CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents used in this study and their sources

All chemicals used were analytical grade or equivalent. The chemicals shown below are listed in groups according to supplier.

Aldrich Chem. (USA)

1,9-Dimethyl-Methylene Blue

Bio-Rad Laboratories (Hercules, CA)

Acrylamide: N,N'-Methylenebisacrylamide Electrophoresis Purity Reagent, Ammonium persulphate (APS), N,N,N',N'-tetra-methyl-ethylenediamine (TEMED), Protein assay (Dye reagent concentrate), Sodium dodecyl sulphate (SDS),

Carlo Erba reagent (Rodano (Mi), Italy)

di-Sodium hydrogen phosphate anhydrous, Potassium sulfate, Sodium chloride, Sodium dihydrogen phosphate monohydrate,

Fisher Chemicals (UK)

Triton X-100, Acetonitrile HPLC grade

Fluka (Buchs, swizerland)

Sodium hydroxide, tri-Sodium citrate dihydrate

Gibco (New York, USA)

Dulbecco's Modified Eagle Medium (DMEM; Cat. No.12100), Penicillin/Streptomycin (10,000 U/10,000 ug/ml)

LAB-SCAN

n-Hexane

Merck (Darmstadt, F.R. Germany)

Acetic acid, Citric acid monohydrate, Ethanol (absolute), Hydrogen peroxide, Potassium chloride, Sodium acetate trihydrate, Sodium chloride, Sodium hydrogen carbonate, Sodium carbonate anhydrous, Methanol

Pharmacia, Biotechnology (Uppsala, Sweden)

Coomassie brilliant blue R-250

Sigma (Sigma Aldrich, St.Louis, MO, USA)

Bovine serum albumin, Cesium chloride, Chondroitin sulfate C, D-Glucuronic acid lactone, Gelatin typeB, glycerol, Hyaluronic acid (from human umbilical cord), Matrix metalloproteinase-2 (MMP-2), Matrix metalloproteinase-9 (MMP-9), papain, polyoxyethylene sorbitan monolaurate (Tween-20), recombinant-Human interleukin-1β, sodium phosphate

USB (OHIO,USA)

Tris (Hydroxymethyl) aminomethane hydrochloride, Glycine

2.1.2 Lists of antibodies used in this study

Peroxidase conjugated anti-biotin monoclonal antibody (Zymed Laboratory, Inc. Ca, USA)

2.2 Methods

2.2.1 Preperation of Plai (*Zingiber cassumunar* Roxb.) extracts

Fresh rhizome of Plai were cut into pieces, dried at 50-60° C and ground. Dried powder of Plai samples were extracted with hexane, 70% ethanol and distilled water. Dried ethanolic and water extracts were obtained after removing the solvent by evaporation under reduced pressure in evaporater, then lyophilized. Dried hexane extract was obtained after removing the solvent by evaporation and dry at 37°C. Dried residue was weight and stored at -20°C (51). The extracts were used in all experiments were from the same plant materials. However the HPLC fingerprint of each extract was recorded for further reference.

The HPLC system for isocratic elution

Column : Apollo C18 5 μ, 250 X 4.6 mm

Guard column : Apollo C18 5 μ, 7.5 X 4.6 mm

Mobile phase : 50% acetonitrile

Flow rate : 1.0 ml/min

Injection volume: 10 μl

Run time : 30 min

Detection : 267 nm

2.2.2 Cartilage Explants Culture

Articular cartilage was dissected from the metacarpophalangeal joints of pigs aged 20-24 weeks. Cartilage discs (3 mm^2) were biopsied from the weight-bearing region of the articular surface. Randomly selected explant discs (3 per well, approximately 30 mg total) were cultured in a 24-well tissue culture plate with serum-free medium (Dulbecco's modified Eagle's medium, DMEM) containing 200 units/ml penicillin and 200 μg/ml streptomycin. The explants were maintained in culture in a humidified incubator with 5% CO_2 at 37°C.

Explants were maintained in media without serum for 24 hours prior (day 0 media) to the first of three treatment days. Conditioned media were collected on day 3 of culture, stored at -20°C until analysed for indicators of degradation. Retinoic acid (RetA), (10 μM) or recombinant human Interleukin-1 beta (rHuIL-1β), (50 ng/ml) was added to induce cartilage

degradation. To evaluate the effects of Plai extract (extract with hexane, 70% ethanol or water), with varying concentration 50-200 μg/ml (dissolved in 10%DMSO) or positive control 100 μg/ml (dissolved in 10%DMSO) of Diacereine were added to the cultures. Each experiment contained a control in which neither retinoic acid nor IL-1β was added to the media. Treatments were performed with triplicated wells using tissue from the same animal donor.

2.3 Analytical methods

2.3.1 Uronic acid assay

Hexosamine and uronic acid are the components of the repeating units of all glycosaminoglycans (GAGs). Uronic acid is widely determined as the representative of GAGs in biological substances. This assay measures uronic acid by releasing the monosaccharide using acid hydrolysis. For a standard curve, 0 to 20 μg of glucuronic acid lactone or sample (5 μl) in up to 400 μl of water was added to a test tube. Concentrated sulfuric acid-borate reagent (2.5 ml) was added and to the tube and mixed. The tubes were then incubated at 100^{0}C in a water bath for 5 minutes and cooled to room temperature in ice bath. The solution of 0.1% m-phenylphenol (50 μl) was added and mixed. The uronic acid reaction was incubated at room temperature for 15 minutes. The absorbance of the pink to red color was read in a spectrophotometer at 540 nm against distilled water blank (52).

2.3.2 Dye binding assay

The sulfated glycosaminoglycan (S-GAG) concentrations were determined using a colorimetric dye binding assay modified by Farndale et al. The assay is based on a metachromatic shift in absorption maximal from 690 nm to 535 nm as a complex compound is formed in a mixture of 1,9-dimethylmethylene blue (DMMB) and the sulfated-GAG in the sample and standard. The dye solution was made by adding 16 mg of 1,9-dimethylmethylene blue to 5 ml ethanol to 2 g of sodium formate and 2 ml of formic acid in a total volume of 1 liter at pH 3.5. The maximum absorbance of the dye solution was at 620 nm. This solution was stored at 4^{0}C in dark bottle. Chondroitin 6-sulfate (CS-C) standards (0-40 μg/ml:50 μl) or samples (50 μl) were transferred to a microtitre plate. The dye solution (200 μl) was added immediately to each well and absorbance was measured at 620 nm, a precipitate might form on standing. A standard curve

of CS-C concentration and absorbance 620 nm was plotted. The concentration of CS-C in the samples were calculated from the standard curve. (53).

2.3.3 Enzyme-linked immunosorbent assay

A competitive inhibition based ELISA for HA

Microtiter plates (Maxisorp, Nunc) were coated at 4^0C overnight with umbilical cord HA (100 µl/well) in the coating buffer. Uncoated area was then blocked with 150 µl/well of 1% (w/v) BSA in the incubating buffer for 60 min at 25^0C. After washing, 100 µl of the mixture, sample or standard competitor (HA Healon:range 39.06-10,000 ng/ml) in B-HABPs (1:100), were added. After incubation for 60 min at 25^0C, plates were washed and then the peroxidase-mouse monoclonal anti-biotin (100 µl/well; 1:4,000) was added and incubated for 60 min at 25^0C. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37^0C for 20 min to allow the color to develop. The reaction was stopped by addition of 50 µl of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

2.3.4 Gelatin Zymography

Gelatinolytic activity of proteins from tissue culture media was assayed by electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a vertical gel apparatus according to the method of Laemmli (54), with modification that gelatin was included in the resolving gel . Gelatin type B (Sigma-Aldrich) was co-polymerized at a final concentration of 1 mg/ml into 0.75 mm thick, 10% polyacrylamide gel. Samples were mixed with an equal volume of 2X sample buffer, incubated at room temperature for 30 min. Each sample was loaded to a well and the samples were electrophoresed for 200 min at 90 V. After electrophoresis SDS was removed from the gel by washing 2 x 15 min in 2.5% Triton X-100 at room temperature and incubated for 16 hr at 37^0C in activating buffer. The gels were subsequently stained with 0.2% Coomassie Brilliant Blue R-250 for 5 hours at room temperature, destained with 50% methanol and 10% acetic acid to reveal zone of lysis within the gelatin matrix. The gel was dried on a Whatmam paper. Molecular weight standard markers were

run on each gel. Gelatinolytic activity was detected as clear band against a background of stained, intact gelatin-impregnated acrylamide gel.

Quantification of the gelatinolytic band on the zymogram was performed by densitometry. The image acquisition was done with an Agfa scanner (SNAPscan 1212), by using Adole Photoshop Elements 2.0 program. The zymogram densitometry was achieved with a Acion Image software for PC (Scion Corporation, Frederick, Maryland, USA), working in the Gel Plot 2 mode.

2.3.5 Statistical method

The release of extracellular matrix (ECM) biomolecules from cartilage induced by retinoic acid and interleukin-1β was estimated by calculation:

$$\% \text{ change} = \left(\frac{\text{Day3 medium - Day 0 medium}}{\text{Day 0 medium}} \right) \text{X } 100$$

$$\% \text{ remaining of uronic acid content} = \left(\frac{\text{uronic acid (Day 3 of treated)} \times 100}{\text{uronic acid (Day 3 of control)}} \right)$$

The significance of the differences between groups of data was tested using the one way analysis of variance. Statistical significance was considered when $p < 0.05$.

The release of matrix metalloproteinase (MMP) from cartilage induced by retinoic acid and interleukin-1β was quantified by densitometry and was estimated by the calculation

$$\% \text{ of control} = \frac{\text{density (Day 3 of treated)} \times 100}{\text{density (Day 3 of the untreated control)}}$$

The significance of the differences between groups of data was tested using the one way analysis of variance. Statistical significance was considered when $p < 0.05$.