Serum Keratan Sulphate in Arabian Horses as A Cartilage Degradation Biomarker; the Effect of Daily Training Program

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Abstract

Keratan sulfate (KS) is a glycosaminoglycan found mostly in hyaline cartilage, and it is a potential marker of hyaline cartilage catabolism. It has been recognized as a marker of early cartilage injury because of significant increases in concentration before macroscopic damage occurs. The objective of the current study was to assess the KS concentration in the serum of Arabian horses in the Equestrian Club, King Abdulaziz University, subjected to a training program, and correlate it to the resting baseline values. Serum Keratan Sulphate concentration was measured in twelve Arabian horses with a mean ± SD age of 36 ± 2 months subjected to a training program. The KS concentration was measured using an inhibition enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (mAb) 1/20/5-D-4. The results showed significantly higher values of keratan sulfate compared to the baseline values before training. Levels of serum KS showed a significant rise after 15 and 30 minutes of the training program. However, there was a notable reduction in the concentration of keratan sulphate discovered 60 minutes after exercise. This study showed transiently higher keratan sulphate levels in the serum of Arabian horses, which may be indicative of increased metabolic activities in joint cartilage due to joint overloading.

Keywords: Keratan sulfate; Arabian horses; Cartilage; ELISA

Introduction

The prevalence of osteoarthritis and joint disorders is progressively rising globally. The rising global prevalence of osteoarthritis, along with its considerable economic and social impacts, has led to heightened interest in novel strategies for the early detection of joint disorders, which presents a valuable opportunity to impede disease progression and restore balance in the affected joint ***(Kraus et al., 2024)***. The sequences of events in natural osteoarthritic diseases are well established to be varied ***(Cope et al., 2019)***. However, there may be main and/or secondary roles for the synovial membrane, fibrous joint capsule, articular cartilage, subchondral bone, and

intraarticular ligaments. ***(Crane et al., 2024, Mukherjee and Das, 2024)***. Osteoarthritis leads to an inflammatory response, causing an elevation in levels of inflammatory mediator ***(Mukherjee and Das, 2024)***. This results in the release of various macromolecules into the synovial fluid, which then enter the bloodstream ***(Lohmander and Felson, 1997)***. Chondrocytes are the components that make up articular cartilage, and they are embedded in the extracellular matrix, which is mainly made up of collagen and proteoglycans that play a key role in maintaining the cartilage's appropriate function ***(Wang et al., 2024)***. Keratan sulphate (KS) is a side-chain molecule that is attached to a core protein in proteoglycans ***(van der Kraan, et al,. 2024)***. It has frequently been investigated as a marker of cartilage turnover ***(Pomin, 2015)***. It is primarily found in the extracellular matrix of the joint cartilage, particularly in aggrecans. It is also present, albeit to a lower extent, in the cornea and other tissues ***(Okumura et al., 1997, Caterson and Melrose, 2018)***.

An anti-equine antibody 1/14/16H9 was identified, and an enzyme-linked immunosorbent assay (ELISA) utilizing this antibody was employed to evaluate the KS concentration in blood and synovial fluid. On the other hand, it is not yet known if KS can serve as a marker for cartilage anabolism, cartilage catabolism, or both respectively ***(Okumura et al., 1997)***. Measurement of KS is commonly conducted using the human KS monoclonal antibody (mAb) 1/20/5-D-4. This mouse monoclonal IgG has the ability to recognize KS in natural proteoglycan, as well as in proteoglycan monomer preparations that have been isolated from the hyaline cartilage of humans and a variety of animal species, such as bovine, ovine, and monkey cartilage ***(Thonar, et al., 1985, Okumura et al., 1997, Wu et al., 2022)***. The 1/20/5-D-4 has the ability to specifically identify and attach to KS chains in their native state when they are linked to proteoglycans ***(Caterson et al., 1983)***. Consequently, it may be used to investigate the location and distribution of KS molecules ***(Thonar et al.n 1985).***

Prior research on cartilage catabolic activity, specifically focusing on the use of KS as a marker for cartilage turnover, revealed that foals with joint disorders revealed a notable increase in the concentration of serum keratan sulfate compared to healthy foals in normal growth stages ***(Okumura et al.n 1997)***. One significant factor that contributes to the turnover of cartilage and the release of molecular fragments into synovial fluid and serum is the mechanical loading of both healthy and diseased joints ***(Helal et al., 2007)***. Furthermore, a notable increase in the concentration of cartilage oligomeric matrix protein in the serum of human runners during and after a marathon ***(Neidhart et al., 2000, Hernández-Hermoso et al., 2021)***.

The current study aimed to measure changes in serum KS levels at the start and end of a mechanical loading training program for Arabian horses.

Furthermore, investigate the potential use of KS as a biomarker for cartilage metabolic activity in Arabian horses.

Materials and Methods

Suez Canal University's Faculty of Veterinary Medicine's Institutional Animal Use and Care Committee was responsible for reviewing and approving all of the procedures that were implemented during the experiments (SCU-VET-AREC 2025011)

1. Horses

This study was carried out at King Abdulaziz University's Equestrian Club in the Kingdom of Saudi Arabia. The present study was performed on 12 Arabian horses (7 females and 5 males) aged 36 ± 2 months.

2. Inclusion Criteria

Prior to the study, horses were selected based on the following criteria: Healthy horses without orthopedic disorders as evaluated through clinical examination, radiographic examination, and ultrasonography tendon imaging. If any signs of lameness or joint swelling appeared during the training program, additional diagnostic imaging was performed.

3. Training Program and Sampling

All horses were subjected to a training regime in which the training stage consisted of a daily workout followed by complete rest. The training program for the individual horse took place in one day. Each workout was performed over a constant distance consisting of a 1,000-metre walk, a 1,500-metre trot, a 3,000-metre canter or gallop, and then a 2,000-2,500-metre walk. As the training stage advanced, the running speed (m/s) increased during the canter/gallop stage; the maximum speed ranged from 12.4 m/s to 14.5 m/s. Blood samples were taken from the external jugular vein immediately before (the baseline) each training stage, then at ¼, ½, 1, and 24 hours after the daily workout, serum was collected and kept at -20˚C until the time of the assay.

4. Keratan Sulphate Assay

Inhibition ELISA was used to determine the KS concentration in the serum of Arabian horses using a mAb 1/20/5-D-4 as previously described ***(Okumura et al. 1997, Okumura and Fujinaga 1998)***. mAb 1/20/5-D-4 (Seikagaku Corporation, Tokyo, Japan) was used as a primary antibody to detect the KS epitope in serum. A peroxidase-conjugated rabbit anti mouse IgG1 (Zymed Laboratory, San Francisco, CA, USA) was used as a secondary antibody. Briefly, 100 µL of purified Proteoglycan Monomer (PGm), dissolved in a coating buffer consisting of 20 mM sodium carbonate, 20 mM sodium bicarbonate, and 0.002% sodium azide, with a final pH adjusted to 10, were added to each well at a concentration of 5 µg/ml. The solution was incubated for 2 hours at 20˚C and subsequently for 12 hours at 4°C.

Afterward, 70 µL of diluted standard and/or sample at a final dilution of 1/100 were mixed with an equal volume of monoclonal antibodies for KS, with a final dilution of 5D4 at 1/10,000. The dilution was conducted with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/Tween) and then incubated at 4°C. Coated wells underwent washing with PBS and were then blocked using 100 µL of 1% bovine serum albumin per well. Following the blocking step, 100 µL of the KS-antibody mixture was added to the ELISA plates, which were then incubated at 20˚C for one hour and an additional hour at 4°C. The plates were washed again and incubated for one hour after the addition of 100 µL per well of alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:200,000 in PBS/Tween. Another washing step was performed before adding 100 µL per well of glycine buffer (0.1 mol/L glycine, 1 mol/L MgCl2, 1 mol/L ZnCl2, pH 10.4) containing 1 mg/mL of p-nitrophenyl phosphate (Sigma 104-0). The plates were left for one hour to allow color development, after which an ELISA plate reader was utilized.

5. Statistical analysis

The mean ± standard deviation was used to express quantitative data. The SPSS program, version 22, was used to conduct the statistical analysis (IBM, Armonk, NY, USA). Data were analyzed using the ANOVA test, followed by Tukey's post hoc test. The significance level was set at P value < 0.05. The illustrated graph was created using GraphPad Prism software version 8.0.2 (GraphPad Software, CA, USA).

Results

The amount of KS in the serum of the Arabian horses fluctuated for each horse over the evaluation period **(Fig. 1)**. Compared to the baseline values before training, the KS level in the serum showed a substantial rise after 15 and 30 minutes of the training program, surpassing the initial values (P = 0.002 and 0.009, respectively). There was a substantial decrease in serum KS levels found 60 minutes after exercise, with no difference from the baseline level (P = 0.908). After a 24-hour period following the exercise, the level of serum KS was nearly comparable to the baseline level (P = 1.0) **(Table 1)**.

Table (1): The variation in serum keratan sulfate concentrations (ng/mL) following exercise (mean ± SD) compared to pre-exercise levels (baselines).

	Baseline	After ¼ h	After ½ h	After 1 h	After 24 h
keratan sulfate concentration	176.17±17.022^{c}	207.68±21.94^{a}	203.88±22.00^{ab}	183.13±18.47^{bc}	176.29±18.50^{c}

Different letters between groups indicate P < 0.05, and the same letter indicates P > 0.05

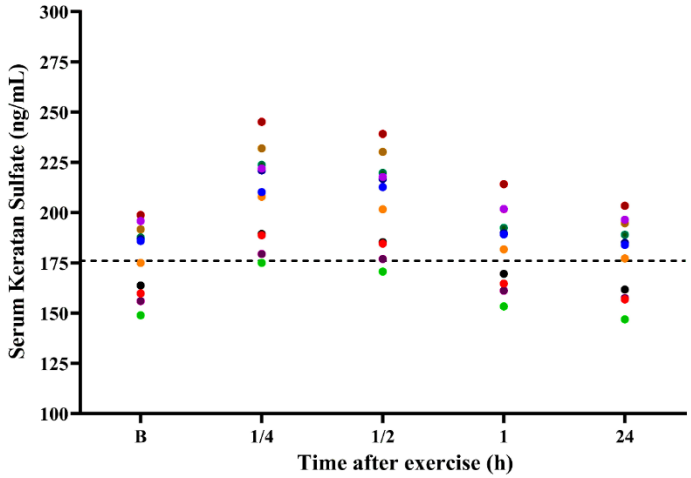

Fig.1: The findings of serum keratan sulfate pre-exercise (B) and at ¼, ½, 1, and 24 hours post exercise. Each colored dot represents the individual measurement, while the dashed line represents the mean baseline value of serum keratan sulfate.

Discussion

The current study used the mAb 1/20/5-D-4 as a valuable marker to investigate the catabolic process of articular cartilage in Arabian horses' serum. The daily training in this investigation imposed significant stress on the articular cartilage of the Arabian horses, leading to temporary degradation of the ephemeral cartilage. These findings align with earlier reports, indicating an increase in the KS levels in the serum of healthy individuals after exercise ***(Roos et al., 1995, Martel-Pelletier et al., 2021).*** The affinity of the antibody for KS in horses remains unclear; however, the concentration of KS in serum and synovial fluid was measured using the 1/20/5-D-4 monoclonal antibody. Nevertheless, the levels of KS in serum and synovial fluid are minimal, and the selected immunoassay employing this antibody is more sensitive ***(Okumura and Fujinaga, 1998)***.

In our investigation, we observed that the baseline levels of serum KS in all horses were lower than the levels seen after training. Furthermore, these levels rose significantly 15 minutes and 30 minutes after exercise. However, the KS levels were declined to baseline values after one hour. These findings highlight that a training program or excessive strain on joint cartilage increases the activity of cartilage metabolism, causing the release of KS molecules into the bloodstream. This leads to fluctuations in serum levels before and after daily training, following an increase in the synovial fluid. ***Okumura et al. (2002)*** proposed that in Thoroughbred horses, the

concentration of KS in the blood is likely to rise in response to mechanical stress on the articular cartilage or changes in cartilage metabolic activity.
In the present study, although KS was not measured in urine, the rapid restoration of serum KS levels to their pre-exercise values might be attributed to its excretion in urine. In 2006***, Misumi et al.*** conducted a study investigating the detection of Cartilage Oligomeric Matrix Protein (COMP) as a joint biomarker in urine. The study found that COMP molecules, which are released from the joints' extracellular matrix, can be enzymatically fragmented in the synovial fluid. However, these fragments are less likely to be degraded during blood circulation. Although there is no explanation for the mechanism by which large COMP fragments may get past the glomerular basement membrane, it is possible that these fragments undergo a reduced level of degradation throughout the process of filtering from the blood into the urine.
This study validated the results from previous research on Thoroughbred horses ***(Okumura et al., 2002)***, which indicated that the restoration of KS concentration to its pre-training levels following a temporary rise implies the possibility of proteoglycan release from the bloodstream into the liver, urine, or any other tissues for a short duration. Moreover, the persistent elevation of the levels of serum KS may suggest the continuous degradation of cartilage in the joints or other cartilaginous tissues.

Conclusion

This study, to the best of our knowledge, is the first trial to assess the levels of serum keratan sulphate in healthy Arabian horses using the monoclonal antibody 1/20/5D4 to identify any variations in keratan sulphate concentration in the serum resulting from daily training activity. The temporary elevation of serum keratan sulphate levels in all horses may pointed out an increase in metabolic processes in joint cartilage caused by excessive stress and overloading on the joints.

Conflict of Interest

The author declares no conflict of interests for this study.

Acknowledgments

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الملخص العربي

التغيرات في مستوى كبريتات الكيراتان في مصل الدم في الخيول العربية كمؤشر حيوي لتدهور الغضروف: تأثير برنامج تدريبي يومي

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قسم الجراحة، التخدير والأشعة، كلية الطب البيطري، جامعة قناة السويس، الطريق الدائري ، الإسماعيلية، 41522، مصر.

كبريتات الكيراتان هي جليكوس امينوجليكان توجد غالبا في الغضروف الزجاجي، والتغير في تركيزها يعتبر علامة محتملة على القصور في الغضروف الزجاجي خاصة غضاريف المفاصل. تم التعرف على كبريتات الكيراتان كعلامة لإصابة الغضروف المبكرة بسبب الزيادات الكبيرة في التركيز في الدم قبل حدوث الضرر العياني. هدفت الدراسة إلي تقييم تركيز كبريتات الكيراتان في مصل الدم للخيول العربية في نادي الفروسية بجامعة الملك عبد العزيز والتي خضعت لبرنامج تدريبي ومقارنة القياسات قبل التدريب بقيم خط الأساس في الراحة. تم قياس تركيز كبريتات الكيراتان في مصل الدم في اثني عشر حصانا عربيا (7 إناث و5 ذكور) بمتوسط عمر 36 ± 2 شهرا يخضعون لبرنامج تدريبي. تم قياس تركيز كبريتات الكيراتان باستخدام مقايسة الممتز المناعي المرتبط بالإنزيم المثبط (ELISA) باستخدام الجسم المضاد وحيد النسيلة (4-D-5/20/1). أظهرت النتائج قيما أعلى بكثير من كبريتات الكيراتان مقارنة بالقيم الأساسية قبل التدريب. وكانت مستويات كبريتات الكيراتان في مصل الدم مرتفعة جدا بعد 15 و30 دقيقة من البرنامج التدريبي. ومع ذلك، كان هناك انخفاض ملحوظ في تركيز كبريتات الكيراتان التي تم اكتشافها بعد 60 دقيقة من التمرين. الخلاصة: أظهرت هذه الدراسة ارتفاعا مؤقتا في مستويات كبريتات الكيراتان في مصل الخيول العربية، مما قد يكون مؤشرا على زيادة أنشطة التمثيل والتغير المؤقت في غضروف المفاصل بسبب الحمل التدريبي الزائد للمفاصل.