

The Effects of Growth and Disease in Serum Keratan Sulfate Concentration in Dogs

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ABSTRACT. The purpose of this study is to investigate keratan sulfate (KS) concentration in the serum of puppies and the effects of age, body weight, breed and diseases. Serum samples from six neonatal dogs (4 Beagles, 2 Labrador Retrievers), and from 127 adult dogs with various diseases were collected at a Teaching Animal Hospital. Canine serum KS concentration was measured by inhibition enzyme-linked immunosorbent assay (ELISA). Samples from puppies were evaluated for growth-related changes, and samples from patients were evaluated for age, body weight, breed and disease-related changes. Serum KS concentration was high in puppies from birth to 4 months of age. KS values started to decrease from 4 months to 9 months of age, and then gradually reached to the plateau. Though in the small sample, mean KS concentration in a Labrador Retriever was higher than in Beagles during the first 10 months. The values of serum KS showed body weight-related increase within retrievers among teaching hospital population and there was significant increase in body weight-related change. Cartilage metabolism is high in canine immature joint and that activity continues for 5 months, and that higher in Labrador Retrievers rather than in Beagles. There was no effect from other factors, including age, body weight, breed and disease in all patients. Serum KS concentration of Retrievers is higher than Beagles, and that value increased with gain of body weight. We suggest that Retriever have higher cartilage metabolism with growth or ageing.

KEY WORDS: canine, growth, keratan sulfate, puppies, serum.

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During growth, the epiphyseal and growth plate cartilages undergo interstitial and endochondral ossification. In these processes, the extracellular matrix of the cartilage is degraded and results in release of cartilage containing products into blood [11]. Extracellular matrix components consist of a core protein to which glycosaminoglycans, mainly chondroitin sulfate (CS) and keratan sulfate (KS), are covalently attached, proteoglycan fragments and glycosaminoglycans are present in synovial fluid and detected in peripheral blood [10, 21]. In arthritis, the extracellular matrix of cartilage is damaged, resulting in the release of its components into the synovial fluid and the blood [13, 21]. The degraded proteoglycan components in the joint are rapidly degraded into smaller fragments, and diffused into the body fluids that are in contact with the cartilage [13, 21]. Thus, a measurement of blood KS concentrations is used in monitoring cartilage catabolism in human or veterinary practice.

Hip dysplasia and osteochondrosis are common joint diseases in young dogs. Early diagnosis of osteoarthritis (OA) is an important factor to prevent aggravation to secondary OA. Previous studies showed the usefulness of joint markers, such as CS, KS and cartilage oligomeric matrix protein, in monitoring cartilage metabolism and for early detection of joint diseases [2, 8, 9, 12]. In children, foals and dogs/puppies, serum KS concentration has reported higher than the adult [13, 17, 22, 23]. However, to the authors' knowledge, there are no reports describing serum markers in dogs during growth.

The objectives of this study is to determine the changes of KS concentration in serum of puppies at the growing stage, and to investigate the effects of other factors, including body weight, breed and disease.

MATERIALS AND METHODS

Dogs and Serum sample: Six neonatal dogs, 4 Beagles (2 males, 2 females) and 2 Labrador Retrievers (male and female was each one) were randomly selected from our laboratory. These puppies underwent physical and radiographic examinations for evaluation of normal growth. Blood was collected immediately after birth, and then weekly up to the age 7 months. Monthly collection was then done until 15 months.

In addition, serum samples (n=127) were provided at the Teaching Animal Hospital, University of Kagoshima. These samples were sorted into some categories by dividing clinical state, osteoarthritis (n=22), neurological disease (n=13), trauma and fracture (n=7), tumor (n=30) and others (n=55). The sampled serum were centrifuged at 3,500 G for 15 min at 4°C, and the clear supernatant was collected, aliquoted and stored at -70°C until assay.

Preparation of canine cartilage proteoglycan monomer (PGm): Cartilage PGm was purified from canine cartilage according to a method previously reported [6, 17, 21, 22]. Hyaline cartilage was collected from the normal joints of adult dogs which were euthanized for reasons without joint problem. The cartilage was rinsed in saline solution and cut into small pieces. These diced cartilage fragments were extracted with 4 M guanidine HCl–0.05 M sodium acetate, pH 5.8, containing protease inhibitors (0.1 M 6-aminohexanoic acid, 0.005 M benzamidinium HCl, 0.01 M EDTA) for 24 hr at 4°C, with stirring. The extracts were separated from the cartilage residue by centrifugation at 15,000 × g for 30 min at 4°C, and the supernatant was dialyzed for 24 hr 4°C against 9 volumes of 0.05 M sodium acetate, pH 6.5, containing protease inhibitors. Canine cartilage PG monomers

(A1D1 fraction) were obtained after CsCl equilibrium density gradient centrifugation ($194,000 \times g$ at 10°C) under 2 conditions at densities of 1.65 (40 hr) and 1.50 (48 hr), respectively. The A1D1 fraction was dialyzed against 100 volumes of deionized water, 0.1 M NaCl, at 4°C , washed twice in deionized water, and then frozen and dried. The isolated A1D1 fraction was stored until used.

Antigen: The A1D1 fraction for the ELISA system was purified as described previously [17, 21].

Antibodies: A previously described monoclonal IgG1 antibody (1/20/5–D–4 Seikagaku-kogyo, Japan.) [6] that specifically recognizes an antigenic determinant in the polysaccharide structure of both corneal and skeletal keratin sulfate was used as the source of anti-KS antibody in the ELISA. Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., U.S.A.) was used as the second antibody.

Quantification of serum KS concentration by inhibition ELISA: We measured KS concentration in the serum of canine with an inhibition ELISA, designed by Thonar *et al.* [21] and Okumura *et al.* [17] with some modifications. One hundred μl of prepared canine A1D1, in a coating PBS solution (20 mM sodium carbonate, 20 mM sodium bicarbonate, 0.02% sodium azide, pH 10) was placed into each well of 96-well microtitre ELISA plates (Sumitomo Bakelite Co., Ltd., Japan) at $0.16 \mu\text{g/ml}$, incubated for 1 hr at 37°C and then overnight at 4°C . KS standards were prepared to give a range of $2.5 \mu\text{g/ml}$ – 2.44 ng/ml by doubling dilutions in PBS solution, pH 7.0, containing 0.05% Tween20 (PBS/Tween, pH 7.0). Serum was diluted 1/10 in the same buffer. Diluted standards (60 μl) and serum (60 μl) were mixed with 60 μl of monoclonal antibody 5D4 (diluted 1/10,000 in PBS/Tween, pH 7.0, 1% BSA) and incubated overnight at 4°C .

ELISA technique: Coated wells were washed 3 times with PBS solution, pH 7.0 and blocked with 100 μl /well of 5% BSA in PBS/Tween, pH 7.0, for 1 hr at room temperature. Blocked plates were washed 3 times with PBS/Tween, pH 7.0, and incubated with 100 μl /well of the inhibition mixture (monoclonal antibody plus KS standard or serum) for 1 hr at 37°C and then for 1 hr at 4°C . The plates were washed 3 times with PBS/Tween, pH 7.0 and incubated with 100 μl /well of alkaline phosphatase conjugated goat anti-mouse IgG diluted 1/20,000 in PBS/Tween, pH 7.0, 1% BSA for 1 hr at 37°C . After three times washes with PBS/Tween, pH 7.0, and one time washes with PBS solution. One hundred μl of glycine buffer (0.1 M glycine, 1 mM MgCl_2 , 1 mM ZnCl_2 , pH 10.0) containing 1 mg/ml of p-nitrophenyl phosphate (Sigma Chemical Co., U.S.A.) was placed into each well for 1 hr at 37°C . The plates were read at a wavelength of 405 nm, using an ELISA plate reader (Bio-Rad Laboratories, U.S.A.). A semi-log standard graph, where \log_{10} [concentration of KS standards] were plotted against the readings, was constructed, and the concentration of antigenic KS in the serum was calculated using the linear portion of the standard curve corresponding to the reliable range of KS concentration in this assay.

Statistical analyses: All quantitative data were expressed as mean \pm SD. The statistical differences in KS concentration were analyzed by factorial analysis of variance and Scheffe's method was used for simultaneous multiple comparisons. A *p*-value less than 0.05 was considered significant.

RESULTS

The inhibition ELISA system: Reliability of this assay system was established at concentrations between 4.88 ng/ml and $0.13 \mu\text{g/ml}$ (Fig. 1).

Concentration of serum KS in growing puppies: In all puppies, serum KS concentration showed high values from birth to 4 months of age. KS values started to decrease from 4 months to 9 months of age, and then gradually became stable. Also, during the first 5 months, mean KS concentration in the Labrador Retrievers was higher than in Beagles (Fig. 2). In the mean value of serum KS in growing Labrador Retrievers and Beagles, there were significant differences between the ages of 1 month of age versus 7, 9, 11, 12, 14, 15 months of age, 2 or 3 months of age versus 7–15 months of age and 4 months of age versus 7, 9, 11, 12, 14 months of age (Fig. 3).

The relationship between serum KS concentration and age, body weight, breed, diseases: The serum KS concentration did not show significant age, gender and body weight-related changes. There were no significant differences between the breed of patients. However, there was significant increase in body weight-related increase in Retrievers (Labrador Retrievers; $n=7$, Golden Retrievers; $n=23$, total; $n=30$), ($p=0.024$) (Fig. 4). Among disease groups, our result did not revealed any significant differences (Fig. 5).

DISCUSSION

KS concentrations in serum and synovial fluid in humans

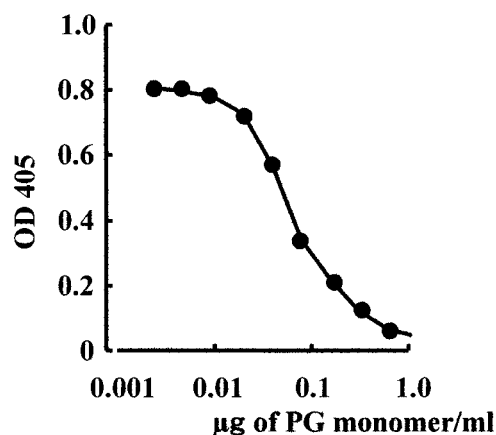


Fig. 1. Standard curve for the inhibition ELISA, used for measuring karatan sulfate concentrations. The available standard curve = 4.88 ng/ml to $0.13 \mu\text{g/ml}$.

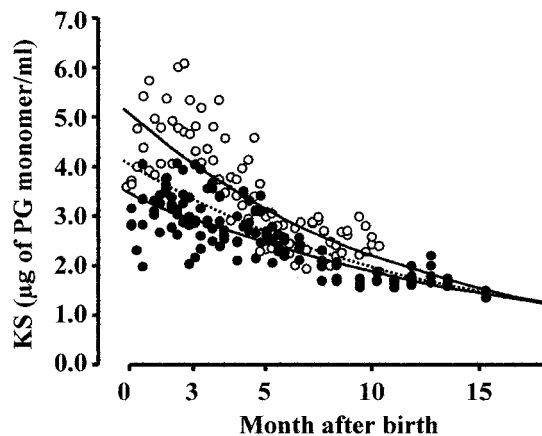


Fig. 2. Serum keratan sulfate concentration in puppies. Open circle is Labraor Retrievers and closed circle is Beagles. Upper line is Labraor Retrievers, lower line is Beagles and middle broken line is mean value of all puppies.

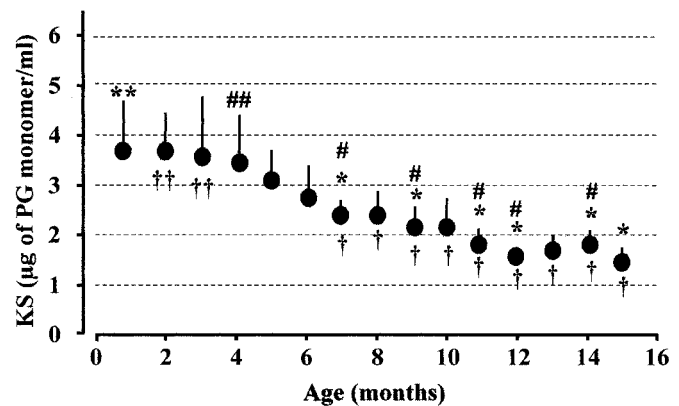


Fig. 3. Mean serum keratan sulfate concentration in puppies measured monthly between birth and 15 months. ** value is significantly ($p < 0.05$) different from * value. ## value is significantly ($p < 0.05$) different from # value. †† is significantly ($p < 0.05$) different from † value.

and animals has been measured in normal or diseased joint by an ELISA-inhibition assay [2–4, 8–10, 12, 14, 15, 17, 19–23, 26]. The measurement of KS concentration has been reported as a biomarker of OA. Although the relationship between aggrecan and aging [7, 16, 24, 25] and also the relation between KS and growth [17, 22, 23] have shown in few studies, it is still unknown in the dog.

In this study, we investigated that longitudinal serum KS concentration in dogs from birth to 15 months and evaluated the relationship between serum KS concentration and other factors, including body weight, breed and diseases. The serum KS concentration increased for 4 months after birth during the rapid growth phase, and it started to decrease 9 months after birth. The serum KS concentration became stable after it reached the adult value. Okumura *et al.* reported that the change of serum KS concentration in foals was high from 1 week to 3 months after birth and decreased rapidly from 3 months to 5 months of age [17]. They added that KS concentration in male foals is significantly higher than that in female foals. Thoner *et al.* described age related changes of serum KS concentration in children [22]. Our results of KS concentration in this study had a little difference in the period of peak, in the pattern of decrease, and in the age when reached to plateau. However, the studies in both foals and children showed that the serum KS concentration is high in the species growth phase and decreases to adult value with the decrease of growth rate. The serum KS concentration in the Labrador Retrievers was higher than in Beagles in this study. However, it was unclear whether the difference of breed in normal turnover or the presence of early cartilage and/or synovial lesion in osteoarthritis predisposing breed from our data. In young dogs, osteochondrosis, osteochondritis dissecans and hip dysplasia are joint diseases during developmental stage. And it is important that further investigation of serum KS concentration in these diseases to determinate whether serum KS concentration is

significantly different with these joints disorder. Alwan *et al.*, Tohdhunter *et al.* had reported that blood KS concentration was high in horses with osteochondrosis previously [1, 23].

In serum KS concentration from 127 canine patients, there were no significant differences with gender, age, body weight and breed. These results show it is difficult to detect a dog with OA by serum KS concentration alone [3, 5, 21, 23]. Some reports showed that KS concentration is high in early stage of OA and decrease with going to chronic stage in human and animals [8, 9, 12, 14, 15, 19, 20, 23, 26]. And also KS in human rheumatoid osteoarthritis (RA) has a low level [3, 20]. We suspect the reason that OA group did not show significant high value may be because we included several stages of OA and RA in our data and dogs with early OA lesion that did not show sign of OA might be included other disease category. The differences of serum KS concentrations in the OA group also could be affected by size of the keratan sulfate chains and number of epitopes recognized by the keratin sulfate antibody, because of continuing proteolysis after release from cartilage to appear in blood. Further study to investigate an accurate stage of OA is need to evaluate the relation between canine OA and serum KS.

Serum KS values had an age and body weight-related increase in the Retriever group. There was significant difference between serum KS and body weight. Retrievers are a breed associated with OA and perhaps these results show presence of occult cartilage or synovial lesion without clinical signs. The risk of OA in Retriever may increase with growth and age. The fact that the clinically normal Labrador Retriever showed high serum KS concentration may support this hypothesis. In humans, the relationship of body/mass index and OA has been shown, and obesity may be an important factor [18]. Although it will be difficult to configure applicable body/mass index for all breed due to various body sizes in dogs, it will be possible in some spe-

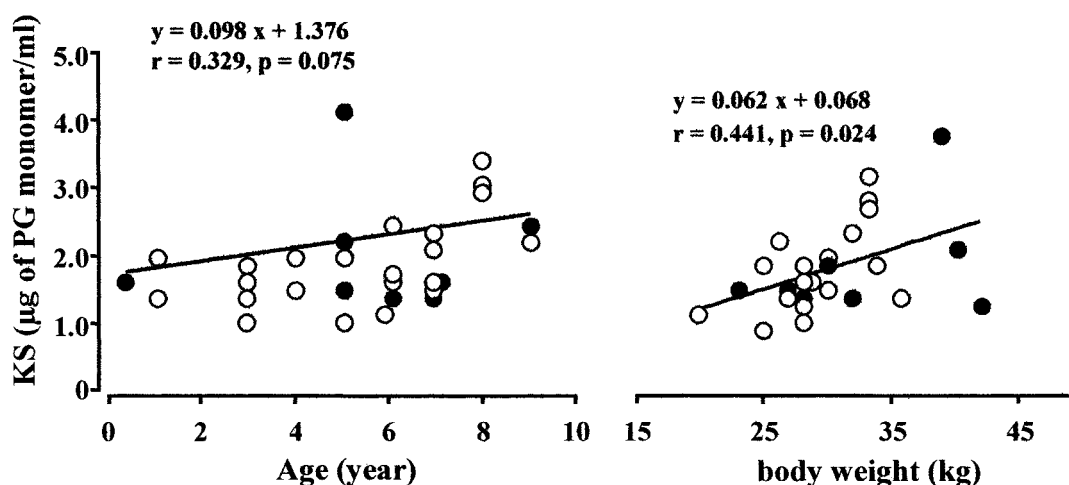


Fig. 4. The changes of serum keratan sulfate concentration in age and body weight of Retriever group. Closed circle is Retriever with osteoarthritis, open circle is Retriever with other disease.

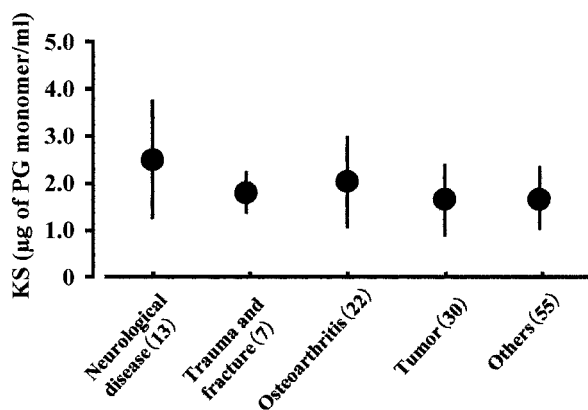


Fig. 5. Mean serum keratan sulfate concentration in patients' categories for animals with known diseases. The figure was shown by mean \pm SD value.

cific (osteoarthritis predisposed breed) breeds. This index may be useful to compare biomarkers in canine joint diseases. We suspect that serum KS concentration in obese dogs will be high because the cartilage suffers from the excessive weight loading. In these dogs, the risk of OA might increase with age as reported in humans [7, 16, 24].

This study showed that serum KS concentration in puppy dog is increased for 4 months after birth. It supports high cartilage metabolism in the immature canine joint which continues for 4 months with growth. During this time, serum KS concentration of the Labrador Retriever is higher than the Beagle. In the adult Retriever with other diseases present the serum KS value tended to have a body weight-related increase. This may suggest that Retrievers have a higher rate of cartilage degeneration with growth or ageing than other breeds. Further study needs to investigate the relationship between cartilage, synovial degeneration and some biomarkers in canine OA.

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