CHAPTER III
MATERIALS AND METHODS

MATERIALS

All reagents were analytical grade. The following chemicals are listed in groups according to suppliers.

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

TRIZMA hydrochloride (Tris[hydroxtmethyl]-aminomethane hydrochloride), polyoxyethylene sorbitan monolaurate (Tween 20), bovine serum albumin (BSA), o-phenylene-diamine (OPD), sodium azide, sulfuric acid, and 35% hydrogen peroxide

Carlo Erba reagenti (Rodano (Mi), Italy)

Di-sodium hydrogen phosphate anhydrous, potassium sulfate, sodium chloride, and sodium-dihydrogen phosphate

Merck (Darmstadt, F.R. Germany)

Sodium acetate trihydrate, sodium hydrogen carbonate, sodium carbonate anhydrous, citric monohydrate, and potassium chloride

Bio-Rad (Bio-Rad®, USA)

Coomassie® Brilliant Blue G – 250 dye (dye reagent)

Monoclonal antibody

WF6 monoclonal antibody derived from Bone and Joint Research Laboratory, Faculty of Medicine, Chiang Mai University

IgM-specific peroxidase conjugated anti-mouse immunoglobulin (μ chain specific, Sigma Aldrich, St. Louis, MO, USA)

METHODS

Study subjects and Research design

Horses age between 2 to 9 year olds in Chiang Mai, Nakornratchasima, Saraburi, and KhonKhaen were chosen to be subjects of this research. Study subjects were divided into 2 groups as follow.

1. Normal group

The subjects were 62 clinically normal horses. All of horses had no history of previous joint disease. Physical examination revealed that they were healthy, had no abnormal clinical sign, showed no sign of lameness during lameness examination and had normal hematologic value, liver and kidney functions from blood examination.

2. Abnormal group

All of subjects in this group were 50 horses which were classified into 3 groups as follow.

1)	Arthritic group	6	horses
2)	Osteochondral (chip) fracture group	12	horses
3)	Osteoarthritic group	32	horses

Each horse had passed the diagnostic processes by history taking, physical examination, lameness examination and radiography respectively. Lameness examination consists of 3 processes as follow.

1. Assessment of physical abnormalities of leg or joint.
2. Grading of lameness as shown in table 3.

   If the horses showed sign of lameness in grade 1 or more, palpation and flexion test were required.

3. Palpation, Flexion test for identifying the point of problem joint.

And then radiographic examination was applied at the located joint to diagnose arthritis, osteochondral (chip) fracture and osteoarthritis as inclusive criteria.

Table 3 The degree of lameness (52).

Grade of Lameness	Clinical appearance
1	Lameness is not observed at walk but is recognizable at trot. If exercised on hard surface, an audible difference in sound is appreciated with increasing hoof sounds emanating from the sound limb.
2	Alteration in gait is noted at walk but no overt head movements. At a trot the lameness becomes obvious.
3	Lameness is obvious at both a walk and trot.
4	Non - weight bearing lameness is present.

Inclusive criteria

The radiographic criterias of arthritis, osteochondral fracture and osteoarthritis consisted of that was shown below (53-54) and in figure 9-12.

1) Arthritis

The radiographic finding;

- soft tissue swelling
- no chip fragment or bone abnormalities

2) osteochondral (chip) fracture

The radiographic finding;

- soft tissue swelling
- chip fragment
- no sign of osteoarthritis

3) osteoarthritis

The radiographic finding with one or more of these criterias;

- soft tissue swelling / thickening
- periarticular marginal osteophytes
- bony proliferation
- subchondral bone sclerosis
- subchondral bone lysis
- narrowing of joint spaces

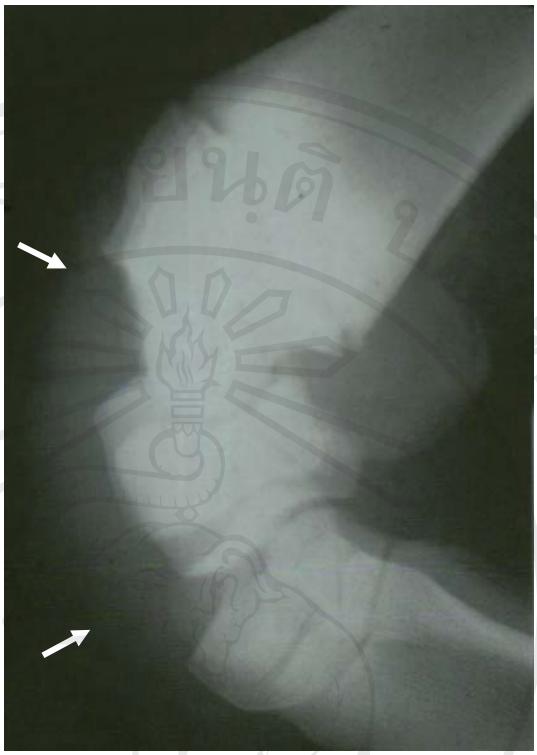

Figure 9 Extracapsular soft tissue swelling. Diffuse swelling on the dorsal aspect of carpus on flexed lateromedial positioning (46).

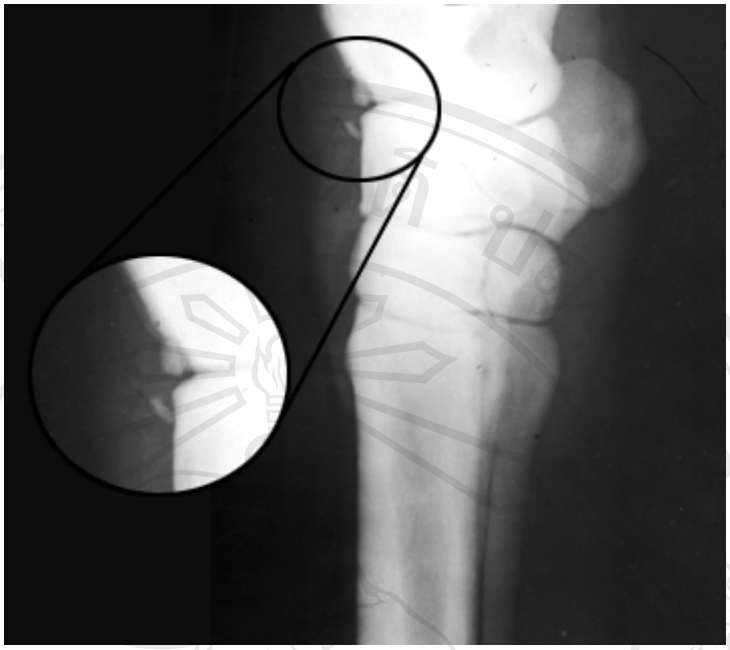

Figure 10 Multiple chip fractures along the distal aspect of the radius (55).

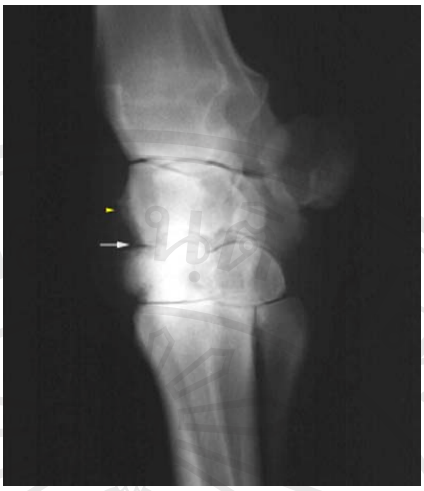

Figure 11 Moderate osteoarthritis. The change in contour of the distal dorsomedial margin of the radial carpal bone and the proximal dorsomedial margin of the third carpal bone (arrow). In addition, there is enthesiophyte present on the area of joint capsule attachment on the dorsomedial margin of the radial carpal bone (short arrowhead) (56).

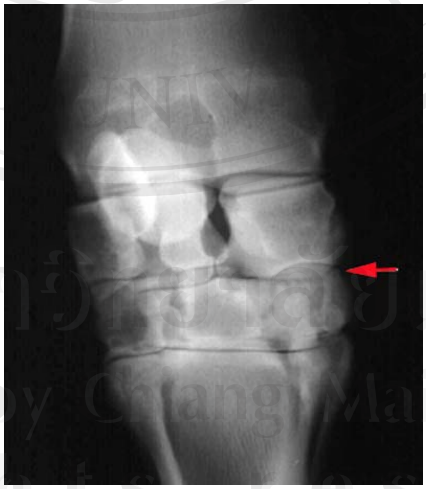

Figure 12 Moderate osteoarthritis 2. There is narrowing of the medial aspect of the middle carpal joint on the dorsopalmar radiographic projection (arrow) (56).

The research design was approved by the animal welfare committee, faculty of veterinary medicine, Chiang Mai university.

This research was divided into 3 parts as follow

Study A

This study was designed to compare the level of chondroitin sulfate epitope (WF6 epitope) in serum between normal horses and horses with arthritis, osteochondral (chip) fracture (OC) or osteoarthritis (OA).

Study B

The objective of this study was to compare the level of serum chondroitin sulfate epitope (WF6 epitope) from horses with osteochondral (chip) fracture (OC) between before and after treatment by arthroscopic surgery.

This study consisted of 3 horses with OC, were treated by arthroscopic surgery, which selected from osteochondral (chip) fracture group. Blood samples were collected before treatment (week 0) and after treatment at week 4, 8 and 12 respectively.

Study C

The objective of this study was to compare the level of chondroitin sulfate epitope (WF6 epitope/total protein) in synovial fluid between abnormal and contralateral normal joints in horses with osteochondral fracture or osteoarthritis.

This study consisted of 2 horses with OC and 1 horse with OA, which were selected from osteochondral fracture group and osteoarthritic group respectively.

Blood collection

After, the horses were examined by history taking, lameness examination and radiographic examination for diagnostic confirmation. Two ml. of blood was collected from each normal horse in everyday morning and each abnormal horse by EDTA vacutainer tube with 18G 1 ½ inch needle and 10 ml. of blood was collected by plain vacutainer tube with the same needle. All tubes were kept in 4°C cooler box during the transportation to the laboratory. Blood samples were analyzed within 24 hours including complete blood count (CBC), measurement of Aspartate aminotransferase (AST) and creatinine level, in order to check hematologic value, liver and kidney function respectively. The blood samples that had AST and creatinine level higher than reference value (57) were excluded. The remaining serum samples with normal AST and creatinine levels, were aliquot (1 ml.) in each microcentrifuge tube and stored at – 20°C until further analyzed (Figure 13). The serum samples were analyzed at biochemical laboratory. Total protein concentration and the level of chondroitin sulfate epitope (WF6 epitope) were measured by microtitre plate technique and competitive inhibition ELISA.

Synovial fluid collection

Approximate 2 ml. of synovial fluid was collected from each of abnormal and contralateral normal joints into plain tube by aseptic arthrocentesis. Samples of synovial fluid were centrifuged and then the supernatants were stored in microcentrifuge tube at – 20°C until assay was carried out. The synovial fluid samples were analyzed at biochemical laboratory. Total protein concentration and the level of chondroitin sulfate epitope (WF6 epitope) were measured by microtitre plate technique and competitive inhibition ELISA (Figure 14).

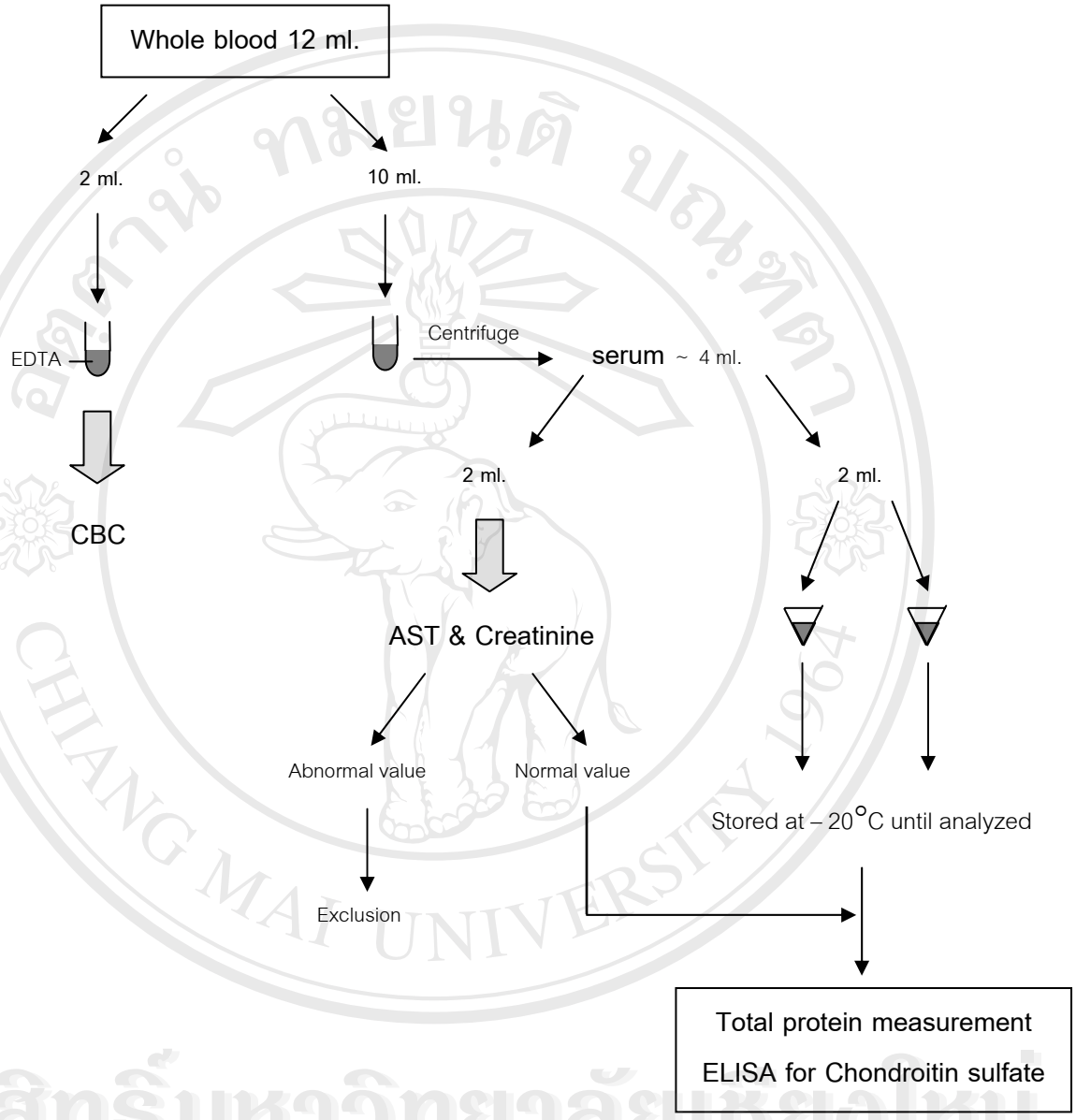

Figure 13 Blood collection procedure

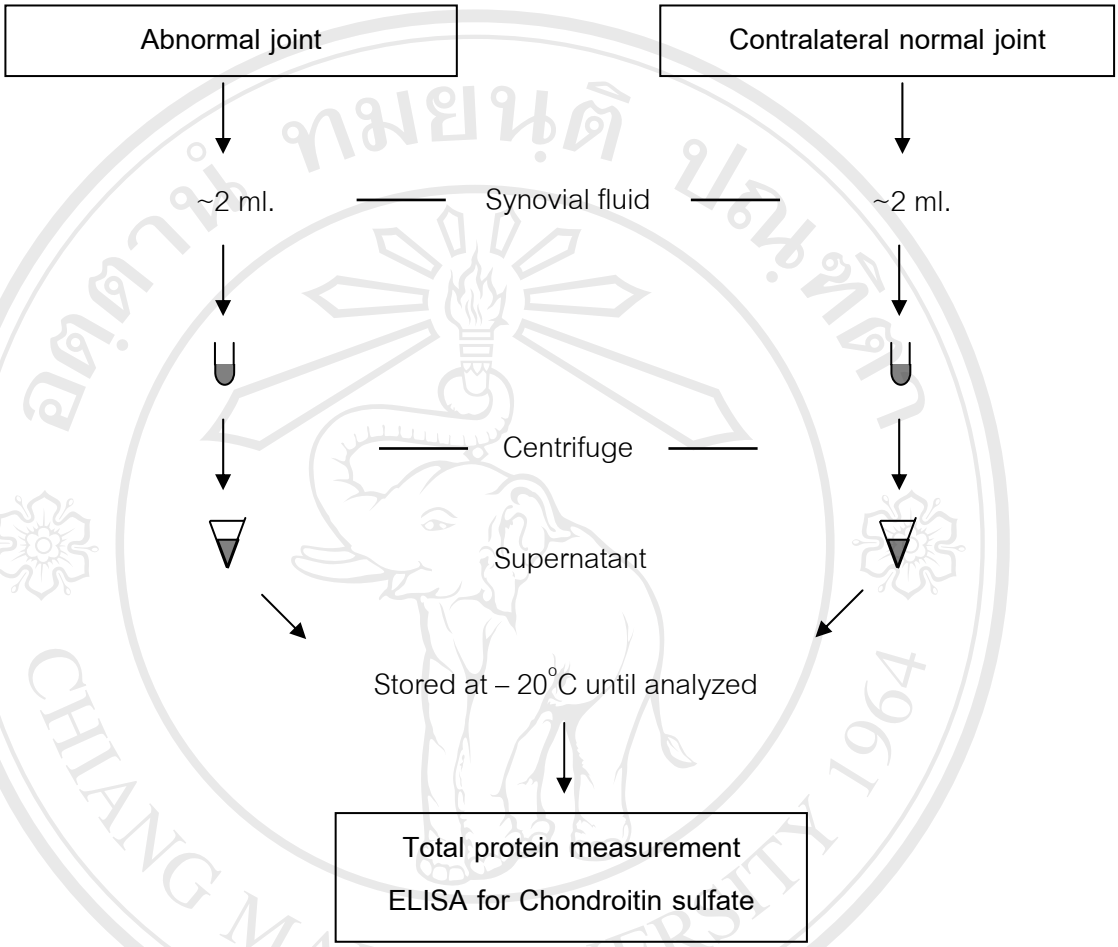

Figure 14 Synovial fluid collection procedure

Laboratory methods

Chondroitin sulfate epitope determination

Chondroitin sulfate epitope (WF6 epitope) in serum and synovial fluid were measured by competitive inhibition ELISA using similar method reported by Peansukmanee (42).

Principle of Competitive Inhibition ELISA

Enzyme-link immunosorbent assay, sometimes called ELISA, is one of the immunoassay. It combines the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assay by using antibodies of antigen coupled to an easily assayed enzyme.

In the competitive inhibition method, plates are coated by known coating antigen. Unknown or known amounts of antigen are allowed to react with a primary antibody. The excess antibody will be coupled with coating antigen. Then the secondary enzyme-labeled antibody is added in order to react with the remainder primary antibody. After the complex is washed in buffer, the substrate is added, and enzyme activity is measured.

A competitive inhibition ELISA for WF6

In competitive inhibition ELISA for WF6, shark proteoglycan (A1-fraction) is used as coating antigen, shark proteoglycan (A1D1-fraction) as competitor, WF6 monoclonal antibody (mAb WF6) as primary antibody and the IgM-specific peroxidase conjugated anti-mouse immunoglobulin as secondary antibody.

Microtitre plates (Maxisorp®, Nunc) were coated overnight at room temperature with 10 µg/ml shark proteoglycan (A1-fraction) 100 µl/well in coating buffer. Plates were washed by using Tris-IB (0.1% BSA), 150 µl/well (3 times). Then free sites were blocked with 150 µl/well of 1% BSA (in Tris-IB) and incubated for 60 minutes at 37°C. After washing, 100 µl/well of the mixture, sample or standard competitor (shark PG A1D1-fraction: 19.53-10000 ng/ml) in WF6 mAb (1:200), were added. After incubation for 60

minutes at 37°C, plates were washed and then the IgM-specific peroxidase conjugated anti-mouse immunoglobulin (100 µl/well; 1:2000) was added and incubated 60 minutes, 37°C. Plates were washed again and then the peroxidase substrate (100 µl/well), was added and incubated at 37°C for 3-5 minutes to allow the color to develop. The reaction was stopped by addition of 50 µl/well of 4M H_2SO_4. The absorbance ratio at 492/690 nm was measured by using the Titertek Multiskan® MCC/340 multiplate reader.

Laboratory quality control

Standard was analyzed in every ELISA plates for standardization of WF6 concentration. Intra-assay was determined by using 20 replicated analysis in control horse serum. Inter-assay was determined by using triplicate measurement of different plates.

Total protein determination (Protein assay)

Total protein (TP) concentrations in serum and synovial fluid were determined by using Microtitre plate technique. 10 µl/well of synovial fluid sample and standard (BSA: range 15.625-1000 µg/ml) were added in microtitre plates (Maxisorp®, Nunc). Then dye reagent (Coomassie® Brilliant Blue G – 250 dye) was diluted with DDI (1:5). After that, diluted dye reagent was added (200 µl/well) and incubated at room temperature for 5 minutes. The absorbance at 620 nm was measured by using the Titertek Multiskan® MCC/340 multiplate reader.

Statistical analysis

SPSS® for Windows version 11.0 (SPSS Inc., Illinois, USA) software was used for statistical calculation. Significant difference was considered at the level of $p < 0.05$ for all analyses. WF6 epitope concentrations were not normally distributed for all study groups. Therefore, data were transformed to natural log scale to use for further analyses.

In study A, level of serum chondroitin sulfate epitope (WF6 epitope) from normal horses were compared with arthritic horses, horses with osteochondral (chip) fracture or osteoarthritic horses using Student T- test or the analogues nonparametric test. And Analysis of variance (ANOVA) was used to test whether age, breed, or working status significantly affected the serum WF6 epitope concentrations in clinically normal horses (univariate analysis). Multivariable analysis using multiple regression was performed with $p < 0.15$ from univariate analysis.

In study B and C, level of chondroitin sulfate epitope (WF6 epitope) in serum and synovial fluid from horses with osteochondral fracture and osteoarthritic horse were not analyzed because the sample number was not enough for a valid statistic test.