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**CHONDROPROTECTIVE ACTIVITY:
SURGICALLY-INDUCED EXPERIMENTAL OSTEOARTHRITIS
IN GUINEA PIGS**

SUBSTANCE FR-91

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REPORT N° CD-92/3087F

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SUBSTANCE FR-91

1. OBJECTIVE

The objective of this study was to evaluate the chondroprotective activity of the substance FR-91 using the method of experimental osteoarthritis induced by partial medial meniscectomy in the guinea pig.

2. JUSTIFICATION

Surgical induction of a degeneration of cartilage by partial medial meniscectomy produces a rapid and progressive lesion in the knee.

This experimental model shows, in late stages of the lesion, characteristics similar to those of human osteoarthritis.

3. EXPERIMENTAL DESIGN

The animals remained 5 days in an acclimatization period before the beginning of the experiment.

All animals (except those of the Blank Control group) were anesthetized with sodium pentobarbital (35 mg/kg, 10 ml/kg, i.p.) and were subjected to a partial medial meniscectomy.

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A small incision was made on the median face of the right knee and the median collateral ligament was exposed by blunt dissection.

The transection of this ligament permits localization of the medial meniscus, of which the distal half was carefully extracted, avoiding damage to cartilage surfaces.

Sulphathiazol powder was then sprinkled on the wound and the animal was sutured.

Four groups of ten animals each were formed at random. To be able to substitute an animal in case of necessity, more animals were operated on and two additional animals were added to each group.

The experimental groups were as follows:

Group	Treatment	Dose (ml/kg/day)	Color
A	BLANK CONTROL	0.75	White
B	OPERATED CONTROL	0.75	White/black
C	REF	0.375	Yellow
'D	FR-91	0.125	Blue

- a) The Blank and Operated Control groups received physiological saline.
- b) The treatments were administered daily for 12 weeks, beginning the day after partial medial meniscectomy.

The treatment was administered by intramuscular injection, alternating between the right and the left legs.

At the end of the treatment period, the animals were sacrificed and the

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femur and tibial joints were extracted and placed in a decalcifying mixture consisting of 10 ml formic acid, 5 ml formol and 100 ml distilled water, for seven days. Subsequently, the joints were fixed in neutral buffered formol for 24 hours. Afterwards, and after observing that the decalcification had been insufficient, the bones were returned to the decalcifying mixture for three days. Finally, the joints were again fixed in neutral formol for 24 hours and washed in running water for the following 24 hours.

The preparations were then stained with hematoxylin-eosin and a histological evaluation was made of the changes observed, according to incidence and intensity criteria.

4. EVALUATION OF RESULTS

The mean (MED), standard deviation (SD) and standard error of the mean (SEM) have been calculated of the body weights of the animals in each group. They have been compared statistically by single-factor variance analysis and when statistically significant differences were found, the Duncan-Kramer multiple comparison test was performed.

The severity of the lesions found was evaluated microscopically using the following scoring indices:

STRUCTURE OF ARTICULAR CARTILAGE

- 0: Normal
- 1: Irregularity in joint surface and/or fissures
- 2: Erosion to the intermediate zone

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- 3: Erosion to the radial zone
- 4: Erosion to the calcified zone, focal, with or without eburnation
- 5: Widespread disorganization of joint cartilage

CELLULAR PROLIFERATION

- 1: Diffuse hypercellularity
- 2: Foci of proliferating chondrocytes
- 3: Hypocellularity
- 4: Widespread areas of disappearance of cartilage

TOLUIDINE BLUE STAINING

- 1: Slight reduction of staining
- 2: Moderate reduction
- 3: Severe reduction
- 4: No detectable staining
- 5: Widespread areas of disappearance of cartilage

The structure of joint cartilage and cellular proliferation were evaluated using two different scoring criteria, but they were added for the statistical evaluation, as they are parameters providing complementary information.

The loss of staining with toluidine blue indicates a loss of proteoglycans which occurs in degenerative changes of the cartilage.

5. RESULTS

For the evaluation of the results, 10 animals were analyzed for each group, discarding the two reserve animals in each group.

STRUCTURE OF JOINT CARTILAGE AND CELL PROLIFERATION

The overall results are shown in Table I.

a) In the Blank Control group in which neither leg was operated on, the mean scores obtained were 4.5 and 4.4 for right and left legs, respectively.

These scores are a consequence of cartilage degeneration produced by spontaneous osteoarthritis, given the age of the guinea pigs.

b) In the Operated Control group in which experimental osteoarthritis was induced by partial medial meniscectomy in the right leg, the score for this leg rose to 7.4. It was also observed that the score for the left leg was higher than that for the Blank Control group, reaching 5.4. This increase is due to the fact that the guinea pigs, as they have been operated on in the right leg, support themselves selectively on the left leg, thus increasing spontaneous osteoarthritis.

c) In the group treated with the REF substance at a dose of 0.375 mg/kg, the score in the right leg descended in comparison to the Control groups, to give a score of 6.6, implying an inhibition of 27.6% , while in the left, unoperated leg, the score obtained (5.2.) was the same as that for the same leg in the Control groups.

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In the group treated with FR-91 at a dose of 0.125 mg/kg, a score reduction was observed of 31 % in the right leg and of 37.5 % in the left leg (6.5 and 4.9, respectively) in comparison with the Control groups. Statistically significant differences were found between the Blank Control and the Operated Control group.

TOLUIDINE BLUE STAINING

The overall results are shown in Table I.

The scoring criteria used are found in Section 4.

- a) In the Blank Control group, the mean score obtained for the right and left legs were 2.6 and 1.7, respectively. These scores indicate a moderate to severe loss of the proteoglycans present in the joint, provoked by a spontaneous degeneration of the cartilage due to the age of the guinea pigs.

- b) In the Operated Control group, it was observed that in the joint of the operated knee, the loss of proteoglycans was from severe to practically complete in some of the animals, reaching a mean score of 3.3. In the left joint in the majority of animals, a moderate to severe reduction was observed in the amount of proteoglycan, equal to that of the Blank Control group, with a mean score of 2.3.

- c) In the group treated with REF at a dose of 0.375 ml/kg, a severe reduction of proteoglycans was observed in the right joint, with a mean score of

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3.2, while in the left joint the mean was 2.3, similar to that of the Blank Control group.

d) In the group treated with FR-91 at a dose of 0.125 mg/kg, the mean scores in the right and left joints were 2.8 and 2.5, respectively, showing that in the operated right joint the proteoglycan loss was less than in the Operated Control and the REF groups.

CONCLUSIONS

The chondroprotective activity of the substance FR-91 has been evaluated.

Experimental osteoarthritis was induced by partial medial meniscectomy in the rear right leg of the guinea pig.

In these experimental conditions, the administration of substance FR-91 at a dose of 0.125 ml/kg showed appreciable chondroprotective activity, in the right (operated) leg as well as in the left (unoperated) leg.

TABLE I
 Project N° CD-92/3087 F
 Species: Albino guinea pig
 Sex: Male
 Route: im

Treatment		n	EVALUATION OF LESIONS							
			LEFT Structure + cell proli- feration	LEG % inh	Staining	% inh	RIGHT Structure+ cell pro- liferation	LEG % inh	Staining	% inh
Blank control	MED	10	4.5		2.6		4.4		1.7	
	DE		2.22	-	1.35	-	2.22	-	1.16	-
Operated control	MED	10	7.4		3.3		5.2		2.3	
	DE		1.78	-	1.06	-	2.30	-	0.95	-
REF 0.375 ml/kg	MED	10	6.6		3.2		5.2		2.3	
	DE		1.07	27.6	1.14	14.3	1.87	0	0.82	0
FR-91 0.125 ml/kg	MED	10	6.5		2.8		4.9		2.5	
	DE		2.07	<u>31.0</u>	1.4	<u>71.4</u>	2.42	<u>37.5</u>	0.85	<u>33.3</u>

TABLE II
 Project M CD-92/3087 F
 Species: Albino guinea pig
 Sex: Male

CHONDROPROTECTIVE ACTIVITY
 Body weight (g)
 Mean ± SEM

Group	Treatment	Dose (im) ml/kg/day	n	Day 0	5 Nov 92	9 Nov 92	12 Nov 92	16 Nov 92	19 Nov 92	23 Nov 92	26 Nov 92	30 Nov 92
A	Blank control	0.750	10	589.7±11.03	611.9±12.03	634.8±10.71	648.9±12.33	671.0±11.73	696.8±12.76	719.1±13.46	730.4±14.53	752.9±15.27
B	Opreted control	0.750	10	595.1±7.76	585.2±8.14	621.8±8.62	652.7±8.69	678.7±9.88	710.7±10.06	740.6±9.81	741.8±13.04	770.2±18.01
C	REF	0.375	10	605.1±7.68	591.9±6.90	631.4±9.57	649.6±10.87	686.9±9.78	695.6±8.14	741.6±7.29	749.8±7.95	772.4±6.61
D	FR-91	0.125	10	579.9±9.07	581.6±8.01	608.6±8.48	634.9±7.74	649.0±13.30	674.9±13.29	706.2±12.60	720.4±11.48	752.8±11.89
	Single-factor variance analysis			NS	NS	NS	NS	NS	NS	NS	NS	NS

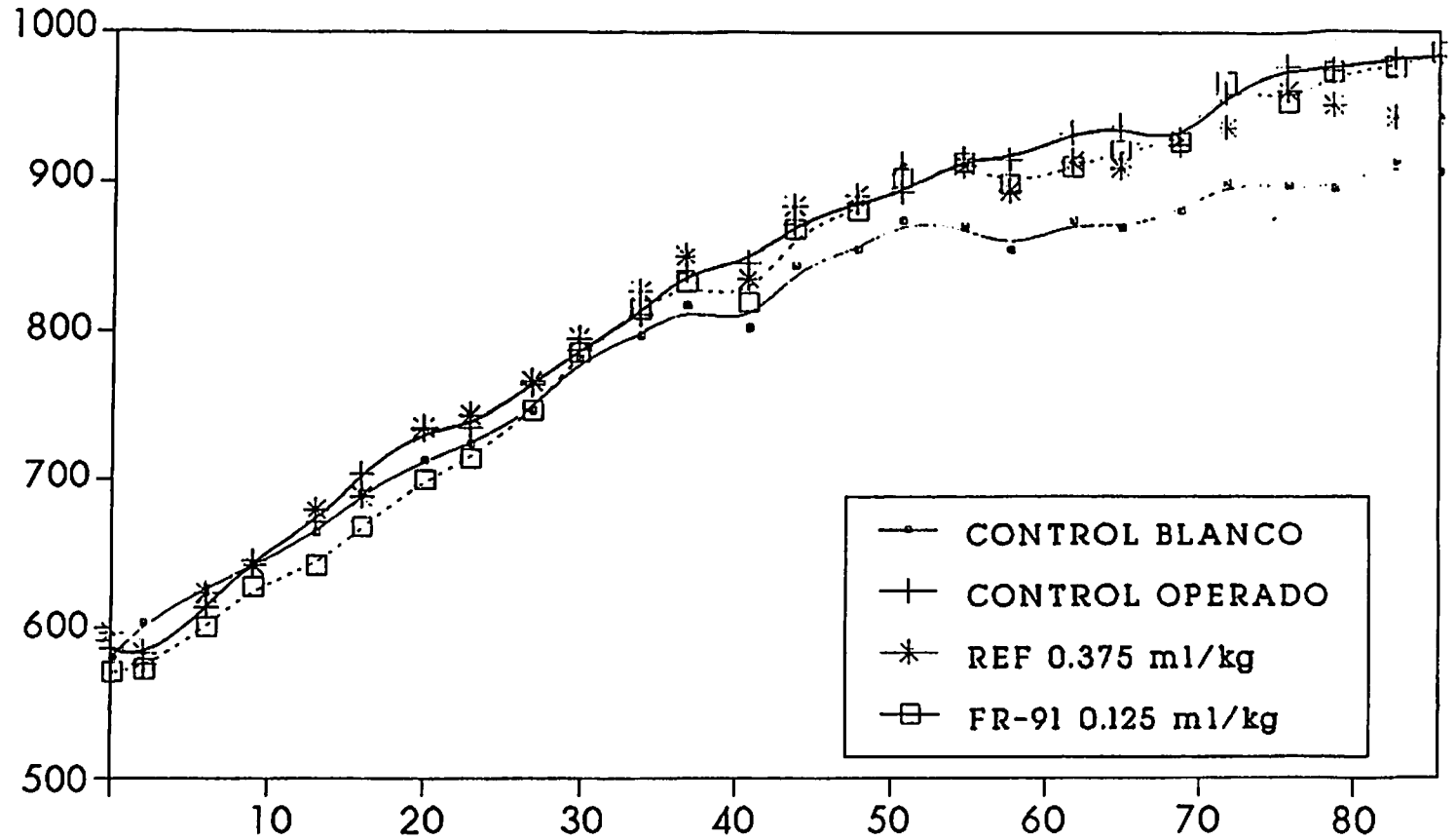
TABLE II (cont.)
 Project N CD-92/3087 F
 Species: Albino guinea pig
 Sex: Male

CHONDROPROTECTIVE ACTIVITY
 Body weight (g)
 Mean \pm SEM

Group	Treatment	Dose (ml) ml/kg/day	n	3 Dec 92	7 Dec 92	10 Dec 92	14 Dec 92	17 Dec 92	21 Dec 92	24 Dec 92	28 Dec 92	31 Dec 92
A	Blank control	0.750	10	787.4 \pm 14.62	802.4 \pm 14.86	822.0 \pm 15.89	807.2 \pm 17.07	848.4 \pm 17.58	859.7 \pm 18.79	878.4 \pm 18.34	874.8 \pm 21.30	859.6 \pm 22.07
B	Operated control	0.750	10	793.1 \pm 18.35	815.8 \pm 17.89	854.8 \pm 17.11	851.0 \pm 19.43	876.6 \pm 16.17	890.7 \pm 19.01	897.9 \pm 19.32	920.7 \pm 18.83	919.7 \pm 18.83
C	REF	0.375	10	800.5 \pm 7.34	831.9 \pm 7.55	855.4 \pm 6.45	840.2 \pm 9.43	888.4 \pm 6.27	895.6 \pm 9.44	916.6 \pm 7.26	915.9 \pm 7.80	899.1 \pm 7.79
D	FR-91	0.125	10	791.3 \pm 10.55	819.4 \pm 15.02	837.7 \pm 11.25	823.7 \pm 13.79	872.8 \pm 16.62	885.1 \pm 14.43	907.2 \pm 16.03	917.5 \pm 15.26	903.1 \pm 16.73
	Single-factor variance analysis			NS	NS	NS	NS	NS	NS	NS	NS	NS

CHONDROPROTECTIVE ACTIVITY
Evolution of body weight (g)

Body weight (g)



Blank control
Operated control
REF 0.375 ml/kg
FR-91 0.125 ml/kg

Day of
operation

Day of
sacrifice

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HYPOTHESIS ON THE MECHANISM OF ACTION

Working hypothesis and scientific basis of the study:

The etiopathology of osteoarthritic disease (arthrosis) is unknown and it is not possible, with the current state of knowledge, to attribute it to any obvious underlying cause.

However, hypotheses for the pathogeny of the disease do exist and the activity of FR-91 should be related to one of these hypotheses.

It was understood that it was necessary to evaluate the chondroprotective activity as well as its activity in animal models, to be able to relate this activity to current hypotheses on osteoarthritis relative to the knowledge of the types of collagen, alterations in the cartilage matrix, metabolic activity of the chondrocyte, proinflammatory activity, etc. That is, to demonstrate biological activity on the cellular structures implicated in osteoarthritis which allow FR-91 to be related to some of the currently-existing hypotheses of osteoarthritis.

According to current science, the chondrocytes are responsible for the synthesis of proteoglycans (PG) and of collagen fibers joined by the PG. The synoviocytes also secrete interleukin 1 and tumor necrosis factor (TNF). (I. Marques *et al.*, Rheumatology Treatise, JIMS, Barcelona, Spain, 1994)

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In cartilage affected by osteoarthritis, it is known that the concentration of enzymes which degrade the matrix (collagenases and PGases) is elevated and that these enzymes are derived neither from the synovial membrane nor from the joint liquid; the origin of the supposed inducers of these degradatory enzymes is unknown (1, Marques).

Normal cartilage possesses inhibitors of these degrading enzymes.

One of the working hypotheses most often proposed is that, in osteoarthritis, an altered stoichiometric relationship exists between inducers of degradatory enzymes and inhibitors of these substances (2, Harrison, Principles of Internal Medicine, 12th Ed., Vol. II, p. 1711), and that this alteration can be stimulated by various causes, including mechanical causes of pressure overload on the affected joints (2, p. 1712).

The scientific studies carried out with FR-91 permit the conclusion, in the models used, that:

- a) the in vitro proliferation of synoviocytes is stimulated
- b) a chondroprotective activity in the chondrocyte matrix is produced.

These results correlate extremely well with the working hypothesis, since:

1. It is known that the factors secreted (interleukin 1, TNF, etc.) by the synoviocytes impede the failure of the chondrocyte matrix which occurs in the arthritic process. This probably occurs by an activator/inhibitor control on the process of degradatory enzyme synthesis (2, Harrison).

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2. The chondroprotective activity measured (see study in the report) indicates that FR-91 permits maintenance of correct proteoglycan synthesis, responsible for the cohesion of collagen fibers and, hence, for the maintenance of the joint cartilage structure. This is so (see study conclusions) because in the model used, the concentration of proteoglycans is measured using toluidine blue staining. This measurement is performed in animals subjected to surgery and treated with FR-91 as well as in operated, untreated animals (Operated Control) and in unoperated and untreated animals (Blank Control). The results are clear and extremely favorable for FR-91. In our case, the inhibition of the loss of staining, demonstrating the maintenance of the proteoglycan structure, was considerable with respect to the Operated Control group and approximated the values of the unoperated, untreated group (Blank Control). The model is that most commonly used in the scientific bibliography in this area. Other existing models are being slowly abandoned, as the induction of osteoarthritis by chemical substances which damage the cartilaginous matrix cannot be extrapolated to the human phenomenon. The model used permits observation of osteoarthritis by:

- a) Surgical induction (partial meniscectomy) in the operated knee
- b) Mechanical induction by weight overload of the animal on the unoperated knee

An excellent result is obtained in both knees.

CONCLUSIONS

In conclusion, the preclinical results obtained permit the formulation of a working hypothesis, that FR-91 affects the stoichiometric equilibrium of the activators/inhibitors

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of the degradatory enzymes of the chondrocyte matrix and which it helps to maintain, by impeding progression of matrix degradation (maintenance of proteoglycan concentration). It also permits normalization (synoviocyte and chondroprotective activity) of this altered equilibrium.

To test the proposed hypothesis is not feasible at present in clinical terms, without taking recourse to invasive tests which permit, using histochemical methods, evaluation of the preclinical effects observed in the various models. In clinical parameters, the impidence of progression of the cartilaginous matrix destruction and the normalization of the affected structures should be manifest by an improvement in mobility, reduction of pain and favorable evolution of the radiologic images in the patients treated. This would demonstrate the efficiency of FR-91, since no clinical laboratory biochemical study using other indicator elements, proteoglycans, glucosaminoglycans, synovial liquid, etc., has been shown to be of utility (2, Harrison, 12th Ed., p. 1714).