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Journal

Inflammopharmacology, 32(2)

ISSN

0925-4692

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Publication Date

2024-04-01

DOI

10.1007/s10787-024-01443-w

Inflammopharmacology (2024) 32:1239–1252
https://doi.org/10.1007/s10787-024-01443-w

Inflammopharmacology

ORIGINAL ARTICLE

Intraarticular monomethyl fumarate as a perspective therapy for osteoarthritis by macrophage polarization

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Received: 29 October 2023 / Accepted: 24 January 2024 / Published online: 13 March 2024

Abstract

Background Osteoarthritis (OA) is a chronic disease that may lead to joint structure degeneration, cartilage destruction, osteophyte formation, subchondral bone disruption, and pain. In this scenario, a higher proportion of the proinflammatory macrophage type 1 (M1) than the anti-inflammatory macrophage type 2 (M2) could be highlighted as a hallmark of OA progression. The balance between these two macrophage types emerges as a new therapeutic target in OA. This study aimed to evaluate the analgesia and macrophage profile in the treatment of experimental osteoarthritis (EOA) with systemic dimethyl fumarate (DMF) or local intra-articular monomethyl fumarate (MMF).

Results DMF via gavage or MMF via intra-articular in the right knee of EOA rats showed improvements in gait parameters and the nociceptive recovery of the mechanical threshold assessment by adapted electronic von Frey treatment on the twenty-first day (long-lasting phase). DMF treatment decreased proinflammatory TNF-α while increasing anti-inflammatory IL-10 cytokines from the macerated capsule on the fifth day (inflammatory phase). MMF treatment showed joint capsule mRNA extraction downregulating iNOS and TNF-α gene expression while upregulating IL-10 and MCP-1. However, CD206 was not significant but higher than untreated EOA rats' joints on the seventh day (inflammatory phase).

Conclusions Our studies with EOA model induced by MIA suggest a new perspective for human treatment committed with OA based on macrophage polarization as a therapeutic target, switching the proinflammatory profile M1 to the anti-inflammatory profile M2 with DMF systematic or by MMF locally treatment according to the OA severity.

Keywords Pain · Osteoarthritis · Macrophages · Polarization · Dimethyl · Monomethyl fumarate

Abbreviations

OA	Osteoarthritis
EOA	Experimental osteoarthritis
MS	Multiple sclerosis
CNS	Central nervous system
PNS	Peripheral nervous system

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M1	Macrophage type 1
M2	Macrophage type 2
IL	Interleukin
Arg-1	Arginase-1
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
TGF-β	Transforming growth factor
DMF	Dimethyl fumarate
MMF	Monomethyl fumarate
Nrf2	Nuclear transcription factor derived from erythroid type 2
bZIP	Basic leucine zipper protein
Keap1	Kelch-like ECH-associated protein
MMP	Matrix metalloproteinases
NSAIDs	Non-steroidal anti-inflammatory drugs
PGE2	Prostaglandin-E2
DAMPs	Damage associated with molecular pattern
PAMPs	Pathogens associated with molecular patterns
LPS	Lipopolysaccharide
INF-γ	Interferon-gamma
TNF-α	Tumor necrosis factor alpha
Nf-κß	Nuclear factor-kappa B
ROS	Reactive oxygen species

Background

Osteoarthritis (OA) is a chronic disease that affects the worldwide population, causing pain (Lin et al. 2018). The impairments from this disease might be seen in different age phases, mainly in older adults, and it is considered a multifactor disease caused by genetic factors, obesity, aging, metabolic dysfunction, or senescence (Anandacoomarasamy and March 2010).

These impairments may drive OA progression, resulting in the inflammatory process, synovium inflammation, osteophyte formation, cartilage destruction, subchondral disruption, and pain. During this progression, macrophages are linked to this proinflammatory process triggered by a higher proportion of macrophage type I (M1) than macrophage type II (M2) during OA progression (Kennedy et al. 2011).

The macrophages have an essential role in promoting homeostasis, eliminating pathogens (M1), and healing tissue (M2) (Kennedy et al. 2011). M1 profile has a classical activation, characterized by the proinflammatory cytokines releases such as TNF-α, IL-6, and IL-1β, generating nitric oxide (NO) and reactive oxygen species (ROS) (Wu et al. 2020). The M2 profile has an alternative activation characterized by the anti-inflammatory releases of IL-10 and TGF-β.

Unbalancing macrophage phenotype leads to the OA progression due to increased monocyte immigration and polarization to the M1 profile at the local damage, releasing more proinflammatory products (Wu et al. 2020; Warmink et al. 2023). During OA, macrophages also recognize the damage-associated molecular patterns (DAMPs) or debris tissue from OA, driving to the M1 phenotype and impacting the healing process by the M2 phenotype (Murray and Wynn 2011; Zhang et al. 2020).

In searching to improve patients with OA conditions, different treatments have focused on pharmacologic therapies to enhance patients' health by reducing the inflammatory process and relieving pain. Despite some benefits during the treatment, they may compromise patients' lives based on the side effects associated with long treatments (Coimbra et al. 2019). These side effects are related to immune suppression (corticoids), hepatotoxic (paracetamol), cardiovascular issues (non-steroid\anti-inflammatory drugs—NSAIDs), and some of them may cause addiction (opioids) (Grässel and Muschter 2020; Ayhan et al. 2014).

Despite these treatments promoting some benefits by relieving pain, they do not promote macrophage polarization to the M2 profile, an essential step to act during OA progression. Upregulating the M2 polarization profile could boost the healing process, enabling the pro-chondrogenic environment and reducing cartilage disruption triggered by the M1 profile during OA progression (Da Costa et al. 2021; Warmink et al. 2023).

Emerging therapies using macrophage polarization as a therapeutic target to mitigate damages caused by the M1 profile are of great interest in promoting tissue healing in OA joints (Thomson and Hilkens 2021). This way, dimethyl fumarate (DMF) is a pro-drug converted in the active metabolite monomethyl fumarate (MMF), metabolized in the liver by methyl esterases. In multiple sclerosis, DMF has been suggested to polarize astrocytes along the treatment, being a possibility to investigate its role in OA; once DMF and MMF are drugs available for human treatment that could be a new option for many other chronic diseases by switching macrophages M1 to M2 profile (Yulin Chen et al. 2020; Pålsson-McDermott and O'Neill 2020).

Therapeutic properties related to DMF are associated with its active metabolite MMF. MMF upregulates the transcription nuclear factor erythroid 2 (Nrf2), promoting several productions of anti-oxidant genes able to suppress proinflammatory pathways like NF-κB, which is a master regulator of the inflammatory response in the M1 profile (Lategan et al. 2021). In addition, Nrf2 starts accumulating in the presence of oxidative process or stress and driving to the nucleus, forming a heterodimer that links to the Maf protein. This complex links to the specific region in DNA and upregulates the anti-oxidant genes while eliminating ROS and reducing inflammation (Saha et al. 2020).

Therefore, DMF or MMF properties may prevent the oxidative damages triggered by joint injuries and inflammation response during OA progression. It drives macrophage

polarization to the M2 profile, a crucial step for reverting chaos observed in OA and the higher macrophage M1 polarization (Singh et al. 2018). Moreover, reverting the inflammatory environment by increasing M2 polarization may induce a pro-chondrogenic environment, reducing several factors related to cartilage degeneration, such as metalloproteinase, aggrecanases, proinflammatory cytokines, and ROS. These products are associated with OA progression, inducing cartilage disruption and chondrocyte death, impacting the maintenance of cartilaginous tissues within joints by producing collagen and the extracellular matrix (Zhang et al. 2020; Lv et al. 2019).

Observing the OA scenario and the therapeutic possibility of the DMF and MMF in macrophage polarization, this study has aimed to evaluate the oral administration of DMF and its active metabolite MMF by intra-articular pathway in experimental osteoarthritis (EOA) induced by the administration of monoiodine acetate (MIA) in the right knee of male rats.

Materials and methods

Animals

The ethics committee approved (CEUA number 5965-1/2021) this study using 150 male *Wistar* rats, 6–8-week-old, weighing 230.40 ± (g) that were provided by the University's Multidisciplinary Center for Biological Research (CEMIB-UNICAMP). Rats were kept in polypropylene cages at the Pain Study Center at UNICAMP, with a temperature of 22 ± 2 °C, 12 h dark/light cycle, and receiving appropriate feed for the species (Nuvital®) and water ad libitum.

Monoiodine acetate as a model for experimental osteoarthritis

The experimental osteoarthritis (EOA) model used in this study was induced by the intra-articular injection of monoiodine acetate (MIA) (Xu et al. 2020). First, 1.5 mg of MIA was freshly prepared and diluted in saline NaCl (0.9%) and injected 50 µL via intra-articular in the right knee of Wistar. In the operating room, rats were anesthetized by inhalation pathway with isoflurane (3%). After ensuring the anesthetic effect, their knees were kept at 90º to make the patella identification and the fingertip for the gap beneath the patella easier. The Hamilton high precision syringe coupled to a cannula at one end and to a 26G 1//2 hypodermic needle at the other (0.45 mm x 13 mm) was inserted with no resistance and vertically for around 5 mm to inject into the joint cavity the MIA diluted (Udo et al. 2016). Rats' EOA after being intra-articularly injected with MIA in the right knee has demonstrated symptoms since the first day of hyperalgesia, wearing-deficits, and inflammatory responses (Udo et al. 2016). During the fourth to the seventh day, a peaky of proinflammatory cytokines released by macrophages M1 is associated with this period, representing the inflammatory or acute phase, while following the 10th to the 14th day represents the chronic or late phase of EOA, observing subchondral disruption, cartilage degeneration, and chondrocytes deaths (Udo et al. 2016).

Dimethyl fumarate and monomethyl fumarate drugs

Dimethyl fumarate (DMF, 97% purity) is a pro-drug that was administrated daily via the gavage pathway. The main active metabolite monomethyl fumarate (MMF, 97% purity) was injected in a single dose by intra-articular pathway, following the same procedure as in the MIA injection protocol. Both drugs were acquired and described from Sigma-Aldrich. The dose of 30 mg/kg of DMF was prepared and diluted in the vehicle (DMSO 5% and saline NaCl 0.9%). The MMF followed the same procedure, except for the administration pathway that was via intra-articular (280 ng in 50 µL). The intervention in EOA with DMF was administrated by gavage pathway or MMF single injected in the right knee 3 days post-MIA injection, being evaluated by mechanical and dynamic behavioral tests, respectively, by nociceptive mechanical pain threshold via adapted electronic von Frey and Catwalk, improving animals performance (Pitarokoili et al. 2015).

Mechanical threshold assessment by adapted electronic von Frey

The nociceptive behavioral analyses were performed according to the methodology described by Guerrero et al. (2006). On the elevated horizontal wire mesh stand, rats were previously acclimated for 60 min in acrylic cages (23 cm × 20 cm × 18 cm). After this period, the experimenter performed behavioral tests using an adapted von Frey device or digital analgesia meter (Insight®, Brazil) to measure mechanical hyperalgesia from the right hind paws. This adapted electronic von Frey consists of a modification at the plastic tip, which was sectioned 1 cm from the top of the 200 µL tip (Kasvi®; K8-200F-1) with an original area of 0.5 mm^2 to a new area of 4.15 mm^2 [25]. The rats were acclimated, and the modified tip was fitted to the portable force transducer. A gentle perpendicular force for pressuring and lifting the paw was done on the metatarsal pads by a larger tip, with a progressive and constant movement. This movement induces the flexion of the tibiofemoral joint, causing a withdraw paw displaying in grams of gram-force (g) on the electronic device screen. The measures were collected five times for each EOA joint in 3 min for the same rat. These

procedures were applied during 0 (basal), and post-MIA injection 1, 3, 5, 7, 14, and 21 days. The difference between the basal and treatment values represents the hyperalgesia standardized in percentages.

Gait analyses by the Catwalk system

The dynamic evaluation was performed using the Catwalk XT device (CatWalk System, Noldus Inc., Wageningen, The Netherlands). This device has an automated walkway system that allows rats to walk freely on a glass platform illuminated by a green light crossing horizontally, and a red light from the top illuminates the glass walkway. These lights highlight the rat's paw when in contact with the walkway floor, allowing the high-speed camera under the glass walkway to record the rat's movement (Kara et al. 2021). On Catwalk software XT 10.5 (Noldus Inc.), the experimenter set up the parameters for analyzing the EOA animals. These parameters were green LED intensity of 0.35, green light intensity of 16.5, red light intensity (ceiling) of 17.8, maximum velocity allowed of 60%, camera gain of 25.01, minimum time of 0.5 s, and maximum time of 0.8 s. The experimenter calibrated the camera using a section of 20 × 10 cm on the glass platform to start recording when animals start crossing this section (Vieira et al. 2020). For each rat, three runs were recorded during the days: 0 (basal), post-MIA injection 1, 3, 4, 5, 7, 14, and 21. Rats were placed gently on the glass platform to acclimate by walking for 5 min, one by one. At the end of the experiment, the OA parameters analyzed in catwalk software were stand (cm^2), swing (s), max intensity At (%) (cm), print area (cm^2), terminal dual stance (s), and the mean intensity of the 15 most Intense Pixels (Lakes and Allen 2016). The chosen CatWalk parameters are described according to the manual of the equipment producer as follows:

The stand is the duration in seconds of contact of a paw with the glass floor; the **swing** is the duration in seconds of no contact of a paw with the glass floor; the **print area** is the surface area of the complete print. Print Area is, by definition at least as large as the Maximum Contact Area; **max intensity at (%)** is the max intensity at s relative to the stand of a paw; **mean intensity of most 15 prints** is defined as the mean intensity of the 15 pixels with the highest intensity of a paw; **terminal Dual Stance** is the last time in a Step Cycle of a hind paw that the contralateral hind paw also makes contact with the glass-floor.

Extraction of the knee joint capsule

The joint capsule was extracted on the 7th (inflammatory phase) and 21st (chronic phase) days post-MIA injection. These EOA animals were initially treated with DMF or MMF 3 days post-MIA injection and then euthanized with isoflurane (5%) during the inflammatory and chronic phase of EOA. The right knee region was cleaned using cotton soaked in 70% alcohol, and the joint capsule was carefully harvested using a surgical blade for each group. All the joint capsule collected was instantly frozen in liquid nitrogen and stored in a − 80 ºC freezer for posterior processing to quantify pro- and anti-inflammatory cytokines and mRNA genes related to macrophage profile (Guerrero et al. 2006).

Cytokine quantification by enzyme-linked immunosorbent assay (ELISA)

According to the R&D system procedure for ELISA, pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-10) were quantified from the right knee joint capsule. The capsule tissue was transferred to a vial with a solution composed of ethylenediaminetetraacetic (EDTA—0.4%), phosphate-buffered saline (PBS), protease inhibitor (1%), benzethonium chloride (0.004%), albumin (0.5%), tween 20 (0.05%). In addition, a porcelain bead was added to the vial, followed by fast-prep agitation for 05 s at a velocity of 5.0 m/s. This procedure is repeated thrice with 5 min between the cycles. At the end of the process, the supernatant from the vial was collected to quantify cytokines.

Isolation and culture of bone marrow derivated macrophages (BMDM)

BMDM cells were collected from the femur of rats previously anesthetized with isoflurane (5%) (Kobayashi et al. 2016). After the rat's euthanasia, femur epiphysis was cut and flushed with 10 mL of sterile PBS in the bone. The experimenter filtered the solution of bone marrow stem cells in a cell strainer (70 µm), and the filter cells were homogenized in a falcon tube (50 mL). Sterile ammonium chloride buffer was added to the medium for 5 min to lyse the red blood cells. After, 10 mL of sterile PBS was added to stop the lyse reaction, and the falcon tube was homogenized and centrifuged (4 ℃, 15 min at 1800 rpm). Then, cells were resuspended and homogenized in a culture medium of 20 ML of RPMI (ThermoFisher Scientific). These cells were counted and plated in 24 wells plate (5×10^5 cells/well), added to RPMI culture medium macrophage colony-stimulating factor (MC-SF; 20 ng/mL), and bovine fetal serum (10%), 37 ℃, 5% of CO2. The culture medium was replaced on the third and fifth days. On the fifth day, was stimulated with lipopolysaccharide (LPS—100 ng/mL) plus interferon-gamma (INF-γ—20 ng/mL) to polarize macrophages in M1 profile for 1 h and 30 min and treated with DMF (50 µM) and MMF (50 µM) for 4 h and 30 min (Gillard et al. 2015). For each group of this experiment, the triplicate wells were performed. The DMF and MMF drugs were diluted in pure DMSO, representing a percentage of 5% in medium culture

for both treatments (De Abreu Costa et al. 2017). At the end of the experiment, the cells were driven to RT-PCR analyses to check macrophage profile genes.

mRNA quantification by real-time polymerase chain reaction (RT-PCR)

Samples from the extraction capsule joint and in vitro BMDM culture were driven to extract mRNA using 500 µL of Trizol (Tri-Reagent, T9424; Sigma-Aldrich®). For the capsule joint, 500 µL of Trizol with porcelain beads was added for tissue defragmentation by agitation in fast-prep for 05 s at a velocity of 5.0 m/min and intervals of 5 min between the agitation periods, repeating this process thrice. The quantification of mRNA samples showed a reading ratio of 260 nm/280 nm and 260 nm/230 nm around 1.9–2.0. The cDNA reaction was followed according to the quantitative real-time kit SYBR® Green using 30 ng/cDNA to amplify target genes (mRNA fold change) associated with macrophage profiles, using β-actin (ACTβ) as housekeeping. The cDNA quantified in the BMDM culture plate was Arg-1 and iNOS genes enzymes, while in the capsule joint was MCP-1, iNOS, TNF-α, CD163 related to M1 profile, and CD206 e IL-10 related to M2 profile. The primer sequences are shown in Table 1.

Note: mRNA fold change expresses the genes related to macrophage polarization after MMF treatment in EOA animals for the M1 and M2 profiles. The sequences correspond to Monocyte Chemoattractant Protein-1 (MCP-1); Tumor Necrosis Factor-alpha (TNF-α); Inducible Nitric Oxide Synthase (iNOS); Hemoglobin Scavenger Receptor (CD163); Mannose-1 Receptor (CD206); Interleukin-10 (IL-10); β-actin (ACTβ).

Table 1 Primers for quantitative real-time PCR sequencing

Targets genes	Forward sequence/reverse sequence
MCP-1	*5′-ACCTGGATCGGAACCAAATGAG-3′* *5′-GAAGTGCTTGAGGTGGTTGTGG-3′*
TNF-α	*5′-AGAGTGAACCGACATGGC-3′* *5′-GGGTAATAAAGGGATTGGG-3′*
iNOS	*5′-CGAGTTGTGGATTGTTCTTCGC-3′* *5′-CGTCTCTTCCGTGGCAAAGC-3′*
CD163	*5′-ATGGAGTCACAGCGACTGCG-3′* 5′-GAGGAAGGCAATGAGAAGGACC-3′
CD206	*5′-CTGGAAGACATCATACTGCAATG-3′* *5′-CAGTCTCGATGGAAACCAGG-3′*
IL-10	*5′-TGATGCCACCAGCTTGTC-3′* *5′-TTAATGTCACGCACGATTTCCC-3′*
ARG-1	*5′-CCAGTATTCACCCCGGCTAC-3′* *5′-GTCCTGAAAGTAGCCCTGTCT-3′*
ACTβ	*5′-TGGCATTGTGATGGACTCCG-3′* *5′-AAGCAATGCTGTCACCTTCCC-3′*

Statistical analyses

The statistical analysis of the data was performed using GraphPad Prism (GraphPad Software, version 8.0.1, La Jolla, CA, USA). All data were expressed as the mean ± SEM by one-way ANOVA (and nonparametric or mixed) from the macerated capsule and in vitro studies or two-way ANOVA (or mixed model) for behavioral studies, being considered as significant the value of $p < 0.05$.

Results

The EOA animals were analyzed during the inflammatory and chronic phases of the disease to investigate DMF and MMF drugs as potential treatments for OA (Pitarokoili et al. 2015).

Throughout the treatment, the weight of the EOA animal was closely monitored, and no significant effects were observed with either MMF or DMF treatment. These rats were weighed at the beginning of the experiment (day 0) and at various intervals after MIA injection, specifically on days 1, 3, 5, 7, 14, and 21 (supplementary figure). During this time, behavioral tests were assessed using catwalk (gait analyses) and an adapted electronic von Frey (Ferreira-Gomes et al. 2008; Gillard et al. 2015). At the end of the inflammatory and chronic phase, the capsule tissue from the right knee was collected for cytokine quantification by ELISA and mRNA quantification by RT-PCR.

After 3 days of MIA injection, EOA rats were treated daily with 30 mg/kg of DMF via gavage. After 6 days of receiving the MIA injection, the EOA treated with DMF showed an initial recovery from pain sensitivity during the inflammatory phase. This was assessed by measuring the mechanical threshold using an adapted electronic von Frey device. On the 21st day of treatment with DMF, hyperalgesia was significantly reduced (Fig. 1A). Interestingly, a single injection of 280 ng MMF reduced hyperalgesia and led to earlier nociceptive recovery by the fourth day. Recovery continued until baseline on the 21st day (Fig. 1B).

The Catwalk behavioral test indicated that EOA animals that were treated with DMF reduced sensitivity based on analysis of their right hind paw. An increased permanence time in EOA mice was absent in treated rats, whose permanence time followed a similar trajectory to vehicle-treated control rats. These data suggest that the rats might avoid movement in response to pain, stimulating nociceptive neurons (Fig. 2A) (Ferreira–Gomes et al. 2008). During the seventh day of the inflammatory phase, an observation was made in the footprints of rats. Rats that were not treated showed partial foot contact on the glass platform, while the EOA rats treated with DMF showed total foot contact, as suggested by Fig. 2B. This suggests that the

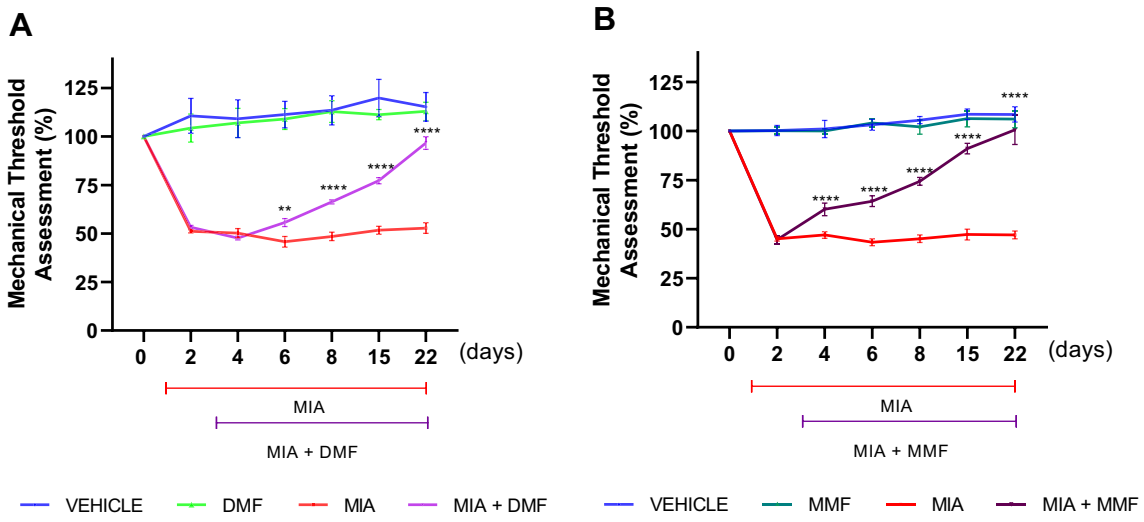


Fig. 1 Mechanical threshold assessment by adapted electronic von Frey in EOA animals induced by MIA. Nociceptive analyses were made in EOA animals induced by 1.5 mg of MIA solution (50 µL) via intra-articular in the right knee joint. **A** After 3 days, EOA animals received 30 mg/kg of DMF daily via gavage until 22 days; **B** After 3 days, EOA animals received a single dose of 280 ng (50 µL) of MMF via intra-articular. Dilutions for each group: vehicle (saline 0.9% + DMSO 5%), 1.5 mg of MIA (saline 0.9%), 30 mg/kg of DMF (saline 0.9% + DMSO 5%), and 280 ng of MMF (saline 0.9%) + DMSO 5%); these nociceptive statistical analyses were performed using two-way ANOVA (or mixed model) with Tukey's multiple comparison test with $p < 0.05$. $N = 05$. Symbols: **** = 0.0001; *** = 0.008; ** = 0.012

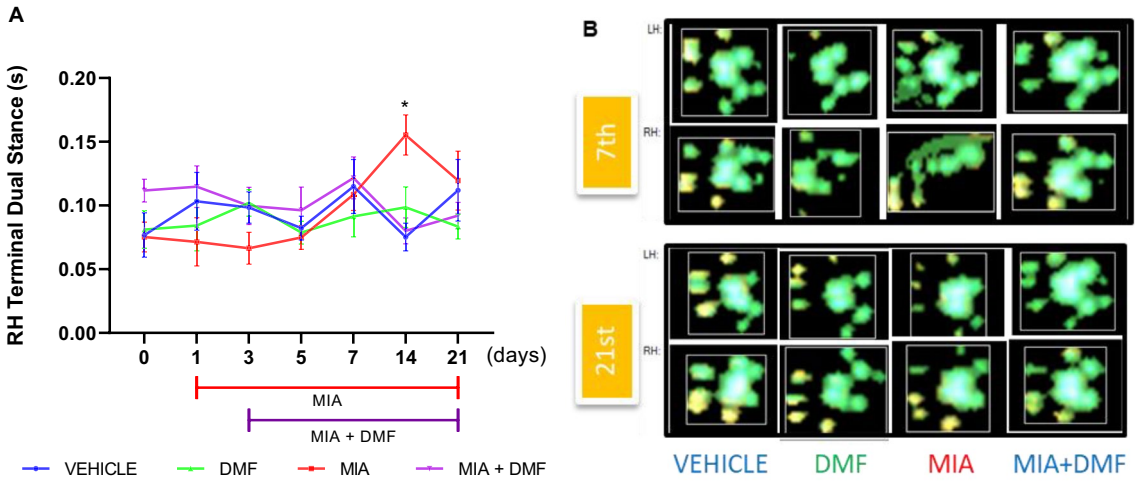


Fig. 2 Dynamic gait analyses of EOA animals induced by MIA and treated with DMF. EOA rats were induced by 1.5 mg of MIA solution (50 µL) via intra-articular in the right knee joint and analyzed by Catwalk device the right hind gait parameter. After 3 days, EOA rats received 30 mg/kg of DMF via gavage daily until 22 days. **A** RH terminal dual stance represents the EOA parameter showing the time spent in this position for each group; **B** footprint represents the inflammatory phase (7th day) and chronic phase (21st day). Dilutions for each group: vehicle (saline 0.9% + DMSO 5%), 1.5 mg of MIA (saline 0.9%), and 30 mg/kg of DMF (saline 0.9% + DMSO 5%). Statistical analyses were performed using two-way ANOVA (or mixed model) with Tukey's multiple comparison test with $p < 0.05$. $N = 05$. Symbol * = 0.0405

EOA rats experienced higher hyperalgesia, which may be linked to the adapted electronic von Frey assessment and the pain caused by the movement of the knee (Gillard et al. 2015; Vieira et al. 2020).

After the 14th day (chronic phase), the MIA group showed signs of a healing process in comparison to the MIA + DMF group, as evidenced by the total foot contact on the glass platform. This impression is due to the natural

fibrosis process that protects the knee joint from the damage caused by MIA, which leads to an inflammatory process, cartilage disruption, and ultimately, fibrosis over time, but that does not reduce pain according to the adapted electronic von Frey (Fig. 1) (Thomson and Hilkens 2021; Udo et al. 2016).

Observing this EOA progression, the literature suggests that the inflammatory process is highly associated with a proinflammatory macrophage profile. This results in hyperalgesia, which is demonstrated by our behavioral tests in animals with early-onset osteoarthritis (Mushenkova et al. 2022; Thomson and Hilkens 2021).

Although we did not separate macrophages from the macerate joint capsule, we could quantify the levels of proinflammatory TNF-α and anti-inflammatory IL-10 cytokines in the macerate joint capsule of EOA rats. Our findings showed that on the fifth day post-MIA injection, the levels of TNF-α were higher in EOA rats without intervention compared to those treated with DMF during the inflammatory phase (Fig. 3A). Previous studies have suggested that M1 macrophages release higher levels of TNF-α during OA progression (Mushenkova et al. 2022). The levels of IL-10 were also increased in EOA rats who received DMF treatment (Fig. 3B) (Teixeira et al. 2017). These pro- and anti-inflammatory cytokines were not detected during the chronic phase.

After observing improvements in EOA treatment with DMF administered orally, which was evident from the gait analyses, adapted electronic von Frey, and cytokine quantification by ELISA, indicating macrophage modulation, the next step was to evaluate the role of MMF administered via an intra-articular pathway.

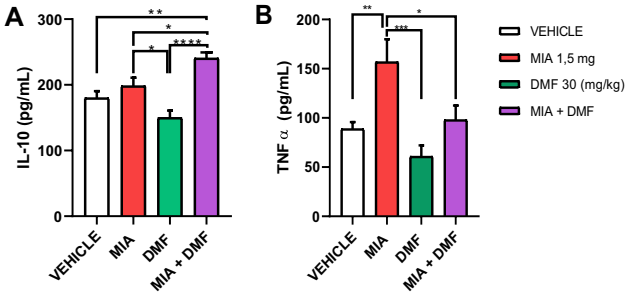


Fig. 3 Cytokine quantification from a macerated capsule of the right knee of EOA animals treated with DMF. EOA rats induced by 1.5 mg of MIA solution (50 µL) via intra-articular in the right knee joint. After 3 days, they received 30 mg/kg of DMF daily via gavage until the fifth day, representing the inflammatory phase. **A** Analyses of anti-inflammatory cytokine IL-10; **B** Proinflammatory cytokine TNF-α. Dilutions for each group: vehicle (saline 0.9% + DMSO 5%), 1.5 mg of MIA (saline 0.9%), and 30 mg/kg of DMF (saline 0.9% + DMSO 5%). Statistical analyses were performed using one-way ANOVA (nonparametric or mixed) with Tukey's multiple comparison test with $p < 0.05$. $N = 10$. Symbols: **** = 0.0001; *** = 0.008; ** = 0.012 * = 0.04

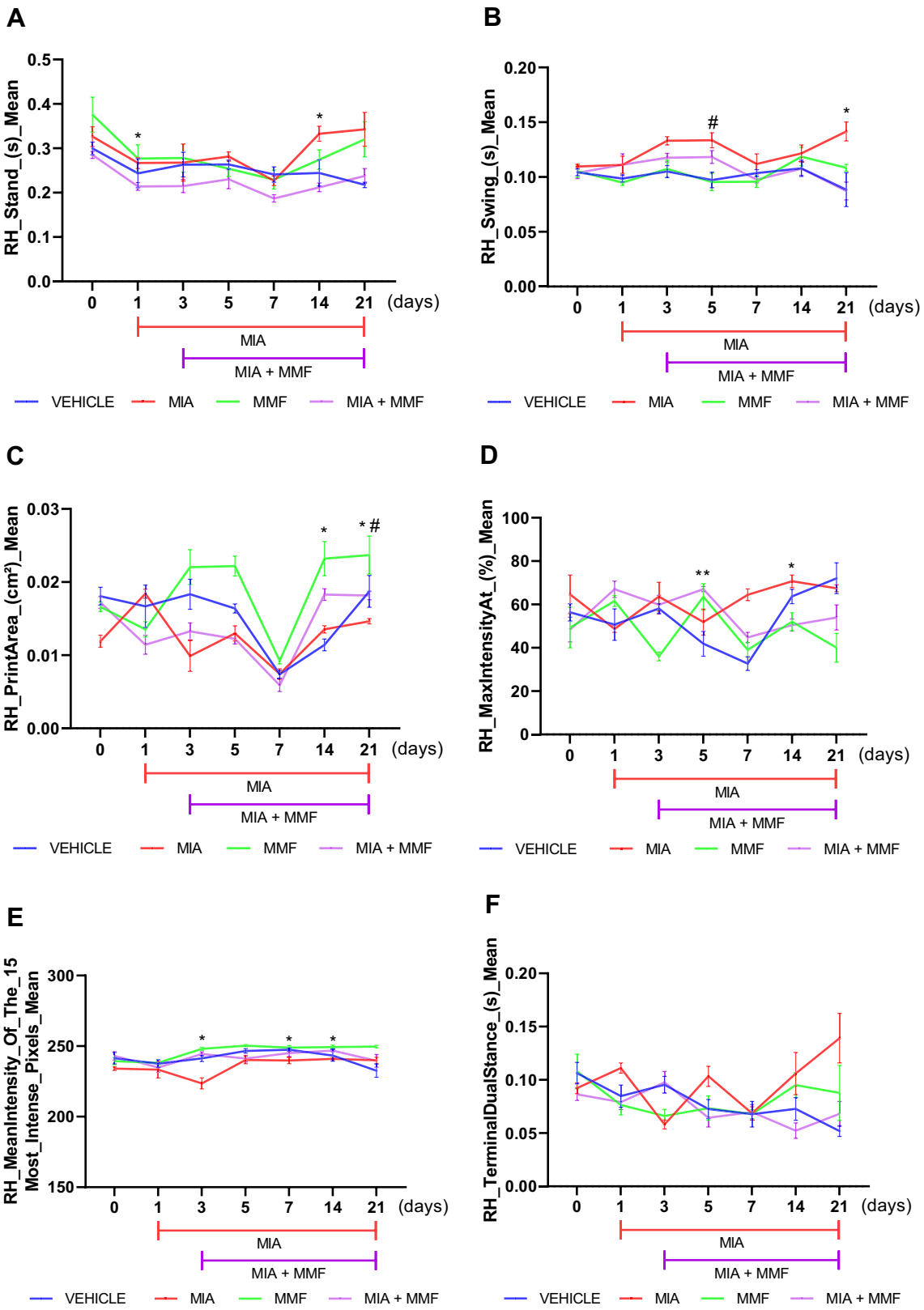
Posterior receiving a single dose of 280 ng MMF, rats with EOA displayed significant improvements in several OA gait parameters, indicating a reduction in pain sensitivity and movement-evoked hyperalgesia (Fig. 4) compared to rats treated with 30 mg/kg of DMF (Fig. 2) (Jones et al. 2019). The OA gait parameters (catwalk) were also assessed based on the right hind paw were stand (s) (Fig. 4A), swing (s) (Fig. 4B), print area (cm^2) (Fig. 4C), max intensity At (%) (Fig. 4D) and mean intensity of the 15 most intense pixels (Fig. 4E).

The study found that the EOA animals treated with MMF had similar stand (Fig. 4 A) and swing (Fig. 4B) parameters (time in seconds) to the control groups. This suggests that the animals experienced reduced pain sensitivity. On the other hand, the EOA rats that were not treated with MMF seemed to avoid movement-evoked pain (Fig. 4A–F), resulting in a longer time to complete a swing cycle on the glass floor (Fig. 4B–D). This indicates an increase in pain sensitivity. In addition, the untreated rats showed a reduced footprint area (Fig. 4C), further suggesting increased pain sensitivity. Although there was no statistical difference in the terminal dual stance parameter (Fig. 4F), the EOA rats that were not treated with MMF tended to avoid movement-evoked pain as observed on the adapted electronic von Frey (Fig. 1B) (Ferreira-Gomes et al. 2008; Vieira et al. 2020; Garrick et al. 2021).

These data indicate that EOA rats without DMF or MMF treatment exhibit deficits in wearing by avoiding contact on a glass platform and hyperalgesia signals (Tateiwa et al. 2019; Koda et al. 2016; Rim and Ju 2021). Although max intensity at (%) parameter in EOA animals not treated with MMF (Fig. 4D), this recovery might be related to the fibrosis process during the chronic phase to protect the knee joint from the damage according to the physical movement (Rim and Ju 2021).

The study showed that DMF and MMF treatments improved rats' performance with EOA by analyzing their behavior. In addition, DMF treatment reduced the proinflammatory cytokine TNF-α and increased the anti-inflammatory cytokine IL-10 in the joint capsule on the fifth day after MIA injection. These cytokine genes and markers related to macrophage profiles in the joint tissue of EOA animals were analyzed to evaluate the MMF treatment on the seventh day (Fig. 5) (Zhang et al. 2020).

On the fifth day after the MIA injection, there was a peak in cytokines likely released by macrophages. On the seventh day, mRNA analyses were performed on genes from the joint tissue capsule: monocyte recruitment (MCP-1) (Fig. 5A); m1 profile for TNF-α (Fig. 5B) and iNOS (Fig. 5C); m2 profile for CD163 (Fig. 5D), CD206 (Fig. 5E) and IL-10 (Fig. 5F). This study suggests a


A
RH_Stand_(s)_Mean
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RH_Swing_(s)_Mean
0.20
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RH_TerminalDualStance_(s)_Mean
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◂**Fig. 4** Dynamic gait analyses of EOA animals induced by MIA and treated with MMF. EOA animals were induced by 1.5 mg of MIA solution (50 µL) via intra-articular in the right knee joint and analyzed by Catwalk device the right hind gait parameter. After 3 days, EOA animals received a dose of 280 ng of MMF via intra-articular in the right knee joint. **A** Stand and **B** Swing represent, respectively, the time spent in the same position and the time spent for right hind swing; **C** print area, **D** max intensity At, and **E** mean intensity of the 15 most intense pixels represent the complete right hind contact on the glass platform; **F** terminal dual stance idem of item (**A**). Dilutions for each group: vehicle (saline 0.9% + DMSO 5%), 1.5 mg of MIA (saline 0.9%), and 280 ng of MMF (saline 0.9% + DMSO 5%). Statistical analyses were performed using two-way ANOVA (or mixed model) with Tukey's multiple comparison test with $p < 0.05$. N = 5. Symbols: ** = 0.013; * = 0.0405

downregulation in the proinflammatory genes and markers associated with the M1 profile while upregulating the M2 profile post-intra-articular MMF treatment (Koda et al. 2016, Ni et al. 2020, Yulin Chen et al. 2020).

Analysis in vitro observed iNOS (Fig. 6A) and Arg-1 gene express (Fig. 6B) with mRNA extracted from BMDM culture plate where macrophages were previously polarized to M1 (LPS 100 ng/mL + INF-γ) for 1 h and 30 min and after treated with DMF (50 µM) and MMF (50 µM) for 4 h and 30 min (Tardito et al. 2019; Kourakis et al. 2020). The relative proportion of iNOS and Arg-1 suggests macrophage polarization from M1 to the M2 profile (Fig. 6C). This study suggests macrophage polarization from M1 to M2, linking to what was observed in the macerated capsule tissue.

The mRNA fold change quantification of the joint capsule suggests upregulating MCP-1 gene expression in EOA animals treated with MMF. This gene is responsible for the recruitment of monocytes, leading to monocyte differentiation and polarization to the M2 profile due to the bioavailability of MMF in the cavity joint. EOA animals without treatment have a higher presence of DAMPs, leading to M1 polarization (Thimmulappa et al. 2006; Yulin Chen et al. 2020).

These thoughts are supported by downregulating the proinflammatory genes such as TNF-α and iNOS while upregulating the anti-inflammatory gene IL-10 in EOA animals treated with MMF. Moreover, despite no statistical difference for CD163, this gene tended to be higher than in non-treated groups, linking to the EOA improvements by behavioral analyses during the inflammatory phase (Conaghan et al. 2019).

Our results with MMF and DMF may suggest an essential role in macrophage polarization in the joint region by intra-articular or oral gavage pathways, respectively. However, more studies are required to fully understand this process in EOA treatment and how these drugs are attenuating or promoting the pro-chondrogenic environment for cartilage regeneration.

Discussion

Drugs already available for human medicine have been investigated to treat other chronic diseases. DMF and MMF are examples of medications used to treat multiple sclerosis that could have therapeutic properties in OA. Our studies suggest a perspective for human treatment committed by OA once the behavioral performance was well succeeded, reducing hyperalgesia and inflammatory process, one of the hallmarks of OA progression triggered by the M1 profile. This study indicates a possibility for treating OA based on the improvements observed in EOA (Ma et al. 2021).

As well known, OA is a chronic and common bone disease that has as a hallmark the inflammatory process triggered by an unbalanced macrophage M1 profile causing cartilage disruption and chronic pain (Fernandes et al. 2020). The present study indicates that macrophage polarization could be the first treatment line to revert OA progression, preventing these symptoms that are affecting people's quality of life around the world, reducing hyperalgesia because pain-feeling tends to continue once cartilage regeneration in the joint is limited, and decreasing chondrocytes activity along OA progression induced my macrophages (Cui et al. 2020).

Despite some pharmacology treatments that might help OA to relieve pain, many of these treatments are correlated with several side effects in long-term use that may cause organ function problems but cannot reverse cartilage damage (Yusuf 2016). These treatments, in general, do not focus on macrophage polarization, which is an essential cell involved in OA progression. Studies indicate that reverting this inflammatory process to the anti-inflammatory scenario by upregulating the M2 profile will not only suggest a decrease in the M1 profile but also support a pro-chondrogenic environment for chondrocytes (Zhang et al. 2020; Wang and He 2022).

Our investigation of DMF suggests therapeutic properties by oral administration in OA (Kourakis et al. 2020). MMF would be a new perspective for OA treatment by intra-articular pathway treatment according to the OA severity (Lategan et al. 2021). Both studies indicate that DMF and MMF are used for MS but could also be extrapolated for other diseases (Singh et al. 2018; Carlström et al. 2019). This extrapolation might be seen during the present study that proposed EOA animals induced by MIA in the knee demonstrated therapeutic properties by reducing hyperalgesia, indicating the macrophage polarization from the M1 to M2 profile along the treatments, downregulating M1 profile and might upregulate M2 profile could improve EOA animals performance (Kobayashi et al. 2016; Wang and He 2022).

In these studies, daily doses of 30 mg/kg of DMF and a single dose of 280 ng of MMF demonstrated by behavioral

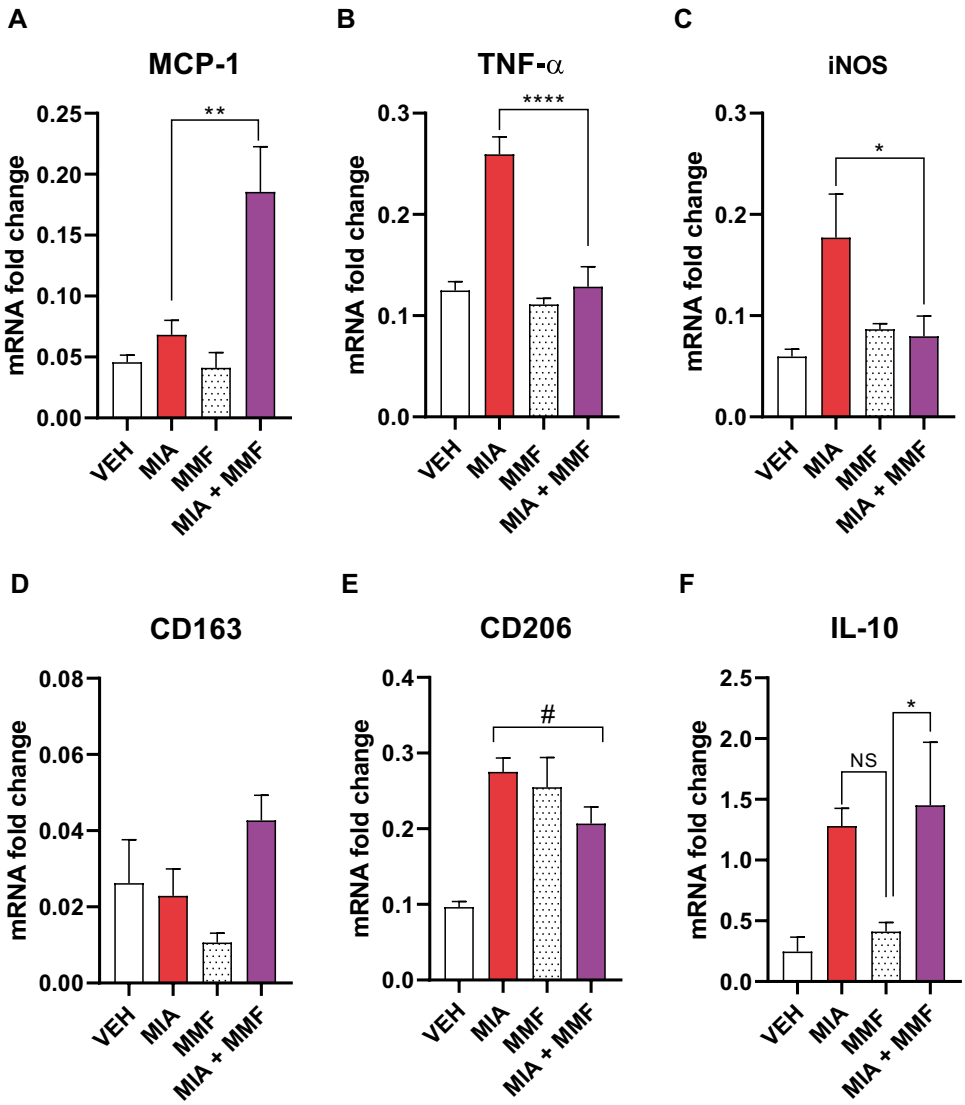

Fig. 5 mRNA fold change quantification from the capsule of the right knee of EOA animals treated with MMF. EOA animals were induced by 1.5 mg of MIA solution (50 µL) via intra-articular in the right knee joint and analyzed by Catwalk device the right hind gait parameter. After 3 days, EOA animals received a dose of 280 ng of MMF via intra-articular in the right knee joint and collected capsule joint on the seventh day. On the seventh day, the capsule was extracted to analyze mRNA genes and markers related to macrophage profile. **A** Monocyte Chemoattractant Protein-1 (MCP-1); **B** Tumor Necrosis Factor-alfa (TNF-α); **C** inducible nitric oxide synthase (iNOS); **D** hemoglobin-haptoglobin receptor (CD163); **E** mannose receptor (CD206); **F** interleukin-10 (IL-10). Dilutions for each group: vehicle (saline 0.9% + DMSO 5%), 1.5 mg of MIA (saline 0.9%), and 280 ng of MMF (saline 0.9% + DMSO 5%). Statistical analyses were performed using two-way ANOVA (or mixed model) with Tukey's multiple comparison test with $p < 0.05$. Duplicate samples. $N = 6$. Symbols: **** = 0.0001; *** = 0.008; ** = 0.012. # = 0.0101 comparison with veh (vehicle)

analyses that mechanical nociceptive hyperalgesia assessment was recovered via adapted electronic von Frey in debility rats, an experienced reduced pain (Kuner 2010; Vieira et al. 2020). Interestingly, MMF recovered earlier than the mechanical nociceptive hyperalgesia assessment in a single injection, reducing the inflammatory phase and impacting the gait assessment, which demonstrated that both medicines could improve EOA rat's performance (Teixeira et al. 2017; Vieira et al. 2020).

Studies by Gao et al. (2022) using von Frey filaments indicate an important role of DMF by upregulating the gene nrf2 and reducing hyperalgesia (secondary hyperalgesia) in EOA animals induced by MIA, which could alleviate the experienced pain behavior during the chronic phase (14th-day post-MIA injection). Similarly, our studies by adapted electronic von Frey also indicated that DMF and MMF could reduce hyperalgesia (primary hyperalgesia) and improve rats' performance by daily treatment with DMF or a single dose injected with MMF. These drugs are important for acting during the inflammatory phase and might prevent cartilage and the damage associated with macrophage M1 profile. However, studies by Gao et al. (2022) suggest an analgesic effect during the chronic phase.

Nonetheless, the therapeutic properties are based on the metabolite-active MMF. Thus, MMF showed more significant OA gait parameters than DMF treatment, indicating the intra-articular pathway as a better option for OA treatment. This is also observed in the literature that evaluated patient symptoms (Mora et al. 2018; Jones et al. 2019; Wang and He 2022).

In other words, restricting the bioavailability of MMF to the lesion place could reduce the inflammatory phase established in this scenario earlier after post-MIA injection, which is similar to the OA symptoms. This can improve rats' performance of EOA animals that experienced reduced pain sensitization on right hind stand time, and recover the

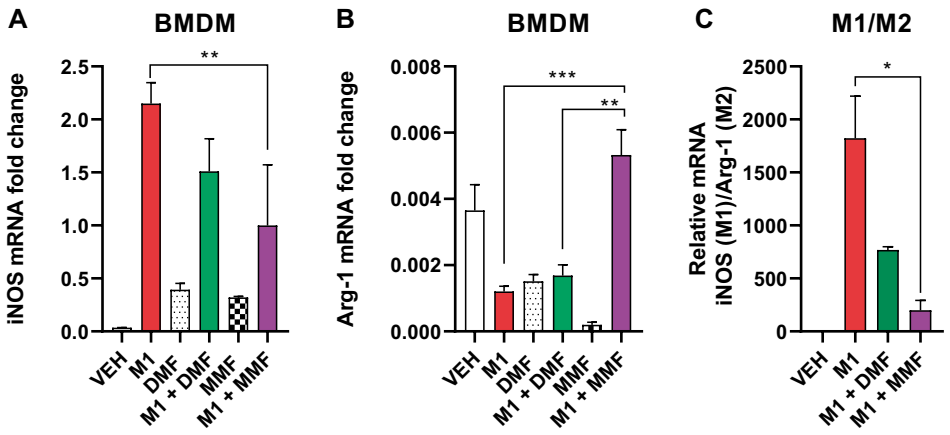

Fig. 6 mRNA fold change quantification from BMDM plate polarized to M1 and treated with DMF and MMF. Bone marrow cells were extracted from the femur of male *Wistar* rats and plated with 500,000 cells per well. The culture medium was supplemented with fetal bovine serum (10%) and MC-SF (20 ng/mL). After the third day, the culture medium was replaced, repeating the same procedures. On the fifth day, the culture medium was replaced, and macrophages were polarized to the M1 profile by LPS (100 ng/ml) + IFN-γ (20 ng/ml). After 1h 30, the culture medium was replaced with a new one. In this new culture medium, doses of 50 µM of MMF or 50 µM of DMF were added for 4 h and 30 min. **A** mRNA quantification of the expressed gene iNOS found change; **B** mRNA quantification of the expressed gene Arg-1 found change; **C** analysis of the proportion of M1(iNOS) by M2 (Arg-1). Drugs were diluted in pure DMSO, achieving 5% when applied in a culture medium. VEH = vehicle (DMSO 5%). Statistical analyses were performed using one-way ANOVA (nonparametric or mixed) with Tukey's multiple comparison test. Duplicate samples. $N = 3$. $p < 0.05$. Symbols: *** = 0.008; ** = 0.012

total footprint contact on the glass floor, once the animal pain-relieve is associated with the higher upregulating of macrophage M1 polarization during the inflammatory phase, cartilage disruption and nociceptive sensitization (Teixeira et al. 2017; Wang and He 2022).

As known, macrophages are orchestrating the inflammatory phase in EOA (Zhang et al. 2020). Our study during the fifth day of the inflammatory phase, post-MIA injection, the macerated joint capsule of EOA animals treated with DMF showed reducing proinflammatory cytokine TNF-α while increasing anti-inflammatory cytokine IL-10, indicating therapeutic actions by reprogramming of macrophage profiles (Kobayashi et al. 2016). Intervening in this inflammatory phase suggests a possibility to reduce hyperalgesia in EOA rats and promote better performance, as suggested by behavior analyses of adapted electronic von Frey and gait parameter analyses (Catwalk).

Despite these methods seeming to be applied in human analyses, at least the drugs could be driven to improve human quality of life once it is complicated to find the correct diagnosis and treatment for humans committed with OA, even with advanced technology (Felson and Neogi 2018; Thysen et al. 2015). This study provides information that would bring a new perspective of treatment based on macrophage polarization (M2) to reduce pain by upregulating anti-inflammatory products.

Our studies suggest downregulating M1 and upregulating M2 mRNA from the macerated capsule joint tissue in EOA animals treated with MMF until the seventh day. The proinflammatory genes were downregulating, decreasing TNF-α and iNOS gene expression while increasing anti-inflammatory genes like IL-10, with relatively higher upregulating in the CD163 marker, being a signature for M2 profile (Kobayashi et al. 2016; Wang and He 2022). In addition to this analysis, culture plates polarized for the M1 profile and treated with MMF have also indicated downregulation of iNOS and upregulation of Arg-1 gene expression, suggesting an essential role of MMF in macrophage polarization (Kobayashi et al. 2016).

These data support what has been discussed about the improvements observed in behavioral analyses and put forward the possibility of carrying out these studies for human treatment. As well human as EOA animals, M1 and M2 profiles are unbalanced and could be an essential step to revert OA progression. Our study with MMF indicates a possibility to revert the inflammatory process by upregulating the M2 profile after increasing the MCP-1 expression and polarizing the monocytes recruited for the anti-inflammatory profile M2 when achieving the lesion place in regards to the DAMP stimulation that drives macrophages to the M1 profile during OA progression (Shapouri-Moghaddam et al. 2018; Ni et al. 2020).

In sum, our studies suggest therapeutic properties in EOA rats' joint knees, as much as DMF and MMF, bringing benefits that might be extrapolated for chronic disease OA by acting in the macrophage polarization in humans. However, more studies are fully required to understand whether macrophage polarization to the M2 profile might

promote the pro-chondrogenic place by enhancing chondrocyte activity and cartilage regeneration for experiencing reduced pain post-treatment, once the literature shows the importance of this cross-talk among macrophages and chondrocytes (Akkiraju and Nohe 2015, De Sousa 2019, Fernandes et al. 2020).

Conclusion

DMF and MMF are drugs already approved for human treatment in the USA that might be extrapolated for other therapies based on their active metabolite with therapeutic properties for OA. In this study, not only DMF but also MMF was demonstrated to reduce inflammatory scenarios triggered by the M1 profile via gavage and mainly via MMF treatment via the intra-articular pathway, bringing a new perspective to polarize macrophages in joints to the M2 profile, enhancing the pro-chondrogenic environment and promoting cartilage regeneration. However, more studies are required to fully understand the role of macrophage polarization (M2) and the possibility of stimulating the cartilage regeneration process.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10787-024-01443-w.

Acknowledgements The present project 88882.461722/2019-01 was supported by the Higher Education Personnel Improvement Coordination—CAPES. The authors gratefully acknowledge the support of the Laboratory of Nerve Regeneration at the Institute of Biology (IB/Unicamp) and the Department of Translational Medicine (FCM/Unicamp).

Author contributions Douglas Menezes de Souza: conceptualization, methodology, validation, formal analysis, investigation, writing—original draft, writing—review and editing, visualization, and funding acquisition; Catarine Massucato Nishijima contributed by supporting in vitro and behavioral analyses, drugs dilution, and manuscript preparation; Kauê Franco Malange contributed by teaching behavioral experiments, study design and manuscript preparation; Bruno Henrique de Melo Lima contributed teaching and performing behavioral experiments in catwalk, and manuscript preparation; Vinícius Capetini performed RT-PCR experiments and supported in manuscript preparation; Alexandre Leite Rodrigues de Oliveira contributed to the methodology and resources for the catwalk procedure; Gabriel Forato Anhê contributed to the methodology and resources for RT-PCR; Claudia Herrera Tambeli contributed writing, reviewing, and editing the manuscript; Carlos Amilcar Parada contributed in the conceptualization, resources, writing—original draft, writing—review and editing manuscript, supervision, project administration, and funding acquisition.

Funding The present project 88882.461722/2019-01 was supported by the Higher Education Personnel Improvement Coordination—CAPES, Brazil. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, 88882.461722/2019-01, Douglas Menezes de Souza

Data availability The datasets generated during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interests The authors declare that they have no competing interests.

Ethical approval and consent to participate The experiment was approved by the Ethics Committee on the Use of Animals (CEUA for protocol 5965-1/2021 on March 21st, 2022. All experiments were conducted and performed according to the guidelines of the National Council for Animal Experimentation Control (CONCEA).

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