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Suppression of Human Cartilage Degradation and Chondrocyte Activation by a Unique Mineral Supplement (SierraSil™) and a Cat's Claw Extract, Vincaria®

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ABSTRACT

Cartilage degradation, a hallmark of both rheumatoid arthritis and osteoarthritis, contributes to the dysmobility, pain and compromised quality of life associated with these conditions. We investigated the hypothesis that the unique clay-based mineral supplement SierraSil™ alone, and in combination with an extract of cat's claw, Vincaria,® could limit human cartilage degradation-activated chondrocytes. The investigative model used was human cartilage tissue, obtained at the time of knee surgery, studied in vitro. Cartilage explants were used to quantify cartilage matrix degradation, and for obtaining human chondrocytes that were evaluated in cell culture. SierraSil was subjected to neutral, alkali, and acid washes, followed by neutralization before addition to cartilage explants or cultured chondrocytes (0.05, 0.1, and 0.2 μg/ml). Vincaria, an alkaloid depleted aqueous extract of cat's claw (*Uncaria guianensis*) was studied in combination with SierraSil (final concentrations of 2.5, 5 and 10 ng/ml). Chondrocytes were activated with the addition of the inflammatory cytokine interleukin-1β (IL-1β, 5 ng/ml). Measured outcomes were media nitrate/nitrite levels as an index of nitric oxide production, and media glycosaminoglycan (GAG) concentrations as an index of matrix breakdown. Following neutral or alkali washes, SierraSil was ineffective in reducing nitric oxide release, although a small reduction in GAG release was observed with neutral extracts ($p<0.05$). However, the combination of SierraSil + Vincaria significantly reduced both GAG and nitric oxide release under these conditions. Following an acid wash to mimic passage of the material through the stomach, SierraSil alone significantly reduced IL-1β-induced GAG release by 68-73% ($p<0.01$) and SierraSil + Vincaria by 58-77% ($p<0.01$). Production of NO by human chondrocytes was also reduced by acid-washed SierraSil alone ($p<0.05$) and was more pronounced with the SierraSil + Vincaria combination ($p<0.01$). IL-1β-induced nitric oxide production and GAG release is known to reflect the activation of inducible pathways (inducible nitric oxide synthase and matrix metalloproteases). The attenuation of these events suggests that this herbo-mineral combination limits cartilage destruction by curtailing these transcriptional events in chondrocytes. Results suggest that this nutraceutical-based therapeutic agent may offer a new approach to limiting joint destruction and dysmobility associated with arthritis.

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INTRODUCTION

Rheumatoid and osteoarthritis are inflammatory states that exert catabolic actions on cartilage.[1,2] Activation of chondrocytes by various inflammatory signals results in the digestion of cartilage matrix and release of the fragments, leading to an overall reduction in cartilage tissue. A primary initiator of cartilage destruction is the cytokine IL-1β, which is produced by activated synovium, infiltrating macrophages, and activated chondrocytes.[3,4] IL-1β is a potent catabolic agent in arthritis, and approaches that can attenuate these catabolic pathways are sought as potential treatments in the management of arthritis and joint dysfunction.[5,6]

Diverse cascades of events are activated by IL-1β in chondrocytes. One well-described pathway is enhanced NO production, which results in transcriptional activation of inducible nitric oxide synthase (iNOS).[7,8] Matrix metalloproteases (MMPs)[9-11] are also transcriptionally regulated and activated by IL-1β. MMPs are enzymes that actively digest the cartilage matrix, releasing degraded glycosaminoglycans.

The nutraceuticals glucosamine and chondroitin are also cartilage matrix elements, essentially bricks in the wall that is cartilage. The popularity of glucosamine and chondroitin as therapeutic agents for arthritis reflects the contention that oral ingestion and absorption of these matrix elements will replace that which is lost to the catabolic inflammatory process.[12] However, this replacement process is not well regulated by the simple ingestion of substrate, and is fighting against a tide of catabolic release. It is assumed that ingested elements will be absorbed and transported to the site of destruction and inserted into the cartilage at a rate that exceeds the rate of loss. For this reason, therapeutic approaches with improved response rates, faster onset of benefits, and enhanced overall effectiveness are desirable.[12-14]

We have shown in a double-blind placebo-controlled trial[15] that Vincaria, an extract of *Uncaria guianensis*, which is devoid of immune-enhancing alkaloids, is an effective treatment for osteoarthritis. In this study, benefits were significant within a week and were further enhanced with continued administration. This was an extension of a number of pre-clinical studies that demonstrated that cat's claw extracts were cytoprotective and anti-inflammatory agents.[16-19] What was clearly evident was that these actions resulted from the ability of cat's claw to negate redox events, particularly activation of the transcription factor NF-κB.

NF-κB regulates over 30 genes associated with inflammation, including chemokines and adhesion molecules, but those of particular relevance to cartilage are inducible nitric oxide synthase (iNOS) and MMPs. We had previously shown that cat's claw was an effective inhibitor of iNOS expression and thereby suppresses the elevation of NO production associated with inflammation.[20-22] We were also the first group to demonstrate that selective inhibition of iNOS could prevent chronic inflammation and associated cell death and tissue destruction.[23,24] Perhaps even more impressive is the observation that cat's claw is the most potent natural substance for preventing the activation of tumor necrosis factor a (TNFα),[16,19] which has a primary role in rheumatoid arthritis and is the focus of antibody-based therapies (Remicade® and Enalabrel®). However, while NF-κB also regulates formation and activity of MMPs, a direct link between cat's claw and MMPs had not been reported.

The human cartilage explants and chondrocyte culture models were chosen because they (a) accurately reflect the inflammatory processes in the disease setting, (b) could be well controlled, and (c) had a significant background perspective with other botanical-based transcriptionally-active agents like green tea polyphenols.[25,26] We had shown that the epigallocatechin gallate (EGCG) was a potent inhibitor of IL-1β-induced MMPs activity, nitric oxide production and iNOS induction, cyclo-oxygenase 2 induction (COX2), and various transcription factors in this model.[27] The important ramifications of these observations were then confirmed in animal models of arthritis.[25] This detailed background allowed us to not only directly test the hypothesis but also to frame these results in a manner that would assist in follow-up clinical trials.

NF-κB and related transcription factors are activated by oxidants, and for this reason are deemed to be redox sensitive —the redox state of a molecule reflecting its oxidative state. Minerals, particularly transition metals, are redox active and can both promote and attenuate free radical and oxidant events,[28,29] depending on how electron transfer is manipulated. Anecdotal evidence suggested that the mineral-rich clay product SierraSil was effective in alleviating the symptoms of arthritis and inflammation. Further, there were suggestions that the onset benefits occurred with some relative rapidity, i.e., days/weeks as opposed to months for glucosamine and chondroitin. These observations suggested that a locus of action was different between these therapeutic approaches. This suggestion, combined with the appreciation that minerals can affect oxidant processes, led us to hypothesize that SierraSil may attenuate redox-based transciption events, which then formed the basis of our working hypothesis.

MATERIALS & METHODS

Reagents

Culture medium and reagents were purchased from either Cellgro (Mediatech, MD, USA) or GIBCO BRL (Bethesda, MD, USA). SierraSil™ (SM317) was obtained from the manufacturer, Sierra Mountain Minerals (Vancouver, Canada) and Vincaria® (RN180) cat's claw extract was supplied by Rainforest Nutritionals, Inc. (Phoenix, AZ). Recombinant IL-1β was purchased from R&D Systems (St. Paul, MN,).

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Culture of Human Chondrocytes

Human osteoarthritis cartilage samples were procured through the Cooperative Human Tissue Network with prior approval of the Institutional Review Board of University Hospitals of Cleveland. Chondrocytes were prepared by the enzymatic digestion of femoral head cartilage as previously described.[30,31] Chondrocytes were plated (1 x 10^6 cells/ml) in 35 mm culture dishes (Becton-Dickinson, Mountain View, CA, USA) in complete Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and were allowed to grow for 72 hours at 37°C and 5% CO2 in a tissue culture incubator. Chondrocytes were serum-starved overnight and then treated with IL-1β (5 ng/ml) and different doses of SierraSil (0.05, 0.1 and 0.2 μg/ml) or SierraSil + Vincaria, with final Vincaria concentrations of 2.5, 5, and 10 ng/ml. Media was collected after 12 hr for determination of nitrite and nitrate levels.

Culture of Cartilage Explants

Full thickness slices (20-25 mg) were obtained from human cartilage samples using sterile scalpel blades (Feather Razor Co., Osaka, Japan). Four to five cartilage pieces were transferred to each well of a 24-well, flat-bottomed plate (NUNC A/S, Roskilde, Denmark) containing DMEM supplemented with antibiotics and 10% fetal calf serum. These were repeated 2–3 times.The cartilage explants were treated with IL-1β alone or in combination with SierraSil (± Vincaria) for 72 hours. Controls included the culture of explants without either IL-1β or SierraSil. SierraSil was tested in the absence of IL-1β for any basal activity.

Quantitation of Glycosaminoglycans

At the conclusion of the explant culture period, the culture medium was collected from each group. A 50 μl aliquot was used to estimate total glycosaminoglycan concentration by a colorometric method employing DMMB as previously described (Farndale et al., 1986). Color intensity was read spectrophotometrically at 535 nm, and values derived from a standard curve derived from a different concentration of chondroitin sulfate. Results are expressed as micrograms of glycosaminoglycan released per mg of cartilage tissue.

Determination of Nitric Oxide Production

Media from cultured chondrocytes in the various treatment groups were collected and used to measure nitrite content using a commercially available kit (R&D Systems).

Wash/Extract Procedures

Preparation of acidic extracts of SierraSil ± Vincaria were made by mixing 0.4 g of the material with 0.1 N HCl (0.4% solution) followed by shaking for 30 minutes at room temperature. The pH was then restored to 7.0 by adding 1M NaOH and the final volume adjusted to 100 ml with deionized water.

Alkaline washes were made similarly to the acidic extracts. SierraSil ± Vincaria 0.4 g was mixed with 0.05 M NaOH and shaken for 30 minutes at room temperature. The pH was then adjusted to 7.0 with 1 M HCl. Neutral washes were made with deionized water.

All washes were centrifuged at 10,000 rpm for 1 minute to pellet all insoluble material and then sterilized by passage through a 0.45 micron filter under vacuum.

Statistical Analysis

Results were compared by a One-Way Analysis of Variance; when there was a significant variation between groups, then post-hoc evaluations were made using the Dunnett's Multiple Comparisons test. A significant difference was inferred if the p value was less than 0.05.

RESULTS

Neutral Washes

Cartilage explants and cultured chondrocytes were tested under basal and IL-1β-stimulated conditions where SierraSil ± Vincaria (0.05 and 0.1 μg/ml) were solubilized under neutral pH conditions. The results, summarized in Table 1, indicate that SierraSil alone had no effect on GAG release at the 0.05 μg/ml dose and a small but significant reduction at the 0.1 μg/ml dose. In contrast, SierraSil elevated NO release above that evident with IL-1β at both doses ($p<0.01$). The combination of SierraSil + Vincaria significantly attenuated NO production when compared to SierraSil alone at both concentrations ($p<0.01$) and was significantly different from IL-1β at the 0.1 μg/ml dose ($p<0.01$). Basal NO or GAG release was unaffected by SierraSil or SierraSil + Vincaria, at the highest concentration tested (data not shown).

Table 1. Influence of SierraSil and SierraSil + Vincaria on NO production and GAG release from human cartilage under neutral pH extraction procedures.

	Control	IL-1β	IL-1β + SierraSil 0.05 μg/ml	IL-1β + SierraSil 0.1 μg/ml	IL-1β + SierraSil + Vincaria 0.05 μg/ml	IL-1β + SierraSil + Vincaria 0.1 μg/ml
NO	0.06 ± 0.03	0.105 ± 0.001	0.122 ± 0.001*	0.156 ± 0.001*	0.107 ± 0.001	0.097 ± 0.001*
GAG	0.980 ± 0.043	1.400 ± 0.138	1.438 ± 0.053	1.286 ± 0.025*	1.121 ± 0.017*	0.930 ± 0.006*

* $p<0.05$ vs. IL-1β

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Alkaline Washes

Cultured chondrocytes treated with alkali-extracted SierraSil were examined at the 0.05, 0.1, and 0.2 μg/ml concentrations for NO release. SierraSil alone had negligible effect on IL-1β- induced NO release, apart from a small but significant increase at the 0.1 μg/ml dose (Fig. 1). On the other hand, SierraSil + Vincaria significantly reduced NO release at the 0.1 and 0.2 μg/ml concentrations ($p<0.01$). This suppression of IL-1β-induced nitric oxide formation represented a 25 and 39% reduction respectively (Fig.1). Neither SierraSil nor SierraSil + Vincaria in the absence of IL-1β affected basal NO release at the highest concentration tested (data not shown).

Because of the minor effects on NO production, an evaluation of alkaline extracts of SierraSil on GAG release was not performed.

Acid Extraction

Acid washes to mimic passage through the stomach resulted in enhanced bioactivity of SierraSil (Fig. 2 and 3). In cultured human chondrocytes, acid washes of SierraSil resulted in a reduction in IL-1β-induced nitric oxide release at all concentrations tested ($p<0.01$). As observed with the other experimental conditions, the combination of SierraSil with Vincaria resulted in a further suppression of IL-1β-induced NO formation ($p<0.01$ vs. SierraSil alone and $p<0.001$ vs. IL-1β).

Cartilage breakdown induced by IL-1β was determined by GAG release and a significant suppression was observed at all concentrations examined for both SierraSil and SierraSil + Vincaria ($p<0.01$, Fig. 3). However, a clear dose-dependency was not evident over the range examined (0.05–0.2 μg/ml). The protection of cartilage breakdown approximated 68–83% for SierraSil and 58–77% for SierraSil + Vincaria.

DISCUSSION

Arthritic joints have high levels of pro-inflammatory cytokines that contribute to the pathophysiology by promoting cell recruitment, cell activation, and release of destructive matrix metalloproteases, and by preventing chondrocyte proliferation and extracellular matrix synthesis. These processes culminate in a loss of cartilage matrix and a progressive destruction of joint architecture, and together with symptoms (pain, discomfort) contribute to a compromised quality of life. Despite the enormous size of the osteoarthritis problem and pharmaceutical market, estimated at 12.38 billion dollars annually, current approaches provide symptomatic relief and do not arrest the disease process and loss of cartilage matrix. Based on the need for therapeutic alternatives, nutraceuticals are also used for treating osteoarthritis and represent approximately 640 million dollars in sales in the USA annually. The best known of these complimentary medicine approaches is glucosamine and chondroitin. However, this approach suffers from a slow onset of action, limited efficacy, and a low response rate.[12-14] Other approaches are less well appreciated by the consumer and health care providers but mechanistically offer greater appeal. For example redox-active botanicals possess the ability to prevent the activation of a litany of genes involved in the inflammatory process.

One of these classes of agents is the green tea polyphenols which affect signal transduction and limit the catabolic pathways described in this study.[16] Several papers extend these observations to various animal models.[26] Cat's claw is also a redox-sensitive transcription inhibitor, which limits the formation of Th1 cytokines like TNFa and oxidant-induced cell death.[16-19] Administration of alkaloid-depleted cat's claw has also been shown by us to be an effective clinical strategy in osteoarthritis, with benefits evident within a week as expected for an intervention that works by suppressing gene expression.[15] Indeed vincaria cat's claw is the most potent natural product inhibitor of TNFa formation reported to date with a EC_{50} of approximately 10 ng/ml.[16,19] This remarkable potency was confirmed in the present conditions where Vincaria cat's claw was combined with SierraSil over a concentration range of 2.5–10 μg/ml.

Despite this, however, cat's claw is sometimes erroneously termed an immune stimulant;[32-34] this misconception may account for its reduced popularity in treating osteoarthritis. There is limited evidence for any immune-activating actions of cat's claw at therapeutic doses, and this assertion has largely been attributed to the oxindole alkaloid fraction present in *Uncaria tomentosa*. In this study, we used an alkaloid-depleted cat's claw extract (Vincaria) to avoid this chemical confusion and to better link the present observations to the previous clinical observations in osteoarthritis.[15]

Matrix metalloproteases and inducible nitric oxide synthase are two pathways that lead to cartilage catabolism that are regulated at the transcriptional level by redox-sensitive transcription factors.[5-11,21,22] We had previously described Vincaria cat's claw as an effective inhibitor of iNOS gene expression and nitric oxide production, actions confirmed in this study. The present study also confirms that cat's claw is an effective inhibitor of cartilage degradation via MMPs. This assertion is indirect, although it is well appreciated that IL-1β-induced GAG release results from the formation and activation of MMPs.

SierraSil is a relatively new nutraceutical with little background information on efficacy and mechanisms of action, although there is anecdotal evidence that it is beneficial to arthritic patients. SierraSil is a mineral-rich clay product, similar but not identical to bentonite. Considering the variety and amount of its mineral constituents, we hypothesized that it may be acting via redox-sensitive pathways. The present results confirmed that hypothesis with a

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reduction in IL-1β-induced nitric oxide formation and GAG release. It was also evident that the SierraSil's active components, which currently are unknown, are more readily released into solution under acidic conditions than either alkaline or neutral pH conditions. Vincaria cat's claw was effective following acidic, neutral, and alkaline washes, although it was slightly less effective following alkaline washes. Clay products are noticeably insoluble in water, and these extraction processes may have released different amounts of the bioactives accounting for the variations in bioactivity. We interpret the current results as being reflective that doses of SierraSil that are used by consumers (2-3 g/day) will provide ample bioavailable bioactives for limiting joint destruction.

We appreciate that the present results were obtained in vitro, requiring that caution be used in extending these observations to the clinical setting. However, Vincaria has been shown to be effective in a double-blind placebo-controlled trial.[15] Both SierraSil and SierraSil + Vincaria were effective in this in-vitro model. Additionally, other interventions that limit these processes have also been effective in the clinical or experimental model setting.[25] Thus, within the constraints of caution, it is reasonable to assume that SierraSil may limit cartilage destruction and chondrocyte activation in clinical osteoarthritis. A clinical trial is currently underway to confirm this assertion, including its combination with Vincaria cat's claw.

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Figure 1. Effects of alkaline-extracted SierraSil and SierraSil + Vincaria on the production of nitric oxide from human chondrocytes. The * depicts a significant difference between the group and the IL-1β controls.

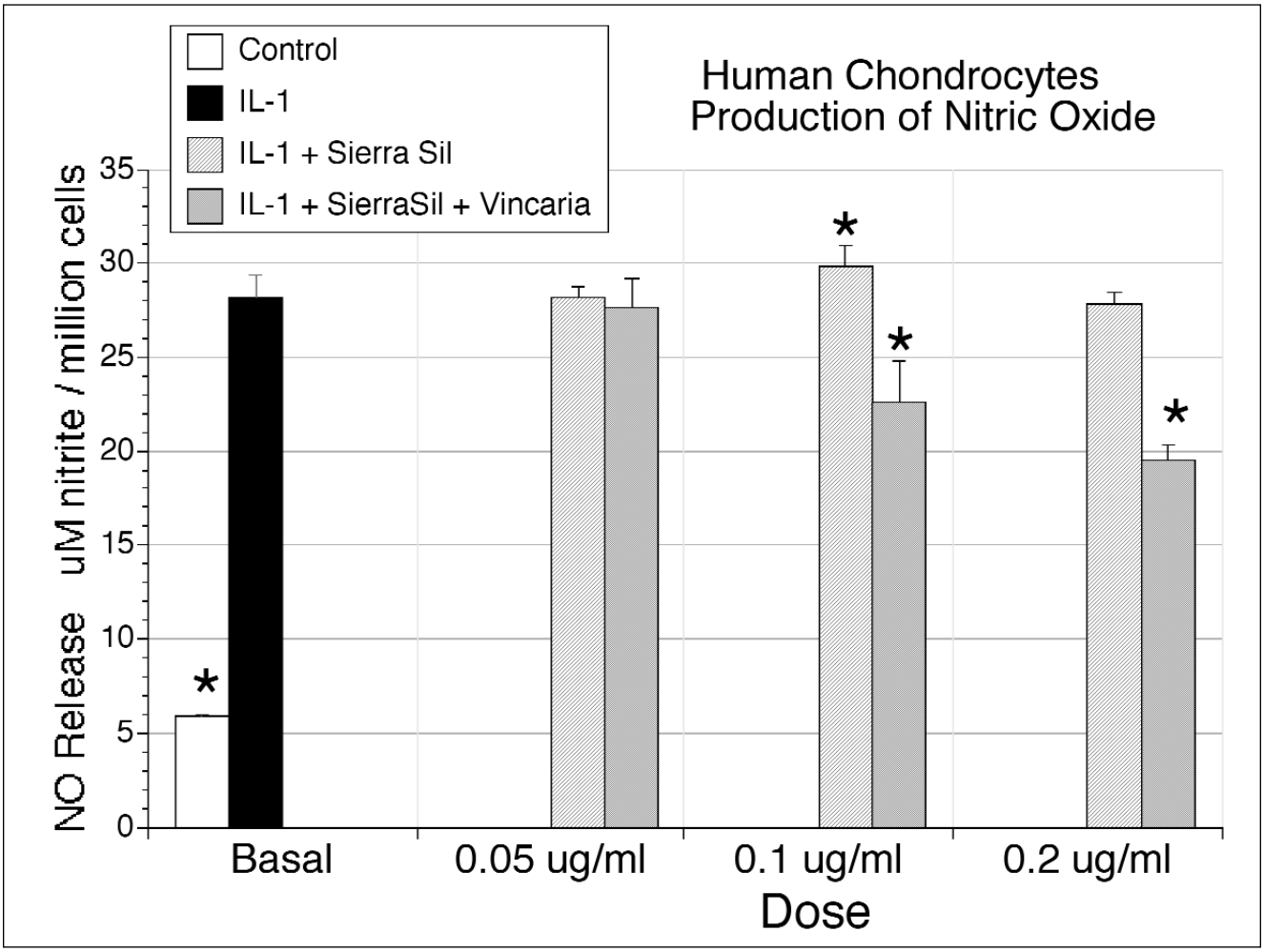

Figure 2. Effects of acid extracted SierraSil and SierraSil + Vincaria on the production of nitric oxide from human chondrocytes. The * depicts a significant difference between Il-1b and all groups ($p<0.01$), and ** a significant difference between SierraSil and SierraSil + Vincaria at comparable concentrations ($p<0.01$).

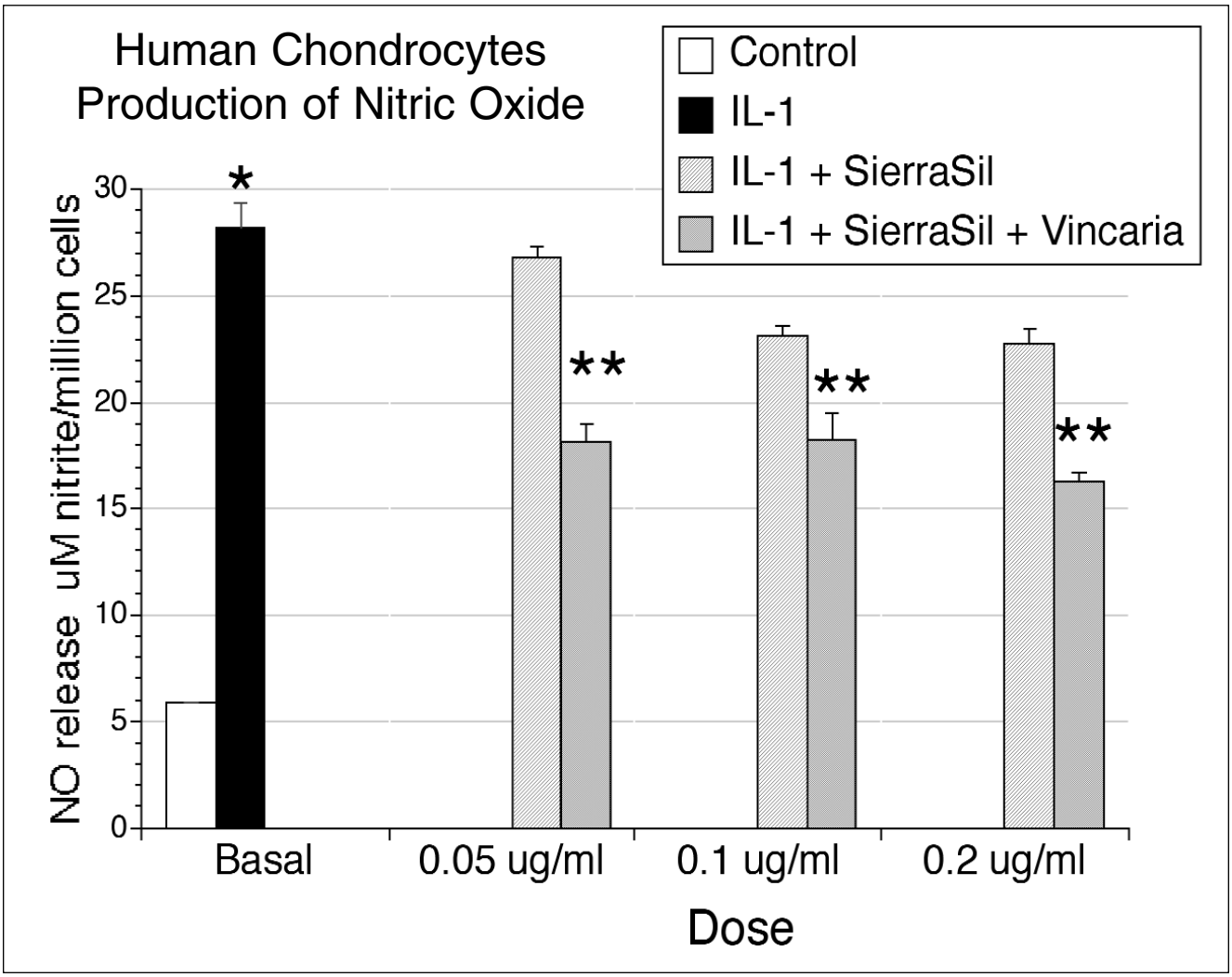

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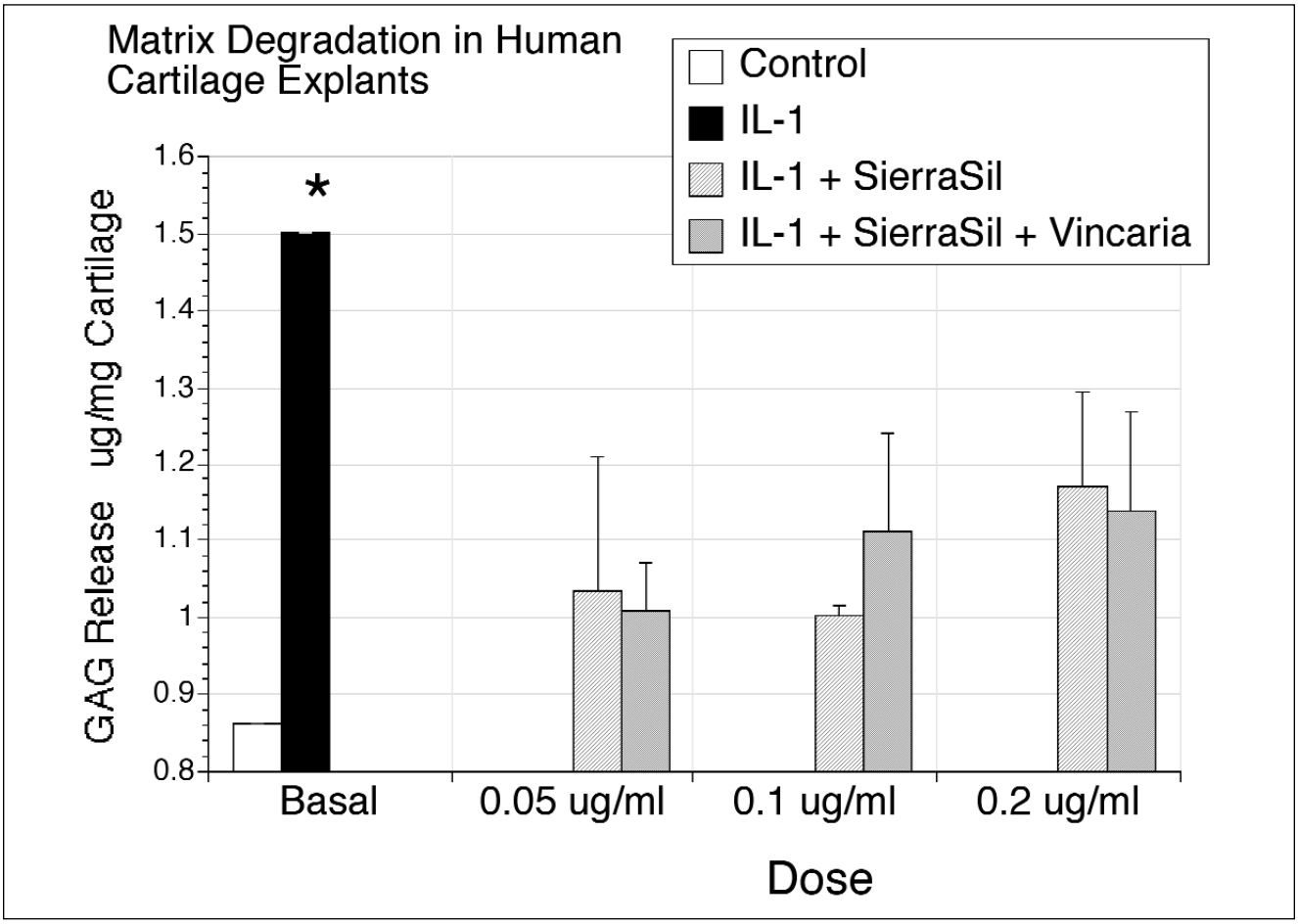
Figure 3. Effects of acid-extracted SierraSil and SierraSil + Vincaria on the release of GAG from human cartilage explants. The * depicts a significant difference between IL-1β and all other groups ($p<0.01$)



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