CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents used in this study and their sources

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

Carlo Erba reagent [Rodano (Mi), Italy]

Di-Sodium hydrogen phosphate anhydrous, Potassium sulfate, Sodium chloride, Sodium dihydrogen phosphate monohydrate.

Fluka (Buchs, swizerland)

2,4-Dinitrophenyl hydrazine, Sodium hydroxide, tri-Sodium citrate dehydrate, glutaraldehyde

Gibco (New York, USA)

Dulbecco's Modified Eagle Medium (DMEM; Cat. No.12100),

Penicillin/Streptomycin (10,000 U/10,000 µg/ml).

I.T.Baker

Sulfuric acid, Hydrochloric acid.

LAB-SCAN

Ethyl acetate, *n*-Hexane, *n*-Propanol, Perchloric acid **.**

Merck (Darmstadt, F.R. Germany)

Acetic acid, Citric acid monohydrate, di-sodium hydrogen phosphate dodecahydrate, Ethanol (absolute), Hydrogen peroxide, Potassium chloride, Potassium dihydrogen

phosphate monohydrate, Sodium acetate trihydrate, Sodium chloride, Sodium hydrogen carbonate, Sodium carbonate anhydrous, Methanol.

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

Alamarblue, Bovine serum albumin, Calbazole, Cesium chloride, Chondroitinase ABC (E.C. 4.2.2.4) from *Proteus vulgaris*, DAB substrate, D-Glucuronic acid, glycerol, Hyaluronic acid (from human umbilical cord), o-phenylenediamine dihydrochloride (OPD) substrate , *p*-dimethyl amino benzaldehyde, papain, polyoxyethylene sorbitan monolaurate (Tween-20), Sodium phosphate, Sodium pyruvate.

USB (OHIO,USA)

Tris (hydroxymethyl) aminomethane hydrochloride, Glycine.

Zymed Laboratory (Ca, USA)

Peroxidase conjugated anti-biotin monoclonal antibody.

2.2 Methods

2.2.1 Chondrocyte isolation, expansion and passaging

Equine articular cartilage was obtained from a fetlock joint with no pathological lesions and history of inflammatory joint disease and without known infectious disease. Cartilage sample was first washed twice in a phosphate buffer solution containing antibiotics and then minced. Extracellular matrix was digested overnight at 37 ^{o}C by incubation of the tissue in 2.5 mg/ml type I collagenase (Roche, Mannheim, Germany) in Dulbecco modified Eagle medium (DMEM) containing 5% fetal calf serum. After centrifugation the chondrocytes were resuspended in DMEM, cell culture medium supplemented with 10% fetal calf serum. Chondrocytes were seeded as primary cell culture in 75 cm^2 culture flasks at low density (5×10^3 cells/cm^2) and incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 (67). Culture medium was changed every two to three days until the cells become confluent on the culture plate, the primary cell cultures were trypsinized using trypsine EDTA and passaged.

2.2.2 Monolayer culture and treatments

2.2.2.1 Dose effect of TGF-β1 on HA accumulation

2.0×10^5 passaged cells were seeded in 6-well culture plates, incubated in 10% FCS-DMEM for 48 h. After this period 10% FCS-DMEM was removed and replaced with 10% FCS-DMEM containing TGF-β1(Peprotech Asia, Israel) at concentration of 0, 2.5, 5.0 10.0 ng/ml for 3 days (n=3 for each TGF-β1 concentration). Culture medium was collected for ELISA HA analysis and cell was harvested for determination of DNA content.

2.2.2.2 Time effect of TGF-β1 on HAS-2 gene expression

The 80% confluents of cells were starved for 24 h before treatment with TGF-β1 (2.5ng/ml) for indicated time points (0, 3, 6, 9, 15, 24, 36, 48 h) then cell was harvest for mRNA extraction and gene expression analysis by semi-quantitative RT-PCR technique.

2.2.2.3 Dose effect of TGF-β1 on HAS-2 gene expression

The 80% confluent of cells were starved for 24 h before treatment with TGF-β1 for indicated concentrations (0, 2.5, 5.0, 10.0 ng/ml) for 3 h. Cells were harvested for mRNA extraction and gene expression analysis by semi-quantitative RT-PCR technique.

2.2.3 3-dimensional cultures and treatments

Gelatin based scaffold (Spongostan standard, Johnson & Johnson), a resorbable gelatin sponge and biodegradable matrix scaffolds composed of gelatin derived from porcine skin. It was cut in size of 5.0 mm×2.5 mm. Gelatin based scaffold were sterilized by autoclave. All scaffolds were presoaked in serum-free DMEM under sterile conditions prior to seeding. Equine chondrocytes $1x10^6$ cells were resuspende in 25 µl medium and seeded in upper side of gelatin based scaffold. The constructs were incubated for 4 hours to allow the cells to diffuse into and attach to the scaffold before 1 ml of medium was added. After 24 hours, each construct was transfered to a blank 24-well plate and replenished with 1.0 ml of chondrogenic media, 10% FCS-DMEM supplementing with ITS (PAA Laboratories, Austria, 10 µg/ml insulin, 5.5 mg/ml transferrin, 5 ng/ml selenium), 25 µg/ml ascorbic acid, 10^{-7}

M dexamethasone and with or without 10 ng/ml TGF-β1 (5). Chondrogenic media were changed every two to three days. The constructs remained in culture for 21 days and were measured the cell proliferation on day 7, 14, 21. The constructs were harvested and washed with 1XPBS. The construct for determination of HA content, uronic acid content and DNA content were digested at 60 ^{o}C with 2U papain for 24 h.

2.3 Analytical methods

2.3.1 Enzyme-linked immunosorbent assay

The HA level in conditioned medium and papain-digested scaffold were measured using a competitive inhibition-based-ELISA. Briefly, culture media samples and papain-digested scaffold sample containing unknown amounts of HA (175 ml), as well as a standard containing known concentrations (1.9-10,000 ng/ml) of a highly purified HA preparation (Healon®) were placed in small polypropylene tubes with appropriate concentrations of biotinylated-HA binding proteins (B-HABPs) (175ml) and incubated at room temperature (25°C) for 1 h. Aliquots (100 µl) of this reaction mixture were applied to umbilical cord HA-coated and BSA-blocked 96-well-plate and incubated at 25°C for 1 h. The wells were then washed with phosphate buffered saline solution (0.05% Tween-20), then, the appropriate dilution (1:2000 in PBS) of HRP-conjugated anti-biotin (Sigma, USA) was added to each well, incubated at 25°C for 1 hour and washed, then peroxidase substrate (OPD, o-phenylenediamine) was added. After the color development at 25°C for 20 minutes, the reaction was stopped by the addition of 50 ml 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using a Thermo scientific Multiskan EX microplate reader. The levels of HA in the culture media samples were determined by their abilities to inhibit color

development in the assay relative to a standard curve generated from the purified HA preparation (68).

2.3.2 Uronic acid content assay

The uronic acid content was measured in papain-digested scaffold by colorimetric assay using carbazole method. Glucuronic acid lactone was used as standard. Concentrated sulfuric acid-birate reagent (300 µl) was added into both sample and standard (0.48-2.40 µg) and incubated at 100°C for 10 min and cooled down to room temperature. Then 12 µl of carbazole solution (50 mg carbazole in 40 ml ethanol) was added and incubated at 100°C for 10 min. The absorbance of the pink to red color was read at 540 nm. The uronic acid content in scaffold was determined using the standard curve (69).

2.3.3 DNA content assay

The DNA content was also determined from aliquots of the papain digest using the Hoechst dye 33258 assay (Sigma-Aldrich) and fluorometry (emission wavelength: 365 nm and excitation wavelength: 458nm), as described previously (70, 71). The standard curve for the analysis was generated using calf thymus DNA (50-1,600 ng/ml) (Sigma-Aldrich), and the results were used to normalize the amount of HA and Uronic acid. For the measurement of the DNA amount, 50 µl extract was added to the dye solution. The total amount of DNA in the sample was determined using the standard curve.

2.3.4 **Reverse Transcription Polymerase Chain Reaction** (RT-PCR)

RNA was extracted from monolayer cells using NucleoSpin® RNA II (Machere-Nagel, Germany). Reverse transcription reaction was performed using 1.0 μg of total RNA and reverse-transcribed into cDNA using RevertAid^{TM} First Strand cDNA synthesis kit, (MBI Fermentas, Germany). Semi-quantitative PCR was perform using Taq DNA polymerase (Fermentas, Germany) with primer for equine GAPDH, the upstream primer (5'-TGGTATCGTGGAAGGACTCAT-3') and the downstream primer (5'-GTGGGTGTCGCTGTTGAAGTC-3') annealing for 1.15 mins at 55 ^{o}C, primer for equine HAS-2 the upstream primer (5'-AGAGAAGTCATGTACACGG-CCTTC-3') and the downstream primer(5'-GGTCTGCTGGTTTAACCATCTGAG-3') annealing for 1.15 mins at 57.8 ^{o}C (72). The amplified products were electroporesed on 1.5% (w/v) agarose gel, stained with ethidium bromide, and then imaged using a Bio-Rad Gel-Doc fluorescent image analysis analyzer. The integrated densities were then calculated using Totallab 1D gel analysis software and normalized to the house-keeping gene GAPDH (glyceraldehydes-3- phosphate dehydrogenase) to permit semi-quantitative comparisons in mRNA levels as previously described.

2.3.5 **Cell proliferation assay**

Cell viability and proliferation were assessed by AlamarBlue assay following the vendor's instructions. AlamarBlue is a non-toxic aqueous fluorescent dye that does not affect phenotype, viability or proliferation of the cell. The AlamarBlue assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction

(REDOX) indicator that both fluorescences and changes of color in response to chemical reduction of growth medium resulting from cell growth (73, 74).

2.3.5.1 Cell proliferation assay in monolayer culture

EAC were seeded in 24-well plate (10,000 cells/well) and incubated in 10% FCS-DMEM containing TGF-β1 at the concentration of 0, 2.5, 5.0, 10.0 ng/ml. They were measured the proliferation rate by Alarmablue assay on day 0, 4, 7 of culture. The condition media were removed and replaced with medium supplemented with 10% (v/v) AlamarBlue fluorescent dye for 4 h at 37 °C. One hundred microliters of medium from each sample was read absorbance at 540 and 630 nm in the Multiskan EX microplate reader.

2.3.5.2 Cell proliferation assay in 3-D culture

The gelatin scaffolds seeded with chondrocytes were incubated in chondrogenic media. The cells in scaffolds were measured proliferation on day 7, 14 and 21 of culture. The condition media were removed and replaced with medium supplemented with 10% (v/v) AlamarBlue fluorescent dye for 4 h at 37 °C. One hundred microliters of medium from each sample was read absorbance at 540 and 630 nm in the Thermo scientific Multiskan EX microplate reader.

2.3.6 Scanning Electron Microscopy (SEM)

The gelatin scaffolds were harvested following 21 days of culture, washed twice by phosphate buffer saline (PBS) and immersed in PBS for 3 times. They were fixed in 2.5% glutaraldehyde (pH 7.4) overnight at 4 °C (75). They were then dehydrated in increasing concentrations of ethanol (from 50% , 75% , 90% to 100%)

followed by vacuum drying. Dry scaffolds were sputter-coated with gold at 40 mA prior to observing under SEM.

2.3.7 Histological and immunohistochemical analysis

The gelatin scaffolds were harvested following 21 days of culture, fixed in 4% paraformaldehyde at 4 °C, overnight, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at a thickness of 4-5 μm (74). For histological analysis, sections were then deparaffinized in xylene two times, rehydrated using a graded series of ethanol. For histological sections were stained with hematoxylin and eosin (H&E). GAGs were stained with alcian blue 8GX at pH 2.5 (76). For immunohistological verification of HA synthesis, sections were blocked endogenous peroxidase with 3% H_2O_2 and non-specific sites were blocked with 3% BSA. Sections were incubated with a biotinylated-HA binding proteins; B-HABPs for 24 h and followed with incubation of HRP- conjugated anti-biotin for 1 h. After washing with PBS, DAB solutions were added into section until see the brown color precipitation occur on the sections. The sections were mounted with mounting medium and left at room temperature until dry.

2.3.8 Statistical analysis

All data are presented as means ± standard deviation (SD) for n = 3. Statistical comparisons were performed using Student's *t*-test. *p*-values < 0.05 were considered statistically significant.