

High Plasma Concentrations of Sclerostin, an Inhibitor of the WNT Signaling Pathway, in Young Horses Affected by Osteochondrosis

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Abstract

Osteochondrosis (OC) is a developmental disease in horses with a significant impact on the horse's welfare and performance. The early disturbance of enchondral ossification progresses to inflammatory and healing process in older horses. Metabolic pathway analysis showed an obvious dysregulation of several signaling pathways related to cartilage formation and cartilage repair such as Wnt/ β -catenin, Indian hedgehog and TGF- β signaling pathways. Other regulated genes appeared to be involved in high carbohydrate diet, abnormal insulin metabolism or inflammation. Sclerostin is an osteocyte-secreted soluble antagonist of the Wnt/ β -catenin signaling pathway. It is crucial for osteoblast development and activity and is increased in naturally occurring lesions of equine osteochondrosis. The aim of this study is to compare the circulating sclerostin levels between OC-affected (n = 20) and healthy horses (n = 19). A significant linear regression between plasma sclerostin and age is observed especially in the healthy young horses. The mean plasma sclerostin concentration is significantly higher in young horses suffering from osteochondrosis compared to the control horses. These results reinforce the possible role of the Wnt/ β -catenin signaling pathway in the OC pathogeny. The inhibition of this essential pathway could disturb the osteo-chondral differentiation. More studies are currently needed to define the eventual clinic interest of plasma sclerostin as future biomarker in bone and cartilage diseases.

Keywords

Sclerostin, Osteochondrosis, Wnt/ β -Catenin Pathway, Horse, Equine Osteochondrosis

1. Introduction

Osteochondrosis (OC) is a developmental disease of growing horses. It is defined as a disturbance in the process of endochondral ossification [1] which can lead to the formation of detached fragments (osteochondrosis dissecans (OCD)), fissures, or subchondral bone cysts at the epiphyseal growth cartilage [1] [2]. OCD is a frequent cause of pain, lameness and reduced performance in young athletic horses [3] [4]. The disease appears to be multifactorial in origin, including skeletal growth rates, nutrition, endocrinological factors, exercise, biomechanics, and genetic effects [5].

In a previous study, we analyzed the transcript profile of leukocytes from horses affected with OC using the high throughput sequencing method digital gene expression analysis [6]. Metabolic pathway analysis showed an obvious deregulation of several signaling pathways related to cartilage formation and cartilage repair such as Wnt/ β -catenin, Indian hedgehog- and TGF- β signaling pathways. Other regulated genes may play a role in high carbohydrate diet, abnormal insulin metabolism or inflammation [6]. Power and others [7] demonstrated that sclerostin protein is increased in naturally occurring lesions of equine osteochondrosis.

Sclerostin is a small protein specifically expressed by osteocytes which antagonizes canonical Wnt/ β -catenin signaling by binding to Wnt co-receptors, low-density lipoprotein receptor-related protein 4, 5 and 6 [8] (**Figure 1**). Sclerostin coded by the SOST gene in osteocytes. Sclerostin is mainly produced in response to mechanical stress applied to the skeleton. It appears to play an important role in the regulation of bone remodeling [9]. Recent studies have shown that the SOST gene is also expressed by articular chondrocytes and that modulation of its activity may have effects on articular cartilage and subchondral bone [10].

In humans, serum sclerostin levels are gender dependent and increase with age as well as in several pathologies such as diabetes, obesity and osteoporosis [11]-[13]. In cases of juvenile idiopathic arthritis (JIA), soluble antagonists of the canonical Wnt/ β -catenin signaling were measured in serum and subsequently induced inhibition of osteoblast proliferation, maturation, and progenitor differentiation. A significant correlation between the sclerostin serum concentration and the number of tender and swollen joints supports the hypothesis that chondrocytes and cells of the subchondral bone may contribute to circulating sclerostin in JIA [14].

The aim of the present study is to demonstrate the implication of the Wnt/ β -catenin signaling pathway by measuring in young horses suffering from osteochondrosis, the concentration of circulating sclerostin and to compare these values to a group of healthy horses without any suspect radiologic signs of osteochondrosis.

2. Materials & Methods

2.1. Population

Thirty-nine young Belgian Warm blood horses, with a mean age of 268 (± 63.55) days, were selected for this study which followed a “case control study” model. Selection was made between 125 examined horses in 2014 on the base of the uncountable results of the radiographies. The foals were presented at the European Center of the horse (Vielsalm, Belgium) by the breeders in the context of the orthopedic diseases screening program promoted by the Wallonia Region. All the foals were weaned and fed a diet based on hay or haylage and concentrates. The OC affected group was composed of 6 females and 14 males. The control group was composed of 9 females and 10 males. Although veterinary examinations were performed routinely, the ethics committee of the University of Liege has agreed to the study protocol.

2.2. Radiographic Exam

All horses were sedated for the radiographic examination using detomidine (0.01 mg/kg IV) alone or combined with butorphanol (0.02 mg/kg IV). The following views were taken: lateromedial views of the 4 fetlocks, lateromedial and plantarolateral-dorsomedial oblique views of the hocks and a lateromedial view of the stifles. Extra views were taken when necessary. Horses were diagnosed on the basis of characteristic lesions of abnormal endochondral ossification located in the metacarpo and metarso-phalangeal, tibiotarsal, and femoro-patellar joints [15]. The foals presenting one lesion in the fetlock joint such as fragmentation and irregularity observed

on the dorsal aspect of the sagittal ridge and the condyles of the metacarpus or metatarsus or dorsal, palmar or plantar fragmentations at the margin of the proximal phalanx were not included in this study. Concerning the stifle joint, variably sized irregularity or flattening of the lateral trochlear ridge of the femur with sometimes partial calcification of the tissue within the defect were considered as OC-lesions (**Figure 2(a)**). Concerning the hock, OC-lesions were identified as fragments still in place (intermediate ridge) (**Figure 2(b)**) or surface irregularities of the trochlear ridge(s) or malleolus. Horses with osteoarthritis and other radiological abnormalities were also excluded of this study.

The OC-affected group included 20 horses and 19 horses without any evidence of OC lesions were analyzed as control groups. At the moment of the radiographic exams, blood samples were collected from each horse on EDTA or dry tubes. Plasma and serum were frozen at -20°C for later analysis.

2.3. Sclerostin Assay

Serum sclerostin concentrations were determined by a sandwich ELISA kit designed and validated specifically

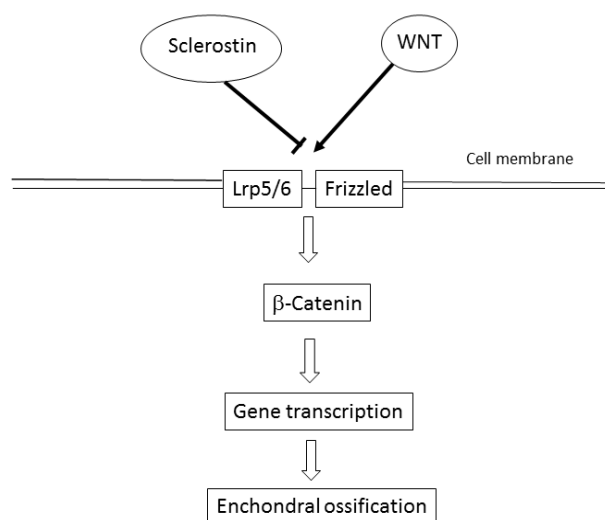


Figure 1. Modulation of the Wnt B-catenin pathway by sclerostin (modified from M. Lewiecki, 2011, discovery medicine) The binding of Wnt proteins to the LRP5/6-Frizzled co-receptor on the cell membrane leads to stabilization of intracellular β -catenin and regulation of gene transcription that promotes ossification. Sclerostin by binding to LRP5/6 antagonizes Wnt signaling and inhibits enchondral ossification.

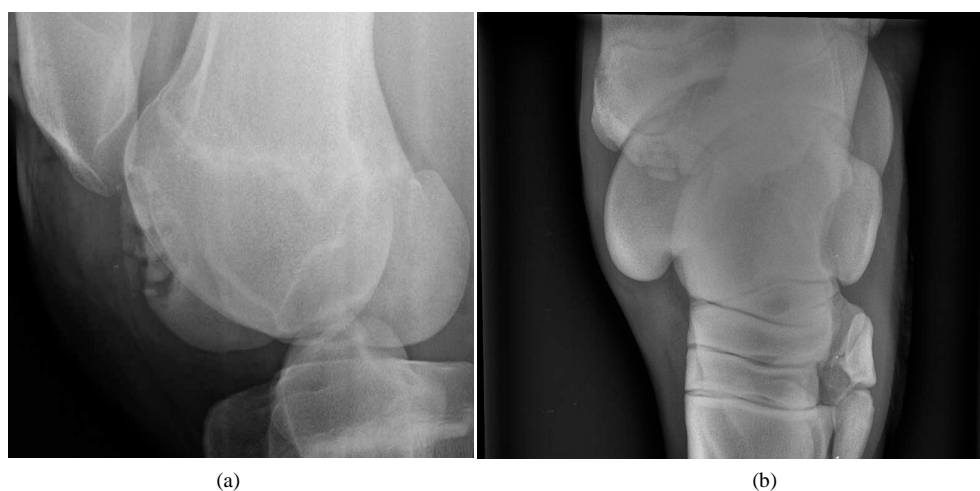


Figure 2. OC lesion in the lateral ridge of the femoral trochlea (a) and OC lesion of the intermediate ridge of the tibial trochlea as examples of the lesions found during the radiographic exams.

for equine samples (Horse Sclerostin (SOST) ELISA Kit, MyBioSource, BiopTis). Sclerostin standards ranging from 31.2 to 1000 pg/ml and undiluted serum were added (50 μ l) into the wells of a microplate then 100 μ l of HRP-conjugate reagent was added to each well. The microplate was incubated for 60 minutes at 37°C. After incubation, the microplate was washed 4 times with the wash solution of the kit and 100 μ l of the TMB-peroxidase substrate (mix of chromogen solutions A and B) was added to reveal the peroxidase activity of the HRP conjugate. The plate was incubated for 15 minutes at 37°C then 50 μ l of the acidic stop solution was added. The absorbance of the yellow color was read at 450 nm with the Multiscan Ascent plate reader. Each sample was assayed twice and the mean value was calculated. According to the firm, the sensitivity of the kit is 5.0 pg/ml.

2.4. Statistical Analysis

Equality of variances was assessed by Levene's test. Linear regressions were calculated between the values of sclerostin and age in all horses and then in each group. A global linear model was used to compare the mean values of plasma concentrations of sclerostin (variable) between the OC-affected group and the control group (factor A) taking into account the sex (factor B) and the age (covariate). Significance was set at $P \leq 0.05$. Medcalc software, Ostend, Belgium.

3. Results

The Levene's test was not significant assuming the equality of variances. A significant linear relation existed between the plasma sclerostin values and the age ($R^2 = 0.12$; $P = 0.03$). **Figure 3** illustrates the results of the linear regression calculation for the two groups: the OC-affected horses and the control group. A significant linear relationship was observed only in the control group ($R^2 = 0.28$; $P = 0.01$).

The global linear model showed a significant effect of the OC-status ($P = 0.035$) and a non-significant effect of the sex ($P = 0.75$). Mean values of sclerostin (\pm SE) in the OC-affected horses were 71.6 ± 3.3 pmole/L and 61.5 ± 3.2 pmole/l in the control horses. **Figure 4** illustrates the box and whisker plot.

4. Discussion

To our knowledge, this is the first time that the circulating levels of sclerostin are measured in horses. A recent study shows that sclerostin in humans varies according to sex and age [16] and that sclerostin levels were higher in men than in women independently of their age. This was explained by the higher bone mass that men usually have. Sclerostin is mainly expressed by osteocytes, so individuals with higher bone mass, consequently, have a

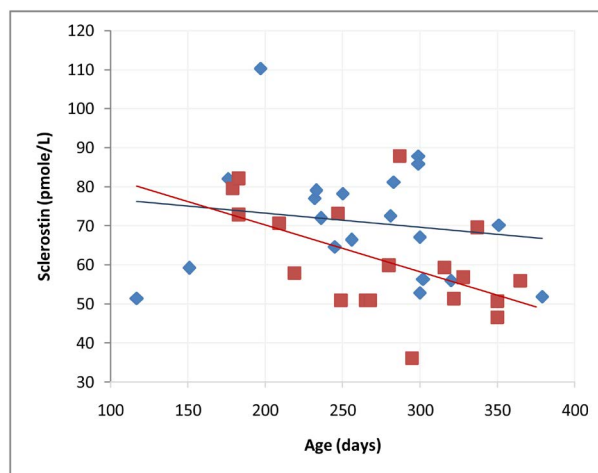


Figure 3. Linear relationship between of sclerostin levels in the OC (blue) and in the control group (red) related to the age. Both groups together show a significant ($P = 0.03$) decrease in the sclerostin concentration with age. When considered separately, the linear relation is only significant in the healthy group ($P = 0.01$).

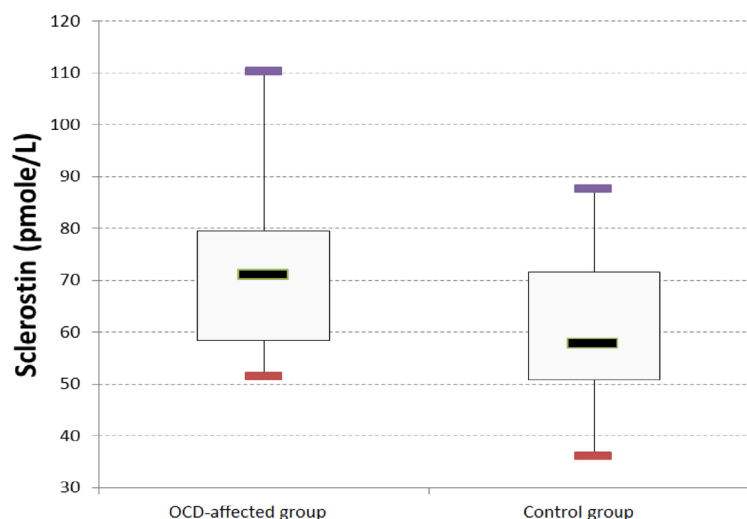


Figure 4. Box and Wisker plot comparing the OC and the control group. The OC affected group has a significant ($P = 0.035$) higher concentration of sclerostin than the control group.

higher number of osteocytes. Sclerostin levels in humans are rising with age, but studies concerned people aged from 20 to approximately 60 years. In our study we found that sclerostin levels are decreasing with the age but our study included only young horses during their growth. The adult human normal values of sclerostin in serum were between 10 and 40 pmole/L [16]. Until now, there were no reference values in horses. Our control group (not affected by osteochondrosis) had a mean value of 61.5 pmole/L. The affected group had a mean value of 71.6 pmole/L in serum. For this reason, we have chosen to perform a case control study and two groups of young horses of the same age were selected. The horses of the control group did not show any signs of OC in the fetlocks, hocks and stifles. For the OC-affected horses we have chosen only cases with radiological lesions associated that were undoubtedly osteochondrosis in the hocks or the stifles. Indeed, some lesions in the fetlocks are not clearly associated with osteochondrosis such as the first phalanx dorsal or plantar fragments [17]. Furthermore, all the horses were weaned and examined during the winter period in order to minimize the environmental effects. A study performed in 2014 shows that sclerostin levels are influenced by season in human [18]. In winter season, sclerostin concentration is 20% higher than the all-year mean.

Several limitations of our study came from the fact that we did not know the exact composition of the diet, the housing conditions, the body condition scores and intensity of daily exercise. All these factors may influence the expression of the disease [19]-[21].

Based on a digital gene expression analysis, our group analyzed the transcript profile of leukocytes from horses affected with OC using the high throughput sequencing method digital gene expression analysis [6]. Metabolic pathway analysis showed an obvious dysregulation of several signaling pathways related to cartilage formation and cartilage repair such as Wnt/ β -catenin, Indian hedgehog- and TGF- β signaling pathways. Other genes regulated may play a role in high carbohydrate diet, abnormal insulin metabolism or inflammation [6]. Furthermore, the transcript profile of leukocytes demonstrated that OC-related genes are differentially expressed in horses of different ages when compared to their age-matched controls and are differently expressed according to the affected joints in young foals (<12 months) [22] [23]. Recently a miRNAs profile study in cartilage samples showed that miRNAs might be involved in equine OC physiopathology and in the cellular response to the biomechanical stress in cartilage and bone [24].

Wnt/ β -catenin signaling pathway is a major regulator of the bone activity [25]. The activation of this pathway leads to the formation and differentiation of osteoblastic cells, which create and increase the bone mass. This pathway starts when the appropriate Wnt peptide binds to the receptors LRP4, 5 and 6 (lipoprotein receptor related-protein) [26]. Sclerostin is an antagonist peptide of Wnt/ β -catenin pathway that binds to these receptors acting as a negative regulator of the bone formation.

Concerning the possible implication of Wnt/ β -catenin pathway in the osteochondrosis, Power and others [7] demonstrated that sclerostin was increased in naturally occurring lesions of growth cartilage. They concluded

that given that sclerostin protein is increased in lesions of OC, and that OC is associated with retained cartilage, it is possible that sclerostin may be having an effect on the cartilage via protease action, as proposed in OA. In that study samples were taken from stifle cartilage. In our study, the sclerostin concentrations were measured in peripheral blood.

Besides the effects on bone metabolism and the growing awareness of the role of sclerostin in human osteoporosis, the Wnt/ β -catenin pathway and sclerostin are also involved in certain inflammatory and metabolic diseases such as obesity and diabetes [27] [28]. For example, humans suffering from type 2 diabetes have higher sclerostin levels than the controls [27].

Several omics studies underlined also the role of an energetic metabolism dysfunction in the osteochondrosis development. Desjardin and others [29] observed abnormal mitochondria and endoplasmic reticulum in the deep zone of OC cartilage. The mitochondrial dysfunction could be partially explained by the altered Wnt/ β -catenin signaling pathway also known as a key regulator of mitochondrial function [6] [7] [30].

Sclerostin is expressed by the SOST gene and a modulation of sclerostin levels appears to be a finely tuned mechanism by which osteoblast, chondrocytes and progenitor cells coordinate regional and local osteogenesis [31]. Methylation of bone SOST, its mRNA, and serum sclerostin levels correlate strongly with fracture risk in postmenopausal women [32]. Delgado-Calle and others [33] showed that the SOST is subject to an epigenetic regulation. In that study osteoblasts were hypermethylated and osteocytes were hypomethylated in a CpG-rich region in the SOST proximal promoter.

5. Conclusion

To conclude, based on the recent studies in equine and human medicine, we can support the concept that genetic and epigenetic changes in SOST gene influence its bone mRNA expression and serum sclerostin levels in osteochondrosis. These results reinforce the possible role of the Wnt/ β -catenin signaling pathway in the OC pathogenesis. However, as in human medicine, more studies are needed to define an eventual clinic interest of plasma sclerostin as future biomarker in bone and cartilage diseases.

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