

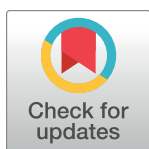
RESEARCH ARTICLE

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

Paula A. T. S. Castro^{1*}, Germanna M. Barbosa¹, Dafiner H. Machanocker², Raphael S. Peres², Thiago M. Cunha², Jonathan E. Cunha¹, Francisco F. B. Oliveira¹, Fernando Silva Ramalho², Thiago L. Russo¹, Fernando Q. Cunha², Tania F. Salvini¹

1 Department of Physical Therapy, Center of Biological Sciences and Health (CBSH), Federal University of São Carlos (UFSCAR), São Carlos, Brazil, **2** Department of Pharmacology, Center for Research in Inflammatory Diseases (CRID), Ribeirão Preto Medical School, University of São Paulo (USP), Ribeirão Preto, Brazil

* paula.soupat@gmail.com



OPEN ACCESS

Citation: Castro PATS, Barbosa GM, Machanocker DH, Peres RS, Cunha TM, Cunha JE, et al. (2022) Clinical-like cryotherapy in acute knee arthritis of the knee improves inflammation signs, pain, joint swelling, and motor performance in mice. PLoS ONE 17(1): e0261667. <https://doi.org/10.1371/journal.pone.0261667>

Editor: Rafael Ximenes, Universidade Federal de Pernambuco, BRAZIL

Received: April 19, 2021

Accepted: December 7, 2021

Published: January 21, 2022

Copyright: © 2022 Castro et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This research was funded by FAPESP, grant number 2015/26567-2 and CNPq, grant number 2011/22122-5 and 2013/301344-2. The responsible for receiving the funding studies is the authors PATSC, FAPESP, grant number 2015/26567-2. The responsible for receiving the funding studies is the authors TFS, CNPQ, grant number 2011/22122-5 and 2013/301344-2.

Abstract

To assess the effects of clinical-like cryotherapy on inflammatory signs (*in vivo* neutrophil migration, cytokines, and joint inflammation), pain, joint swelling, balance, and motor coordination in mice with knee arthritis. Young C57BL/6 mice were randomly divided into three groups (8 to 10 mice per group): Control group: mice with no intervention; antigen-induced arthritis (AIA) group: mice sensitized and immunized with intra-articular (i.a.) injection of methylated bovine serum albumin (mBSA); and AIA + cryotherapy group: mice sensitized, immunized with i.a. injection of mBSA, and submitted to a clinical-like cryotherapy protocol. After 21 days of sensitization, AIA and AIA + cryotherapy groups received i.a. injection of mBSA (100 µg/joint) to induce joint inflammation, and a clinical-like cryotherapy protocol was applied to AIA + cryotherapy group (crushed ice bag, two cryotherapy sessions of 20 min every two hours). Experimental analysis was conducted in the initial (immediately after i.a. injection of mBSA) and final periods (two hours after the second cryotherapy session). The number of synovial fluid neutrophils, cytokine levels, joint histology, pain, joint swelling, and motor performance were also analyzed. Our results showed that clinical-like cryotherapy in mice with acute knee arthritis reduced inflammatory signs, pain, and joint swelling, and improved balance and motor coordination.

Introduction

Arthritis is characterized by infiltration of inflammatory cells and cartilage and destruction of bone, clinically manifested as pain, swelling, and stiffness in affected joints [1]. Inflammatory cytokines and chemokines play a pivotal role in local and systemic inflammation in patients with arthritis, contributing to development and progression of the disease [2]. Although not

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: AIA, Antigen-induced arthritis; mBSA, Methylated bovine serum albumin; CFA, Freund's complete adjuvant; PBS, Phosphate-buffered saline; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; H&E, Hematoxylin and eosin.

well studied, neutrophils also participate in disease progression, and evidence indicates that neutrophil influx occurs during disease recurrence [3]. The experimental model of antigen-induced arthritis (AIA) is suitable, reproducible, and exhibits histopathological findings similar to human rheumatoid arthritis [4,12].

Several drug classes are used to treat arthritis, relieve symptoms, and avoid disease progression [5]. Although some components of the arthritic inflammatory response still need to be elucidated, significant developments have been observed in recent decades, including noninvasive therapeutic agents targeting inflammation. Cryotherapy is often used as non-pharmacological intervention to decrease pain, swelling, and inflammation [6]; its physiological effects decrease local blood flow by vasoconstriction, tissue metabolism, oxygen use, and muscle injury [7]. Therefore, studying the possible effects of cryotherapy in an arthritis model may be useful for clinical practice. For example, the ice pack was considered well-tolerated [8,37] and effective in reducing joint and muscle damage in chronic models of anterior cruciate ligament transection [9].

Thus, this study aimed to assess the effects of clinical-like cryotherapy on inflammatory signs (i.e., *in vivo* neutrophil migration, cytokines, and joint inflammation), pain, joint swelling, balance, and motor performance in mice with knee arthritis. We hypothesized that clinical-like cryotherapy reduces inflammatory signs, pain, and joint swelling and improves balance and motor coordination in mice with acute knee arthritis.

Materials and methods

This study was conducted following the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and the International Association for the Study of Pain. The protocol was approved by the research ethics committee of University of São Paulo (Protocol Number: 9197020816) and Federal University of São Carlos (Protocol Number: 1124010316/2015). Trained professionals blinded to experimental groups conducted all procedures. The study was designed to detect a between-group difference of one second in paw withdrawal latency based on a previous study with similar methods [10]. A statistical power ($1-\beta$) of 80%, alpha of 5%, and effect size of 0.52 were considered for sample calculation, resulting in a total of 30 mice (8 to 10 per group). The G*Power software (version 3.1; University of Trier, Trier, Germany) was used for sample size calculation and power analysis [11].

Animals and experimental design

The study was performed using 30 young male C57BL/6 mice (20 to 25 g), maintained in temperature-controlled rooms (22 to 25°C), and provided with water and food *ad libitum*. Mice were randomly distributed into three experimental groups (8 to 10 mice each): control group, mice with no intervention; AIA group, mice were sensitized and immunized with i.a. injection of mBSA; AIA + Cryotherapy group, mice were sensitized, immunized with i.a. injection of mBSA, and submitted to the clinical-like cryotherapy protocol. After intra-articular (i.a.) injection of methylated bovine serum albumin (mBSA, 100 µg/joint) to induce joint inflammation, this study was divided into two periods of experimental analysis: initial, immediately after i.a. injection of mBSA; and final, two hours after the second cryotherapy session.

Induction of antigen-induced arthritis

AIA and AIA+Cryotherapy mice were immunized as previously described [12–14] and sensitized with 500 µg of mBSA in 0.2 mL of an emulsion containing 0.1 mL of saline and 0.1 mL of Freund's Complete Adjuvant (1 mg/mL of inactive *Mycobacterium tuberculosis*). The application was administered by subcutaneous injection in the dorsal region (near the tail) on day 0.



Fig 1. Position of mice during clinical-like cryotherapy. * crushed ice bag; → right paw.

<https://doi.org/10.1371/journal.pone.0261667.g001>

Mice were boosted with the same preparation on days 7 and 14. Control mice were neither sensitized nor immunized with i.a. injection of mBSA. Twenty-one days after initial injection, arthritis was induced in immunized mice with i.a. injection of mBSA (100 µg/joint), dissolved in 10 µL of saline into the right tibiofemoral joint. Mice were manipulated with extreme caution and anesthetized (O₂: 2.0 L/m, 2% isoflurane) during one min for each immunization to minimize pain. AIA and AIA+Cryotherapy mice immunized with i.a. injection of mBSA were isolated and placed in a separate cage. Mice from all groups were anesthetized with O₂ 2.0 L/m, 2% isoflurane and euthanized by cervical dislocation in the final period of experimental analysis.

Clinical-like cryotherapy protocol

This protocol was adapted to simulate clinical practice [37]. First, mice were anesthetized (O₂ 2.0 L/m, 2% isoflurane) and maintained in dorsal decubitus on a table with right knee in lateral position (Fig 1). Then, clinical-like cryotherapy (plastic bag filled with crushed ice) was applied directly to the right knee immediately after i.a. injection of mBSA. Two cryotherapy sessions were performed for 20 min every two hours.

In vivo neutrophil migration

In the final period of experimental analysis, the articular cavity of immunized (AIA group, AIA + Cryotherapy group) and non-immunized (control group) mice were analyzed. Neutrophil migration was determined through synovial fluid, using a previously established method (with modifications) [15,16]. Articular cavities were washed twice with 3.3 µL of phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and diluted to a final volume of 50 µL with PBS/EDTA to assess leukocyte migration. Neutrophils were counted in a Neubauer chamber with Turk's solution, and results were expressed as number of neutrophils (mean ± standard error of mean [SEM]) per joint cavity.

Cytokine levels

Concentrations of IL-1β, IL-10, TNF-α, and IL-6 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (DuoSet; R&D Systems, Minneapolis, MN, USA). In the final period of experimental analysis, mice were terminally anesthetized, and

tibiofemoral joints and synovial membranes were removed and homogenized in 300 μ L of buffer with protease inhibitors, as previously described (with modifications) [17,18]. Results were expressed as pg/ml of each cytokine.

Evaluation of joint inflammation

Tibiofemoral joints were collected in the final period of experimental analysis, fixed in 4% (vol/vol) buffered formalin, and decalcified in 10% EDTA for two to three weeks. Tissues were then trimmed, dehydrated in ethanol, and embedded in paraffin for histological preparation. Joint sections were stained with hematoxylin and eosin (H&E) to analyze synovitis (i.e., inflammatory cell influx and synovial hyperplasia). Synovitis severity was classified by measuring thickness of synovial cell layer on a scale from 0 to 3 (0 = 1 to 2 cells, 1 = 2 to 4 cells, 2 = 4 to 9 cells, and 3 = ≥ 10 cells) and cellular density in the synovial stroma on a scale from 0 to 3 (0 = normal cellularity, 1 = slightly increased cellularity, 2 = moderately increased cellularity, and 3 = greatly increased cellularity) [19]. Three histological sections were assessed separately, obtaining an average value for each mice.

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

Articular nociception of the tibiofemoral joint was assessed in the final period of experimental analysis using a previously established method (with modifications) [9,20]. Mice were placed in acrylic cages (12 \times 10 \times 17 cm) with a wire grid floor in a quiet room 15 to 30 min before testing for environmental adaptation. Stimulations were applied when animals were quiet, not exploring, defecating, or resting on their paws. An electronic pressure-meter and hand-held force transducer fitted with polypropylene tip (Insight EFF 301, Brazil) were used in these experiments. For this model, a large tip (4.15 mm²) was attached to the probe. An increasing perpendicular force was applied to the central area of plantar surface of the hind paw to induce flexion of 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 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. Flexion-elicited mechanical threshold was expressed in grams (g).

Joint swelling (edema)

Joint thickness of the knee was analyzed under anesthesia (O₂ 2.0 L/m, 2% isoflurane) using an electronic digital caliper (Mitutoyo Absolute Digimatic 150 mm, Japan) and measured in the initial and final periods of experimental analysis in the same conditions. Three measurements were performed in the same area for each joint, and results were expressed in mm [12,21,22].

Knee skin temperature

Knee skin temperature was analyzed under anesthesia (O₂ 2.0 L/m, 2% isoflurane) using an infrared digital thermometer (-18 to 260°C; Wurth Temp, Cotia, Brazil) in the initial and final periods of experimental analysis. Three measurements were performed in the same area for each joint, and results were expressed in °C [15,21].

Gait function—balance and motor coordination

Gait function (balance and motor coordination) was assessed using a previously established method (with modifications) [23,24]. 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 rotarod. Fall time was recorded in seconds (i.e., latency time). Mice that did not fall off the rotarod received the maximum score of 300 s. Each mouse performed low rotation (8 rpm) for 300 s, 48 hours before the initial period of experimental analysis to adapt and attain stable performance in the rotarod. On the experimental day, low speed (10 rpm) was established, and mice performed the test until 300 s or falling [24]. Analysis was performed with 10 mice from each group simultaneously, with no repetition on the experimental day.

Statistical evaluation

Analyses were performed using the Statistical Package for the Social Sciences software (SPSS 22.0 Inc, Chicago, IL). Homogeneity of variance and normality distribution were verified using Levene and Shapiro-Wilk tests, respectively. Ordinary one-way ANOVA compared between-group differences of *in vivo* neutrophil migration, cytokines levels, synovitis score, nociception, and balance and motor coordination. Two-way ANOVA with repeated measures compared skin temperature and joint swelling with interaction between group (control, AIA, AIA + Cryotherapy) and initial and final periods of experimental analysis. Tukey's HSD post-hoc test was performed when needed ($\alpha = 5\%$ and 95% confidence interval). Data were expressed as mean \pm SEM.

Results

In vivo neutrophil migration

Acute phase of AIA groups exhibited increased neutrophil recruitment into knee joint in the final period of experimental analysis compared with control group (mean difference: 6.46 neutrophils $\times 10^3$ /i.a., Power = 0.99, $F_{2,27}$: 14.00, $P < 0.0001$). AIA + Cryotherapy presented increased neutrophil recruitment into knee joint compared with control group (mean difference: 2.37 neutrophils $\times 10^3$ /i.a., Power: 0.78; $F_{2,27}$: 14.00, $P > 0.05$) and decreased compared with AIA group (mean difference: 4.09 neutrophils $\times 10^3$ /i.a., Power: 0.75; $F_{2,27}$: 14.00; $P < 0.0001$, Fig 2).

Cytokines levels

In the final period of experimental analysis, concentrations of IL-1 β (mean difference: 1331 pg/ml, Power = 0.99, $F_{2,22}$: 24.64, $P < 0.0001$), IL-10 (mean difference: 68.68 pg/ml, Power = 0.99, $F_{2,22}$: 15.91, $P < 0.0001$), and IL-6 (mean difference: 134.9 pg/ml, Power = 1.00, $F_{2,22}$: 34.01, $P < 0.0001$) increased in AIA group compared with control group (Fig 3A–3C). In AIA + Cryotherapy group, IL-1 β (mean difference: 635.9 pg/ml, Power = 0.79, $F_{2,22}$: 24.64, $P < 0.0001$), IL-6 (mean difference: 83.34 pg/ml, Power = 0.98, $F_{2,22}$: 34.01, $P < 0.0001$), and TNF- α (mean difference: 31.02 pg/ml, Power = 0.89, $F_{2,22}$: 5.814, $P = 0.0094$) decreased compared with AIA group. IL-10 (mean difference: 90.25 pg/ml, Power = 0.99, $F_{2,22}$: 15.91, $P < 0.0001$) and TNF- α (mean difference: 32.38 pg/ml, Power = 0.99, $F_{2,22}$: 5.814, $P = 0.0094$) also decreased in the AIA + Cryotherapy group compared with control group (Fig 3B).

Evaluation of joint inflammation

Histological analysis of joint sections showed severe synovitis score in AIA group compared with control group (mean difference: 3.288, Power = 1.00, $F_{2,21}$: 30.31, $P < 0.0001$, Fig 4A). Neutrophil infiltration in synovium and peri-articular tissues in the final period of experimental analysis showed a moderate synovitis score in AIA + Cryotherapy group compared with AIA (mean difference: 1.088, Power = 0.68, $F_{2,21}$: 30.31, $P < 0.0001$) and control group (mean

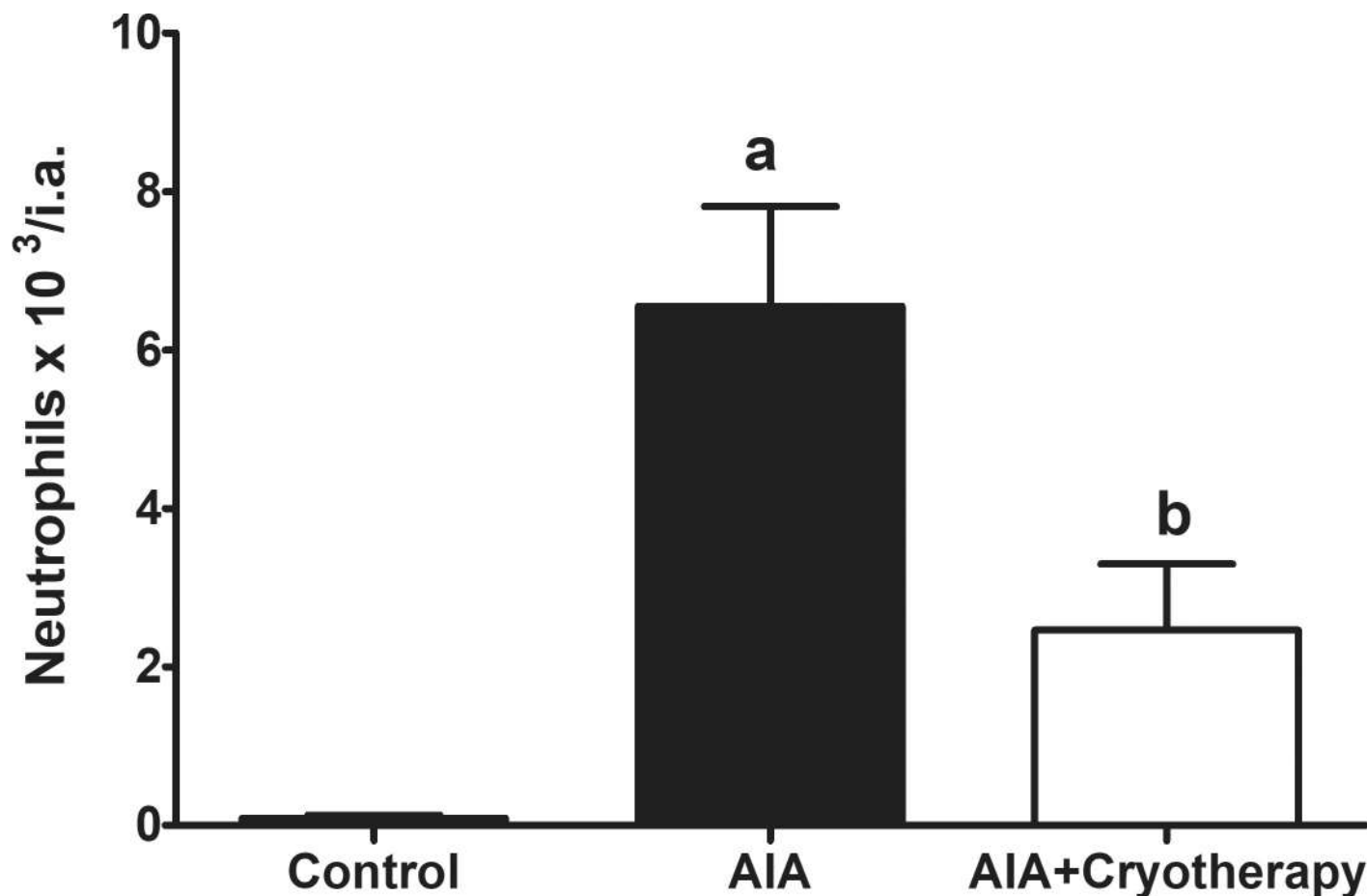


Fig 2. *In vivo* neutrophil migration in AIA model. Immunized C57BL/6 mice received i.a. injection of 100 µg of mBSA per joint and were treated with clinical-like cryotherapy (crushed ice bag). *In vivo* neutrophil migration was assessed in the final period of experimental analysis using a Neubauer chamber. Data expressed as mean ± SEM (n = 10). (a): Compared to control group; (b): Compared to AIA group.

<https://doi.org/10.1371/journal.pone.0261667.g002>

difference: 2.200, Power = 0.99, $F_{2,21}$: 30.31, $P < 0.0001$, Fig 4A). Morphologically, scores indicated increased synovial layer thickness and cellular density caused by inflammatory leukocyte infiltration (Fig 4B).

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

Mechanical threshold reduced in AIA group compared with control group (mean difference: 2.200 g, Power = 0.96, $F_{2,27}$: 9.758, $P = 0.0006$). On the other hand, mechanical threshold of AIA + Cryotherapy group increased compared with AIA group (mean difference: 1.800 g, Power = 0.87, $F_{2,27}$: 9.758, $P = 0.0006$, Fig 5).

Joint swelling (edema)

Knee joint swelling increased in AIA group compared with control group (mean difference: 0.52 mm, Power = 0.89, $F_{2,27}$: 3.01, $P < 0.01$) in the final period of experimental analysis. Joint swelling was reduced in AIA + Cryotherapy group compared with AIA group (mean difference: 0.55 mm, Power = 0.95, $F_{2,27}$: 3.01, $P < 0.001$), but no differences were observed compared with control group (mean difference: 0.03 mm, Power = 0.05, $F_{2,27}$: 3.01 $P > 0.05$, Fig 6).

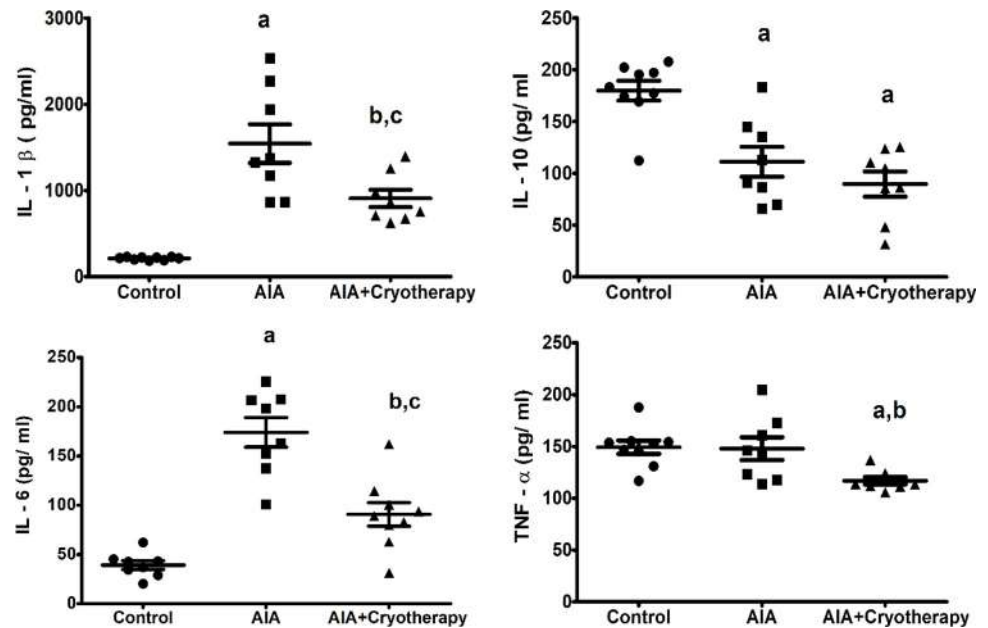


Fig 3. Levels of IL-1 β , IL-10, IL-6, and TNF- α in AIA model. Immunized C57BL/6 mice received i.a. injection of 100 μ g of mBSA per joint and were treated with clinical-like cryotherapy (crushed ice bag). Concentrations of (A) IL-1 β , (B) IL-10, (C) IL-6, and (D) TNF- α were assessed in the final period of experimental analysis using ELISA Kit. Data expressed as mean \pm SEM (n = 10). (a): Compared to control group; (b): Compared to AIA group; (c): Compared to AIA + Cryotherapy group.

<https://doi.org/10.1371/journal.pone.0261667.g003>

Knee skin temperature

No difference in skin temperature of right knee joint was observed among groups ($F_{2,27}$: 1.38, $P > 0.05$).

Gait function—balance and motor coordination

Latency time was shorter in AIA group than in control group (mean difference: 45.11 s, Power = 0.55, $F_{2,26}$: 4.45, $P = 0.02$). In AIA + Cryotherapy group, latency time was higher than in AIA group (mean difference: 45.11 s, Power = 0.55, $F_{2,26}$: 4.45, $P = 0.02$) and similar to control group (Mean difference: 0.00 s, Power = 0.05, $F_{2,26}$: 4.45, $P = 0.02$, Fig 7).

Discussion

Cryotherapy improved balance and motor coordination and reduced inflammatory signs (synovitis severity), pain, and joint swelling in mice with acute knee arthritis. Inflammatory signs (i.e., neutrophil migration, cytokines levels, and joint inflammation [synovitis severity]) are related to systemic inflammatory response of arthritis [38]. Moreover, the AIA experimental model is adequate for experimental studies and reproducible, with several histopathological characteristics similar to human rheumatoid arthritis [3,25].

Cryotherapy reduced neutrophil migration to synovial fluid. In humans, joints with arthritis present high levels of neutrophils, which may damage cartilage, bones, and tissue through secretion of proteases and metabolites. In addition, neutrophils stimulate inflammation by releasing cytokines, chemokines, leukotrienes, and prostaglandins [26–29]. The decrease in neutrophil migration was accompanied by pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in synovial fluid, confirming the beneficial effect of clinical-like cryotherapy in controlling

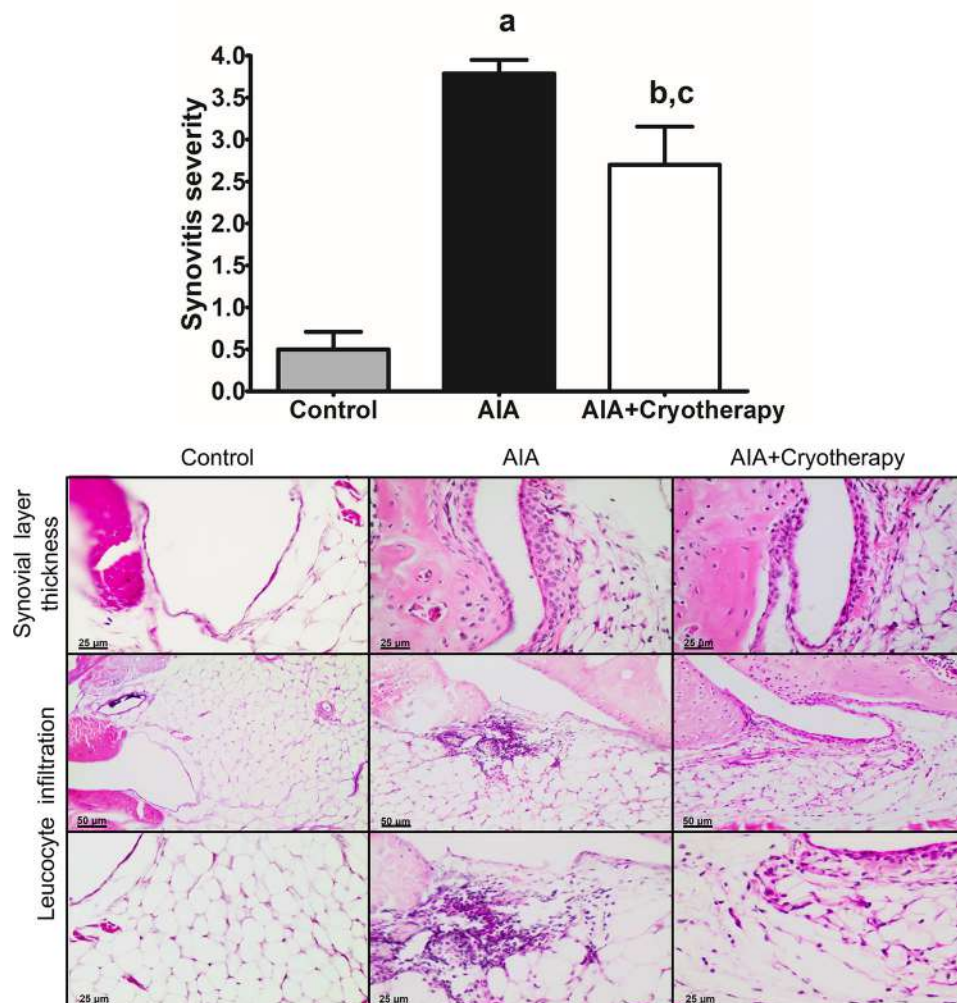


Fig 4. Synovitis scores in AIA model. A: Synovitis scores of acute joint pathology and synovial inflammation in the final period of experimental analysis. Data expressed as mean ± SEM (n = 10). (a): Compared to control group; (b): Compared to AIA group; (c): Compared to AIA + Cryotherapy group. B: Representative images of knee joint sections stained with H&E and respective histopathological scores.

<https://doi.org/10.1371/journal.pone.0261667.g004>

acute inflammation in knee arthritis. Cryotherapy may also downregulate important enzymatic pathways; for example, cytokines, cartilage-degrading enzymes, and proangiogenic factors involved in joint inflammation and destruction [7]. Cryotherapy did not decrease IL-10 levels in the present study, suggesting its action against progression of acute joint inflammation and demonstrating that IL-10 is beneficial as anti-inflammatory cytokine; however, the action pathway of IL-10 needs to be better investigated. Cytokines level (members of IL-1 family, IL-6 and its receptor, IL-17, IL-20, IL-21, and IL-23) increases in early stages of arthritis and decreases during regression of inflammation [30,31]. Histological analysis revealed that cryotherapy also reduced the inflammatory state by reducing leucocyte infiltration and reducing thickening of the synovial membrane.

Our results also showed the benefits of clinical-like cryotherapy in reducing pain and joint swelling. Pain resulting from AIA in mice depends on a cytokine cascade [18]. Joint swelling is common in several types of arthritis and is caused by synovial inflammation, increasing permeability of endothelial cells and plasma extravasation [32,33].

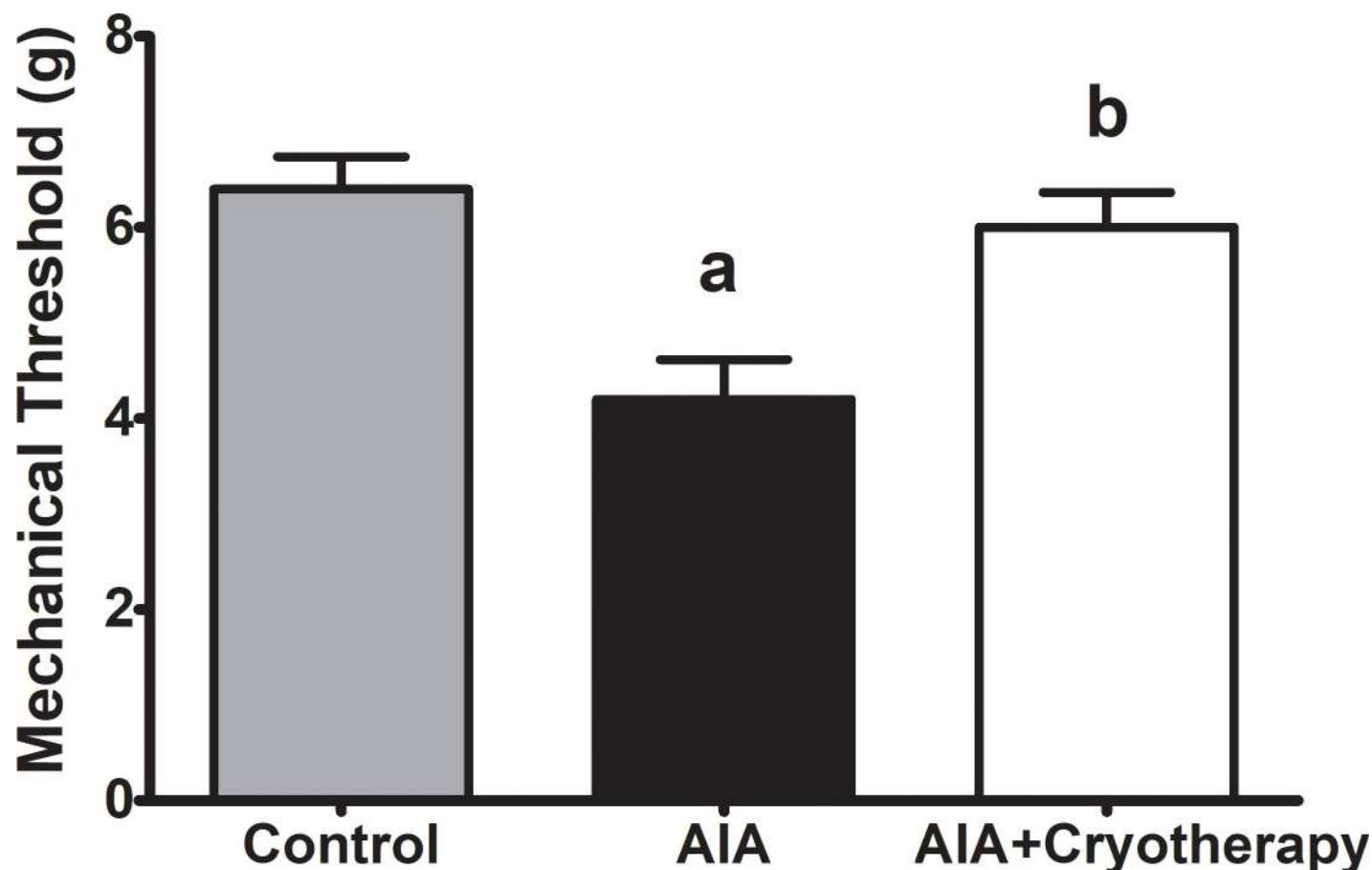


Fig 5. Mechanical threshold in AIA model. Immunized C57BL/6 mice received i.a. injection of 100 μ g of mBSA per joint and were treated with clinical-like cryotherapy (crushed ice bag). Mechanical threshold was assessed using an electronic Von Frey esthesiometer in the final period of experimental analysis. Data expressed as mean \pm SEM (n = 10). (a): Compared to control group; (b): Compared to AIA group.

<https://doi.org/10.1371/journal.pone.0261667.g005>

Inflammatory and nociceptive effects of arthritis may induce balance and motor abnormalities [34], despite no association between motor coordination and pain was found in our study. Therefore, we suggest that the effects of clinical-like cryotherapy contributed to preserving gait function. Cryotherapy has been used in clinical rehabilitation to reduce joint pain, swelling, degeneration, and inflammation in patients with arthritis induced by post-sports injury and several rheumatic joint diseases [35–38]. In addition, cryotherapy can be prescribed in isolation or associated with other therapies and may be well-accepted by patients with knee osteoarthritis [37]. According to Ogura et al., [37], the 20-min ice pack protocol simulates clinical treatment, and the effect of short-term cryotherapy benefits pain intensity.

Most studies investigating the effects of cryotherapy on acute arthritis used a protocol of longer duration or higher intensity than performed in clinical practice. Results of our cryotherapy protocol (based on clinical practice) indicate that new protocols should be developed, and other types of cryotherapy might be associated with treatment of rheumatoid arthritis. Several action mechanisms have been proposed to explain the effects of cryotherapy on inflammatory process of joints [7,8] and should be further studied. In the study by Ramos et al. [38], cryotherapy (three sessions of 30 min every two hours) decreased inflammatory processes (mRNA

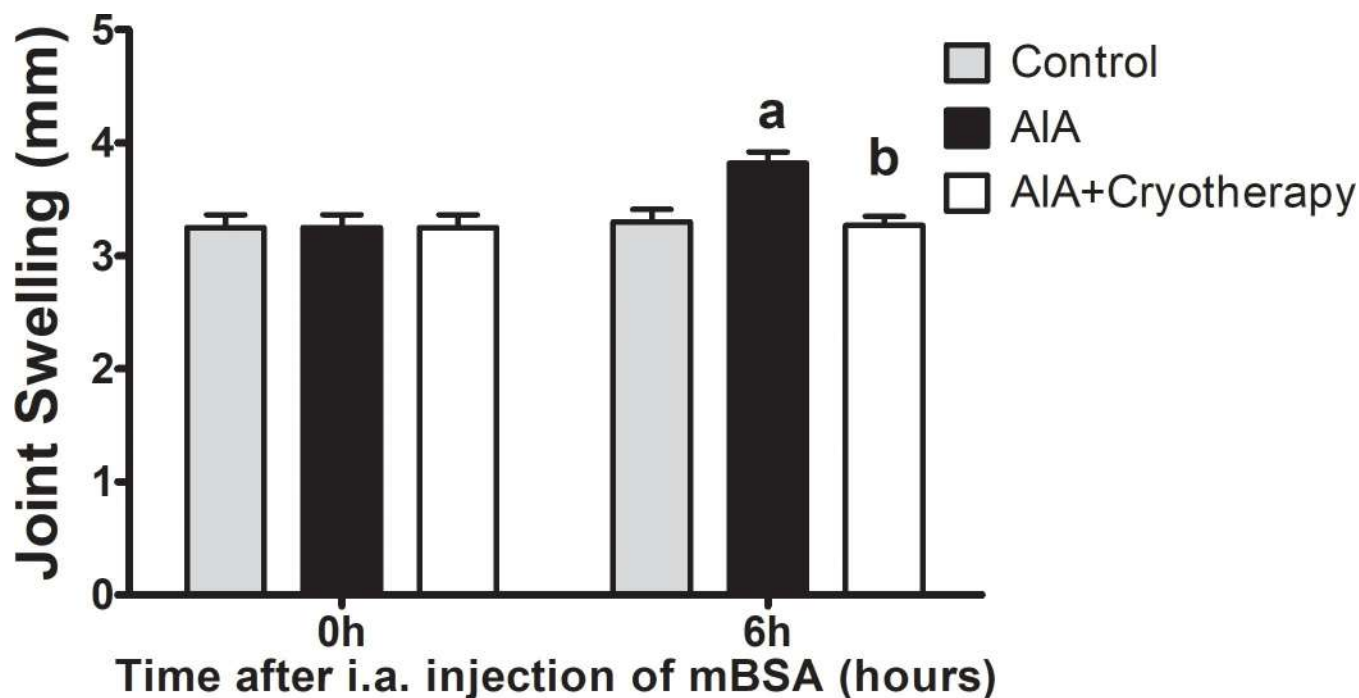


Fig 6. Joint swelling in AIA model. Immunized C57BL/6 mice received i.a. injection of 100 μ g of mBSA per joint and were treated with clinical-like cryotherapy (crushed ice bag). Joint swelling (mm) was assessed using a digital caliper in the initial and final periods of experimental analysis. Data expressed as mean \pm SEM (n = 10). (a): Compared to control group; (b): Compared to AIA group.

<https://doi.org/10.1371/journal.pone.0261667.g006>

levels of TNF- α , NF- κ B, TGF- β , MMP-9, and macrophage percentage) in the first 48 hours after injury. Also, Siqueira et al. [39] demonstrated that multiple applications of cryotherapy after muscle injury (30 min three times per day, on the day of the injury, and for two days after injury) reduced oxidative stress in rats. In another study [40], ice packs (30 min once per day for 10 days) reduced cell infiltrate and synovial hyperplasia in a rabbit model of zymosan-induced arthritis. Clinical-like cryotherapy also reduced joint synovial inflammation of anterior cruciate ligament transection-induced knee osteoarthritis [9]. Cryotherapy activates efferent parasympathetic neurons to release acetylcholine. These ligand-receptor interactions may inhibit NF- κ B pathway and subsequently decrease pro-inflammatory cytokines, oxidative stress agents, and gene transcription of adhesion molecules [41–44]. Therefore, molecular, pro-inflammatory, and cytokine pathways should be studied to understand the anti-inflammatory properties in inflammatory rheumatic diseases.

Our study provides a new contribution on the benefits of clinical-like cryotherapy for treating acute knee arthritis in an animal model. Cryotherapy is also used empirically as complementary treatment for rheumatoid arthritis and present good tolerance compared with corticosteroids or non-steroidal anti-inflammatory drugs; however, protocols were not standardized yet [7].

Conclusions

Two 20-min clinical-like cryotherapy sessions (the first session applied immediately after i.a. injection of mBSA and the second applied after 2 hours after the first session) decreased inflammatory signs (i.e., neutrophil migration and synovitis severity), pain, and joint swelling, and improved balance and motor coordination in mice in the acute phase of arthritis.

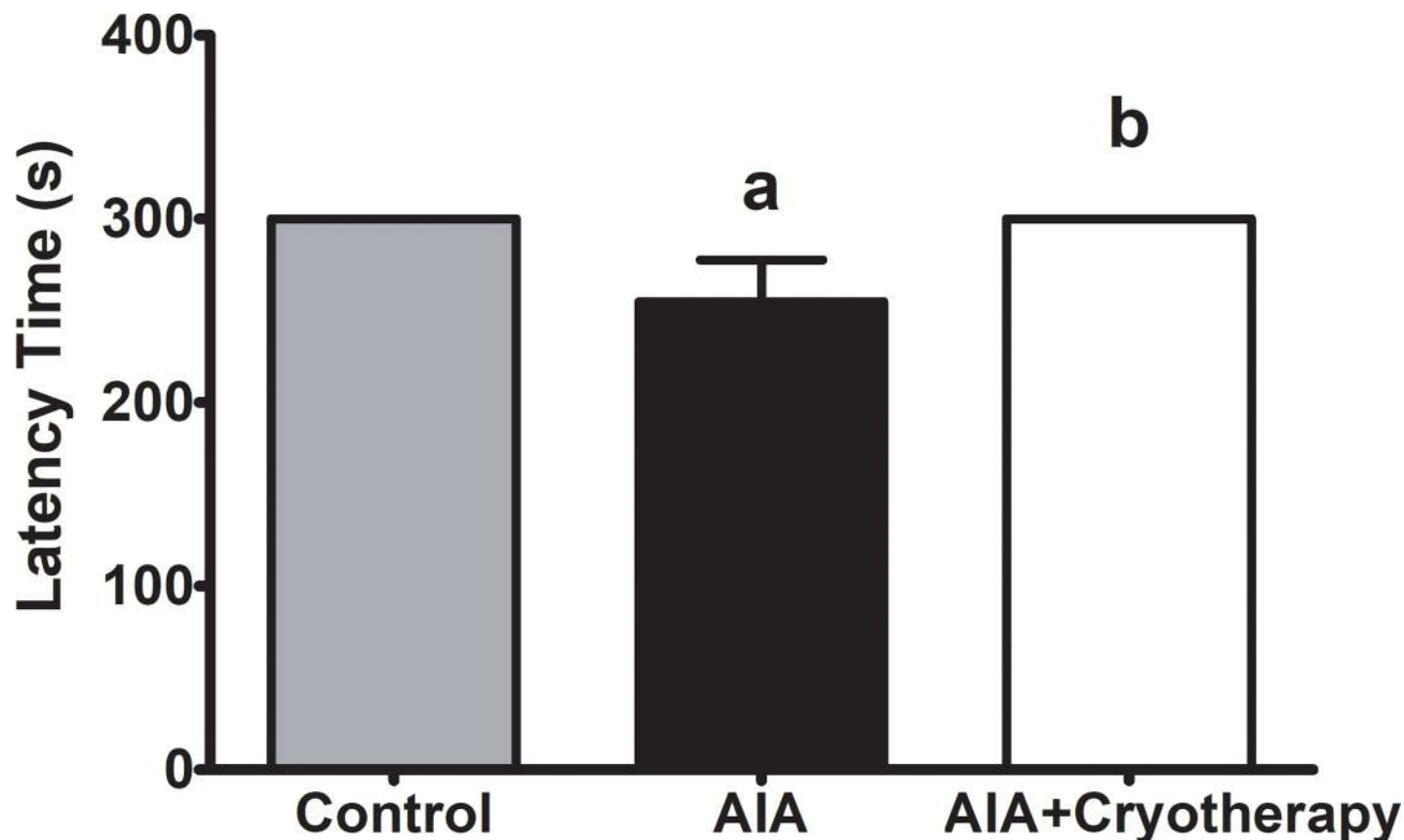


Fig 7. Gait function—balance and motor coordination in AIA model. Immunized C57BL/6 mice received i.a. injection with 100 μ g of mBSA per joint and were treated with clinical-like cryotherapy (crushed ice bag). Balance and motor coordination were assessed using the rotarod test in the final period of experimental analysis. Data expressed as mean \pm SEM (n = 10). (a): Compared to control group; (b): Compared to AIA group.

<https://doi.org/10.1371/journal.pone.0261667.g007>

Study limitations

This study is not free of limitations. No sham or placebo group was included in the experimental analysis. Second, pain assessment at baseline and knee temperature immediately after ice pack removal were not performed and could clarify the role of pain and skin cooling after cryotherapy. Third, we did not compare cryotherapy protocol with other protocols for arthritis. Therefore, future studies are needed to clarify these topics in animal models.

Author Contributions

Conceptualization: Paula A. T. S. Castro, Tania F. Salvini.

Data curation: Paula A. T. S. Castro.

Formal analysis: Paula A. T. S. Castro.

Funding acquisition: Thiago M. Cunha, Tania F. Salvini.

Investigation: Paula A. T. S. Castro, Germanna M. Barbosa, Dafiner H. Machanocker, Raphael S. Peres, Jonathan E. Cunha, Francisco F. B. Oliveira, Thiago L. Russo.

Methodology: Paula A. T. S. Castro, Germanna M. Barbosa, Dafiner H. Machanocker, Raphael S. Peres, Jonathan E. Cunha, Francisco F. B. Oliveira, Fernando Silva Ramalho, Thiago L. Russo.

Project administration: Paula A. T. S. Castro.

Resources: Paula A. T. S. Castro, Thiago M. Cunha, Thiago L. Russo, Fernando Q. Cunha, Tania F. Salvini.

Supervision: Thiago M. Cunha, Fernando Q. Cunha, Tania F. Salvini.

Writing – original draft: Paula A. T. S. Castro.

Writing – review & editing: Paula A. T. S. Castro, Germanna M. Barbosa, Tania F. Salvini.

References

1. Aletaha D., Neogi T., Silman A.J., Funovits J., Felson D.T., Bingham C.O. et al. Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Ann Rheum Dis*. 2010; 69(9): 1580–8. <https://doi.org/10.1136/ard.2010.138461> PMID: 20699241
2. Raza K., Falciani F., Curnow S. J., Ross E.J., Lee C.Y., Akbar A.N., et al. Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. *Arthritis Res Ther*. 2005; 7(4): R784–95. <https://doi.org/10.1186/ar1733> PMID: 15987480
3. Dominical V.M., Bertolo M.B., Almeida C.B., Garrido V.T., Miguel L.I., Costa F.F., et al. Neutrophils of rheumatoid arthritis patients on anti-TNF-alpha therapy and in disease remission present reduced adhesive functions in association with decreased circulating neutrophil-attractant chemokine levels. *Scand J Immunol*. 2011; 73(4): 309–18. <https://doi.org/10.1111/j.1365-3083.2011.02503.x> PMID: 21208248
4. Oliveira M. C., Tavares L.P., Vago J.P., Batista N.V., Queiroz-Junior C.M., Vieira A.T., et al. Tumor Necrosis Factor, but Not Neutrophils, Alters the Metabolic Profile in Acute Experimental Arthritis. *Plos One*. 2016; 11(1): e0146403. <https://doi.org/10.1371/journal.pone.0146403> PMID: 26742100
5. Smolen J. S., Landewé R., Bijlsma J., Burmester G. R., Dougados M., Kerschbaumer A., et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. *Annals of the rheumatic diseases*. 2020; 79(6): 685–699. <https://doi.org/10.1136/annrheumdis-2019-216655> PMID: 31969328
6. Lubkowska A., Szygula Z., Chlubek D., Banfi G. The effect of prolonged whole-body cryostimulation treatment with different amounts of sessions on chosen pro and anti-inflammatory cytokines levels in healthy men. *Scand. J. Clin. Lab. Invest*. 2011; 71(5): 419–425. <https://doi.org/10.3109/00365513.2011.580859> PMID: 21574854
7. Guillot X., Tordi N., Mourot L., Demougeot C., Dugué B., Prati C., et al. Cryotherapy in inflammatory rheumatic diseases: a systematic review, *Expert Review of Clinical Immunology*. 2014; 10(2): 281–294. <https://doi.org/10.1586/1744666X.2014.870036> PMID: 24345205
8. Guillot X., Martin H., Seguin-Py S., Maguin-Gaté K., Moretto J., Totson P., et al. Local cryotherapy improves adjuvant-induced arthritis through down-regulation of IL-6/IL-17 pathway but independently of TNFα. *Plos One*. 2017; 12(7): e0178668. <https://doi.org/10.1371/journal.pone.0178668> PMID: 28759646
9. Barbosa G. M., Cunha J. E., Cunha T. M., Martinho L. B., Castro P. A. T. S., Oliveira F. F. B., et al. Clinical-like cryotherapy improves footprint patterns and reduces synovial inflammation in a rat model of post-traumatic knee osteoarthritis. *Scientific Reports*. 2019; 1(9): 14518.
10. Sluka K.A., Christy M.R., Peterson W.L., Rudd S.L., Troy S.M. Reduction of pain-related behaviors with either cold or heat treatment in an animal model of acute arthritis. *Arch Phys Med Rehabil* 1999; 80:313–7. [https://doi.org/10.1016/s0003-9993\(99\)90143-0](https://doi.org/10.1016/s0003-9993(99)90143-0) PMID: 10084440
11. Beck T.W. The importance of a priori sample size estimation in strength and conditioning research. *J Strength Cond Res*. 2013; 27:2323–37. <https://doi.org/10.1519/JSC.0b013e318278eeaa0> PMID: 23880657
12. Quadros A. U., Pinto L. G., Fonseca M.M., Kusuda R., Cunha F.Q., Cunha T. M. Dynamic weight bearing is an efficient and predictable method for evaluation of arthritic nociception and its pathophysiological mechanisms in mice. *Scientific Reports*. 2015; (5): 1–11. <https://doi.org/10.1038/srep14648> PMID: 26511791
13. Brackertz D., Mitchell G. F., Mackay I. R. Antigen-induced arthritis in mice. I. Induction of arthritis in various strains of mice. *Arthritis Rheum*. 1977; (20): 841–50. <https://doi.org/10.1002/art.1780200314> PMID: 857805

14. Kawazaki Y., Zhang L., Chegn J. K., Ji R. R. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci*. 2008; (28): 5189–94.
15. Pinto L.G., Talbot J., Peres R.S., Franca R.F., Ferreira S.H., Ryffel B., et al. Joint production of IL-22 participates in the initial phase of antigen-induced arthritis through IL-1beta production. *Arthritis Res Ther*. 2015; (17): 235. <https://doi.org/10.1186/s13075-015-0759-2> PMID: 26330334
16. Peres R. S., Santos G. B., Cecilio N. T., Jabor V. A., Niehues M., Torres B. G., et al. Lapachol, a compound targeting pyrimidine metabolism, ameliorates experimental autoimmune arthritis. *Arthritis Res Ther*. 2017; 19(1), 47. <https://doi.org/10.1186/s13075-017-1236-x> PMID: 28270195
17. Cunha T.M., Verri W.A Jr., Silva J.S., Poole S., Cunha F.Q., Ferreira S.H. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. *Proc Natl Acad Sci USA*. 2005; (102): 1755–60. <https://doi.org/10.1073/pnas.0409225102> PMID: 15665080
18. Cunha T.M., Verri W.A. Jr., Schivo I.R., Napimoga M.H., Parada C.A., Poole S., et al. Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. *Journal of leukocyte biology*. 2008; 83(4): 824–832. <https://doi.org/10.1189/jlb.0907654> PMID: 18203872
19. Lewis J. S., Hembree W.C., Furman B.D., Tippetts L., Cattel D., Huebner J.L., et al. Acute joint pathology and synovial inflammation is associated with increased intra-articular fracture severity in the mouse knee. *Osteoarthritis Cartilage*. 2011; (19): 864–73. <https://doi.org/10.1016/j.joca.2011.04.011> PMID: 21619936
20. Cunha T. M. et al. An electronic pressure-meter nociception paw test for mice. *Braz J Med Biol Res*. 2004; 37(3): 401–407. <https://doi.org/10.1590/s0100-879x2004000300018> PMID: 15060710
21. Barbosa G. M., Cunha J. E., Russo T. L., Cunha T. M., Castro P., Oliveira F., et al. Thirty days after anterior cruciate ligament transection is sufficient to induce signs of knee osteoarthritis in rats: pain, functional impairment, and synovial inflammation. *Inflammation research: official journal of the European Histamine Research Society*. 2020; 69(3): 279–288.
22. Cunha J. E., Barbosa G. M., Castro P., Luiz B., Silva A., Russo T. L., et al. Knee osteoarthritis induces atrophy and neuromuscular junction remodeling in the quadriceps and tibialis anterior muscles of rats. *Scientific reports*. 2019; 9(1): 6366. <https://doi.org/10.1038/s41598-019-42546-7> PMID: 31019213
23. Carter R. J., Morton J., Dunnett S.B. Motor coordination and balance in rodents. *Curr Protoc Neurosci*. 2001; Cap. 8, unid 8, p.12. <https://doi.org/10.1002/0471142301.ns0812s15> PMID: 18428540
24. Song Y. N., Li H. Z., Zhu J. N., Guo C. L., Wang J. J. (2006). Histamine improves rat rota-rod and balance beam performances through H(2) receptors in the cerebellar interpositus nucleus. *Neuroscience*. 2006; 140(1): 33–43. <https://doi.org/10.1016/j.neuroscience.2006.01.045> PMID: 16533576
25. Otero M., Lago R., Gomez R., Lago F., Dieguez C., Gomez-Reino J. J., et al. Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis. *Ann Rheum Dis*. 2006; 65(9):1198–201. <https://doi.org/10.1136/ard.2005.046540> PMID: 16414972
26. Kehoe O., Cartwright A., Askari A., El Haj A. J., Middleton J. Intra-articular injection of mesenchymal stem cells leads to reduced inflammation and cartilage damage in murine antigen-induced arthritis. *Journal of Translational Medicine*. 2014; (12):157. <https://doi.org/10.1186/1479-5876-12-157> PMID: 24893776
27. Wright H.L., Moots R.J., Bucknall R.C., Edwards S.W. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology*. 2010; (49):1618–1631. <https://doi.org/10.1093/rheumatology/keq045> PMID: 20338884
28. Wipke B.T., Allen P.M. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J Immunol*. 2001; 167(3):1601–8. <https://doi.org/10.4049/jimmunol.167.3.1601> PMID: 11466382
29. Chou R.C., Kim N.D., Sadik C.D., Seung E., Lan Y., Byrne M.H., et al. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. *Immunity*. 2010; 33(2): 266–78.
30. Burmester GR, Feist E, Dörner T. Emerging cell and cytokine targets in rheumatoid arthritis. *Nat Rev Rheumatol*. 2014; (10):77–88. <https://doi.org/10.1038/nrrheum.2013.168> PMID: 24217582
31. Mabey T, Honsawek S. Cytokines as biochemical markers for knee osteoarthritis. *World J Orthop*. 2015; 6(1):95–105. <https://doi.org/10.5312/wjo.v6.i1.95> PMID: 25621214
32. Middleton J., Americh L., Gayon R., Julien D., Aguilar L., Amalric F., et al. Endothelial cell phenotypes in the rheumatoid synovium: activated, angiogenic, apoptotic and leaky. *Arthritis Res Ther*. 2004; (6):60–72.
33. Ushiyama T., Chano T., Inoue K., Matsusue Y. Cytokine production in the infrapatellar fat pad: another source of cytokines in knee synovial fluids. *Annals of the Rheumatic Diseases*. 2003; 2(62):108–112. <https://doi.org/10.1136/ard.62.2.108> PMID: 12525378

34. Saldanha A. A., Vieira L., de Oliveira F. M., Lopes D. O., Ribeiro R., Thomé R. G., et al. Anti-inflammatory and central and peripheral anti-nociceptive activities of α -asarone through the inhibition of TNF- α production, leukocyte recruitment and iNOS expression, and participation of the adenosinergic and opioidergic systems. *Inflammopharmacology*. 2020; 28(4): 1039–1052. <https://doi.org/10.1007/s10787-019-00679-1> PMID: 31865494
35. Deal D. N., Tipton J., Rosencrance E., Curl W. W., Smith T. L. Ice reduces edema. A study of microvascular permeability in rats. *J. Bone Joint. Surg. Am.* 2002; (84):1573–1578. PMID: 12208913
36. Bleakley C., McDonough S., MacAuley D. The use of ice in the treatment of acute soft-tissue injury: a systematic review of randomized controlled trials. *Am. J. Sports Med.* 2004; (32):251–261. <https://doi.org/10.1177/0363546503260757> PMID: 14754753
37. Dantas L.O., Breda C.C., Serrão P.R.M. S., Aburquerque-Sendín F., Jorge A.E. S., Cunha J.E., et al. Short-term cryotherapy did not substantially reduce pain and had unclear effects on physical function and quality of life in people with knee osteoarthritis: a randomised trial. *Journal of Physiotherapy*. 2019; (65):215–221.
38. Ramos G.V., Pinheiro C.M., Messa S.P., Delfino G.B., Marqueti R.C., Salvini T.F., et al. Cryotherapy Reduces Inflammatory Response Without Altering Muscle Regeneration Process and Extracellular Matrix Remodeling of Rat Muscle. *Scientific Reports*. 2016; 4(6):18525.
39. Siqueira A. F., Vieira A., Ramos G. V., Marqueti R. C., Salvini T. F., Puntel G. O., et al. Multiple cryotherapy applications attenuate oxidative stress following skeletal muscle injury. *Redox report: communications in free radical research*. 2017; 22(6): 323–329. <https://doi.org/10.1080/13510002.2016.1239880> PMID: 27750503
40. Farry P.J., Prentice N.G., Hunter A.C., Wakelin C. A. Ice treatment of injured ligaments: an experimental model. *The New Zealand medical journal*. 1980; 91(651): 12–14. PMID: 6928055
41. Yenari M.A., Han H.S. Influence of hypothermia on post-ischemic inflammation: role of nuclear factor kappa B (NFkappaB). *Neurochem Int*. 2006; 49(2):164–169. <https://doi.org/10.1016/j.neuint.2006.03.016> PMID: 16750872
42. Webster C.M., Kelly S., Koike M.A., Chock V.Y., Giffard R.G., Yenari M.A. Inflammation and NFkappaB activation is decreased by hypothermia following global cerebral ischemia. *Neurobiol Dis*. 2009; 33(2): 301–312. <https://doi.org/10.1016/j.nbd.2008.11.001> PMID: 19063968
43. Pavlov V.A., Tracey K.J. The cholinergic anti-inflammatory pathway. *Brain Behav Immun*. 2005; 19: 493–499. <https://doi.org/10.1016/j.bbi.2005.03.015> PMID: 15922555
44. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest*. 2007; 117(2): 289–296. <https://doi.org/10.1172/JCI30555> PMID: 17273548