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Title: Treatment with Adenosine Receptor Agonist Ameliorates Pain Induced by Acute and Chronic Inflammation

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Running title: Anti-inflammatory and antinociceptive action of LASSBio-1359

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ABBREVIATIONS: A_{2A}-R, A_{2A} adenosine receptor; ANOVA, Analysis of variance; DMSO, dimethyl sulfoxide; CFA, Complete Freund's Adjuvant; d, day; g, gram; GAPDH, glyceraldehyde-3-phosphate; i.p., intraperitoneal injection; iNOS, inducible nitric oxide synthase; LASSBio-1359, [(E)-N'-(3,4-dimethoxybenzylidene)-N-methylbenzohydrazide]; p.o., pathway oral, RA, rheumatoid arthritis, s, seconds; TNF- α , tumor necrosis factor alpha.

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Abstract

Rheumatoid arthritis is an inflammatory autoimmune condition and tumor necrosis factor- α (TNF- α) plays an important role in its pathophysiology. In vitro, (E)-N'-(3,4-dimethoxybenzylidene)-N-methylbenzohydrazide (LASSBio-1359) has exhibited anti-TNF- α properties and in vivo these effects are mediated via activation of adenosine receptor. This work investigates the antinociceptive action of LASSBio-1359 in murine models of acute and chronic inflammatory pain. Male mice received an intraperitoneal injection of LASSBio-1359 and then were evaluated in formalin- and carrageenan-induced paw edema assays. Complete Freund's Adjuvant (CFA) was used to induce a mouse model of monoarthritis. These mice were treated with LASSBio-1359 by oral gavage to evaluate thermal and mechanical hyperalgesia. TNF- α and iNOS expression, as well as histological features were also analyzed. The time of reactivity to formalin in the neurogenic phase was reduced from 56.3 ± 6.0 s to 32.7 ± 2.2 s and 23.8 ± 2.6 s following treatment with LASSBio-1359 at doses of 10 mg/kg and 20 mg/kg, respectively. A reversal of the antinociceptive action of LASSBio-1359 was observed in inflammatory phase following treatment with ZM 241385, an adenosine A_{2A} antagonist. Carrageenan-induced thermal and mechanical hyperalgesia were reduced after treatment with LASSBio-1359. Similarly, CFA-induced thermal and mechanical hyperalgesia were reduced following treatment with LASSBio-1359 (25 and 50 mg/kg). Levels of TNF- α and iNOS expression increased in the monoarthritis model and were normalized in animals treated with LASSBio-1359, which was also associated with beneficial effects in the histological analysis. These results suggest that LASSBio-1359 represents an alternative treatment for monoarthritis.

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Introduction

Pain is an unpleasant feeling that is often caused by intense or damaging stimuli. However, it is an essential sensation that usually signals that tissue injury has occurred as a result of external and internal damaging events. Tissue injury can lead to the activation of nociceptors (sensitizing peripheral sensory neurons) by mediators whose identification has contribute to the understanding of the pathophysiology of pain (Woolf and Ma, 2007; Pavin et al., 2011). In the 1970s, it was described that pain and immune system may be associated beyond an acute response based on the clinical observations that patients with chronic pain exhibit other symptoms in addition to hyperalgesia. Moreover, these other symptoms paralleled the classical systemic sickness response that is commonly observed and which includes lethargy, depression, and anxiety. Therefore, the concomitant presence of sickness behaviors and chronic pain is considered suggestive of an underlying immune activity (Grace et al., 2014; McMahon et al., 2015).

Cytokines are small regulatory proteins that are produced by white blood cells and a variety of other cells, including cells of the central nervous system (CNS). The activation or dysregulation of cytokine production is implied in a variety of disease states, including acute (bacterial, viral, fungal and parasitic infections; tissue necrosis; tissue injury by foreign bodies; hypersensitivity reactions) and chronic inflammatory (Sommer and Kress, 2004; Staud, 2015). In particular, the latter includes rheumatoid arthritis (RA).

Tumor necrosis factor- α (TNF- α) is a principal pro-inflammatory cytokine that is produced by the immune system, and it is also produced in the peripheral and the CNS under pathological conditions (Zhang et al., 2011). Several studies have correlated high tissue levels of TNF- α with the pain and hyperalgesia that is associated with a number of diseases (Barnes et al., 1992; Shafer et al., 1994; Tak et al., 1997; Lindenlaub and Sommer, 2003; Uceyler et al., 2015; Yunus, 2015).

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RA is a chronic disease that leads to inflammation in the thin synovial membrane that surrounds joints. This membrane normally produces lubricating and nutritive synovial fluid. However, during RA the synovial layer is invaded by neutrophils, mast cells, dendritic cells, macrophage, and T and B cells. Following extensive angiogenesis and the proliferation of synoviocytes during, disease progression, hyperplastic synovial tissue becomes invasive and destroys cartilage and bone. In the chronic phase of RA, the TNF- α and interleukin 1 (IL-1) are responsible to maturation of the osteoclasts and bone resorption that impaired the rheumatic joint (Strand et al., 2007; Schett and Teitelbaum, 2009; Komatsu and Takayanagi, 2015).

Chronic pain can occur during RA, and the inflammation involved is usually followed by increased behavioral responses to innocuous (allodynia) and noxious (hyperalgesia) stimulation (Pinto et al., 2007; Edwards et al., 2011). Hyperalgesia is induced by the action of mediators released in response to inflammation in the inflamed tissue. Patients with RA show increased TNF- α levels and consequently, it is hypothesized that blocking the release of TNF- α represents a strategy for reducing acute and chronic inflammation. (Cunha et al., 2005; Verri et al., 2006; Strand et al., 2007; Silveira et al., 2013; Walsh and McWilliams, 2014; Buttgerit et al., 2015; Wei et al., 2015).

Currently, our research group is studying a series of new *N*-acylhydrazone derivatives that exhibit potent anti-inflammatory and analgesic activities. These compounds were synthesized via molecular simplification of the lead compound, (LASSBio-294) by replacing the rings linked to the acyl or imine subunits and/or modifying the stereoelectronic behavior of the acylhydrazone group (previously described as a pharmacophore group for analgesic and anti-inflammatory) by *N*-alkylation (Kummerle et al., 2012).

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One of these analogues, LASSBio-1359 [(E)-N'-(3,4-dimethoxybenzylidene)-N-methylbenzohydrazide], exhibited anti-TNF- α activity *in vitro* and *in vivo* (Kummerle et al., 2012) and was described as an agonist of adenosine receptor (Alencar et al., 2013; Moura et al., 2015). Therefore, the antinociceptive and anti-inflammatory activities of LASSBio-1359 were further investigated in this study, with the goal of reversing the mechanical and thermal hyperalgesia that can be induced in an animal model of RA (monoarthritis).

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Material and Methods

Animals

Experimental protocols were approved by the Animal Care and Use Committee of the Universidade Federal do Rio de Janeiro, Brazil CEUA (DFBCICB069). Male *Swiss* mice (25–35g) were housed under controlled temperature ($21\pm 1^{\circ}\text{C}$) and humidity (60%) conditions with a 12-hour light/dark cycle. Food and water were available *ad libitum*. Animals were acclimated for at least 30 min prior to the beginning of the experiment.

Drugs

LASSBio-1359 (LASSBio/UFRJ, Brazil) was synthesized and characterized as previously described (Kummerle et al., 2012). LASSBio-1359, morphine sulphate (Cristália, Brazil), ZM241385 (Tocris, USA) and acetylsalicylic acid (ASA, Sigma, USA) were dissolved in dimethyl sulfoxide (DMSO, Cristália, Brazil). Ethylenediaminetetraacetic acid (EDTA, Sigma, USA) and formaldehyde (Isofar, Brazil) were dissolved in distilled water. Carrageenan (Sigma, USA) was dissolved in saline. Indomethacin (Sigma, USA) was dissolved in a mixture of ethanol:Tween 80:saline (2:8:90).

Formalin-induced hind paw licking

Thirty minutes after receiving an intraperitoneal (i.p.) injection of either vehicle, morphine (10 mg/kg), acetylsalicylic acid (150 mg/kg), or LASSBio-1359 (5, 10 or 20 mg/kg), mice were administered via intraplantar (i.pl.), 20 μL of formalin (2.5%). The nociceptive behavior in response to formalin injection is characterized as the licking or biting the paw, which causes a classical biphasic nociceptive response (Abbott et al., 1995). The initial acute neurogenic phase (0–5 min) is followed by a quiescent period (5–15min), before a prolonged tonic inflammatory response (15–30 min). The effect of adenosine pathway was investigated by the pre-treatment with specific antagonist of the

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adenosine A_{2A} receptor, ZM 241385 (3 mg/kg, i.p.), which was injected 20 min prior to the LASSBio-1359 administration.

Carrageenan-induced pain

Sub-acute paw inflammation in mice was induced by injection of 20 µL of carrageenan (1%, i.p.). Antinociceptive effects of LASSBio-1359 (10 or 20 mg/kg, i.p.) and indomethacin (4 mg/kg, i.p.) were evaluated 150 min after the injection of carrageenan.

CFA-induced monoarthritis

Chronic inflammation was induced by two subcutaneous injections of 15 µL of complete Freund's adjuvant (CFA), containing 5 µg/µL heat-killed *Mycobacterium butyricum* (Becton Dickinson, Franklin Lakes, USA). Mice under anesthesia (sevoflurane 2%, Cristália, Brazil) were injected with CFA in the vicinity of the tibio-tarsal joint (Chillingworth and Donaldson, 2003). The control group received injection of incomplete Freund adjuvant (IFA, without bacteria). After 7 days, mice were treated with vehicle (DMSO), LASSBio-1359 (10, 25, 50 or 100 mg/kg) or ASA (300 mg/kg) by oral gavage.

Paw immersion test

Thermal hyperalgesia observed in mice after carrageenan and CFA injection was evaluated through the paw immersion test as previously described (Lolignier et al., 2011). Briefly, the injected hind paw was immersed in a 46 °C water bath until withdrawal was observed. Control latency was determined as the average of 3 observations. In the sub-acute inflammation model, the latency of each animal was obtained every 15 min during the first 2 h and measured 24 h after carrageenan injection. However, in the chronic inflammation model, the latency was observed every 3-4 days until the end of treatment.

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Paw pressure test

Mechanical hyperalgesia was investigated through the paw pressure test using the Randall–Selitto device (Ugo-basile analgesimeter, Italy) in both sub-acute and chronic models. Increasing pressure (expressed in grams) was applied to the hind paw until vocalization or paw withdrawal. The maximal pressure was set at 250 g (Almela et al., 2009).

Membrane preparations and Western blot analysis

Treated animals were euthanized on day 21 and soft paw tissues were removed, stored in lysis buffer containing protease inhibitors, and frozen in liquid nitrogen. Tissues were homogenized in an ice-cold lysis buffer consisted of 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM MgCl₂, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 1 mM dithiothreitol, 1 µg/mL polypeptide protease inhibitor solution (pepstatin, chymostatin, aprotinin, leupeptin, and antipain; Sigma), sodium dodecyl sulfate (1% SDS), and 1% Triton X-100 (Bio-Rad,USA). Homogenate was centrifuged for 5 min at 1000 G and supernatant (~ 0.5 mL) was collected and stored at - 80°C (Okorokov and Lehle, 1998). The total protein concentration for each sample was determined spectrophotometrically by using the Lowry method (Lowry et al., 1951).

Proteins (14 µg) were separated by electrophoresis in 10% SDS- PAGE gels and were transferred onto nitrocellulose membranes (Bio-Rad, USA). Membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 and then were incubated with primary antibodies against TNF-α and iNOS (Abcam, Cambridge, USA), and GAPDH (Cell Signaling, Danvers, USA). The secondary antibodies used were anti-rabbit and anti-mouse, both of which were HRP-labeled (Abcam, Cambridge, USA). Bound antibodies were visualized with a Super Signal West Pico Chemiluminescence Kit (Pierce, Rockford, IL, USA) and an Amersham Imager 600 (GE Healthcare UK).

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The density of each band was determined and normalized to GAPDH using ImageJ software (Research Service Branch, NIH, USA).

Histological analysis of inflammation

Animals were sacrificed at the end of the treatment and their hind paws were immediately removed, fixed in 10 % neutral buffered formalin, and decalcified in 10 % EDTA (pH 7.2) over 14 days. Tissues were dehydrated overnight in 70% ethanol, then three times (40 min each) in absolute alcohol, followed by three times (40 min each) in xylol. After that, tissues were embedded in paraffin (60°C) and sections (3 µm thick) were stained with hematoxylin and eosin or safranin O/fast green FCF, followed by the examination by blinded experts in a light microscopy.

Statistical analysis

All data were expressed as the mean \pm standard error of the mean (SEM). Differences among groups were considered statistically significant when the *P* value was <0.05 using either one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's test or two way ANOVA followed by Tukey's test.

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Results

The effect of LASSBio-1359 in formalin-induced hind paw-licking test

Swiss mice received a single i.pl. injection of 20 μ L formalin. When LASSBio-1359 was administered prior to formalin, the animals spent less time licking/biting their paws during the neurogenic (0–5 min) and inflammatory (15–30 min) phases. In the neurogenic nociception phase, the administration of LASSBio-1359 (10 mg/kg and 20 mg/kg) resulted in an attenuation of the licking/bite time response of the control group (DMSO) from 56 ± 6 s to 37 ± 2 s and 24 ± 2 s, respectively. In the inflammatory phase, the animals treated with LASSBio-1359 (10 mg/kg and 20 mg/kg) exhibited a decrease in licking/bite time from 307 ± 44 s (for the control group) to 129 ± 21 s and 140 ± 16 s, respectively. Pre-treatment with ASA, a non-steroidal anti-inflammatory agent, significantly reduced reactivity in the second phase, but not in the neurogenic phase (Figure 2). The administration of the A_{2A} adenosine receptor antagonist, ZM 241385 (3 mg/kg i.p.), inhibited the antinociceptive effect of LASSBio-1359 during the inflammatory phase (Figure 3).

The effect of LASSBio-1359 in thermal hyperalgesia of carrageenan-induced pain test

Initially, carrageenan-induced thermal hyperalgesia was evaluated for Swiss mice based on their paw withdrawal latency in response to a 46 °C water bath. The thermal hyperalgesia threshold was reduced from 13.5 ± 0.3 s to 5.6 ± 0.6 s 150 min after the injection of carrageenan. When the mice were treated with LASSBio-1359 (10 mg/kg and 20 mg/kg), the carrageenan-induced thermal hyperalgesia thresholds were 11.5 ± 1.5 s and 13.8 ± 0.6 s, respectively. These effects were observed at different time points of the evaluation. Similarly, treatment with indomethacin attenuated carrageenan-induced thermal hyperalgesia (Figure 4).

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The effect of LASSBio-1359 in the mechanical hyperalgesia of carrageenan-induced pain test

Treatment with LASSBio-1359 (10 mg/kg and 20 mg/kg, i.p.) reduced the mechanical hyperalgesia induced by carrageenan, with the threshold increasing from 79.7 ± 6.6 g to 206.7 ± 17.7 g and 197.8 ± 21.6 g, respectively, 150 min after the injection of carrageenan. Indomethacin produced total reversal of carrageenan-induced mechanical hyperalgesia as well as LASSBio-1359 (Figure 5).

The effect of LASSBio-1359 in the monoarthritis

CFA induced chronic inflammation around the tibio-tarsal joint of the Swiss mice after 7 d and this was accompanied by reduced thresholds for both thermal and mechanical hyperalgesia from 13.6 ± 0.3 s to 6.9 ± 0.6 s, and from 245.7 ± 1.9 g to 109.9 ± 8.7 g, respectively. Significant reductions in both thermal and mechanical hyperalgesia were observed during 13 days of treatment. In contrast, injections of incomplete Freund's adjuvant did not reduce either threshold. Animals that received CFA and were subsequently treated with DMSO exhibited low thresholds for mechanical and thermal hyperalgesia (Figures 6 and 7). In contrast, the mice that were treated with LASSBio-1359 at doses of 25, 50, and 100 mg/kg were found to exhibit improved thermal and mechanical hyperalgesia over the treatment period. Similar results were observed after treatment with ASA which increased thermal and mechanical threshold in mice with monoarthritis.

The effect of LASSBio-1359 on TNF- α and iNOS expression in paw tissues of the monoarthritis mouse model.

In a Western blot analysis of paw tissues obtained from the monoarthritis animal model, higher levels of TNF- α expression were detected (Figure 8A). In contrast, the monoarthritis animals that received LASSBio-1359 expressed lower levels of TNF- α

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detected in their paw tissues. A similar expression profile was observed for iNOS in the paw tissues without and with LASSBio-1359 treatment, respectively (Figure 8B). However, TNF- α and iNOS were overexpressed in paws of animals with monoarthritis treated with ASA.

Histopathological evaluations of the paw tissues obtained from the monoarthritis mice

Sections of the tibio-tarsal joint from the animals treated with IFA were stained with hematoxylin/eosin and were evaluated histologically (Figure 9A). IFA-treated mice (e.g., the normal group) exhibited a minimal inflammatory response around the cartilage and bone in the synovium tissues. In contrast, the CFA group that was treated with DMSO (Figure 9B) exhibited classical features of arthritis. Specifically, a severe inflammatory response was observed that included synovial cells hyperplasia, moderate to severe fibrosis, pannus formation, cartilage destruction, and extensive bone lysis. In Figures 9D and 9E, oral treatment with LASSBio-1359 at doses of 25 mg/kg and 50 mg/kg resulted in a significant suppression of histopathological changes in the cartilage, preservation of bone, and an absence of pannus or fibrosis. In addition, minor inflammatory cells were observed. Despite these results, an oral treatment regimen of 10 mg/kg LASSBio-1359 was found to be associated with an infiltrate of inflammatory cells (Figure 9C), suggesting that a transient inflammatory response occurred in the treated group.

Cartilage tissues were also analyzed (Figure 10), and the thickness of the cartilage tissue from the monoarthritis group treated with DMSO (Figure 10B) was less than that of the control group that was treated with IFA (Figure 10A). For the animals treated with LASSBio-1359 (50 mg/kg), no decrease in cartilage thickness was observed, thereby suggesting that cartilage degradation was reversed (Figure 10D). The area of cartilage

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stained with safranin was reduced in approximately 40% in CFA-induced monoarthritis group using the ImageJ program (NIH, Bethesda, USA), indicating a severe proteoglycans (in red) deficiency, which was prevented after treatment with LASSBio-1359 (50 mg/kg) prevented this reduction.

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Discussion

This study demonstrated that the novel agonist of adenosine A_{2A} receptor (A_{2A} -R), LASSBio-1359 reversed the hyperalgesic response induced by stimulation of peripheral receptor modulated by activation of inflammatory process in mice. A decrease in the expression levels of both TNF- α and iNOS or damage of tibio-tarsal joint in the paw of animals with CFA-induced monoarthritis was noted after treatment with LASSBio-1359.

The behavioral response of mice to formalin was evaluated following i.pl. injection of formalin which produced a biphasic behavioral response that involved both central and peripheral components. The first phase includes a direct stimulation of nociceptors by formalin, while the second phase may be associated with the release of inflammatory mediators into tissues (Yano et al., 2006). In the present study, treatment with LASSBio-1359 inhibited both the first and second phases of formalin-induced nociception. Similarly, morphine has been reported to inhibit nociception in both phases of the formalin test (Shibata et al., 1989).

When the mechanisms associated with the antinociceptive action observed during the second phase of the formalin test were evaluated, pretreatment with an antagonist for the A_{2A} -R, ZM 241385, was found to reverse the antinociceptive effect of LASSBio-1359. LASSBio-1359 has also been reported to be an A_{2A} -R agonist with a capacity to stimulate adenylate cyclase activity (Alencar et al., 2013). As a result, ATP is converted to cAMP, thereby leading to the intracellular accumulation of this second messenger and the activation of protein kinase A. This in turn leads to inhibition of the formation and release of pro-inflammatory cytokines such as TNF- α and IL-1 β (Cronstein, 2006; Jacobson and Gao, 2006; Varani et al., 2010)(Jacobson and Gao, 2006; Varani et al., 2010; Chen et al., 2013), and also leads to inhibition NF κ B nuclear translocation (Mediero et al., 2013). Those anti-inflammatory effects were observed after treatment with LASSBio-1359.

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The inflammation in the hind paw of the animals caused by carrageenan usually is used to investigate antinociceptive and anti-inflammatory activities (Randall and Selitto, 1957; Sugishita et al., 1981; Henriques et al., 1987; Hargreaves et al., 1988; Shibata et al., 1989; Petersson et al., 2001; Mendes et al., 2009; Shah and Shah, 2015; Sudo et al., 2015). Carrageenan may induce inflammation in two phases. In the early phase (1–2 h after the injection of carrageenan), inflammation is mostly mediated by histamine, serotonin, and the increased synthesis of prostaglandins in the surrounding damaged tissues. The later phase of inflammation (2 h after the injection of carrageenan), prostaglandins are released by polymorphonuclear cells and macrophage are engaged in process of vascular permeability (Gupta et al., 2006; Prajapati et al., 2014). Usually, the local inflammation is elevated with pro-inflammatory cytokines TNF- α , IL-1, and IL-6 (Cuzzocrea et al., 1999; Ogata et al., 1999). Besides inflammation, carrageenan can induce hyperalgesia (Zhang et al., 2004).

The TNF- α has been shown to initiate inflammatory responses and have important role in the pain and central sensitization (Zhang et al., 2011). The decreased sensitization induced by high levels of TNF- α (secreted by macrophage) needs to be reduced. Reduced TNF- α expression has been demonstrated as responsible for the anti-inflammatory responses observed in animal models (Xu et al., 2012; Wang et al., 2015a). In the present study, thermal and mechanical hyperalgesia induced by carrageenan were suppressed after treatment with LASSBio-1359. This approach has been widely employed to assess the effects of new analgesic and anti-inflammatory drugs (Posadas et al., 2004; Radhakrishnan et al., 2004; Quintao et al., 2005).

To mimic a chronic inflammatory pain state that is accompanied by increased behavioral responses to noxious (hyperalgesia) stimulation, monoarthritis was induced by CFA. This model differs from neuropathic pain because the hyperalgesia is deeply

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associated with inflammation and furthermore there is no time for healing of the pathological tissues. CFA-induced behavioral changes are usually observed in animals after three days (early phase), and then they typically continue for at least three weeks (Ohsawa et al., 2000; Hong et al., 2009; Aoki et al., 2014b). In the present study, a strong hypersensitivity to heat and mechanical responses were characterized by a reduction in paw withdrawal latencies between day 3 and day 7. Moreover, both mechanical and thermal hyperalgesia were attenuated following treatment with LASSBio-1359.

Nociceptors may be activated through noxious thermal, mechanical or chemical responses. Some studies report that heat threshold for cutaneous nociceptors are sensitized first in monoarthritis (Danziger et al., 1999; Morell et al., 2014). After that, joint and surrounding deep nociceptors are involved in the sensitization in the chronic process (Danziger et al., 1999; Morell et al., 2014). The joint nerves have thick myelinated A β , thinly myelinated A δ and a high proportion unmyelinated C fiber. When a joint undergoes an inflammatory process, the fibers, A β and A δ , are more sensitive to applied pressure and to movements of the joint. C fibers, which modulate the hyperalgesia and become sensitized to respond to light pressure and movements in the working range of the joint. These changes provide a mechanical afferent sensory response that indicates joint pain (Cline et al., 1989; Schaible and Grubb, 1993; Aoki et al., 2014a). Exacerbated mechanical and thermal hyperalgesic responses were observed in the arthritis murine model and antinociceptive action of LASSBio-1359 was demonstrated.

Previous studies characterized LASSBio-1359 as an A_{2A}-R agonist, that is coupled G protein stimulatory (Scott and Kingsley), culminating in an increase of adenylyl cyclase activity mediating an increase of cAMP accumulation. The A_{2A}-R are widely expressed in immune system cells and have a crucial role in inflammatory conditions such as arthritis, Huntington and Parkinson diseases (Ohta and Sitkovsky, 2001; Bilzer and Gerbes, 2002;

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Sebastiao and Ribeiro, 2009; Neumann et al., 2014). Studies describe a high expression of A_{2A} -R in patients suffering rheumatoid arthritis. TNF- α produced by macrophage, monocytes, and T cells, have an important role in the appearance, spreading and systemic manifestations of the disease and its increased levels might increase the expression and activity of iNOS (Choy and Panayi, 2001; Scott and Kingsley, 2006; Hasko et al., 2008; Moore et al., 2008; Gonzalez-Gay et al., 2009; Nowak et al., 2010; Varani et al., 2011; Vincenzi et al., 2013). Additionally, the activation of A_{2A} -R can inhibit NF κ B nuclear translocation and osteoclast differentiation, preventing the bone destruction. This event has a beneficial effect for inflammatory diseases such as arthritis (Hasko and Cronstein, 2013; Mediero et al., 2013). LASSBio-1359 binding and stimulating A_{2A} -R can lead to the inhibition of the release of pro-inflammatory cytokines, e.g. TNF- α , providing a decrease in the transcription of inflammatory proteins, e.g. iNOS, preventing bone destruction.

Pain is the principal complain in patients with RA which is a common cause of disability, with more than one-third of patients eventually experiencing work disability. Medications for the treatment of pain in inflammatory arthritis are currently non-steroidal anti-inflammatory drugs with acetaminophen prescribed as a first-line therapy. Meanwhile, the use of antidepressants as an analgesic therapy for inflammatory arthritis remains controversial (Heiberg et al., 2005; Allaire et al., 2008; Saag et al., 2008; Lee, 2013) It is also important to note that some patients experience adverse effects due to the drugs used to treat arthritis and this can include gastrointestinal toxicity mainly during treatment with NSAID (1991; Farrell et al., 1991; Carrasco-Pozo et al., 2011; Guthrie, 2011; Carrasco-Pozo et al., 2016).

The adenosine receptors which are distributed in the spinal cord and some brain areas are involved in the modulation of pain (Ribeiro et al., 2002; Sawynok and Liu, 2003; Morelli

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et al., 2009; Burnstock et al., 2011; Zylka, 2011) because their activation by adenosine or others agonists can inhibit pain. Activation of the A_{2A} -R in the spinal cord, precisely in lamina II neurons, promotes inhibition NMDA currents and could modulate antinociceptive activity (Guntz et al., 2008) and its blockade by antagonists reversed this effect (DeLander et al., 1992; De Lander and Keil, 1994; Borghi et al., 2002; Yoon et al., 2005; Yoon et al., 2006; Lee et al., 2010; Ng et al., 2015; Wang et al., 2015b). Intrathecal administration of the A_{2A} R agonist was effective to attenuate mechanical allodynia and thermal hyperalgesia in pain model (Loram et al., 2009). Additionally, the activation of A_{2A} -R on microglia and astrocytes cells can control chronic pain. Thus, adenosine pathway could represent an important target for the suppression of pain, anti-inflammatory action and neuroprotection. (Eisenach et al., 2003; Hasko et al., 2005; Loram et al., 2009; Loram et al., 2013; Melani et al., 2014; Sawynok, 2015).

In conclusion, this study provided evidence that the novel adenosine A_{2A} agonist, LASSBio-1359, effectively reversed pain response associated to an inflammatory reaction modulated by production and release of cytokine in the CFA-induced arthritis in mice.

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Authorship Contributions

Participated in research design: Montes, Hammes, Sudo and Zapata-Sudo.

Conducted the experiments: Montes, Hammes and Montagnoli.

Contributed to the design and synthesis of new compounds: da Rocha, Fraga and Barreiro.

Performed data analysis: Barreiro, Fraga, Sudo, Zapata-Sudo.

Contributed to the edition of the manuscript: Montes, Sudo and Zapata-Sudo.

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Legends

Figure 1. (E)-N'-(3,4-Dimethoxybenzylidene)-N-methylbenzohydrazide (LASSBio-1359)

Figure 2. Effects of i.p. administration of LASSBio-1359 (5, 10 and 20 mg/kg), morphine (10 mg/kg) and acetyl salicylic acid (150 mg/kg) in formalin-induced hind paw licking test. Data represent time spent by animal licking or biting the formalin-injected paws. Values are expressed as mean \pm SEM (n = 10). **P<0.01 or ***P<0.001 when compared to the vehicle-treated group using one-way ANOVA followed by Dunnet's.

Figure 3. Effects of LASSBio-1359 (10 mg/kg i.p.) on formalin-induced hind paw licking test in the absence and presence of ZM 241385. Data are expressed as mean \pm SEM (n = 6-10). *P<0.05 and *P<0.01 vs control; §P<0.05 vs LASSBio-1359 (10 mg/kg i.p.) using one-way ANOVA followed by Dunnet's test.

Figure 4. Effects of LASSBio-1359 (10 and 20 mg/kg i.p.) or indomethacin (4 mg/kg i.p.) in the paw immersion test. Carrageenan-induced thermal hyperalgesia in the ipsilateral paw after 150 min. Data are expressed as the mean \pm SEM (n =10). **P<0.01 when compared to DMSO using two-way ANOVA followed by Tukey's test.

Figure 5. Effects of LASSBio-1359 (10 and 20 mg/kg i.p.) or indomethacin (4 mg/kg i.p.) in the paw pressure test. Carrageenan-induced mechanical hyperalgesia in the ipsilateral paw after 150 min. Data are expressed as mean \pm S.E.M (n =10). **P<0.01 when compared to DMSO using two-way ANOVA followed by Tukey's test.

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Figure 6. Effects of LASSBio-1359 (10, 25, 50 and 100 mg/kg p.o.) or acetylsalicylic acid (300 mg/kg p.o.) in the monoarthritis model. CFA induced mechanical hyperalgesia in the ipsilateral paw after 3 days. Data are expressed as the mean \pm SEM (n = 7-10). **P<0.01; ***P<0.001; ##P<0.01; ###P<0.001 compared to CFA+DMSO using two-way ANOVA followed by Tukey's test.

Figure 7. Effects of LASSBio-1359 (10, 25, 50 and 100 mg/kg p.o.) or acetylsalicylic acid (300 mg/kg p.o.) in monoarthritis model. CFA induced thermal hyperalgesia in the ipsilateral paw after 3 days. Data are expressed as the mean \pm SEM (n = 7-10). **P<0.01; #P<0.05; ##P<0.01; ###P<0.001 compared to CFA+DMSO using two-way ANOVA followed by Tukey's test.

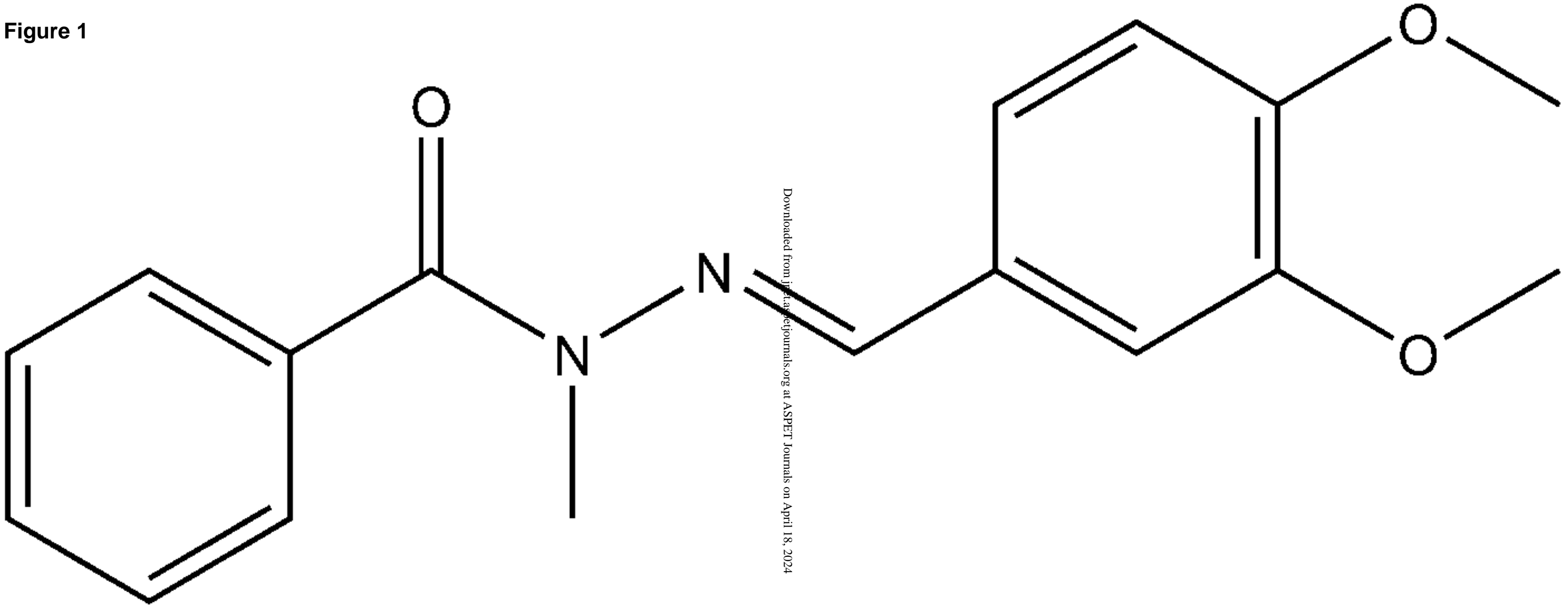
Figure 8. Expression of TNF- α (A) and iNOS (B) in paws from mice treated with IFA or CFA after oral administration of LASSBio-1359 (10, 25, 50 and 100 mg/kg). Graphs show protein quantification and each column represents the mean \pm SEM (n=3-4). *P<0.05 compared to control; #P<0.05 compared to DMSO using one-way ANOVA followed by Dunnett's.

Figure 9. Representative hematoxylin and eosin sections of tibio-tarsal joints (n = 3). Arthritic joints showed synovial hyperplasia (arrow), destruction of cartilage and bone (asterisk) and inflammatory cell infiltrate (double asterisk). A = IFA; B = CFA + DMSO; C = CFA + LASSBio-1359 (10 mg/kg); D = CFA + LASSBio-1359 (25 mg/kg); E = CFA + LASSBio-1359 (50 mg/kg). Cart = Cartilage. Horizontal bar = 100 μ m.

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Figure 10. Safranin O/Fast green FCF staining of articular cartilages (n = 3). Arthritic-joints treated with DMSO showed lower amount of proteoglycans compared to animals injected with IFA. Mice treated with LASSBio-1359 (10 or 25 mg/kg) showed a red-staining pattern indicating partially reversal of proteoglycan loss. A = IFA; B = CFA + DMSO; C = CFA + LASSBio-1359 (10 mg/kg); D = CFA + LASSBio-1359 (25 mg/kg). Dashed line indicates the cartilage (Morelli et al.) border. Horizontal bar = 100 μ m.

Figure 1



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Figure 2

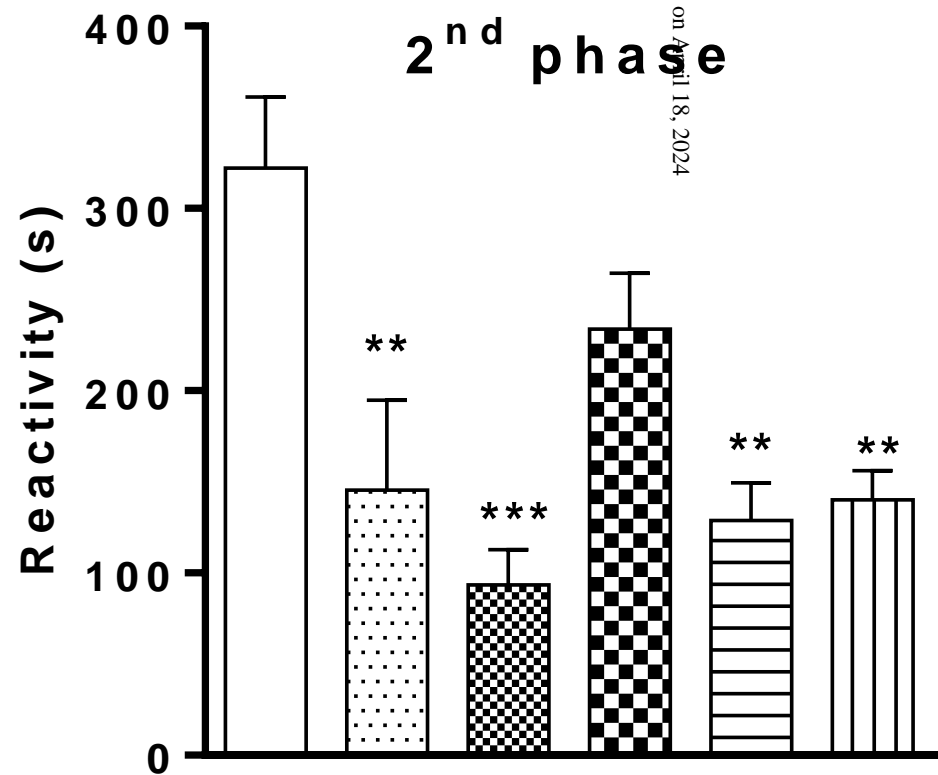
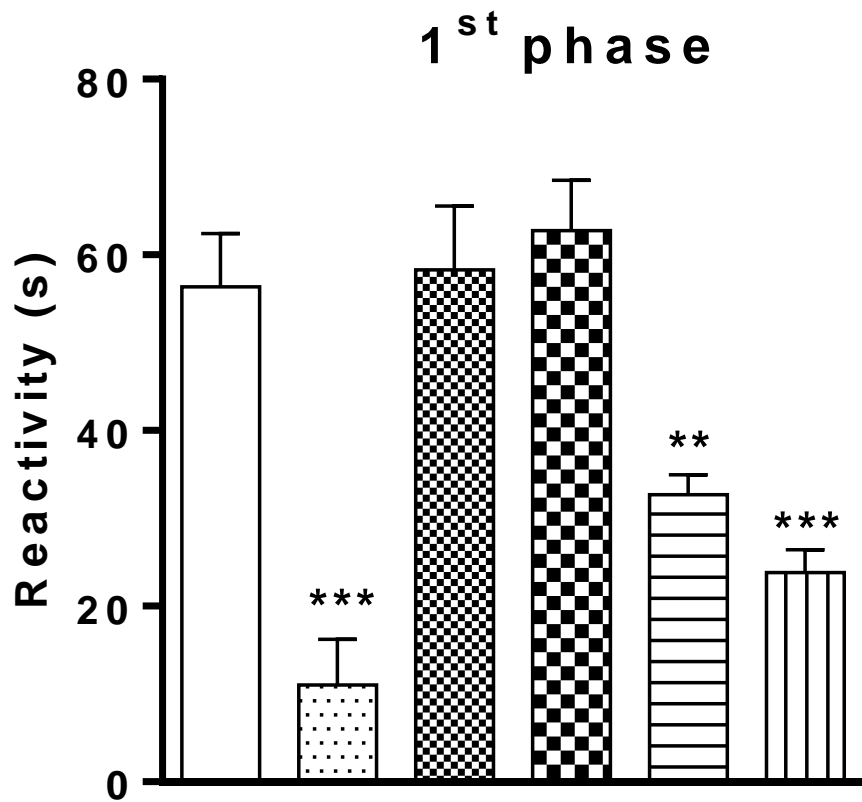
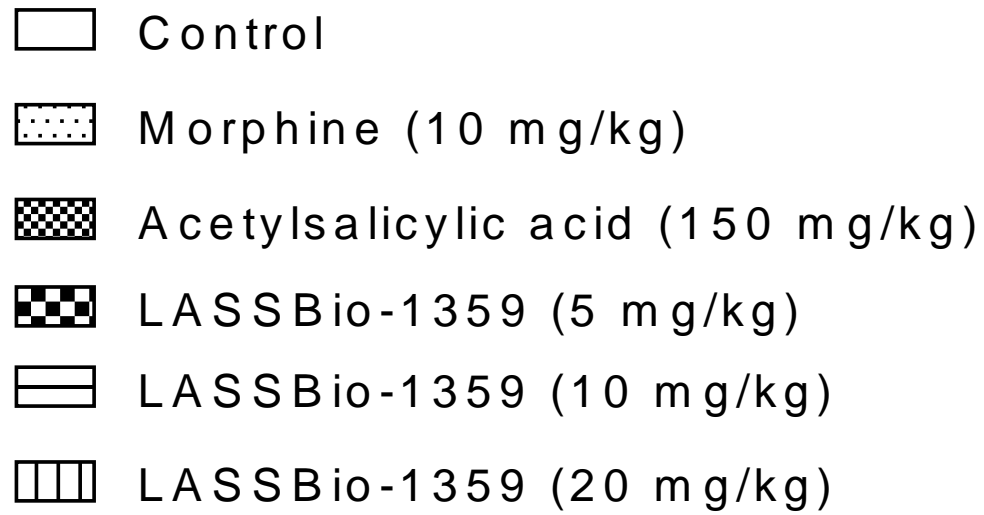
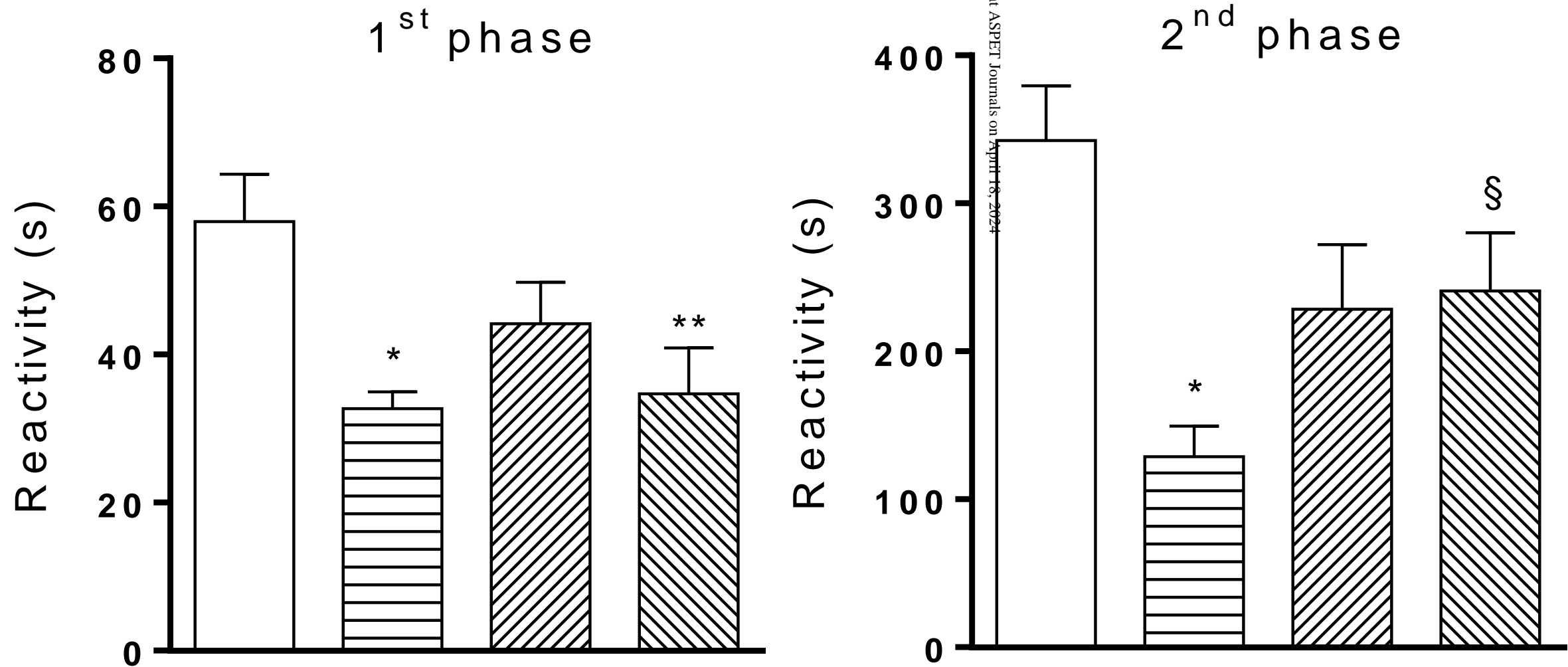


Figure 3

- Control
- LASSBio-1359 (10 mg/kg)
- ZM 241385 (3 mg/kg) + Control
- ZM 241385 (3 mg/kg) + LASSBio-1359 (10 mg/kg)



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Figure 4

- DMSO
- Indomethacin (4 mg/kg)
- ▲ LASSBio-1359 (10 mg/kg)
- LASSBio-1359 (20 mg/kg)

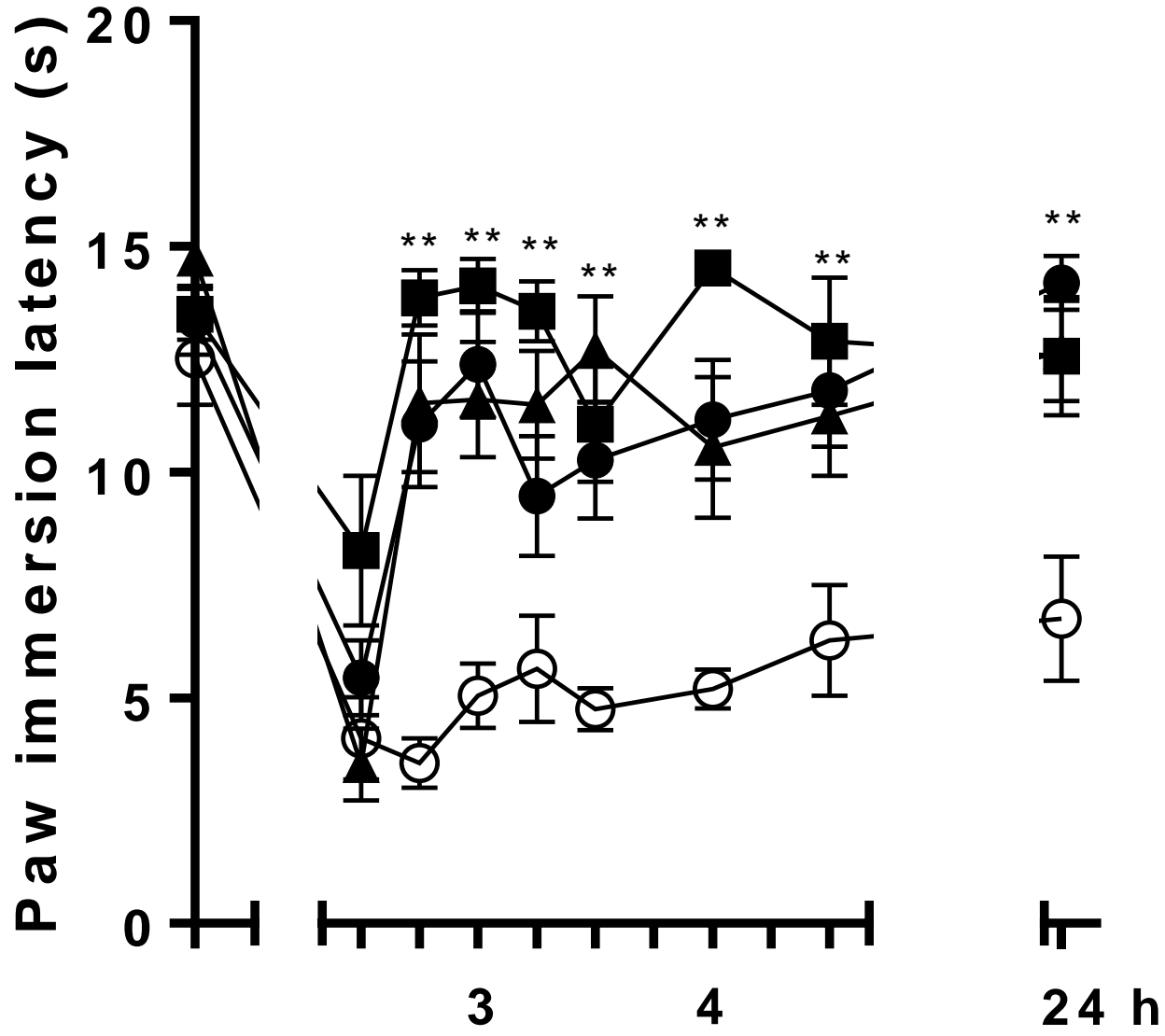


Figure 5

- DMSO
- Indomethacin (4 mg/kg)
- ▲ LASSBio-1359 (10 mg/kg)
- LASSBio-1359 (20 mg/kg)

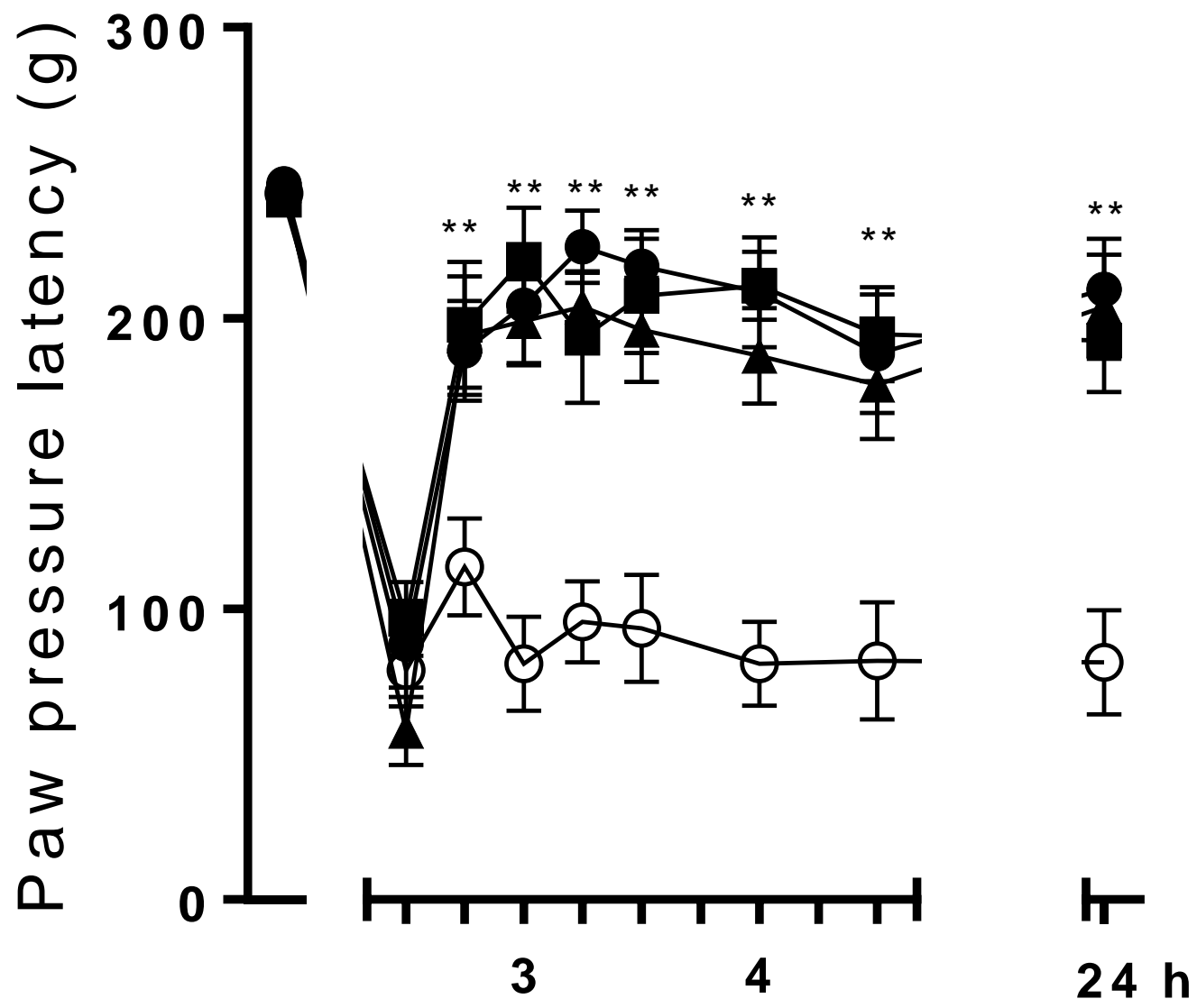
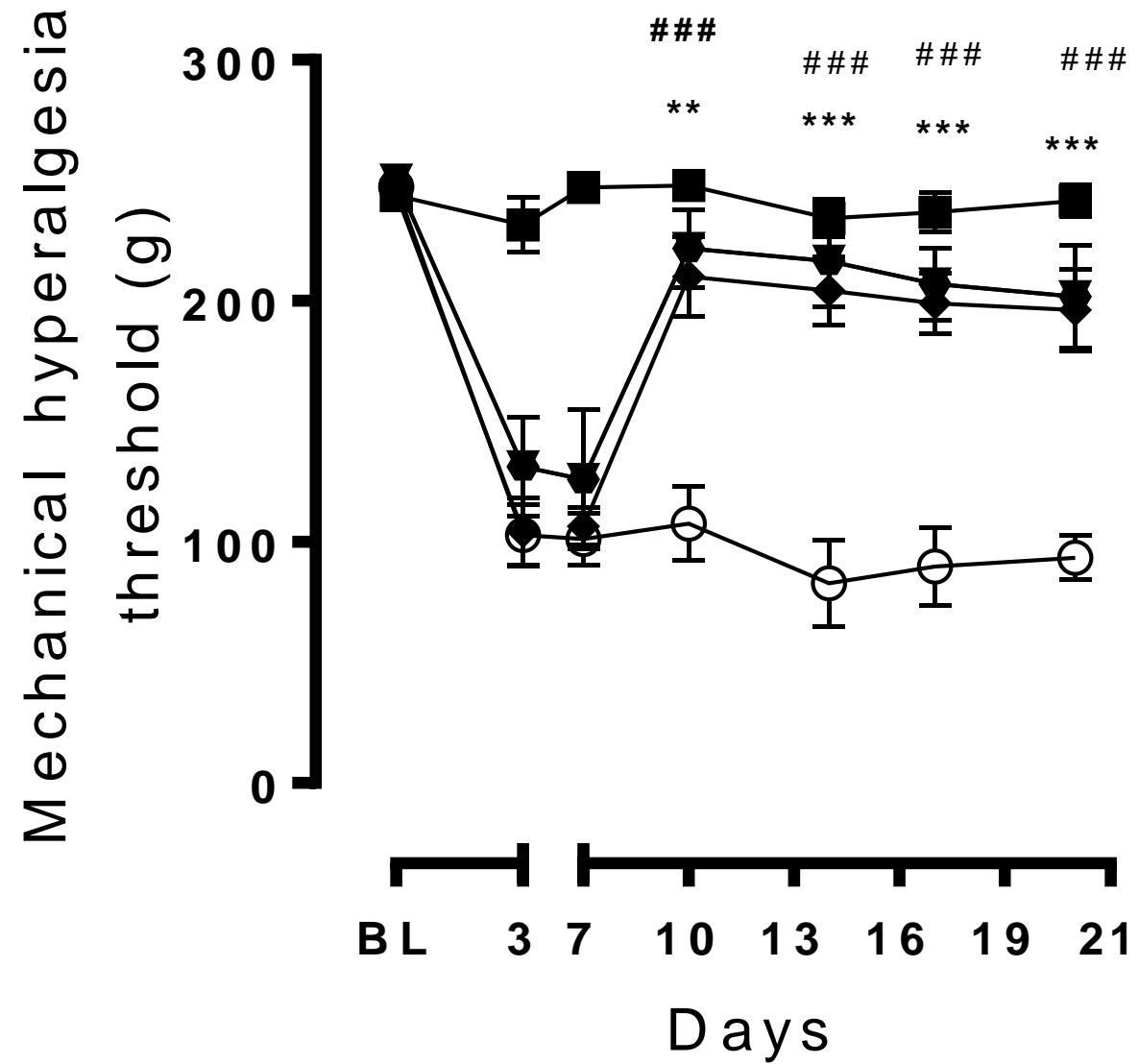


Figure 6

- IFA
- CFA + DMSO
- CFA + ASA (300 mg/kg)
- ▼ CFA + LASSBio-1359 (10 mg/kg)



- IFA
- CFA + DMSO
- ▲ CFA + LASSBio-1359 (100 mg/kg)
- CFA + LASSBio-1359 (50 mg/kg)
- ◆ CFA + LASSBio-1359 (25 mg/kg)

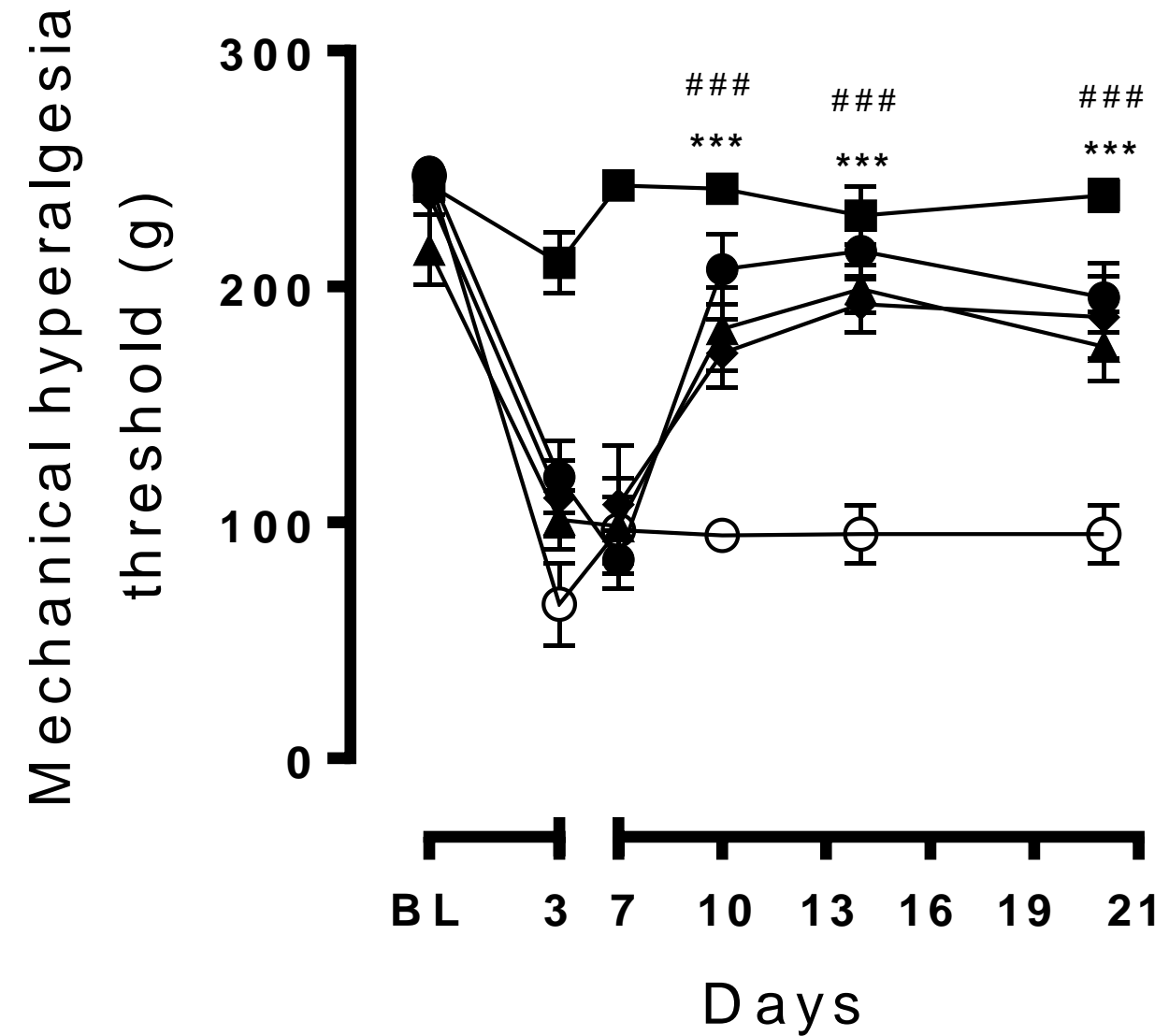


Figure 7

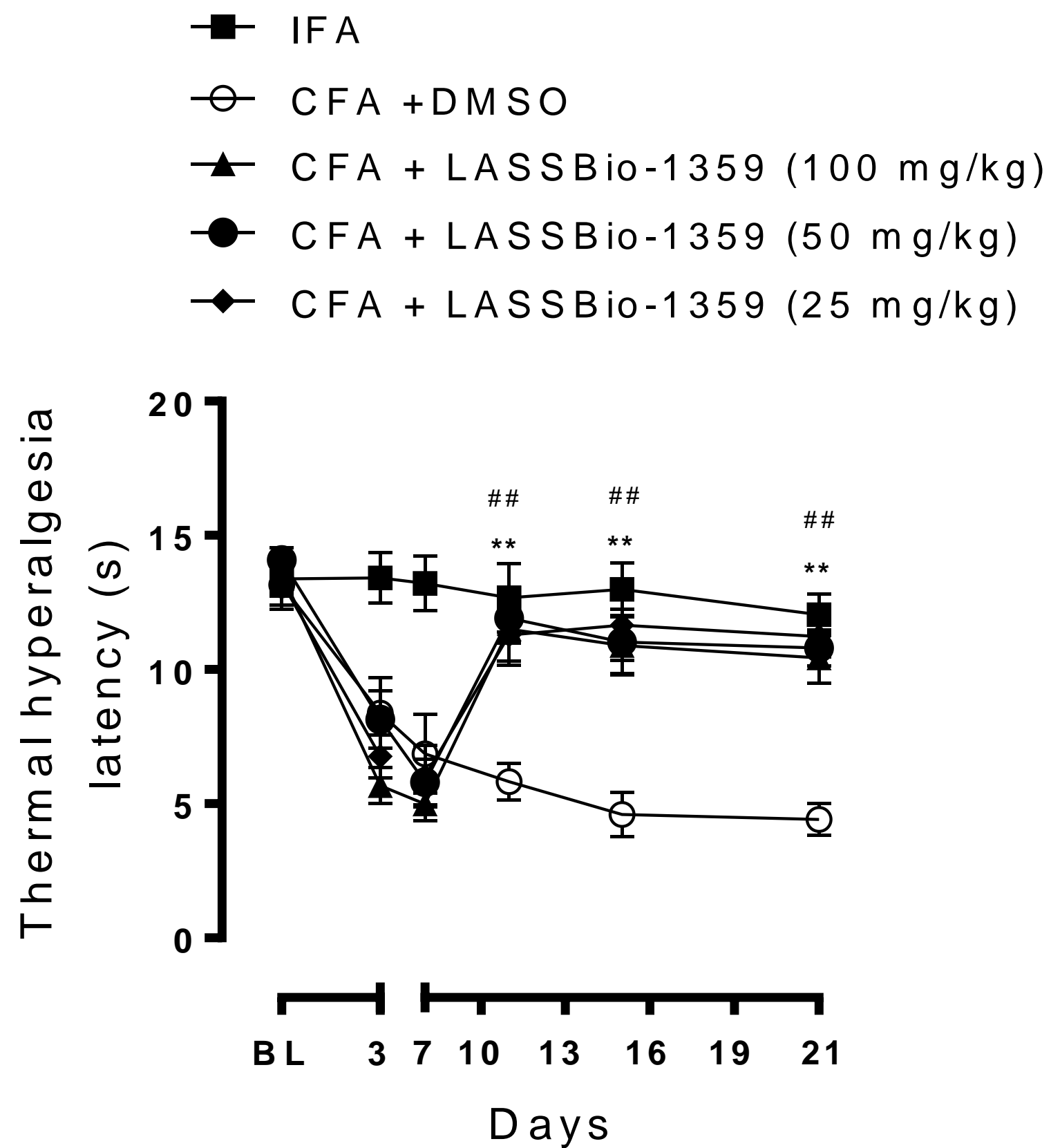
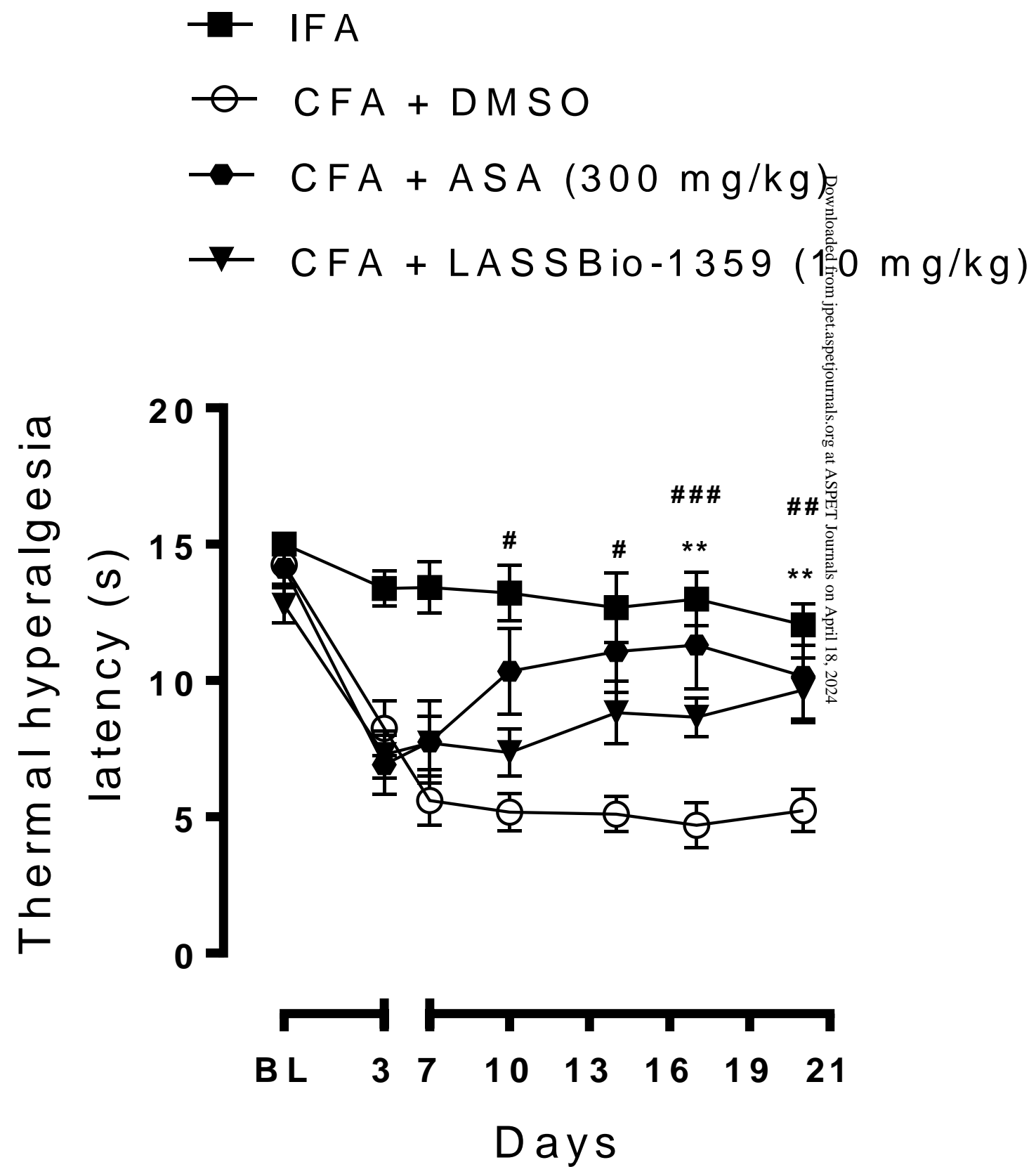


Figure 8

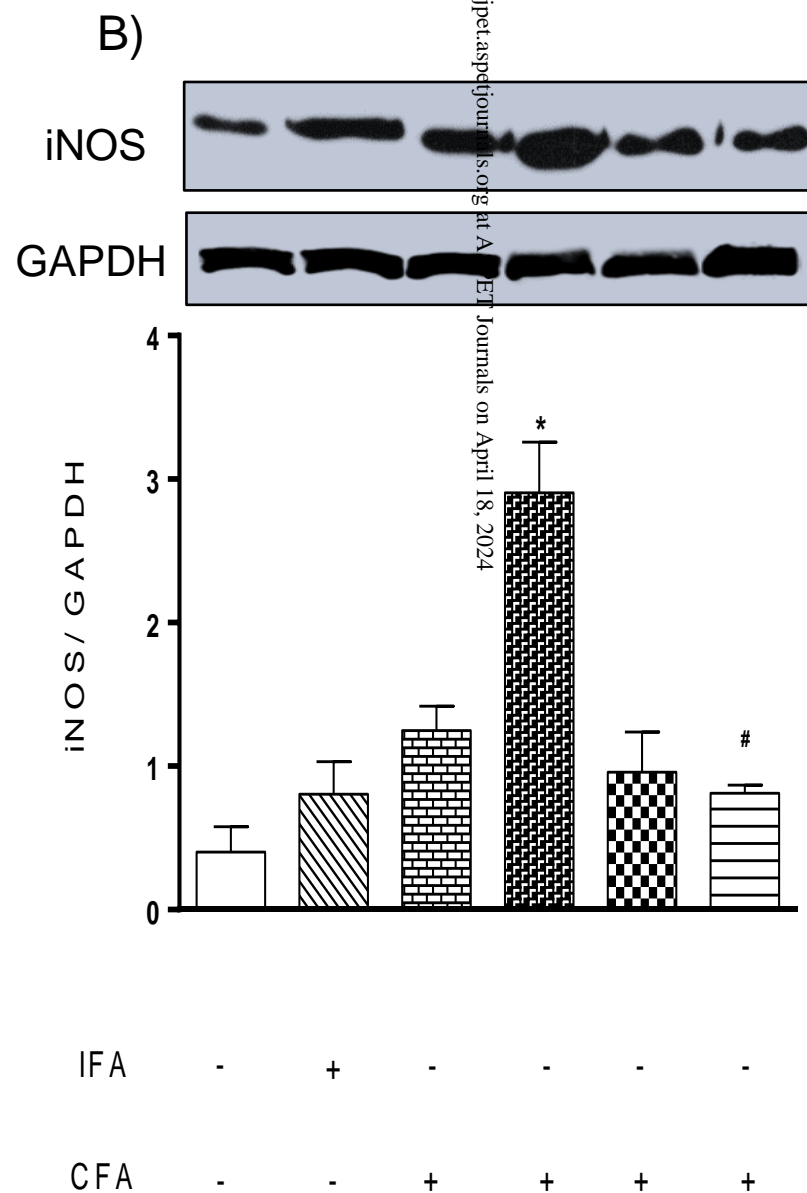
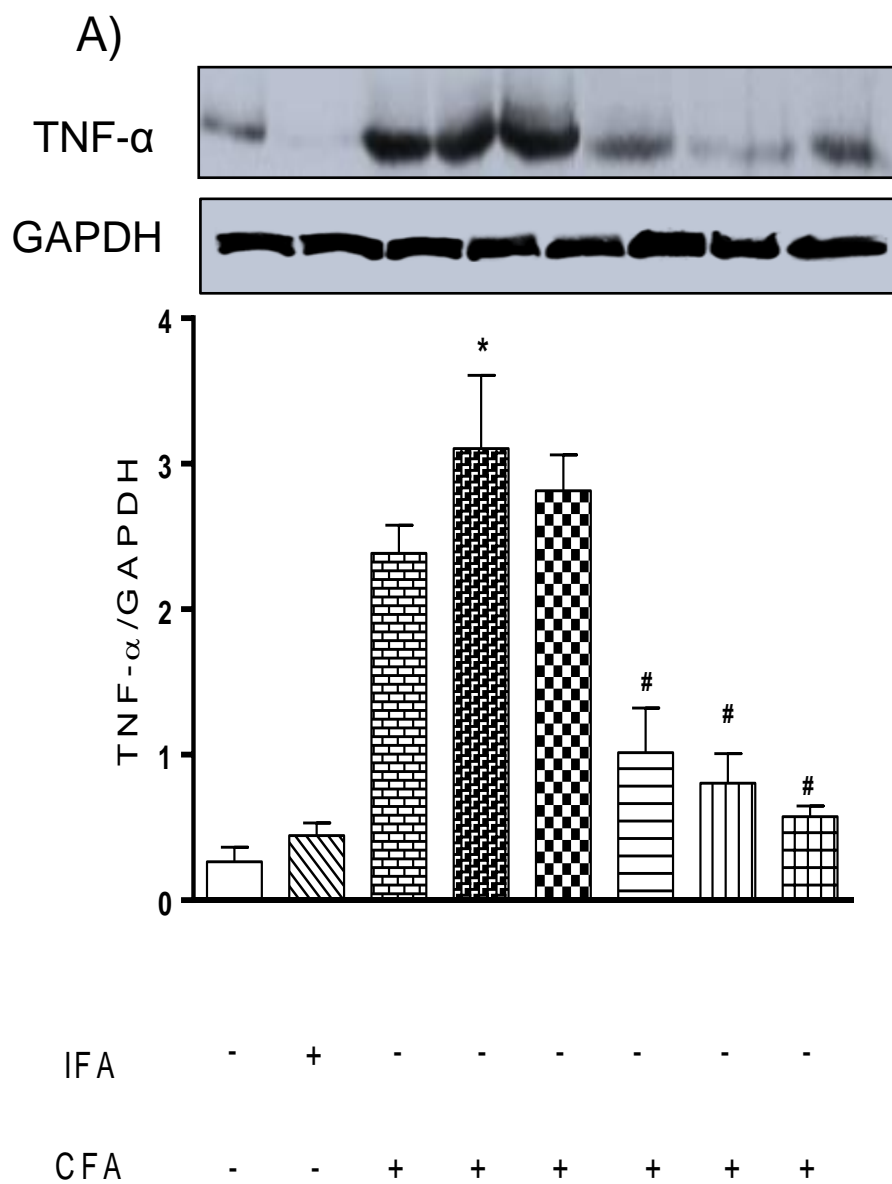
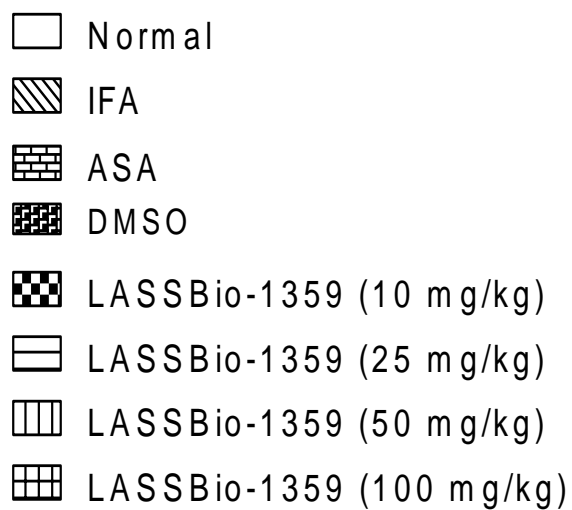
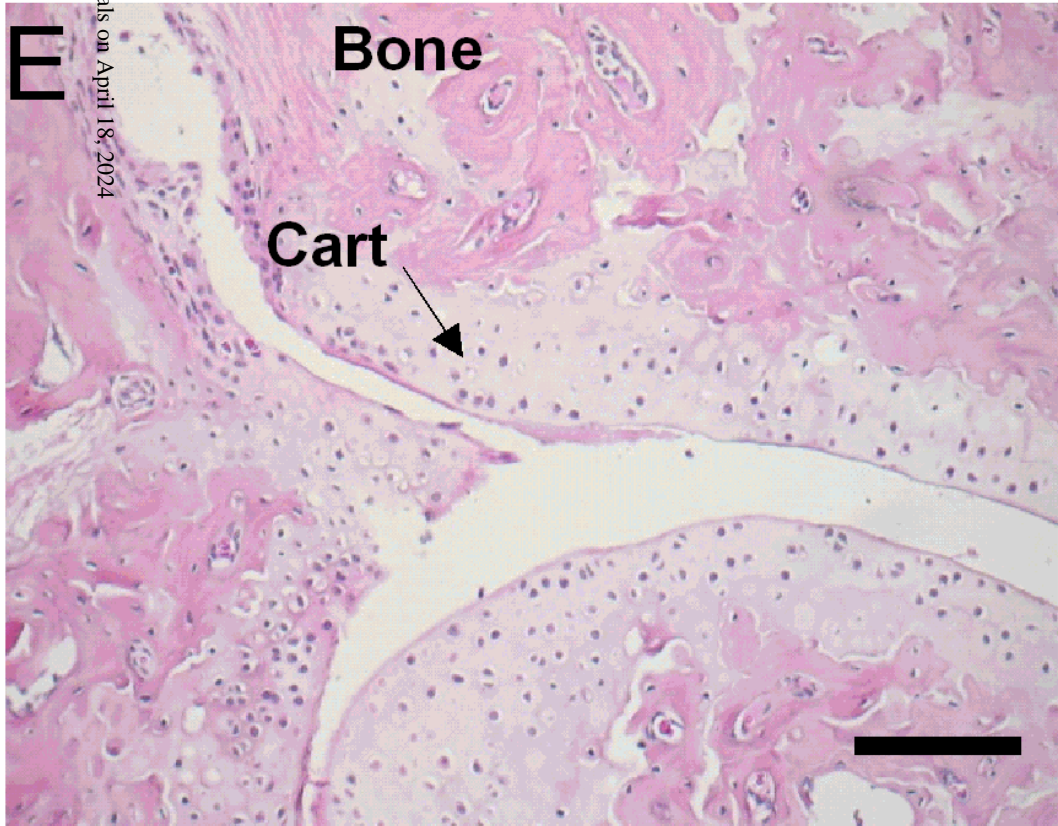
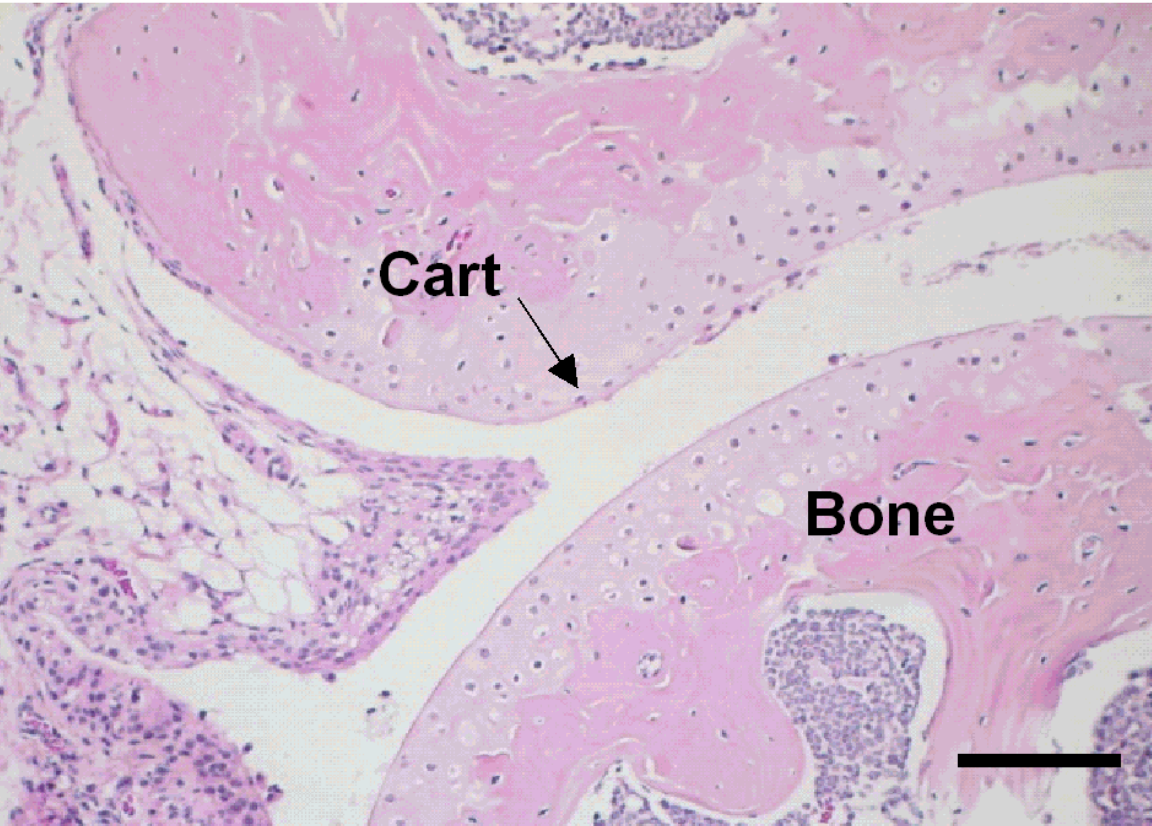
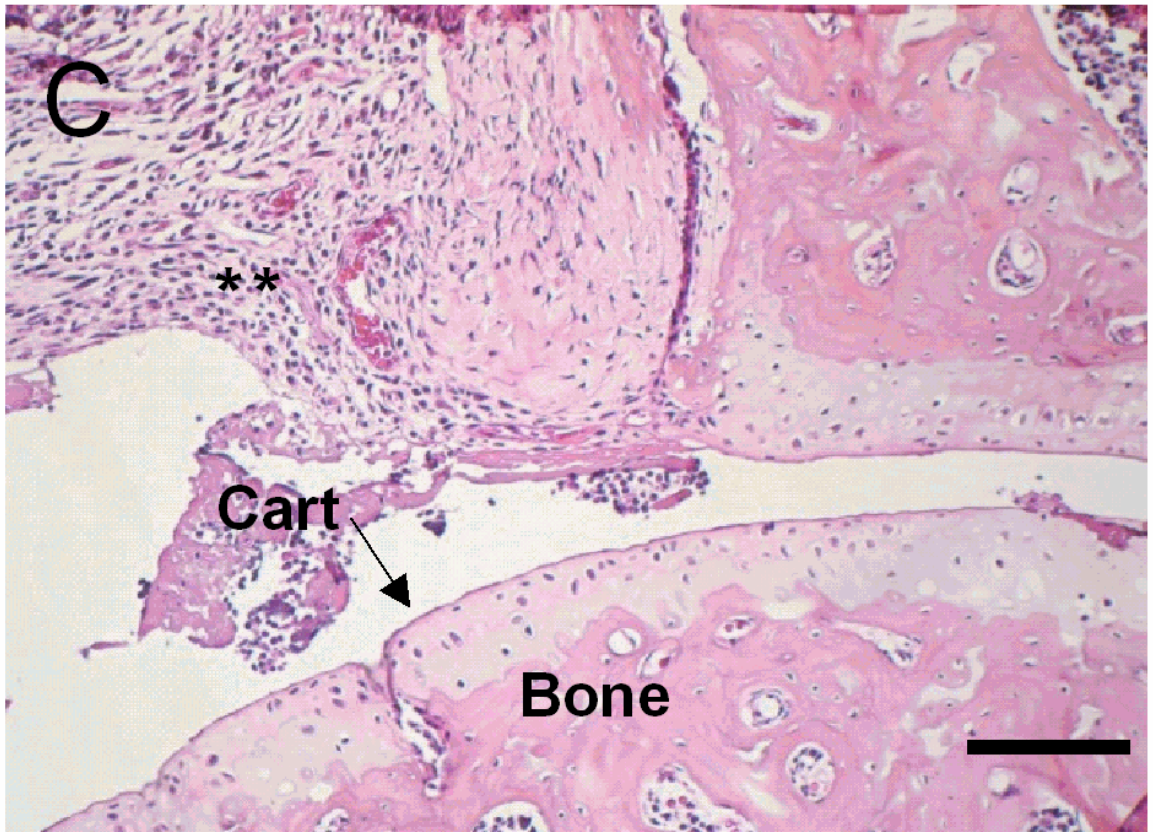
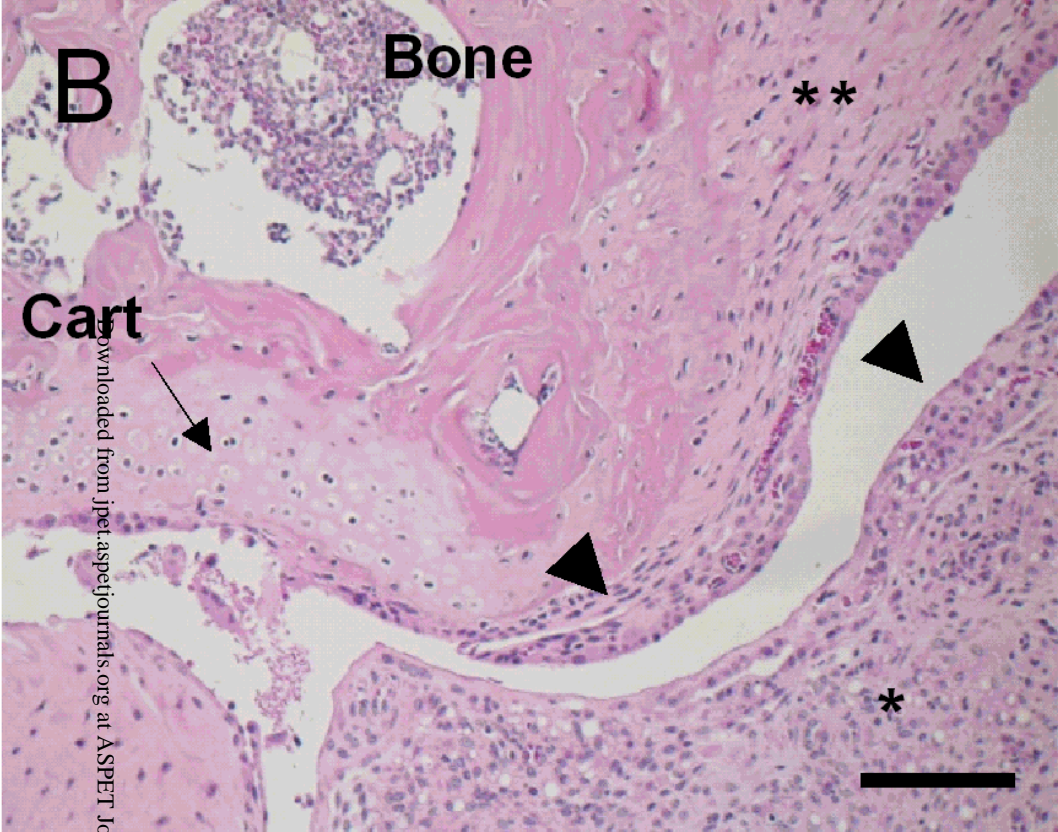
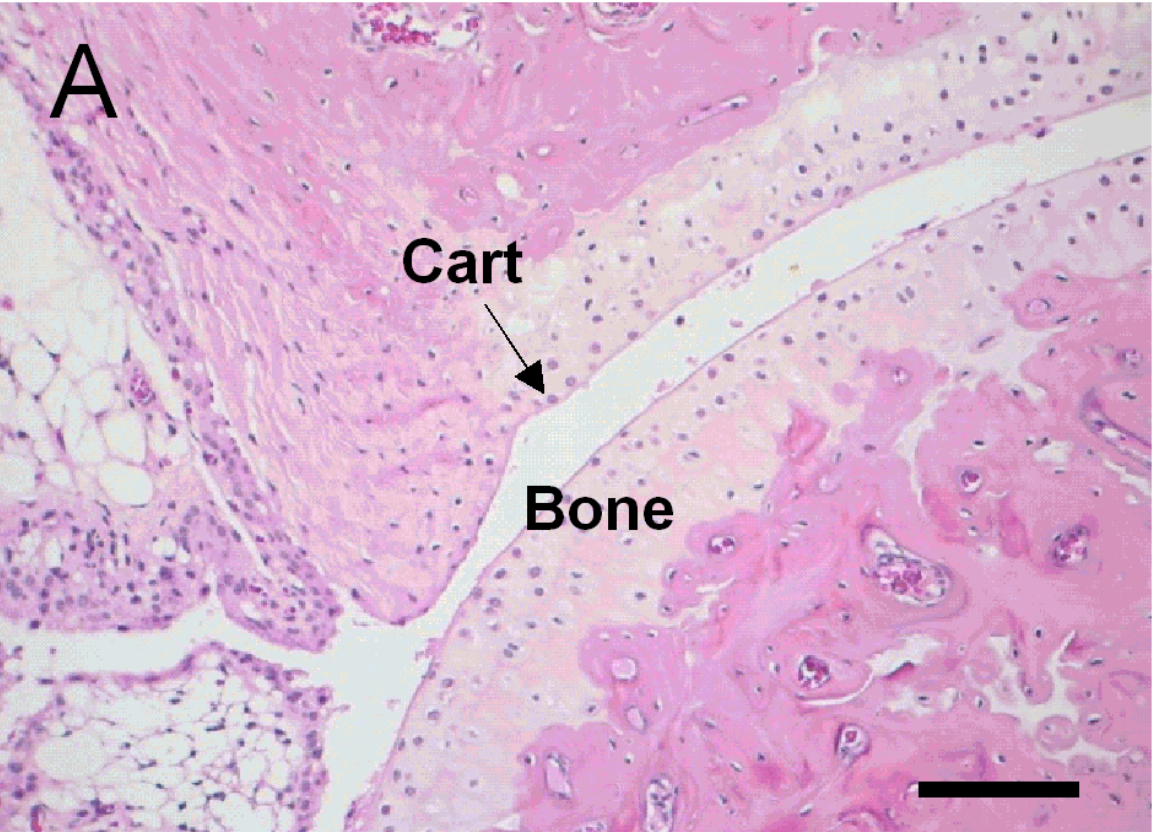


Figure 9



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Figure 10

