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Evaluation of a new enzyme-linked immunosorbent assay to detect keratan sulfate in equine serum

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Abstract

This study aimed to evaluate a system that identifies cartilage turn over and/or degradation through measurement of a new keratan sulfate (KS) epitope concentration in equine sera. Blood samples were collected from 30 horses, 1 (n = 15) and 2 year-olds (n = 15). Serum samples were analyzed for an epitope of keratan sulfate by 1/20/5D4 (KS5D4) and new epitopes of keratan sulfate using high sensitive keratan sulfate (HSKS), measured by two respective enzyme-linked immunosorbent assays (ELISAs). There was no correlation in serum concentration of KS evaluated using 5D4 and HSKS. Age had no significant effect on concentrations of KS measured with KS5D4 while 1 year-old horses showed significantly higher amounts than 2 year-olds with HSKS. Results suggest that HSKS could detect early signs of cartilage metabolic changes.

Key words: cartilage, horse, keratan sulfate

Articular cartilage is the main site of irreparable damage in joint diseases. A particular characteristic of joint disease is that cartilage degradation begins early before clinical signs are apparent. Much work has been done in order to clarify and better understand pathological features of the onset, development and treatment for joint disease in animals and humans. Recently, breakdown products of cartilage structure have been studied as potential markers of events that lead to cartilage wearing^{2,11,27}. Special attention has been given to

the search for such biomarkers in equine body fluids by a number of studies^{1,7,8,19,20}.

Cartilage matrix is formed mainly by a network of type II collagen fibers which contain proteoglycans, namely aggrecans¹². Aggrecans comprise a core protein to which several glycosaminoglycan (GAG) side chains are attached. Each aggrecan is bound to a link protein connected to hyaluronan forming macromolecules immobilized within the intact collagen network¹⁰. Release of these molecules and their fragments into synovial fluid and

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plasma follows the anabolic and catabolic process in the cartilage. The cleaved or released GAGs in the cartilage and synovial fluid are mainly chondroitin and keratan sulfate (KS) produced during damage to articular cartilage.

It is currently difficult to detect early degeneration of articular cartilage. Initial changes of cartilage cannot be detected using conventional X-ray examination or by magnetic resonance imaging. Therefore, biomarkers may be useful in screening tests reflecting cartilage metabolism, for the purpose of detecting articular cartilage damage at the early stage. In 1983, Caterson *et al.*⁵⁾, dealing with hyaline cartilage, followed by Thonar *et al* in 1985²⁴⁾, measured serum KS by using enzyme-linked immunosorbant assay (ELISA) with anti-KS monoclonal antibody 1/20/5D4 (5D4) and suggested its usefulness as a marker of osteoarthritis. Keratan sulfate has since been evaluated as one of the biomarkers for cartilage degradation^{3,4,9,27)}. Several different epitopes have been identified and ELISAs using anti-equine KS antibodies were developed, such as 1/14/16H9^{16,18,19,23)}, demonstrating a variety of sensitivities.

Keratan sulfate has been evaluated using competitive ELISA, which is a highly sensitive technique but is not very consistent^{24,25)}. It is possible to derive different results from the same batch of samples under a different running of the same assay. Other methods of the ELISA system are consistent but not so sensitive, making the development of sensitive antibodies a necessary advance²⁵⁾. However, this measurement has recently been considerably improved, and this new approach, high sensitive keratan sulfate (HSKS)²²⁾, could sensitively detect small KS fragments released during the early stages of damage. It has proved successful in detecting such fragments in serum from several animal species, including humans, but has not yet been tested in horses²²⁾.

The objective of the present study is to evaluate KS concentration in equine serum as

measured by KS5D4 and by HSKS, comparing the two measurement techniques of the same ELISA system.

This study involved 30 healthy Thoroughbred horses located in the facilities of a breeding/training farm in Hokkaido, Japan. None of these horses showed any clinically detectable lameness or orthopedic abnormalities. Fifteen of these horses were approximately 1 year old, and the other 15 were approximately 2 years old.

Blood samples from the jugular vein were collected into tubes without anticoagulants. To avoid peak concentrations of KS immediately after exercise, samples were obtained from all groups between 4 and 12 hr after they finished training. Following centrifugation ($2000 \times g$, 10 min), serum samples were collected and stored at -70°C until they were used for analysis.

Measuring KS by KS5D4 was performed using 50 μl of each sample with no dilution. This technique involved a monoclonal antibody specific to KS5D4 (Keratan Sulfate 5D4, Code No. 280565, Seikagaku Biobusiness, Tokyo, Japan). An antibody-coated microplate was washed 4 times with wash solution, then the first well was filled with blank (0 ng/ml) and the 5 following wells in that column filled in turn with 50 μl of KS standard solutions (at 40, 20, 10, 5 and 2.5 ng/ml). The remaining wells were filled with samples (not diluted) and incubated for 60 min at 37°C . After washing 4 times, 25 μl of horseradish peroxidase-conjugated streptavidin solution and 25 μl of biotinylated antibody solution were added to each well, mixed gently and incubated for 60 min at 37°C . The microplate was then washed 4 times, followed by the addition of 50 μl of substrate solution (3,3',5,5' - tetramethylbenzidine) into each well and incubated for 10 min at room temperature. Finally the reaction was stopped by adding 50 μl of stop solution and mixing gently. The absorbance at 450 nm was then measured using a microplate reader (reference wave length of 630 nm).

Determination of KS epitopes was also estimated by highly sensitive measurement using specific KS antibody (HSKS, Code No.280567, Seikagaku Biobusiness, Tokyo, Japan). Briefly, 100 μ l of buffer solution was added to each well of antibody-coated plate. Then the first well was filled with blank (0 ng/ml) and the 5 following wells in that column filled in turn with 20 μ l of KS standard solutions (at 4, 2, 1, 0.5 and 0.25 ng/ml). The remaining wells were filled with samples, and in this case, diluted 1000 times. The microplate was incubated at room temperature for 60 min, washed 5 times, then 100 μ l of horseradish peroxidase-conjugated antibody solution was added to each well, incubated for 30 min and washed 5 times. A substrate solution of 100 μ l of 3,3',5,5' - tetramethylbenzidine was added to each well and incubated at room temperature for 30 min. Finally, 100 μ l of stop solution were added and gently mixed. The absorbance was measured at 450 nm in a microplate reader (reference wavelength of 630 nm).

Correlation between the results from KS5D4 and HSKS assays was examined using Spearman's rank correlation and correcting for ties. The test was applied to each age group separately and to the entire group. Student's t test was applied to the serum levels of the biomarkers as measured by the two different techniques in order to determine the effects of age on type of test. The significance value was set at 0.05.

There was no correlation in serum concentration of KS evaluated using KS5D4 with that evaluated by HSKS. This was true neither in general (Spearman correlation coefficient $\rho = 0.075$, $p = 0.685$) (Fig. 1), or in the two age groups (1 year-old, $\rho = 0.021$, $p = 0.9361$; 2 year-old, $\rho = 0.102$, $p = 0.7008$)

Age had no significant effect on concentrations of KS measured with KS5D4 ($p = 0.9055$) but there was a significant age effect when HSKS was considered ($p = 0.0009$) (Fig. 2). The mean concentration obtained from 1

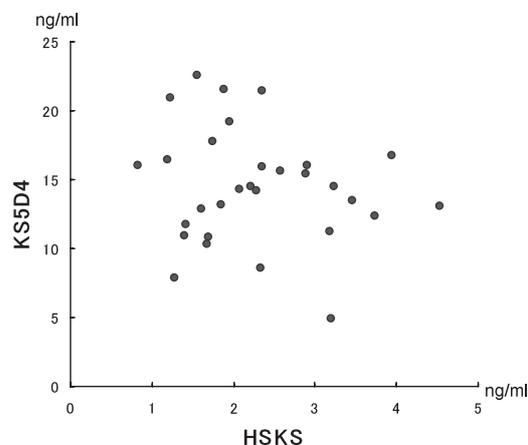


Fig. 1. Correlation between keratan sulfate concentrations found using 5D4 and HSKS, within 1 and 2 year-old horses. *Spearman correlation coefficient = 0.075 P value = 0.685.

year-old horses using HSKS (2.82 ± 0.88 ng/ml, mean and standard deviation) was significantly higher than that for 2 year-olds (1.76 ± 0.59 ng/ml).

Serum concentrations of KS identified by KS5D4 were increased after exercise along with some other biomarkers in subjects regardless of the existence of joint pathology^{6,21,23}. It has been reported that concentration of KS in serum increases immediately after training, decreasing rapidly within 1 hr and reaches initial values from then on¹⁹. Therefore, in order to prevent such peak alterations in any of the KS epitope measurements, all samples in this study were collected from 4 to 12 hr after training. The serum level of the keratan sulfate epitope was found to rise rapidly after the transection of the anterior cruciate ligament in dogs, long before osteoarthritic lesions could be detected²⁵. This is a good indicator that monitoring KS concentration in body fluids could provide indications of recent alterations that may lead to damage in the articular cartilage.

Young horses (3 months of age) hold a larger volume of cartilage in the body when compared to mature, adult ones¹³. Even though such immature cartilage contains lower KS concentration, in quantity per body weight it is equivalent to adults. Further, the younger the

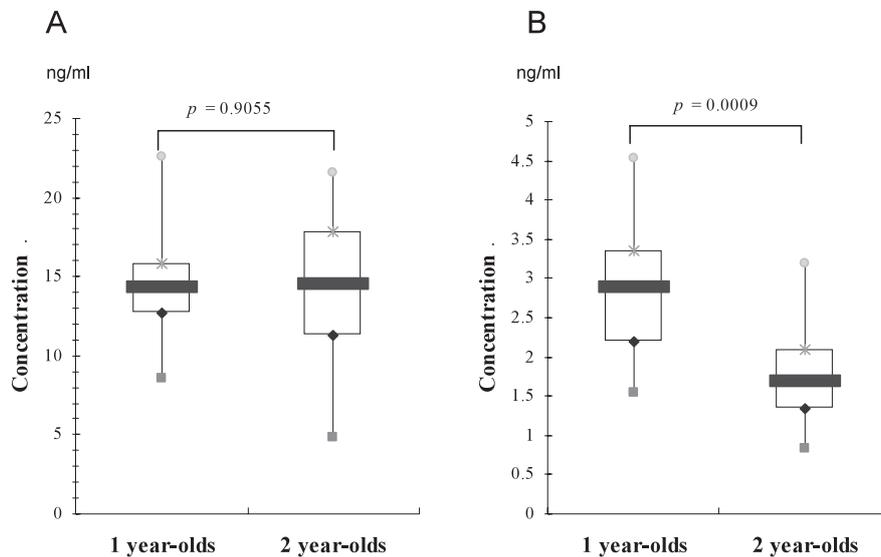


Fig. 2. Keratan sulfate concentrations for KS5D4 (A) and HSKS (B) markers, comparing between levels in 1 year-old and 2 year-old horses. Data are shown as box plots (expressed in ng/ml for KS5D4, and in µg/ml for HSKS). Each box represents the 25th and 75th percentiles. Lines outside the boxes represent the 10th and 90th percentiles (minimum and maximum limits, respectively). Lines inside the boxes represent the median.

animal, the higher the physiological cartilage turn-over rate¹⁴). Therefore, it can be expected that younger horses release higher amounts of keratan sulfate, regardless of the presence of articular cartilage lesions. Thus, it is desirable to perform measurement of keratan sulfate epitopes that would be sensitive enough to still represent the earliest damage taking place.

No correlation was observed between concentration values of KS measured by KS5D4 and HSKS obtained in serum collected from the same group of animals. Different epitopes resulting from the fragmentation of keratan sulfate polymer have been studied previously^{18,25}, which also did not appear to show strong correlation with concentration values found by KS5D4. Therefore, from our current results, it is possible to state that HSKS clearly identifies a different epitope. Antibodies in this system could detect smaller fragments of keratan sulfate as well as lower concentrations of fragments released during initial cartilage damage.

Our findings of no significant difference between 1 year-old and 2 year-old horses in serum KS concentration identified by KS5D4 may possibly be due to KS being degraded into

fragments too small to be detected by this ELISA. This corroborates the observation, using KS5D4, of an initial decrease of synovial fluid KS concentration and inconsistent differences in serum level in serum following cranial cruciate ligament transection in dogs. Levels significantly increased from baseline values at 3 months after surgery³.

Measured by HSKS, serum levels were higher in 1 year-old horses than in 2 year-olds. This phenomenon is possibly a consequence of higher cartilage turn over rate in younger animals. Using KS5D4, foals showed their peak values at 10 weeks of age, as reported by Todhunter *et al*, 1997²⁶). Also, by identifying a different epitope, using 16H9, 3 month-old foals were shown to have higher concentrations when compared to older horses, which rapidly decreased after 5 months of age¹⁷). Identification by 16H9 also showed higher KS levels in 2 year-old horses, when compared to 3 and 4 year-old ones^{15,19}). KS concentrations in healthy horses were noticeably higher in younger animals compared to older ones, at all stages of regular cartilage turn-over involving catabolism and anabolism.

Accordingly, it would be possible to assume that the identification of cartilage lesions by screening serum or synovial fluid using biomarkers would vary depending on a combination of different variables. Once a standard and sensitive evaluation can be established, it would be of major value in screening clinical cases of horse joint impairment, especially in cases where radiographically silent cartilage lesions exist. The results of the present study suggest that HSKS measurement system has potential for identifying cartilage degradation in horses when compared to the conventional identification by KS5D4. Using HSKS, it may be possible to identify even slight, minor changes in cartilage metabolism, particularly in young athlete horses, regardless of the existence of gross joint injury.

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