# Diacerein reduces the level of cartilage chondrocyte DNA fragmentation and death in experimental dog osteoarthritic cartilage at the same time that it inhibits caspase-3 and inducible nitric oxide synthase

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## Abstract

### Objective

*The primary objective of this study was to evaluate the* ex vivo *therapeutic efficacy of diacerein and its active metabolite, rhein, on osteoarthritic (OA) cartilage chondrocyte DNA fragmentation and death in the experimental canine model of OA. The study also aimed to explore the effect of the drug on the level of important factors involved in this phenomenon, i.e., caspase-3 and inducible nitric oxide synthase (iNOS).*

### Methods

*OA knee cartilage was obtained from dogs that had received surgical sectioning of the anterior cruciate ligament (ACL) and were sacrificed 12 weeks after surgery. Cartilage explants were cultured in the presence or absence of therapeutic concentrations of diacerein (20 μg/ml) or rhein (20 μg/ml). Cartilage specimens were stained for TUNEL reaction and immunostained using specific antibodies for active caspase-3 and iNOS. Morphometric analyses were also performed.*

### Results

*In OA cartilage specimens, a large number of chondrocytes in the superficial layers stained positive for TUNEL reaction. Treatment with therapeutic concentrations of diacerein (20 μg/ml) or rhein (20 μg/ml) significantly reduced the level of chondrocyte DNA fragmentation to about the same extent in both treatment groups ($P < 0.006$, $P < 0.002$, respectively). The levels of caspase-3 and iNOS in cartilage explants were also significantly decreased (caspase-3, diacerein $P < 0.04$; caspase-3, rhein $P < 0.0003$; and iNOS, rhein $P < 0.009$, respectively) when compared to the control group.*

### Conclusions

*This study shows that diacerein/rhein can effectively reduce the level of OA chondrocyte DNA fragmentation and death under the present experimental conditions. This effect is mediated by a decrease in the level of caspase-3 expression, which could possibly be related in part to the reduced level of iNOS and secondarily to NO production. These findings provide additional new information about the mechanisms of action of diacerein on the progression of OA.*

**Key words**
Diacerein, chondrocyte death, experimental OA.

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## Introduction

The morphological changes observed in the cartilage lesions of osteoarthritis (OA) include the gradual depletion of the extracellular matrix macromolecules and a decreased number of chondrocytes – the only cell type existing in cartilage (1-3). The loss of chondrocytes in OA is a complex process likely related, at least in part, to both necrosis and apoptosis (4-6). A number of studies have explored the mechanisms possibly involved in the induction of OA chondrocyte death/apoptosis.

Recent studies point to the very significant role of the excess production of nitric oxide (NO) and reactive oxygen species in OA cartilage chondrocyte and synoviocyte death/apoptosis (7-10). A number of studies have demonstrated the essential roles played by NO in OA chondrocyte death *in vivo* (4, 9, 11). This phenomenon seems related to the upregulation of inducible nitric oxide synthase (iNOS) gene expression by inflammatory mediators such as pro-inflammatory cytokines. Treatment with a selective and potent inhibitor of iNOS, N-iminoethyl-1-lysine (L-NIL), was demonstrated to reduce the level of chondrocyte death *in vivo* in the experimental dog model of OA (9). Moreover, this study also demonstrated a positive correlation between the level of active caspase-3 and cell death, which provided an additional proof for the role of apoptosis in OA chondrocyte death. In addition to this finding, a recent *in vitro* study using dog OA cartilage explants also strongly supports the activation of the caspase cascade, namely caspase-9 and -3, as being linked to chondrocyte death/apoptosis (12).

Diacerein has been demonstrated to effectively reduce both the progression of cartilage degradation in experimental OA and joint space narrowing in human hip OA patients (13-15). A number of *in vitro* studies using human OA chondrocytes have demonstrated that the effect of diacerein/rhein could be mediated by the inhibition of a number of catabolic factors, including the synthesis of interleukin-1 (IL-1), metalloproteases and NO (16-18). Based on these findings, there is a very strong rationale for the effect of these drugs to be possibly mediated by the inhibition of chondrocyte death/apoptosis, a phenomenon that is now believed to be involved in the progressive loss of cartilage in OA. Moreover, the fact that diacerein/rhein has been shown to be a potent inhibitor of NO synthesis (17) makes this hypothesis even more attractive. Thus, there are a number of very strong arguments favoring the further exploration of the effect of diacerein/rhein on the pathophysiology of OA by examining the modulation of chondrocyte apoptosis by this drug.

The aims of this study were first to explore *ex vivo* the effect of therapeutic concentrations of diacerein/rhein on chondrocyte death by apoptosis in experimental dog OA cartilage, and secondly to explore the effect of the drug and its active metabolite on the level of iNOS and caspase-3.

## Materials and methods

### *Experimental group*

Nine adult crossbred dogs (2-3 years old), each weighing 20-25 kg, were used. Surgical sectioning of the anterior cruciate ligament (ACL) of the right knee through a stab wound was performed as described (19). Before surgery, the animals were anesthetized intravenously with pentobarbital sodium (25 mg/kg) and intubated. After surgery, the dogs were sent to a housing farm where they were free to exercise in a large pen. The dogs received no treatment and were sacrificed 12 weeks after surgery.

### *Cartilage explant sampling and culture*

Immediately after the dogs were killed, the right knees were dissected aseptically. Each knee was examined for gross morphologic changes. A total of 13 cartilage specimens were obtained by aseptically dissecting full thickness strips of cartilage across the margin of the lesioned areas of articular cartilage from femoral condyles and tibial plateaus, which were processed for culture. Each cartilage specimen was cut into 3 explants (roughly 150 mg each), rinsed several times in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL

Life Technologies, Burlington, ON, Canada), and incubated for 48 hours at 37ºC in a humidified atmosphere of 5% $CO_2$/95% air in a medium containing DMEM, 10% heat inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT, USA), and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin) under the following experimental conditions: (1) absence of the drug; (2) in the presence of therapeutic concentrations (Cmax) of diacerein (20 µg/ml); or (3) rhein (20 µg/ml) (Laboratoires Negma-Lerads, Toussus-Le-Noble, France).

*In situ detection of DNA fragmentation (TUNEL)*

Cartilage explants were fixed in Tissufix #2 (Laboratoire Gilles Chaput Inc., Montreal, PQ, Canada) for 24 hours and embedded in paraffin. Sections (5 µm) of paraffin-embedded explants were placed on Superfrost Plus slides (Fisher Scientific, Nepean, ON, Canada), deparaffinized in toluene, hydrated in a graded series of ethanol, and preincubated with chondroitinase ABC (0.25 units/ml; Sigma-Aldrich Canada, Oakville, ON, Canada) in phosphate buffered saline, pH 8.0, for 60 minutes at 37°C. Slides were further incubated with 0.3% Triton X-100 (30 minutes) followed by a digestion with proteinase K (20 µg/ml; Sigma-Aldrich, Canada) for 15 minutes at room temperature. The sections were washed in phospate-buffered saline (PBS) containing 2% $H_20_2$ for 5 minutes to inactivate endogenous peroxidase.

*In situ* analysis of apoptosis was performed using the Apoptag kit (Intergen Company, Purchase, NY, USA). After washes in PBS, sections were left for 5 minutes in an equilibration buffer. The sections were then incubated at 37ºC for 1 hour with TdT in reaction buffer (ratio of 70% reaction buffer to 30% TdT enzyme). Slides were washed for 10 minutes in stop-wash solution and then incubated for 30 minutes at room temperature with anti-digoxigenin peroxidase conjugate. The detection was performed using diaminobenzidine (Vector Laboratories, Burlingame, CA, USA), and the sections were counterstained with eosin.

*Immunohistochemical studies*

Cartilage explants were processed for immunohistochemical analysis as described (20). Briefly, sections (5 µm) of paraffin embedded explants were placed on Superfrost Plus slides, deparaffinized in toluene, rehydrated in a series of graded dilutions of ethanol, and preincubated with chondroitinase ABC (0.25 units/ml) in PBS, pH 8.0, for 60 minutes at 37°C. For caspase-3, sections were incubated in citrate buffer, pH 6.0 at 65°C for 20 minutes. The sections were washed in PBS, then in 2% hydrogen peroxide/PBS for 30 minutes. Sections were further incubated with universal blocking solution (DAKO Diagnostics, Mississauga, ON, Canada) for 30 minutes at room temperature, then blotted and overlaid for 18 hours at 4°C in a humidified chamber with: (1) a rabbit polyclonal antibody (IgG) against iNOS (200 µg/ml, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA); or (2) a rabbit polyclonal antibody (IgG) anti-caspase-3 (100 µg/ml, dilution 1:200, R&D Systems, Minneapolis, MN, USA), which recognized only the mature form (p20 subunit) of the enzyme.

Each slide was washed three times in PBS pH 7.4 and stained using the avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories). This method entails incubations in the presence of the biotin conjugated secondary antibody for 45 minutes at room temperature followed by the addition of the avidin-biotin-peroxidase complex for 45 minutes. All incubations were carried out in a humidified chamber, and color was developed with 3,3'-diaminobenzidine (Vector Laboratories) containing hydrogen peroxide. The slides were counterstained with eosin.

To determine the specificity of staining, three control procedures were used, according to the same experimental protocol: (1) use of adsorbed immune serum (2 hours at 37ºC) with a 50-fold molar excess of recombinant or purified antigen; (2) omission of the primary antibody; and (3) substitution of the primary antibody with an autologous preimmune rabbit IgG immunoglobulin. The purified antigens used in our study were iNOS blocking peptide (Santa Cruz Biotechnology) and rhcaspase-3 (Upstate Biotechnology, Lake Placid, NY, USA). The primary antibodies used in this study have been shown in previous studies to be immunospecific in dogs (9, 21).

Several sections were made from each block of cartilage, and slides from each explant were processed for immunochistochemical analysis. Each section was examined under a light microscope (Leitz Orthoplan) and photographed with Kodak Ektachrome 64 ASA film.

*Morphometric analysis*

Three sections from each explant were examined using a Leitz Diaplan microscope (x40), and each section was scored separately. These data were then integrated as a mean for each explant. The number of chondrocytes staining positive in the superficial zone (superficial and upper intermediate layers) of cartilage for TUNEL reaction, iNOS, or caspase-3 was estimated as described (9, 20). Briefly, each section was divided into three different areas at the superficial zone of cartilage. For each explant, it was ensured before the evaluation that an intact cartilage surface could be detected for use as a marker to validate the morphometric analysis.

The total number of chondrocytes staining positive were quantified separately. The final results were expressed as the percentage of positive chondrocytes (mean± SEM). The maximum score for each cartilage explant was 100%. Each slide was evaluated by two independent observers under blinded conditions; variations between the observers' findings were < 5%.

*Statistical analysis*

All data were expressed as mean ± SEM and analyzed with Mann-Whitney U test when appropriate. P values < 0.05 were considered significant.

## Results

*TUNEL reaction* (Figs. 1 and 2)

In OA (control) cartilage explants, a large number of chondrocytes showed a positive reaction for TUNEL, particularly in the superficial layers, and to a lesser extent also in the deep layers of OA cartilage. These results are similar

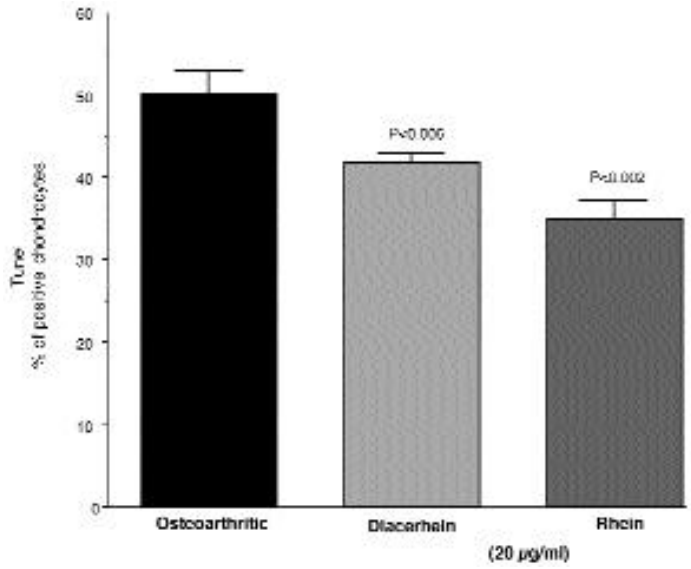


**Fig. 1.** Effect of diacerein/rhein on the level of chondrocyte DNA fragmentation (TUNEL reaction) in OA dog cartilage explants. Each specimen (n = 13) was divided into 3 explants and incubated in the absence (OA) or presence of the drugs at the indicated concentrations. The level of TUNEL positive cells was determined as described in Materials and Methods. Bars show the mean ± SEM percentage of positive chondrocytes. P values indicate the difference versus OA control (Mann-Whitney U test).

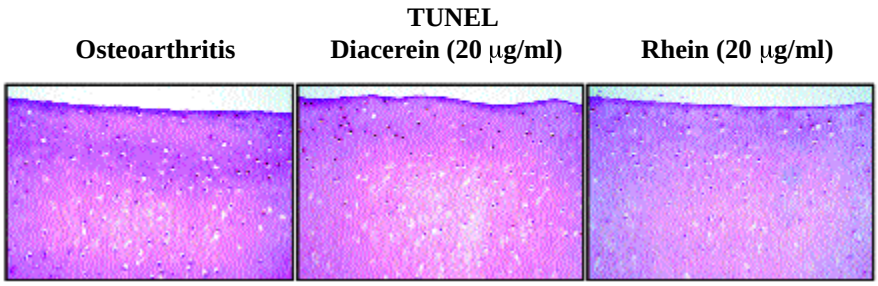


**Fig. 2.** Representative sections of OA cartilage explants showing *in situ* detection of chondrocyte death by TUNEL reaction. Explants were incubated in the absence (OA) or presence of diacerein (20 µg/ml) or rhein (20 µg/ml) (original magnification X 100).

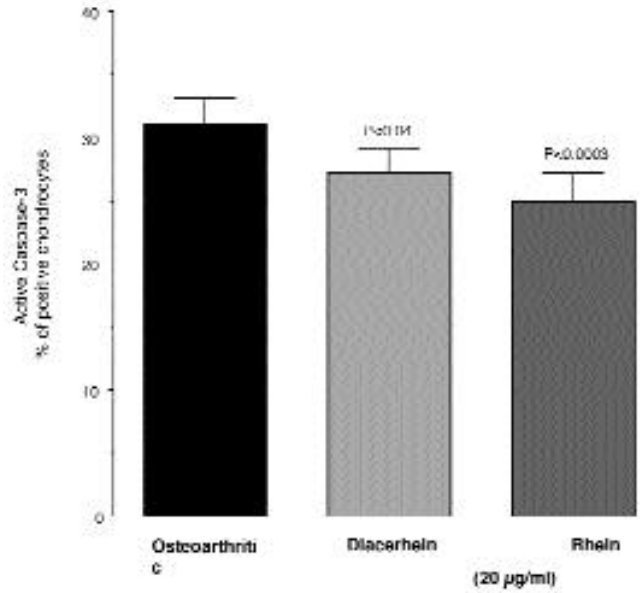


**Fig. 3.** Effect of diacerein and rhein on the level of active caspase-3 in OA dog cartilage explants. Each cartilage specimen (n = 13) was divided into 3 explants and incubated in the absence (OA) or presence of the drugs at the indicated concentrations as described in Materials and Methods. The level of caspase-3 was determined as described in Materials and Methods. Values are mean ± SEM percentage of caspase-3 positive chondrocytes. P values indicate difference versus OA control (Mann-Whitney U test).

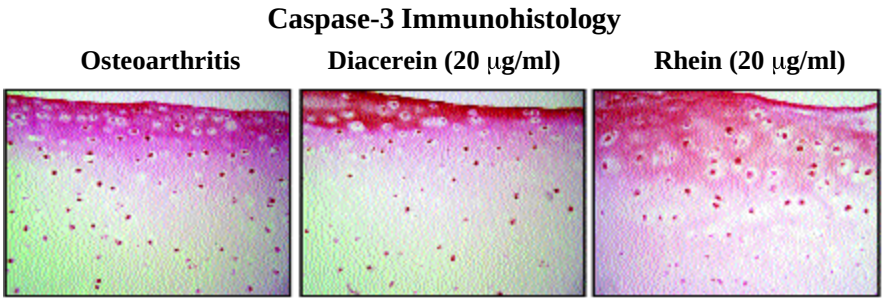


**Fig. 4.** Representative sections of OA cartilage explants showing *in situ* detection of chondrocyte death by active caspase-3. Explants were incubated in the absence (OA) or presence of diacerein (20 µg/ml) or rhein (20 µg/ml) (original magnification X 100).

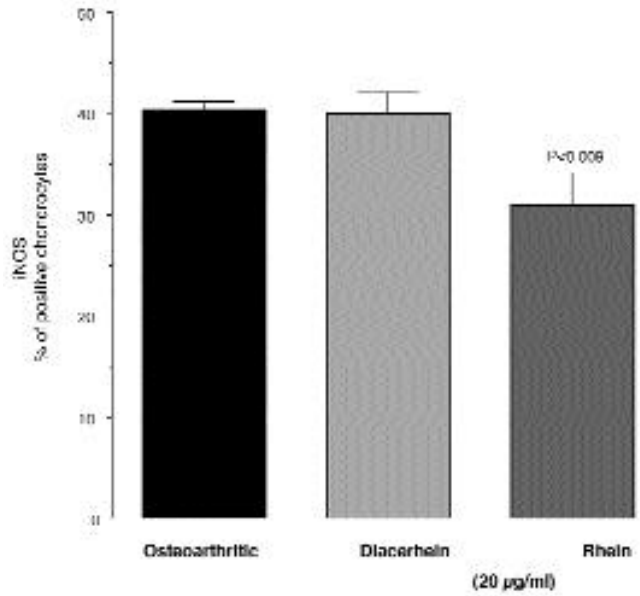


**Fig. 5.** Effect of diacerein and rhein on the level of chondrocyte iNOS in OA dog cartilage explants. Cartilage specimens (n=13) were divided into three explants and incubated in the absence (OA) or presence of the drugs at the indicated concentrations. The level of iNOS apoptosis was determined as described in Materials and Methods. P values indicate the difference versus OA control (Mann-Whitney U-test).

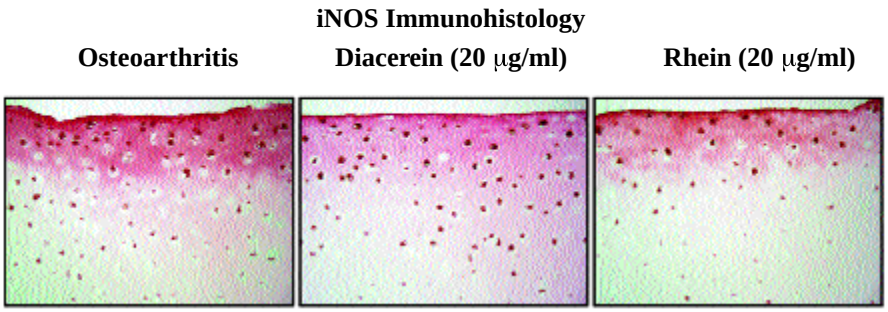


**Fig. 6.** Representative sections of OA cartilage explants showing *in situ* detection of chondrocyte death by iNOS. Explants were incubated in the absence (OA) or presence of diacerein (20 µg/ml) or rhein (20 µg/ml) (original magnification X 100).

to those obtained in our previous studies (9, 12). Moreover, we previously demonstrated that in experimental dog OA cartilage, a much higher level of TUNEL positive cells is found in OA compared to normal cartilage (9). Our present experiments demonstrated that the level of chondrocytes staining positive for TUNEL reaction in the explants was significantly reduced by treatment with either diacerein ($p < 0.006$) or rhein ($p < 0.002$). However, the extent of inhibition was somewhat more pronounced for those explants incubated with rhein.

*Level of active caspase-3*
(Figs. 3 and 4)
A large number of cells staining positive for caspase-3 were found in the superficial layers of the untreated OA (control) explants. The level was found to be similar to the one previously reported under *in vivo* and *ex vivo* conditions in the experimental dog model (9, 12). Treatment with either diacerein or rhein reduced the level of active caspase-3 found in the explants ($p< 0.04$, $p < 0.0003$, respectively).

*Levels of iNOS* (Figs. 5 and 6)
The level of iNOS positive cells in the untreated OA (control) explants was estimated at 40%. Again, this level is very similar to the one reported in previous studies in dog OA cartilage and higher than the level found in normal cartilage (9,12). Rhein, but not diacerein, markedly and significantly reduced the level of iNOS positive cells in the explants ($p < 0.009$).

## Discussion

This study provides new and interesting data about the effect of diacerein/rhein on OA cartilage metabolism. It shows that the drug has the potential to reduce the level of programmed cell death in OA chondrocytes by reducing the level of iNOS and active caspase-3. The finding, in addition to the results of previous studies exploring the effect of diacerein/rhein on the catabolic pathways of OA, provides interesting and insightful information about the mechanisms by which this drug could exert its effects on the OA disease process.
The structural changes taking place during the evolution of OA are multiple and affect all tissues within the joint structure (1). Nevertheless,the progressive degradation of the articular cartilage is one of the most significant changes responsible for not only many of the symptoms of the disease but are also likely intimately involved in the perpetuation of the disease process (1, 2). One of the important reasons explaining the interest in studying the effects of diacerein on the major OA pathophysiological pathways can be found in the recently published three-year study by Dougados *et al.* (13) showing that this drug could reduce the progression of joint space narrowing in hip OA patients.
The degradation of OA cartilage is related to multiple factors. The positive

effects of diacerein/rhein on this process have many potential explanations. A number of studies have been undertaken to explore the effects of this drug on OA pathophysiological pathways. These effects include, among others, the induction of synovial inflammation and the stimulation of proinflammatory cytokine synthesis, the activation of matrix metalloproteases (MMP), and a reduction in the synthesis of major cartilage matrix macromolecules (1). The proteolysis of the extra-cellular MMP by a number of proteases, including MMP, has been extensively documented (2). To that effect, diacerein/rhein has been demonstrated *in vitro* to be able to effectively reduce the synthesis of MMP by chondrocytes (16). Diacerein/rhein has been demonstrated to effectively downregulate the synthesis of the proinflammatory cytokine, IL-1, by human OA synovium, and it can also reduce the production of active IL-1ß by reducing the level of interleukin-1 converting enzyme (ICE) in OA chondrocytes (22).

In addition, the excess production of NO in OA cartilage is also believed to contribute to the catabolism of OA cartilage through a number of mechanisms (23). The increased level of NO and ROS are likely to contribute, although not as a sole factor (10,24),to the death (apoptosis) of chondrocytes. Diacerein/rhein has been demonstrated to reduce *in vitro*, in a cell culture system, the IL-1-induced iNOS synthesis level and activity in OA chondrocytes (17). If present *in vivo*, this effect contributes to decreasing the excess production of NO in OA cartilage.

The role of chondrocyte death by necrosis and/or apoptosis in cartilage degradation has been the subject of a number of interesting publications. The reduction in the number of living cells, particularly in fibrillated areas of cartilage, is likely to be an important local factor contributing to the loss of matrix. The exact importance of programmed cell death in the whole OA process is, however, still a subject of debate (6).

In studies done using OA cartilage specimens, this phenomenon seems to be more significant in areas with more severe lesions, and more specifically, in the superficial layers of cartilage (5, 8, 9). Based on several recent studies, there seems to be a higher level of chondrocyte apoptosis/death in experimental OA when compared to human OA (9,11). The likelihood, for instance, that in experimental dog OA, apoptosis was the predominant mechanism by which the chondrocytes died instead of necrosis was strongly suggested by the close correlation between the level of cell death (TUNEL reaction) and the level of active caspase-3 in these cells (9). This could possibly mean that this phenomenon has a higher contribution to cartilage damage in experimental OA. The exact reasons for this finding remain unknown but could possibly be related to the nature of the process itself, which is obviously a more acute and accelerated phenomenon.

The implication of the caspase cascade in cell death by apoptosis is well documented (25). The cytosolic aspartate-specific protease caspases involve a family of enzymes responsible for the "ordered" disassembly of cells (25). Caspase-8, -9, and -3 are the primary enzymes involved in cell apoptosis. These enzymes induce cell death by a number of mechanisms, including DNA fragmentation and inactivation of the proteins that protect cells against apoptosis (25,26). They are present in the cytoplasm as proenzymes and can undergo self-activation or can be activated in a cascade-like manner by enzymes with similar specificity. Caspase-9 is activated in response to agents or insults that induce the release of cytochrome c from the inner mitochondrial (27). Caspase-8 is activated by the cytoplasmic death domains of the receptors. Caspase-8 and -9 can activate caspase-3, which could amplify the caspase-8 and -9 signals. Upon activation, caspase-3 cleaves vital intracellular proteins and additional caspases.

The findings in the present study concerning the level of chondrocyte death/ apoptosis in dog OA explants are well in line with our previous report (12). Both diacerein and rhein were capable of reducing the level of cell death/ apoptosis, and quite interestingly, the effect of rhein, the active metabolite of diacerein, was more pronounced. This effect was also very well correlated with the capacity of these drugs to reduce the level of active caspase-3. This finding is a very strong argument supporting the effect of diacerein/rhein on the level of chondrocyte death being mainly related to a reduction in the level of programmed cell death (apoptosis).

Treatment with rhein was found to also significantly reduce the level of iNOS and therefore should have also reduced the production of NO. In fact, a previous report has demonstrated the ability of diacerein/rhein to reduce IL-1-induced NO production (18, 21). The finding of the present study showing that diacerein could reduce the level of chondrocyte apoptosis even in the absence of reduction in the iNOS level by the drug may indicate that under the present experimental conditions, diacerein has a preferential effect at the post-translational level. These findings provide additional support for the hypothesis that the reduction in NO production by diacerein/rhein could be one of the mechanisms by which this drug reduces chondrocyte apoptosis. Reports indicate that NO could induce cell death through a number of mechanisms that include direct DNA damage, generation of peroxynitrite, and the inactivation of antioxidant enzymes (28-31). Moreover, and nicely related to our previous finding on active caspase-3, NO might induce apoptosis via mitochondrial damage and cytochrome c release, leading to the activation of the caspase cascade enzymes (32). The reduction in the level of chondrocyte death/apoptosis by diacerein in the absence of a reduction in the level of iNOS may very well indicate that, as reported, NO is probably not the sole factor involved in initiating chondrocyte apoptosis (7, 10, 24).

In summary, this study demonstrated that under the present *ex vivo* experimental conditions, diacerein/rhein can reduce the death/apoptosis of chondrocytes in OA cartilage, which is likely related to the inhibition of iNOS and NO production and secondarily, the activation of the caspase cascade. This phenomenon could be related in part, but not solely, to a reduction in the level

of iNOS. These findings provide interesting new information about potential mechanisms of action of diacerein/rhein on the progression of OA.

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