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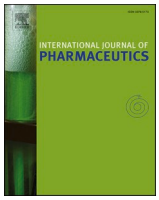

Efficacy of memantine prodrug microemulsion in a Preclinical model of tendinopathy

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ABSTRACT

Tendinopathy is a complex musculoskeletal pathology characterized by pain and dysfunction in the tendon. Tendinopathy management includes pharmacological treatments, which, however, have numerous side effects and low efficacy. New pharmacological approaches are essential to overcome these drawbacks. This study aimed to test the activity of the newly synthesized memantine-prodrug Memit against chronic tendon pain, which releases H_2S and memantine at the therapeutic site, formulated in a microemulsion for *peri*-tendon administration. Memit (1 % *w/w*) was successfully formulated in a microemulsion using the titration method, and the recovery was quantified by liquid chromatography. The characterization of the obtained Memit-loaded microemulsion (Memit-ME) by dynamic light scattering and electron microscopy showed the droplets' homogeneous dimensional dispersion and spherical shape. The formulation was found to be physically and chemically stable. Additionally, Memit-ME provided a prolonged release of the prodrug, essential to maintaining its retention at the therapeutic site. The formulation was administered in a rat model of tendon damage induced by a single intratendon carrageenan injection to evaluate its anti-hyperalgesic and anti-allodynic profile. The study demonstrated that, thanks to its safety profile and effectiveness in significantly reducing spontaneous pain and postural imbalance, the developed Memit-ME represents a promising approach for tendinopathy management.

1. Introduction

Tendons are responsible for transmitting the contractile force between musculoskeletal tissues, connecting muscles to bones by the musculotendinous junction and the osteotendinous junction. Tendons are connective tissues, predominantly containing water (up to 55–70 %), while collagen represents 60–85 % of dry weight. Collagen is arranged in a hierarchical manner parallel to the long axis of the tendon, imparting viscoelastic and sensitive properties to different degrees of strain (Thorpe and Screen, 2016).

Overuse, injury, aging, and health conditions such as arthritis can damage the structure and function of tendons. Additionally, tendon-related disorders are challenging to treat due to the relatively hypovascular and hypocellular structure of tendons, which has limited intrinsic regenerative capacity (Durgam and Stewart, 2017). Regrettably, though, tendon disorders are common in clinical practice, and the treatment includes surgical repair, mechanical stimulation (sports rehabilitation), and anti-inflammatory drugs for topical or systemic use, supplemented by interventions such as physical therapy and tissue engineering (Clayton and Court-Brown, 2008; Nourissat et al., 2015).

Treating acute tendon injuries involves repairing broken tendons with surgical techniques, but chronic tendinopathies are the most challenging to manage from a pharmacological perspective. Patients with chronic tendinopathies show increased perceived pain during

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tendon palpation and report pain during normally pain-free movements, which have been recognized as possible manifestations of hyperalgesia and allodynia (Rio et al., 2014). Treatment of chronic tendon injury to reduce pain is mainly attempted through local or systemic therapies (Li et al., 2021). However, clinical approaches by local or systemic administration are not so efficient, and the systemic ones usually cause side effects to healthy organs. Accordingly, *peri*-tendon injection represents an elective route of administration to treat chronic tendinopathies. Injections should be *peri*-tendinous to avoid injection directly into the tendon substance and consequent tendon rupture. In fact, studies on animal models have shown that intra-tendinous corticosteroids can modify the biomechanical properties of tendons (Hugate et al., 2004).

If side effects are generally reduced with the *peri*-tendon injection, several limitations can be associated with this type of administration route, including the need for repeated administrations, poor drug solubility in the biological environment, scarce drug retention, and enhanced drug clearance from the site of the administration. A very interesting approach to overcoming these limitations is represented by the formulation of nanomedicines, which provide efficacious approaches by enhancing drug retention in a controlled manner at the site of action, preventing burst release, protecting against rapid clearance, and optimizing conventional treatments (Parchi et al., 2016; Vanti et al., 2023, 2022). The literature reports a few cases of nanocarriers applied to tendinopathies, such as organic nanoparticles, specifically lipid-polymer hybrid nanoparticles (Sandra López-Cerdá, 2024)and heparin-polymeric or bovine serum albumin-heparin nanoparticles (Choi et al., 2020; Shen et al., 2022), liposomes (Micheli et al., 2023) and extracellular vesicles (Graça et al., 2022), electrospun fibers (Zhao et al., 2015), inorganic nanoparticles like gold nanoparticles (Dohnert et al., 2012), magnetic nanoparticles (Vinhas et al., 2020) or mesoporous silica nanoparticles (Zhu et al., 2023), and finally a hyaluronic acid-hydrogel (Hsiao et al., 2019) or a nanoparticle-hydrogel (Kim et al., 2022).

To the best of our knowledge, this study is the first attempt at testing a microemulsion for treating tendinopathy, particularly by *peri*-tendon local injection. Microemulsions are transparent, thermodynamically stable colloidal systems with optimal shelf-life (Bilia et al., 2019; Vanti, 2021). We chose an oil-in-water microemulsion because it acts as a solvent for the drug, combining with it the advantages of a nanocarrier, such as the drug loading. Accordingly, the drug is protected in the internal oily phase until it is released by passive diffusion from the globules to the external biological environment. Microemulsions intravenously administered have been shown to decrease the rate of clearance compared to a conventional solution, thus increasing the drug half-life and overall drug exposure (Dubey, 2014). Further, due to the slow and prolonged drug release, microemulsions have also been shown to decrease toxicity and improve efficacy (Date and Nagarsenker, 2008; Dubey, 2014).

Additionally, our attention has been focused on testing a new potential drug. In a previous study, Rapposelli and coworkers synthesized a new hybrid molecule, named Memit, by replacing the memantine amine group with an isothiocyanate functionality as a putative H_2S-donor moiety (Sestito et al., 2019) in order to implement the biological activity in neuroprotection. Indeed, H_2S is a well-known endogenous gasotransmitter that plays a key role in mediating various anti-inflammatory and antioxidant effects across different systems (Kimura, 2014; Wallace, 2007). Memit displayed the ability to release H_2S through a cysteine-mediated mechanism, generating memantine, a well-known N-methyl-D-aspartate receptor (NMDAR) antagonist (Fig. 1). The researchers demonstrated that Memit reduced the self-induced aggregation of Aβ (1–42) and exerted a cytoprotective effect against damage induced by Aβ oligomers in both human neurons and rat microglial cells. Like memantine, the new compound promotes autophagy, a complex process required for cellular homeostasis and cell survival that is altered in neurodegenerative diseases. Memit, among other activities, can protect against neuronal inflammation, reducing ROS production. Although Memit showed several positive effects in neurodegenerative diseases,

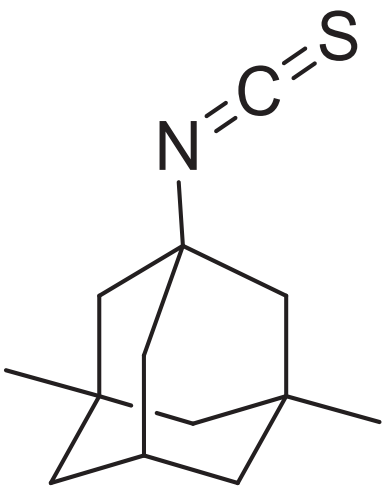

Fig. 1. Chemical structure of Memit. Formula: $C_{13}H_{19}NS$; molecular weight: 221.36 g/mol.

the capability of the molecule to release H_2S and memantine suggested investigating this small molecule as a therapeutic tool for tendinopathy. Indeed, recent research reported that NMDARs are also implicated in tendinopathy, playing a role in pain modulation, inflammation, and cellular responses (Alfredson and Lorentzon, 2005; Wasker et al., 2023).

Since the synthetic molecule is insoluble in water, a microemulsion was developed to provide a dosage form with significant drug loading, retention in the therapeutic site, and controlled release [24]. This study aimed to evaluate the activity against chronic tendon pain of Memit-loaded microemulsion (Memit-ME) administered by the *peri*-tendon route in a rat model of tendon damage induced by a single intra-tendon injection of carrageenan (Micheli et al., 2022).

2. Materials and Methods

2.1. Chemicals

Memit was synthesized as previously reported (Sestito et al., 2019). Vitamin E acetate was bought from A.C.E.F. S.p.a. (Piacenza, Italy). Labrasol ALF and transcutol HP were generously gifted from Gattefossé (Saint-Priest, France). All the organic solvents, namely methanol, acetonitrile, and formic acid, were purchased from Merck Life Sciences Srl (Milan, Italy). Ultrapure water was obtained by a synergy Simplicity UV water purification system provided by Merck Life Sciences Srl (Milan, Italy). Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, PA, USA).

2.2. HPLC-DAD analysis

Quantitative determination of Memit was carried out using a 1100 High-Performance Liquid Chromatograph (HPLC) equipped with a Diode Array Detector (DAD) from Agilent Technologies Italia Spa (Rome, Italy) and a Kinetex EVO C-18 100 Å column with an internal diameter of 5 µm and 150 x 4.6 mm dimensions (Piponski et al., 2020). The analyses were performed using a mobile phase composed of (A) 0.02 % v/v formic acid/water (pH 3.2) and (B) acetonitrile with a 0.5 mL/min flow rate and the following multi-step gradient: 0–15 min 20–100 % B; 15–30 min 100–20 % B; post time was 5 min. The temperature was set at 25 °C. UV–visible spectra were recorded in the 200–600 nm range, and chromatograms were acquired at 246 nm. The calibration curve was prepared by solubilizing Memit in methanol. A linear relationship was obtained in the investigated concentration range of 10–0.1 µg ($R^2 > 0.9999$).

2.3. Preparation of microemulsion and pseudo-ternary phase diagram construction

The microemulsion was prepared using the aqueous titration method (Vanti et al., 2021a). The oily phase (vitamin E acetate) was combined with the mixture (Smix) of surfactant (Labrasol ALF) and cosolvent (Transcutol HP) at various gravimetric ratios (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1) by stirring at 35 °C for 5 min. The selected Smix consisted of Labrasol ALF and Transcutol HP in a 9:1 gravimetric ratio. The lipophilic mixture was successfully mixed and warmed on a magnetic stirrer set at a constant temperature of 35 °C. It was then titrated with ultrapure water dropwise, while the sample was maintained on the heating magnetic stirrer at 700 rpm. The obtained system was allowed to stabilize at 35 °C for 10 min and cool to room temperature (21 ± 2 °C) under gentle magnetic stirring (300 rpm). As a first evaluation method, changes in sample appearance were visually monitored during water addition to determine if a transparent microemulsion, an emulsion, a gel, or turbidity had formed (Abouhosseini Tabari et al., 2022; Bamanna et al., 2025; Soliman et al., 2010). The existing region of the developed microemulsion was determined by visual inspection and light scattering analysis of the sample. The pseudo-ternary phase diagram showing the existing areas of all systems obtained by titration was constructed using the free online platform TernaryPlot.com (TernaryPlot, 2024). The Memit-ME was prepared using the same procedure, with the active ingredient added to the oily phase before warming and titrating.

2.4. Light scattering analysis

The microemulsion was analyzed by the dynamic light scattering (DLS) technique using the Zetasizer Pro Red Label of Malvern Panalytical (Alfatest Srl, Milan, Italy) equipped with a He-Ne gas laser with a maximum output power of 10 mW and a beam wavelength of 632.8 nm, and with an avalanche photodiode (APD) detector. The measurements were performed at 25 °C, with a backscatter detection angle of 173°. The analyses enable the determination of the samples' average hydrodynamic diameter (Size, nm) and polydispersity index (PDI, a dimensionless parameter). All data were processed using the Cumulants method defined in the International Standards and recognized by Health Agencies (ISO 22412, 2017; ASTM E3247-20, 2020). Each measurement was performed using square polystyrene cuvettes for DLS.

2.5. Memit recovery in the microemulsion

The total amount of Memit effectively loaded into the microemulsion and recovered at the end of the preparation procedure is referred to as the recovery. It is obtained by dissolving the microemulsion globule by 100-fold dilution with methanol. The samples were vortexed and immersed in the ultrasonication bath for 5 min at 21 °C to improve the globule dissolution and Memit extraction in the organic solvent. The samples were centrifuged for 5 min at 14,000 rpm to precipitate any undissolved microemulsion components and were analyzed by HPLC-DAD, as described in paragraph 2.2. Recovery is expressed as the percentage of the recovered amount compared with the initial weighted amount of the drug (Vanti et al., 2021a, 2021b).

2.6. Scanning transmission electron microscopy

The microemulsion was also analyzed by the scanning electron microscope (SEM) Gaia 3 (Tescan s.r.o, Brno, Czech Republic), a FIB-SEM (focused ion beam scanning electron microscope), operating in high-vacuum mode with electron beam voltage of 20 kV and bright-field transmission electron microscope (TEM) detector (Vanti et al., 2021a). Gaia 3 was equipped with an EDS-X-ray microanalysis system (EDAX, AMETEK, USA), TEAM EDS Basic Software Suite TEAM™, and was delivered with a STEM (scanning transmission electron microscopy) detector, which provides a complementary method for image acquisition of transmitted electrons. The detector comprises several semiconductor sensors for both bright-field and dark-field imaging. The transmitted electron signal can be collected by placing the detection system below the specimen.

2.7. Memit release from microemulsion

The release profile of Memit from the microemulsion was evaluated by the dialysis bag method using cellulose membranes with selective permeability (Vanti et al., 2022). Memit-ME's exact volume (0.5 mL) was located inside the SpectraPor® Micro Float-A-Lyzer® ready-to-use dialysis device of high-purity biotech grade cellulose ester with a molecular weight cut-off of 8–10 kDa. The Luer® port and 1 mL syringe allow easy loading and in-process sample retrieval (Repligen Europe BV; Breda, The Netherlands). A magnetic bar was fixed below the device's bottom to allow the floating membranes to rotate. The filled device was maintained under magnetic stirring at 100 rpm for 72 h in an exact volume (200 mL) of 1 % *v/v* DMSO/PBS, heated at the controlled temperature of 37 ± 1 °C. The formulation (0.02 mL) was collected and replaced by an equal volume of fresh empty microemulsion at specific time points (5, 10, 15, 20, 25, 30 min and 1, 2, 3, 4, 5, 24, 29, 48, 72 h) up to 72 h. The acceptor medium was replaced every 24 h with a pre-heated fresh medium. All samples were treated as described in paragraph 2.5 to determine Memit recovery by HPLC-DAD analysis. The experiment was performed in triplicate.

2.8. Chemical and physical stability of Memit-ME

The Memit-ME was stored at 25 °C in the dark for one month to evaluate its chemical and physical stability. Specifically, weekly, Memit recovery was determined by HPLC-DAD according to the method described in paragraph 2.5, while Size and PDI were measured by DLS.

2.9. Animals

For all the experiments described below, male Sprague-Dawley rats (Envigo, Varese, Italy) weighing approximately 250–300 g were used. The animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 × 41 cm) and kept at 23 ± 1°C with a 12-hour light/dark cycle, with light on at 7 a.m. They were fed a standard laboratory diet and had access to tap water ad libitum.

2.10. Induction of tendonitis and treatments

The animals were anesthetized with isoflurane (4 % for induction and 1.5 % for maintenance of anesthesia). Tendon damage was induced near the osteotendinous junction of the rat's right Achilles tendon by a single intra-tendon injection of 20 μL of carrageenan 0.8 % after having flexed the paw to form a 45° angle, using a 30 G needle. Carrageenan was solubilized in physiological solution (Lake et al., 2008; Micheli et al., 2022). Control animals were treated with saline solution. Memit-ME (10 mg/mL; 50 μL) or its vehicle was *peri*-tendon injected on days 1, 3, 5, 7, and 10. Behavioral tests were performed on days 3, 5, 7, 10, and 13 after the induction of damage and before the initiation of new treatments.

2.11. Paw pressure test

The nociceptive threshold of rats was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton (Leighton et al., 1988). A constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats

were lightly restrained (Micheli et al., 2019a). Vocalization or withdrawal reflex thresholds were expressed in grams. An arbitrary cut-off value of 100 g was adopted.

2.12. Incapacitance test

Weight-bearing changes were measured using an Incapacitance Apparatus (Linton Instrumentation, UK), detecting changes in postural equilibrium after a hind limb injury. Rats were trained to stand on their hind paws in a box with an inclined plane (65° from horizontal). This box was placed above the Incapacitance apparatus. This allowed us to independently measure the weight that the animal applied to each hind limb. The value reported for each animal is the mean of five consecutive measurements. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, indicating postural equilibrium. In contrast, an unequal weight distribution on the hind limbs indicated a monolateral decreased pain threshold (Crocetti et al., 2020). Data are expressed as the difference between the weight applied to the limb contralateral to the injury and the weight applied to the ipsilateral one (Δ weight) (Bua et al., 2020).

2.13. Beam balance test

Walking impairments were evaluated using the beam balance test, as described in the literature (Ding et al., 2004). The test was done with the rats being placed on a narrow strip of wood (30 cm × 1.3 cm) while balancing, and the scoring standards were as follows: 0 points, the four limbs were all on the wood in a balance situation; 1 point, the limbs of one side were able to grasp the wood or shake on the wood; 2 points, one or two limbs slipped from the wood; 3 points, three limbs slipped from the wood; 4 points, the rat was suspended on the wood and fell over after a struggle (Micheli et al., 2019b).

2.14. Rota rod test

Motor coordination was evaluated using the Rota rod test (Ugo Basile, Varese, Italy). The Rota rod apparatus consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into four equal sections by five disks. Thus, up to four rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed based on the number of falls from the rod for a maximum of 600 s. A cut-off of six falls was used, the test was suspended, and the time was recorded. Each rat was assessed once, and the group mean average score was calculated (Micheli et al., 2019b).

2.15. Statistical analysis

Trained observers were not informed about the specific treatment of each animal group that carried out the tests. Results were expressed as means ± S.E.M., and the analysis of variance was performed by one-way ANOVA test. A Bonferroni's significant difference procedure was used as a post hoc comparison. Statistical comparisons involving a single factor were defined *a priori* for detailed analyses as follows. Data from the carrageenan + vehicle group were compared with those from the vehicle + vehicle group to confirm the validity of the injury model. Data from the carrageenan + vehicle group were also compared with those from the carrageenan + Memit vehicle and carrageenan + Memit ME groups to assess the efficacy of the treatments. Finally, data from the carrageenan + Memit vehicle group were compared with those from the carrageenan + Memit ME group to explore the improvement of Memit ME. P values less than 0.05 and 0.01 were considered significant. Data were analyzed using the "Origin 9.1" software.

3. Results and discussion

3.1. Preparation of Memit-ME

The oil, surfactant, and cosolvent were selected based on their miscibility with the active liquid ingredient. Labrasol ALF (caprylocaproyl polyoxyglycerides) is a liquid nonionic water-dispersible surfactant (HLB 12) for lipid-based formulations that solubilize poorly water-soluble active ingredients and was selected as a microemulsion surfactant (Gué et al., 2016). Transcutol HP (diethylene glycol monoethyl ether) is a high-purity solvent, a cosolvent in surfactant formulations such as microemulsions, and a solubilizer for solubility enhancement (Lamas et al., 2006; Mahmoud et al., 2014). It is currently used as an excipient of formulations for parenteral use, such as medicinal products for the intravenous administration in humans and animals (Sullivan et al., 2014), and was selected as a cosolvent for the development of Memit-ME. Vitamin E acetate is a fat-soluble acetate ester of vitamin E, rapidly converted into vitamin E in the body. A Ph. Eur. grade vitamin E acetate was used as the oily excipient in developing the microemulsion (Ye et al., 2021), according to its well-established use for parenteral administrations.

The pseudo-ternary phase diagram was obtained by the titration method (Fig. 2). The light blue area represents the microemulsion existing region, while the grey area represents the emulsion region. Specifically, microemulsions appeared as clear solutions, whereas emulsions exhibited phase separation. Visual inspection during water addition for diagram construction was enough to discriminate between the two systems. We confirmed the existing microemulsion region through a DLS analysis. The specific composition of the microemulsion was designed to load high concentrations of Memit while maintaining a consistency suitable for *peri*-tendon injection using a 30G needle. The final microemulsion was constituted of 4 % *w/w* Transcutol HP, 36 % *w/w* Labrasol ALF, 4 % *w/w* vitamin E acetate, and 56 % *w/w* water. Memit was added to the microemulsion at a concentration of 1 % *w/w* (Azarbaijani et al., 2021; Morel et al., 2013; Takeda et al., 2009). The obtained formulation was subsequently characterized and used in all *in vivo* studies.

3.2. Technological characterization of Memit-ME

Memit-ME appeared as a clear, uncolored liquid (Fig. 3A). It was analyzed by DLS to gain a first physical characterization in terms of dimensions (Size) and homogeneity (PDI). Specifically, the empty microemulsion had a Size of 36.09 ± 5.710 nm and a PDI of 0.2233 ± 0.01102. In comparison, Memit-ME showed a Size slightly increased to 40.39 ± 6.967 nm (Fig. 3B), probably due to the presence of 1 % *w/w* Memit in the oily internal phase of the microemulsion, and a PDI increased to 0.2621 ± 0.01442. However, the dimensional distribution was still represented by a Gaussian curve, but with a greater width. Memit-ME observation by STEM (Fig. 3C) showed spherical droplets with dimensions around 50 nm in accordance with values obtained by DLS. Concerning the chemical characterization of the formulation, we determined Memit Recovery to estimate the final concentration of the active compound. Memit recovery, quantified by HPLC-DAD analysis, was found to be 99.96 ± 3.213 %, and no variation in the UV spectrum was observed. Therefore, the microemulsion preparation procedure didn't affect the chemical stability of the loaded prodrug. All reported data are expressed as the mean ± standard deviation, with a minimum of n = 3.

3.3. Memit release from microemulsion

We employed the dialysis bag method to estimate the release of Memit from microemulsion droplets by passive diffusion over a 72-hour period. Memit was quantified indirectly by sampling the release medium from the micro-floating dialysis device at specific time intervals, as

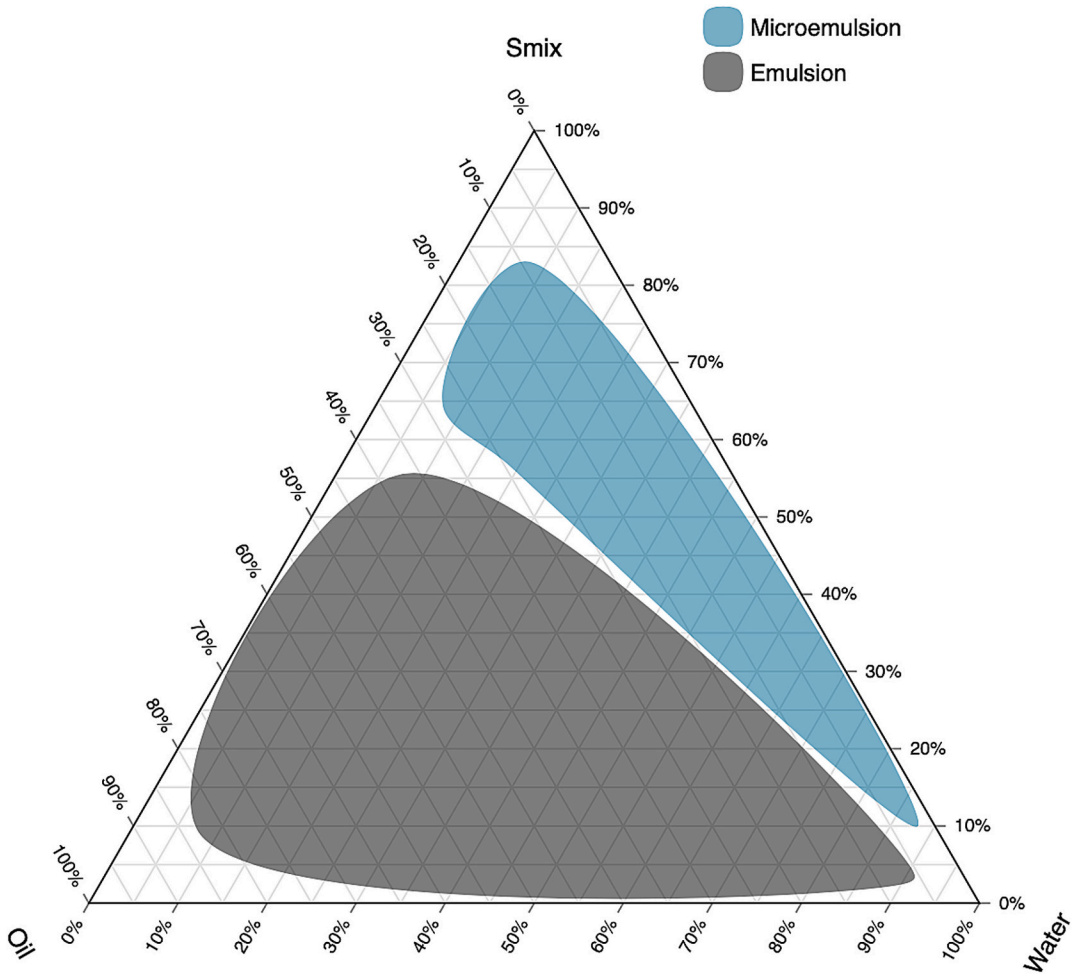

Fig. 2. Pseudo-ternary phase diagram. Oil is vitamin E acetate, Smix is Labrasol ALF plus Transcutol HP in a 9:1 gravimetric ratio.

Memit could not be detected directly during the assay. The experiment was conducted in a 1 % *v/v* DMSO/PBS acceptor medium to solubilize the released Memit. The percentage of DMSO was kept low to reduce interference with the device membrane, as the producer did not report compatibility. In addition, the mixture could solubilize a higher Memit concentration than Memit-ME concentration, providing, together with acceptor medium replacement every 24 h, sink conditions during the assay (a solubility test wasn't performed because the synthetic drug was available in small amounts) and mimicking endogenous clearance of the *peri*-tendon region. The experiment revealed a fast Memit diffusion from the microemulsion during the first 60 min, reaching 49.53 ± 4.428 % (Fig. 4). It ensures an immediate effect after administration. Afterward, the release proceeded more slowly, reaching 86.09 ± 0.7947 % after 48 h and continuing up to 95.59 ± 4.306 % after 72 h. This *in vitro* release behavior can be translated into a sustained Memit release between animal administrations done every 48 h to give, in turn, a continuous H_2S release. In microemulsions, the dispersed phase is surrounded by a fluid film of surfactant and cosurfactant. This structure provides a significant barrier to the drug dissolved in the internal phase against its rapid diffusion to the external phase and beyond, especially when the drug, like Memit, has high solubility in the internal phase but poor solubility in the external phase (Dubey, 2014). We can expect a slightly higher release percentage of the drug *in vivo,* where the microemulsion undergoes the natural clearance of the *peri*-tendon region. However, the prolonged release mechanism of the drug from microemulsions is important precisely to contrast the drug clearance in the *peri*-tendon region, enhancing drug retention at the site of action in a controlled way. Consequently, it helps to reduce the dose frequency, increasing patient compliance.

3.4. Physical and chemical stability of Memit-ME

The stability monitoring of Memit-ME was conducted for up to one month, with the formulation stored at 25 °C and protected from light. The microemulsion's Size, PDI (Fig. 5A), and Memit Recovery (Fig. 5B) were evaluated weekly. Microemulsions are thermodynamically stable systems, and, as we expected, no separation phenomena were observed during the storage. In addition, the dimensional analysis by DLS showed a slight increase in droplet dimensions from approximately 40 nm to 49 nm during the month, without any significant differences in PDI, which remained between approximately 0.26 and 0.28. Overall, Memit-ME preserved the homogeneity in size distribution during storage, which is crucial for maintaining pharmacokinetic properties and the active ingredient concentration, both of which are essential for accurate dosage.

3.5. In vivo studies

The Memit-ME was developed to explore a novel and valid approach for tendinopathy management. This formulation was then tested in a rat model of carrageenan-induced tendinopathy.

H_2S sulfide is a gaseous neurotransmitter that is well-documented to play a key role in various physiological processes in both the peripheral and central nervous systems (Gerasimova et al., 2015). Evidence collected in previous studies demonstrated that the administration of slow-release H_2S donors, simulating the H_2S release conditions *in vivo*,

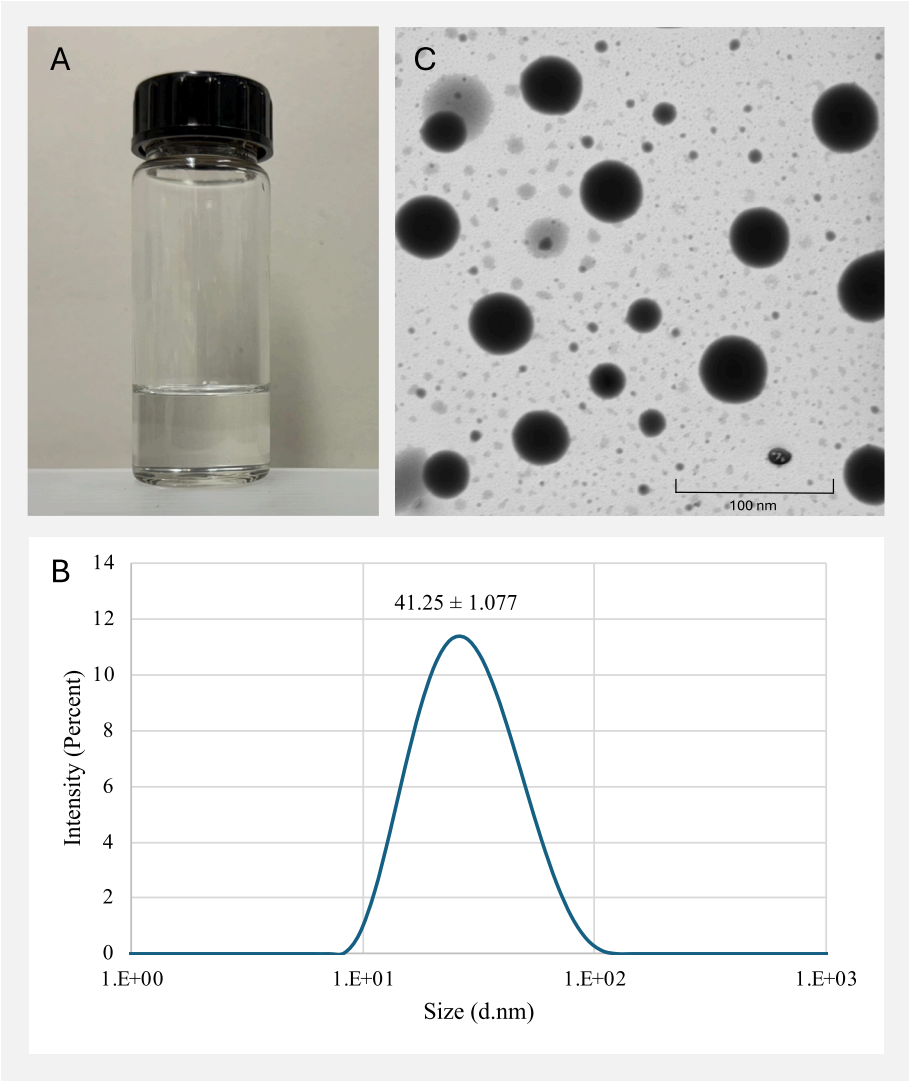

Fig. 3. Picture of Memit-ME (A). DLS analysis: size distribution; Mean ± SD; n = 3 (B). STEM micrograph of Memit-ME. Scale bar = 100 nm (C).

has antihyperalgesic properties during neuropathic pain induced by the administration of chemotherapeutic drugs like oxaliplatin and paclitaxel (Di Cesare Mannelli et al., 2017) or sciatic nerve injury (Lucarini et al., 2018), as well as by osteoarthritis pain (Batallé et al., 2020). Then, H_2S-releasing chemical moieties have been increasingly explored as potential therapeutic agents in various diseases (Wallace et al., 2018).

Memantine, a non-competitive NMDAR antagonist, has demonstrated efficacy in several models of persistent pain, providing a rationale for its potential application in tendinopathy (Pickering and Morel, 2018; Sang, 2000; Takeda et al., 2009; Zhou et al., 2011). Since elevated levels of free glutamate have been detected in patients with tendinopathy (Schizas et al., 2010; Spang et al., 2017), memantine, released from the compound Memit, may help to reduce pain by blocking excitatory neurotransmission, thereby alleviating the symptoms associated with tendon disorders.

However, to date, no data are available on chronic pain specifically related to tendon damage. Some studies have investigated the use of memantine in chronic pain and inflammatory conditions. For example, Takeda and colleagues demonstrated that memantine, administered at 10 mg/kg, effectively reduced chronic pain in a neuropathic pain model involving loose ligation of the sciatic nerve (Takeda et al., 2009b). In another study, Morel et al. used a 20 mg/kg dose in a spinal nerve ligation model and observed significant anti-hyperalgesic effects (Morel et al., 2013). Furthermore, in a rat model of inflammatory pain, memantine was shown to reduce paw edema, leukocyte infiltration, myeloperoxidase activity, and malondialdehyde levels (Azarbaijani et al., 2021). Although these studies report the beneficial effects of memantine in different models of chronic pain, supporting its potential as a new pharmacological treatment, the evidence so far pertains only to systemic administration.

To the best of our knowledge, this is the first study to investigate the *peri*-tendon application of a slow-release H_2S donor combined with memantine as a therapeutic strategy for chronic pain associated with tendon damage. Memit was formulated in a microemulsion to allow for retention at the therapeutic site and controlled release. For the *in vivo* evaluations, we used a model of tendinopathy induced by a single intratendon injection of 20 μL of carrageenan 0.8 % on day 1. As previously reported by our research group, after carrageenan injection, the animals develop mechanical and thermal allodynia, as well as spontaneous pain, over several days (Micheli et al., 2022). The pain threshold alterations are due to a local release of chemical mediators that sensitize the primary afferents (Neil et al., 1987) as well as to the accumulation of neutrophils in the perivascular space (Diehl et al., 1988). Moreover, tendon fibers undergo both morphological and functional alterations following carrageenan injection (Korkmaz et al., 2008; Szabo et al., 2004). The pain-relieving profile of Memit-ME was evaluated following five *peri*-tendon treatments performed on days 1, 3, 5, 7, and 10.

This protocol, along with the estimated Memit-controlled release

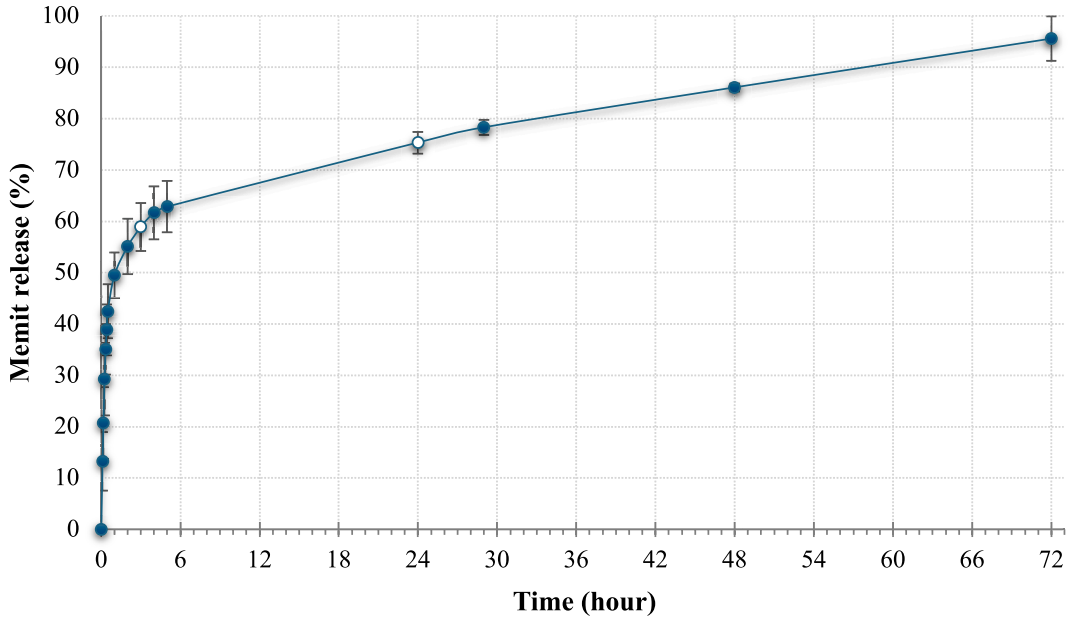

Fig. 4. Memit-ME *in vitro* release. Data are shown as Mean ± SD; n = 3.

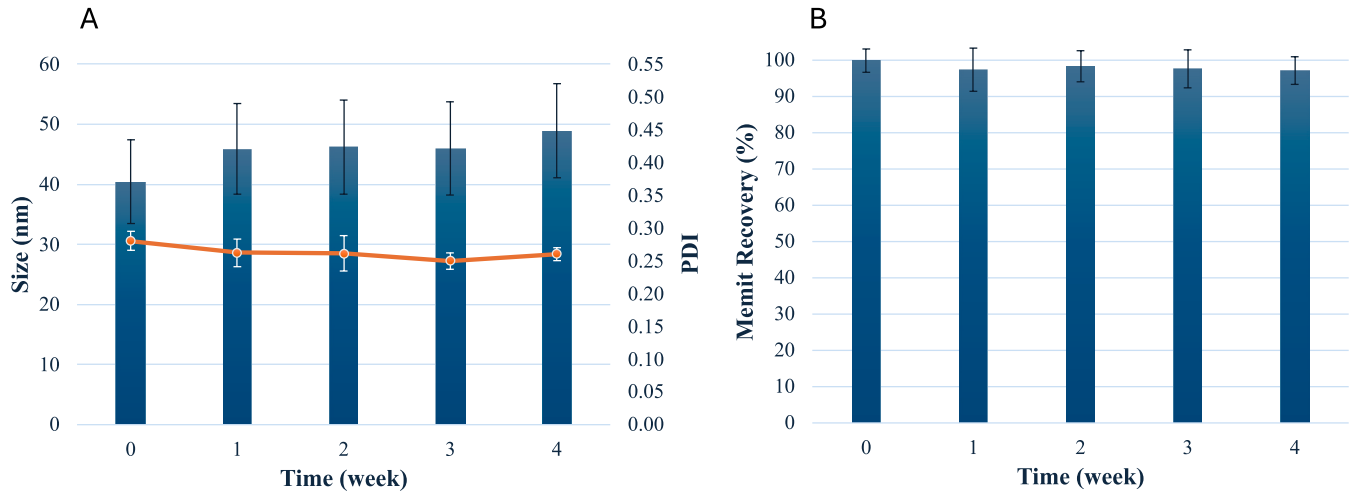

Fig. 5. Memit-ME physical stability in terms of Size and PDI (A). Memit-ME chemical stability in terms of Memit Recovery (B). Data are shown as Mean ± SD; n = 3.

from the microemulsion, ensures a continuous release of H_2S throughout the entire experiment. The behavioral measurements were performed over a time course to highlight the effect of Memit-ME in animals challenged with a noxious stimulus (Paw pressure test). Moreover, the effect on spontaneous pain (Incapacitance test) and motor skills and coordination (Beam balance and Rota rod tests) was also analyzed. The measurements were always performed before the new daily treatment. We used the *peri*-tendon injection as a route of administration since it is frequently used to manage chronic tendinopathies; indeed, it allows us to avoid tendon damage that could arise following intra-tendon administration (Fredberg and Ostgaard, 2009). As shown in Fig. 6, Memit-ME significantly increased the weight burdened by the rat on the ipsilateral posterior paw in comparison to the carrageenan + vehicle-treated group. The effect arose already on day 3 (after 1 per-tendon injection) and remained stable during the entire experimental session (Fig. 6). The microemulsion alone (Memit vehicle) was inactive.

Carrageenan injection also determines the development of spontaneous pain, as measured by hind limb weight-bearing alterations using the Incapacitance test. The difference between the weight placed by the rat on the contralateral and ipsilateral paws (expressed as Δ weight) was significantly increased in animals treated with carrageenan in comparison to the group of rats that received vehicle (saline solution). This difference slightly continued to increase from the day of the damage, reaching values of about 40 g on days 10 and 13 (Fig. 7). Memit-ME *peri*-tendon administrations reduced the postural imbalance of the animals, halving the Δ weight to 15—20 g. It is noteworthy that the effect of Memit-ME is significant even after a single administration, while its vehicle (Memit vehicle) was inactive, as already highlighted in the Paw pressure test.

Pain is not the only symptom of tendinopathies; muscle wasting and weakness are not uncommon clinical observations, and these may introduce variability in measurements of movement and motor control (Gibson et al., 2006). Achilles tendinopathy affects the patient's daily activity and sporting performance (Sussmilch-Leitch et al., 2012). Motor deficits, including reduced plantarflexion torque, have been reported in people with, and preceding the onset of Achilles tendon pain (Mahieu et al., 2006; O'Neill et al., 2014). However, the mechanisms underpinning these deficits are not clear.

Motor skills and coordination are also affected in the animal model of carrageenan-induced tendinopathy, as already reported in our previous

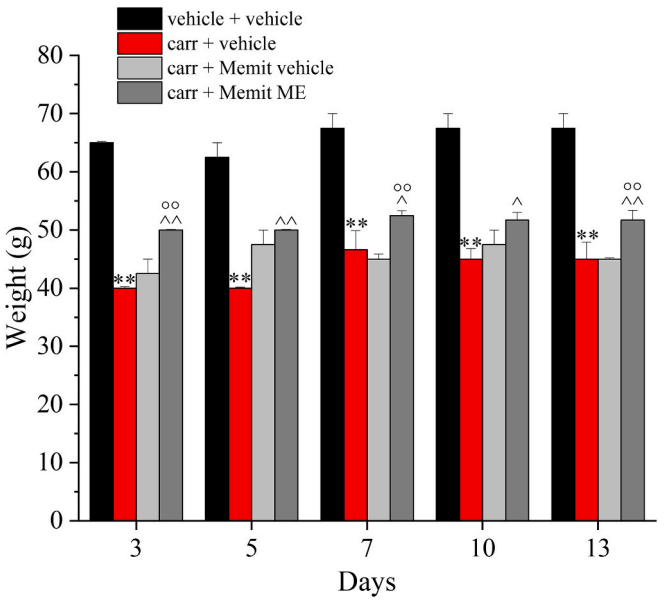

Fig. 6. Effect of repeated *peri*-tendon injections of Memit-ME on carrageenan-induced hyperalgesia in the rat. Tendon damage was induced by a single intra-tendon injection of 20 μL of carrageenan 0.8 % solubilized in saline solution after having flexed the paw to form a 45° angle, using a 30 G needle. Memit-ME (10 mg/mL; 50 μL) or its vehicle (Memit vehicle) were *peri*-tendon injected on days 1, 3, 5, 7 and 10. Control animals were treated with vehicle (saline). The paw pressure test was performed on days 3, 5, 7, 10, and 13 after the damage induction and before the new daily treatments. Each value represents the mean ± S.E.M. of 5 rats. Statistical analysis is one-way ANOVA followed by Bonferroni's post hoc comparison. **P < 0.01 vs vehicle + vehicle treated animals;^P < 0.05 and^^P < 0.01 vs carr + vehicle-treated rats; °°P < 0.01 vs carr + Memit vehicle.

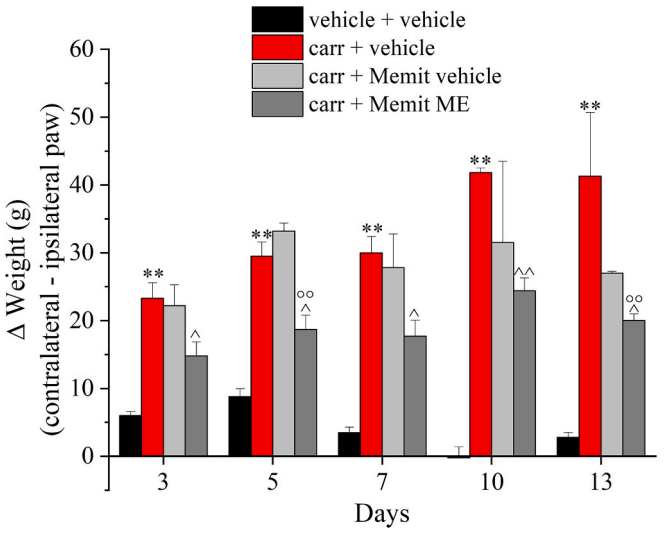

Fig. 7. Effect of repeated *peri*-tendon injections of Memit-ME on carrageenan-induced spontaneous pain in the rat. Tendon damage was induced by a single intra-tendon injection of 20 μL of carrageenan 0.8 % solubilized in saline solution after having flexed the paw to form a 45° angle, using a 30 G needle. Memit-ME (10 mg/mL; 50 μL) or its vehicle (Memit vehicle) were *peri*-tendon injected on days 1, 3, 5, 7 and 10. Control animals were treated with vehicle (saline). Incapacitance test was performed on days 3, 5, 7, 10, and 13 after the damage induction and before the new daily treatments. Each value represents the mean ± S.E.M. of 5 rats. Statistical analysis is one-way ANOVA followed by Bonferroni's post hoc comparison. **P < 0.01 vs vehicle + vehicle treated animals;^P < 0.05 and^^P < 0.01 vs carr + vehicle-treated rats; °°P < 0.01 vs carr + Memit vehicle.

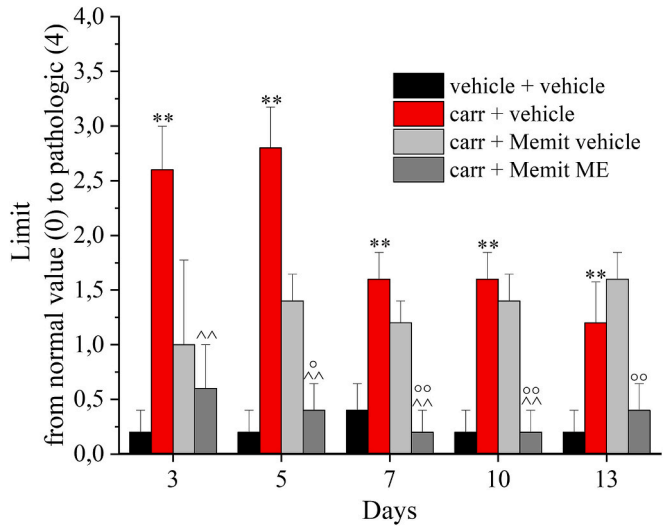

Fig. 8. Effect of repeated *peri*-tendon injections of Memit-ME on carrageenan-induced spontaneous pain in the rat. Tendon damage was induced by a single intra-tendon injection of 20 μL of carrageenan 0.8 % solubilized in saline solution after having flexed the paw to form a 45° angle, using a 30 G needle. Memit-ME (10 mg/mL; 50 μL) or its vehicle (Memit vehicle) were *peri*-tendon injected on days 1, 3, 5, 7 and 10. Control animals were treated with vehicle (saline). The beam balance test was performed on days 3, 5, 7, 10, and 13 after the damage induction and before the new daily treatments. Each value represents the mean ± S.E.M. of 5 rats. Statistical analysis is one-way ANOVA followed by Bonferroni's post hoc comparison. **P < 0.01 vs vehicle + vehicle treated animals;^^P < 0.01 vs carr + vehicle-treated rats; °P < 0.05 and °°P < 0.01 vs carr + Memit vehicle.

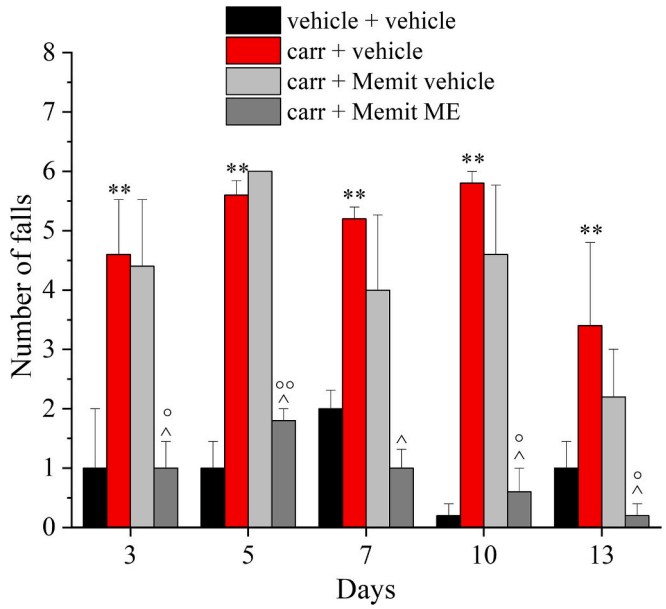

Fig. 9. Effect of repeated *peri*-tendon injections of Memit-ME on carrageenan-induced spontaneous pain in the rat. Tendon damage was induced by a single intra-tendon injection of 20 μL of carrageenan 0.8 % solubilized in saline solution after having flexed the paw to form a 45° angle, using a 30 G needle. Memit-ME (10 mg/mL; 50 μL) or its vehicle (Memit vehicle) were *peri*-tendon injected on days 1, 3, 5, 7 and 10. Control animals were treated with vehicle (saline). Rota rod test was performed on days 3, 5, 7, 10, and 13 after the damage induction and before the new daily treatments. Each value represents the mean ± S.E.M. of 5 rats. Statistical analysis is one-way ANOVA followed by Bonferroni's post hoc comparison. **P < 0.01 vs vehicle + vehicle treated animals;^P < 0.05 vs carr + vehicle-treated rats; °P < 0.05 and °°P < 0.01 vs carr + Memit vehicle.

works (Micheli et al., 2023, 2022). The pathological score is increased in carrageenan-treated animals in comparison to the control group (Fig. 8) to indicate the presence of motor alterations, which are particularly evident in the days following the damage (days 3 and 5). Memit-ME significantly improved the animals' motor skills by more than halving the alterations induced by carrageenan (Fig. 8) up to day 10. As already recorded in the other experiments, the Memit vehicle was inactive.

Moreover, Memit-ME repeated *peri*-tendon injections significantly reduced the number of falls of the rat compared to the carrageenan + vehicle-treated group when challenged in the Rota rod test (Fig. 9). Thus, it indicates a protective effect exerted by Memit formulated in microemulsion in tendinopathy-evoked motor impairments.

4. Conclusions

Tendinopathy, the clinical syndrome of pain and dysfunction in a tendon, is often a chronic condition. Tendinopathy is a complex musculoskeletal pathology, frequently sport-related and affecting a young and active population (Hägglund et al., 2011; Järvinen et al., 2005; Littlewood et al., 2013).

Tendinopathy management aims to reduce symptoms, particularly pain, promote tendon healing, and improve patient function. It consists of active modalities, such as tendon loading exercises and patient education, and passive modalities, mainly pharmacological treatments and surgical intervention. Currently, exercises, referred to as tendon loading programs, remain the most effective conservative approach in treating tendinopathy. However, therapies are usually used in combination (Cardoso et al., 2019; Millar et al., 2021). Among pharmacological therapies, topical glyceryl trinitrate is considered a safe and reliable treatment. Glycerol trinitrate patches release nitric oxide, which may promote collagen synthesis. The most common side effects are headache and contact dermatitis, and the treatment is contraindicated in some patient categories (Assem and Arora, 2015; Cardoso et al., 2019; Cumpston et al., 2009; Hunte and Lloyd-Smith, 2005; Millar et al., 2021; Saltychev et al., 2022). Other approaches include corticosteroid injection around the tendon (dexamethasone, triamcinolone, methylprednisolone, and hydrocortisone), which has been a mainstay for tendon-related disorders for many years as a short-term pain-relieving medication. However, corticosteroids had detrimental effects on tendon function, and they impaired the physiological healing response of local tissues in the following months (Cardoso et al., 2019; Millar et al., 2021; Yang and Chen, 2020). Oral or topical non-steroidal anti-inflammatories (i.e., ibuprofen) may provide a short-term analgesic effect, but they may adversely affect tendon healing at the bone-tendon junction, besides further adverse effects when taken systemically (Amin et al., 2015; Cardoso et al., 2019; Weinfeld, 2014). Several studies have investigated the use of platelet-rich plasma based on changing the molecular environment by providing a super-physiological concentration of platelets at the lesion site to simulate the initial stage of healing (Lu et al., 2023; Masiello et al., 2023; Millar et al., 2021). Another strategy is the autologous blood injection into an affected tendon, which aims to increase the concentration of growth factors to promote tendon healing (Cardoso et al., 2019; De Vos et al., 2010; Nourissat et al., 2013). However, these approaches are still controversial due to their side effects and drawbacks. Interest in stem cell therapy is also increasing due to its high effectiveness in treating musculoskeletal disorders. Stem cells have been assumed to promote tendon healing and tissue regeneration over scarring, regulate inflammation, and reorganize the extracellular matrix (Millar et al., 2021; van den Boom et al., 2020; Wang et al., 2023).

In this tricky context, new pharmacological approaches are essential to overcome the numerous side effects and contraindications of existing therapies and provide an efficacious, safe, and compliant cure for tendon injuries.

In the present study, we focused on testing the newly synthesized memantine prodrug, Memit, as a potential treatment for tendinopathy, due to its various biological activities. Memit releases H_2S through a cysteine-mediated mechanism and generates memantine. The research aimed to explore, for the first time, the activity against chronic pain related to tendon damage of Memit formulated in a microemulsion. Specifically, the formulation was administered by the *peri*-tendon route in a rat model of tendon damage induced by a single intra-tendon carrageenan injection. Microemulsions are very attractive formulations that can improve conventional treatments. They can load high concentrations of drugs that are poorly soluble in water, and, thanks to their biocompatibility and biodegradability, they have desirable properties for many administration routes, including the *peri*-tendinous one. Memit was successfully formulated in an o/w microemulsion to enhance its solubility, reaching a concentration of 1 % *w/w*. The Memit-ME dispersed globules had, approximately, sizes of 40 nm and a PDI of 0.26. Besides showing their spherical shape, the STEM micrographs confirmed the dimensions and homogeneous distribution of droplets. Memit-ME exhibited optimal physical and chemical stability during the one-month storage period at 25 °C. The slow *in vitro* release (about 40 % after 48 h) is also promising for an equally controlled drug release at the therapeutic site. The prolonged release protects the loaded drug from degradation processes until it is released and contrasts with the drug clearance in the *peri*-tendon environment. Moreover, Memit-ME was tested in a rat model of carrageenan-induced tendinopathy to evaluate the efficacy of the slow H_2S and memantine release against chronic pain related to tendon damage and, consequently, the anti-hyperalgesic and anti-allodynic profile of Memit-ME. The formulation significantly increased the weight burdened by the rat on the ipsilateral posterior paw, reduced the postural imbalance of the animals even after a single administration, improved the animals' motor skills by more than halving the alterations induced by carrageenan, and reduced the number of falls, indicating a protective effect exerted by the Memit-ME in tendinopathy-evoked motor impairments.

In conclusion, thanks to its safety profile, the controlled release, which can reduce the dose frequency, increasing patients' compliance, and effectiveness in reducing spontaneous pain and postural imbalance, the developed Memit-ME represents a promising approach for tendinopathy management.

CRediT authorship contribution statement

Giulia Vanti: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Laura Micheli:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Lorenzo Di Cesare Mannelli:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Clementina Manera:** Methodology, Investigation. **Simona Sestito:** Methodology, Investigation. **Maria Camilla Bergonzi:** Resources, Funding acquisition. **Simona Rapposelli:** Writing – review & editing, Resources, Project administration, Conceptualization, Funding acquisition. **Carla Ghelardini:** Writing – review & editing, Resources, Funding acquisition, Project administration. **Anna Rita Bilia:** Writing – review & editing, Resources, Project administration, Conceptualization, Funding acquisition.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Simona Rapposelli reports financial support was provided by International Society of Drug Development, srl (ISDD, Milan). Simona Sestito

and Simona Rapposelli has patent # WO2019129403 (A1) issued to INTERNATIONAL SOC FOR DRUG DEVELOPMENT S R L. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Studies in animals

All animal manipulations were carried out according to Directive 2010/63/EU of the European Parliament and of the European Union Council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 333-2020-PR) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Data availability

Data will be made available on request.

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