PREVENTIVE EFFECT OF FISH COLLAGEN PEPTIDE IN CFA INDUCED ARTHRITIC RATS

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5.1 Introduction

Arthritis is a form of joint disorder that involves inflammation of one or more joints. There are different forms of arthritis. The most common form, osteoarthritis (degenerative joint disease) is a result of trauma to the joint, infection of the joint, or age (Felson *et al.,* 2000; Leyland *et al.,* 2012). Osteoarthritis (OS) is characterized by progressive destruction of joint cartilage and its associated structures (bone, synovial and fibrous joint capsules), remodeling of the periarticular bone, and inflammation of the synovial membrane (Blagojevic *et al.,* 2010). This disorder is basically produced by an imbalance between the synthesis and degradation of the articular cartilage. This imbalance leads to the classic pathologic changes of wearing away and destruction of cartilage. (Kuptniratsaikul *et al.,* 2002; Loeser *et al.,* 2012)

Rheumatoid arthritis (RA) is characterized by inflammation of the synovial membrane of diarthrodial joints. Early indications of RA are swelling and pain of the proximal inter-phalangeal and later, the larger joints become affected, especially those of the knee, elbow and ankle. (Mc Innes and O'Dell, 2010) Hyperplasia or

thickening of the synovial membrane is promoted by cytokines and growth factors released from migrating cells. The synovial membrane becomes revascularised making it redder than normal. The cytokine enriched environment produced by pro-inflammatory cytokines (IL-1α, IL-6 and TNF-α) results in the aberrant growth of complex vessels known as pannus which invades the cartilage resulting in the degradation of the articular surfaces (Astusi *et al.,* 2005; McInnes and Schett, 2011).

Rheumatoid arthritis progresses in three stages. The first stage is the swelling of the synovial lining, causing pain, warmth, stiffness, redness and swelling around the joints. Second is the rapid division and growth of cell, or pannus, which causes the synovium to thicken. In the third stage, the inflamed cell releases enzyme that may digest the bone and cartilage, often causing the joints to lose its shape and alignments, more pain and loss of movements (Scott *et al.,* 1998; Mc Innes and Schett, 2011).

There are different classes of anti-arthritic drugs available like non-steroidal anti-inflammatory drugs (NSAIDS), Monoclonal antibodies, uricosuric agents, gold compounds, anti-cytokinine immunosuppressant like glucocorticoids, etc. Though the goal of these drugs has been to relieve pain and to decrease joint inflammation, these drugs are known to produce various side effects including gastrointestinal disorders, organ damages, immunodeficiency and humoral disturbances. (Harirforoosh and Jamali, 2009; Roth, 2005) Selective COX-2 inhibitors make alternative approach to arthritic treatment with reduced GI side effects, but on long term treatment leads to serious cardiovascular and thrombotic side effects. Accordingly, reducing side effects should be considered while designing improved therapeutics for arthritis, besides enhancing medicinal effectiveness (Moore, 2007).

The extracellular framework and two-thirds of the dry mass of adult articular cartilage are polymeric collagen. Treatment with chondroprotectives, such as glucosamine sulfate, chondroitin sulfate, hyaluronic acid, collagen hydrolysate, or nutrients, such as

antioxidants and omega-3 fatty acids are being increasingly recognized as an alternate approach to arthritic treatment (Henrotin *et al.,* 2012; Jerosch, 2011). Numerous clinical studies have demonstrated that the targeted administration of selected micronutrients leads to a more effective reduction of OA symptoms, with less adverse effects. Their chondroprotective action can be explained by a dual mechanism: (1) as basic components of cartilage and synovial fluid, they stimulate the anabolic process of the cartilage metabolism; (2) their anti-inflammatory action can delay many inflammation-induced catabolic processes in the cartilage. These two mechanisms are able to slow the progression of cartilage destruction and may help to regenerate the joint structure, leading to reduced pain and increased mobility of the affected joint (Sawitzke *et al.,* 2010).

Rat adjuvant arthritis is a chronic, polyarticular, erosive type of arthritis induced by an injection of killed mycobacterium (Pearson and Wood, 1959). It is an experimental model that shares some features with human rheumatoid arthritis. One of the most important features of adjuvant arthritis is the chronic synovitis, including inflammatory cell infiltration, pannus formation, cartilage destruction and bone erosion. Adjuvant arthritis is widely used for studying the pathogenesis of rheumatoid arthritis and for searching new drugs for treatment of rheumatoid disease (Greenwald, 1991; Zheng and Wei, 2005).

The present research work was aimed at the scientific validation of the anti arthritic effect of Fish Collagen Peptide (FCP) of fish skin origin in CFA induced rat model system. Freund's complete adjuvant induced arthritis in rat model which is the best and most widely used experimental model for arthritis with clinical and laboratory features which closely mimic the clinical features of human rheumatoid disease. This model is sensitive to anti-inflammatory and immune inhibiting medicines and considers being relevant for the study of pathophysiological and pharmacological control of inflammation process as well as for the evaluation of anti-

arthritic potential of drugs (Greenwald *et al.,* 1991; Simoes *et al.,* 2005).

Various experimental parameters like changes in Paw edema, Body weight, Arthritic index evaluated during the course of treatment and at the end of the study X ray radiographs, various blood parameters relevant in the arthritic condition, bone histopathology of synovial joints analysis were performed.

5.2 Materials and methods

5.2.1 Animals used

Wistar strain male albino rats, weighing130 ± 20 g, were selected for the study. The animals were kept in polypropylene cages (with stainless steel grill top) under hygienic and standard environmental conditions (temperature of 22 ± 2°C, humidity 60-70%, and 12 hr light/dark cycle). The animals were allowed a standard diet (procured from M/s Sai feeds, Bangalore, India) and water ad libitum. The study was conducted with the approval from the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

5.2.2 Toxicity study

For toxicity studies, two groups of rats with 6 animals each were taken. One group observed as control and the other group is treated with FCP.For acute oral toxicity study, FCP at a single dose of 2g/kg body weight was given orally to test group whereas an equal volume of water was given to control group. Observations were made and recorded systematically 1, 2, 4 and 6 h after FCP administration. The visual observations were noted. The number of survivors was noted after 24 h and these were then maintained for a further 14 days with once in daily observation. On day 15, all rats were fasted for 16−18 h, then anesthetized with ethyl ether and sacrificed.

For sub acute oral toxicity study, FCP at the dose of 1g/kg body weight for 14 days, whereas an equal volume of water was given to control and kept for other 14 days after treatment. During the period of administration, the animals were weighed and observed daily to detect signs of toxicity. Daily visual observations were made and recorded systematically. At the end of the period, all rats were fasted for 16–18 h, then anesthetized with ethyl ether and sacrificed.

5.2.3 Complete Freund's adjuvant induced Arthritis

Arthritis was induced in rats by the intraplantar injection of 0.1 ml of Complete Freund's Adjuvant (CFA) containing1.0 mg dry heat-killed Mycobacterium tuberculosis per milliliter sterile paraffin oil into a foot pad of the left hind paw of male rats. A glass syringe (1 ml) with the locking hubs and a 26G needle was used for injection. The rats were anesthetized with ether inhalation prior to and during adjuvant Injection, as the very viscous nature of the adjuvant exerts difficulty while injecting. The paw swellings were periodically examined up to 21 days. The diameter of each paws from the ankle were measured using screw-gauge in mm measurement.

5.2.4 Experimental setup

The effect of test drug for the established type of Adjuvant-induced arthritis was carried out by the method of Persico *et al.* (1988). The dosing schedule of the drug and the experimental set up are shown in table 5.1 and fig.5.1 respectively. The animals were divided into five groups of six animals each. Group I served as control (without treatment), Group II served as arthritic control (disease control), Group III was treated with diclophenac sodium (positive control), the standard drug. Group IV & V were treated with different concentrations of test sample, the FCP. The animals in all the groups (except group II) were administered with the treatment by oral route twice a day for 28 days. Animals in group I was administered with

10ml/ kg distilled water. While the animals in group III were treated with the standard drug diclophenac sodium (5mg/kg). Animals of group IV and V were fed with 0.5g/kg and 1.0g/kg FCP.

Table 5.1 Dosing schedule and treatment in different groups

Sl. No	Group	No. of Animals	Treatment	Dose
1	Group 1 Normal Control	6	Neither CFA treated nor drug treated	-----
2	Group 2 Disease Control	6	Freund's adjuvant (CFA)	0.2 ml (3mg/ml)
3	Group 3 Standard Drug Treatment	6	Diclofenac sodium + CFA	10 mg/kg body weight
4	Group 4 FCP treatment	6	Collagen peptide + CFA	0.5g/kg body weight
5	Group 5 FCP treatment	6	Collagen peptide + CFA	1 g/kg body weight

Figure 5.1 Experimental design of arthritis study
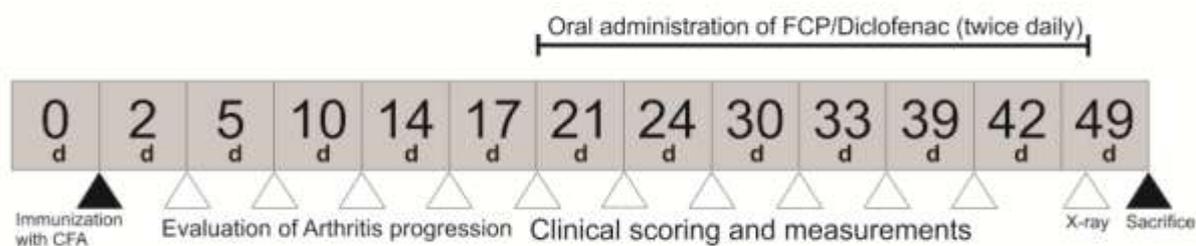
5.2.5 Evaluation of the development of arthritis

Rats were inspected daily for the onset of arthritis characterized by oedema and/or erythema in the paws. The incidence and severity of arthritis were evaluated using a system of arthritic scoring, and measurement of paw oedema every 2 days from the starting day of the experiment. Animals were observed for presence or absence of nodules in different organs like ear, fore paw, hind paw, nose and tail.

5.2.5.1 Paw oedema

Paw size of both hind limbs were recorded on the day of CFA injection, and measured every 2 days beginning on the day of starting the experiment using screw-gauge in mm measurement. The 6[th]day measurement is indicative of primary lesions and13[th]day measurement will aid in estimating secondary lesions. On the 21st day, the secondary phase of rheumatoid arthritis becomes more evident and inflammatory changes spreads systemically and becomes observable in the limb not injected with Freund's adjuvant. The mean changes in injected paw oedema with respect to initial paw size, were calculated on respective days and percentage inhibition of paw oedema with respect to untreated group was calculated using following formula.

Percentage inhibition = 1-Change in paw oedema of untreated group/ change in paw oedema of treated group x 100

5.2.5.2 Body weight

Changes in body weight have also been used to assess the course of the disease and the response to therapy of anti arthritic drugs. Body weight of each animal was measured every 3 days alternatively using electronic balance.

Percentage increase in body weight = (final body weight-initial body weight)/ initial body weight x 100

5.2.5.3 Arthritis score assessment

The incidence and severity of arthritis were evaluated using a system of arthritic scoring every 3 days beginning on the day next to adjuvant injection by two independent observers. Lesions of both hind paws of each rat were graded from 0 to 4 according to its clinical arthritic signs described by Brand *et al.,* (2007). The scoring system is in table 5.2

Table 5.2 Scoring system for subjective evaluation of arthritis severity

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

The total arthritis scores were calculated from the sum of both hind paws, with a maximum possible score of 8 for each rat.

5.2.5.4 Biochemical Analysis

The animals were sacrificed by ether anesthesia at the end of the experiment and the blood was collected by cardiac puncture prior to the sacrifice. The spleen were rapidly removed and washed with ice-cold saline. The tissues were cut into small pieces and homogenised using tris buffer (0.01 M, pH 7.4) at 4°C to give 10% homogenate. The haemolysate was extracted. The collected blood with anti-coagulant was centrifuged to remove the plasma.

Blood samples were collected from a carotid artery into heparinized and dry non-heparinized centrifuge tubes. The heparinized blood was used for hematological study and the serum separated from the non-heparinized blood was assayed for biochemical analysis. For biochemical analysis, blood was centrifuged at 1500 g for 10 min to obtain serum and the following parameters like ALP (marker for bone destruction), ACP (the lysosomal enzyme activity), SGOT, SGPT, CRP, ceruloplasmin, urea, creatinine, were estimated by using respective kits.

5.2.5.5 Anti CCP

Anti Cyclic Citrullinated Peptide Ab has been assayed since it is a convenient immune marker for the inflammation. This test is considered as a novel arthritis detection test commonly employed in humans. The analyses anti-CCP was carried out in the Department of Clinical Immunology, DDRC Ernakulam.

Anti-CCP antibodies were detected using a commercial anti-CCP2 enzyme linked immunosorbent assay kit, following the manufacturer's instructions. Briefly, microtitre plates were incubated for 60 minutes at room temperature with serum samples diluted at 1:100 in phosphate buffered saline. Prediluted anti-CCP standards and positive and negative controls were added to each plate. All assays were done in triplicate. After three washes, plates were incubated for 30 minutes at room temperature with alkaline phosphatase labelled murine monoclonal antibody to human IgG. After three further washes, the enzyme reaction was developed for 30 minutes, stopped with sodium hydroxide-EDTA-carbonate buffer, and the plates were read at 550 nm wavelength. Anti-CCP was considered positive when the absorbance value was higher than the cutoff of the kit (5 U/ml). The concentration of anti-CCP autoantibody was estimated by interpolation from a dose–response curve based on standards. All serum samples with high concentrations of anti- CCP antibodies were further quantified at a greater sample dilution.

5.2.5.6 COX (Cycloxygenase) activity assay

The COX levels in CFA-induced rat paws were determined using Cayman's COX fluorescent activity assay kit. It provides a convenient fluorescent based method for determining COX I and COX ll activities in both crude (tissue homogenates) and purified enzyme preparations. The assay utilizes the peroxidase component of COXs. In this assay, the reaction between PGG2 and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin which can be analyzed using an excitation

wavelength of 530-540 nm and an emission wavelength of 585-595 nm The kit also includes isozyme-specific inhibitors for distinguishing COX ll activity from COX l. The COX concentration is expressed as Fluorophore Units (FU) from the standard curve for resorufin. 1 FU is defined as the amount of enzyme that will cause the formation of 1 nm fluorophore per minute at 22°C.

5.2.5.7 Histological processing and assessment of arthritis damage

The histopathologic assessment was focused on the ankle joints with the most severe joint damage and each joint was evaluated separately. Hematoxylin and eosin-stained sections were observed for inflammation and pannus formation by two independent observers.

Histopathological changes were scored using the following parameters. Infiltration of cells was scored on a scale from 0 to 3, depending on the amount of inflammatory cells in the synovial tissues. Inflammatory cells in the joint cavity were graded on a scale from 0 to 3 and expressed as exudate. A characteristic parameter in Freund's complete adjuvant is the progressive loss of articular cartilage. This destruction was separately graded on a scale from 0 to 3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of the articular cartilage. Bone erosion was scored on a scale ranging from 0 to 3, ranging from no abnormalities to complete loss of tarsus. Cartilage and bone destruction by pannus formation was scored ranging from 0, no change; 1, mild change (pannus invasion within cartilage); 2, moderate change (pannus invasion into cartilage/subchondral bone); 3, severe change (pannus invasion into the subchondral bone); and vascularity (0, almost no blood vessels; 1, a few blood vessels; 2, some blood vessels; 3, many blood vessels).

5.2.5.8 Radiological findings

X-ray radiography analysis has been done in order to display changes in the joints such as bony erosion and variation at joints of

different experimental animals. Before sacrificing the animals; X-rays were taken at the joints of the hind paw of the animals for evaluating the bone damage. Radiographs were taken using X-ray apparatus (Siemens- 60MA, Germany) and industrial X-ray film (Fuji photo film, Japan). The X-ray apparatus was operated at 220 V with a 40 V peak, 0.2 second exposure times, and a 60cm tube-to film distance for leg projection. Radiological visual scoring was performed by two different observers and visual scoring values were calculated based on the following conditions

Erosions: 0-3 (none, mild, moderate, severe),
Joint space narrowing: 0-3(none, minimal, moderate, severe), and
Joint space destruction: 0-3(none, minimal, extensive, ankylosis)

5.2.5.9 Statistical analysis

The results are expressed as Mean ± SE from n=6 observations. The findings were also analyzed for determining significance of difference by ANOVA test followed by pair-wise comparison of various group by LSD. The differences among groups were considered to be significant at $p < 0.05$. The analysis was carried out by using SAS system version 9.3 (SAS Institute Inc., Cary, NC, USA)

5.3 Results and Discussion

A considerable part of the disability caused by arthritis conditions is joint damage. Accordingly, preventing and diminishing joint damage is an important treatment goal in early arthritis. Hence, reliable predictors of joint damage are required.

The immunologically mediated complete Freund's adjuvant (CFA) arthritic model of chronic inflammation is considered as the best available experimental model of arthritis (Williams, 1998) as it has been shown to share a number of clinical and immunological features with human arthritis. Method mimics the human pathophysiological state including chronic swelling in multiple

joints due to accumulation of inflammatory cells, joint cartilage erosion, bone destruction and used to investigate the activity of various potent anti-inflammatory and anti-arthritic agents. Therefore, the findings with this model are considered to have higher clinical reproducibility in arthritis.

In CFA model, macrophages play a central role. After activation they are capable of synthesizing mediators such as PGE2 and cytokines such as TNF-α and IL-1 and they induce the production of a variety of enzymes which initiate cartilage and bone destruction (Hopkins *et al.,* 1990). It is also reported that damage to the cartilage in arthritic joint is associated with the cellular output of toxic agents such as nitric oxide and its oxidizing product (e.g. peroxynitrite),free radicals and products of hydrogen peroxide (e.g. hydroxyl radical and hypochlorous acid).

Augmentation in migration of total leukocyte, lymphocytes and monocytes/macrophages from blood into the synovial cavity influence the arthritic condition of joint (Levy *et al.,* 2006) and these mediators are responsible for the pain, destruction of cartilage and leads to severe disability.

5.3.1 Toxicity study

Acute and sub acute oral toxicity study in male Wistar albino rats shows no evidence of significant adverse effect or health risk toxic effects. According to the hematological, biochemical, and organ weight examinations, some parameters differed in both the male and female rats but none of these appeared to be of toxicological significance, and were slightly higher or lower than those of the controls. Correspondingly, these data are within the normal limits established under laboratory control as determined by Lillie *et al.* (1996). Thus, it can be concluded that FCP is virtually non-toxic. The study results provide an experimental basis for FCP to be safely used as ingredients of functional foods or pharmaceuticals.

Arthritis was induced reproducibly in all animals injected the adjuvant, with onset of injected hind paw (right paw) erythema and swelling (arthritis onset) occurring on day 9, swelling of non-injected hind paw (left paw) began on day 11 and persisted to the end of the experiment.

In the present study the arthritic rats showed a soft tissue swelling that was noticeable around the ankle joints during the acute phase of arthritis and was due to oedema of periarticular tissues such as ligaments and joint capsules (fig.5.2). The swelling has been found to be increasing in the initial phase of inflammation and then becomes constant in 2 weeks. These changes in paw volume have been found to be associated with an increase in granulocytes and monocytes (Arend and Dayer, 1990). Because, the activation of macrophages results in the production of several cytokines including IL-1, IL-6, interferon-γ (IFN-γ) and TNF-α which have been implicated in immune arthritis (Dai *et al.,* 2000).

TNF-α is mainly involved in the perpetuation of the inflammatory cascades in autoimmune diseases, which affect connective tissues where the connective tissues become hyper contracted due to inflammation (Kinne *et al.,* 2000). Furthermore, macrophage derived nitrous oxide may increase vasodialation and vascular permeability at the inflammatory site, which may aggravate the arthritic process (Nissler *et al.,* 2004). Moreover, prostaglandins greatly potentiate exudates by inducing relaxation of arteriolar smooth muscle cells and increasing the blood supply to the tissue (Simon *et al.,* 1965).

Several scientific reports have presented good bioavailability of hydrolyzed collagen, after oral administration by animals and human beings. Oesser *et al.* (1999) discovered that about 95% of orally applied collagen hydrolysate was absorbed within the first 12 h. Zague (2008) described the high safety of eating collagen hydrolysates in an animal model (1.66 g/kg of body weight per day).

5.3.2 Effect on Paw edema

The challenge with CFA (1%, 0.1ml) showed significant increase in paw oedema which has reached to peak on 3rd week and remained constant by the end of the study in arthritis control as compared to normal control. The data indicate that the intradermal injection of CFA may induce arthritis and was characterized by initial swelling of one or more limbs, which resulted in increase of paw oedema of 54.65% in the arthritic control group.

Later characteristics of the disease included gross joint deformation and total loss of joint mobility. The observations are in agreement with the study of Courtenay *et al.* (1980) who also demonstrated the incidence of arthritis in course of time by using complete freunds adjuvant.

Rats fed with FCP (0.5 & 1.0g/kg) showed significant and dose-dependent attenuation in paw oedema from day 30 onwards as compared to Disease Control rats. Rats treated with diclophenac (10mg/kg) significantly decreased ($P < 0.05$) paw volume from day 25 to 50 and the effect is comparable with the test sample, FCP 0.1 g/kg ($P < 0.05$). Also there is significant difference between the two dosages. (Significant difference is shown in table 5.3 and trend of the treatment effect in different groups is shown in fig. 5.4).

5.3.3 Effect on Body weight

As the incidence and severity of arthritis increased, there is significant decrease in the body weights of the arthritis control occurred as compared with normal control during the course of the experimental period. But the results showed the drug treated group could ameliorate the weight loss occurred during arthritis and there is no significant difference in the effect of diclophenac and FCP (1g/kg) ($P < 0.05$) treated groups.

Figure 5.2 Morphological representations of rat paw. *(A)Normal control (B)ArthritisControl.*
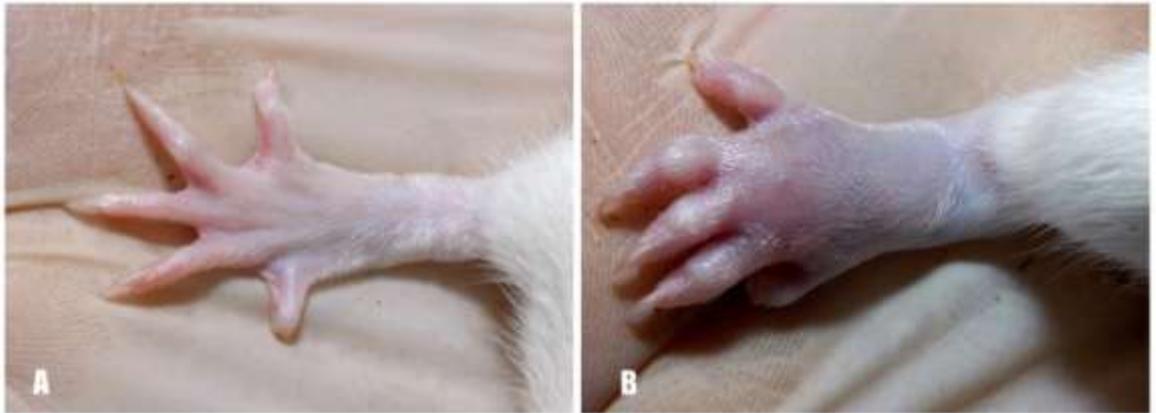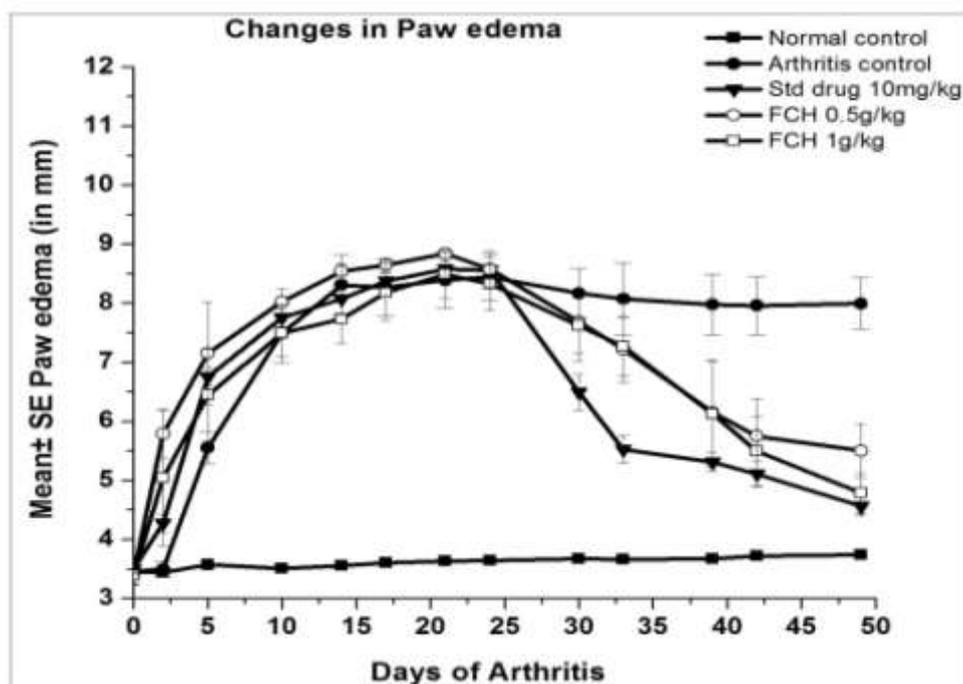

Figure 5.3 Morphological representations of rat paw after the treatment period

Figure 5.4 Mean paws oedema change over time. Values are plotted as the mean ± SE (n=6) in each group

5.3.4 Arthritic score assessment

Arthritic score is a clinical assessment of joint swelling (Funk *et al.,* 2006). In the present study as a result of CFA induced inflammation, the arthritic score was increased till the end of the study ($p < 0.05$) in CFA treated rats when compared with control rats. Treatment with standard drug and FCP beginning on day 21 showed significantly decreased ($p < 0.05$) arthritic score. There is no significant difference in the treatments ($p < 0.05$). The significant difference in each group is shown in table 5.5. The trend of arthritic score is graphically shown in fig.5.6.

The alteration in plasma protein induces the synthesis of proinflammatory cytokines, prostaglandins, leukotrienes and matrix metallo proteinases that caused fluid accumulation in the synovium. This results in an increase in arthritis scores due to damage in joints and bones of the rats paw (Cai *et al.,* 2007).

Table 5.3 Effect of FCP on Paw edema of adjuvant arthritic rats

Treatments	Day 21	Day 24	Day 30	Day 33	Day 39	Day 42	Day 49	% inhibition on 49th day
Normal control	3.63 ± 0.02	3.64 ± 0.02	3.67 ± 0.01	3.66 ± 0.01	3.66 ± 0.02	3.72 ± 0.02	3.74 ± 0.04[a]	
Arthritis control	8.39 ±0. 48	8.44 ± 0.39	8.17 ± 0.41	8.07 ± 0.61	7.98 ± 0.51	7.96 ± 0.49	7.80 ± 0.44[b]	
Std drug	8.58 ± 0.11	8.56 ± 0.09	6.49 ± 0.30	5.53 ± 0.24	5.31 ± 0.16	5.11 ± 0.21	4.56 ± 0.16[c]	90.13
FCP 0.5mg/kg	8.83 ± 0.08	8.57 ± 0.31	7.68 ± 0.53	7.22 ± 0.56	6.15 ± 0.88	5.75 ± 0.64	5.50 ± 0.46[d]	88.11
FCP 1.0mg/kg	8.49 ±0. 40	8.33 ± 0.45	7.62 ± 0.59	7.27 ± 0.49	6.13 ± 0.89	5.49 ± 0.58	4.79 ± 0.33[c]	89.29

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

Table 5.4 Effect of FCP on body weight of adjuvant arthritic rats

Treatments	Day 21	Day 24	Day 30	Day 33	Day 39	Day 42	Day 49	% increase in Body weight
Normal control	157.16 ±0.60	165.00 ± 0.86	170.50 ± 0.85	173.50 ± 0.56	179.66 ± 0.95	188.17 ± 0.91	194.17 ± 1.08[a]	23.55
Arthritiscontrol	152.33 ± 0.61	153.50 ± 0.84	151.66 ± 0.41	147.83 ± 0.61	146.16 ± 0.51	143.83 ± 0.60	142.83 ± 0.83[b]	-6.24
Std drug	140.00 ± 0.85	143.00 ± 0.77	148.00 ± 0.89	149.50 ± 0.76	153.83 ± 0.52	156.67 ± 1.11	161.17 ± 0.87[c]	15.12
FCP 0.5mg/kg	149.50 ± 0.34	151.50 ± 0.42	152.66 ± 0.61	154.33 ± 0.71	156.83 ± 1.13	160.50 ± 1.23	165.00 ± 1.39[d]	10.37
FCP 1.0mg/kg	145.66 ± 0.66	148.66 ± 0.76	152.33 ± 0.84	155.50 ± 0.85	159.00 ± 0.93	163.33 ± 1.16	166.83 ± 1.01[c]	14.53

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

5.3.5 Effect on biochemical parameters

The biochemical profiles of the treated and control groups arepresented in Table 5.6 and 5.7. Some of the biochemical values of the collagen peptide treated rats differed slightly from those of the controlgroups, but some values were in the range of normal and are graphically shown in fig.5.7.

The biochemical estimations of ALP, ACP, and SGPT, SGOT, blood urea, creatinine and total protein were carried out to detect the toxic effect on the liver and kidney. In the present study, no significant changes were observed in biochemical parameters after 42 days of drug treatment compared with normal group.

The toxicity in liver, kidney and heart are the most common adverse effect when we administer nonsteroidal anti-inflammatory drugs, anti-rheumatic drugs and steroids. The cytoplasmic enzymes like AST and ALT serves as indicators and suggestive for disturbances of the cellular integrity induced by pathological conditions. These enzymes are used as sensitive markers for evaluation of protective activity, against persistent inflammation. The increased enzyme activity may result from one of the several mechanisms which include the release of various enzymes from leukocytes, from necrotic or inflamed synovial tissue and production and release of an increased amount of enzymes due to altered synovial tissue (Schellekens, *et al.,* 2000).

AntiCCP assay is a novel and most reliable method for detecting the prognosis of the arthritic inflammation in affected animals. Significant decrease in anti CCP level has seen in group IV and V rats. This clearly indicates the positive effect of collagen peptide against rheumatoid arthritis.

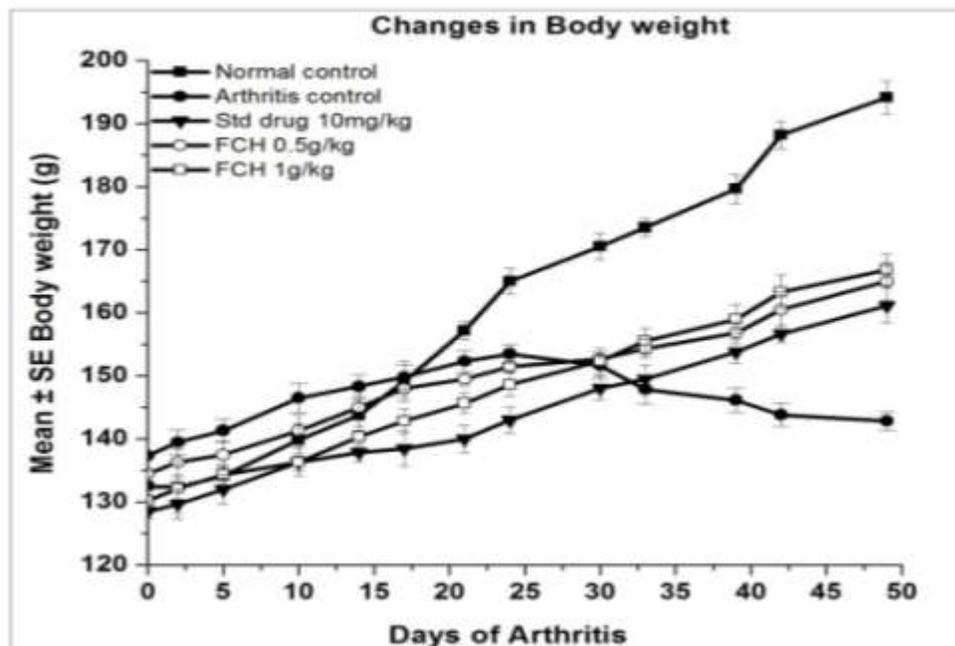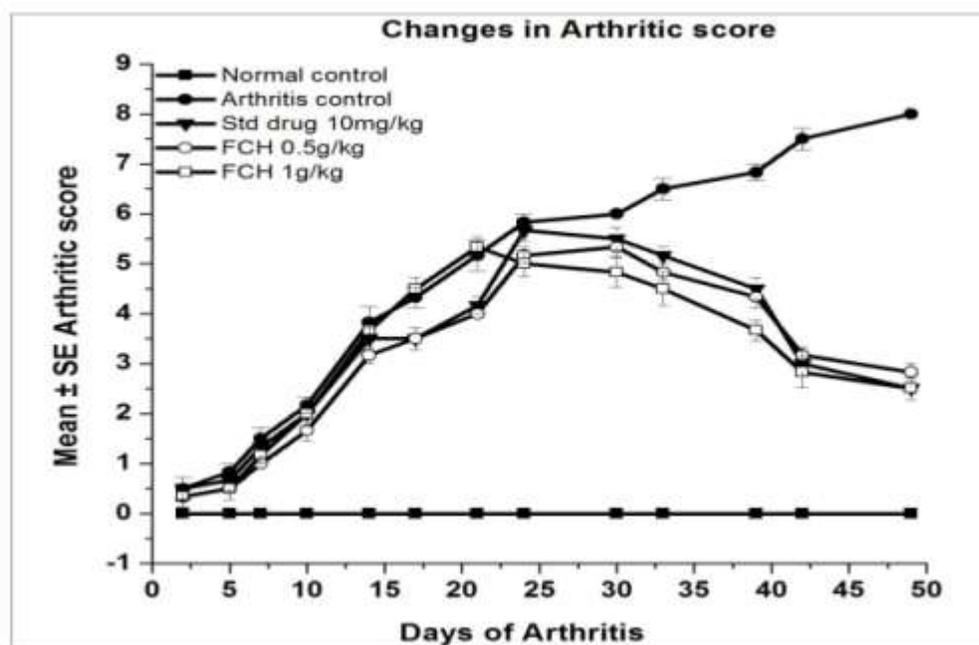*Figure 5.5 Mean body weight change over time. Values are plotted as the mean ± SE (n=6) in each group*

Figure 5.6 Changes in arthritic score over time. Values are plotted as the mean ± SE (n=6) in each group

5.5 Effect of FCP on Arthritic score of adjuvant arthritic rats

| Treatments	Day 21	Day 24	Day 30	Day 33	Day 39	Day 42	Day 49
Normal control	0	0	0	0	0	0	0^a
Arthritis control	5.17 ± 0.31	5.83 ± 0.39	6.00 ± 00	6.50 ± 0.22	6.83 ± 0.17	7.50 ± 0.22	8.00 ± 0.00 [b]
Std drug	4.17 ±0.16	5.67 ± 0.21	5.50 ± 0.22	5.17 ± 0.17	4.50 ± 0.22	3.00 ± 0.26	2.50 ± 0.22 [c]
FCP 0.5mg/kg	4.00 ± 0.00	5.17 ± 0.17	5.33 ± 0.21	4.83 ± 0.17	4.33 ± 0.21	3.17 ± 0.16	$2.83 ± 0.17^c$
FCP 1.0mg/kg	5.33 ± 0.21	5.00 ± 0.26	4.83 ± 0.30	4.50 ± 0.34	3.67 ± 0.21	2.83 ± 0.31	2.50 ± 0.22 [c]

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

As far as enzyme markers are concerned ACP level is a convenient method to predict the prognosis of inflammation status in affected animals. Remarkably ACP level was clearly in favor to predict the anti arthritic effect of collagen peptide and it even does a slight advantage over standard drug.

Table 5. 6 Effect of FCP on biochemical parameters of adjuvant arthritic rats evaluated on 49th day

Treatments	ACCP	ALP	ACP	SGOT	SGPT
Normalcontrol	04.07±0.08^c	120.67±1.45^a	19.67±1.20^a	128.33±0.88^c	44.00±0.57^b
Arthritiscontrol	14.33±0.33^a	136.67±2.33^a	24.00±0.57^a	151.33±6.88^{ab}	28.67±2.03^d
Std drug	05.37±0.09^b	120.33±1.45^b	21.67±1.67^a	167.67±1.45^a	32.67±2.03^{cd}
FCP 0.5mg/kg	07.10±0.30^b	113.33±0.88^b	19.67±1.20^a	152.67±4.33^{ab}	38.00±0.57^{bc}
FCP 1.0mg/kg	06.53±0.15^b	120.67±5.36^b	20.67±1.45^a	143.00±4.16^{bc}	55.67±2.73^a

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test

Table 5.7 Effect of FCP on biochemical parameters of adjuvant arthritic rats evaluated on 49th day

Treatments	CRP	Ceruloplasmin	Urea	Creatinine
Normal control	1.96±0.03 ^{a}	23.00 ± 2.52 ^{a}	32.00 ± 1.73 ^{a}	0.43±0.03 ^{a}
Arthritis control	2.10 ±0.15 ^{a}	23.00 ± 1.15 ^{a}	32.33± 4.81 ^{a}	0.43±0.03 ^{a}
Std drug	2.03±0.14 ^{a}	22.33 ± 0.33 ^{a}	40.67± 2.33 ^{a}	0.43±0.03 ^{a}
FCP 0.5mg/kg	2.13±0.03 ^{a}	20.67 ± 0.33 ^{a}	34.00 ± 2.88 ^{a}	0.33±0.09 ^{a}
FCP 1.0mg/kg	1.27±0.14 ^{b}	20.00 ± 1.15 ^{a}	35.33± 0.33 ^{a}	0.37±0.09 ^{a}

Values are expressed as mean ± SE (n=6). Statistical significance was calculated by ANOVA followed by tukey's studentized range (HSD) test
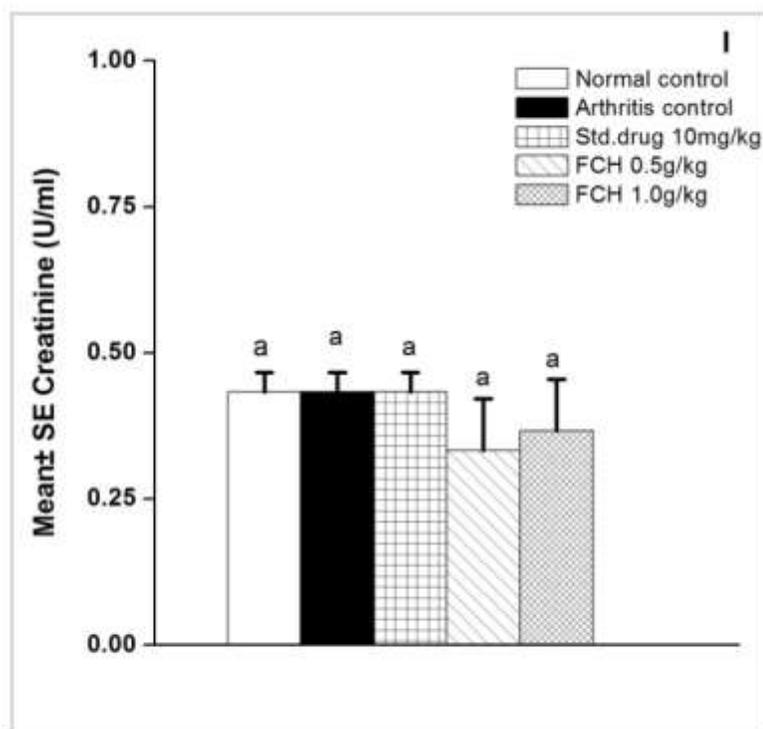
Figure 5.7 Effect of treatment on biochemical parameters of adjuvant arthritic rats (A) Anti CCP antibody (B) ALP (C) ACP (D) SGOT (E) SGPT (F) CRP (G) Ceruloplasmin (H) Urea (I) Creatinine
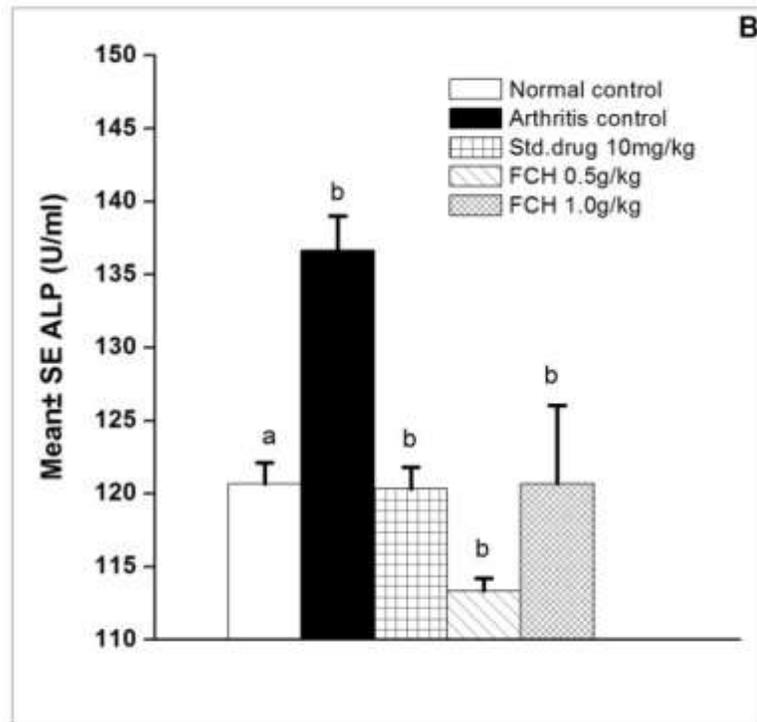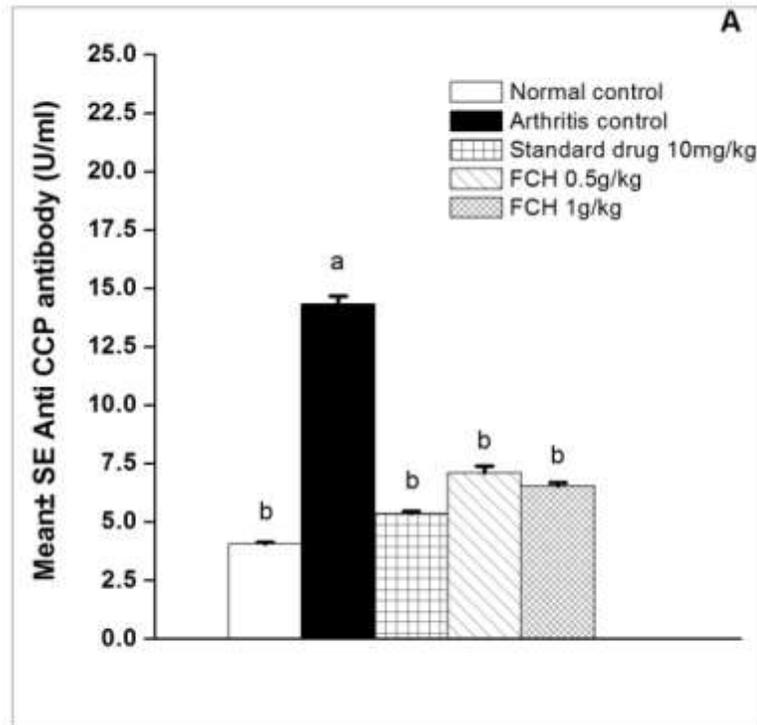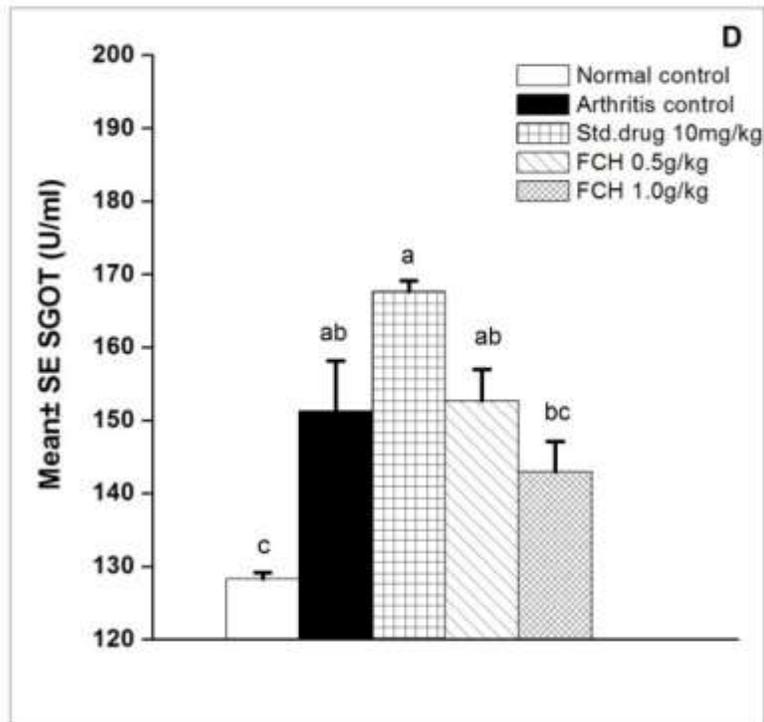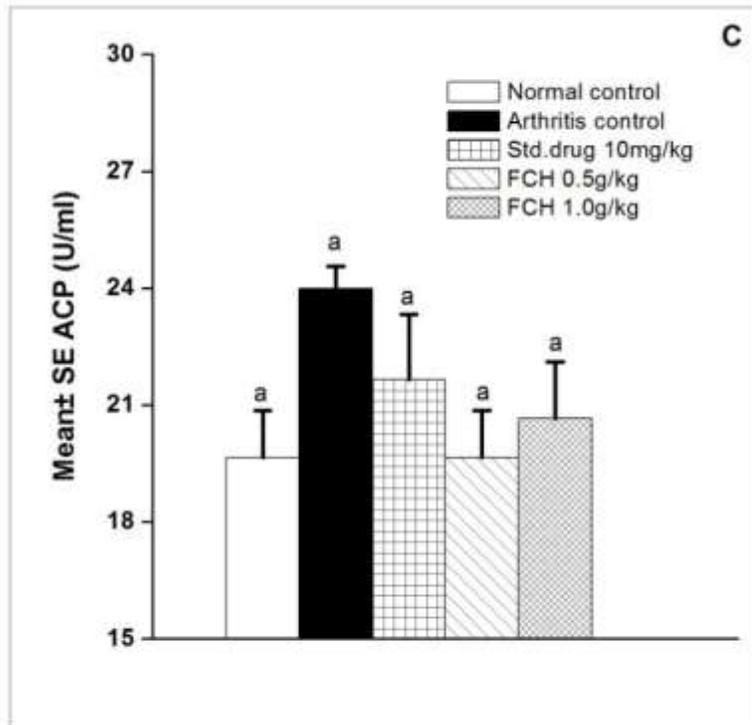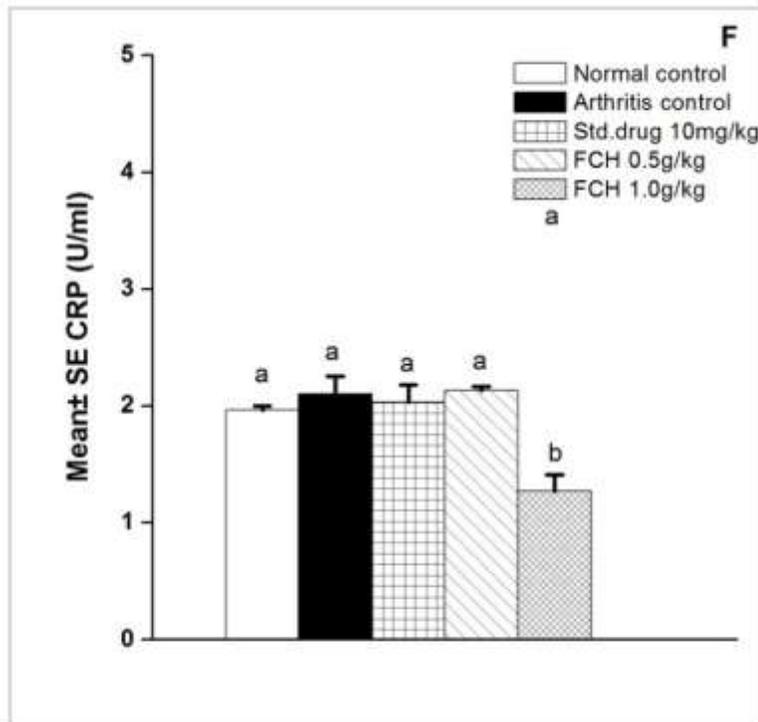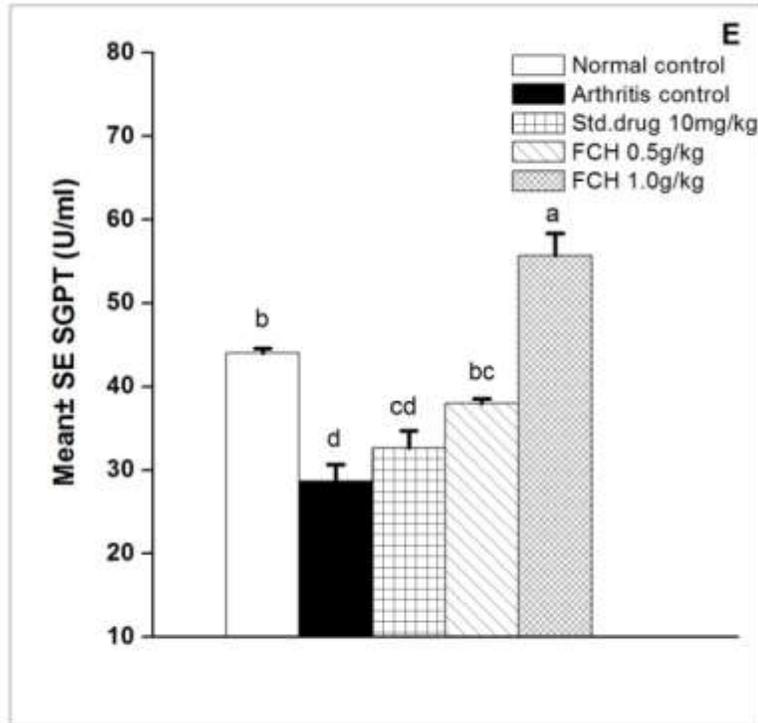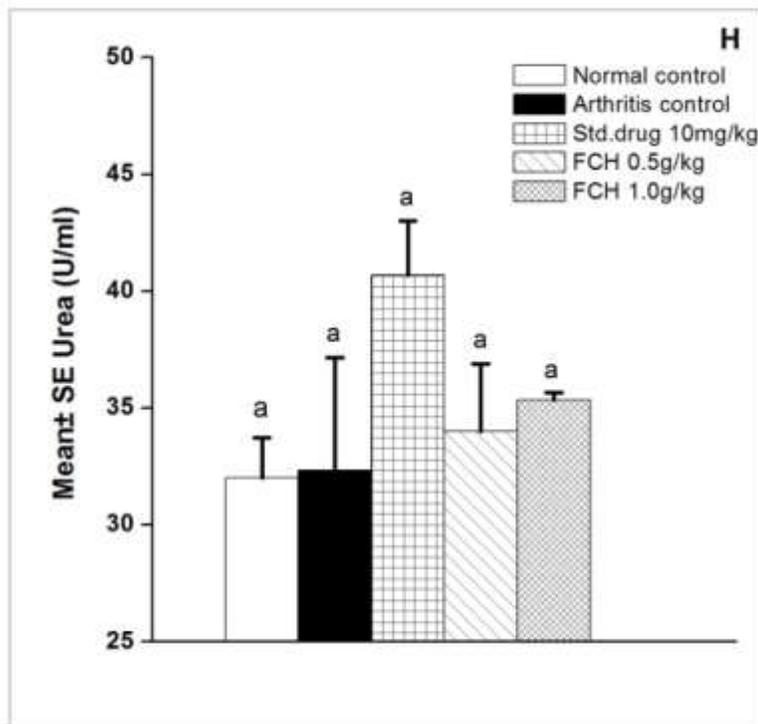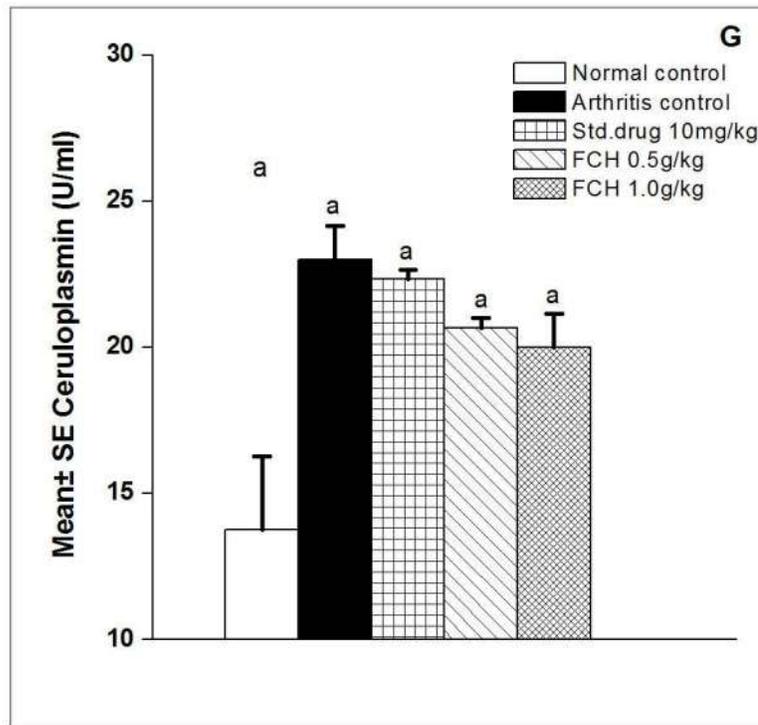
5.3.6 Effect of inhibition of COX

The total COX activity from paw exudate was significantly (p<0.05) increased in arthritic control (57.15%) and FCP treated group (31.25%) meanwhile in drug treated group there was significant reduction in total COX activity. The activities of COX I and COX II were individually monitored in all the groups with the help of enzyme specific inhibitors SC-560 and DUP-697 which are selective COX I and COX II inhibitors. In the treated groups there was significant reduction in the COX II activity compared to arthritic control and the inhibition is more for diclophenac treated group (61.5%). But it shows a negative effect that it not only inhibit COX II but also significantly inhibit biologically important COX I. There is no significant difference between the COX I levels of arthritic control and diclophenac treated group. COX I levels are comparable with normal control and FCP (1g/kg) treated group. The normal control group showed negligible

COX II activities thus confirming the fact that COX II is an inducible form of COX released only during conditions of acute inflammations. The effect is graphically shown in fig.5.8.

FCP is thus highly desirable since inflamed tissues could be targeted without disturbing the homeostatic functions of prostaglandins in noninflamed organs. Theoretically, then, selective COX-2 inhibition should preserve the anti-inflammatory efficacy without causing the associated toxicities of NSAlDs.

Figure 5.8 Effect of treatment on COX activity in adjuvant arthritic rats
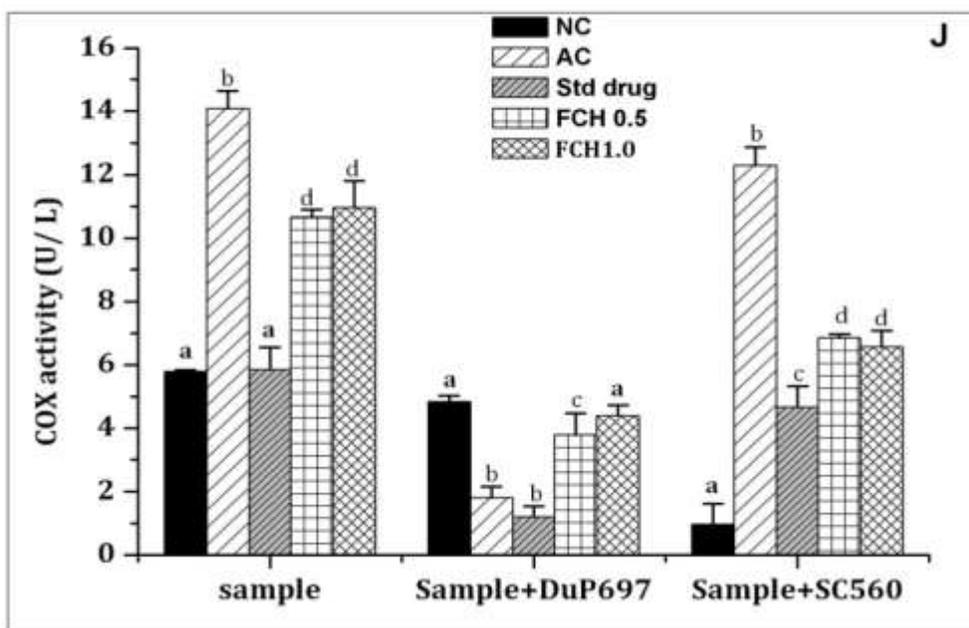
5.3.7 Radiological findings

Bone destruction, which is a common feature of adjuvant arthritis, was examined by radiological analysis. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only

in the developed stages of arthritis (Harris, 1990). The loss of articular cartilage leads to diminished joint space, which may be brought about a variety of pathological mechanisms.

Fig. 5.9 shows radiographic changes in joints of control and treated rats. Arthritis control group had developed definite joint space narrowing of the intertarsal joints, diffuse soft tissue swelling that included the digits, diffuse demineralization of bone, marked periosteal thickening, and cystic enlargement of bone and extensive erosions produced narrowing or pseudowidening of all joint spaces. The degree of bone resorption, diminished joint space and tissue swelling was markedly reduced in test treated groups.

The standard drug diclophenac sodium treated groups, there is considerable reduction in soft tissue swelling and narrowing of the joint space as compared to arthritis control.

Test treated groups (FCP of different concentrations, 0.5mg/kg and 1mg/kg body weight) shows moderate effect on change in joint architecture and it attenuate abnormalities consisted of asymmetric soft tissue swelling and small erosions, periosteal thickening, and minimal joint space narrowing, predominantly localized to the proximal areas of the paws. But there is no observable difference in the two dosages.

Figure 5.9 Radiographic changes in joints of control and treated rats. A- Normal control, B & C – Arthritis control, D – diclophenac treated group, E &F – FCP treated group. No evidence of pathological changes was observed in vehicle control group. Control group showing severe inflammation with diffused joint space and bone erosion. Treated group showing decreased soft tissue swelling with no evidence of bone erosion and inflammation.
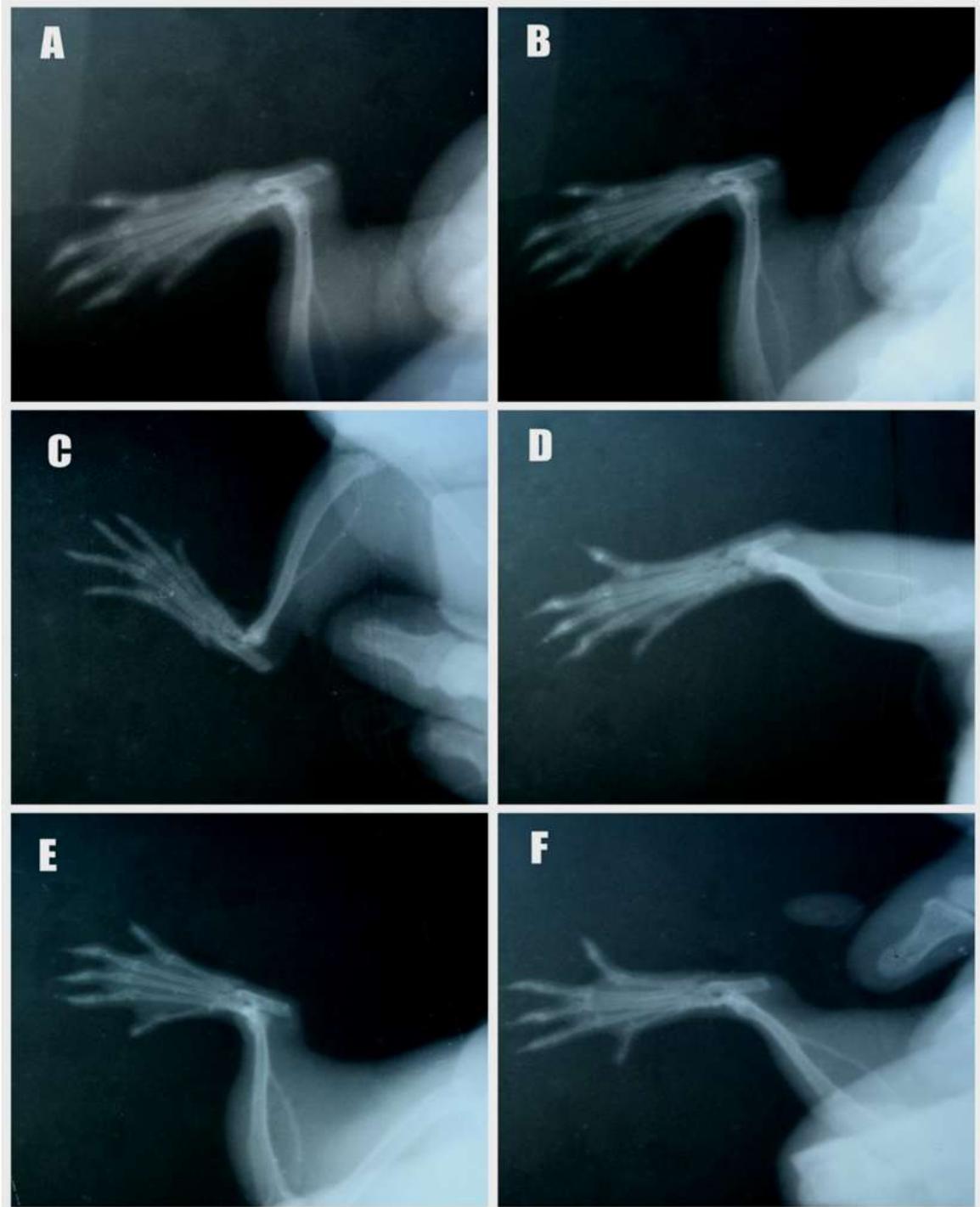
5.3.8 Histopathological changes in hind paw joints

Soft tissue swelling around ankle joint of arthritic rat was considered to be due to oedema of periarticular tissues such as ligament and capsule. Diminished joint space is the hallmark of arthritis, which is due to articular cartilage destruction brought by cytokines such as TNF- α and IL-1, which stimulate the release of proteolytic enzymes such as collagenases, glycohydrolases and neutral proteases. As a result, the pannus invades the joint and sub-chondral bones and eventually the joint is destroyed and undergoes fibrous fusion or ankylosis (Sudaroli and chatterjee, 2007).

Histopathological evaluation of the tibiotarsal joint of Arthritis Control (AC) rat showed massive influx of inflammatory cells, synovial hyperplasia with mono and polymorphonuclear cells accumulation in the joint and oedema associated with granuloma formation. It also shows the presence of higher degree of necrosis and degeneration with partial erosion of the cartilage (Fig.5.10B and 5.11B).

In the tibiotarsal joint Normal rats (NC), there was intact articular cartilage with normal synovial lining and connective tissue structure. It does not show any evidence of lymphocytic infiltration (Fig.5.10A and 5.11A). Treatment with diclophenac (10 mg/kg) showed normal connective tissue of tibiotarsal joint with the presence of lower degree of edema. There was absence of necrosis aswell as lymphocytic infiltration (Fig.5.10C and 5.11C). Tibiotarsal joint of rats treated with FCP (0.5g/kg and 1.0g/kg) showed less inflammatory signs like scanty cellular infiltrateless oedema. It does not show any sign of granuloma formation (Fig. 5.10D and 5.11D). Degeneration of the ankle joint was not observed in any of the drug treated groups when compared with the normal control.

Persistent inflammation produces swollen joints with severe synovitis, decreased nociceptive threshold, and massive subsynovial infiltration of mononuclear cells, which along with angiogenesis leads to pannus formation. Expansion of the pannus induces bone erosion and cartilage thinning, leading to the loss of joint function (Feldmann *et al.,* 1996)

Figure 5.10 Histopathological changes in tibiotarsal joints (10X magnification) A- Normal control, B- Arthritis control, C- Diclofenac treated group, D- FCP treated group.
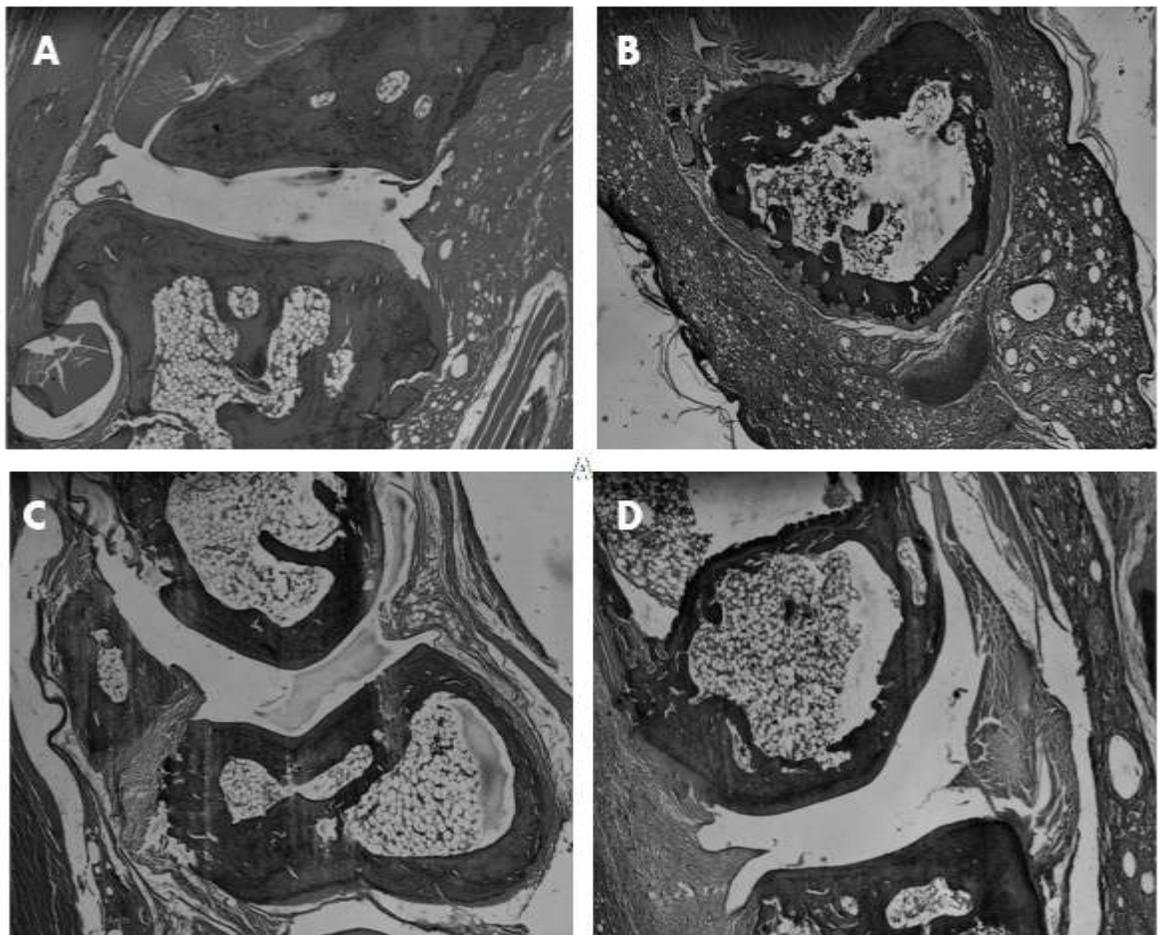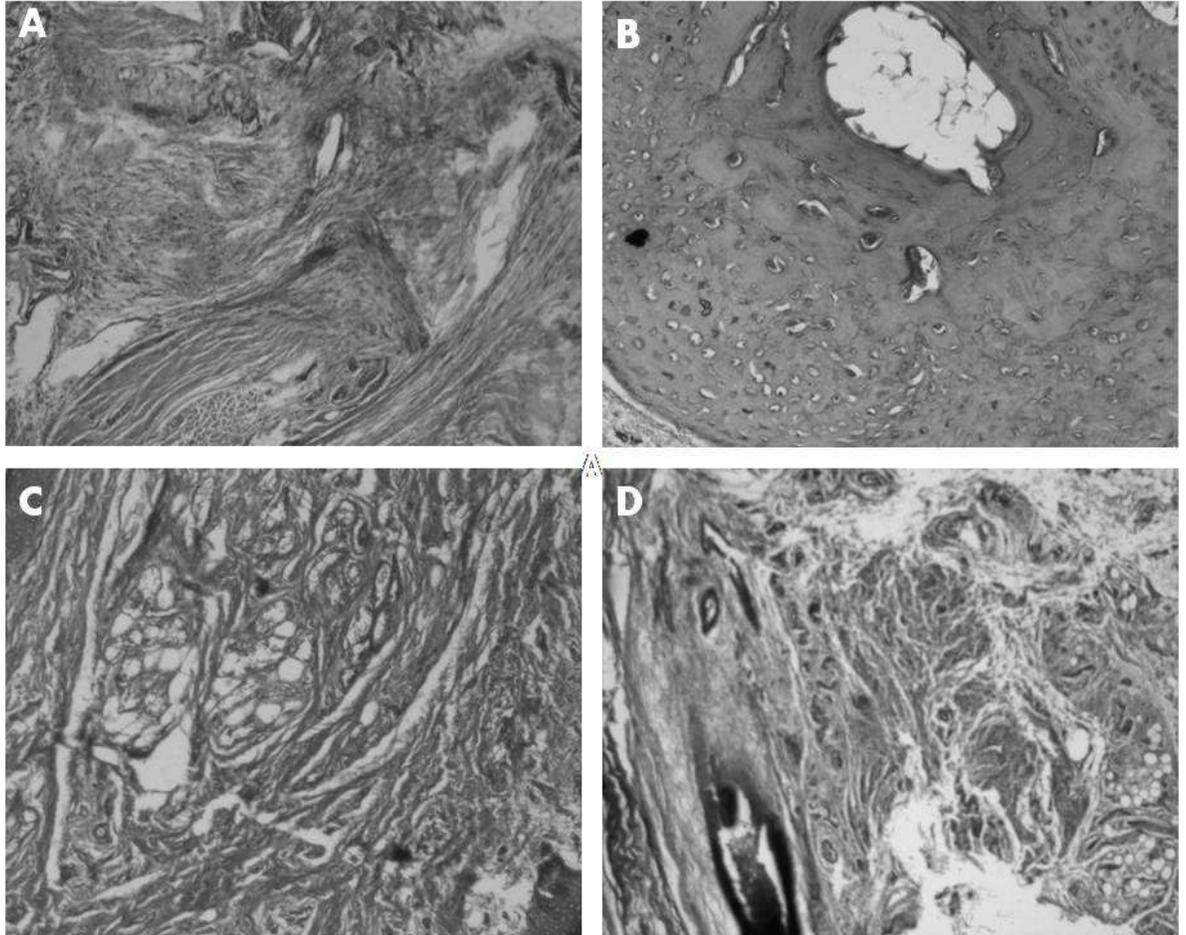
Figure 5.11 Histopathological changes in tibiotarsal joints (100 X magnification) A- Normal control, B- Arthritis control, C- Diclofenac treated group, D- FCP treated group

5.4 Conclusion

The result concluded that the collagen peptide from fish skin exerts potent anti arthritic activity significantly ($p<0.05$) altering the pathogenesis during arthritis without exerting any side effect during the chronic treatment and proved significant for the treatment of arthritis.

The mechanism by which oral administration of hydrolyzed collagen may improve bone formation in rats remain unclear, but has been suspected to be associated with the release and absorption of collagen derived peptides acting on bone metabolism, as observed in cartilage (Oesser *et al.,*1999). This effect could also be mediated by interaction of small collagen-derived peptides with the bone matrix. In this sense, the type I collagen-derived peptide Asp-Gly-Glu-Ala may bind to α1β1 integrin receptors on cell membranes and stimulate osteoblast-related gene expression of bone marrow cells (Mizuno *et al.,* 2001).

Collagen hydrolysate has been used in pharmaceuticals and food supplements for improving skin and cartilage tissues. It is digested and absorbed in the digestive tract, appears in the human blood partly in a peptide form, (Iwai *et al.,* 2005; and Ohara *et al.,* 2007) and is accumulated in skin for up to 96 hours as shown by Oesser *et al.* (1999). Recently, it has been shown that not only amino acids but also oligopeptides are absorbed by the small intestine. Moreover, Pro-Hyp, a dipeptide, was identified as the major constituent of food derived peptides to be detected in human serum and plasma. We hypothesized that in the study that some Pro-Hyp containing peptides reaches the articular cartilage and acts as a bioactive peptide, exertinga chondroprotective effect.

Oesser and Seifert, (2003) in their studies have showed some evidences exist on the ability of collagen hydrolysates to stimulate biosynthesis of type II collagen and proteoglycans in chondrocytes. Raabe *et al,* (2010) have reported the marked effect of a fish collagen hydrolysate on chondrogenic differentiation of equine adipose tissue-derived stromal cells. These studies suggest that effectiveness of collagen hydrolysates on biosynthesis of macromolecules would be based on their unique amino acid composition, very similar to that of type II collagen. Oral administration of collagen hydrolysates would provide high levels of Glycine and Proline, two amino acids essentials for the stability and regeneration of cartilage (Walrand *et al.,* 2008). The therapeutic effect of collagen hydrolysates on osteoarthritis could also be mediated by the effect of specific peptides on gene expression and function of chondrocytes (Ohara *et al.,* 2007).

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