

Computerized photocolometric analysis of the effects of intraarticular betamethasone on the proteoglycan concentration of leporine knee cartilage matrix: influence of the number of intraarticular injections

Análise fotocolométrica computadorizada dos efeitos da betametasona intra-articular sobre a concentração de proteoglicanos da matriz cartilaginosa de joelhos leporinos: influência do número de injeções intra-articulares

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A B S T R A C T

Objective: To study the effects of repeated injections of betamethasone on proteoglycan concentration in the articular cartilage of normal knees of Californian rabbits of both sexes. **Methods:** Eighty animals were randomly divided into eight groups of ten animals each. Three control groups (saline solution injected or not) and five study groups — therapeutic doses, repeated or not, of betamethasone injected into the right knee of each animal at weekly intervals. After eight days from the last injection, sections of articular cartilage from tibial plateaus collected from weight-bearing surfaces were stained with hematoxylin and eosin for light microscopy analysis and with safranin O for the proteoglycan content assay. The staining intensity of safranin O was quantified by histomorphometry using an Olympus BX 50 microscope and a microcomputer with the Image Pro-plus 4.5.0 software. **Results:** Animals receiving one, two and four betamethasone injections showed no differences when compared to normal controls. Animals receiving six and eight injections had a significant decrease in safranin O staining intensity ($p < 0.05$) as compared to the control groups and the other study groups. **Conclusion:** A decrease in the concentration of articular cartilage proteoglycans dependent on repeated betamethasone injection was effectively demonstrated.

Key words: Joint knee. Proteoglycans. Adrenal cortex hormones. Rabbits.

INTRODUCTION

The great shift in the conservative treatment of articular lesions occurred with the introduction of injections of corticosteroids and their analogues. However, the rapid relief of symptoms, as well as the restoration of the functional levels of joints, eventually led to the indiscriminate use of those drugs. Case reports of Charcot's arthropathy, bone necroses and an increase in articular cartilage degeneration were prominent in the years following the introduction of that modality of treatment^{1,2}.

Experimentally, corticosteroids cause lesions in the cartilage tissue by inducing proteoglycan loss, a fact that plays a key role in the degeneration of articular cartilages. Conversely, research studies with animal models have demonstrated a chondroprotective effect³⁻⁶. The

intraarticular application of corticosteroids at low doses blocks the deleterious effects produced by inflammatory processes, thus preventing cartilage degeneration. The intraarticular application of corticosteroids remains controversial as to its safety and efficacy⁷.

Articular cartilages have gained prominence in orthopedics, given their importance to mobility. Countless drug-based treatments with supplementation of elements that constitute the matrix, both orally and through infiltrations, are currently in the foreground⁸.

With the objective of evaluating the effects of the association of short-acting and long-acting corticosteroids on normal hyaline cartilage, the present study was conducted by applying this combination of drugs to rabbit knees at a dosage proportional to that adopted in clinical practice.

Study conducted at the Experimental Surgery Laboratory of the Graduate Program in Clinics and Surgery of the Universidade Federal do Paraná, Curitiba, PR, Brazil.

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METHODS

Eighty adult Californian rabbits (*Oryctolagus cuniculus*), both male and female, weighing between 2950 g and 3300 g (mean 3200 g) were allocated into eight groups of 10 animals each. The animals were kept in cages adequate for the species, and fed ecological rabbit chow.

The dosage of the medication to be administered was calculated based on a mean weight of 65 kg for humans. The rabbits weighed 1/20 of the human body weight equivalent. Each vial of the medication, containing 1 mL of the corticosteroid solution composed of betamethasone dipropionate and betamethasone disodium phosphate, was diluted in 9 mL of 0.9% saline. From the resulting solution, 0.5 mL (0.25 mg dipropionate and 0.1 mg phosphate) was used for infiltration, which corresponds to 1/20 of the dose used for intraarticular applications in human knees. The infiltrations were conducted on a weekly basis, according to the treatment assigned to each study group as described in table 1.

Disposable gloves, needles and 1 mL vaccine syringes marked in 0.1 mL increments were used for the applications. The skin of the right stifle of the animals was prepared with chlorhexidine and 70% alcohol, without the need for shaving.

All rabbits underwent euthanasia eight days after the last infiltration scheduled for the respective group; the no-infiltration control group was sacrificed by the end of the trial. For euthanasia, the animals were first anesthetized with 100 mg/kg ketamine and 8 mg/kg xylazine intramuscularly, and then subjected to an intracardiac injection of 10mL potassium chloride at 19.1% .

The knees were dissected and the tibial plateaus were removed, collected in individual containers with 10% formalin and referred to the pathologic anatomy laboratory.

The tibial plateaus were decalcified and sectioned sagittally down the medial and lateral compartments, and embedded in paraffin. Following microtomy, four slides from the weight-bearing surfaces of the plateaus were obtained, two of the medial aspect, and two of the lateral. Safranin O staining was used in one slide of the medial plateau and one of the lateral plateau; the same procedure was repeated with hematoxylin-eosin staining. The slides stained by safranin O were analyzed on histomorphometry equipment consisting of an Olympus BX 50 microscope connected to a computer running the software Image Pro-plus version 4.5.0 by Media Cybernetics.

Staining intensity by safranin O was measured under standard lighting at 10 X magnification for each slide. The image captured by the equipment was analyzed by the software that quantitated the pixels of the staining (Figure 1).

For statistical analysis, the variable of interest was optical density. The null hypothesis of equal mean optical density values across the groups was tested against the alternative hypothesis that the mean optical density of at least one of the groups was different from the others. To that end, the one-way ANOVA was used, as well as the LSD test for multiple comparisons. To evaluate the homogeneity of variances, Cochran test was applied, and Shapiro-Wilks test was used to assess the normality of the data within groups.

Table 1 – Study groups according to type and volume of the solution injected, number and intervals of applications.

Group	Substance applied	Number of applications	Volume applied	Interval
1	Saline 0.9%	0	0.5mL	——
2	Saline 0.9%	1	0.5mL	——
3	Saline 0.9%	8	0.5mL	Weekly
4	Betamethasone Dipropionate 0.25mg Phosphate 0.1mg	1	0.5mL	——
5	Betamethasone Dipropionate 0.25mg Phosphate 0.1mg	2	0.5mL	Weekly
6	Betamethasone Dipropionate 0.25mg Phosphate 0.1mg	4	0.5mL	Weekly
7	Betamethasone Dipropionate 0.25mg Phosphate 0.1mg	6	0.5mL	Weekly
8	Betamethasone Dipropionate 0.25mg Phosphate 0.1mg	8	0.5mL	Weekly

RESULTS

The plateaus were found to be normal in Groups 1, 2, 3, 4, 5 and 6. Pearly granulation was present in both plateaus in three cases in group 7 and six cases in Group 8. In the three cases that exhibited a volume increase at the time of the operation, there was coarse granulation, more marked in the medial plateau next to the ligament insertion.

In the histological analysis of the slides stained with hematoxylin-eosin, no tissue injuries were found in the studied groups.

The assessment of the staining intensity of the histological sections stained by safranin O, performed through the histomorphometry equipment, yielded results that were laid out as means of optical density provided by the software. The results were compared on a group-versus-group basis, always taking into account the level of significance of 5%. There was a statistically significant difference between Groups 7 and 8 as compared with control Group 3 and Groups 4, 5 and 6, showing progressive proteoglycan loss as the number of applications increased, as seen in figure 2.

DISCUSSION

The idea of assessing the effects of the combined corticosteroids was based on the fact that no studies could be found in the literature testing the association of betamethasone dipropionate and betamethasone disodium phosphate⁸. This combination of corticosteroids could result in greater protein synthesis suppression in the cartilage matrix, leading to earlier degeneration after fewer infiltrations than reported in the literature with drugs administered alone.

This medication is commercially available under different proprietary names, but the same composition and dosage. The less soluble corticosteroids are the most often used for infiltrations, as they remain for up to three weeks within the joints and reduce the systemic effects⁹. The summation of a short-duration and rapid onset of action corticosteroid and a long-duration corticosteroid minimizes inflammatory response effects similar to those of gout and pseudogout triggered by the phagocytosis of corticosteroid crystals by leukocytes.

Betamethasone is a very potent corticosteroid as an anti-inflammatory, and its action is proportionally stronger than several other corticosteroids. Since there were no safety parameters to indicate when that combination was beginning to damage the cartilage, the decision was to find the maximum number of doses equivalent to that administered to humans that would cause a decrease in matrix proteoglycans, thus resulting in structural fragility.

The development of corticosteroid-induced lesions in rabbit articular cartilages is very frequent¹⁰, and most studies demonstrating cartilage degenerative changes due to the action of corticosteroids were conducted with this animal species⁴⁻⁶. In the joint, the effects of proteoglycan

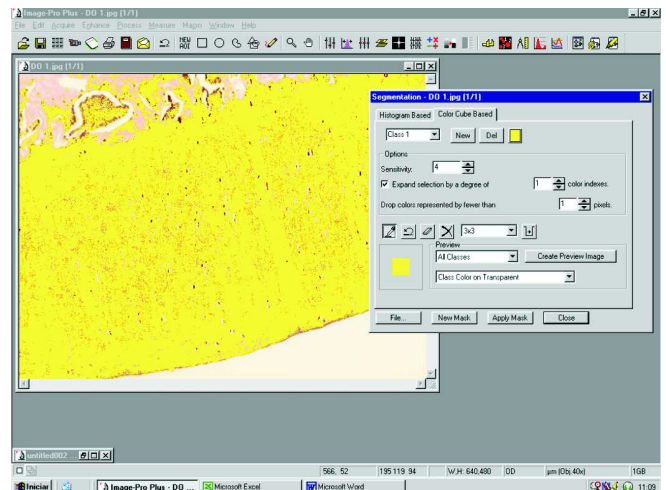


Figure 1 - A screen of the software (Image Pro-Plus 4.5) used to assess safranin O staining intensity.

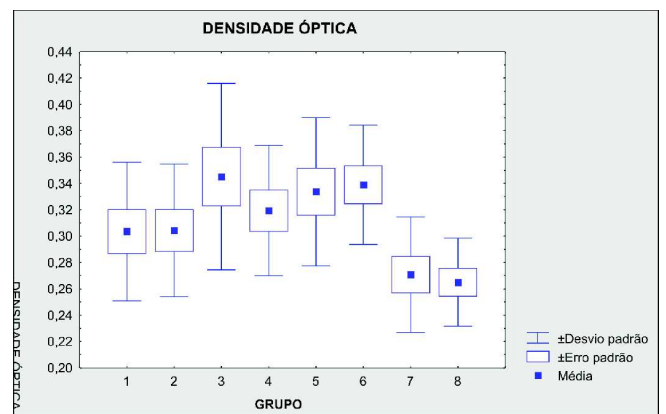


Figure 2 - Optical density of safranin O in each of the groups.

Comparisons with $p < 0.05$:

G3 x G7=0.0018; G3 x G8=0.0008;

G4 x G7=0.0378; G4 x G8=0.0207;

G5 x G7=0.0076; G5 x G8=0.0037;

G6 x G7=0.0040; G6 x G8=0.0019.

loss are determinant for corticosteroid-induced lesions. The resulting structural fragility leads to cartilage collapse in weight-bearing areas. These compounds have a short half-life: approximately eight days. Collagen synthesis is strongly affected; however, since its durability is longer, no *in vivo* decrease is observed given the duration of the trials.

The dosage in milligrams per kilogram was adopted considering that the proportion based on body surface area used for some medications results in a high dose, which is by itself potentially deleterious to cells, as demonstrated by tissue culture¹¹.

Safranin O is a dye that establishes a stoichiometric binding with polyanions, i.e., one safranin O molecule binds to each negative charge of chondroitin sulphate and keratin sulphate, and does not bind to collagen. Thus, it is possible to affirm that the alterations seen in staining intensity derive from a decline in proteoglycans, more specifically keratin sulphate and chondroitin sulphate. The assessment of staining intensity conducted with the aid of

histomorphometry equipment allowed for a more objective measurement of the data, thus precluding possible subjective discordance among observers.

The results show the absence of adverse effects with the application of the combined corticosteroids, which can be demonstrated by colorimetry with one, two and four infiltrations into normal cartilage. Nevertheless, the duration of the experiment evaluating the animals eight days after the last infiltration must be taken into account. The use of a corticosteroid that remains longer within the joint will eventually cause medication buildup, as new infiltrations are performed at a shorter time interval than needed for the medication to wear off. Thus, the study departs from a known starting dose, which is only accurate for the group infiltrated once. With the other groups it is difficult to determine the dose, due to the slow absorption. A decrease was observed in safranin O staining intensity in the cartilage matrix of rabbits infiltrated six and eight times at weekly intervals (figure 2). In three animals of the group infiltrated eight times which presented an increase in articular volume, synovial fluid culture was negative. Macroscopically, the plateaus of these animals revealed a greater accumulation of small areas of roughness on the medial plateau next to the ligament insertion.

In the present study, even though the rabbits were able to move in their cages, they did not engage in physical exercise. There was more intense degeneration in the cartilage tissue of rats whose knees were infiltrated with

corticosteroid and were made to exercise¹². Few studies exist showing the effects of the association of exercise and intraarticular corticosteroids.

The association of short-acting and long-acting corticosteroids did not produce diverging data from those found in the literature, since proteoglycan loss in the matrix occur at times and number of infiltrations comparable to those observed when drugs were administered alone. No cell injury was observed, although the interval between corticosteroid applications into the joint was too short for the type of substance employed, which is in agreement with the observations by other investigators^{13,14}.

The present study clearly demonstrates that one corticosteroid infiltration into the joint does not result in cell and/or matrix injuries. However, the repeated intraarticular use, without the due and necessary interval between infiltrations, leads to articular cartilage surface injury by loss of proteoglycans at the site. New studies considering the potency of the corticosteroid as well as a sufficient time interval for cartilage tissue recovery should be conducted.

In conclusion, the levels of articular corticosteroid conducive to chondroprotective effects can be lower than the dose normally used in infiltrations. The search for a lower dosage that would not impair protein synthesis too heavily and could at the same time block inflammatory processes is imperative for the treatment with this medication.

R E S U M O

Objetivo: Analisar os efeitos da injeção repetida de betametasona na concentração de proteoglicanos da cartilagem articular do joelhos normais de coelhos californianos de ambos os sexos. **Métodos:** Os animais foram randomizados em oito grupos de dez animais cada. Três grupos controle (injeção ou não de solução salina isotônica) e cinco grupos de estudo - doses terapêuticas, repetidas ou não, de betametasona injetadas no joelho direito de cada coelho, com intervalos semanais. Após oito dias da última injeção prevista, cortes histológicos da cartilagem das áreas de apoio dos platôs tibiais foram corados com hematoxilina e eosina para análise por microscopia óptica, e com safranina O para a pesquisa da quantidade de proteoglicanos. A intensidade da coloração da safranina O foi quantificada em aparelho de histomorfometria, composto por microscópio Olympus BX 50 e microcomputador com software Image Pro-plus 4.5.0. **Resultados:** Não houve diferenças nos animais que tiveram seus joelhos injetados com betametasona uma, duas e quatro vezes quando comparados com os grupos controle. Nos animais que receberam seis e oito aplicações a intensidade da coloração com safranina O reduziu-se significativamente ($p < 0,05$) quando comparada tanto com grupos controle quanto com os outros de estudo. **Conclusão:** Foi possível demonstrar redução da concentração de proteoglicanos na matriz cartilaginosa articular dependente do efeito deletério cumulativo das repetidas injeções intra-articulares de betametasona.

Descritores: Articulação do joelho. Proteoglicanos. Corticosteróides. Coelhos.

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