SYNTEX S.A.
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TECHNICAL REPORT

TITLE: Efficacy evaluation of a Low Molecular Chondroitin Sulfate (LMWCS) Triethanolamine Salt (topical preparation) in an induced arthritis in equines.

Authors:
This work was performed at the Clinic Department , Equine Area, Faculty of Veterinarian Sciences, National University of Buenos Aires, Director : Professor Hugo Scipioni DVM.

Objective:
The aim of the work was to evaluate the therapeutic efficacy of an ointment whose active principle is a Low Molecular Weight Chondroitin Sulfate, triethanolamine salt (LMWCS-T), developed and produced by SYNTEX S.A. in the treatment of induced arthritis in equines.

Materials and Methods

Animals: there have been used 9 clinically healthy equines, free of pre-existing joint pathologies. The animals have been submitted to the usual feeding and stabling at the Faculty of Vet. Sciences.

Experimental Arthritis: an aseptic arthritis has been induced to the horses, by inoculating 0.5 ml of Freund's Complete Adjuvant in the Carpus joint.. It's worth of mentioning that such a model had been already used by another authors in similar studies (Hamm E. Et al.Vet. Med. 70:811-816, 1984).
Animals were divided at random in two groups:

1. Control group: 4 animals, without treatment.
2. Treated group: 5 animals, treated.

Treatment:
24 hours after the inoculation, all the equines belonging to the Treated group were subjected to a daily topical application of 20 grams of LMWCS-T creme. This application was performed covering the region of the affected articulation by manual soft massage until the total absorption of the product (that took, according to the environmental humidity grade, between 3 to 5 minutes as maximum). The animals of the control group didn't receive any treatment.

Evaluated parameters:

Every animal has been periodically checked during the assay. With this aim, two kind of parameters have been controlled:

Clinical parameters

Cardiac frequency, breathing frequency and body temperature, all of them tested by the routine methods. This checking was performed twice a week.

Articular parameters

In order to test the evolution of the induced injury, the following parameters have been evaluated twice a week:

a) Articular circumference: It has been measured at the level of the proximal border of the accessory carpal bone by a metallic flexible ribbon. In order to normalize the data, it has been considered the increasing of the articular circumference as an increment percentage (%) on the initial circumference.

b) Strained Flexion: It has been performed measuring the distance in centimeters (cm) between the foot coronary band and the olecranon bone with the leg in strained flexion.

c) Lameness: Lameness degree was evaluated by making the animals trot immediately after a one minute forced flexion of the affected articulation.
Different values to the observed difficulty degrees were assigned in order to quantify the parameter:
Grade 4: highest difficulty; the animal doesn't not rest the foot on the floor.
Grade 3: severe difficulty; the animal rest slightly in clamps .
Grade 2: moderated difficulty; the animal rest the hoof completely but some difficulty.
Grade 1: slight difficulty; good resting but limps in the first steps after flexion .
Grade 0: normal.

d) Analysis of synovial fluid: On day 0 (previous to the induction) and 7, 21 and 35, days after, 2 ml of synovial fluid from the affected carpus joint were extracted by puncture of joint capsule. The obtained sample was fractionated in two glass tubes, one with anticoagulant and another without anticoagulant, and both were destined to analysis. The analysis were performed by the Laboratory of Clinical Analysis of the Faculty of Veterinary . Sciences' Hospital, University of Buenos Aires.
The analysis included:

- *Physical exam*: 1. Quantity, 2. Color, 3. Density, 4. Viscosity and 5. Turbidity.
  1, 2 and 5: Were performed by the direct observation through the liquid placed in a glass tube.
  3: Density measurement has been performed using a refractometer. Some drops of synovial liquid (post-centrifugation) have been placed in a glass tube (taking care of not touching the walls of the tube) , observing the values in a graduated scale.

4: To measure viscosity, a little liquid has been poured between the thumb and the index finger, separating them very slowly. In the liquids with regular viscosity, there must be a continuous thread bigger than 1 cm.

- *Chemical exam*: It included: 1) Mucin clotting and 2) Proteins determination
  1: To perform this test it was used acetic acid at 2.5%. 1 ml of synovial liquid was placed in a glass tube (avoiding to touch the walls) and added 4 ml of acetic acid. They were vigorously mixed and let to settle for some minutes. The result can be, poor or fragmented.
  2: The proteins determination was performed by refractometry, as well as the density measurement. It also was performed using post-centrifugation synovial liquid.

  - *Cytological exam*: It has been done after centrifugation. The white cells were diluted with HCl 10N (1 in 100 ml of de-ionised water). The counting has been performed in Neubauer's chamber. The total number has been calculated based on the dilution factor and the areas counted from the chamber.
  - *Differential cells diagnostic*: After centrifuging the liquid (5 minutes at 1000-2000 RPM, the supernatant has been set apart and a little fraction of it was was allowed to dry and coloured with Diff Quich or Wright.
    They have been examined with the immersion lens, doing the run of the substance in a Greek guard shape. A total of 100 nucleated cells were counted, calculating afterwards the % of the different cellular elements observed.
  - *Radiological control:* A radiological control has been done previous to the induction (day 0) and the days 7, 21 y 35 after induction. The equipment used was a RA-ix 20-90, screen AGFA rare earth, AGFA Chassis and sensitive green film. The control has been performed by the Radiology Service of the Hospital, at Faculty of Veterinarian Sciences of the University of Buenos Aires.
  - *Statistical analysis:* Anova test was performed in a parcels divided design at random, in the data corresponding to the parameter "increase of Articular Circumference and Strained Flexion ". Such analysis was performed by the Statistics Department of the Faculty of Veterinarian Sciences of the University of Buenos Aires.

Results

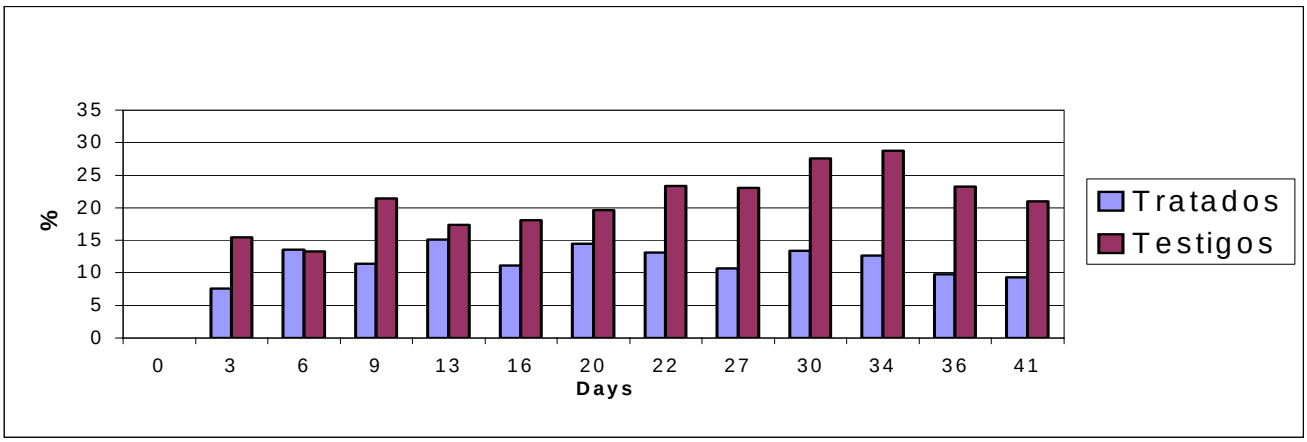
Clinical parameters

All the evaluated constants kept within the values considered regular for the species during the whole assay.

Articular parameters

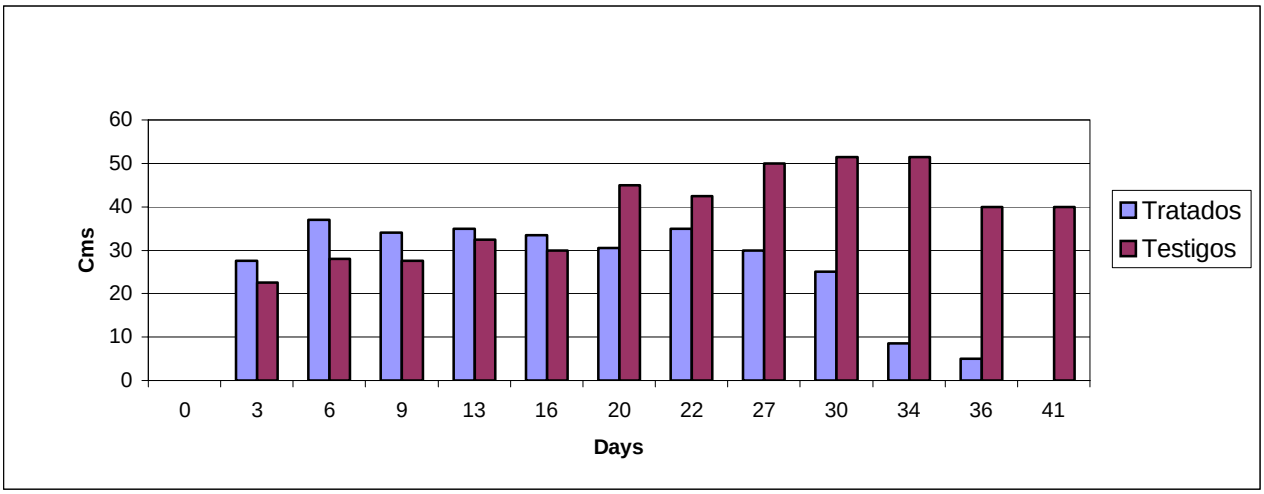
The average of the obtained results for each parameter were the following:

a) Increase of the Joint circumference (%)



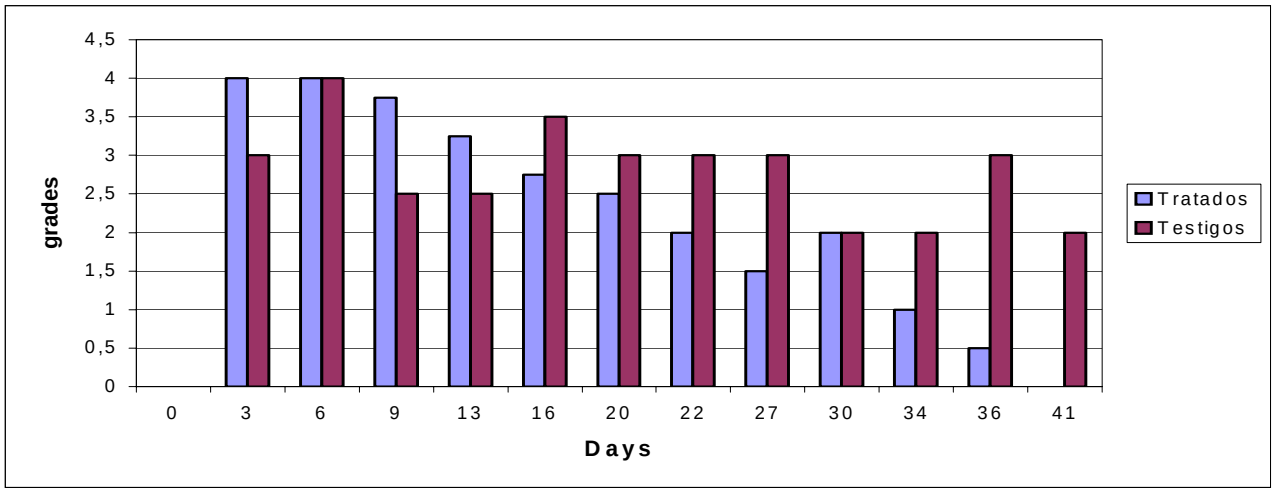
Día	0	3	6	9	13	16	20	22	27	30	34	36	41
Trat.	0	7.6	13.6	11.4	15.1	11.1	14.5	13.1	10.7	13.4	12.7	9.8	9.28
Ctrol.	0	15.5	13.3	21.4	17.4	18.1	19.6	23.3	23.1	27.6	28.8	23.2	21.0

b) Strained Flexion (cm)



Day	0	3	6	9	13	16	20	22	27	30	34	36	41
Trat.	0	27.5	37	34	35	33.5	30.5	35	30	25	8.5	5	0
Ctrol	0	22.5	28	27.5	32.5	30	45	42.5	50	51.5	51.5	40	40

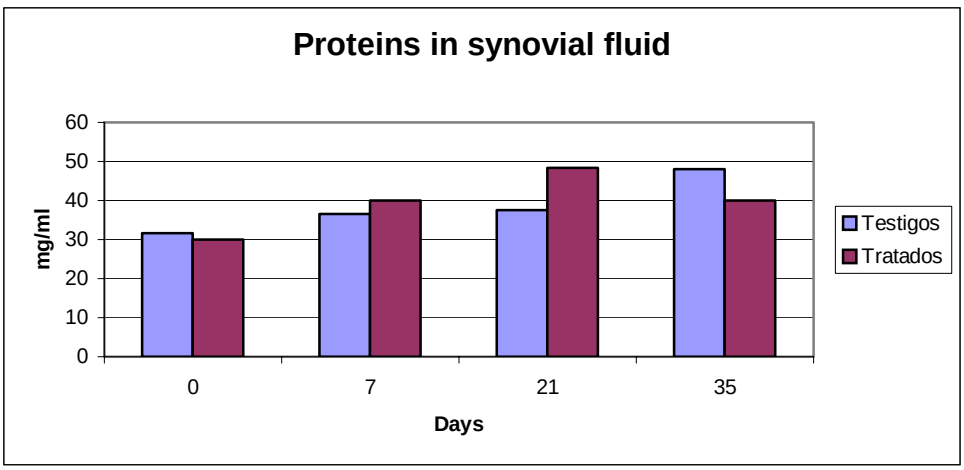
c) Lameness degree

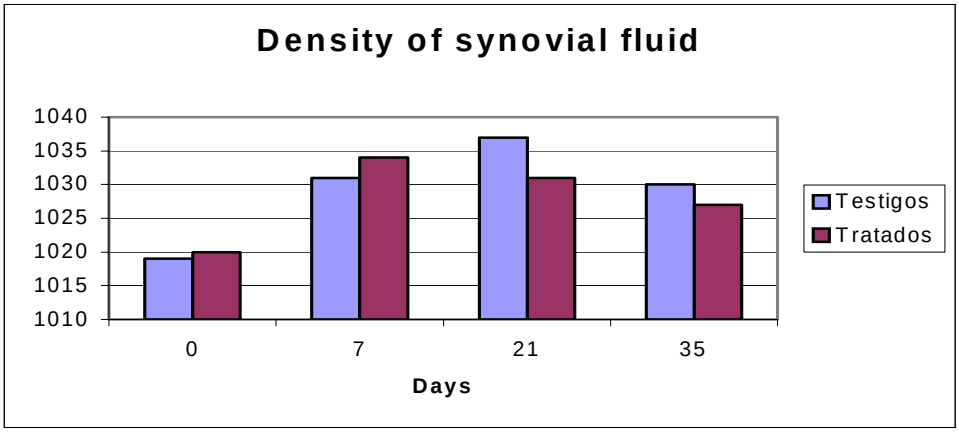


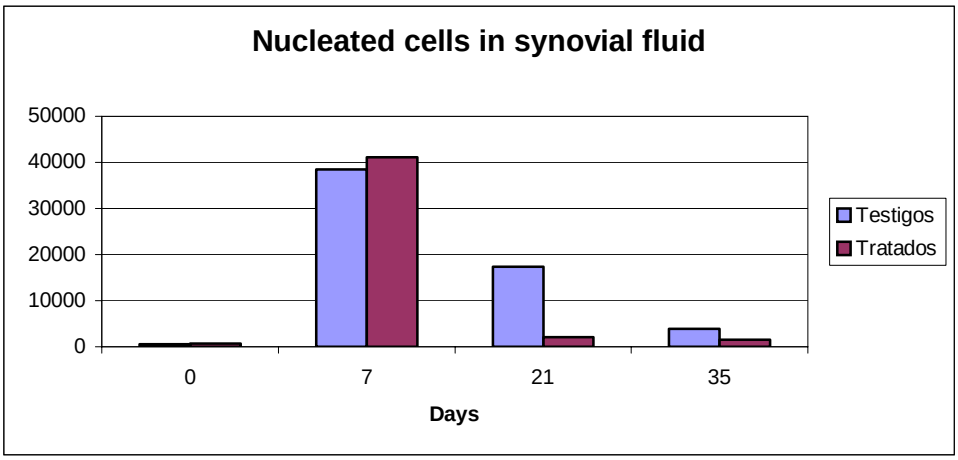
Day	0	3	6	9	13	16	20	22	27	30	34	36	41
Trat.	0	4	4	3.75	3.25	2.75	2.5	2	1.5	2	1	0.5	0
Test.	0	3	4	2.5	2.5	3.5	3	3	3	2	2	3	2

d) Analysis of Synovial fluid

Days	0		7		21		35	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Proteins (mg/ml)	31.6	30.0	36.6	40	37.5	48.3	48	40
Density	1019	1020	1031	1034	1037	1031	1030	1027
Turbidity	Clare	Clare	Slightly. turbid	Slightly. turbid	turbid	Yellow brilliant	turbid	Amarillo brilliant
Viscosity	Good	Good	Bad	Bad	Bad	Regular	Regular	Regular
Mucin	Good	Good	Poor Fragm.	Regular/ Fragm.	Fragm/ Haemorr.	Fragment.	Fragment.	Slightly fragm. Homogenous.
Nucleated Cells	623	650	38533	41100	17340	2150	3850	1500







e) Radiological control

In the equines of the control group, it has been observed a notorious periosteum reaction in distal radius, intermediate carpus y radial carpus, with considerable activity that remained unchanged during the whole considered period. In the animals of the treated group, it has been observed a tumefaction of the soft tissues and a slight periostial reaction, that favorably developed during the considered period.

Discussion

The experimental arthritis was successfully reproduced in all the animals subjected to the assay, as shown by the alteration of all the considered parameters. These data coincide with those of the bibliography for this kind of studies. Taking into account the evolution observed in the treated and control animals, favorable changes can be observed in the treated equines just 15 days after the treatment started.. On that moment, the treated animals got better regarding clinical records, the increment of the articular circumference was significantly higher in the control animals ($p<0,05$), which appeared

at the 9th day of the assay. Regarding lameness and strained flexion, such difference was evident at the day 16 and 20 respectively. As it can be observed in the data belonging to the initial results of the synovial liquid assay, both groups showed regular characteristics for the species regarding proteins, nucleated cells, density, turbines degree, viscosity and mucin clotting. Both groups showed data altered in similar ways until the third week of the assay, where there begins to exist a quantifiable difference in the values. It was observed that they showed a tendency to normality only in the treated animals. It's worth of highlighting that in the case of the animals belonging to the control group , they still showed clinical and laboratory altered signals while the treated animals showed regular values in the most of the studied parameters.

Conclusion

As per the results obtained from the present assay, it can be concluded that the treatment done with the cream whose active principle is the LMWCS-T reverted the inducted pathology, which shows an effective incorporation of the active principle to the articulation by this route. Moreover, the applied dose and the way of application also showed to be suitable to treat this experimental pathology. The use of the mentioned product didn't cause any collateral or secondary effect on the treated animals all throughout the assay. Therefore, this kind of effect in treatments with this product can be disregarded. The results obtained in this severe pathology suggest that the product could be successfully used in natural non- infectious articular pathologies.