Clinical-like cryotherapy in acute arthritis of the knee improves inflammation signs, pain, joint swelling and motor performance in mice

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Research article

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Abstract

Background

Assess the effect of clinical-like cryotherapy on inflammatory signs (in vivo neutrophil migration, cytokines and joint inflammation), pain, and joint swelling in mice with knee arthritis. Balance and motor coordination were also evaluated.

Methods

Young C57BL/6 mice were randomly divided into 3 groups (n = 10/group): control group: animals were not immunized with antigen-induced arthritis (AIA) and no intervention occurred; AIA group: animals immunized with methylated bovine serum albumin (mBSA) for knee arthritis; AIA + cryotherapy group: animals immunized with mBSA and submitted to cryotherapy. After 21days, the study was divided into 6-hour experimental analysis periods after injection-induced joint inflammation with mBSA (100 ug/joint) and a cryotherapy protocol was applied (ice pack, two 20-minute sessions). Number of synovial fluid neutrophils, cytokine levels, joint histology, pain, joint swelling and motor performance were also analyzed.

Results

Our results showed that the application of cryotherapy in mice with acute knee arthritis reduced inflammatory signs and improved the symptoms of pain and joint swelling, as well as motor coordination and balance in the animals.

Conclusions

Clinical-like cryotherapy used in the acute phase of arthritis reduced inflammatory signs and improved the symptoms of pain, joint swelling, motor coordination and balance in the mice.

Background

Arthritis is characterized by an infiltration of inflammatory cells and cartilage, as well as bone destruction, and manifests itself clinically as pain, swelling and stiffness in the affected joints (1). Inflammatory cytokines and chemokines play a pivotal role in the local and systemic inflammation of arthritic patients, contributing to the development and progression of the disease (2). Despite not being well explored, neutrophils also participate in arthritis progression, and evidence indicates that neutrophil influx occurs during recurrence of the disease (3). The experimental model of AIA is a suitable and reproducible experimental model that exhibits several features with histopathological findings similar to those observed in human rheumatoid arthritis (4, 12). Different categories of drugs are routinely used to treat

arthritis, in order to relieve symptoms and avoid progression of the disease (4). Although some components of the arthritic inflammatory response still need to be elucidated, there have been significant developments in recent decades, including noninvasive therapeutic agents that target inflammation. Cryotherapy has been used as a non-pharmacological intervention to decrease pain, swelling and inflammation (5). Its physiological effects promote a decrease in tissue blood flow by causing vasoconstriction, reducing tissue metabolism, oxygen use and muscle injury (6). Studying the possible effects of cryotherapy in an arthritis model may be useful for clinical practice. The ice pack was considered a well-tolerated modality of therapy (7), and it was effective to ameliorate joint and muscle damage in chronic models of transection of the anterior cruciate ligament (8).

The purpose of this study was to assess the effect of clinical-like cryotherapy on inflammatory signs (in vivo neutrophil migration, cytokines and joint inflammation), pain, and joint swelling in mice with knee arthritis. Balance and motor coordination were also evaluated. Our hypothesis is that clinical-like cryotherapy reduces inflammatory signs, pain, and joint swelling as well as improving balance and motor coordination in mice with acute arthritis of the knee.

Methods

All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health and International Association for the Study of Pain (IASP). The research was approved by the Ethics Committee on the Use of Animals - Ribeirao Preto Medical School, University of Sao Paulo (protocol number 9197020816) and the Federal University of São Carlos (protocol number 1124010316/2015). Trained professionals blinded to the identity of the experimental groups conducted all the procedures.

Animals and Experimental Design

The study was performed using male C57BL/6 mice (20–25 g), maintained in temperature-controlled rooms (22–25 °C) and provided with water and food *ad libitum*. This study was divided into 6-hour experimental analysis periods after injection-induced joint inflammation. The mice were randomly distributed into three experimental groups: Control group: animals were not immunized with mBSA and no intervention was applied; AIA group: animals immunized with mBSA for knee arthritis; AIA + cryotherapy group: animals immunized with mBSA and submitted to clinical-like cryotherapy protocol.

Induction of Antigen-Induced Arthritis (AIA)

Mice were immunized as previously described (9, 10, 11). Briefly, the mice were sensitized with 500 ug of mBSA in 0.2 mL of an emulsion containing 0.1 mL of saline and 0.1 mL of Freund's Complete Adjuvant (CFA) (1 mg/mL of inactive Mycobacterium tuberculosis), administered by subcutaneous (s.c.) injection on day 0. The mice were boosted with the same preparation on day 7 and 14. Control mice were not immunized with the antigen. Twenty-one days after the initial injection, arthritis was induced in the immunized animals by intra-articular (i.a.) injection of mBSA (100 µg/joint) dissolved in 10 µL of saline

into the right tibiofemoral joint. At a pre-established time after antigen challenge (6 hours), the controls and AIA group mice were culled and the AIA + cryotherapy group submitted to the cryotherapy protocol.

Cryotherapy Protocol

Cryotherapy consisted of a plastic pack filled with crushed ice applied directly to the right knee. Mice underwent two 20-min sessions of cryotherapy, with a 2-hour rest period between them. The first session was applied immediately after intra-articular injection of mBSA. For cryotherapy, the animals were anesthetized and maintained in the horizontal position on a table with their right knee in the lateral position (Fig. 1).

In vivo neutrophil migration

Immunized (AIA group, AIA + cryotherapy group) or non-immunized (control group) mice were analyzed 6 hours after mBSA injection directly into the articular cavity. Neutrophil migration was determined using synovial fluid, as previously described(12, 13). The articular cavities were washed twice with 3.3 µL of phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and then diluted to a final volume of 50 µL with PBS/EDTA to evaluate leukocyte migration. The neutrophils were counted in a Neubauer chamber diluted in Turk's solution and the results expressed as the number (mean ± SEM) of neutrophils per joint cavity.

Cytokine measurements

At the established time after i.a. injection of the inflammatory stimuli, the animals were terminally anesthetized, and the knee joints or synovial membranes were removed and homogenized in 300 µl of buffer-containing protease inhibitors. IL-1β, IL-10, TNF-α, and IL-6 concentrations were measured by conducting the enzyme-linked immunosorbent assay (ELISA) using commercial kits (DuoSet; R&D Systems, Minneapolis, MN, USA), as previously described (14, 15). The results were expressed as pg/ml of each cytokine. As control, the concentrations of these cytokines were measured in non-immunized animals.

Evaluation of joint inflammation

Tibiofemoral joints were fixed in 4% (vol/vol) buffered formalin and decalcified in 10% Ethylenediamine tetraacetic acid (EDTA) for 2–3 weeks. The tissues were then trimmed, dehydrated in ethanol, and embedded in paraffin for slide preparation. Joint sections were stained with hematoxylin and eosin (H&E) to analyze synovitis (inflammatory cell influx and synovial hyperplasia). The severity of the synovial pathology (i.e., synovitis) was classified using a scoring system that measured the thickness of the synovial cell layer on a scale of 0–3 (0 = 1–2 cells, 1 = 2–4 cells, 2 = 4–9 cells, and 3 = 10 or more cells) and cellular density in the synovial stroma also on a scale of 0–3 (0 = normal cellularity, 1 = slightly increased cellularity, 2 = moderately increased cellularity, and 3 = significantly increased cellularity(16).

Dorsal flexion of the tibiofemoral joint: Assessment using a modified electronic pressure-meter test for mice

Articular nociception of the tibiofemoral joint was also evaluated using a previous method with modifications (9, 17). Mice were placed in acrylic cages (12 × 10 × 17 cm high) with a wire grid floor in a quiet room, 15–30 min before testing, for environmental adaptation. Stimulations were applied only when animals were quiet, not exploring, defecating, or resting on their paws. An electronic pressure-meter was used in these experiments, in addition to a hand-held force transducer fitted with a polypropylene tip (Insight EFF 301, Brazil). For this model, a large tip (4.15 mm^2) was attached to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the tibiofemoral joint followed by paw withdrawal. A tilted mirror below the grid provided a clear view of the hind paw. The electronic pressure-meter automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until three consistent measurements (i.e. variation of less than 1 g) were obtained. The flexion-elicited mechanical threshold was expressed in grams (g).

Joint swelling (edema)

Three measures of knee joint thickness were taken under anesthesia (0_2: 2.0 L/m, 2% isoflurane), using an electronic digital caliper (Mitutoyo Absolute Digimatic 150 mm, Japan) before i.a. injection with 100 µg of mBSA, and the same procedure was repeated 6 hours after the challenge. The results were expressed as the difference between the initial and final value (delta), in mm (9).

Balance and motor coordination

Rotarod tests were used to measure balance and motor coordination(18). Each mouse was placed in an individual compartment of the rotarod (Insight EFF 412, Brazil) and forced to keep walking to avoid falling off the rod. Animals were first habituated to low rotation (8 rpm) for 300 s, followed by10 rpm for a further 300 s. The time taken for the mouse to fall from the rotating rod was recorded. Animals that did not fall off the rotarod were assigned the maximum score of 300 s. Before intra-articular injection, each animal was trained once 48 hours before the test in order to achieve a stable performance on the rotarod.

Results

In vivo neutrophil migration

This acute phase of AIA groups was also characterized by increased neutrophil recruitment into the knee joint after 6 hours compared to group control; however, the AIA + cryotherapy group exhibited a decline in neutrophil recruitment into the knee joint ($p < 0.0001$), Fig. 2.

Cytokine measurements

It was observed that the mBSA challenge into the tibiofemoral joint of immunized mice induced an increase in IL-1β and IL-6 in synovial tissue 6 h after injection (Fig. 3A and 3C), and local (joint) treatment of immunized mice with cryotherapy against reduced IL-1β, IL-6 and TNF-α during arthritis development (Fig. 3A, 3C and 3D). Additionally, the i.a. injection of mBSA in immunized mice decreased IL-10 levels in

the synovial membrane 6 h after the challenge (Fig. 3B). Local joint treatment of immunized mice with cryotherapy did not reduce IL-10 compared to the control group ($p < 0.0001$), Fig. 3B.

Evaluation of joint inflammation

Joint inflammatory parameters after i.a. injection of mBSA, immunized mice were also evaluated. In line with these findings, histological analysis of joint sections resulted in a severe synovitis score in relation to controls, according to neutrophil infiltration in the synovium and peri-articular tissues 6 h after the mBSA challenge (Fig. 4A), and a moderate synovitis score in the -AIA + cryotherapy group compared to the AIA / or control group (Fig. 4A). Morphologically, scores denote greater synovial layer thickness and cellular density caused by inflammatory leukocyte infiltration ($p < 0.0001$), Fig. 4B.

Dorsal flexion of the tibiofemoral joint: Assessment using a modified electronic pressure-meter test for mice

The i.a. injection of mBSA induced mechanical articular hypernociception only in the AIA group, but not the AIA + cryotherapy group, showing a similar mechanical threshold to that of the control group ($p = 0.0006$), Fig. 5.

Joint swelling (edema)

The mice showed joint swelling 6 h after mBSA challenge, with differences between the AIA group and control and between the AIA + cryotherapy and AIA groups ($p < 0.0001$) (Fig. 6).

Balance and motor coordination

There was a decrease in the latency of gait/ balance and motor coordination in the AIA group compared to both the control and AIA + cryotherapy groups ($p = 0.02$) (Fig. 7).

Discussion

Our results showed that the application of clinical-like cryotherapy in mice with acute knee arthritis reduced inflammatory signs and improved pain, joint swelling, motor coordination and balance.

The inflammatory signs (neutrophil migration, cytokines and joint inflammation) analyzed in our study are related to the systemic inflammatory response of arthritis (19, 20). The experimental model of AIA is a suitable and reproducible experimental model that exhibits a number of histopathological findings similar to those observed in human rheumatoid arthritis (4, 12).

One of the beneficial effects of cryotherapy was the decline in neutrophil migration to the synovial fluid. High neutrophil levels are found in human arthritic joints, especially in the joint synovial fluid, with significant potential to inflict damage directly to tissue, bone and cartilage through the secretion of

proteases and metabolites, as well as stimulate inflammation via the secretion of cytokines, chemokines, prostaglandins and leukotrienes(21, 22, 23, 24). The decline in neutrophil migration was accompanied by a decrease in IL-1β, IL-6 and TNF-α cytokine levels in the synovial fluid, demonstrating the beneficial effect of the clinical-like cryotherapy protocol used to control acute joint inflammation. Levels of these cytokines are known to rise in the early stages of arthritis and fall during regression(25, 26). Histological analysis revealed that cryotherapy also improved the inflammatory picture by reducing leukocyte infiltration and thickening the synovial membrane.

In addition to the aforementioned inflammatory signs, the presence of pain and swelling are also characteristic of arthritis. Our results also showed the beneficial effects of clinical-like cryotherapy in reducing pain and joint swelling. The pain resulting from the antigen-induced challenge in mice depends on a cytokine cascade(15). Joint swelling, caused by the release of inflamed synovium through the cells of local blood vessels, is common in different types of arthritis (27, 28).

Two cryotherapy sessions (20 min every 2 hours) improved inflammatory signs, decreased pain and swelling, and enhanced the motor coordination and balance of the animals in the acute phase of arthritis. Cryotherapy has been used in the clinical rehabilitation of patients with post-sports injury arthritis, reducing joint pain, swelling, degeneration and inflammation(29, 30, 31). Our study provides a new scientific contribution on the benefits of clinical-like cryotherapy in the treatment of acute knee arthritis in an animal model. It is also used empirically as a complementary treatment for rheumatoid arthritis, with a good tolerance profile when compared to corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs); however, the protocols have not been standardized(7).

Conclusions

Clinical-like cryotherapy used in the acute phase of arthritis reduced inflammatory signs and improved pain, joint swelling, motor coordination and balance in mice.

Abbreviations

AIA - antigen-induced arthritis; CFA - Freund's Complete Adjuvant; EDTA – ethylenediamine tetraacetic acid; ELISA - enzyme-linked immunosorbent assay ; H&E - hematoxylin and eosin; IASP - International Association for the Study of Pain; mBSA - methylated bovine serum albumin; NSAIDs - non-steroidal anti-inflammatory drugs; PBS - phosphate-buffered saline.

Declarations

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The research was approved by the Ethics Committee on the Use of Animals - Ribeirao Preto Medical School, University of Sao Paulo (protocol number 9197020816) and the Federal University of São Carlos (protocol number 1124010316/2015).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the study are available from the corresponding author on reasonable request.

COMPETITIVE INTERESTS

The authors declare that they have no competing interests.

FINANCING

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AUTHORS' CONTRIBUTIONS

Study conception and design: Castro PATS, Salvini TF;Collection and assembly of data: Castro PATS, Machanocker DH, Cunha JE, Barbosa GM, Perez RS, Oliveira FFB; Analysis and interpretation of the data: Castro PATS, Cunha TM, Cunha FQ, Ramalho FS, Salvini TF;Drafting of the manuscript: Castro PATS, Salvini TF; Critical revision of the article for important intellectual content: All authors; Final approval of the article: All authors; Obtaining of funding: Salvini TF; Castro, P.A.T. takes responsibility for the integrity of the work as a whole (paula.soupat@gmail.com).

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Figures

Figure 1

Animals’ position during the cryotherapy protocol. * ice pack; → right paw.

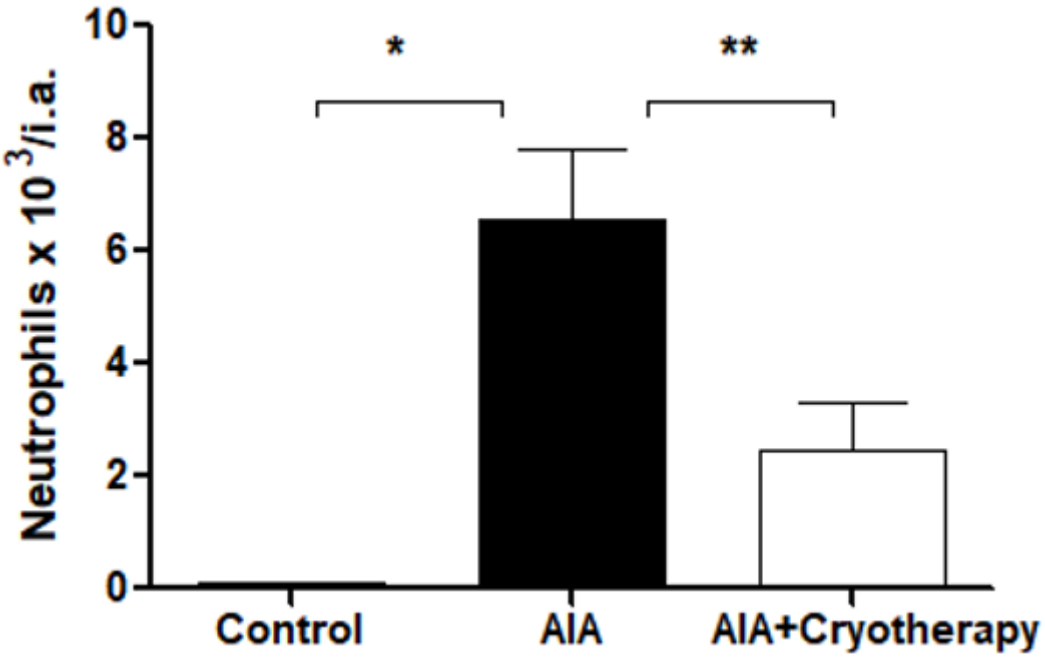

Figure 2

In vivo neutrophil migration in the AIA model. Immunized C57BL/6 mice were challenged with i.a. injection of 100 µg of mBSA per joint and treated with cryotherapy (crushed ice bag). In vivo neutrophil migration was evaluated 6 hours after mBSA injection using a Neubauer chamber. Data are expressed as the mean ± SEM (n = 10). $p<0.0001$: * compared to the control group; ** compared to the AIA group. One-way analysis of variance with Tukey's Multiple Comparison Test.

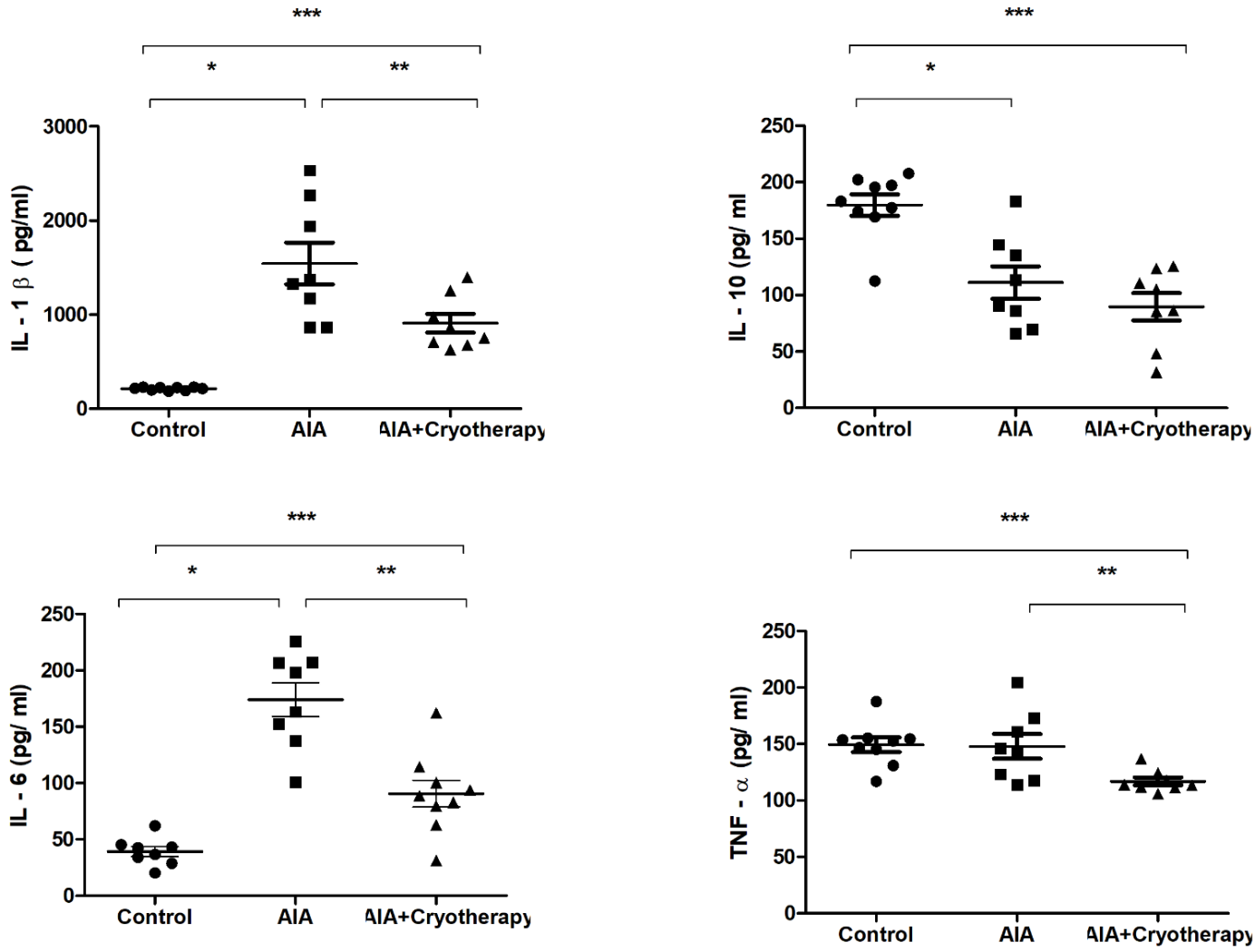

Figure 3

Levels of IL-1β, IL– 10, IL-6 and TNF-α in the AIA model. Immunized C57BL/6 mice were challenged with i.a. injection of 100 µg of mBSA per joint and treated with cryotherapy (crushed ice bag). The concentrations of (A) IL-1β, (B) IL-10, (C) IL-6 and (D) TNF-α were evaluated 6 hours after mBSA injection using the Elisa Kit. Data are expressed as mean ± SEM (n=8). p<0.0001: * compared to the control group; ** compared to the AIA group; *** compared to the AIA+cryotherapy group.

Figure 4

Synovitis scores in the AIA model. A: Synovitis scores of acute joint pathology and synovial inflammation 6h after the challenge. The results are expressed as mean ± SEM (n=8). p<0.0001 *compared to the control group; ** compared to the AIA group; *** compared to the AIA+cryotherapy group. B: Representative images of knee joint sections stained with H&E and respective histopathological scores. Magnification: upper row 400x; lower row 200x.

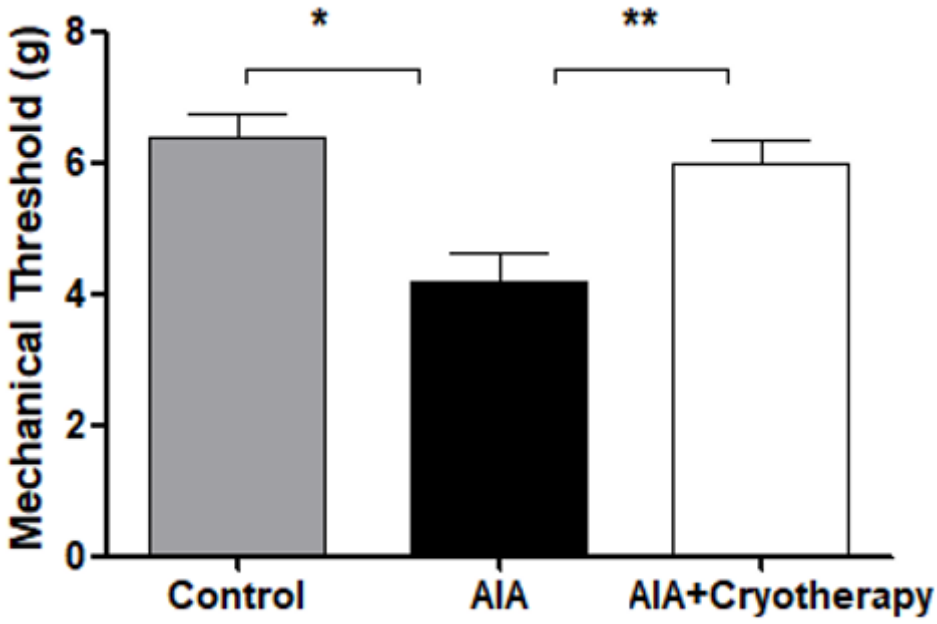

Figure 5

Articular hypernociception in the AIA model. Immunized C57BL/6 mice were challenged with i.a. injection of 100 µg of mBSA per joint and treated with cryotherapy (crushed ice bag). Articular hypernociception was evaluated 6 hours after mBSA injection using an electronic Von Frey esthesiometer. Data are expressed as mean ± SEM (n = 10). p=0.0006: * compared to the control group; ** compared to the AIA group. One-way analysis of variance with Tukey's Multiple Comparison Test.

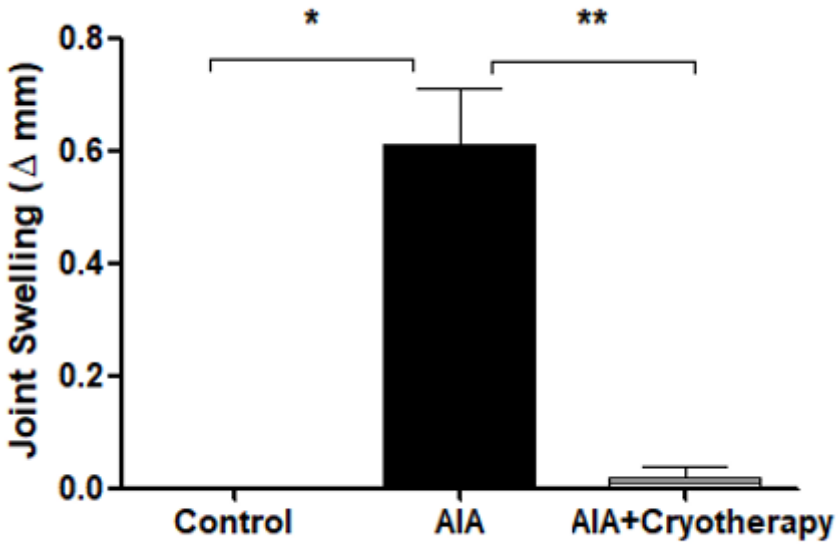

Figure 6

Joint swelling in the AIA model. Immunized C57BL/6 mice were challenged with i.a. injection of 100 µg of mBSA per joint and treated with cryotherapy (crushed ice bag). Joint swelling (Δmm) was evaluated 6 hours after mBSA injection using a digital caliper. Data are expressed as mean ± S.E.M. (n = 10). p<0.0001: * compared to the control group; ** compared to the AIA group. One-way analysis of variance with Tukey's Multiple Comparison Test.

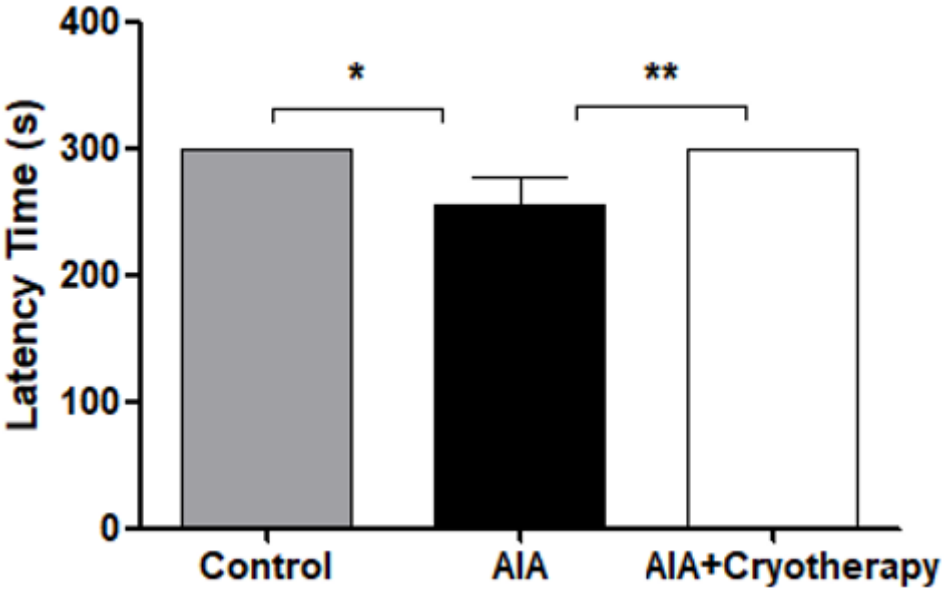

Figure 7

Gait function/balance and motor coordination in the AIA model. Immunized C57BL/6 mice were challenged with i.a. injection with 100 µg of mBSA per treated and treated with cryotherapy (crushed ice bag). Balance and motor coordination were evaluated 6 hours after mBSA injection using the rotarod test. Data are expressed as mean ± S.E.M. (n = 10). p=0.02; * compared to the control group; ** compared to the AIA group. One-way analysis of variance with Tukey's Multiple Comparison Test.