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IN VIVO AND *IN VITRO* STUDIES ON JOINT REGENERATIVE POTENTIAL OF FISH SKIN DERIVED COLLAGEN PEPTIDES

Hema G. Sukumaran*[1], Shyni K.[1], Ganesan B.[1], Joshy C.G.[1], Manu Prasad M.[2], Suseela Mathew[1]

[1]Department of Biochemistry & Nutrition, Central Institute of Fisheries technology, Cochin, Kerala.

[2]Department of Cancer biology, Rajiv Gandhi center for Biotechnology, Trivandrum, Kerala.

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***Corresponding Author**
Hema G. Sukumaran
Department of Biochemistry & Nutrition, Central Institute of Fisheries technology, Cochin, Kerala.

ABSTRACT

Marine fish skin derived bioactive peptides, based on their structural properties with specific sequence of amino acids represent an important group of molecules having various biological functions. This study was designed to investigate the joint regenerative potential of fish skin derived collagen peptides (FCP) using animal models and *in vitro* studies with human osteoblast cells. *In vivo* studies, arthritis was induced in male Wistar albino rats by intradermal injection of Freund's complete adjuvant (CFA). CFA induced rats were treated with oral administration of FCP developed from grouper fish skin. During treatment period, the parameters like paw oedema, arthritic index were measured. The results showed that treatment with FCP markedly reduced paw swelling and arthritis index. The external healing effects were supported by the biochemical analysis after the treatment. The anti arthritic effect of peptide was confirmed by the analysis of bone histopathology and X-ray radiographs of affected paws. In this scenario, it is hypothesized that the antiarthritic effect of collagen peptide is related to the stimulating effect on collagen biosynthesis in arthritic joints. For elucidating the effect, *in vitro* cell line studies were conducted. Human osteoblast cell lines (HOS) were treated with FCP in both time and dose dependent manner. Quantification of collagen by chromogenic precipitation with Sirius red showed increased collagen production in FCP treated cells. Western blot and immunocytochemistry analysis further confirmed the FCP-mediated stimulatory action on collagen biosynthesis in cell culture. I*n vivo* and *in vitro* studies concluded the bone regenerative potential of FCP.

KEYWORDS: Fish collagen peptide, Freund's complete adjuvant, Human osteoblast cell lines, Confocal imaging.

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 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).

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, and 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).

The present research work is aimed at the scientific validation of the anti arthritic effect of FCP in CFA induced rat model system. Various experimental parameters like arthritic index, changes in paw edema and body weight were 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 were performed.

An *in vitro* study was designed to investigate the biological effects of FCP on human osteoblast cell lines (HOS). To elucidate this novel function regarding collagen synthesis, we treated human osteoblast cells with collagen peptide in both a time and dose dependent manner followed by measurements on biosynthesis and secretion of type 1 collagen. Quantitative measurement of collagen was done by sirius red staining, western blot analysis and immuno fluorescence analysis.

MATERIALS AND METHODS

Animals used: Wistar strain male albino rats, weighing 130 ± 20 g, were selected for the study. 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).

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. 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.

Table 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

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.

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.

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).

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. 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.

Anti CCP (ACCP)

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.

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.

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).

***In vitro* studies with HOS cell lines**

Osteoblast culture

An osteoblast cell line derived from a human osteosarcoma, (HOS) were cultured under standard conditions.

Quantification of collagen from FCP treated cells

Osteoblast cells were treated with FCP at different concentrations ranging from 0.5 mg/mL to 1 mg/mL for different time intervals of 6 to 24 hours. To investigate whether the peptides present in the collagen hydrolysate can stimulate the synthesis of collagen upon treatment, we measured the total collagen present in the cells protein extract.

1. Collagen chromogenic precipitation with Sirius Red

Collagen extract from treated cells (100µl) were put in eppendorf tubes and was precipitated with 1 ml of a solution of dye sirius red in 0.5 M acetic acid. After shaking, the tubes were maintained in rest for 30 minutes at room temperature and then centrifuged for 30 minutes at 30,000g. The supernatant was disposed and the pellet then washed consecutively with distilled water and 0.01N HCl to remove unbound dye. The bound dye was solubilized by incubation in 1 mL of 0.1N KOH for 15 minutes, in room temperature. Then absorbance of the solution was determined in spectrophotometer of 540 nm wavelength. Optical densities obtained were interpolated in a curve of absorbance, using collagen type I from calf skin soluble in acetic acid as standard.

2. Western blotting and densitometric analysis

The HOS cells were seeded in 60 mm culture dishes at a density of 1×10^5 cells and were cultured with or without FCP. Cells in the FCP group were incubated with an optimal concentration based on the MTT assay. After different time interval (6hr, 12hr and 24hr), the cells were retrieved with a rubber policeman. The cells in each group were lysed in a lysis buffer (1M HEPES, 10% NP-40, 1mM NaF, 1mM Na_3VO4, 0.5M EDTA, 100% protease inhibitor cocktail, deionized water) and then they were sonicated with a cell disrupter for 1 min in icecold water. After centrifugation of the lysates at 1,000 rpm for 10 min at 4°C, the supernatants were subjected to a western blot analysis.

The proteins were denatured by boiling in Lammeli sample buffer for 5 min at 203°F, separated by SDS–PAGE. The proteins were transferred onto polyvinylidene difluoride membranes. The membranes were incubated with 5% (w/v) nonfat milk powder in TBS for 1 h to prevent nonspecific binding. Following the blocking, the membranes were incubated overnight at 4°C with a primary antibody (Anti-Collagen 1 antibody). The specific antibody binding was detected with a horseradish peroxidase-conjugated secondary antibody and visualized using the enhanced chemiluminescence plus Western blotting detection reagents. To confirm equal loading conditions membranes were stripped and reprobed with β-actin antibody. The band density of each group was quantified by a densitometric analysis using the Scion Image software program. The ratio of the densitometric value of the FCP group to the value of the control group was then calculated.

3. Immunocytochemistry (Confocal imaging)

HOS cells were plated at 50% confluence on a 4-chamber glass slide (Nunc, Lab-Tek) in 15% FBS medium for 1h at 37°C. FCP is treated at concentrations from 0.3mg/mL to 1.0 mg/mL to the cells for 24h time. After treatment, the cells were fixed by adding 0.5 mL of 4% PFA to each chamber and incubating for 15 min. After washing with PBT, the cells were incubated for 30 minutes in 0.5 mL of 1% BSA. All remaining steps are performed in the dark to protect fluorescent markers.

After washing, the cells were incubated with primary antibody (Anti-Collagen 1 antibody), (1/1000: in 3% BSA/ PBS) for 12h at 4°C. An FITC-conjugated goat polyclonal to rabbit IgG was used at dilution at1/160 as secondary antibody. Then the cells were counterstained with DAPI for staining nucleus. Immunofluorescence was visualized using Nikon AIR confocal imaging system and Andor Revolution XD Spinning Disc Microscope with and orixon 897 EMCCD cameras.

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).

RESULTS AND DISCUSSION

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 (Fig. 1).

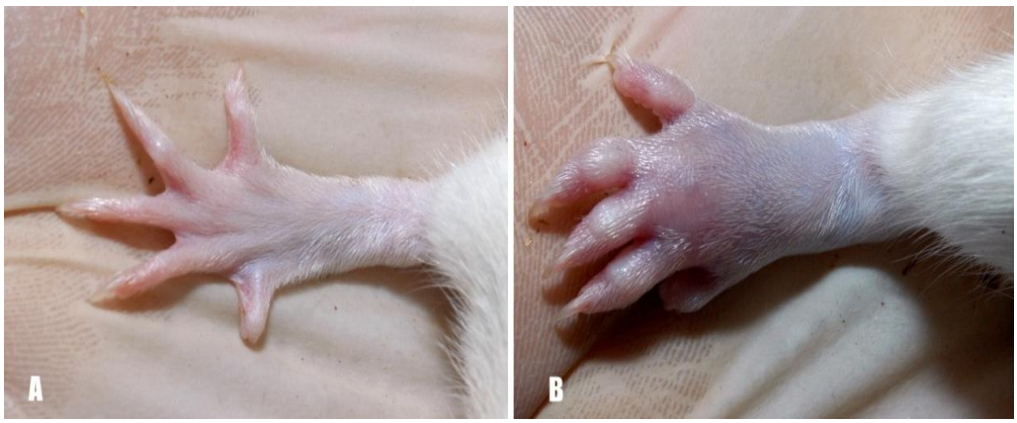


Figure 1. Morphological representations of rat paw. (A)Normal Control (B) Arthritis Control.

Effect on Paw edema

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. 2).

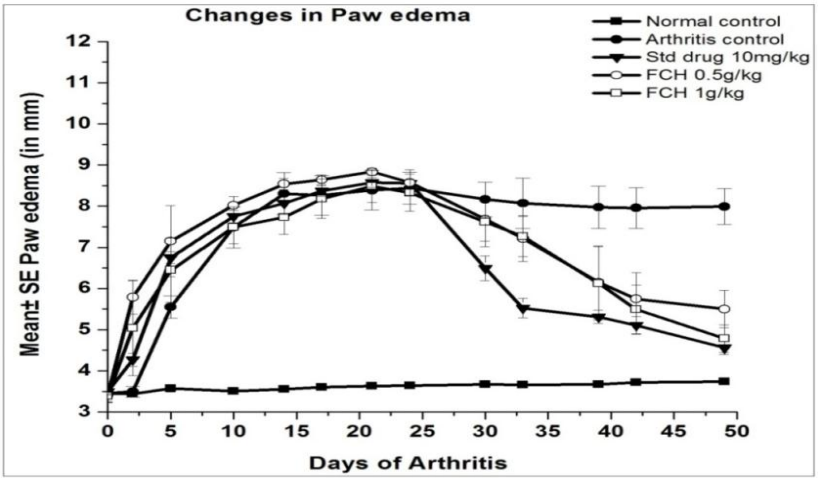


Figure 2. Mean paws oedema change over time. Values are plotted as the mean ± SE (n=6) in each group

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. 3.

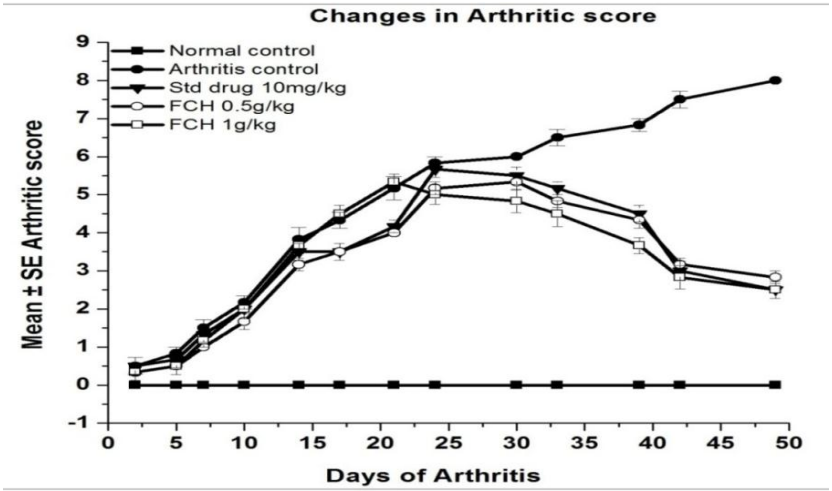


Figure 3. Changes in arthritic score values over time. Values are plotted as the mean ± SE (n=6) in each group.

Effect on biochemical parameters: The biochemical profiles of the treated and control groups are presented in Table 2 and 3. Estimations of ACCP SGPT, SGOT, ALP, 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. ACCP assay is a novel and most reliable method for detecting the prognosis of the arthritic inflammation in affected animals. Significant decrease in ACCP level has seen in group IV and V rats. This clearly indicates the positive effect of collagen peptide against arthritis.

Table 2: 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 3: 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

Radiological findings

Bone destruction, which is a common feature of adjuvant arthritis, was examined by radiological analysis. Fig. 4 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, marked periosteal thickening, and cystic enlargement of bone and extensive erosions produced narrowing or pseudo widening of all joint spaces. The degree of bone resorption, diminished joint space and tissue swelling was markedly reduced in test treated groups. There is considerable reduction in soft tissue

swelling and narrowing of the joint space in drug treated group 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.

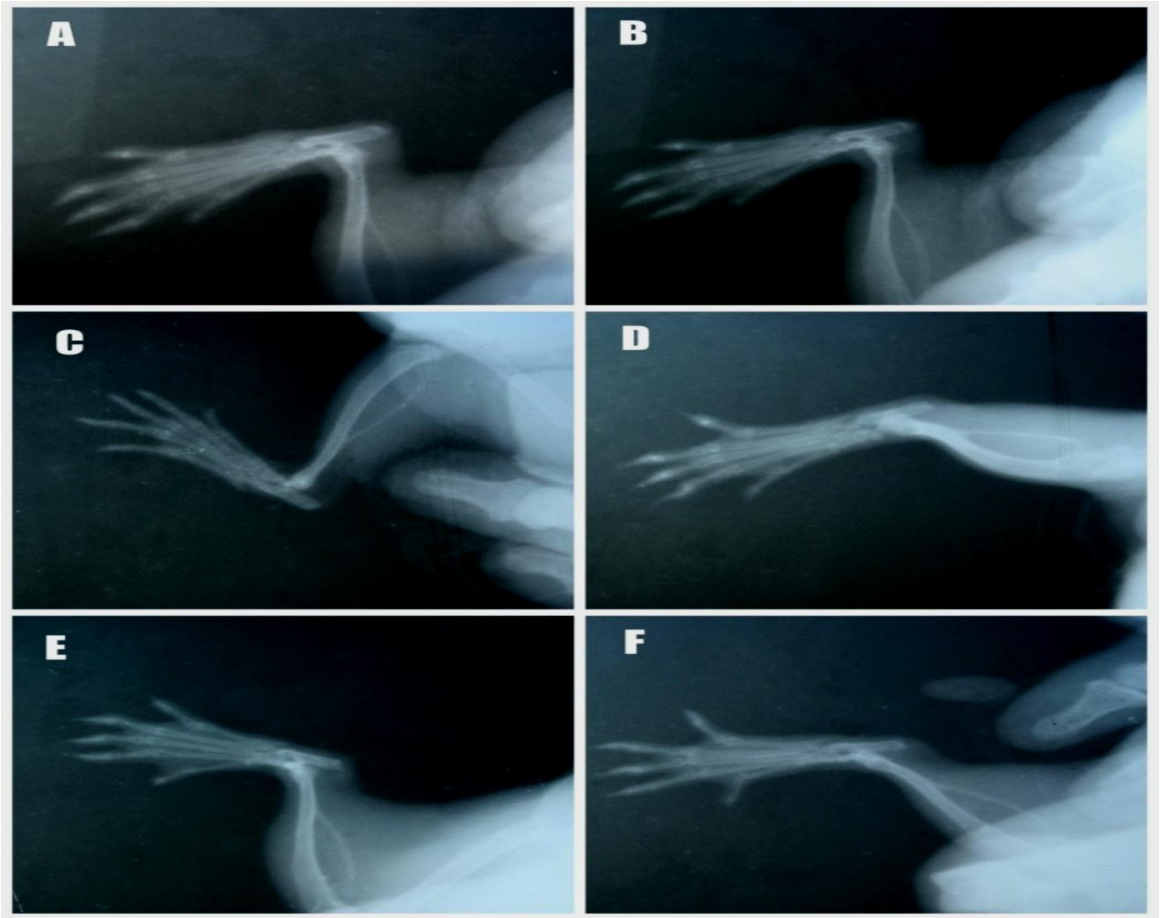


Figure 4. Radiographic changes in joints of control and treated rats. A- Normal control, B & C – Arthritis control, D – diclophenac treated group, E &F – FCP treated group.

Histopathological changes in hind paw joints: 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 and 6).

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. Treatment with diclophenac (10mg/kg) showed normal connective tissue of tibiotarsal joint with the presence of lower degree of oedema. There was absence of necrosis as well as lymphocytic infiltration.

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. Degeneration of the ankle joint was not observed in any of the drug treated groups when compared with the normal control.

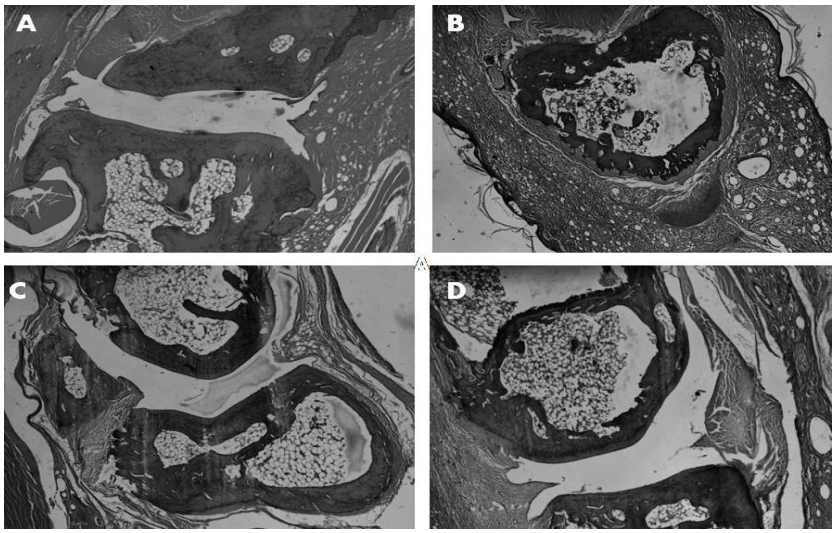


Figure 5. Histopathological changes in tibiotarsal joints (10X magnification) A- Normal control, B- Arthritis control, C- Diclofenac treated group, D- FCP treated group.

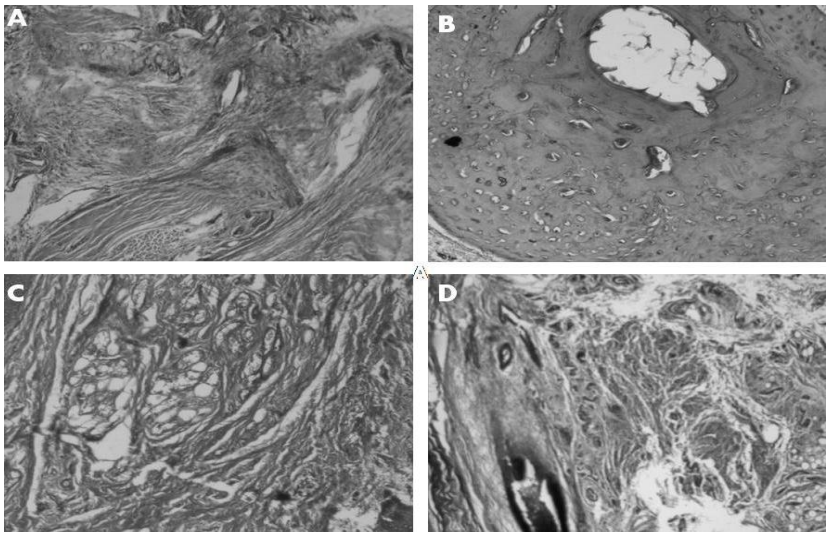
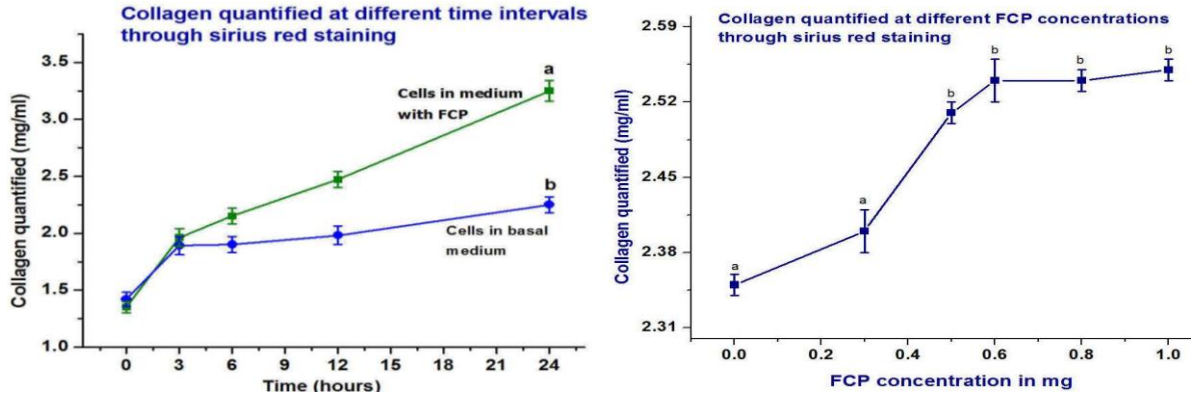


Figure 6. Histopathological changes in tibiotarsal joints (100X magnification) A- Normal control, B- Arthritis control, C- Diclofenac treated group, D- FCP treated group

Quantification of Type 1 collagen from FCP stimulated cells

1. Picro Sirius red staining: The treatment of cultured osteoblast cells with 0.6 mg/ml FCP over a culture period of 24 hours induced a marked increase in collagen secretion into the culture medium with significantly high when compared to the control cells receiving no FCP supplement (Fig. 7, A). At the end of the experimental period (24 hours), collagen secretion was almost 1.5 fold higher in FCP stimulated cultures in comparison with the control cells. Fig. 7 B shows increased concentration s of FCP in the culture medium induced a dose dependent stimulation of collagen secretion in 24 hours of time. From concentrations above 0.5 mg/mL of FCP, there is a significant enhancement of collagen secretion could be observed compared to untreated cells.



(A) **(B)**

Figure 7. Collagen quantified (A) at different time intervals & and (B) at different FCP concentrations through Sirius red staining. The results were expressed as mean ± SE ($p<0.05$). Data were analyzed by one way ANOVA followed by post-hoc procedure using SAS 9.3.

2. Western blotting

Blotting was done to confirm the result of stimulatory effect of FCP on HOS cells analyzed through sirius red staining. The immune reaction was done by using rabbit polyclonal antibodies against collagen type 1 antibody. Two immune reactive bands were detected by using anti collagen antibody (Fig. 8). The two bands obtained correspond to α and β chains of type 1 collagen. Expression levels of type 1 collagen, from cell lysate of FCP treated cells, showed that there is dose dependent increase in type 1 collagen synthesis. Results are expressed in arbitrary densitometric units normalized for the expression of β actin in each sample (mean±SD of five separate experiments).

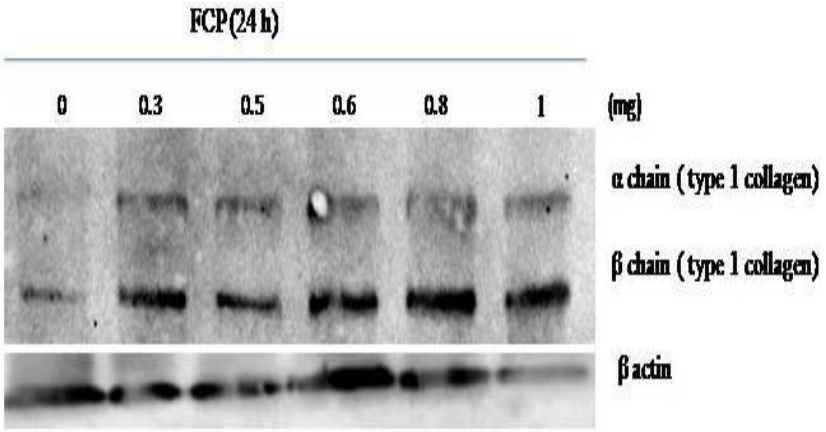


Figure 8. Western blotting using antibodies against type 1 collagen. Lane 2, 3, 4, 5and 6 are respectively of the 0.3, 0.5, 0.6, 0.8 and 1 mg/ml FCP treated cell's protein extract. Lane 1 corresponds to cell lysate of control cells.

3. Immunocytochemistry: The stimulation of type 1 collagen was fluorescently visualized by means of immunocytochemistry (Fig 9). After 24 hours of incubation, osteoblast cultures treated with FCP 0.5, 0.6 and 0.8 mg/mL concentrations significantly deposited tight nets of collagen fibers pericellularly (green fluorescence), where as in normal cultures the measurable amount of cell associated type 1 collagen was considerably reduced.

The specificity of the effect of FCP on collagen biosynthesis in osteoblasts was investigated using native collagen and non collagenous protein hydrolysate. But supplementation of the cell medium with 0.6 mg/ml native type 1 collagen or with a collagen free hydrolysate 0.6 mg/ml induced no stimulation in collagen biosynthesis in the osteoblast cells.

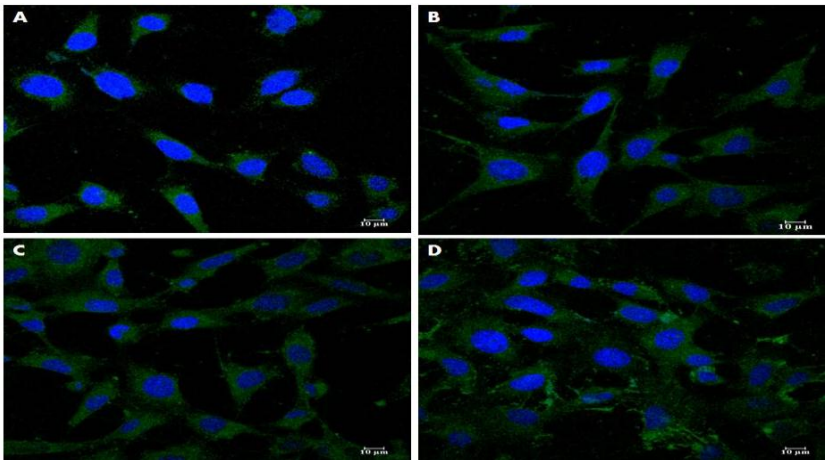


Figure 9. Confocal visualization of type 1 collagen (green fluorescence pericellularly) secreted by HOS cells. The nuclei were counterstained with DAPI (blue fluorescence). A, is control cultures grown in media without FCP; B, C and D are cultured in media with 0.5, 0.6, 0.8 mg/ml FCP respectively. Scale bar = 10 µm.

CONCLUSION

In vivo animal studies reveals 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. *In vitro* cell line studies confirms that the developed collagen product stimulates the joint matrix to synthesise collagen and helping to maintain the structure of the joint and potentially aiding joint comfort. So it is concluded that the fish collagen peptide developed can make a formulation of pharmaceutical drug for joint regeneration. Thorough clinical studies are essential and mandatory to confirm the safety aspects of FCP before recommending it as a pharmaceutical supplement for human applications.

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