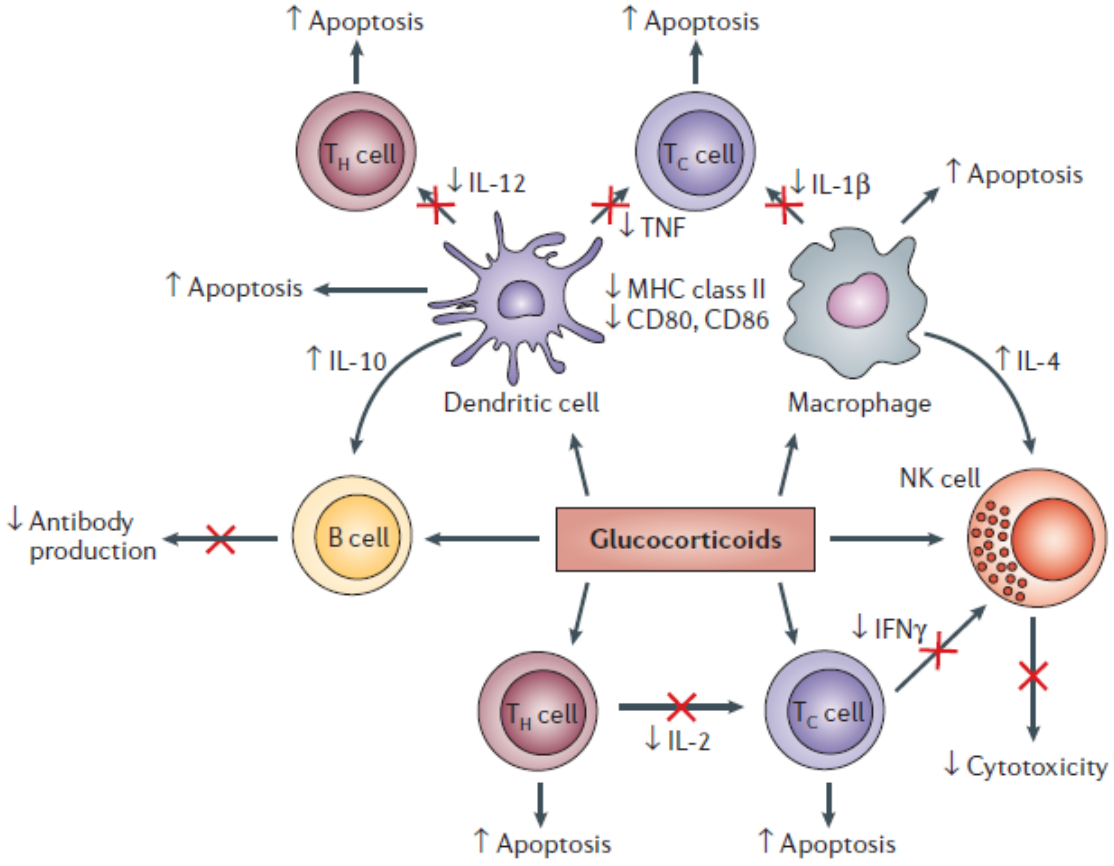

Figure 3.3 : Effects of glucocorticoids on immune-cell populations. IFNγ, interferon-γ; NK cell, natural killer cell; TC, cytotoxic T cell; TH, T helper cell. [Esther et al, 2006]

Their anti-inflammatory and immunosuppressive effects provide relief for many patients and are especially useful for those patients refractory to treatment with NSAIDs. Unfortunately, corticosteroid therapies often accompanied by numerous side effects, including bone loss, increased susceptibility to infection, osteoporosis, and peptic ulcers. Additionally, weaning patients from corticosteroids can be difficult and relapses of articular degeneration are frequent once the steroid is discontinued [Dennison et al,1998]. Another major problem with the use of glucocorticoid is steroid resistance.

3.10 GLUCOCORTICOID RESISTANCE.

Impaired glucocorticoid control of inflammation might also result from lack of glucocorticoid responsiveness, or glucocorticoid resistance, of target cells or tissues, and might contribute to autoimmune, inflammatory and allergic diseases [Derijk et al,2000; Leung et al,1997; Adcock et al, 1995; De rijk et al, 2004; Winsen et al, 2005; Tower et al, 2005]. Glucocorticoid resistance might result from mutations or polymorphisms of glucocorticoid receptors [Derjik et al, 2000; Lee et al, 2004] or impaired interactions of the receptor with one of the >200 cofactors, such as corticosterone-binding globulin [Mingrone et al,1999] and multidrug resistance-1 [Diaz- Borjon et al, 2000], that are required for glucocorticoid- receptor function. In addition, expression of glucocorticoid receptor, an inactive form that does not bind ligand or activate gene transcription [Derijk et al,2002; Oaklely et al,1996] is induced during chronic inflammation, and might result in relative glucocorticoid resistance in such states.

Moreover, pathogens might themselves induce glucocorticoid resistance. Recently it has been shown that *Bacillus anthracis* lethal toxin selectively and potently represses the activity of glucocorticoid receptor and other nuclear hormone receptors in a non-competitive fashion, by preventing glucocorticoid-receptor binding to DNA through interactions with one or more of its cofactors or accessory proteins [Webster et al, 2003]. Indeed, although the precise mechanisms of this effect are unclear, interference with glucocorticoid production, such as by adrenalectomy, render otherwise anthrax lethal-

toxin-resistant mouse strains highly susceptible to rapid death from this toxin [Moayeri et al, 2005]

3.10.2 ROLE OF P-GLYCOPROTEINS

During recent years it has become clear that the access of gluococorticoids to certain cells and tissues is hampered by the efflux transporter P-glycoprotein (Pgp), a member of the family of ABC-transporter proteins [Borst et al, 2002]. The Pgp is encoded by the multi-drug-resistance or mdr1 genes: MDR1 in humans, and mdr1a and mdr1b in rodents. Expression data and substrate specificity suggest that the two rodent protein together serve the same function as the human MDR1 gene product. Pga actively transports a broad range of substrates from the intracellular compartment to the extracellular space. Pgp is expressed on leukocytes, fibroblasts and epithelial membranes in the intestinal epithelium, hepatocytes, renal proximal tubular cells, adrenal gland and capillary endothelial cells comprising the blood-brain and blood-testis barrier.

The synthetic glucocorticoid, dexamethasone, is a good substrate for Pgp and, accordingly, the accumulation of dexamethasone in the brain of mdr1a knockout mice is increased compared with wild-type mice [Schinkel et al, 1996], as is binding of dexamethasone to the glucocorticoid receptor in the brain [Meijer et al, 1998]. Other Broad ranges of structurally unrelated compounds are transported by P-gp, including antineoplastic agents, HIV protease inhibitors, prednisone, gold salts, methotrexate, colchicine as well as several antibiotics causing development of their resistance.

For the endogenous steroids corticosterone and cortisol there is a striking difference in Pgp transport. Corticosterone is not a substrate for either the mouse mdr1a [Meijer et al, 2002] or the human MDR1 Pgp [Karssen et al, 2001]. It is a rather weak substrate for the murine mdr1b Pgp [Bourgeios et al, 1993] which is not, however, expressed at the blood–brain barrier [Jette et al, 1993; Schinkel et al, 1997]. In contrast, the main endogenous

glucocorticoid in humans, cortisol, is a substrate for both mouse mdr1a and mdr1b, and human MDR1 [Ueda et al, 1992; Jette et al, 1993; Schinkel et al, 1997]
Up-regulation of expression of Pgp expression or activity is a theoretical mechanism for the development of further glucocorticoid and other drugs resistance which is great problem in various autoimmune disorders including rheumatoid arthritis.

3.10.3 CLINICAL STUDIES REGARDING SUPPRESSION OF P-GLYCOPROTEIN IN RHEUMATOID DISEASE

Pgp inhibitors or substrates may decrease this relative glucocorticoid and other drug resistance by facilitating the uptake of glucocorticoids inside the cells in various diseases,

Multidrug resistance-I (MDR-I) was previously studied in rheumatic autoimmune disorders. Increased P-glycoprotein activity in lymphocytes from systemic lupus erythematosus patients was found which might affect steroid requirements for disease control. [Yvonne et al, 2003]

Expression of multidrug resistance P-glycoprotein on lymphocytes from children with steroid dependent nephritic syndrome treated with cyclosporine A and ACE-inhibitor was measured to study the effect of them on p-glycoprotein inhibition and its effect on steroid intracellular concentration. P-glycoprotein substrate cyclosporine A are effective in suppressing its expression and thus optimization of steroid therapy can be achieved with less adverse effects. [Anna et al, 2007]

P-glycoprotein expression on lymphocytes in refractory rheumatoid arthritis patients was also studied. Patients partially responding to steroids and DMARDs were shown high p-glycoprotein expression in lymphocytes. Moreover, p-glycoprotein substrate, Tacrolimus were found to reduce this resistance. The results indicate that overcoming drug resistance

by p-glycoprotein substrates can be useful for effective steroid treatment. [Tsujimura et al, 2008]

3.10.4 ROLE OF P-GLYCOPROTEIN IN ANIMAL MODELS OF ARTHRITIS

Reports stated that in adjuvant arthritis in rats, TNFα induced both a strong time-dependent diminution of MDR1 mRNA and a significant decrease in the efflux function of P-gp in Caco-2 cells. The expression and activity of intestinal P-gp may thus be altered in AA rats. Another study reported that Intestinal P-glycoprotein (P-gp) activitiy at upper segment was also signiˆcantly decreased in AA rats to 60z of that in normal rats, and the other segments (middle and lower) of intestine also exhibited tendencies toward decrease in P-gp activity. levels of mdr1a mRNA and P-gp protein were also in AA rats. Morever, hepatic P-gp activity was decreased due to reduction of the expression level of the hepatic P-gp [Meguru et al, 2002]. Therefore pharmacokinetics and bioavailabilities of drugs whose membrane permeation is limited by intestinal P-gp may be altered in adjuvant arthritis model and rheumatic diseasses.

3.10.5 INTERACTION OF P-GLYCOPROTEINS BY ANTIDEPRESSANTS

Similar facts have achieved in case of p-glycoprotein on blood brain barrier as recently suggested in relation to antidepressants. Antidepressants were found to increase glucocorticoid receptor function by modulating steroid transporters p-glycoprotein. Later, fluoxetine was also found to suppress p-glycoprotein in intestinal caco-2 cell lines also. [Pariante et al, 2003]

Another in vitro study was carried out on effects of various antidepressants on inhibition of P-Glycoprotein in two different cell systems: L-MDR1 cells (model for human Pgp) and primary porcine brain capillary endothelial cells (pBCECs, model for the blood-brain barrier). Fluoxetine was found to suppress P-glycoprotein activity. The fact that some of the compounds tested exert Pgp inhibitor effects at similar concentrations as quinidine

suggests that pharmacokinetic drug- drug interactions between the newer antidepressants and Pgp substrates required to be thoroughly studied in vivo. [Johanna et al, 2003]

3.11 ANTI-INFLAMMATORY ACTIVITIES OF SELECTIVE SEROTONIN REUPTAKE INHIBITORS- FLUOXETINE

In spite of the selective serotonin reuptake inhibitors and in particular fluoxetine have became first line drugs in the pharmacotherapy of depression, fluoxetine has a numerous anti-inflammatory activity.

Studies on anti-inflammatory activity of the non-tricyclic, pro-serotoninergic, antidepressant drug fluoxetine on paw oedema had been carried out.[Omar et al, 2004] Fluoxetine at the dose of 20 mg/kg daily suppressed carrageenan-induced paw inflammation in the rat after 14 days of the induction.

Fluoxetine significantly and dose dependently reduced the swelling induced by the injection of 10% brewer's yeast suspension in the hindpaw. Pretreatment with the corticotropin-releasing hormone antagonist α-helical CRH-(9–41) did not interfere with the anti-inflammatory action of fluoxetine. Moreover, the drug induced a significant increase of corticosterone plasma concentrations in vivo, whereas, in vitro, it did not stimulate β-endorphin release from anterior pituitary cells. These data suggest that fluoxetine exerts a potent anti-inflammatory action by inducing pituitary-adrenocortical activation via serotonin.

Fluoxetine has found to repress NF-κB and AP-1 activities in a lung epithelial cell line. This may account for their anti-inflammatory effects in the animal model of asthma. Preventive treatment with fluoxetine markedly reduced TNF-α production and NF-κB activity as well as mortality in the LPS-induced septic shock model.[Caroline et al, 2007]

Fluoxetine have anti-inflammatory and antioxidants effects in acetic induced colitis in wistar rats. It was found to inhibit the release of TNF alpha and IL- beta at dose of 10

mg/kg in experimental induced colitis. The cAMP-dependent protein kinase A (PKA) pathway is responsible for suppress TNF alpha production from various cells. [Aida et al, 2004]

Fluoxetine, has negative immunoregulatory effects through suppression of the interferon-g (IFN-g)/ interleukin-10 (IL-10) production ratio, which is of critical importance for the determination of the capacity of immunocytes to inhibit or activate monocytic/lymphocytic functions. [Aida et al, 2004]

Antidepressant fluoxetine enhances glucocorticoid receptor function in vitro by modulating membrane steroid transporters p-glycoprotein. Therefore, Inhibition of drug efflux transporters in vivo should increase concentration of endogenous glucocorticoids some in target cells and may account for the anti-inflammatory properties of antidepressants.

3.12 CO- OCCURANCE OF CHRONIC PAIN, INFLAMMATION AND DEPRESSION

In patients with rheumatoid arthritis, depressive symptoms were significantly associated with negative health and functional outcomes as well as increased health services utilization [Kojima et al, 2006] Depression is one of the most common problems experienced by patients with chronic pain.[Dickens et al, 2001]

In addition, Link among Depression, inflammation, and pain in patients with rheumatoid arthritis was investigated. Depression scores were mildly and positively correlated with the CRP level. Both the depression score and the C Reactive Protein level were significantly associated with pain, even after adjustment for clinical covariates in regression analysis. In logistic analysis, the combined effects on the risk of severe pain (pain score in the upper tertile) increased with depression scores and CRP levels linearly. The results conclude that Depression severity and inflammation were associated clearly

with each other and appeared to have independent effects on perceived pain.[Kojima et al, 2006]

In the randomized, double-blind, parallel study, fluoxetine was found an effective analgesic based on pain intensity and pain relief score at antidepressant dose.

Thus, Link between inflammation and depression suggest the use of fluoxetine in combinations of conventional analgesics and anti-inflammatory agents. In such cases, drug –drug interactions among these agents must have to be the point of focus for therapeutic use.

3.12.1 COMBINATIONS OF SSRIS INCLUDING FLUOXETINE AND NSAIDS

Animals studies stated that Fluoxetine co-administered with indomethacin , celecoxib or rofecoxib before carrageenan reduced the anti-oedema effect of indomethacin or celecoxib, but had additive effect to that of rofecoxib. The anti-oedema effect of fluoxetine was partially suppressed by the opioid antagonist naloxone. [Omar et al, 2004]

SSRIs and NSAIDs are normally both have the tendency to produce gastric ulceration and stimulation of gastric acid secretion. Normally, when serotonin from blood platelets is released it augments platelet aggregation, the process where blood cells clump together to prevent bleeding. Evidence suggests that SSRI antidepressants may inhibit uptake and storage of serotonin by platelets. When serotonin uptake is reduced in blood platelets, platelet aggregation also is reduced, which may predispose patients to bleeding. [Dalton et al, 2003]

Combinations of SSRI and NSAIDs leads to generate a problem: more severe gastroinstestinal bleeding and gastrointestinal ulcerations. The fact is revealed from Meta-Analysis on Gastrointestinal Bleeding (GI) due to Interaction Between Selective Serotonin Uptake Inhibitors and Non-Steroidal Anti-Inflammatory agents. [Loke et al, 2008]

Therefore this combination proved itself a potentially lethal and should be avoided for therapeutic use. [Loke et al, 2008]

3.12.2 COMBINATIONS OF SSRIS LIKE FLUOXETINE AND GLUCOCORTICOIDS :

Based on three evidences,

1. Co-occurance of depression and inflammation in arthritis patients
2. Interaction of fluoxetine with p-glycoprotein in vitro
3. Antiinflammatory activities in various animal models

Combination of fluoxetine and dexamethasone must have been studied in vivo to evaluate its efficacy in treating rheumatoid arthritis.

3.13 ANIMAL MODELS OF ARTHRITIS

3.13.1 ADJUVANT ARTHRITIS

Rat adjuvant arthritis is an experimental model of polyarthritis which has been widely used for preclinical testing of numerous anti-arthritic agents [Pearson et al, 1956; Benslay et al, 1991]

The hallmarks of this model are reliable onset and progression of robust, easily measureable, polyarticular inflammation, marked bone resorption and periosteal bone proliferation. Cartilage destruction occurs but is disproportionally mild in comparison to the inflammation and bone destruction that occurs. [Bendele et al, 2001]

Induction of adjuvant disease can be done with either Freunds complete (FCA) supplemented with mycobacterium or by injection of the synthetic adjuvant N, Ndioctylddecyl- N', N-bis(2-hydroxy-ethyl) propanediamine (LA). Adjuvant can be injected at the base of the tail or in one of the foot pads. If injection is into the footpad, it allows study of the acute inflammatory reaction in that local area as well as the immunological reaction that develops approximately 9 days later in the contralateral paw and various organs.

The pathogenesis/reasons for development of adjuvant disease are not fully understood despite the fact that numerous studies have contributed to the understanding of various possibilities including reactivity to cartilage proteoglycans, heat shock proteins and interactions with intestinal flora[Langerijit et al, 1994; Chang et al, 2008; Bendele et al, 1999].

The oil component and, more so, the mycobacteria, activate the Multinucleated Phagocytic Cells (MPC) system, including the various categories of Dendritic Cells (DC). This results in overall enhanced phagocytosis of particulate material [Nicol et al, 1966] and secretion of monokines [Mussener et al, 1995]. A correlation of this being, the

transient, increased aspecific enhancement of resistance to infection [Castro et al, 1993]. This, in turn, results in stronger-than-normal polyclonal activation and proliferation of T lymphocytes, which, regardless of their being autoantigen-specific but precisely as a result of their status of being activated, tend to infiltrate into tissues despite physical impediments such as the blood-brain barrier [Rabchevsky et al ,1999; Hickey et al, 1991; Hickey- Hsu et al, 1991].

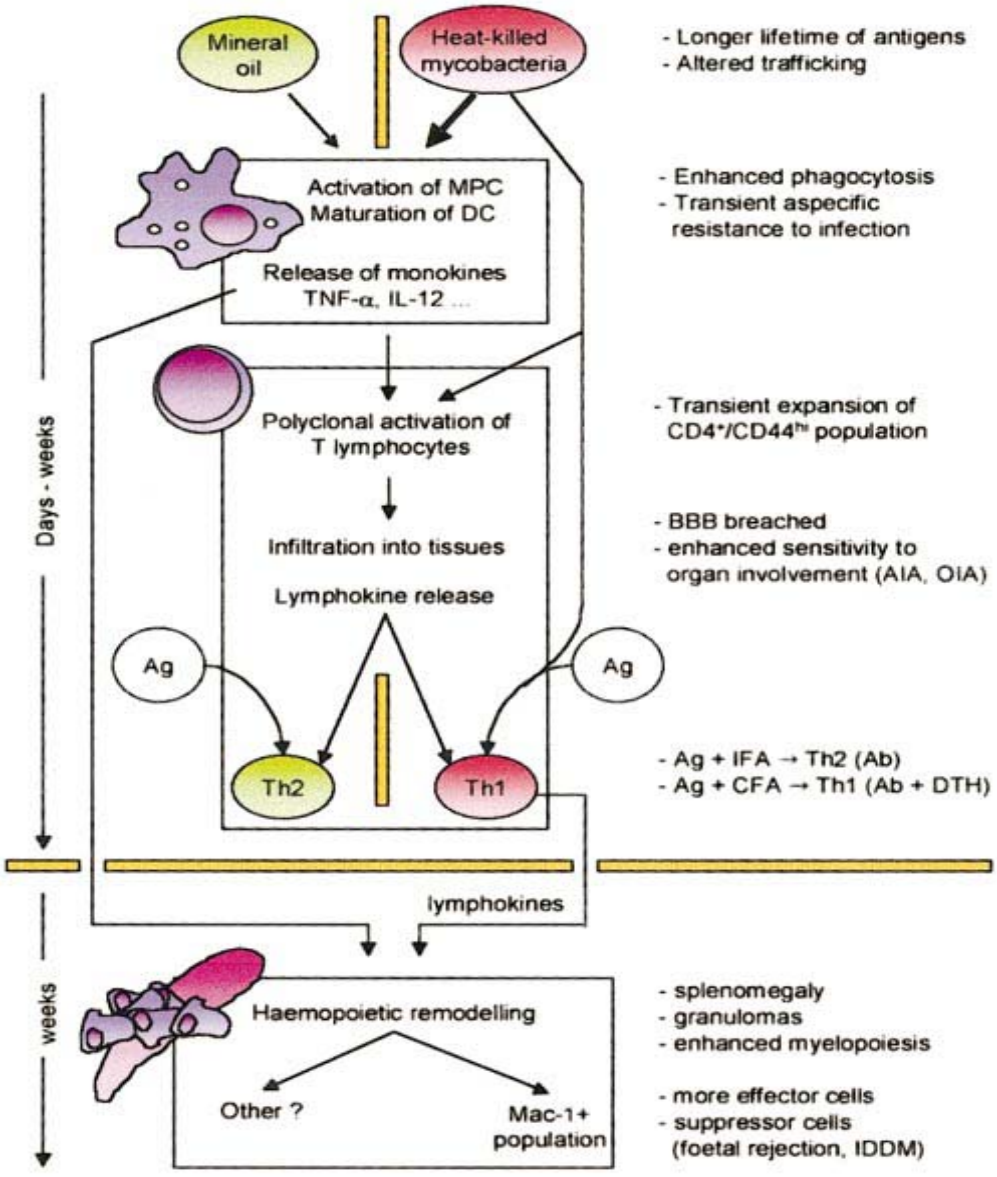

Figure 3.4. Synopsis of action mechanisms of Freund's adjuvants in facilitating experimental autoimmune disease.

The activated T lymphocytes also produce sets of lymphokines, representing a type 1 or type 2 helper activity depending on whether the immunization was done with protein antigen only (i.e., antigen in IFA) or with antigen in conjunction with mycobacteria (i.e., antigen in CFA) [Yip et al, 1999; Heeger et al, 2000]. In both instances, an increased antibody response is generated, but DTH develops preferentially in the second instance. Until this stage, one could say that for inducing autoimmunity and autoimmune disease, Freund adjuvants use the same mechanisms as those by which they enhance specific immune responses to foreign antigens. However, in the case of CFA, the heat-killed mycobacterial cells embedded in oily excipient persist for weeks or even months at the injection site and in phagocyte-rich organs such as lung and liver.

As time elapses, they thus constitute an unabated stimulus for the production of monokines and Th1 lymphokines. These late-produced cytokines may play a role in bringing about the arrest of T-cell expansion and activation but also in the gradual build-up of myelopoiesis, most explicitly revealing itself in splenomegaly and disruption of spleen histology. The Mac-11 cells generated by this haemopoietic activity can fulfill different roles in further evolution of the disease. On the one hand, they may constitute a source of additional effector cells that after maturation and activation, add to the tissue damage. [Alfons et al, 2001]

Conversely, there is evidence for the emerging Mac-11 population to act as suppressor of T-cell activation [McInerney et al, 1991]. Which of these opposing roles predominate may depend on the time of their emergence relative to the other events in disease pathogenesis. In other models, however, such as diabetes in NOD mice, the opposite is the case.

Polyclonal activation of lymphocytes is probably the crucial event in models where administration of IFA or CFA without intentionally added autoantigen causes autoimmune disease. To explain induction of AIA by mycobacterium- containing CFA, cross-reactivity of antimycobacterial antibodies or T-cell receptors with epitopes of host

proteins have been invoked. Yet the identity of these epitopes so far remains unknown, and other mechanisms may need to be considered. In the case of Oil Induced Arthritis, no proteinaceous antigen is used; nevertheless, the disease is T-cell-mediated, suggesting that arthritogenic T-cell clones are constitutively present whose pathogenic activity is only marginally controlled by mechanisms of peripheral tolerance. Induction of disease by oil adjuvant could then be a result of abrogation of such tolerance.

Being suspended in oil, Understanding how Freund's adjuvants facilitate induction of experimental autoimmune disease allows us to define crucial elements in the pathogenesis of these models and, by extrapolation, in the naturally occurring human diseases. Whereas, intuitively, the role of adjuvants could be explained by their facilitating effect on the generation of autoantigen-recognizing lymphocyte clones, the scenario evoked here rather stresses the importance of increased aspecific activation of MPCs, DCs, and T lymphocytes and the excess generation of aspecific effector and regulator cells. The implication is that these mechanisms may also deserve more attention in deciphering the mechanisms of emergence, remission, and recrudescence of natural autoimmune diseases in man. Further research on the cellular and molecular mechanisms underlying adjuvant action in experimental models is therefore warranted

Thus, use of the adjuvant model offers an opportunity to study pathological changes in a variety of tissues other than the joints. Splenomegaly occurs as a result of profound induction of extramedullary hematopoiesis in the red pulp in conjunction with pyogranulomatous inflammation in the red pulp and capsule. These changes are usually in association with mild to marked lymphoid atrophy. Ideally, an agent active in adjuvant disease should restore the spleen weights and morphology to normal as is the case with methotrexate treatment. Hepatomegaly also occurs as a result of hypertrophy of hepatocytes and should be beneficially affected by treatment. Also fairly consistently present in these animals is an anterior uveitis which may be histologically evaluated for treatment effects.

3.13.2 ANTIGEN (METHYLATED BOVINE SERUM ALBUMIN) INDUCED ARTHRITIS

The pathogenesis involves an Arthus reaction on the articular cartilage as antibodies to the positively charged antigen that is injected form complexes that activate complement locally and result in cartilage destruction. There is evidence that T cells specific for the inducing antigen play a critical role in AIA[Langerijt et al, 1994]. There are also histological similarities between AIA and human rheumatoid arthritis that include hyperplasia of synovium forming pannus, a predominantly mononuclear cell infiltration with formation of secondary lymphoid aggregate and cartilage erosion.

The advantage of monoarticular models is that direct comparisons can be made between the treated and untreated joints within the same animal, thus drug effects can be isolated to the inflammatory process. Comparisons between control and inflamed joints of vehicle-treated and drug-treated MA animals can also be made to investigate drug effects on normal joint tissues. It is important to include vehicle-treated negative and positive controls, if only to determine the effect of the procedure and drugs on body growth, and thus patellar size and joint width. In antigen-induced MAR control antigen-free sensitized animals should be used.

The morphologic differences between antigen-induced arthritis in animals and human rheumatoid synovitis may be due also to the greater chronicity of the human disease, as [Loewi et al, 1969]. **A** mechanism that may sustain mBSA-induced arthritis would be the presence of persisting antigen, possibly in the form of immune complexes, in the intraarticular collagenous tissues [Glynn et al, 1968; Cooke et al, 1966] There were the possibilities of a self-perpetuating autoimmune reaction to antigens derived from the inflamed connective tissues [Phillips et al, 1966].

The presence of macrophages suggests that these cells may cause connective tissue degradation in antigen induced arthritis in mice. Increased activity of hydrolytic and other

enzymes has been found in chronically inflamed tissues including rheumatoid joints [Page et al, 1974; Weismann et al, 1966]. And immune complexes, or lymphokines produced after mitogen or antigen activation of lymphocytes, can interact with macrophages and influence their functions [Cardella et al, 1974; Pantalone et al, 1974]. Furthermore, macrophages activated by lymphokines synthesize and release collagenase [Wahl et al, 1974; Wahl S M et al, 1974]. The recruitment of the macrophages into the sites of delayed-type hypersensitivity reactions depends upon the secretion of leuko-attractants and migration inhibitory factors which retain mononuclear phagocytes at the site of inflammation and antigen deposition. Hence the histologic differences between antigen-induced arthritis in mice and human rheumatoid synovitis may merely reflect a more active stage of the chronic inflammatory process in the arthritic knee joints and a more rapid breakdown of the connective tissue. [Brackertz et al, 1977]

4. MATERIALS AND METHODS

4.1 PROTOCOL

The protocol of the experiment is approved by institutional animal ethics committee as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of social justice and Empowerment, Government of India under protocol number **IPS/PCOL/MPH10/002**

4.2 MATERIALS

All chemicals and drugs: Complete freund's adjuvant, methylated bovine serum albumin (Sigma), fluoxetine, dexamethasone, Superoxide dismutase standard, epinephrine bitartarate, sodium carbonate, Dithiobis nitro benzoic acid, Trichloroacetic acid, Sodium citrate, Thiobarbituric acid, Trichloroacetic acid, sodium dihydrogen phosphate, Rheumatoid Factor kit, C-Reactive protein kit. All kits were purchased by Labcare Dignostics Pvt Ltd.

4.3 ANIMALS

Wistar rats of either sex with an initial body weight of 200 to 280 g are used The animals (three per cage) were kept under a 12 h light:12 h darkness cycle and with full access to food and water ad libitum for the following 14 weeks. Each group of animals was individually monitored for water and food intake. Wistar rats are divided randomly in Following groups (Treatment Protocol):

Freund's adjuvant Arthritis	**Number of animals**	**Abbrevation**
Control Groups		
Plain Control	6	PC
Dexamethasone Control (0.1 mg/kg)	6	DC
Fluoxetine Control (2 mg/kg)	6	FC
Dexamethasone (0.05 mg/kg) + Fluoxetine (2 mg/kg) Control	6	DC.05

Freund's adjuvant Arthritis	**Number of animals**	**Abbrevation**
Dexamethasone (0.1 mg/kg) + Fluoxetine (2 mg/kg) Control	6	DC.1
Dexamethasone (0.3 mg/kg) + Fluoxetine (2 mg/kg) Control	6	DC.3
Diseased groups		
Disease Control	6	CD
Disease Fluoxetine (2 mg/kg) Treated	6	DF
Disease Dexamethasone (0.3 mg/kg) Treated	6	DD
Disease Dexamethasone (0.05 mg/kg) + Fluoxetine (2 mg/kg) treated	6	DD.05
Disease Dexamethasone (0.1 mg/kg) + Fluoxetine (2 mg/kg) treated	6	DD.1
Disease Dexamethasone (0.3 mg/kg) + Fluoxetine (2 mg/kg) treated	6	DD.3

Table 4.1: Groups and number of animals in Complete Freund's Adjvuant Induced Arthritis

Antigen induced arthritis groups	**Number of animals**	**Abbrevation**
Normal Control	6	PC
Dexamethasone Control (0.1 mg/kg)	6	DC
Fluoxetine Control (2 mg/kg)	6	FC
Fluoxetine + Dexamethasone (0.1 mg/kg) Control	6	DC.1
Disease Control	6	CD
Disease Dexamethasone (0.1 mg/kg) Treated	6	DD
Disease Fluoxetine (2 mg/kg)Treated	6	DF
Disease Dexamethasone (0.1 mg/kg) + Fluoxetine (2 mg/kg) treated	6	DD.1

Table 4.2: Groups and number of animals in Methylated Bovine Serum Albumin Induced Arthritis

4.4 INDUCTION OF ADJUVANT ARTHRITIS AND TREATMENT PROTOCOL

Wistar rats with an initial body weight of 200 to 280 g are used. On day 0, they are injected into the subplantar surface of the left hind paw with complete Freund's adjuvant. Dosing with the test compounds or the standard is started on the day 1 and continued for 21 days. Paw volumes and body weight were recorded on the day of injection, whereby paw volume was measured plethysmographically. On day 5, the volume of the injected paw was measured again, indicating the primary lesion and the influence of therapeutic agents on this phase. Blood would be collected from retro orbital plexus for measurement of Serum C –reactive protein as an indicative of inflammation. Body weight measurements were carried out weekly. On day 21, Rats were killed and following parameters were measured as described by Vogel et al.

Assessments:

- Physical Parameters :Arthritic index, Paw oedema, Body weights and spleen weight to body weight ratio
- Biochemical and Haematological Factors : Serum Rheumatoid Factor, C- reactive protein, Erythrocyte sedimentation Rate
- Ulcer index (Number of Pateches)
- Oxidative Stress markers in synovial tissue
  Lipid peroxidation
  Reduced glutathione levels
- Histopathology of ankle : Cartilage degradation, pannus formation ,erosions

4.4.1 PHYSICAL PARAMETERS

4.4.1.1 ARTHRITIC INDEX

Arthritic index is the mean of the score given to severity of inflammation on the hind paws, fore paws, ears ,nose and tail.

SCORE

ears: absence of nodules and redness 0
presence of nodules and redness 1

nose: no swelling of connective tissue 0
intensive swelling of connective tissue . . 1

tail: absence of nodules 0
presence of nodules 1

forepaws: absence of inflammation 0
inflammation of at least 1 joint 1
hind paws:absence of inflammation 0
slight inflammation 1
moderate inflammation 2
marked inflammation 3

Evaluation :

An arthritic index is calculated as the sum of the scores as indicated above for each animal. The average of the treated animals is compared with the control group

4.4.1.2 PAW OEDEMA

Paw volumes were measured by digital plethysmometer.

For primary lesions: The percent difference of paw volume of the injected left paw over vehicle control is measured on day 5.
For secondary lesions: The percentage difference of paw volume of the non-injected right paw over controls is measured on day 21.

Paw volumes are physical indicators of the inflammation in early as well as chronic phase of the disease.

4.4.1.2 BODY WEIGHT

Body weights of each animal were recoreded on the day of Freund's adjuvant administration and later weekly till day 21.

4.4.1.3 SPLENOMEGALY

Spleen weight to body weight ratio was assessed to evaluate involvement of spleen.

4.4.2. BIOCHEMICAL AND HAEMATOLOGICAL PARAMETERS

4.4.2.1 C-REACTIVE PROTEIN (CRP-TURBILATEX)

In vitro quantitative measurement of CRP-Turbilatex concentration in serum was done by using diagnostic kit (Lab Care Diagnostics, India Limited.).

Principle:

The CRP-Turbilatex is a quantitative turbidimetric test for the measurement of C-reactive protein (CRP) in human serum or plasma. Latex particles coated with specific anti-human CRP are agglutinated when mixed with samples containing CRP. The agglutination cause an absorbance change, dependent upon the CRP content of the patient sample that can be quantified by comparison from a calibrator of known CRP concentration.

Procedure:

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Sample (CRP) as shown below: **Working reagent (Rw)-** Mix 1 ml Latex Reagent + 9 ml Diluent

	Blank	Standard	Sample
Rw (µl)	1000	1000	1000
Standard (µl)	-	5	-
Sample (µl)	-	-	5

Adjust the instrument to zero with distilled water. Mix and read the absorbance at 540 nm after 10 Seconds (A1) and after 2 minutes (A2) of the sample addition.

4.4.2.2 Erythrocyte Sedimentation Rate (ESR)

Erythrocyte sedimentation rate is measured by Westergren's Method.

Procedure:

A sample of blood (3 ml) was obtained and is mixed with 3.8% sodium citrate solution in proportion of four parts of blood to one part of citrate solution. The mixing of blood was done by rotating this sample gently between the palms of hands.

The blood is sucked slowly up to the mark zero in the Folin Wu tube.

The tube is set upright in the Westergren's stand, taking care that no blood escapes. The tube is fixed with the help of screw cap.

At the end of one hour and two hours, the upper level of red blood cell column is read. It will indicate mm of clear plasma or ESR.

4.4.2.3 SERUM RHEUMATOID FACTOR (RF-TURBILATEX)

In vitro quantitative measurement of RF-Turbilatex concentration in serum was done by using diagnostic kit (Lab Care Diagnostics, India Limited.).

Principle :

The RF-Turbilatex is a quantitative turbidimetric test for the measurement of Rheumatoid Factor in human serum or plasma. Latex particles coated with specific anti-human RF are agglutinated when mixed with samples containing RF. The agglutination cause an absorbance change, dependent upon the RF content of the patient sample that can be quantified by comparison from a calibrator of known RF concentration..

Procedure:

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Sample (CRP) as shown below: **Working reagent (Rw)-** Mix 1 ml Latex Reagent + 9 ml Diluent

	Blank	Standard	Sample
Rw (µl)	1000	1000	1000
Standard (µl)	-	10	-
Sample (µl)	-	-	10

Adjust the instrument to zero with distilled water. Mix and read the absorbance at 650 nm after 10 Seconds (A1) and after 2 minutes (A2) of the sample addition.

4.4.3 ULCER INDEX

Each lesion of stomach was measured along its greatest length and breath. For circular lesion, diameter was measured and finally area was calculated. In case of petechies, five of them were considered to be equivalent to 1 mm^2 of ulcerated area. The total area of the stomach mucosa and that of ulcerated mucosa were calculated. The ratio of total area of the stomach mucosa and that of ulcerated mucosa were calculated and then it was divided by 10 to obtain ulcer index.

$$X = \frac{\text{Total area of stomach mucosa}}{\text{Total area of ulcerated mucosa.}}$$

Ulcer Index = 10/X

The area of ulcerated portion was calculated as per the following formula:

Area of circular lesion $=\pi D^2/4$

Area of linear lesion =L*B

Area of stomach mucosa $=\pi D^2/8$

Where D =Diameter of the stomach mucosa

4.4.4 OXIDATIVE STRESS MARKERS IN SYNOVIAL JOINT TISSUE

Preparation of tissue Homogenates:

At the end of experiment, hind limbs were removed and maintained at 0^oC. Joint cartilage tissues were removed quickly and frozen until assay. On the day of analysis, cartilage samples were washed in ice cold 20 nM Tris-HCL, pH 7.4. The samples were homogenized with Tris-HCl buffer and supernant was collected for various antioxidant assessments.

4.4.4.1 REDUCED GLUTATHIONE:

Reduced of glutathione (GSH) was estimated by the method of Moran et. al, 1979.

Principle:

Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), A disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which measured colorimerically at 412 nm.

Reagents:

1. Trichloroacetic acid (10%):
   Dissolve 10 gm of TCA in 100 ml of distill water.
2. Dithiobis nitro benzoic acid (DTNB) :
   Dissolve 40 gm of DTNB in1% Sodium citrate solution.
3. Phosphate buffer (0.2 M, pH 8.0) :

Dissolve 1.36 gm of KH_2PO_4 in 100 ml of distill water and dissolve in 0.8 gm NaOH in 100 ml distill water.

4. Reduced of glutathione standard :

Dissolve 10 gm of GSH standard in 100 ml of distill water (100µg/ml).

Procedure:

Blank	**Test**
1 ml of D.W.	1 ml of Homogenate
1 ml of TCA (10%)	1 ml of TCA (10%)
Cool for 10 min and centrifuged at 2000 rpm take 0.5 ml of supernatant	
0.5 ml of ST	0.5 ml of ST
2 ml sodium hydrogen phosphate	2 ml sodium hydrogen phosphate
0.25 ml DTNB	0.25 ml DTNB

Mix well keep for at RT read the absorbance against blank at 412 nm using spectrophotometer.

Calculation: **Y = 0.0002X + 0.0049**

X = Conc. of reduced of glutathione

Y = Abs of test sample.

Units: µg of GSH / mg of protein.

4.4.4.2 LIPID PEROXIDATION

Malondialdehyde formation (MDA) was estimated by the method of Ohkawa et al., 1979**.**

Principle:

The method estimates Malondialdehyde (MDA), a product of lipid per oxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under

mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured colorimetrically at 535 nm.

Reagents:

1. Thiobarbituric acid (1% in Tris hydrochloride, pH 7):
   1 gm of thiobarbituric acid was dissolved in 100 ml of Tris hydrochloride buffer pH 7.
2. Trichloroacetic acid (10%):
   10 gm of trichloroacetic acid was dissolved in distilled water.
3. SLS (8%)
   8 gm of SLS in 100 ml of water.

Procedure:

Blank	**Test**
0.2 ml of D.W.	0.2 ml of Homogenate
0.2 ml of SDS	0.2 ml of SDS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml DW	0.6 ml DW
Heated for 45 min in water bath at 95^0 C and cool	
2ml mixture + 2 ml TCA	2ml mixture + 2 ml TCA
Centrifuge on 1000 rpm for 5 min	
Pink color measure at 532 nm	

Calculations :A = a * b * c

A = abs.

a = mol. Extinction coefficient ($1.56 * 10^5$ cm^{-1})

b = Path length (1 cm^2)

c = con of sample

Units: nm of MDA / gm of tissue.

4.4.5 HISTOPATHOLOGICAL ANALYSIS OF ANKLE JOINTS

Rats were killed on day 21 by euthanasia. Hind limbs were removed and fixed in formalin. The limbs were decalcified in 5% formic acid, processed for paraffin embedding, sectioned at 5 μm thickness and subsequently stained with haematoxylin-eosin for examinations under a light microscope. Sections were examined for cartilage destructions, hyperplasia of synovium, pannus formation and destruction of joint space. The biopsy studies of synovial joints (Ankle) of all the groups were carried out at "Accutest Research Laboratories India Pvt Ltd".

4.5 INDUCTION OF ANTIGEN INDUCED ARTHRITIS AND TREATMENT PROTOCOL

Immunization

On day -14 rats were subcutaneously injected with a total volume of 2.0 ml of a suspension containing 0.5 mg/ml mBSA in equal volumes of phosphate- buffered saline (PBS) and Freund's complete adjuvant (2 mg MT/ml; Griffiths et al, 1992).

Induction of arthritis

On day 0, 0.1 mg mBSA in 50 µl of PBS were intraarticularly injected into the right knee (Griffiths et al, 1992). The left knee joint was injected with PBS only and used as control. treatment before and during the peak of primary arthritis (150mg/kg from day -1 to day 6; n=6); On day 7 following parameters are assessed, the animals were killed and dissected for histological examination.

Measurements:

- Joint Diameter
- Haematological Parameters :Erythrocyte sedimentation Rate, White Blood Cell count
- Body weight
- Oxidative stress markers in synovial tissue
  Superoxide dismutase
  Lipid peroxidation
  Reduced malonaldehyde levels
  Catalase levels
- Histopathology of the knee joint : cartilage degeneration, pannus formation, granulomatous erosions

4.5.1 JOINT DIAMETER

Joint diameter is important disease marker for clinical evaluation of rheumatoid arthritis. In Antigen induced monoarthritis, Swelling of diseased joint can be calculated by measuring difference in joint diameter from that of normal joint of the same animal. Measurements are done with the help of Caliper.

4.5.2. HAEMATOLOGICAL PARAMETERS

4.5.2.1 WHITE BLOOD CELL COUNT

Total white blood cell count is performed by heemocytometric method using Neubauer's chamber.

Requirements: Haemocytometric set- Neubauer's counting chamber, WBC dilution pipette; Thomas coverslip, microscope, WBC dilution fluid, 70% alcohol

Composition of WBC dilution fluid:

Substance	Amount	Purpose
Glacial Acetic acid	2 ml	Destroys RBCs
Gentian Violet (1%)	1 ml	Stains nuclei of WBCs
Water	Up to 100 ml	Diluent

Procedure:

Blood for the total leukocyte determination was collected on day 7 post treatment from the retro-bulbar plexus of the medial canthus of the eye, by carefully inserting a micro-capillary tube into the medial canthus of the eye to puncture the plexus and enable outflow of blood into the sample bottle containing EDTA. This was shaken gently to prevent clotting.

The blood is sucked in WBC pipette upto the mark 0.5. Immediately the dilution fluid sucked exactly upto mark 11. Pipette is then brought to horizontal position and the finger is placed over the tip of piptte. The pipette is rolled between the palms to mix the blood with dilution fluid.

Few drops are discarded and then the pipette is held at an angle of 45 to the surface of counting chamber and tip is applied to the narrow slit between the counting chamber and the coverslip. A drop is allowed to come out the pipette. The fluid will run into the capillary space because of capillary action and it is filled. The drop should not flow into the moat.

The fluid is allowed to settle for three minutes on the stage of microscope. The WBC chamber is located and WBCs in 16 smallest squares of WBC are counted. They are counted in 4 squares chambers.

Calculations :

Total Cell counts in 4 chambers : N
Total WBC count in blood = N * 50/cmm

4.5.2.2 ERYTHROCYTE SEDIMENTATION RATE

On day 7 ,ESR is measured by Westergren's method as described earlier in the section 4.4.2.2

4.5.3 BODY WEIGHT

Assessements of the body weights were performed daily from day 1 to day 7 to evaluate any chances of unwanted effects of the tested drug combinations.

4.5.4 OXIDATIVE STRESS MARKERS IN SYNOVIAL JOINT TISSUE

Preparation of tissue Homogenates:

At the end of experiment, hind limbs were removed and maintained at 0^oC . Knee joint cartilage tissues were removed quickly and frozen until assay. On the day of analysis, cartilage samples were washed in ice cold 20 nM Tris-HCL, pH 7.4. The samples were homogenized with Tris-HCl buffer and supernant was collected for various antioxidant assessments.

4.5.4.1 Reduced glutathione and Lipid peroxidation

Assessments were carried out as the method described earlier in the section 4.4.4

4.5.4.2 Catalase levels

Catalase was estimated by the method of Aebi et al, 1987.

Principle:

In the ultra-violet range H_2O_2 shows a continuous increase in absorption with decreasing wavelength. The décor position of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in the absorbance per unit time is a measure of the catalase activity.

Reagent:

1. Phosphate buffer (50 m mol/L pH 7)

   a) Dissolve 6.81 gm of KH_2PO_4 in distill water and make up volume to1000 ml with distill water.

   b) Dissolve 8.9gm of Na_2HPO_4 in distill water and make up volume to 1000 ml with distill water.

2. Hydrogen peroxide (30 n mol/L)

Procedure:

Blank	**Test**
2910 uL of phosphate buffer pH 7	2910 uL of phosphate buffer pH 7
50 uL of Distilled water	50 uL of Diluted Homogenate.
40 uL of hydrogen peroxide solution	40 uL of hydrogen peroxide solution

Add H_2O_2 just before taking OD at 240 nm; take the reading for 3 min. with 15 second interval.

Calculation:

Log A1/A2 × 229.7 (factor) A1= initial Absorbance, A2= final Absorbance.

Units = units / mg of protein.

4.5.5 Histopathological Evaluations of knee joints:

Histopathological evaluations are very important for antigen induced arthritis as it involved most relevant pathway of cartilage destruction to human disease. On day 7, rats are killed and right hind paws are isolated and fixed in formalin. The limbs were decalcified in 5% formic acid, processed for paraffin embedding, sectioned at 5 μm thickness and subsequently stained with haematoxylin-eosin for examinations under a light microscope. Sections were examined for cartilage destructions, hyperplasia of synovium, pannus formation and destruction of joint space. The biopsy studies of synovial joints (knee) of all the groups were carried out at "Accutest Research Laboratories India Pvt Ltd".

4.6 STATISTICAL ANALYSIS

All the values are expressed as mean ± S.E.M. Statistics was applied using SPSS software version 12.0. Statistical significance between normal control and diseased control groups was tested using student's t-test. T- test was used also to determine the statistical significance between diseased and test groups as well as the statistical significance between dexamethasone treated group and combination treated groups. Differences were considered to be statistically significant when $p < 0.05$.

5.1 FREUND'S ADJUVANT ARTHRITIS

5.1.1 PHYSICAL PARAMETERS

5.1.1.1 ARTHRITIC INDEX AND PAW OEDEMA

Administration of Freund's adjuvant induced severe chronic disease of arthritic index in disease control group which was significantly different from plain control. Dexamethasone treated group reduced significantly arthritic score compared to disease control. Combination groups at 0.3 mg/kg and 0.1 mg/kg dexamethasone produced significant reduction of arthritic score which were statistically similar to dexamethasone treated group.($P<0.05$)

Freund's adjuvant induced high increase of percentage paw volume on day 5 on injected paw in disease control groups which was significantly different from plain control. Control treated groups did not show any significant change. Dexamethasone treated group showed significant reduction in % paw volume difference. Fluoxetine treated group showed significant reduction in % paw volume difference. All Combination treatment groups at all doses of dexamethasone (0.3, 0.1 and 0.05 mg/kg) produced significant dose dependent reduction in % paw volume difference. All combination treatment did not produce statistically effect from dexamethasone treated group.($P<0.05$).

High increase of percentage paw volume on day 21 on noninjected paw in disease control groups which was significantly different from plain control. Dexamethasone treated group experienced significant reduction in % paw volume difference. Treatment with Fluoxetine (2 mg/kg) did not show any significant reduction in % paw volume rise in fluoxetine treated group. Combination treated groups at 0.3mg/kg and 0.1 mg/kg doses of dexamethasone produced significant dose dependent reduction in % paw volume difference and have comparable effects to that of dexamethasone treated group. ($P<0.05$).

5.1.1.2 BODY WEIGHT

Gradual but statistically significant reduction in body weight was obtained in disease control groups while gradual rise in body weight was observed in plain control group. Treatment with Dexamethasone (0.3 mg/kg) raised percentage weight loss significantly in both dexamethasone control and dexamethasone treated group compared to plane control and disease control group respectively. Percentage weight loss also was seen in fluoxetine control and treated group not initially but on day 14 and day 21 compared to plain control and disease control group.

Combination groups at all doses of dexamethasone produced significant % body weight loss in both combination control and treated groups. However % weight loss in combination groups of O.1 mg/kg and 0.05 mg/kg dexamethasone treated and control groups were statistically similar to 0.3 mg/kg dexamethasone control and treated groups respectively. Combination group with dexamethasone 0.3 mg/kg showed statistically significant % weight loss compared to that of dexamethasone treated group. ($P<0.05$)

5.1.1.3 SPLEEN WEIGHT TO BODY WEIGHT RATIO (MG/GM)

Administration of Freund's adjuvant increased spleen weight to body weight ratio in disease control groups which was significantly different from plain control. Dexamethasone treated group reduced significantly the ratio compared to disease control. Fluoxetine treated group showed significant reduction in ratio compared to disease control. Combination groups at all does of dexamethasone produced significant reduction of the ratio compared to disease control as well as compared to dexamethasone treated group.($P<0.05$).

5.1.2 BIOCHEMICAL AND HAEMATOLOGICAL PARAMETERS

5.1.2.1 C-REACTIVE PROTEIN LEVEL (CRP) (MG/L) AND ERYTHOCYTE SEDIMENTATION RATE (MM/HR)

Administration of Freund's adjuvant raised levels of serum CRP in disease control groups which was significantly different from plain control. Dexamethasone treated

group reduced significantly serum CRP compared to disease control. Combination treated groups at all does of dexamethasone (0.05, 0.1 and 0.3 mg/kg) produced significant reduction in CRP Fluoxetine treated group did not produced any significant reduction in c-reactive protein levels. Combination groups at 0.3 mg/kg and 0.1 mg/kg dexamethasone gave statistically similar effect to that of dexamethasone treated group. ($P<0.05$) Administration of Freund's adjuvant increased ESR on day 21 in disease control group which was significantly different from plain control. dexamethasone treated group reduced significantly the ratio and fluoxetine treated group showed reduction in ESR to significantly compared to disease control group. Combination treated groups at all does of dexamethasone (0.05, 0.1 and 0.3 mg/kg) produced significant reduction of ESR compared to disease control. Combination groups at highest dose produced statistically similar effect to that of dexamethasone alone. ($P<0.05$).

5.1.2.2 SERUM RHEUMATOID FACTOR LEVEL (IU/ML)

Administration of Freund's adjuvant showed presence of high levels of serum Rheumatoid factor levels in disease control groups which was significantly different from plain control. Dexamethasone treated group reduced significantly serum Rheumatoid factor levels compared to disease control group. Combination treated groups at all does of dexamethasone (0.05, 0.1 and 0.3 mg/kg) produced significant reduction of Rheumatoid factor levels. Combination group at dexamethasone 0.3 mg/kg dose produced statistically significant effect from dexamethasone 0.3 mg/kg treated group.($P<0.05$)

5.1.3 ULCER INDEX

Treatment of dexmethasone resulted in significantly high incidence of ulceration in treated group compared to disease control. The effect also observed in fluoxetine control group compared to plain control.

Combination groups both dexamethasone 0.3 mg/kg treated and control produced high degree of ulceration significant compared to dexamethsone treated group.

However, combination groups at 0.1 mg/kg and 0.05 mg/kg in treated as well as control show statistically similar ulcer index to dexamethasone 0.3 mg/kg treated and control groups respectively. ($P<0.05$).

5.1.4 OXIDATIVE STRESS MARKERS IN SYNOVIAL JOINT TISSUE

5.1.4.1 TISSUE MALONDIALDEHYDE LEVELS (NMOL/MG PROTEIN)

Disease control group showed significant amount of maldondialdehyde levels compared to plain control group. Dexamethasone 0.3 mg/kg treated groups showed significant reduction in malondialdehyde levels compared to disease control. Combination treated groups at all doses showed significant dose dependent reduction in malondialdehyde levels compared to disease control. However, effect in combination groups was statistically similar compared to dexamethasone treated group. ($P<0.05$)

5.1.4.2 REDUCED GLUTATHIONE LEVELS (µG/MG PROTEIN)

Disease control group showed significant reduction of reduced glutathione compared to plain control group. Dexamethasone 0.3 mg/kg treated groups showed raised in reduced glutathione levels compared to disease control group. Combination treated groups at all doses showed significant dose dependent increase in reduced glutathione levels compared to disease control. Effect of 0.1 mg/kg and 0.05 mg/kg dose dexamethasone carrying combination were comparable to dexamethasone treated group. Combination group at dexamethasone 0.3 mg/kg showed significant effect compared to dexamethasone treated group.($P<0.05$)

5.1.5 HISTOPATHOLOGICAL EVALUATIONS OF ANKLE JOINTS

Histopathological evaluations of ankle joints of disease control groups showed destroyed architecture of synovial tissue. Fluoxetine treated group and combination groups with 0.05 mg/kg dexamethasone showed disrupted architecture and breaking

of synovial lining. However, desruption of joint tissue was less in Dexamethasone treated and combination treated groups at 0.3 and 0.1 mg/kg dexamethasone compared to disease control groups in sections of ankle.

5.2. METHYLATED BOVINE SERUM ALBUMIN INDUCED ARTHRITIS

5.2.1 JOINT SWELLING (MM)

Intra-articular injection of methylated bovine serum albumin causes swelling of joints in diseased control group compared to plain control group. Dexamethasone 0.1 mg/kg significantly reduced joint swelling compared to disease control however no significant effect of observed in fluoxetine treated group. Combination treated groups showed similar reduction in joint swelling compared to that of dexamethasone treated group.($P<0.05$)

5.2.2 HAEMATOLOGICAL PARAMETERS

5.2.2.1 TOTAL WHITE BLOOD CELL (WBC) COUNT (NO. PER CMM)

Antigen administration raised total WBC count in the blood in disease control group animals. Dexamethasone 0.1 mg/kg reduced WBC count in both treated and control groups significantly from disease control and plain control groups.. Reduction in WBC count was seen in fluoxetine treated group. However, combination treated group showed similar reduction as do the dexamethasone treated group. ($P<0.05$)

5.2.2.2 ERYTHROCYTE SEDIMENTATION RATE

Disease control groups showed significant raise in ESR compared to plain control group. Dexamethasone 0.1 mg/kg showed reduction in ESR however it was not statistically significant. Fluoxetine and combination groups showed statistically significant reduction in ESR compared to disease control group. ($P<0.05$)

5.2.3 BODY WEIGHT

Plain control and disease control groups showed weight gain during period of 7 days while dexamethasone and combination treated groups showed significant weight loss. Weight loss was less in fluoxetine groups. Combination treated groups showed comparable weight loss to dexamethasone treated group. ($P<0.05$)

5.2.4 OXIDATIVE STRESS MARKERS IN SYNOVIAL JOINT TISSUE

5.2.4.1 MALONDIALDEHYDE AND REDUCED GLUTATHIONE LEVELS

Arise in the oxidative stress was observed in synovial tissues of disease control groups compared to plain control animals. Dexamethasone treatment significantly correct malondialdehyde and reduced glutathione levels compared to disease control. Combination treatment animals showed statistically lower malondialdehyde and higher reduced glutathione levels compared to dexamethasone treated group ($P<0.05$)

5.2.4.2 CATALASE LEVELS

Injection of methylated bovine serum albumin suppressed catalase levels in the local joint after 7 days compared to plain control group. Dexamethasone treated group showed significant improvement in catalase levels compared to disease control group. However, such effect was absent in fluoxetine treated group. Combination of dexamethasone and fluoxetine significantly improved catalase levels in synovial tissues compared to disease control as well as dexamethasone treated group. ($P<0.05$)

5.2.5 HISTOPATHOLOGICAL EVALUATIONS OF KNEE JOINTS

Haemtoxylin-eosin sections of injected knee joints of disease control rats showed synovial hyperplasia and cartilage tissue damage. Fluoxetine treated group showed similar destruction and hyperplasia. However, hyperplasia and tissue damage is greated reduced in dexamethasone and combination treated groups.

Discussion and Conclusion :

Immunologic mechanisms contribute to the pathogenesis of rheumatoid arthritis is evidenced serologically by antiglobulin antibodies (rheumatoid factors) and histologically by lymphocytes, plasma cells and macrophages in the rheumatoid synovium. [Brackertz et al, 1977] Combining fluoxetine with the standard drug- dexamethasone would be the preferred approach for studying antiarthritic activity as they are already included in a common regimen for Rheumatoid arthritis.

In adjuvant arthritis, combination therapy with 0.3 mg/kg dexamethasone suppressed rheumatoid factor as well as splenomegaly and improved antioxidant assessments and histopathological changes significantly compared to dexamethasone 0.3 mg/kg treatment but showed aggrevation of stomach ulcers and weight loss. Combination group having 0.1 mg/kg dexamethasone also produced similar effect on C-reactive protein, arthritic index, paw oedema, antioxidant assessments, serum rheumatoid factor and histopathological evaluations. Fluoxetine treated group showed reduction only in splenomegaly and primary lesions. In antigen induced arthritis, combination group with 0.1 mg/kg dexamethasone significantly suppressed antioxidant assessments, ESR and histopathological menifestations compared to dexamethasone 0.1 mg/kg while effects on WBC count and joint diameter were found similar.

Combination therapy of dexamethasone and fluoxetine are more effective than dexamethasone alone in Freund’s adjuvant and Methylated bovine serum albumin induced arthritis. Further investigations are warranted to examine the possible involvement of P-glycoprotein suppression for improving dexamethasone pharmacokinetics and its therapeutic effect as well as to evaluate the role of fluoxetine on communication between inflammatory and central nervous system through serotonin.

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