CHAPTER III

MATERIALS AND METHODS

MATERIALS

Study subjects

Horses in Chiang-Mai, Nakornratchasima, and Sababuri were chosen to be subjects of this research because of their numerous populations. Research was divided into 2 parts as follow:

Study A

This study has an objective to investigate the normal value of chondroitin sulfate epitopes (3B3 epitope, and WF6 epitope) and hyaluronan (HA) in serum in each group of horses depending on age. The samples were 149 normal healthy horses with no history of previous joint injury. They had normal conformation and actually showed no sign of lameness during lameness examination.

These normal horses were classified into 5 groups as follow:

1. New born to less than two-year-old foals, 25 blood samples were collected from foals at Porn-anan stud farm[1].
2. Horses age between two to less than five years old, 25 blood samples were collected from horses at Darachai farm[2] and Boon-anan farm[3].
3. Horses age between five to less than nine years old, 29 blood samples were collected from horses at Calvary center[4] and Boon-anan farm.
4. Horses age nine to less than fifteen years old, 33 blood samples were collected from horses at Lanna-Riding club[5], Calvary center, Porn-anan stud farm, S.T. farm[6], and Darachai farm.

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[1] Porn-anan stud farm is located in Pakchong district, Nakornratchasima province, Thailand.

[2] Darachai farm is located in Saraburi province, Thailand.

[3] Boon-anan farm is located in Pakchong district, Nakornratchasima province, Thailand.

[4] Calvary center is located in Saraburi province, Thailand.

[5] Lanna-Riding club is located in Chiang Mai province, Thailand.

[6] S.T. farm is located in Wang Maung district, Saraburi province, Thailand.

5. Horses age from fifteen to twenty five years old, 37 blood samples were collected from horses at Lanna-Riding club, Calvary center, Porn-anan stud farm, S.T. farm, and Darachai farm.

Each horse was collected 10 ml of blood by plain vacutainer with size 18G needles. The small amount (0.5-1 ml) of serum was separated from each whole blood sample within 24 hours after collection in order to keep for detecting AST and creatinine level to check liver and renal function respectively. Samples that have AST and creatinine level higher than reference value were excluded. Whole blood and serum samples were kept in 4°C cooler box until these samples were transported to the laboratory. Then blood samples were centrifuged at 2600g for 10 minutes to separate the rest of serum. Serum samples were aliquoted 0.5 ml in each microcentrifuge tube and stored at –20 °c until analyzed.

Study B

The objective of this study is to compare the value of chondroitin sulfate epitopes (3B3 epitope, and WF6 epitope) and hyaluronan (HA) between normal horses and horses with osteoarthritis.

This study consists of 2 groups of horses. One is non-osteoarthritic (non-OA) horse group and another is osteoarthritic (OA) horse group. The 54 non-OA samples were collected from horses age 2-8 years old of study A. The serum samples of 23 OA horses were obtain from cases at Larged animal hospital, Faculty of Veterinary Medicine, Chiang Mai University. The lame horses that showed signs of lameness in grade 1 or more were checked to point the problem joint (grading the lameness was shown in table 4). And then radiographic examination was applied at the located joint to identify osteoarthritis. The radiographic criteria of osteoarthritis consist of that was show below (Widmer and Blevins 1994):

- Periarticular marginal osteophytes
- Narrow joint space, symmetric or asymmetric
- Subchondral bone sclerosis

- Subchondral bone cysts
- Subchondral bone lysis, sliding and gliding joint

Horses that had one or more of these criterias would be classified to osteoarthritic group (figure 10). As in study A, serum samples that had abnormal value of creatinine or AST were excluded. The rest of serum samples were aliquoted and stored at –20 °c.

Table 4 Grading of Lameness in horse (Stashak ,1987)

Grade of Lameness	Clinical appearance
1	Lameness not observe at a walk but recognize at a trot
2	Obviously lame at trot and alteration in gait at a walk but no overt head movement
3	Obviously lame at both a walk and trot
4	Non-weight bearing lameness

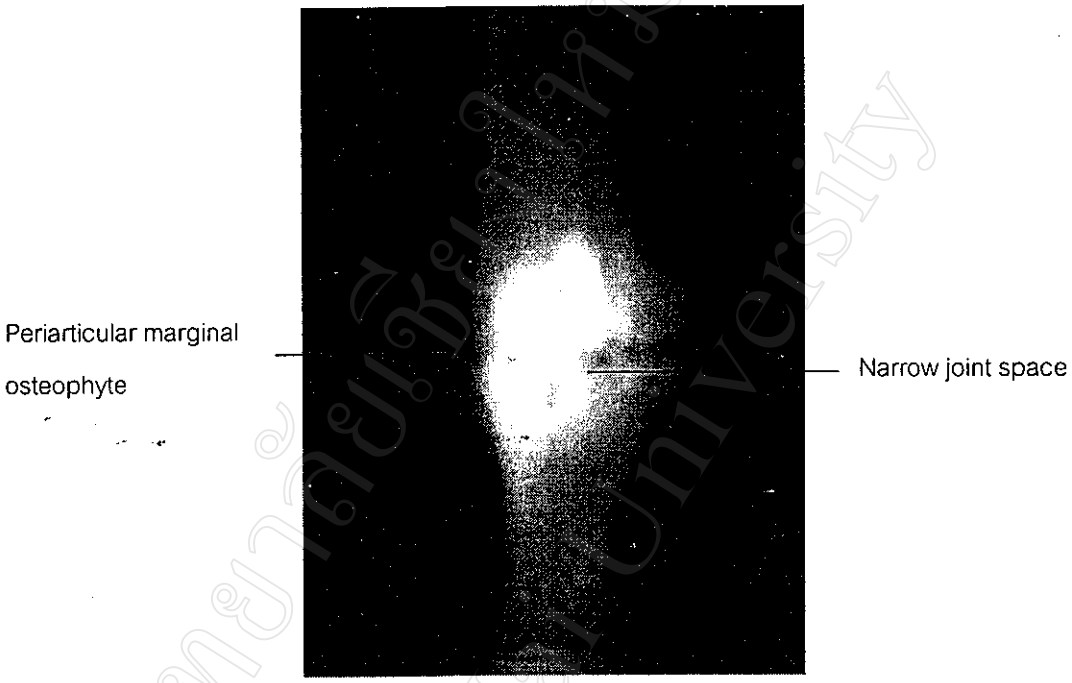

Figure 11 Radiographic view of an osteoarthritic horse at left fetlock (metacarpophalangeal) joint in dorsolateral-palmaromedial view

Reagents and their sources

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[hydroxymethyl]-aminomethane hydrochloride), polyoxyethylene sorbitan monolaurate (Tween 20), hyaluronic acid (from human umbilical cord), bovine serum albumin, o-Phenylenediamine (OPD), sodium azide, sulfuric acid, 35%hydrogen peroxide, and chondroitinase ABC

Pharmacia&Upjohn (Uppsala, Sweden)

Healon® (sterile preparation of sodium hyaluronate)

Carlo Erba reagenti (Rodano (Mi), Italy)

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

Merck (Darmstadt, F.R. Germany)

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

Monoclonal antibodies

3B3 monoclonal antibody, anti proteoglycan (Di-6S: Seikagaku Corporation, Tokyo, Japan)

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

Biotinylated hyaluronan binding protein (b-HABP) produced by 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)

Peroxidase-mouse monoclonal anti-biotin (Zymed Laboratory, Inc. CA, USA)

METHODS

Chondroitin sulfate epitopes (3B3 and WF6 epitope) and hyaluronan in serum were measured by competitive ELISA. The condition of this method applied from Pothachareon (Pothacharoen 2000) as shown in table 5

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, an unknown or known amounts of antigen are allowed to react with a specific antibody. The excess antibody will be coupled with coating antigen. Then the secondary enzyme-labeled antibody are 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.

Table 5 The condition of developed competitive ELISA

Reagent	3B3(+) Assay	WF6 assay	HA assay
Coating antigen (concentration)	Pig PG chondroitinase core (150 ng/ml)	Shark PG (A1-fraction) (10000 ng/ml)	Umbilical cord HA (100 µg/ml)
Competitor (range)	Pig PG chondrotinase core (4.53-2000 ng/ml)	Shark PG (A1D1-fraction) (19.53-10000 ng/ml)	HA Healon (19.53-10000 ng/ml)
Primary antibody (dilution)	3B3 mAb (1 : 20000)	WF6 mAb (1 : 20000)	b-HABP (1 : 200)
Secondary antibody (dilution)	Anti-IgM peroxidase (1 : 1000)	Anti-IgM peroxidase (1 : 4000)	Anti-biotin (1 : 4000)

A competitive Inhibition ELISA for 3B3

Microtitre plates (Maxisorp®, Nunc) were coated overnight at room temperature with 154 ng/ml pig proteoglycan chondroitinase core (100 µl/well) in coating buffer.

Free sites were then blocked with 150 µl/well of 1%(w/v) BSA in incubating buffer for 60 minutes at 37°C. After washing, 100 µl/well of the mixture, sample or standard competitor (pig PG chondroitinase core: range 4.53-2000 ng/ml) in 3B3 mAb (1:20000) 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; 1:1000) was added and incubated for 60 minutes, 37°C. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37°C for 15 minutes to allow the color develop. The reaction was stopped by addition of 50 µl of 4M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

A competitive Inhibition ELISA for WF6

Microtitre plates (Maxisorp®, Nunc) were coated overnight at room temperature with10 µg/ml Shark proteoglucan, A1 (100 µl/well) in coating buffer. Free sites were then blocked with 150 µl/well of 1% (w/v) BSA in incubating buffer for 60 minutes at 37° C. After washing, 100 µl/well of the mixture, sample or standard competitor (Shark PG A1D1: range 19.53 -10000 ng/ml) in WF6 mAb (1:20000), 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; 1:4000) was added and incubated for 60 minutes, 37°C. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37°C for 5 minutes to allow the color to develop. The reaction was stopped by addition of 50 µl of 4M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

A competitive Inhibition ELISA for HA

Microtitre plates (Maxisorp®, Nunc) were coated overnight at 4°C with umbilical cord HA (100 µl/well) in coating buffer. Free sites were then blocked with 150 µl/well of 1% (w/v) BSA in incubating buffer for 60 minutes at 25°C. After washing, 100

µl/well of the mixture, sample or standard competitor (HA Healon: range 4.53-2000 ng/ml) in b-HABP (1:200), were added. After incubating for 60 minutes at 25°C, plates were washed and then the peroxidase-mouse monoclonal anti-biotin (100 µl/well, 1:4000) was added and incubated for 60 minutes, 25°C. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37°C for 20 minutes to allow the color to develop. The reaction was stopped by addition of 50 µl of 4M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

Laboratory quality control

Control serum was collected from one healthy horse at Faculty of Veterinary Medicine. These serum samples were aliquoted and kept at –20°C until used. Control sample was analyzed in every ELISA plates in order of laboratory quality control.
Intra-assay: determined by using 20 replicated analysis in control horse serum.
Inter-assay: determined by using triplicate measurement on different plates.

Statistical analysis

The serum HA, 3B3 epitope, and WF6 epitope of normal and osteoarthritic horses were expressed in each group as mean and standard deviation in table form and as median in figure form. Comparison of these epitopes among normal horse groups was evaluated by ANOVA. And comparison between normal and osteoarthritic horse group were analyzed by unpaired t – test. Analysis for detecting the differentiation of age between normal and osteoarthritic horse had also been done by application of unpaired t -test. All of these analysis, $p < 0.05$ was considered significance. Statistical calculations were performed by application of SPSS for Windows version 10.0 software.