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Intra-articular mucilages: behavioural and histological evaluations for a new model of articular pain

Laura Micheli, Carla Ghelardini, Elena Lucarini, Carmen Parisio, Elena Trallori, Lorenzo Cinci and Lorenzo Di Cesare Mannelli

NEUROFARBA – Pharmacology and Toxicology Section, Department of Neuroscience, Psychology, Drug Research and Child Health, University of Florence, Florence, Italy

Keywords
articular pain; Complete Freund's Adjuvant; *Linum usitatissimum*; monoiodioacetate; mucilages

Correspondence
Lorenzo Di Cesare Mannelli, NEUROFARBA – Pharmacology and Toxicology Section, Department of Neuroscience, Psychology, Drug Research and Child Health, University of Florence, Viale Gaetano Pieraccini 6, 50139 Florence, Italy.
E-mail: lorenzo.mannelli@unifi.it

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Abstract

Objectives The creation of a new valid preclinical model of articular pain by the intra-articular (i.a.) injection of mucilages for the screening of new treatments against arthritis.

Methods A single intra-articular injection (20 μl) of mucilages (from *Althaea officinalis roots* and *Linum usitatissimun seeds*) or vegetal components (*Amorphophallus konjac* gum powder and β-glucan, used as reference standard) were assessed in the rat. The pathology progression was monitored by behavioural measurements (paw pressure test, von Frey test, incapacitance test and beam balance test) and compared to that induced by the i.a. injections of monoiodioacetate (MIA) and Complete Freund's Adjuvant (CFA), well-recognized models of osteoarthritis and rheumatoid arthritis, respectively.

Key findings Among all, the mucilage of *L. usitatissimun* showed the best proalgic profile inducing a painful long-lasting condition. Hypersensitivity was characterized as a mixed form of inflammatory and neuropathic pain by the responsiveness to ibuprofen (100 mg/kg, p.o.) and pregabalin (30 mg/kg, p.o.). The histological evaluation of joint showed a damage that represents both MIA and CFA features.

Conclusions In conclusion, a single i.a. injection of *L. usitatissimun* mucilage can represent a valid model to assess articular pain in the rat for the screening of new treatments against arthritis.

Introduction

Arthritic pain is common and is associated with worse functional outcomes and poorer quality of life when compared with a range of other chronic conditions.[1] A bewildering array of guidelines and other evidence-based resources are available, but the variability and the lack of therapeutic responses can lead to frustration and disappointment for both patients and health professionals.[2] Although the therapeutic armamentarium has increased considerably as a result of targeted modulation of factors central to immunological regulation, around one-third of patients respond insufficiently to any of the current treatments.[3] The lack of effective treatments for arthritic pain can be related also to the unsatisfactory available preclinical models to test the validity of treatments. The heterogeneity in clinical presentation requires animal models that can reproduce the pathophysiology of the human pathological condition for preclinical screening purposes.[4]

The aim of animal models that evoke arthritis is to mimic in a controlled way the scale and progression of joint damage. An ideal animal model is relatively inexpensive and displays reproducible disease progression with a magnitude of effect large enough to detect differences within a short period of time. If the model progresses too rapidly to end-stage degeneration, intermediate time points, which are representative of osteoarthritis or rheumatoid arthritis pathophysiology, may not be obtainable and in the absence of this information, subtle effects of potential interventions may be missed.[5] Another important aspect is the manageability and the essential safety, for operators and for the environment, of the substance used to induce the pathology. Stemming from the existence of models using substances which are safe when ingested or used in the food

and medical fields, but which cause inflammation if injected, we identified a few such substances and assessed their inflammatory potential upon intra-articular injection.

The aim of the present study was to assess a new animal model of articular pain in the rat to be used in parallel with the established model of osteoarthritis and of rheumatoid arthritis induced by intra-articular (i.a.) injection of MIA (monoiodoacetate) and CFA (Complete Freund's Adjuvant), respectively. The intra-articular injection allows to reproduce a localized pain and not systemic which can thus be compared to the human pathological condition. Moreover, this new model should share both inflammatory then neuropathic features being fundamental for the screening of new pharmacological treatments for the management of articular pain states. Starting from the safety requirements of the substance to be used, we looked for starting materials similar to carrageenan, a polysaccharide of known safety in food and medical settings,[6] high manageability, which induces 'foreign body' reaction when injected.[7] Similarly, safe polysaccharides are present in the mucilage of *Althaea officinalis* and *Linum usitatissimum*, as well as in *Amorphophallus konjac* gum powder. β-glucan, having reported action on the immune system activation was used as a reference polysaccharide. The hypersensitivity to mechanical noxious and non-noxious stimuli as well as motor functions were measured for 1 month. Histological evaluation was performed on the compound that showed the best proalgic profile and compared histologically to MIA and CFA.

Materials and Methods

Plant materials

Althaea mucilage change in has been produced from *Altaea officinalis* L. dried roots, cultivated and harvested in Italy by Aboca SpA Società Agricola. Linum mucilage change in has been produced from *Linum usatissimus* L. seeds, cultivated and harvested in Italy by Aboca SpA Società Agricola. *Amorphophallus glucomannan* gum powder (classification according to commission regulation (EU) No 231/2012: E425i) has been produced from *Amorphophallus konjac* K. Koch fresh tuber, cultivated in Cina and supplied by Xi'an Lanyor Biotech Co. Ltd, Shaanxi, China. (code KE90S).

Vegetal extracts production

The production process is characterized by extracting the roots of *A. officinalis* and the seeds of *Linum usitatissimun* with purified water (D/S 1:10). The production of *A. konjac* is characterized by milling the tuber followed by ethanol 20–70% (v/v) extraction (D/S 1/20-26). More details in the Supporting Information.

UHPLC-Q-ToF fingerprint method

Fingerprint analysis was performed by means of an UHPLC (Agilent 1290 Infinity II) coupled with *Dual AJS ESI* source Q-ToF (Agilent 6545). The UHPLC is equipped with a vacuum degasser, a binary pump, a Peltier thermostated autosampler at 10 °C, a Peltier thermostated column compartment. More details are provided in the Supporting Information.

Description of the instruments and of the instrumental conditions

The analysis of saccharides was performed on a gas chromatograph instrument equipped with a capillary column DB1 30 m, 0.25 mm, 0.25 μm (Agilent Technologies, Santa Clara, CA, USA) and with a FID detector (Agilent Technologies). More details are provided in the Supporting Information.

Determination of soluble and insoluble dietary fibre, (according to *AOAC 993.19 ed 17th 2003*)

The soluble and insoluble dietary fibre was performed according to AOAC 993.19 and AOAC 991.42, respectively. More details are provided in the Supporting Information.

Determination of saccharides

The samples (0.5 g) were extracted by means of hot water under stirring for 30 min. After filtration, the extract (20 mg) was added to the internal standard (inositol for monosaccharides, threalose for disaccharides) water was removed under vacuum and the dry residue was derivatized by means of trimethylsilylimidazole (100 μl) and analysed by means of GC-FID. The calibration curve range was from 25 to 1000 mg/l for both the saccharides. The detection limit was 0.1/100 g.

Determination of organic acids

The samples were extracted by means of water under stirring for 30 min at room temperature. After filtration, the extract was opportunely diluted and analysed by means of HPLC-DAD. The calibration curve range was till 400 mg/l for both the organic acid. The detection limit was 0.01 g/100 g.

Preparation of the solution for test on animals

The samples were prepared in a glass beaker using a mechanical stirrer (RW 20.n; IKA Labortechnik, Milan,

Italy). Each raw material was added slowly to the solvent, at room temperature, to obtain a homogeneous solution. The *Althaea mucilage* solution was prepared in saline solution at the concentration of 40% (p/p). The *Linum mucilage* solution was prepared in PBS solution at the concentration 10% (p/p). The *Amorphophallus glucomannan* solution was prepared in PBS solution at the concentration 1% (p/p).

Animals

For all the experiments described below, male Sprague Dawley rats (Envigo, Varese, Italy) weighing approximately 200–250 g at the beginning of the experimental procedure were used. Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines.[8] More details are provided in the Supporting Information.

Monoiodoacetate-induced osteoarthritis

Unilateral osteoarthritis was induced by injection of monoiodoacetate (MIA; Sigma-Aldrich, Milan, Italy) into the tibiotarsal joint.[9,10] More details are provided in the Supporting Information.

Complete Freund's Adjuvant-induced inflammatory arthritis

Articular damage was induced by injection of CFA (Sigma-Aldrich) into the tibiotarsal joint.[11,12] More details are provided in the Supporting Information.

Compounds-induced articular damage

Articular damage was induced by a single injection of *Althea* mucilage solution, *Linum* mucilage solution, *Amorphophallus* solution and β-glucan into the tibiotarsal joint. More details are provided in the Supporting Information.

Drug treatment

For the acute treatments, ibuprofen (100 mg/kg) and pregabalin (30 mg/kg) were suspended in 1% carboxymethylcellulose sodium salt (CMC; Sigma-Aldrich) and per os (p.o.) administered on days 3, 7, 14, 21, 28 from the intra-articular injection of compounds. Behavioural measurements were performed in time course every 15 min for 1 h. In the graphs are reported only the measures performed at 30 min after drugs administration.

Paw pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy) according to the method described by Leighton et al.[13] More details are provided in the Supporting Information.

von Frey test

The animals were placed in 20 × 20 cm plexiglass boxes equipped with a metallic meshy floor, 20 cm above the bench. A habituation of 15 min was allowed before the test. An electronic von Frey hair unit (Ugo Basile) was used: the withdrawal threshold was evaluated by applying force ranging from 0 to 50 g with a 0.2-g accuracy.[14] More details are provided in the Supporting Information.

Incapacitance test

Weight-bearing changes were measured using an incapacitance apparatus (Linton Instrumentation, Norfolk, UK) to detect changes in postural equilibrium after a hind limb injury.[15] More details are provided in the Supporting Information.

Beam balance

A balance beam test consisted of the rats being placed on a narrow strip of wood (30 × 1.3 cm) while balancing and the scoring standards were as follows: 0 point, the four limbs were all on the wood in a balance situations; 1 point, limbs of one side were able to grasp the wood or shake on the wood; 2 points, one or two limbs slipped from the wood; 3 points, three limbs slipped from the wood; 4 points, suspended on the wood and fell over after struggle.[16]

Rota rod test

Rota Rod apparatus (Ugo Basile) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into four equal sections by five disks. Thus, up to four rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod for a maximum of 600 s. After a maximum of six falls from the rod, the test was suspended and the time was recorded.

Histological evaluations

Histological evaluations were performed according to Snekhalatha *et al.*[17] More details are provided in the Supporting Information.

Statistical analysis

Each value represents the mean ± SEM of ten rats per group, performed in two different experimental sets. Groups were as shown in the figures. Behavioural and histological evaluations were performed by investigators who were blinded to the experimental groups. The analysis of variance was performed by ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison. *P* values of less than 0.05 were considered significant. Data were analysed using the 'Origin8.1' software.

Results

Extracts characterization

Althaea mucilage, *Linum* mucilage and *Amorphophallus* glucomannan were characterized by means of UHPLC-q-ToF acquiring the fingerprint of both negative and positive ions. As is possible to observe, negative ions are more abundant than positive ones; *Althaea* mucilage (Figure S1) and *Linum* mucilage (Figure S2) have more metabolites contrariwise to that of Amorphophallus glucomannan (Figure S3). This behaviour can be explained by the production process of the different matrices. *Amorphophallus* glucomannan is a high purified material responding to the regulatory specifications for food additive E425i.

The study of the composition of *Altheae* mucilage, *Linum* mucilage and *Amorphophallus* glucomannan is represented in Table 1. Compounds are rich in fibres, particularly the soluble one, than contained saccharides (especially sucrose) and organic acids as lactic acid and citric acid. *Altheae* mucilage and *Linum* mucilage contained 14.4% and 40.1% of fibres, 18.4% and 7.4% of saccharides and 3.8% and 6.8% of organic acids, respectively. *Amorphophallus* glucomannan contained 88.7% of fibres but no saccharides or organic acids (Table 1). Some small molecular weight metabolites present in the fingerprints of each matrices were identified and their concentration is reported in Table S1. It is possible to observe that their concentration is very low, below 0.03%. The matrices are very complex so actually is challenging the identification of all the molecular species, also because most of the compounds of our mucilage are polysaccharides, non-detectable by LC-MS and traced by the soluble fibre test (results reported in Table 1).

Table 1 Composition's study of vegetal extract used

Components	Compounds		
	Althaea officinalis roots mucil.	*Linum usitatissimum* seeds mucil.	*Amorphophallus konjac* flour
Fibres, total	14.4	40.1	88.7
Soluble	13.8	36.8	79.8
Insoluble	0.6	3.3	8.9
Saccharides, total	18.4	7.4	0.0
Sucrose	15.9	2.0 ± 0.1	0.0
Fructose	1.4	1.2	0.0
Glucose	1.0	0.0	0.0
Organic acids, total	3.8	6.8	0.0
Lactic acid	0.8	6.2	0.0
Citric acid	3.0	0.6	0.0

Behavioural measurements

All compounds (*Altheae* mucilage; *Linum* mucilage; *Amorphophallus* glucomannan; β-glucan; MIA and CFA) were intra-articularly (i.a.) injected once on day 0. Behavioural measurements were performed from day 0 (before i.a. administrations) until day 28. In Figure 1a the response to a noxious mechanical stimulus (paw pressure test) is shown. MIA and CFA significantly lowered rats' pain threshold from day 2 until day 28 in comparison to the control group (vehicle). *Linum* mucilage showed a similar algic profile, reducing the weight tolerated on the posterior paw for 28 days. In particular, the articular pain evoked by the i.a. injection of *Linum* mucilage was significantly higher with respect to MIA-treated rats on days 2, 4 and 22. *Althaea* mucilage significantly reduced the pain threshold from day 2 until day 14, then a recovery from articular damage was observed. β-glucan injection evoked an inflammatory state of the joint that prevented the measure with the paw pressure for the first week; starting from the second week, the algic profile evoked was similar to that induced by MIA injection. *Amorphophallus* glucomannan slightly reduced the pain threshold of the animals without reaching the statistical significance (Figure 1a). As measured by von Frey apparatus, MIA and CFA lowered the pain withdrawal threshold in response to a non-noxious mechanical stimulus until day 28 after i.a. injection. MIA peaked in severity on day 2 to progressively recover in the next days while CFA showed a constant pain withdrawal threshold from the day of injection until the end of the experiment (Figure 1b). *Linum* mucilage highlighted a similar allodynic profile to CFA, reducing the paw withdrawal threshold of the animals from day 2 to day 28. The articular pain evoked by i.a. injection of β-glucan was measurable from day 7 and remain statistically different from the control group until day 28, reproducing an articular pain comparable to CFA.

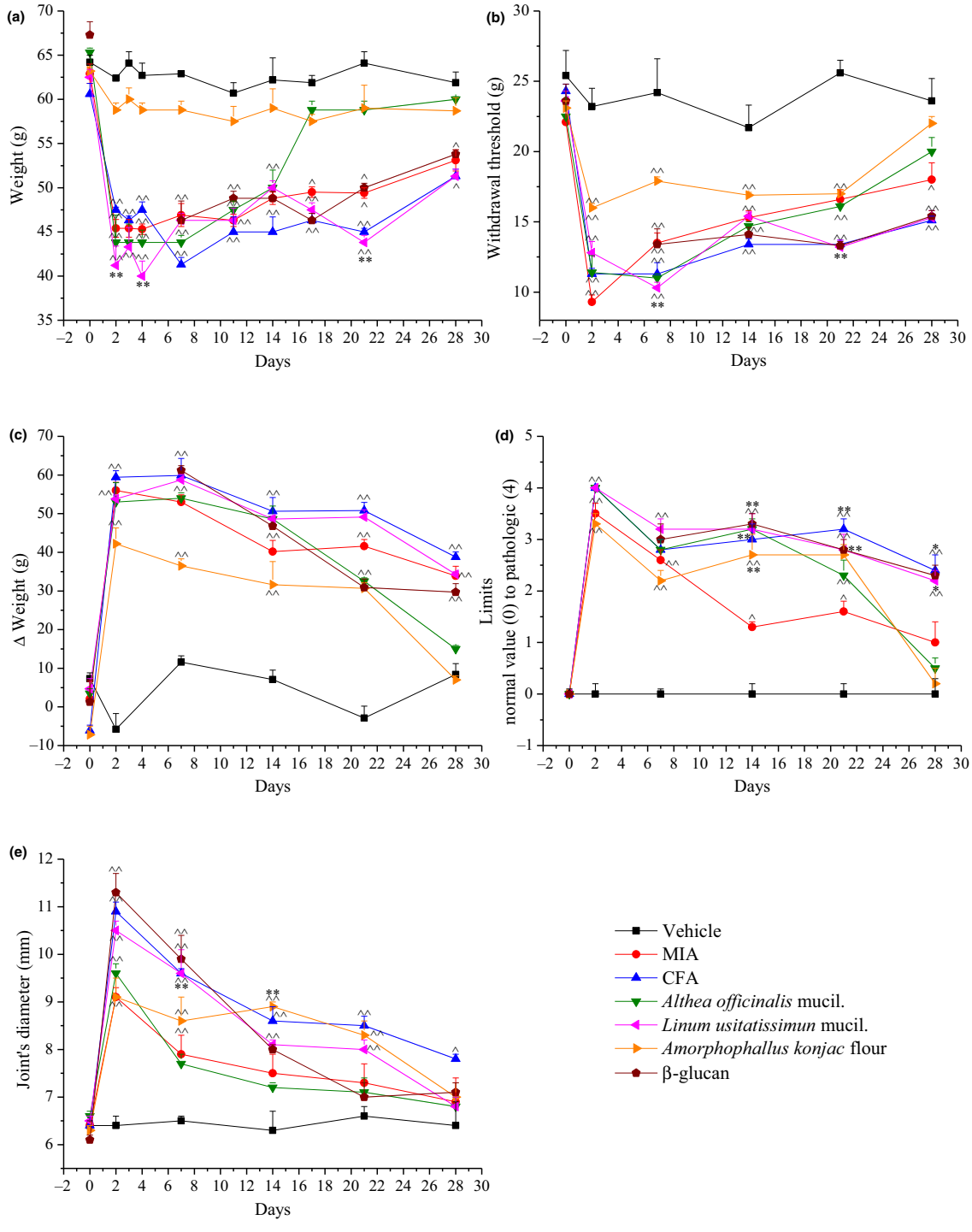

Figure 1 Articular pain, behavioural measurement. (a) Effect of intra-articular injection of compounds in the rat, response to a noxious mechanical stimulus (Paw pressure test); (b) effect of intra-articular injection of compounds in the rat, response to a non-noxious mechanical stimulus (von Frey test); (c) effect of intra-articular injection of compounds in the rat, measure of postural imbalance related to pain (Incapacitance test); (d) effect of intra-articular injection of compounds in the rat, measure of motor abilities related to pain (beam balance test); (e) effect of intra-articular injection of compounds in the rat, measure of the joint diameter. Twenty microliters of *Altheae* mucilage, *Linum* mucilage, *Amorphophallus glucomannan* and β-glucan were injected into the tibiotarsal joint of the rat on day 0. On the same day, two other groups of rats were intra-articularly injected with monoiodioacetate (2 mg/25 μl) and Complete Freund's Adjuvant (50 μl). Pain alterations were evaluated on days 0, 2, 3, 4, 7, 11, 14, 17, 21 and 28 after the intra-articular injection. Each value represents the mean of 10 rats per group performed in two different experimental set. $^{}P < 0.05$ and $^{^}P < 0.01$ vs vehicle group; $^{**}P < 0.01$ vs monoiodioacetate group.

Articular pain evoked by *Althaea* mucilage peaked between 2 and 7 days after injection and was reduced during the next days. *Amorphophallus* glucomannan slightly reduced the pain threshold of the animals during the first 2 weeks after injection in comparison to the others compounds (Figure 1b). Similar results were obtained in the Incapacitance test (Figure 1c). Both MIA and CFA increased the postural unbalance of the animals in comparison to the control group starting on day 2 until day 28 after treatment although CFA is more harmful. The Δ weight between the contralateral and the ipsilateral paw was also increased in rats intra-articularly injected with *Linum* mucilage, *Althaea* mucilage and β-glucan. The postural unbalance induced by *Linum* mucilage is comparable to that evoked by CFA and remained statistically significant in comparison to the control group till the end of the study (day 28). *Althaea* mucilage and β-glucan started to lose their effect in comparison to CFA from the 3rd week after injection. *Amorphophallus* glucomannan increased the postural unbalance from day 2 to day 28 without reach the values of MIA- and CFA-treated animals (Figure 1c). *Linum* mucilage, β-glucan and CFA decreased the motor coordination and ability of the rats from day 2 until the end of the experiments. Indeed, the score assigned to these groups was statistically increased with respect to the control animals. The effect evoked by *Amorphophallus* glucomannan and *Althaea* mucilage was statistically significant until day 21 to disappeared on day 28. The effect of MIA peaked on the 1st week then the animals started to regain their motor skills (Figure 1d). Similar results were obtained with the rota rod test and are reported in Table S2. Moreover, we evaluated the swelling of the ipsilateral paw measuring the joint diameter on days 0, 2, 7, 14, 21 and 28 after the injection of compounds (Figure 1e). CFA, *Linum* mucilage and β-glucan induced the higher inflammatory effect with respect to the other compounds that peaked during the 1st week after injection. The following days, the swelling decreased and the joint diameter returned to the value of control animals on day 21 except for the CFA-treated animals. *Amorphophallus* glucomannan and *Althaea* mucilage increased the joint diameter after their injection with a slight effect in comparison with the other compounds. In particular, the increase was induced by *Amorphophallus* glucomannan remained statistically significant from day 2 to day 21 while *Althaea* mucilage showed a peak only on day 2. MIA slightly increased the swelling of the paw reaching the statistical significance on until day 7 (Figure 1e). To better characterize the type of pain that the i.a. injection of compounds induced, we acutely administered ibuprofen (100 mg/kg, per os) and pregabalin (30 mg/kg, per os) to the animal treated with MIA, CFA, *Althaea* mucilage, *Linum* mucilage and β-glucan (Figure 2). Ibuprofen and pregabalin are reference drug currently used to counteract inflammatory and neuropathic pain, respectively.[18,19] These treatments were acutely performed on days 3, 7, 14, 21 and 28, and the analgesic effect was measured by the paw pressure test (Figure 2 shows the measure 30 min after administration). On MIA-treated rat, ibuprofen and pregabalin were both active on days 3, 7 and 14 to reduce articular pain 30 min after treatment in comparison to the pretest (white column). On day 21, only pregabalin was effective while on day 28 MIA-treated rats spontaneously recovered from the pain state (Figure 2a). On the contrary, on CFA-treated animals, only ibuprofen was effective on days 21 and 28 while, during the previous days, both the reference drugs were active (Figure 2b). Ibuprofen counteracted *Althaea* mucilage-induced articular pain on days 3 and 7 while on day 14 the effect decreased. Pregabalin partially reduced the mechanical hyperagesia on days 3, 7 and 14. No measurements were performed on days 21 and 28 because the pro-algesic effect of *Althaea* mucilage was almost vanished (Figure 2c). On day 3, 7 and 14, ibuprofen completely reversed the articular pain induced by *Linum* mucilage reaching the values of control animas at the paw pressure test. The analgesic efficacy slightly decreased the next weeks. Pregabalin was partially effective each time that was tested (Figure 2d). The articular swelling evoked by β-glucan prevented the measure with the paw pressure for the first week and the effect of ibuprofen and pregabalin were tested from day 7 until day 28. Both drugs increased the weight tolerated by the animals on the ipsilateral paw without differences about their efficacy. On day 28, pregabalin and ibuprofen were inactive (Figure 2e). *Amorphophallus* glucomannan-injected animals were not treated with the reference drugs because their capacity to induced articular damage was less effective.

Morphological analysis of the tibiotarsal joint

The morphological analysis was performed on day 28 after treatment and only on *Linum* mucilage-treated animals because it shown the best algic profile. Intra-articular injection of *Linum* mucilage showed a typical profile of rheumatoid arthritis characterized by hyperplastic synovium, that partially occupied the joint space, and by the presence of an inflammatory infiltrate. At the same time were presented several foci of articular cartilage and bone erosions showing a profile compatible with a joint affected by osteoarthritis. Joint space appears in part ablated and in part replaced with hyperplastic sinovium (Figure 3). The histological analysis was made in comparison with a model of rheumatoid arthritis (CFA) and osteoarthritis (MIA). CFA was characterized by the total ablation of the joint space that appears replaced by hyperplastic tissue. Articular cartilage was completely disappeared or present only in little foci even though

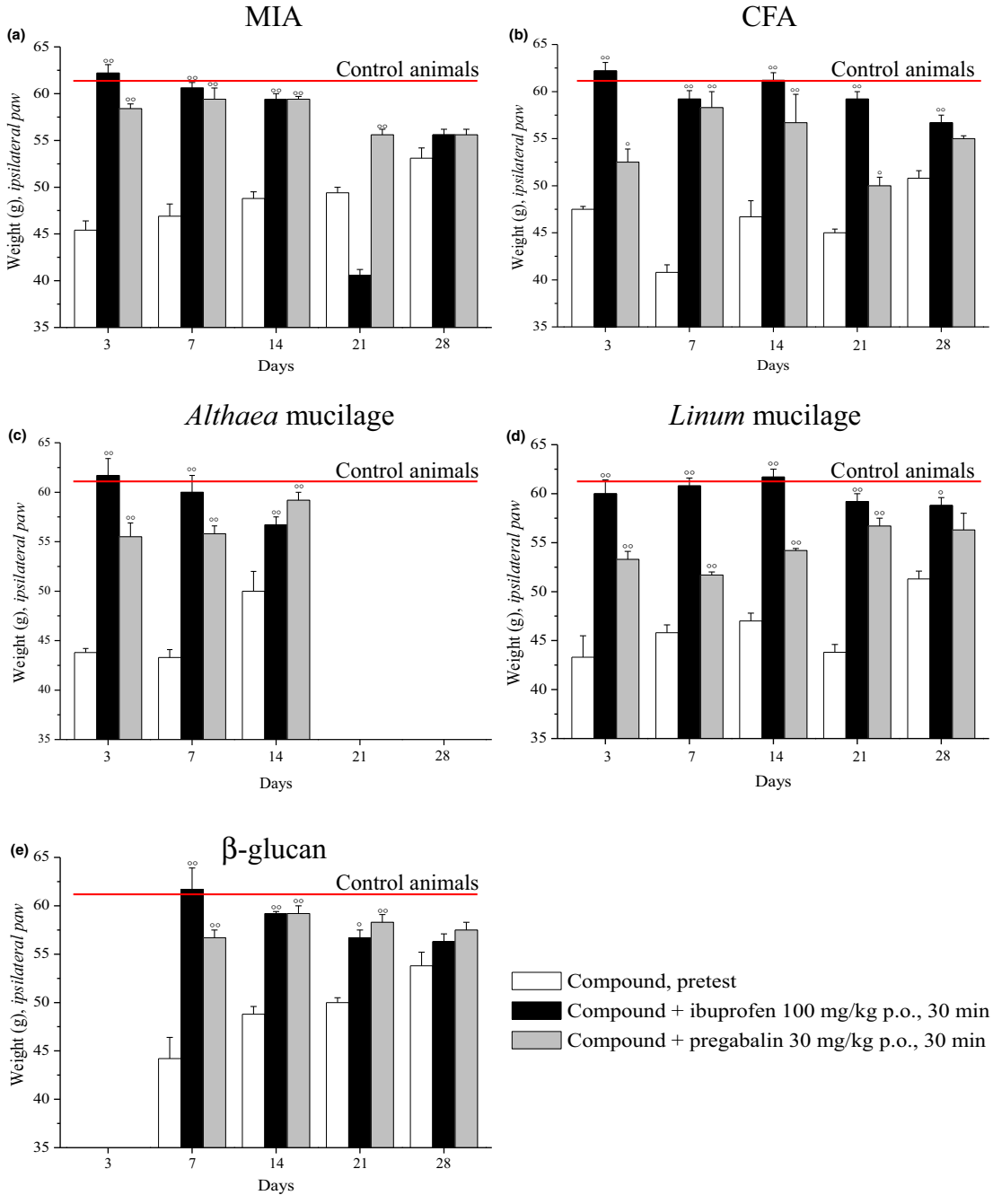

Figure 2 Effect of acute administration of ibuprofen and pregabalin in rats intra-articularly injected with *Altheae* mucilage (c), *Linum* mucilage. (d), β-glucan (e), monoiodioacetate (a) and Complete Freund's Adjuvant (b). Ibuprofen (100 mg/kg) and pregabalin (30 mg/kg) were *per os* acutely administered on days 3, 7, 14, 21 and 28 after the intra-articular injection of compounds. Pain threshold was measured by the paw pressure test 30 min after Ibuprofen and pregabalin administration. Each value represents the mean of 10 rats per group performed in two different experimental set. $^{\circ}P < 0.05$ and $^{\circ\circ}P < 0.01$ vs the pretest of the same group. [Colour figure can be viewed at wileyonlinelibrary.com]

this tissue appears degenerated and damaged. The treatment with MIA caused fibrin accumulation and the presence of considerable articular cartilage and bone erosions. Foci of bone erosion, as well as in *Linum* mucilage, were not characterized by the presence of bone proliferation areas indicating a repair strategy. In addition, even osteophytes were not visible. Moreover, the intraarticular space markedly decreased in comparison

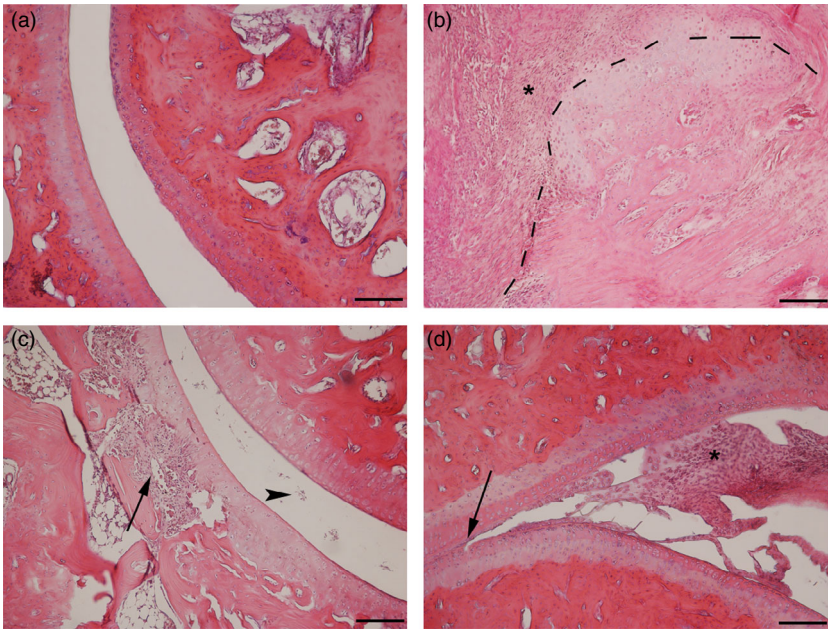

Figure 3 Histology of tibiotarsal joints. Representative micrographs of tibiotarsal joint from the four experimental groups. (a) Control animal. (b) Animal treated with Complete Freund's Adjuvant; dashed line indicates the hypothetical localization of joint space; asterisk indicates the fibrous tissue replacing the joint space. (c) Animal treated with monoiodioacetate; black arrows indicates bone erosions; arrow head indicate fibrin deposition in joint space. (d) Animal treated with *Linum* mucilage; black arrow indicate the fusion of tibia and tarsus. Asterisk indicates the fibrous tissue that infiltrates the joint space. Calibration bar 100 μm.

with the control joint. Both CFA and MIA treatment induced the presence of an abundant inflammatory infiltrate (Table 2).

Discussion

The present study investigated the articular damage induced by a singular i.a. injection of *Altheae* mucilage, *Linum* mucilage, *Amorphophallus* glucomannan and the polysaccharide β-glucan in comparison to that induced by MIA and CFA. The purpose was to create a new animal model of articular pain to accompany those existing. Assessment of pain in animal models of arthritis is pivotal to the interpretation of a model's utility in representing the clinical condition, and enabling accurate translational medicine. Unfortunately, just as no animal model perfectly mimics the complex processes involved in the initiation and progression of articular pain as well as arthritis-associated symptom in humans.[20] MIA and CFA are two of the most animal model of articular damage used to reproduced human osteoarthritis and rheumatoid arthritis, respectively.[21–23] Intra-articular injection of MIA, a chemical which inhibits glycolysis, disrupts chondrocyte homeostatic balance and produces cartilage degeneration and subsequent subchondral bone loss.[22] The development of OA pathology and pain in the MIA model typically occurs within 1–2 weeks and is dose-dependent.[24,25] In addition to joint degeneration and pain, the MIA model also generates acute and transient inflammation. Although the MIA model is ideal for assessing OA pain and peripheral neuropathy, a limitation of this model is that the structural histopathology of the joint itself is severe and does not recapitulate all of the physical features commonly associated with human OA. Following intra-articular MIA injection, the joint becomes hyperaemic, oedematous and there is an infiltration of circulating leucocytes.[15] By days 5–7 the inflammation subsides and remains at low levels throughout the subsequent development of MIA-induced OA.[15]

The heat killed mycobacterium arrangement of CFA is similar to proteoglycan, and arthritis can be developed by a cross-reactive CD_4 T cell clone that recognizes an epitope present on bacterial heat shock proteins and cartilage proteoglycans.[26] In the CFA arthritis model, the animal develops chronic swelling and pain in and around the joints with a release of proinflammatory cytokines from inflammatory cells, which causes the cartilage erosion and bone destruction.[27] In order to induce articular damage to create a new model of pain, we used compounds that are able to mimic an inflammatory state resembling human articular pain when i.a. injected, although they are safe when given orally.

Table 2 Morphological analysis of the tibiotarsal joints

Morphological parameters	Groups Score: from 0 (absent) to 3 (severe)			
	Vehicle	MIA	CFA	*Linum usitatissimum* mucil.
Inflammatory infiltrate	0 ± 0	1.3 ± 0.3	1.7 ± 0.3	1.3 ± 0.3
Synovial hyperplasia	0 ± 0	1.7 ± 0.3	2.0 ± 0.1	1.6 ± 0.
Fibrin deposition	0 ± 0	2.0 ± 0.3	3.0 ± 0.1	1.8 ± 0.4
Synovial vascularity	0 ± 0	1.7 ± 0.3	1.6 ± 0.2	1.5 ± 0.5
Cartilage erosion	0 ± 0	1.3 ± 0.3	2.0 ± 0.1	1.7 ± 0.3
Bone erosion	0 ± 0	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.3
Joint space	0 ± 0	1.7 ± 0.4	3.0 ± 0.1	1.7 ± 0.2

Histological evaluation of the tibiotarsal joints. Rats were intra-articularly injected with *Linum usitatissimum* mucilage (20 μl), MIA (2 mg/25 μl) and CFA (50 μl) on day 0. On day 28, animals were sacrificed and the tibiotarsal joints were collected for the histological evaluations. Sections were observed and an histological score (0: absent; 1: mild; 2: moderate; 3: severe) was attributed to the following morphological parameters: (1) inflammatory infiltrate; (2) synovial hyperplasia; (3) fibrin deposition; (4) synovial vascularity; (5) cartilage erosion; (6) bone erosion; (7) joint space. Each value represents the mean ± SEM of 10 rats per group performed in two different experimental set.

Of all compounds tested, *Linum* mucilage showed the best algic profile with several similarities to CFA model. The articular pain peaked during the first week after the mucilage injection matching with the maximum swelling of the paw. However, the algic conditions remained stable until the end of the experiment. To strength the idea that saccharides are responsible of the articular damage, we reported the effect of β-glucan, a polysaccharide, in comparison to the effect of *Amorphophallus* glucomannan that did not contain saccharides. *Amorphophallus* glucomannan slightly reduced the pain threshold of the animals while β-glucan is more painful. On the other hand, the mucilage *Althaea* mucilage that contains the 15.90% of saccharides with respect to the 6.23% of *Linum* mucilage lost its efficacy after the 2nd week. These evidences suggested that the nociceptive action is due not only to saccharides but required the action of other components. In particular, *Linum* mucilage, previously mentioned as the most harmful, contained the 6.23% of lactic acid with respect to 0.76% of *Althaea* mucilage, and the acidosis of the articular joint space can exacerbate the damage induced by saccharides. As reported by literature, rheumatoid arthritis is also known as a progressive acidic illness as acids build-up in the connective and fatty tissues that lead to joint destruction and functional disability if the patient has an acidic lifestyle and diet.[28,29] Moreover, in some people with rheumatoid arthritis, the retention of dietary, metabolic, respiratory and/or environmental acids leads to chronic inflammation and destruction of the cartilage, bone, and ligaments, causing deformity of the joints.[28,29]

In order to determine if the articular pain induced by these compounds shares more similarities with neuropathic or inflammatory pain, we evaluated the efficacy of antinociceptive drugs specifically used for these type of pain. If MIA and CFA models acquire, respectively, a neuropathic and inflammatory characteristic with the progression of the pathology, *Althaea mucilage* and β-glucan were sensible to both pregabalin and ibuprofen, the reference drugs used for the treatment of neuropathic and inflammatory pain, respectively, indicating a type of multifactorial pain. Also, *Linum* mucilage responded to both drugs but, as CFA, only ibuprofen was active on day 28. The result indicated a greater preponderance of the inflammatory state with respect to the neuropathic.

The histological analysis highlighted that in animals treated with *Linum* mucilage, the tibiotarsal joint showed intermediate characteristics between the two reference models. These morphological evaluations suggested that this model represents a complex pathology with a dual nature: one characterized by a joint damage from an anomalous load as in human osteoarthritis and the other based on an immune-inflammatory reaction such as in rheumatoid arthritis. Thus, mucilage appeared able to induce pain with both neuropathic and inflammatory bases. We are among the first to describe a bridge model between two clinically well distinct pathologies and this kind of model offers the possibility of studying mechanisms involved in the genesis of both inflammatory and immune-mediated pathologies. Moreover, our model may give the possibility to early screening the efficacy of novel compounds on a single set of animals representing a double-faced pathological tool.

Conclusion

Safe compounds for ingestion can mimic an inflammatory state when intra-articularly injected, possible due to their complexity of chemical and physical–chemical characteristics. *Linum* mucilage showed best results. This mucilage-induced articular damage shows common features with the osteoarthritis and rheumatoid arthritis evoked by MIA and CFA, respectively. The saccharides and the lactic acid present in the mucilage are the most responsible for the joint damage, and an accurate characterization of the extract is necessary for the reproducibility of the date in order to use *Linum* mucilage as a new animal model for articular pain. The creation of this new hybrid and valid preclinical model of articular pain can be useful for the screening of new pharmacological treatments for the management of articular pain states.

Declarations

Conflict of interest

All Authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary materials and Figures.

Figure S1. UHPLC-q-ToF fingerprints of Althaea mucilage.

Figure S2. UHPLC-q-ToF fingerprints of Linum mucilage.

Figure S3. UHPLC-q-ToF fingerprints of Amorphophallus glucomannan gum powder (classification according to commission regulation (EU) No 231/2012: E425i).

Table S1. Some metabolites identified in *Althaea* mucilage, *Linum* mucilage and *Amorphophallus* glucomannan.

Table S2. Rota rod test.