

EVALUATION OF EXPRESSION OF COLLAGEN PROTEINS IN OSTEOLAST CELLS UPON TREATMENT WITH FISH COLLAGEN PEPTIDES (FCP)*6.1 Introduction**6.2 Materials and methods**6.3 Results and Discussion**6.4 Conclusion***7.1 Introduction**

Osteoblasts are cells responsible for the synthesis and mineralization of bone during both initial bone formation and later bone remodeling. Osteoblasts form a closely packed sheet on the surface of the bone, from which cellular processes extend through the developing bone. They arise from the differentiation of osteogenic cells in the periosteum, the tissue that covers the outer surface of the bone, and in the endosteum of the marrow cavity (Cooper, 2000; Spreafico, 2006).

Collagen is the major component of the extracellular matrix in multicellular organisms, including humans, and is an important component of ligaments, cartilage and bone. It accounts for about 30% of the total proteins in the body. Type I collagen is the most common type of collagen, and is abundant in bone (Bello, A.E. and Oesser, S. 2006).

Arthritis is the most common musculoskeletal disorder. Treatment of arthritis includes analgesics and anti-inflammatory agents, lubricating, cushioning agents and nutritional supplements (Wu, 2004). Advances in treatment of arthritis include new and safer compounds (e.g., glucosamine, chondroitin sulphate, or methyl-

sulfonyl-methane) capable of repairing damaged articular cartilage or at least decelerating its progressive degradation (Brief *et al.*, 2001)

Collagen hydrolysates are safer compounds that could provide, with less overall toxicity, a greater symptomatic relief than pharmaceutical drugs. Hye Kyung Kim *et al.* (2013) reported osteogenic activity of bovine collagen peptide via ERK/MAPK pathway mediated boosting of collagen synthesis and its therapeutic efficacy in osteoporotic bone. Oral administration of collagen hydrolysate was also demonstrated to reduce bone loss by increasing the quantity of type 1 collagen and proteoglycan in the bone matrix of ovariectomized (OVX) rats (De Almeida Jackix *et al.*, 2010). Collagen peptides have been used therapeutically as a dietary supplement to improve conditions of joints.

Collagen hydrolysate is absorbed in its high-molecular weight form, containing peptides of 2.5-15 kDa (Oesser *et al.*, 1999). Studies reported detection of peptides such as prolyl-proline (Pro-Pro), alanyl-hydroxypropyl-glycine (Ala-Hyp-Gly), prolyl-hydroxyproline (Pro-Hyp), prolyl-hydroxypropyl-glycine (Pro-Hyp-Gly), isoleucyl-hydroxyproline (Ile-Hyp), leucyl-hydroxyproline (Leu-Hyp) and phenylalanyl-hydroxyproline (Phe-Hyp) in human venous blood after ingestion of collagen hydrolysate. Pro-Hyp was the most prevalent among those peptides (Iwai *et al.*, 2005 and Ohara *et al.*, 2007).

Many reports indicated that various peptides obtained from collagen hydrolysate shows biological activity. For example, the Asp-Gly-Glu-Ala tetrapeptide regulated the expression of osteoblast treated genes in the bone marrow (Mizuno *et al.*, 2001). Correspondingly, a hydrogel containing peptides from collagen has been used as a scaffold for a true cartilage like extracellular matrix in regenerative medicine for effective

and lasting repair of articular cartilage (Kisiday *et al.*, 2002). These findings suggest that collagen hydrolysate contains bioactive peptides that affect cartilage homeostasis.

Experimental studies have suggested that some collagen-derived peptides orally administered are absorbed intact in the intestine. Subsequently, these peptides would accumulate preferably in cartilage, where finally may stimulate cartilage metabolism (Oesser *et al.*, 1999). Some evidences exist on the ability of collagen hydrolysates to stimulate biosynthesis of type II collagen and proteoglycans in chondrocytes (Oesser *et al.*, 2003).

Oral administration of collagen hydrolysates would provide high levels of glycine and proline, two amino acids essentials for the stability and regeneration of cartilage. 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 (Walrand, *et al.*, 2008).

In the previous chapter we have evaluated the anti arthritic effect of fish collagen peptides in CFA induced arthritic model. The results suggest that the peptide is having a positive role in alleviating the symptoms of arthritis. In this scenario we are hypothesizing that the antiarthritic effect of collagen peptide may be due to the stimulating effect on collagen biosynthesis in arthritic joints.

Accordingly, an *in vitro* study was designed to investigate the biological effects of fish collagen peptide (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. MTT assay was done to check the cytotoxic effect of FCP on the growth of cells. During treatment with FCP, the collagen synthesis is quantitatively screened in the culture cells. Quantitative

measurement of collagen was done by sirius red staining, western blot analysis and immune fluorescence analysis.

7.2 Materials and Methods

7.2.1 Osteoblast culture

An osteoblast cell line derived from a human osteosarcoma, (HOS) were cultured under standard conditions: Dulbecco's modified Eagles culture medium was supplemented with 10% (v/v) foetal bovine serum, antibiotics: penicillin (100 U/ml) and streptomycin (100 mg/m), and an antifongotic (0.25 mg/ml Amphotericin B).

Cells were incubated in a temperature controlled, humidified incubator with 5% CO₂ at 37°C. Cells were used between passage numbers five and 20 and grown in the complete culture medium, in 75cm² culture flasks and subcultured by trypsinization (0.05% trypsin). Culture medium was changed every 2-3 days.

Both serial cultures of fresh or cryopreserved cells were used for the determination of the following parameters:

- (i) time to reach 50% confluency in the standard culture flasks of 75 cm²
- (ii) Number of dead cells floating on top of the culture medium expressed as percentage of total seeded cells, obtained by trypsinization. Approximately 5x10⁴ cells were seeded at every passage in each well.
- (iii) the total number of cells at saturation density determined after trypsinization on the Coulter counter, at increasing passages

7.2.2 MTT assay

Cell viability was assessed using the MTT colorimetric assay. MTT was taken up into cells by endocytosis or protein-facilitated mechanism and reduced, mainly by mitochondrial enzymes, to yield a purple formazan product which is largely impermeable to cell membranes, thus resulting in its accumulation within living cells. Solubilization of the cells results in the liberation of the purple product which can be detected using a colorimetric measurement. The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of cell number/ proliferation/ viability/ survival/toxicity.

Operatively, 100µl of cell suspension was inoculated to each well of 96-well plates at the density of 2×10^4 cells/well (the area of each well was 0.32 cm²). After 24 h of culture, the medium was removed by aspiration and replaced with 100µl FCP at 0.1–10 mg/mL concentrations for 1, 2, 4, and 12 h.

After incubation, cells were observed under a contrast phase microscope before adding MTT solution, prepared fresh as 5mg/ml in H₂O, filtered through a 0.22µm filter, and kept for 5 min at 37°C. MTT solution (10µl) was added to each well, and the plates were incubated in the dark for 4h at 37°C in CO₂ incubator. After suction removal of the solution, 100 µl of DMSO were added to each well, and the plate was vortexed for 10 min. Absorbance of the solution was measured at 540 nm.

7.2.3 FCP treatment and Protein extraction

The HOS cells were seeded in 60mm culture dishes at a density of 1×10^5 cells and left in the CO₂ incubator for 24 hours for the cells to adhere. Cells were incubated with an optimal concentration based on the MTT assay. The cells were treated with different concentrations of fish collagen peptide for different time periods (6, 12 and 24 hours). The cells were then scraped with

scraper in 1 mL of 1X PBS and were centrifuged at 5000rpm for 5 minutes at 4°C. The spent medium was centrifuged in an eppendorf at 5000rpm for 5 minutes at 4°C. The cells in each group were lysed in a lysis buffer (1M HEPES, 10% NP-40, 1mM NaF, 1mM Na₃VO₄, 0.5M EDTA, 100% protease inhibitor cocktail, deionized water) and then they were sonicated with a cell disrupter for 1 min in ice cold water. The cells were centrifuged at 14000rpm for 10 minutes at 4°C to get the cell lysate. The supernatant was collected and stored at -80°C for evaluation.

7.2.4 Quantification of collagen from FCP treated cells

7.2.4.1 Collagen chromogenic precipitation with Sirius Red

Sirius Red specifically binds to the [Gly-X-Y]_n helical structure of fibrillar collagens such as type I to V collagen, and is used for detecting all types and species of collagen. The assay can be used in the range from 1 to 50 micrograms of protein. The method is specific, sensitive, simple, and rapid. Total amount of collagen synthesized and deposited by the cells after 72 h were measured, using the colorimetric procedure with picro-Sirius red staining. This method has the advantage to estimate not only freshly synthesized collagen but also the total amount of collagen accumulated by the cells during the culture period (Keira *et al.*, 2004).

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 potassium hydroxide 0.1N 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.

7.2.4.2 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 Na₃VO₄, 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 protein concentrations in each group were determined by Bradford assay.

For Bradford assay a set of standards was prepared from a stock of protein (BSA) with stock concentration of 1mg/mL. Each sample was added into 96 well plates in triplicates. The sample volume was 50µL. The protein solution of unknown concentration was diluted by 100 times and added in triplicates to the well. Bradford reagent (200µL) was added to all the wells and was incubated in dark for 5 minutes and absorbance was taken at 570 nm. A standard graph was plotted with the known concentration on X-axis and the corresponding absorbance on Y axis. The concentration of the unknown protein was obtained from the graph.

The proteins were denatured by boiling in Lammeli sample buffer for 5 min at 203.00°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.

7.2.4.3 Immunocytochemistry (Confocal imaging)

Confocal Slide Preparation: Plate cells at about 50% confluence on a 4-chamber glass slide (Nunc, Lab-Tek) in 15% FBS medium for 1h at 37°C. All remaining steps are performed at room temperature with the slides being rocked during incubations. While still in growth media, remove all but 500µL medium and add collagen peptide treatment (0.3mg/mL to 1.0 mg/mL) to the appropriate chambers for 24h time.

Pour off the media, wash each chamber twice quickly with 0.5 mL PBS, and then fix the cells by adding 0.5 mL of 4% PFA to each chamber and incubating for 15 min. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each, and then incubate the cells 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 at 1/160 as secondary antibody.

After incubation and washing, the cells were counterstained with DAPI for staining nucleus. After washing, allowed the slide to dry and removed the plastic chamber piece and sealer holding it in place completely. Placed one drop of mounting media (80% glycerol) on each sheet of cells, and covered with a No. 1.5 thickness cover slip. Gently pushed out if any air bubbles that formed underneath the cover slip and sealed the edges with clear nail polish. Stored slides at 4°C in the dark before and in between viewing under fluorescence.

Immunofluorescence was visualized using Nikon AIR confocal imaging system and Andor Revolution XD Spinning Disc Microscope with and orixon 897 EMCCD cameras.

7.2.5 Statistical analysis

The results are expressed as Mean \pm SE from n=3 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 9.1 (SAS Institute Inc., Cary, NC, USA)

7.3 Result and Discussion

7.3.1 MTT assay

MTT assay measured the reduction in cell viability. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. Absorbance values that are higher than the control cells indicated a percentage inhibition of growth cells.

Results (Table 6.1) shows, there is no significant retardation in the proliferation of cells up to 1.5 mg/ml of FCP treatment and 6 hours of incubation time. But the concentrations of FCP above 2.0 mg/ml significantly affected the growth of cells.

Table 6.1 MTT assay.

Concentration of FCP ($\mu\text{g/mL}$)	% inhibition on growth of cells
0.0	09.23 \pm 0.35
0.2	09.41 \pm 0.57
0.4	09.51 \pm 0.42
0.5	10.15 \pm 0.23
0.6	12.56 \pm 0.47
0.8	12.51 \pm 0.53

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1.0	14.54±0.38
1.5	21.21±0.40
2.0	30.51±0.63*
5.0	58.32±0.74*
10.0	60.51±0.29*

Values are the average of three separate experiments in quadruplicate and are expressed as mean ± SD. * $p < 0.05$.

Figure 6.1 Human osteoblast cells in culture dishes

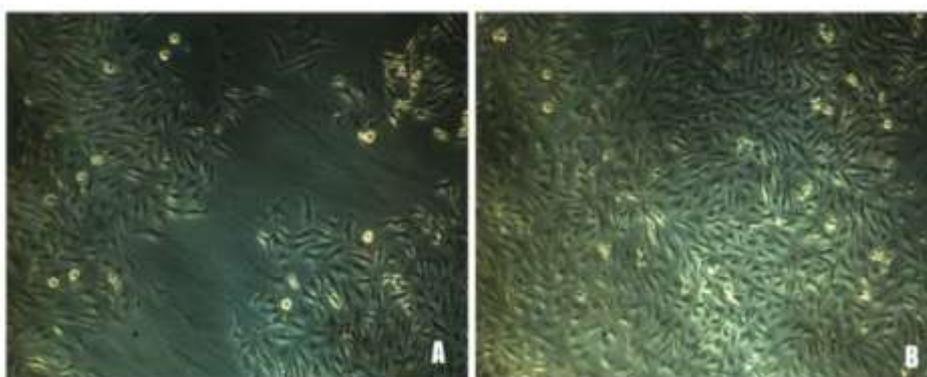
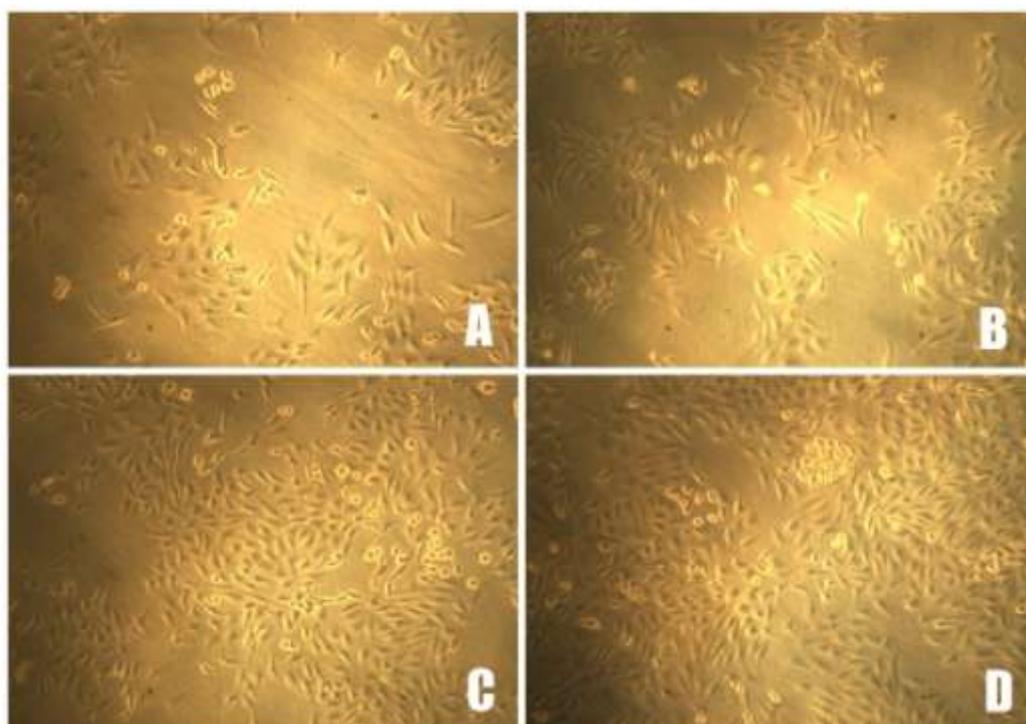


Figure 6.2 Photographs of the HOS cells after the different lengths of incubation time. (A. HOS cells before FCP treatment B. 6 hours after FCP treatment C. 12 hours after FCP treatment D. 24 hours after FCP treatment)



7.3.2 Quantification of Type 1 collagen from FCP stimulated 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 (Fig 6.2). 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.

7.3.2.1 Picro Sirius red staining

Collagen content was determined at different concentrations of FCP and at different time intervals by colorimetric analysis using picro-sirius red staining (Fig.6.3). The precipitated collagen was dissolved in 0.5N acetic acid and quantitatively measured colorimetrically.

Figure 6.3 Conic tubes (1.8mL) with the steps of collagen quantification (chromogenic precipitation reaction with dye Sirius Red)(1) Addition of dye (2) 30 minutes after addition of dye, with filaments (complex collagen-dye) in red (3) post centrifugation, with complex collagen-dye precipitated in the bottom of the tube 4) post-elution with KOH 0.1 N solution.



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. 6.4, 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. 6.4, 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. Oesser *et al.* (1999) reported that chondrocytes treated with collagen hydrolysate at concentrations ranging from 0.5 mg/mL up to 5mg/mL secreted a maximum of 2.2 fold more collagen than the untreated cells.

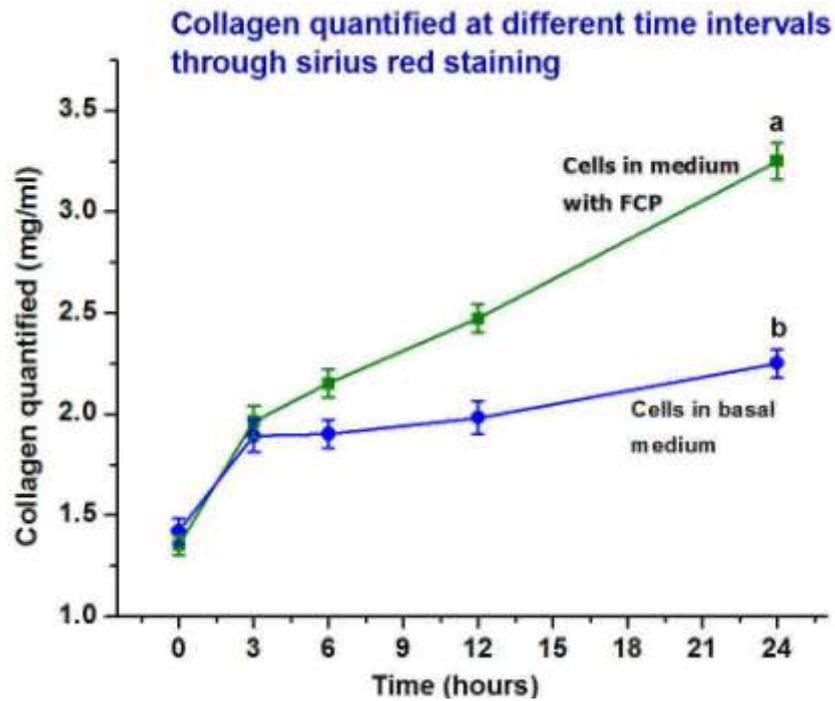
7.3.3.2 Western blotting

Western blotwas done to confirm the result of stimulatory effect of FCP on HOS cells analysed through Sirius red staining. The immune reaction was done by using rabbit polyclonal antibodies against collagen type 1 antibody. Yoshihito Ishida *et al.* (2006) used the same antibodies to detect type 1 collagen from fibroblast cells.

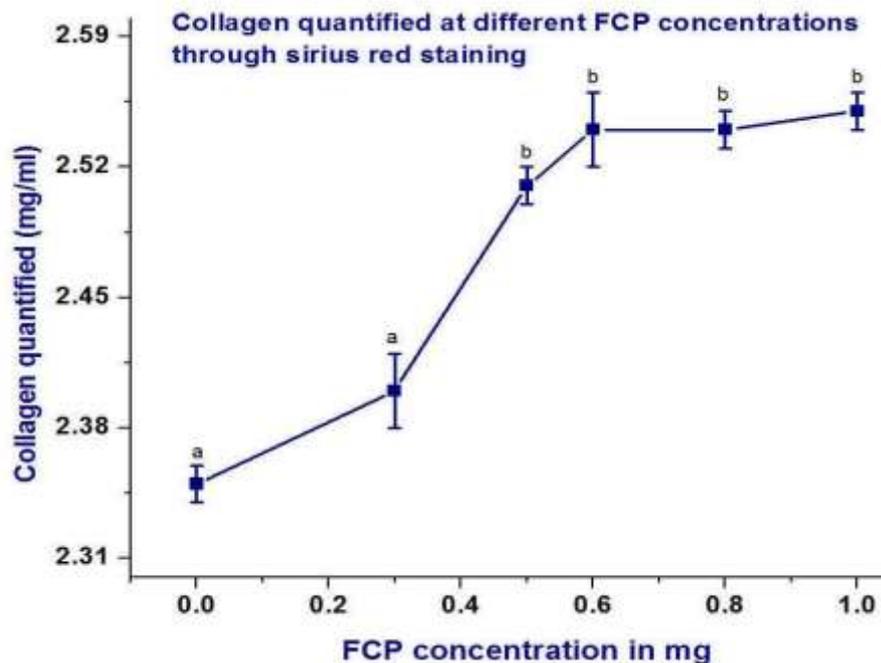
Two immunoreactive bands were detected by using anti collagen antibody (Fig.6.5A). The two bands obtained corresponds to the α and β chains of type 1 collagen.Expression levels of type 1 collagen, from cell lysate of FCP treated cells, showed that there is dose dependent increase in type 1 collagen synthesis.

Figure 6.4 Collagen quantified (A) at different time intervals & and (B) at different FCP concentrations through Sirius red staining The results were expressed as mean \pm SE . Data were analyzed by one way ANOVA followed by post-hoc procedure using SAS 9.3. There is significant changes ($p < 0.05$) in collagen content observed between cells treated with FCP and cells in basal medium with collagen free protein hydrolysate.

(A)



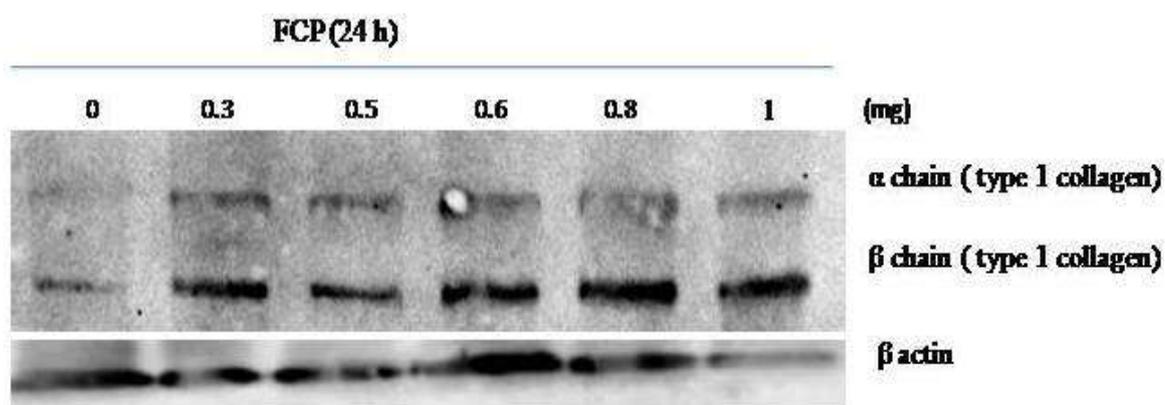
(B)



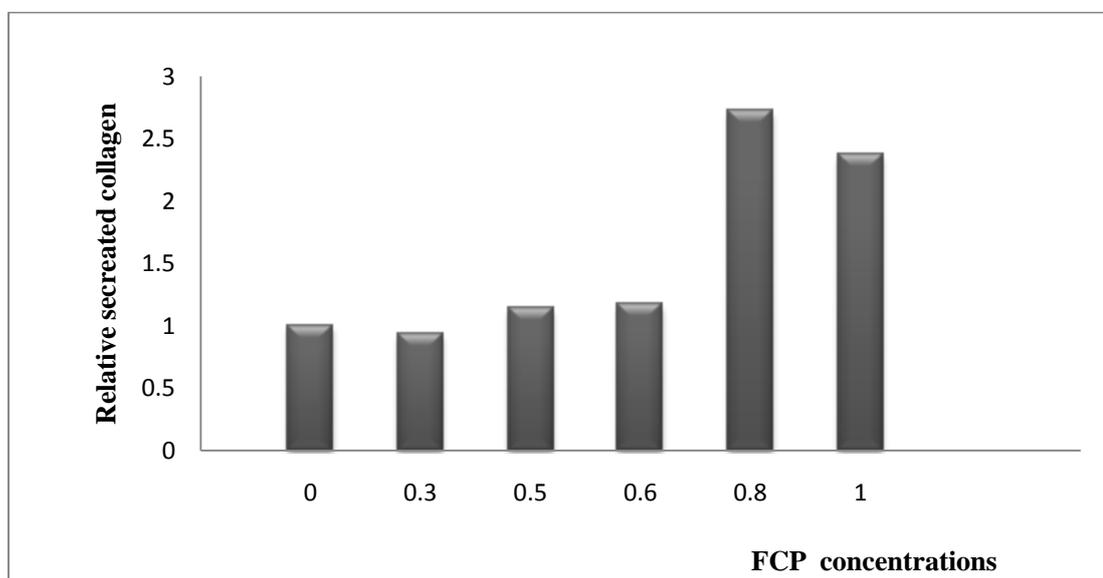
Evaluation of expression of collagen proteins in osteoblast cells upon treatment with fish collagen peptides

Figure 6.5 Western blotting using antibodies against type 1 collagen. The expression levels are normalized to the densitometric value of β actin using image lab software.

(A) Lane 2, 3, 4, 5 and 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.



(B) Densitometric analysis



7.3.3.3 Immunocytochemistry

The stimulation of type 1 collagen was fluorescently visualized by means of immunocytochemistry (Fig 6.6). 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.

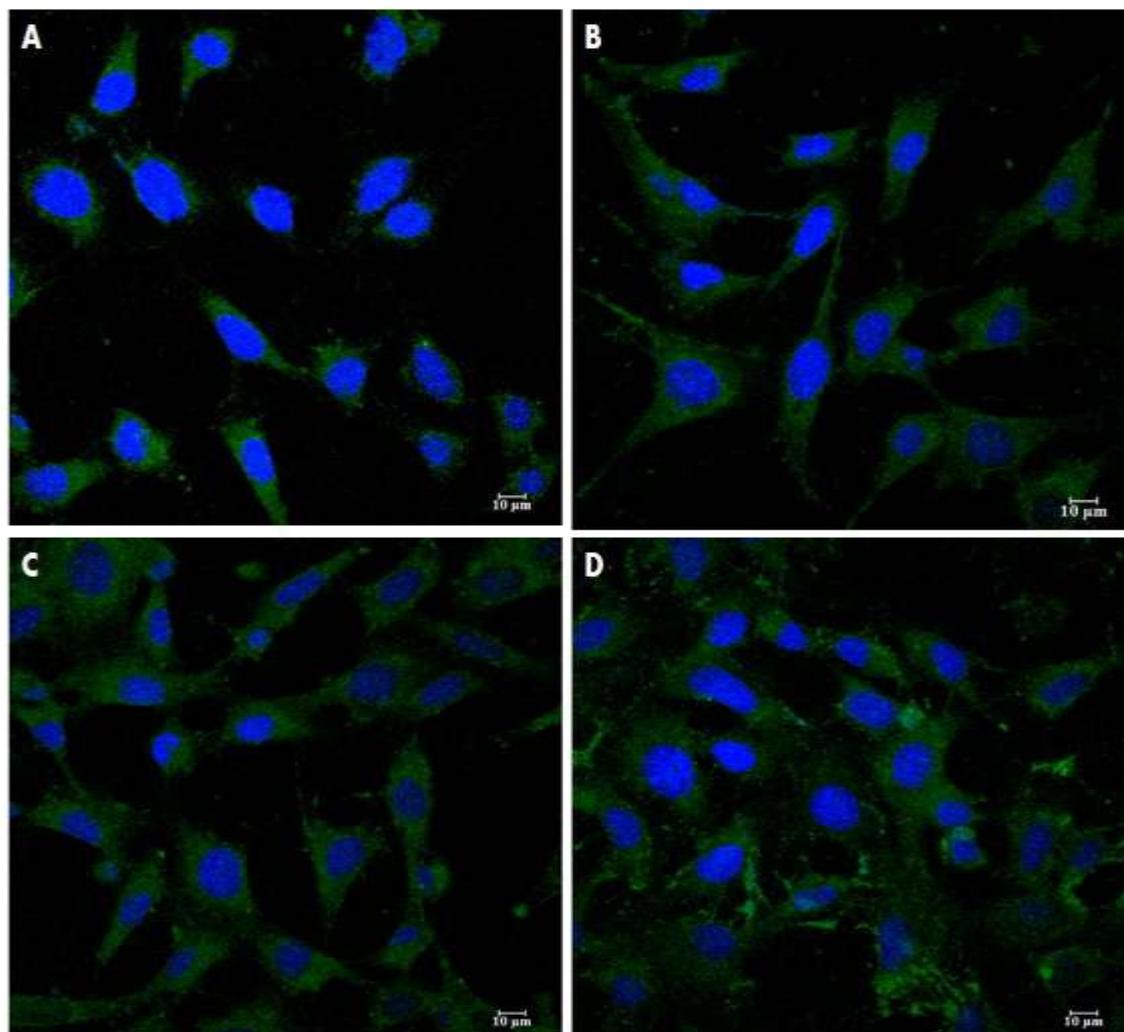
The specificity of the effect of FCP on collagen biosynthesis in osteoblasts was investigated using native collagen and non collagenous protein hydrolysate. But supplementation of the cell medium with 0.6 mg/ml native type 1 collagen or with a collagen free hydrolysate 0.6 mg/ml, induced no stimulation in collagen biosynthesis in the osteoblast cells. Hasegawa *et al.* (2010) and Ishida *et al.* (1981) used the immuno cytochemical techniques for showing the increased expression of type 1 collagen by cells upon induction with synoviolin. Oesser *et al.* (1999) in his investigations examined the stimulatory effect of bovine collagen hydrolysate in the metabolism of chondrocytes using immunocytochemical techniques.

Coll1 α 1 is the most abundant protein synthesized by active osteoblasts and is essential for mineral deposition, and its expression therefore represents the start of osteoblast differentiation (Franceschi and Iyer, 1992). As bone health supplements, bovine CP compounds are thought to stimulate increased synthesis of collagen (Yamada *et al.*, 2013).

MMPs and extracellular signal-regulated kinases (ERKs) play important roles in osteoblast proliferation and differentiation (Zhang *et al.*, 2002). Recently, it was reported that treatment with collagen hydrolysate from porcine skin gelatin significantly increased the collagen content and expression of the Coll1 α 1 gene through phosphorylation of ERK (Kim *et al.*, 2013).

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Figure 6.6 Immunocytochemical visualization of type 1 collagen (green fluorescence pericellularly) secreted by HOS cells. Staining was performed using type 1 collagen specific antibody (AB765P). 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. The results were derived from 3 independent experiments. Scale bar = 10 μ m.



7.4 Conclusion

In the present study, a primary cell culture model was used to investigate the influence of fish collagen hydrolysate on the type 1 collagen synthesis by osteoblast cells. To elucidate this function regarding collagen synthesis, we have treated the cells with FCP in

both a time and dose dependent manner. After 6, 12 and 24 hours of culture, the number of FCP-treated cells increased significantly compared with untreated cells. Quantification of collagen by chromogenic precipitation with Sirius red showed increased production of collagen from treated cells. The stimulatory effect was confirmed by western blot and immunocytochemistry analysis. The results clearly indicated that the presence of fish collagen hydrolysate led to a dose dependent increase in collagen synthesis by osteoblast cells. However native collagens or collagen free protein hydrolysate failed to stimulate the production of collagen in osteoblast cells.

Present study was carried out in order to understand the molecular mechanisms of the possible structure modifying effect of collagen hydrolysate. The *in vitro* study provides the demonstration of the stimulatory effect of fish collagen hydrolysate on the biosynthesis of collagen by human osteocytes cultures.

Saskia Schadow *et al.* (2010) demonstrated that collagen hydrolysates from various sources differ significantly with respect to both their chemical composition of oligopeptides representing collagen fragments as well as their effect on human articular cartilage. Nomura *et al.*, 2005 showed that administering shark skin gelatin to ovariectomized rats increased not only the bone mineral density of femurs but also the content of type I collagen and glycosaminoglycan in the epiphysis.

Since marked effects on human osteocytes were observed in our *in vitro* study it is suggested that FCP from grouper skin can be used as nutraceutical effective in the treatment of arthritic joints. Hence it is concluded that enzymatically hydrolyzed collagen preparations might contain therapeutically useful peptides. The biomedical properties of FCP have to be studied thoroughly in clinical trials before being applied as a safe and effective nutraceutical in human beings.