

Therapeutic and hematological effects of native and low molecular weight chondroitin sulphate administered orally in horses with experimental arthritis (Efectos terapéuticos y hemáticos de condroitin sulfato nativo y de bajo peso molecular administrado por vía oral en caballos con artritis experimental)

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SUMMARY

The objective of the present study was to analyse the therapeutic and secondary effects of 2 types of chondroitin sulphate (CS) administered orally over three months in horses with arthritis. Ten horses with an arthroscopically created osteochondral defect of the carpal joint were divided into 3 groups. Group A consisted of 4 animals receiving a daily 2.5 g oral dose of low molecular weight chondroitin sulphate (LMWCS). Group B comprised 4 animals receiving a daily 2.5 g oral dose of native chondroitin sulphate (NCS). Group C (control) comprised 2 animals receiving an oral placebo solution. Therapeutic and secondary effects were evaluated weekly, by changes in clinical parameters and through measurement of synovial mediators/substances related to inflammation. Significant beneficial changes were obtained on clinical signs of inflammation. There were also significant reductions in synovial fluid PGE₂ and MMP-3 concentrations in the CS treated groups. These compounds also significantly affected temporal profile of total GAGs concentration. LMWCS and NCS, administered orally for 10 weeks to arthritic horses, alleviated clinical signs by reducing the synthesis and/or release of PGE₂ and MMP-3. Its anti-inflammatory and chondroprotective

effect is further indicated by an inhibition of GAGs degradation without any significant effect on blood parameters including those reflecting the normal functioning of the coagulation cascade.

Key Words: Chondroitin sulphate; arthritis; equine; oral; nutraceuticals

RESUMEN

El objetivo del presente estudio es analizar los efectos terapéuticos y secundarios de 2 tipos de sulfato de condroitin (CS) administrados vía oral durante tres meses en caballos con artritis. Diez caballos con un defecto osteochondral creado del empalme carpal fueron divididos en 3 grupos. El grupo A lo conforman 4 animales que reciben una dosis oral diaria de 2,5 g del sulfato bajo del condroitin del peso molecular (LMWCS). El grupo B abarcó 4 animales que recibieron una dosis oral diaria de 2,5 g del sulfato nativo del condroitin (NCS). El grupo C (control) abarcó 2 animales que recibieron una solución oral del placebo. Los efectos terapéuticos y secundarios fueron evaluados semanalmente, por los cambios en los parámetros clínicos y con la medición del indicadores relacionado con la inflamación en el líquido sinovial. Los cambios positivos significativos fueron obtenidos en muestras clínicas de la inflamación. Había también

reducciones significativas en el líquido sinovial PGE2 y las concentraciones Mmp-3 en el CS trataron a grupos. Estos compuestos también afectaron

perceptiblemente el perfil temporal de la concentración total de la mordaza. LMWCS y NCS, administrado oral por 10 semanas a los caballos artríticos, muestras clínicas aliviadas reduciendo la

síntesis y/o el lanzamiento de PGE2 y de Mmp-3. El efecto antiinflamatorio y

condroprotector está indicado más a fondo por una inhibición de la degradación de la mordaza sin ningún efecto significativo sobre parámetros de la sangre incluyendo éstos que reflejan el funcionamiento normal de la coagulación.

INTRODUCTION

The term chondroprotective or disease modifying agent is used to describe substances which increase the synthetic function of chondrocytes and synoviocytes; it has been proposed that they act by decreasing the action of degradative enzymes (metalloproteinases) in the joint and preventing the formation of periarticular fibrin thrombi (1).

Chondroitin-4-sulphate is the most abundant GAG in growing mammalian hyaline cartilage. Chondroitin chains are secreted into the extracellular matrix covalently bound to proteins as proteoglycans, including aggrecan. Proteoglycans, as a consequence of their ability to increase osmotic pressure, induce the movement of water into cartilage, leading to swelling and expansion of the matrix. The characteristic load bearing properties of cartilage are attributable to the compressive resilience and affinity for water of these compounds. As animals age, chondrocytes secrete decreased amounts of chondroitin-4-sulphate and increased amounts of other GAGs, such as keratan sulphate. This change in the type of GAG in the cartilage matrix has been implicated in the initiation and progression of degenerative processes within cartilage.

CS subserves not only an important structural role, but also, as a chondroprotective agent, it exerts metabolic effects, involving competitive inhibition of the actions of many degradative enzymes that break down cartilage matrix and alter synovial fluid composition in OA (2).

In vitro studies on CS actions have revealed three principal effects; a) inhibition of the action of metalloproteinases, thereby decreasing the degradation of collagen and proteoglycans (3); b) stimulation of proteoglycan production by healthy cells (4) and; c) Prevention of fibrin thrombi formation in synovial or subchondral microvasculature (5). For all oral chondroprotective agents, and specifically for CS, there is considerable current scientific debate concerning both bioavailability and bioactivity following absorption (6 - 7). *In vivo* studies are therefore required to elucidate further the roles and mechanisms of action of such agents.

The objective of the present study was to investigate potential therapeutic and secondary effects of 2 types of CS administered orally during a three-month period in arthritic horses.

MATERIALS AND METHODS

Animals were ten standard-breed mares weighing 450 ± 15 kg. Inclusion criteria were : over 2 years of age; acceptable nutrition status; non-gestating mares; no previous joint surgery procedures; no current or previous systemic immune disease; not receiving any type of chronic medication; no history of joint problems.

Chronic Arthritis Experimental Model

Animals were anaesthetised with halothane after induction with xylazine- guaiacolate glyceryl ether-thiopental. Using arthroscopy, an osteochondral defect was created on the proximal surface of the carpal radial bone on the dorsolateral margin. Defects were semi-circular with a diameter of 0.75 cm, and extended through the whole depth of the cartilage to the subchondral bone plate (8).

Dosing schedule

Six days after surgery, animals were randomly divided into 3 groups:

- Group A consisted of 4 animals receiving a daily 2.5 g oral dose of LMWCS, MW 9600 Kda, 6% sulphating grade, in a 25 % solution (manufactured by Syntex S.A., Argentina, Lot 010602).
- Group B comprised 4 animals receiving a daily 2.5 g oral dose of NCS, MW 25000 Kda, 6% sulphating grade, in a 25 % solution (manufactured by Syntex S.A., Argentina, Lot 010710).
- Group C (placebo group) consisted of 2 animals receiving an oral placebo solution (formulation lacking of active principle) with the same dose scheme used in the treated groups.

Blood and synovial fluid sampling

Blood sample collection

Blood samples were obtained weekly from the left jugular vein before and up to 82 days after drug or placebo administration. These samples were divided into two aliquots: the first was collected in a test tube containing EDTA (4ml) for a complete haematology screen. The second 4 ml sample was collected in tubes with no anticoagulant, to evaluate clotting.

Blood fractions were kept on ice for subsequent centrifugation at 2200xg for 10 min at 4° C.

Synovial fluid sample collection

A volume of 2 ml of synovial fluid was withdrawn weekly by syringe and needle before and up to 82 days after administration of drugs or placebo.

Samples were fractionated as follows: 0.25 ml was placed in test tubes containing EDTA for cytology tests. The remainder was placed in test tubes and centrifuged (12000xg for 5min) to obtain cell-free synovial fluid. This aliquot was sub-fractionated: 0.25 ml for total protein; 0.25 ml for PGE₂ assay, 0.5 ml to measure total glycosaminoglycans content. The remaining sample was used to measure MMP-3 (stromelysin) activity. All fractions were stored frozen at -20°C until all analysis was performed.

Evaluation of anti-inflammatory and haematological effects

Before administration and up to 82 days after initiation of the study, weekly control measurements were made (blindly by the same observer), to provide baseline clinical signs and evaluation procedures.

The following clinical parameters were evaluated

Stride length. Stride length was determined by walking the horse on a hard surface and measuring the distance between footprints of the affected limb. A total of ten strides was measured and the first and last eliminated, the average of the eight remaining strides being taken.

Circumference of inflamed joint. Joint circumference was measured at the upper joint margin, using a flexible tape, with the limb extended (normal upright position).

Rest angle and maximum flexion angle. These measurements were made with a goniometer. First, the standing flexion angle of the inflamed limb was measured. Then, the limb was gradually flexed until a pain reaction was obtained. This manoeuvre enabled graded calculation of the flexion parameters.

Haematological measurements

PVC was determined by microhematocrit centrifugation. Leukocyte and erythrocyte counts, haemoglobin concentration, mean corpuscular volume and mean haemoglobin concentration were determined by use of an automated analyser.

Coagulation procedures

The prothrombin time (PT) and partial prothrombin time (PTT) were determined using a fibrometer and standard clinical-pathologic laboratory techniques.

Bleeding time measurement was performed as described by Jergens et al. (9) at the times of blood sampling.

Synovial fluid procedures

Total protein concentration was measured by the Lowry method, as modified by Schacterle and Pollack (10). Concentrations of PGE₂ in synovial fluid were evaluated by enzyme immunoassay using a commercial kit (Biotrak RPN 222, Amersham Biosciences, Argentina).

Total glycosaminoglycan concentration was evaluated by Farndale's method, modified by Alwan (11 - 12).

MMP-3 concentration was measured by ELISA, using a commercial kit (Biotrack 2613, Amersham Biosciences, Argentina).

Radiological evaluation

X-rays were taken on the day prior to treatment and thereafter once monthly up to the last day of treatment.

A portable Kents 2080 machine was used for taking X-rays, with an Orthoplast enhance screen and conventional film.

The following views were used:

Dorso-palmar (DP) ; Latero-medial (LM); Dorso-lateral medial-oblique (DLMO) and Dorso-medial lateral-oblique (DMLO)

Radiographs obtained with each view were assessed on the following scale:

GRADE	DESCRIPTION
0	No signs of lesion
1	Very small osteophytes, with no clear margins
2	Well-defined osteophytes, without chondral or subchondral bone involvement.
3	More than 2 defined osteophytes, mild sclerosis of subchondral bone.
4	Defined and numerous osteophytes, with obvious sclerosis of subchondral bone.

Statistical Analysis

Clinical parameters were standardised before the inferitive analysis, according to individual baseline values of each experimental animal. Standardisation involved correcting modifications from baseline values as percentage change (100% corresponding to baseline value).

Because of the different number of animals in each group, in the descriptive analysis, the arithmetic mean and the mean standard error were calculated.

For the inferitive analysis, since the objective was to analyse modifications induced by treatments over a relatively long period of time, a scheme for repeated samples (13) was applied, based on comparison of the areas under the curve percentage change from baseline vs. time. The area under the curve was calculated by adding the areas under the graph between each pair of consecutive observations. If we have measurements y_1 and y_2 at times t_1 and t_2 , then the area under the curve between those two times is the product of the time difference and the average of the two measurements $(t_2 - t_1) (y_1 + y_2)/2$. This is known as the trapezium rule because of the shape of each segment of the area under the curve. If we have $n+1$ measurements y_i at times t_i ($i=0, \dots, n$) then the area under the curve (AUC) is calculated as :

$$AUC = 0.5 \sum_{i=0}^{n-1} (t_{i+1} - t_i) (y_i + y_{i+1})$$

Where t is time of sample taking and y is the observed measurement.

RESULTS

CLINICAL PARAMETERS

Stride Length

Measurement of stride length was carried out weekly for the 10 weeks of the study. All the animals, regardless of the treatment, improved their stride length during the study. Analysis of standardised results, that is, the percentage of change from baseline values (Fig.1) showed an increase in the stride length in groups treated with CS, in particular the group that received LMWCS. Conversely, the control group showed a relative shortening of stride length, especially during weeks 1, 2 and 3, but remaining high in subsequent weeks.

The area under the curve (AUC) percentage of change as a function of time (Fig.1, Inner Box) showed numerical differences among the treated groups; this parameter was highest in the group treated with LMWCS, and lowest in the control group. Even though these differences did not reach statistical significance (possibly due to variability among experimental subjects) it is possible to infer that treatment with CS decreases the observed shortening of stride length in the animals receiving placebo.

Circumference of the inflamed joint

In all three experimental groups, carpal diameter showed an increase in the first weeks after surgery. This increase was both smaller and less persistent in the group treated

with LMWCS, reaching 4% during the first week of treatment (15 days post surgery), and was greater (7%) in the group receiving NCS.

The control group showed a much greater increase in circumference than the other two, in the range of 8-13%, that persisted up to week 4 (30 days post-surgery) (Fig. 2). Thereafter, the NCS group had circumference values similar to baseline. The increase in the control group persisted for the whole study, above values obtained in both groups treated with CS.

The AUC percentage change in carpal diameter from baseline as a function of time (Fig. 2 Inner Box) was 7751.28 ± 51.43 %/week for the control group, while values were lower in the groups treated with LMWCS and NCS (7529.03 and 7448.26 %/week, respectively). Statistical analysis of AUCs did not show significant differences between the groups. However, as was the case with stride length, a numerical difference was observed between the groups treated with CS on the one hand and the control group on the other.

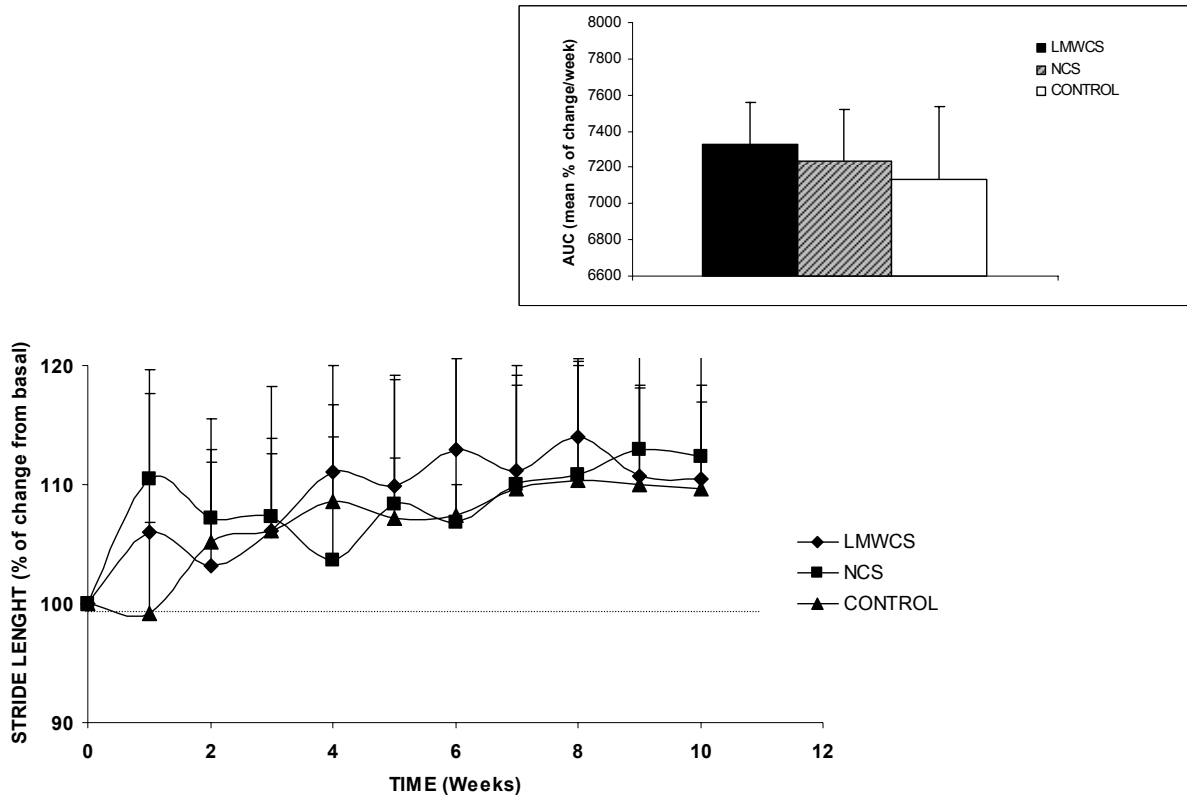


Figure 1: Plot against time of mean \pm SEM percentage change from basal stride length in arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. *Inner Box*: Mean \pm SEM of the area under the curve (AUC) percentage change vs. time for stride length on the three experimental groups.

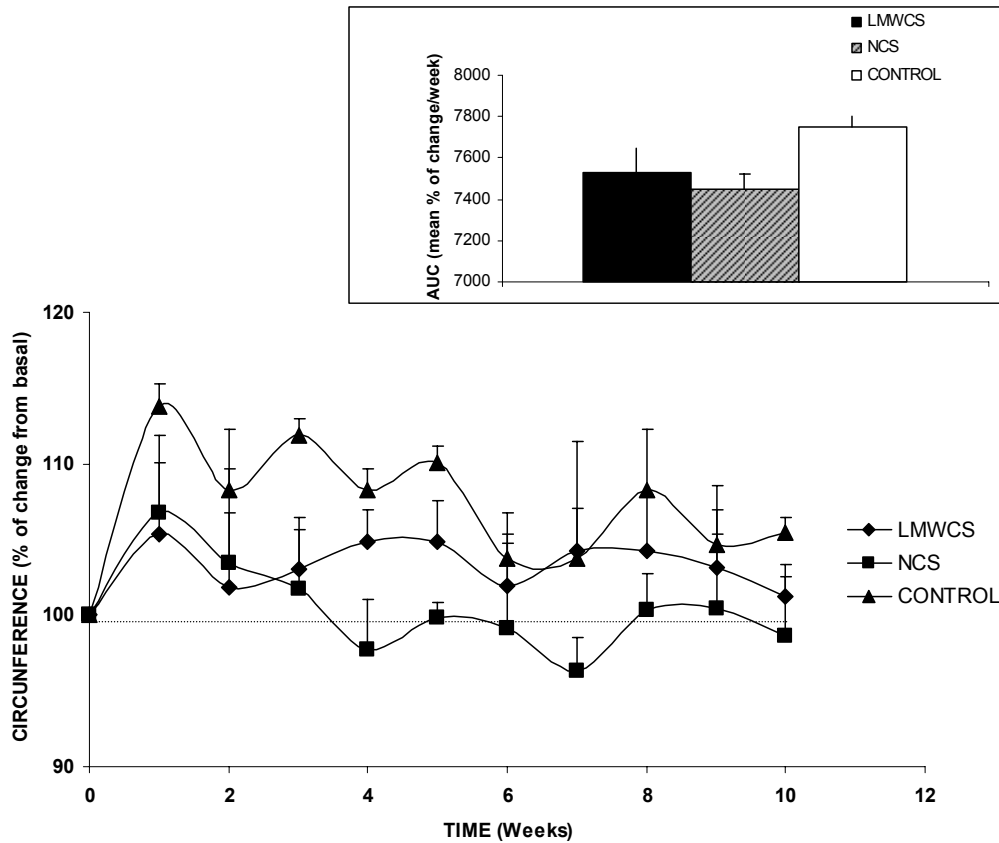


Figure 2: Plot against time of mean \pm SEM percentage change from basal circumference of the inflamed joint in arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. *Inner Box*: Mean \pm SEM of the area under the curve (AUC) percentage change vs. time for circumference of the inflamed joint on the three experimental groups.

Rest Angle

Differences for this parameter among the three experimental groups were minimal. In all experimental groups, a decrease in the rest angle was observed during the first 2 weeks of the study, returning in all cases to baseline values on week 4 (Fig. 3). Statistical analysis of differences among groups in the AUC percentage change from baseline vs. time (Fig. 3 Inner Box), did not show significant differences between treatments.

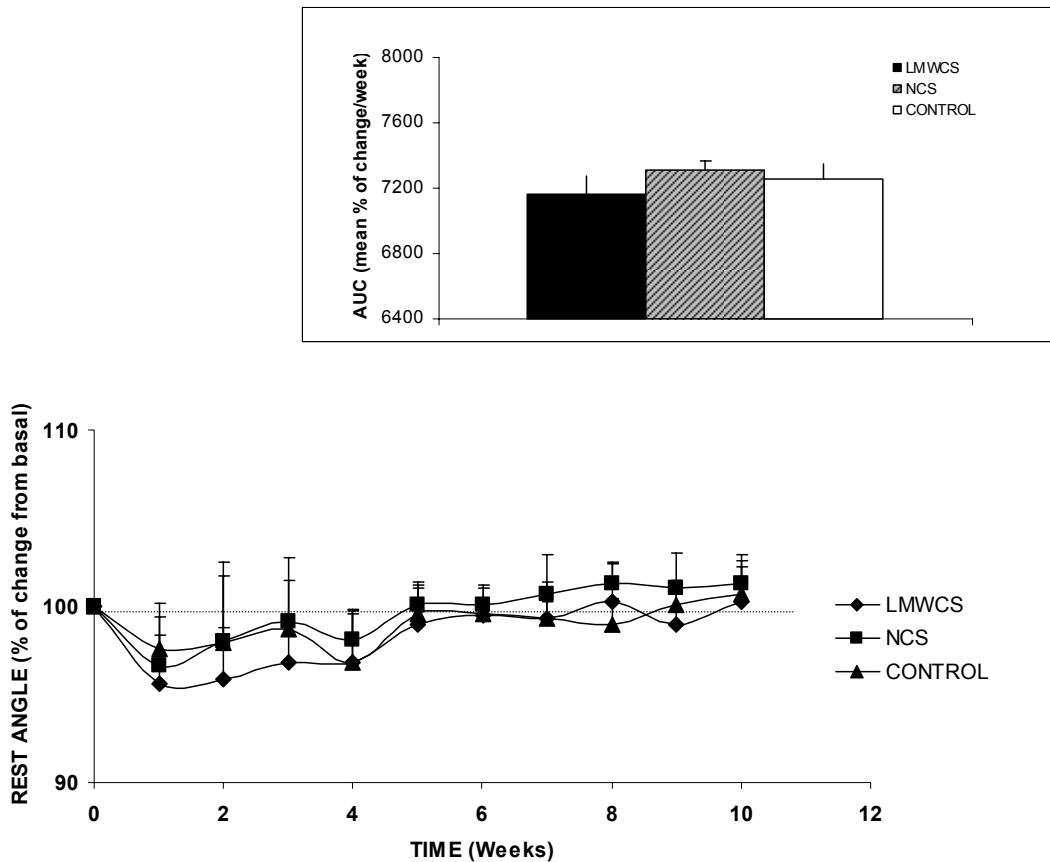


Figure 3: Plot against time of mean \pm SEM percentage change from basal rest angle in arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. *Inner Box*: Mean \pm SEM of the area under the curve (AUC) percentage change vs. time for rest angle on the three experimental groups.

Maximum Flexion Angle

Maximum flexion angle provides an indication of the presence of pain in the joint. In this study, subjects in the three experimental groups showed a clear increase of this parameter in the first weeks after surgery. This increase was more significant in the group treated with LMWCS, compared to the group receiving NCS (Fig. 4). The control group showed a greater and more sustained increase than the groups treated with CS. This is clearly seen when comparing the values of AUC percentage change from baseline as a function of time. The control group mean was 13018 ± 83.02 %/week, while for the groups treated with LMWCS and NCS it was 7448.26 ± 72.07 and 7529.03 ± 114.96 %/week, respectively.

Statistical analyses of AUCs indicated significant differences ($p < 0.05$) between the groups treated with CS and the control group. No statistically significant differences were observed between the LMWCS and NCS groups. These results demonstrate a significant

degree of analgesia in animals receiving CS.

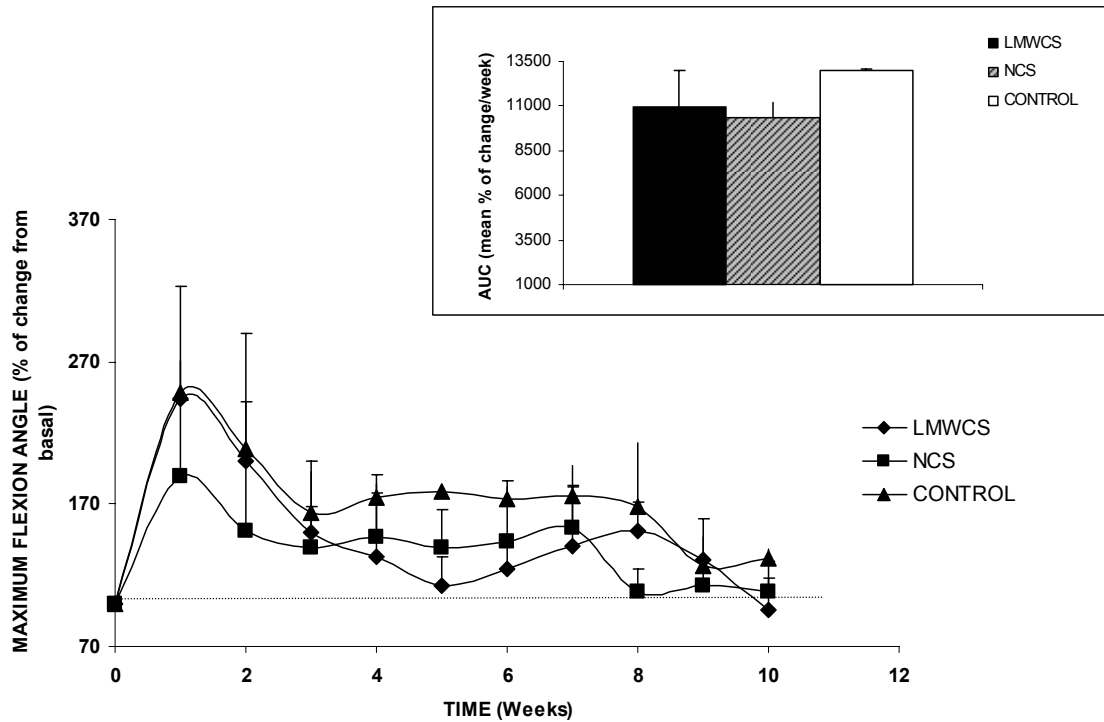


Figure 4: Plot against time of mean \pm SEM percentage change from basal maximum flexion angle in arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. *Inner Box*: Mean \pm SEM of the area under the curve (AUC) percentage change vs. time for maximum flexion angle on the three experimental groups. [(a) LMWCS vs Control $p < 0.05$; (b) NCS vs Control $p < 0.05$]

SYNOVIAL BIOCHEMICAL PARAMETERS

Glycosaminoglycans

The distribution of GAGs concentration in synovial fluid show temporal differences between the three experimental groups (Fig 5). The control group showed a low level of GAGs during the first 5 weeks of the study, with a subsequent gradual increase that reaches a peak approaching 400 $\mu\text{g/ml}$ on week 8. Thereafter, GAGs concentrations decreased to baseline levels on week 10.

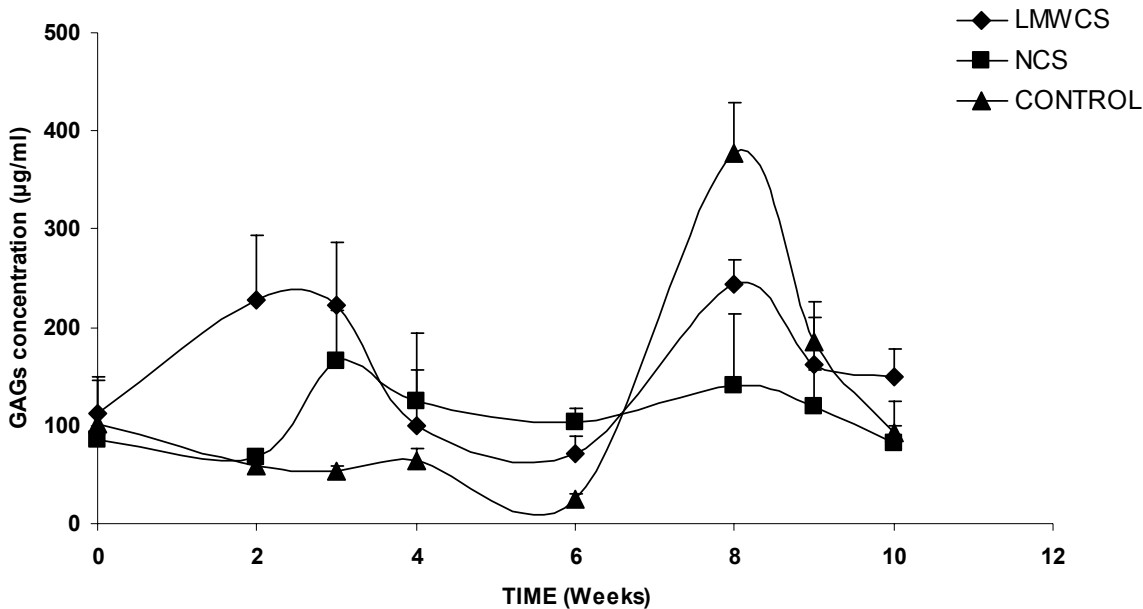


Figure 5: Plot against time of mean \pm SEM glycosaminoglycan concentration in synovial fluid from arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g.

In groups treated with CS, regardless of type, synovial GAGs concentrations were higher than those obtained in the control group during the first 6 weeks of treatment. The group treated with LMWCS had the highest GAGs concentration on week 1, increasing to a peak of 228 $\mu\text{g/ml}$ on week 2, and then decreasing to week 6. After week 6, GAGs concentrations increased to produce a second peak on week 8, coincident with although less pronounced than in the control group.

In the group treated with NCS, GAGs concentration was increased during the first weeks of administration; however, the rate and extent of increase were smaller than in the group treated with LMWCS, with a peak of 160 $\mu\text{g/ml}$ on week 3. Subsequently, concentrations decreased following the same pattern as the LMWCS group, with a trough concentration on week 6; however, this trough was 50% greater than that in the LMWCS group. After 6 weeks, GAGs concentrations began to increase again, with a peak at week 8.

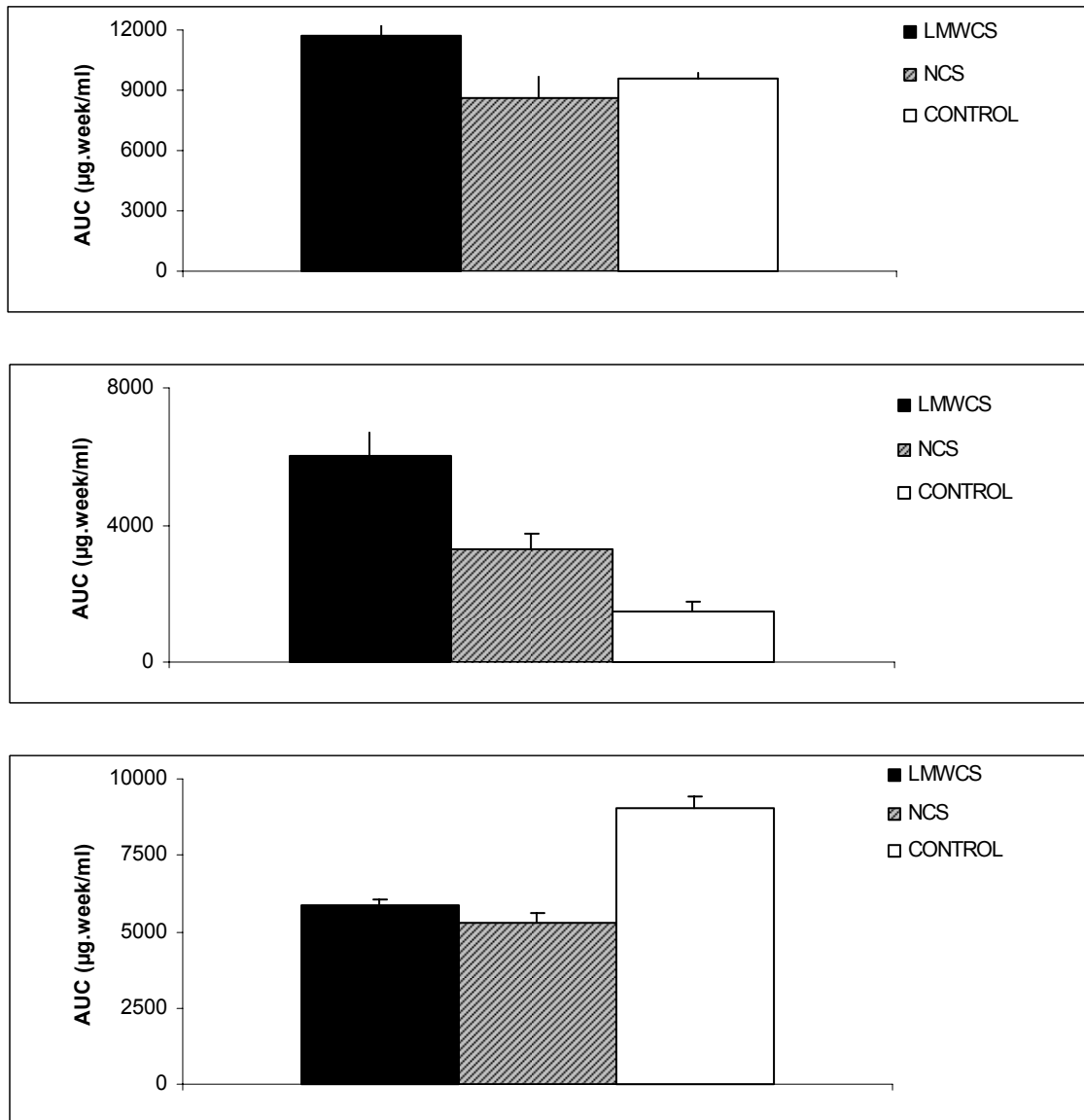


Figure 6: (a) Total [0-10week] (b) partial [0-6 week] and (c) partial [6-10 week] areas under the curve GAGs concentration vs. time in synovial fluid from arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. [(a) LMWCS vs Control $p < 0.05$; (b) NCS vs Control $p < 0.05$; (c) NCS vs LMWCS $p < 0.05$]

Statistical analysis, using AUC GAGs concentration as a function of time, up to week 10, showed significant differences between the LMWCS group and the control and NCS groups, while no difference was seen between the latter two groups (Fig. 6a).

However, these results may be misleading, since they do not consider separately the two GAGs peaks. To clarify temporal differences in GAGs concentrations amongst the three groups, partial AUCs from time 0 (beginning of administration) to week 6, and also the

AUC from week 6 to week 10 were calculated. The 0-6 AUC in the group treated with LMWCS (Fig. 6b) was almost double the AUC calculated for the NCS group (6001.84 and 3272.64 $\mu\text{g}\cdot\text{week}/\text{ml}$, respectively) and almost 5 times greater than that observed in the control group (1469.30 $\mu\text{g}\cdot\text{week}/\text{ml}$). The 6-10 AUC (Fig. 6c) in LMWCS and NCS groups were similar ($\approx 5500 \mu\text{g}\cdot\text{week}/\text{ml}$), and significantly lower than those observed in the control group (9037.54 $\mu\text{g}\cdot\text{week}/\text{ml}$).

Analysis of these partial AUCs showed significant differences ($p < 0.05$) between the control group and the groups treated with CS during the two periods. Significant differences ($p < 0.05$) were also found between the low molecular weight and the native group in the 0 to 6 weeks period.

Proteins

Protein concentration in synovial fluid followed a similar temporal pattern in the three experimental groups. Concentrations increased gradually during the first weeks, with a peak at week 3 (Fig. 7). Although the highest peak was seen in the control group (34.68 mg/ml), concentrations in the groups treated with LMWCS (30.93 mg/ml) and NCS (24.84 mg/ml), were not statistically different. Protein concentrations then decreased in all groups, and attained the baseline level on week 10 after commencement of dosing (week 12 after surgery).

Analysis of AUC protein concentration as a function of time did not reveal statistically significant differences among experimental groups (Fig. 7 Inner Box). This result was anticipated, since the model was one of chronic inflammation. Increases in synovial protein concentration are more common in acute inflammation models.

Prostaglandin (PG) E₂

PGE₂ concentrations in synovial fluid showed different profiles in the groups treated with CS compared to the control group.

In the control group, PGE₂ concentrations were significantly higher than in the groups treated with CS. The apparent decrease in concentration in the placebo group in week 6 is a consequence of missed a sample in one horse together with the low value found in the second animal. Otherwise PGE₂ concentrations remained high until the end of the study, and did not return to baseline values (Fig. 8).

PGE₂ concentrations in groups treated with CS were lower than those in the control group, in particular in the group treated with LMWCS.

Statistical comparison of AUC synovial concentration of PGE₂ as a function of time (Fig. 8 Inner Box) indicated that PGE₂ concentrations in the groups treated with LMWCS ($p < 0.01$) and NCS ($p < 0.05$), were significantly lower than those in the control group (5333.13, 5716.67 and 11014.50 $\text{pg}\cdot\text{week}/\text{ml}$, respectively). These data suggest that CS inhibits the synthesis of PGE₂ at the articular level.

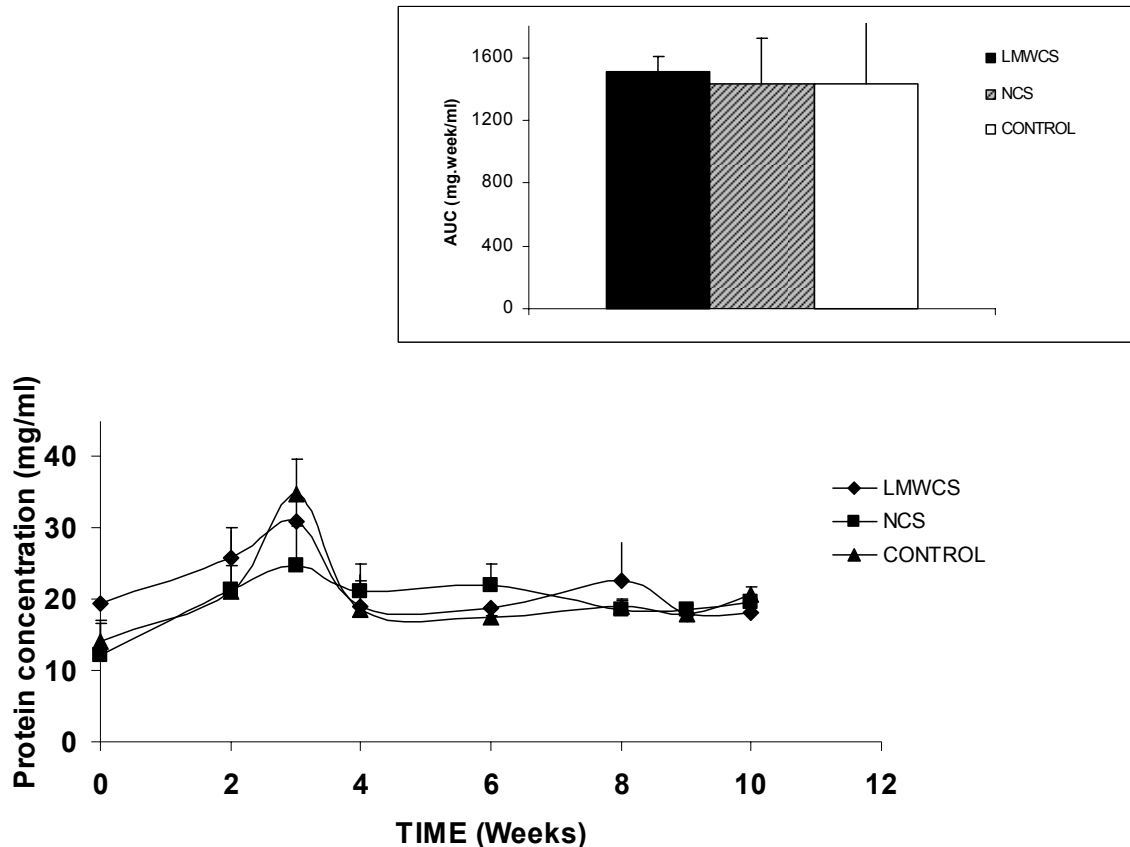


Figure 7: Plot against time of mean \pm SEM protein concentration in synovial fluid from arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. *Inner Box*: Mean \pm SEM of the area under the curve (AUC) synovial fluid protein concentration vs. time on the three experimental groups.

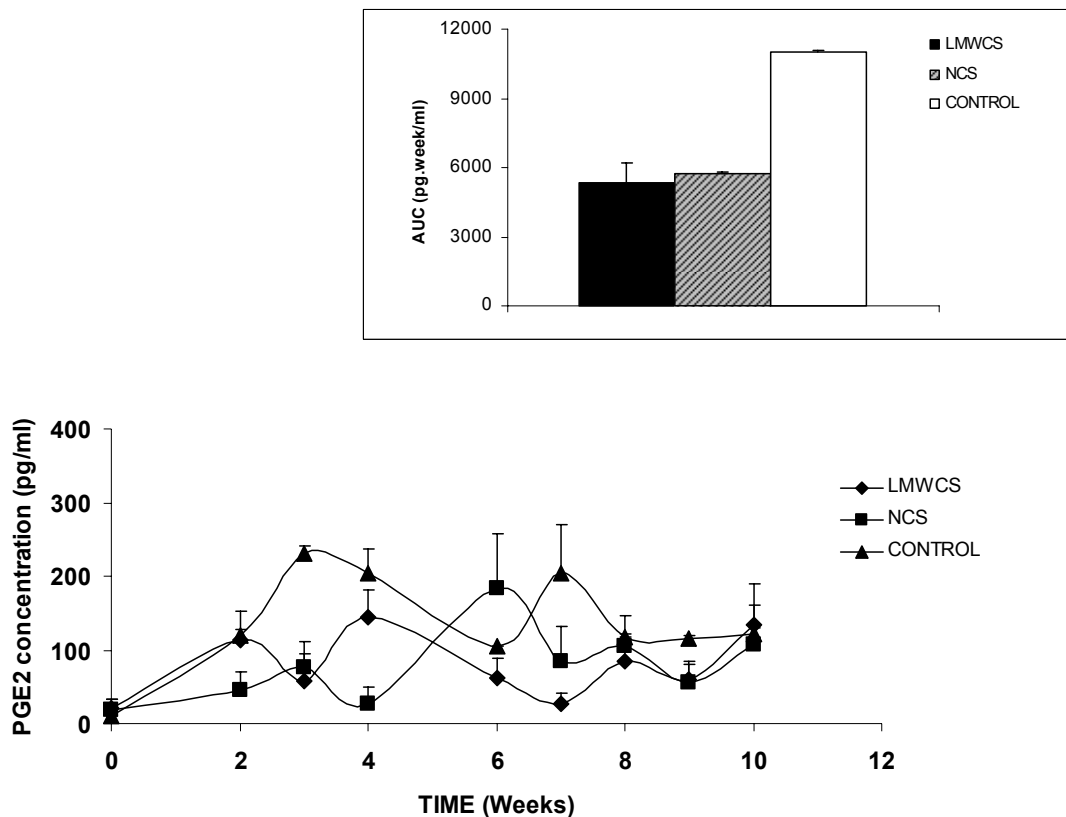


Figure 8: Plot against time of mean ± SEM PGE₂ concentration in synovial fluid from arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. *Inner Box*: Mean ± SEM of the area under the curve (AUC) synovial fluid PGE₂ concentration vs. time on the three experimental groups. [(a) LMWCS vs Control p< 0.01; (b) NCS vs Control p< 0.05]

Matrix Metalloproteinase - 3 (Stromelysin) (MMP-3)

MMP-3 was detected only in synovial fluid from control group animals (Fig. 9). MMP-3 concentrations were detectable only after surgery, that is, after induction of the inflammatory stimulus. MMP-3 concentrations in this group were in the range of 113 - 197 ng/ml during the first 8 weeks, slightly decreasing by week 10. At the time of the last sampling (week 10), MMP-3 was still detectable in the two control group animals, with an average concentration of 46.2 ng/ml.

In horses treated with LMWCS, only low levels of MMP-3 were detected, on isolated occasions and in individual animals, otherwise no trend was observed.

In samples taken from animals treated with NCS, MMP-3 was detected in a single subject and in a single occasion (Week 4).

These findings demonstrate that CS inhibits the synthesis and/or release of MMP-3.

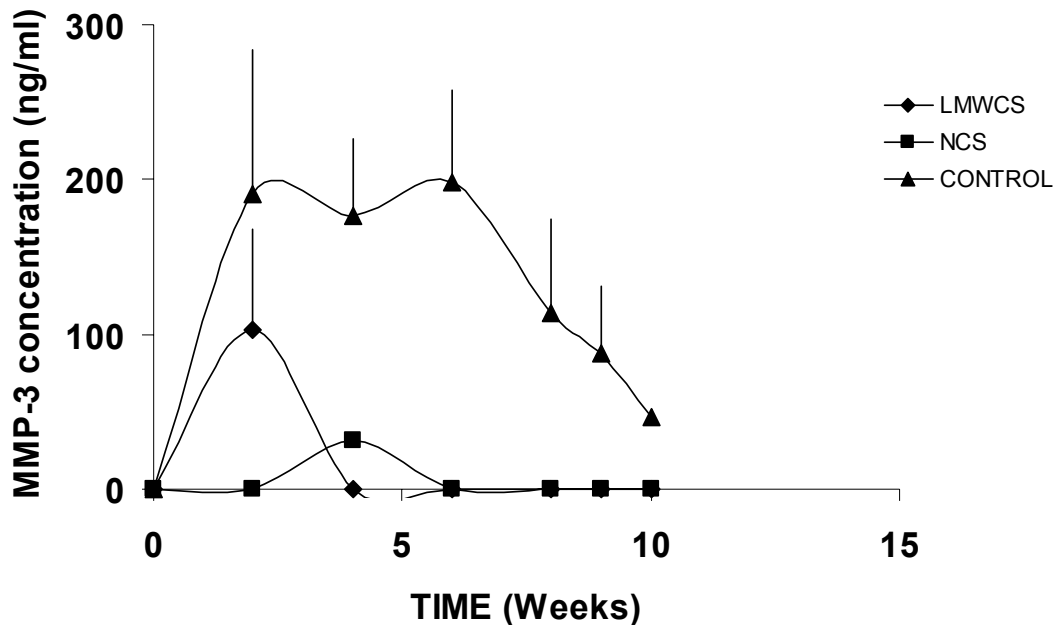


Figure 9: Plot against time of mean \pm SEM MMP-3 concentration in synovial fluid from arthritic horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g.

RADIOLOGICAL DIAGNOSIS

Evaluation of radiological images obtained before and after induction of the cartilage defect, as well as differential changes among the different treatment groups, was made in accordance with the scale presented in the Materials and Methods section.

Initial evaluation consisted of carpal joint assessment before induction of the carpal surgical lesion. This evaluation showed that none of the experimental animals presented cartilage lesions.

Evaluation of radiological changes in each animal was performed following the time sequence, carefully examining the surgical lesion region. Comparison between individual animals was not attempted due to the differences in the surgically induced lesions. Total scores resulting from the radiographic evaluation are presented in Fig. 10. Although all groups treated with CS of either type showed lower scores when compared to the control group, differences were statistically not significant. However, the observations show a less severe radiological evolution of lesions when compared with the control group

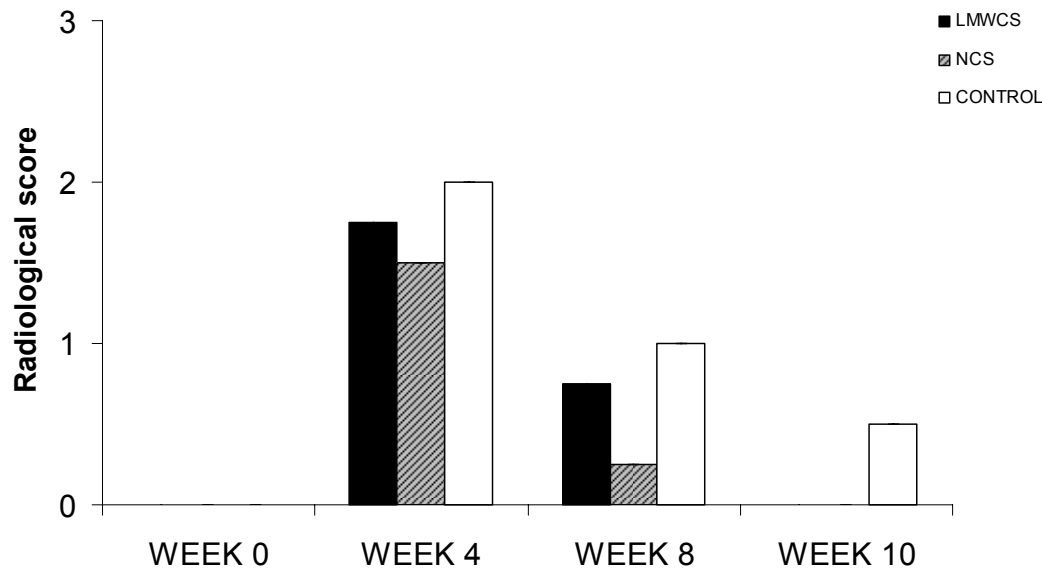


Figure 10: Radiological scores for inflamed joint in horses after 10 week oral administration of LMWCS, NCS and placebo at a daily total dose of 2.5 g. (0 : No signs of lesion; 1:Very small osteophytes, with no clear margins; 2: Well-defined osteophytes, without chondral or subchondral bone involvement.; 3: More than 2 defined osteophytes, mild sclerosis of subchondral bone; 4: Defined and numerous osteophytes, with obvious sclerosis of subchondral bone).

HAEMATOLOGICAL PARAMETERS AND MARKERS OF COAGULATION

None of the blood parameters evaluated showed values outside the normal range for horses. Table I shows average values before and 1, 5 and 10 weeks after beginning of the treatment.

Due to the heparinoid character of CS, particular attention was paid to evaluation of parameters that reflect functioning of blood coagulation.

Coagulation time remained relatively content and within the normal range for the species, and no differences were observed between the CS treated groups and the control group.

Prothrombin time, partial prothrombin time and thrombin activity remained throughout within the normal range. No significant differences occurred between the three treatment groups. Although there were variations in some measurements, these did not follow any trend, and were present both in CS treated groups and in the control group.

Table 1: Mean values (\pm SEM) for haematological data in horses prior and at 1, 5 and 10 weeks of daily oral administration of NCS, LMWCS or placebo at a total dose of 2.5 g.

VARIABLE	PRE -TREATMENT			WEEK 1			WEEK 5			WEEK 10		
	NCS	LMWCS	CONTROL	NCS	LMWCS	CONTROL	NCS	LMWCS	CONTROL	NCS	LMWCS	CONTROL
Hct (%)	37.8 \pm 2.1	32.5 \pm 6.8	42.5 \pm 3.5	37.0 \pm 4.8	34.5 \pm 4.1	40.5 \pm 0.7	34.8 \pm 1.7	35.3 \pm 5.3	36.0 \pm 4.9	30.8 \pm 4.9	29.0 \pm 2.2	30.5 \pm 2.1
Hb (g/dl)	10.5 \pm 2.4	8.93 \pm 0.9	14.1 \pm 1.2	12.4 \pm 1.6	11.4 \pm 1.4	13.4 \pm 0.2	12.4 \pm 0.9	12.7 \pm 1.2	13.0 \pm 0.6	10.0 \pm 1.7	9.73 \pm 0.7	10.1 \pm 0.8
RBC($10^6/\mu$ l)	8.49 \pm 0.4	7.31 \pm 1.5	9.56 \pm 0.8	8.32 \pm 1.1	7.76 \pm 0.9	9.10 \pm 0.1	7.82 \pm 0.4	7.93 \pm 1.1	8.10 \pm 0.3	6.75 \pm 1.2	6.54 \pm 0.5	6.85 \pm 0.5
WBC($10^3/\mu$ l)	5.36 \pm 0.6	6.10 \pm 0.7	5.62 \pm 2.4	7.05 \pm 1.8	6.76 \pm 1.4	4.32 \pm 2.6	6.23 \pm 1.0	6.01 \pm 1.6	7.32 \pm 0.3	7.40 \pm 1.0	8.38 \pm 1.1	7.77 \pm 1.4
PT (sec)	12.0 \pm 0.8	12.5 \pm 1.0	12.0 \pm 0.0	14.7 \pm 1.3	15.5 \pm 2.6	15.5 \pm 0.7	11.7 \pm 0.5	11.7 \pm 1.2	12.5 \pm 0.7	11.2 \pm 0.9	12.0 \pm 0.8	12.0 \pm 0.0
APTT(sec)	34.0 \pm 1.4	34.2 \pm 4.5	37.5 \pm 3.5	46.0 \pm 7.1	43.5 \pm 8.4	39.0 \pm 0.0	65.7 \pm 11	56.2 \pm 2.2	60.5 \pm 6.3	56.7 \pm 3.3	53.7 \pm 2.7	56.5 \pm 3.5
Pact(%)	95.0 \pm 10	88.3 \pm 20	100 \pm 0.0	59.3 \pm 14	64.1 \pm 16	51.2 \pm 5.3	99.4 \pm 1.2	93.3 \pm 11	98.7 \pm 1.7	100 \pm 0.0	100 \pm 0.0	100 \pm 0.0

Hct : hematocrit; Hb: haemoglobin concentration; RBC : red blood cell concentration; WBC: white blood cell concentration; PT : prothrombin time; APTT : partial activated prothrombin time; Pact : prothrombin activity.

DISCUSSION

A controversial issue relating to CS, as well as other compounds classified as Slow Acting Drugs in Osteoarthritis (SADOA's) (14), is its classification as a nutraceutical. The term nutraceutical is used to describe a nutrient when supplied as a food supplement (15). Being nutraceuticals, there is no necessity to provide the extensive body of evidence required to establish the pharmacological and toxicological properties of agents seeking approval by legal bodies when registering drugs for which medicinal claims are made. This unfortunate fact has allowed, in the case of CS, the introduction of a wide range of products containing a heterogeneous range of compounds, of varying and uncontrolled composition and with different manufacturing and pharmacological profiles. However, there are a number of products which have established good quality not only in terms of raw material source but also in the manufacturing procedures used.

The present study utilised such good quality products; therefore it is not advisable to generalise from the results presented in this article to the whole range of CS containing products.

The number of animals in the placebo treated group was small. However, it was sufficient to serve as a control for potential changes. Moreover, in view of the arthritis model used (permanent), it would be unethical to include more individuals in this group.

The present study has indicated the ability of two types of CS, administered orally for 10 weeks, to suppress the signs of arthritis by modifying inflammatory mediators. Therefore, these results imply the adequate absorption of CS administered orally in horses. This is a point of controversy, since there have been numerous contradictory reports addressing the issue of CS oral absorption. Morrison (16) reported low bioavailability (between 0 and 8%), while in other studies (17 - 18 - 6) using radiolabeled preparations report a bioavailability in animals and humans up to 70%, being around 8.5% of the radioactivity associated with intact molecules of CS. In the present study bioavailability was not determined. However, the results suggest that a pharmacologically active amount of CS was absorbed and accessed to the inflamed joint.

The capacity of CS to reduce clinical signs associated with OA has been demonstrated in a range of species (19 - 20 -21 - 22 - 23), including horses (24). In the present study there was a reduction in clinical signs in the two groups treated with CS. However, due to the inter-animal variation only one of the measured clinical parameters, maximum flexion angle, showed statistically significant differences from the control group. Since the maximum flexion angle is an indicator of pain (25) the present results demonstrate an analgesic effect of CS.

It is interesting to highlight the subjectivity of clinical parameters in the evaluation of the effects of antiarthritic drugs. In fact, other authors have reported the lack of reliability of findings on efficacy for this type of compound when clinical parameters alone are used as markers of efficacy (26 - 27).

Protein concentration in synovial fluid from all experimental groups revealed no statistically significant differences. This is expected since the inflammatory model used in the present study does not produce an acute inflammatory reaction. In fact the model

applied is, compared to endotoxin-induced models, more representative of spontaneous occurring joint disease in the horse. In endotoxin induced models most of the pathological changes reflects predominantly a synovitis (28) with vasodilatation and protein plasma leakage into the joint space, changes being observed only in the late stages of spontaneously occurring joint disease in the horse (29).

Prostaglandin E₂ concentration in the control group of horses was in the range reported for clinical cases of osteoarthritis (30). Both types of CS studied showed the ability of long term dosing to reduce PGE₂ synovial fluid concentration, although the mechanism of action is unclear.

Conflicting data have been reported *in vitro* for an effect of CS on PGE₂ release. Orth et al. (31) report no effect of CS on PGE₂ release induced with LPS in cartilage explants. However, in an elegant study Basleer et al.(32) have shown the influence of incubation time on the effect of CS on the release of PGE₂ induced by IL-1 from chondrocytes cultivated in clusters during over periods (0-16 days and 16-32 days). These authors reported a decrease in PGE₂ release from chondrocytes in the period 0-16 days, which was however more significant in the period 16-32 days. This fact may reconcile conflicting data, since most *in vitro* studies are too short to allow demonstration of the effect of CS on PGE₂ synthesis. In the present study decreased PGE₂ concentration was clearly evident from week 4, reflecting a likely slow onset of action of this compound. The relatively slow onset of action of CS has also been shown in a clinical study comparing the anti-inflammatory effect of this compound with diclofenac sodium (19). The authors found that the action of CS increased gradually, being fully apparent around day 30 of treatment, at which time the effect of CS was not different from that of diclofenac sodium.

Since prostaglandin E₂ has been proposed as one of the contributors of arthralgia (33), the reduction on this eicosanoid is likely to at least partially explain the analgesic effect observed through the increased maximum flexion angle.

Analysis of the temporal profile of synovial concentration of GAGs is of interest. Differences in GAGs profiles between the two treated groups and the control group might be due to the origin of measured GAGs. The first GAGs peak may be a consequence of the penetration of CS given orally; in this case, the LMWCS may have greater bioavailability than the NCS. The second peak in the groups treated with CS, coincided with the GAGs peak in the control group, and this might have originated from destruction of the joint cartilage, with the ensuing release of GAGs into the synovial fluid; if this is the case, this could be considered a "catabolic" peak. In fact, Ismaiel et al. (34) reported that the reduction of GAGs content in arthritic articular cartilage explants incubated with IL-1 require at least 14 days exposure. We hypothesise that both the LMWCS and the NCS would have the ability to inhibit GAGs catabolism on week 8. However, the LMWCS, possibly because it is more bioavailable, may increase the availability of GAGs in the early stages, and thus, might provide greater protection of articular structures. Since the techniques used to measure GAGs in this study are quantitative but not qualitative, it is difficult to be certain that the 2nd peak in groups treated with CS is exclusively due to a catabolic process; it is possible that it reflects the sum of the administered GAGs and those released from the cartilage.

Matrix metalloproteinase-3 was not detected on the CS treated groups, reflecting the capacity of CS to inhibit its release and or synthesis. This observation agrees with the *in vitro* results reported by May et al. (35). The capacity of CS to inhibit metabolic degradation of collagen and proteoglycan has been previously reported (36 - 37 -21 - 38). *In vitro* inhibitory actions of Arteparon (PSGAG) and sodium pentosan polysulphate on MMP-3 release have also been reported (39).

The capacity of CS to inhibit MMP-3 may explain the reduction on the 2nd peak of synovial GAGs concentration observed in the present study as well as the reported chondroprotective effect of this compound.

Regarding possible secondary effects of long-term oral administration of the two types of CS studied here, none of the analysed haematological variables was significantly modified. Particular attention was paid to the coagulation cascade, because of the heparinoid nature of CS. However, the variation observed in the indicators of the coagulation process was minimal and always within the normal range reported for horses (40).

It is concluded that LMWCS and NCS after 10-week oral administration in arthritic horses alleviate clinical signs by reducing the synthesis and/or release of PGE₂ and MMP-3. Its anti-inflammatory and chondroprotective effect is further reflected by an inhibition of GAGs degradation without any significant effect on blood parameters including those reflecting the normal functioning of the coagulation cascade.

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Trabajo recibido el 10/11/2005, nº de referencia 010605_RED VET. Enviado por sus autores. Publicado en [Revista Electrónica de Veterinaria REDVET®](#), ISSN 1695-7504 el 01/01/06. [Veterinaria.org®](#) - [Comunidad Virtual Veterinaria.org®](#) - Veterinaria Organización S.L.®

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