

Anti-inflammatory action of the *Ovis aries* lipidic fraction associated to therapeutic ultrasound in an experimental model of tendinitis in rats (*Rattus norvegicus*)

Ação anti-inflamatória da fração lipídica do *Ovis aries* associado ao ultrassom terapêutico em modelo experimental de tendinite em ratos (*Rattus norvegicus*)

Marcelino Martins^{1,2}, Antonio L. M. Maia Filho^{1,2}, Charlyton L. S. Costa¹, Nayana P. M. F. Coelho^{1,2}, Maricília S. Costa³, Regiane A. Carvalho⁴

Abstract

Background: Studies have demonstrated the beneficial effects of topical application of fatty acids as healing agents. The lipid fraction of *Ovis aries* have an anti-inflammatory action that accelerates the healing process. Ultrasound increases blood flow and the extensibility of collagen structures and tendons. **Objectives:** To assess the anti-inflammatory action of the *Ovis aries* lipid fraction associated to pulsed therapeutic ultrasound and friction in an induced tendinitis model. **Methods:** Fifty Wistar rats were divided into four groups: control that consisted of *Ovis aries* gel for topical use; pulsed ultrasound plus oil free sterile lotion; pulsed ultrasound plus *Ovis aries* gel; and oil free sterile lotion for topical use alone. To induce tendinitis a 10 μ L intratendinous injection of collagenase was injected into the right Achilles tendon of rats. Treatment consisted of daily applications of ultrasound using the following parameters: 10% pulsed mode, 10% pulsed frequency of 1 MHz and intensity of 0.5 W/cm² for seven or fourteen days. **Results:** After 7 days of treatment, only the *Ovis aries* plus ultrasound group showed statistically significant difference when compared to the control group. The variation in the number of inflammatory cells on animals treated for fourteen days for the control, ultrasound plus oil free, ultrasound plus *Ovis aries*, *Ovis aries* plus massage and massage plus oil free groups were statistically significant different, p<0.01. It was observed in animals treated for seven days that the ultrasound plus *Ovis aries* group was statistically significant better than the control group, p<0.05. **Conclusion:** It can be concluded that treatment using ultrasound plus *Ovis aries* is more effective than other treatments as it produces significantly better reduction on the number of inflammatory cells at 7 and 14 days.

Keywords: physical therapy; rehabilitation; movement; ultrasound; inflammation; tendinitis.

Resumo

Contextualização: Estudos demonstram o efeito benéfico da aplicação tópica de ácidos graxos como agentes cicatrizantes. A fração lipídica do *Ovis aries* apresenta uma ação anti-inflamatória que acelera o processo de cicatrização. O ultrassom aumenta o fluxo sanguíneo bem como a extensibilidade das estruturas de colágeno e tendões. **Objetivos:** Analisar a ação anti-inflamatória da fração lipídica do *Ovis aries* associado ao ultrassom terapêutico (UST) pulsado e à fricção em modelo de tendinite induzida. **Métodos:** Cinquenta ratos Wistar foram distribuídos nos seguintes grupos: controle, gel *Ovis aries* – uso tópico – UST pulsátil + loção estéril (*oil free*), UST pulsátil + gel *Ovis aries*, loção estéril (*oil free*) – uso tópico. Para induzir a tendinite, utilizou-se uma injeção intratendínea de 10 μ L de colagenase no tendão do calcâneo direito. O tratamento consistiu em aplicações diárias de ultrassom, com os seguintes parâmetros: modo pulsado 10%, frequência de 1 MHz, pulsátil a 10% com intensidade de 0,5W/cm², durante sete ou 14 dias. **Resultados:** A variação do número de células inflamatórias, para os animais tratados por 14 dias, com relação aos grupos controle, UST + *oil free* e UST + *Ovis aries*, apresentou resultados significativos p<0,001. O grupo *Ovis aries* + massagem e o grupo massagem + *oil free* apresentaram resultados significativos, p<0,01. Nos animais tratados por sete dias, observou-se que o grupo UST + *Ovis aries*, em relação ao controle, é estatisticamente significativo, p<0,05. **Conclusão:** Pode-se concluir que o tratamento com UST + *Ovis aries* é mais efetivo que os outros tratamentos, visto que consegue reduzir o número de células inflamatórias no tempo de sete e 14 dias.

Palavras-chave: fisioterapia; reabilitação; movimento; ultrassom; inflamação; tendinite.

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¹ Collegiate of Physical Therapy, Faculdade Integral Diferencial (FACID), Teresina, PI, Brazil

² Universidade Estadual do Piauí (UESPI), Teresina, PI, Brazil

³ Universidade Vale do Paraíba (UNIVAP), São José dos Campos, SP, Brazil

⁴ Universidade Nove de Julho (UNINOVE), São Paulo, SP, Brazil

Correspondence to: Marcelino Martins, Coordenação de Fisioterapia, FACID, Av. Rio Poty, 2381, Horto Florestal, CEP 64049-410, Teresina, PI, Brasil, e-mail: marcelinomartins@facid.com.br

Introduction ⋮⋮⋮

Tendon injuries are common in sports practice and it is assumed that it corresponds from 30 to 50% of total injuries¹. Such injuries are major health problem in industrialized countries because current occupations often demand a continuous series of repetitive movements². Tendons are fibrous structures with cylindroid edges or edges shaped as resistant tapes and made of dense connective tissue³. Tendon injury can occur due to various factors, such as overload, when the athlete makes an effort beyond his/her capacity, or repetitive effort of the same movement, that can cause an inflammatory process. Tendon overuse may lead to degenerative changes affecting the practice of activities because of the development of calcifications on the tendon sheath⁴.

Fatty acids are compounds containing a long hydrocarbon chain and a carboxyl terminal cluster and have three main functions on the human body. They are structural component of biological membranes; they play the role of intracellular precursors of messages and, when oxidized, they generate energy - ATP (adenosine triphosphate)^{5,6}. There are several studies demonstrating the beneficial effects of topical application of fatty acids in the treatment of wounds. They have low cost and are widely used as healing agents as part of the popular culture of different countries; and have the property to serve as a protective barrier against micro-organisms, preventing tissue dehydration, beyond the important immunomodulator character⁷.

Therapeutic ultrasound (US) is a non-invasive treatment acting on the repair of tissue injury. Pulsed US is the US modality most commonly chosen by researchers because of the beneficial effects, especially, of its low intensities⁸. The thermal effect of ultrasound generated by tissue intermolecular friction occurs by agitation of the interstitial fluids' electrolyte environment that is composed of water and solutes. This thermal effect generated by continuous ultrasound wave is contraindicated in acute inflammatory processes, recent traumas, ischemic areas or on sensitivity disorders. The ultrasound response on the pulse mode have a decreased thermal effects and can be used in acute and subacute inflammation, neuropathic pain and swelling⁹.

The ultrasound favors the transcutaneous penetration of various substances in animals such as corticosteroids, dexamethasone, hydrocortisone and indomethacin anti-inflammatory drug; and in healthy humans, such as methyl nicotinate vasodilator^{10,11}. The phonophoresis is a favorable alternative since it is a non-invasive technique. The thermal and mechanical effects of the US lead to physical and chemical changes of biological tissues, favoring the penetration of active principles present in topical substances. The heating of the area being treated may increase the absorption of the drug with the

increase in blood flow, dilation of hair follicles, decrease in skin resistance and increase in the kinetic energy of the drug. The mechanical effects of US are present even if the parameters are set to produce heating¹².

Some authors have shown that US is capable of increasing the penetration of some drugs applied topically. This action of the US associated with drugs has promoted researches in various fields of Medicine¹³. Researches conducted in recent decades have demonstrated that tendon injuries are a major cause of suffering of manual workers and of workers compensation claims². The high incidence of this condition is due to the current work environment high productivity and quality demands that overcome the workers health demands. In most cases, the condition develop as consequence of a lack of control of rhythm and speed of movements associated with machinery and furniture ergonomically incorrect¹⁴.

The aim of the study was to histologically analyze the anti-inflammatory action of the *Ovis aries* lipid fraction applied using US on an experimental model of tendinitis in rats.

Methods ⋮⋮⋮

Fifty adult male rats, of the *Rattus norvegicus* specie, Wistar variety (200-250g), aged over 30 days, were included in this study. All rats were allowed to eat and drink water as desired. Half rats (25) received treatment for 7 days and the other half received treatment for 14 days. The experiment was conducted at the Laboratory of Physiology of the Faculdade Integral Diferencial (FACID), Teresina, PI, Brazil. The study was approved by the Ethics Committee of the FACID under the protocol n° 492/2008, on 11/11/2008, according to resolution n° 196/96, of 10 October 1996, of the National Health Council (CNS).

Experimental groups

The animals from the 7 and 14 treatment groups were randomly divided into five subgroups, with a total of ten animals per subgroup:

- Group 1: control;
- Group 2: *Ovis aries* gel, topical use (massage);
- Group 3: US + sterile lotion (oil free);
- Group 4: US + *Ovis aries* gel;
- Group 5: sterile lotion (oil free), topical use (massage).

Tendinitis induction

Initially, the animals were anesthetized with 0.1 ml per 100 grams of the animal's body weight of ketamine hydrochloride

at 10%, associated with the same dose of hydrochloride and xylazine at 2%, intramuscularly, with the help of two collaborators to hold the animal.

To induce tendinitis experimentally, an intratendineous injection of 10 μ L of collagenase (10mg/ml; SIGMA; C6885) was applied in the right Achilles tendon using a 30G needle. Collagenase was dissolved in a buffered sterile saline solution of phosphate¹⁵.

Preparation of the extract of the *Ovis aries* lipid fraction

The preparation procedure of the *Ovis aries* extract described below was performed at the Laboratory of Natural Products of the Department of Chemistry of the Universidade Federal do Piauí (UFPI), Teresina, PI, Brazil. The fat/protein material obtained from *Ovis aries* (sheep) was submitted to mechanical manual trituration, yielding 1 kg of triturated material and debris with dimensions not to exceed 2 cm. The fat/protein material was transferred to an extraction bottle and 2 liters of hexane P.A., Vetec brand were added to it. Every two days the material was filtered through preparative filter paper and a new solvent was added to the material. A total of three extractions were performed and each were kept in room temperature and with low luminosity. At the end of each step of extraction, the filtrate was concentrated under reduced pressure at 55° C, in a rotary evaporator. At the end of the solvent concentration process the lipid extract of *Ovis aries* used in this study was obtained¹⁶.

Preparation of the lotion at 5% of *Ovis aries* lipids

A lotion loaded with 5% of lipid extract of *Ovis aries* was prepared in order to allow a homogeneous application on the injured sites. The oil free lotion was purchased from a compounding pharmacy and lipid extract of *Ovis aries* was added to the oil to establish a concentration of 5% mass/mass. The consistency of the preparation was adjusted by adding sufficient amount of distilled water. The material was stored in an opaque bottle under refrigeration until use.

Treatment of tendinitis

The ultrasound (Ibramed brand, sonopulse model) was used with the following parameters: 10% pulsed mode, frequency of 1 MHz, 10% pulsed with an intensity of 0.5 W/cm², direct method of coupling with oscillatory movements in 1 cm² ERA for 2 minutes. This protocol is in line with recommended parameters for the use of US^{17,18}. The device was properly

calibrated by a specialized company, before and after the proposed treatment, in order to verify the maintenance of intensity during treatment¹⁹. All animals were treated daily, respecting the period of seven and 14 days. Treatment was initiated 24 hours after tendinitis induction.

Histological analysis

The animals were sacrificed after treatment respecting the healing period of seven and 14 days. Tendons were dehydrated after fixation, included in paraffin and then 150 fields were prepared to be cut in microtome, in a semi-serial manner, with sections of 5 μ m in thickness, five sections per animal, to be stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT). Histomorphometry was performed using a binocular optical microscope, with the acquisition of photos in a 40X objective, for cellular differentiation of the total number of inflammatory cells of the observation field, intratendineous, using the Image J[®] computer program in its *cell counter* function. It is worth considering that all photos presented in the results had the same increase. Cell differentiation was not performed but total cells were counted.

Statistical analysis

The collected data were assessed in regards to the coefficient of variation and the sample distribution to determine the statistical test, considering the significance level of 5% ($p < 0.05$)²⁰. A Kolmogorov-Smirnov test demonstrated that data was normally distributed. Statistical analysis of the variation of the number of inflammatory cells obtained in the treated and untreated groups was conducted, using ANOVA, with Tukey's Post Hoc Test on the statistical program *GraphPad Prism*[®], version 3.0. Standard error of measures were used to construct graph error bars.

Results

The results presented on Figure 1 for the assessment after 7 days of treatment, demonstrated that the number of inflammatory cells was statistically significant different between the group treated with US and *Ovis aries* when compared to the control group. However, there was no significant difference between the other treatment groups and the control group.

The results presented on Figure 2 for the assessment conducted after 14 days of treatment, showed that the number of inflammatory cells was significantly reduced when comparing all groups to the control group.

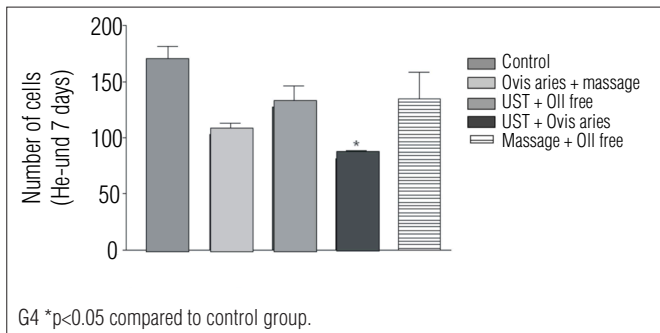


Figure 1. Graph comparing the variation (Δ) of the number of inflammatory cells after seven days of treatment.

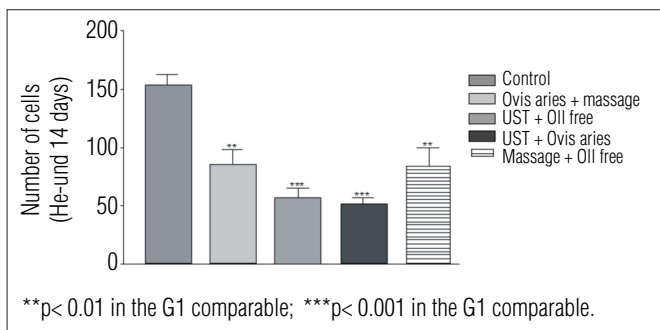


Figure 2. Graph comparing the variation (Δ) of the number of inflammatory cells after fourteen days of treatment.

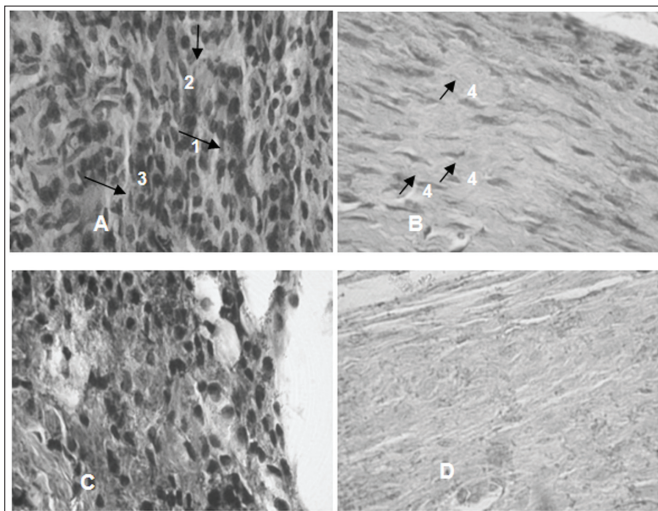


Figure 3. Microscopy of the rat Achilles tendon. Group (3) treated with pulsed US+ sterile Lotion (Oil free). Photo (A) intratendineous 7 days, stained with H&E and a 40X objective Photo (B) intratendineous 14 days, stained with H&E and a 40X objective, showing large cellularity at the expense of inflammatory cells (1), fibroblasts (2) and edema (3), demonstrating that it was a newly formed granulated tissue, showing a decrease in cellularity due to the decrease of inflammatory cells. The fibroblasts now are predominant and are more ordered (4). Photo (C) intratendineous 7 days, stained with Trichrome to Massom using a 40X objective. Photo (D) intratendineous 14 days, stained with Trichrome to Massom using a 40X objective. The last two photos show the maturation of granulation tissue with increased deposition of extracellular matrix, as seen on photo D compared with C.

Figure 3, Photo A, stained with H&E shows that for animals treated for seven days with US and sterile lotion there was the presence of maturing granulation tissue, swelling, and newly formed vessels, but in less quantity than in the newly formed granulation tissue, with more organized vessels and proliferation of fibroblasts with deposition of extracellular matrix (EM). At 14 days of treatment, as shown on Photo B, stained with H&E, there was organizing fibrosis, proliferation of fibroblasts and more organized vessels and in less quantity than in the granulation tissue, but in even greater numbers than in the mature fibrous tissue.

In animals treated for seven days with US and sterile lotion, Photo C stained with TM demonstrated that there was the deposit of EM in a disorganized way, with thinner fibers than in normal conjunctive tissue. In Photo D stained with TM, for the same patient population after 14 days of treatment there were areas of fibrosis with blue areas (deposited EM) in greater quantities than in the red area (proliferated fibroblasts). The fibrosis was in process of organization.

Analyzing Figure 4, of animals treated for seven days with US and *Ovis aries*, Photo A stained with H&E shows maturing granulation tissue, swelling and neovascularization, but in less quantity than in the newly formed granulation tissue. The vessels were also more organized than in the newly formed granulation tissue. There was proliferation of fibroblasts and deposition of EM. After 14 days of treatment Photo B stained with H&E showed organizing fibrosis, fibroblast proliferation, with more organized vessels and in less quantity than in the

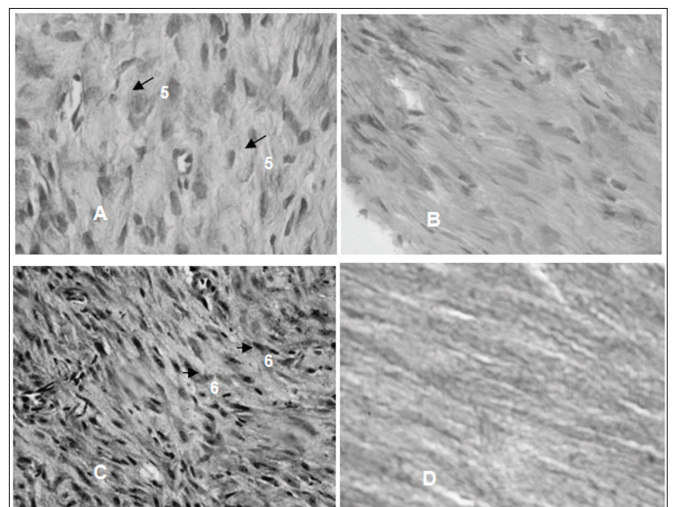


Figure 4. Microscopy of the Achilles tendon of rats, group (4) treated with US + gel *Ovis aries*. Photo (A) intratendineous 7 days, stained with H&E and a 40X objective, viewing capillaries (5). Photo (B) intratendineous 14 days, stained with H & E and a 40X objective. Photo (C) intratendineous 7 days, stained with Trichrome to Massom using a 40X objective, viewing the extracellular matrix - elastic tissue (6). Photo (D) intratendineous 14 days, stained with Trichrome to Massom using a 40X objective.

granulation tissue, but still in less quantity than in mature tissue. Photo C stained with TM of patients treated for seven days showed thinner and disorganized fibers than in normal conjunctive tissue. For rats treated for 14 days, Photo D stained with TM, showed areas of fibrosis and red areas (proliferated fibroblasts) and organizing fibrosis.

Discussion ::::

Therapy with US and *Ovis aries* showed significant better inflammatory process than the control group after treatment for seven days. At 14 days of treatment, it was observed a larger presence of fibroblasts on the treatment groups when compared to the control group, with more organized collagen tissue, suggesting the anti-inflammatory action of these therapies.

It has been reported that oleic and linoleic acids present in the *Ovis aries* lipid fraction can be used as anti-inflammatory agents during the first phase of the healing process, thus effectively accelerating tissue repair⁷.

Other studies have reported the positive influence of topical administration of α -linolenic (n-3), linoleic (n-6) and oleic acids (n-9) in the process of wound healing in rats. It has been observed that from the 5th day of topical treatment with oleic and linoleic acids, there was a significant reduction of the wound. In the first 48 hours there was inhibition of nitric oxide in the site²¹.

In this study, an important healing action of linoleic and oleic acids, found in the lipid fraction of *Ovis aries*^{22,23} was observed. This healing action could be observed through the number of inflammatory cells as presented on Figure 1. The results obtained showed that there was a decrease in the number of inflammatory cells and an acceleration of the inflammatory process, which is consistent with the literature^{22,23}.

Fatty acids have been shown to accelerate the healing process, acting as chemotactic agents for leukocytes promoting angiogenesis as a wound moisturizer^{24,25}.

The effects of US depend on many physical and biological factors, such as: intensity, application time, physiological state of the area to be treated and spatial and temporal structure of the ultrasonic field²⁶. The identification of these variables are necessary for the understanding of the US mechanisms of action on the biological tissue. Ultrasonic irradiations have an important role in the cutaneous healing process, accelerating tissue repair and promoting an accelerated healing and scarring of the tissue in regards to tissue quality²⁹. These changes include increase in the synthesis of protein, mastocytes,

granulation, calcium absorption and mobility of fibroblasts which according to several studies could accelerate the healing process²⁷⁻³⁰.

The results of the present study showed that a 7 day ultrasound therapy associated with *Ovis aries* have significantly better anti-inflammatory effect on the acute phase of the inflammatory process (Figure 1) when compared to the control group. During this period of acute inflammation a vascular response occurs initially, with production of vasoconstriction by the action of norepinephrine and contraction of the endothelium followed by vasodilatation and migration of inflammatory cells (leukocytes and neutrophils) to the injured area. At this time, macrophages remove cellular debris and extracellular changed components and fibroblasts initiate the collagen synthesis²⁸⁻³⁰.

Studies have been conducted to provide a better understanding of polyunsaturated fatty acids on the immune system and the dynamics of eicosanoids derived from arachidonic acid in the modulation of inflammatory responses and immunity, important for the organism and for the cells³¹.

The author of a previously published study³² reported the positive effects of US phonophoresis compared with the effects of topical application of hydrocortisone in the repair of rat's Achilles tendon after tenotomy. US phonophoresis was found to be the most efficient treatment, and authors concluded that the US stimulates the acceleration of tissue repair and induces transcutaneous penetration of hydrocortisone.

This study assessed the application of the *Ovis aries* gel with pulsed ultrasound in the acceleration of the inflammation process. Concomitant use of topical anti-inflammatory gel and US is becoming a common practice in rehabilitation services because it facilitates the penetration of substances transcutaneously¹³.

Conclusion ::::

The model used on the present study showed that the therapeutic effect of the *Ovis aries* lipid fraction associated with pulsed ultrasound and friction positively interfered on the healing process of the tendon. There was a statistically significant decrease in the number of inflammatory cells for the animals treated for 7 and 14 days when compared to the control group.

Further studies are needed to explain the mechanism of action of ultra sound and phonophoresis and to validate the parameters used in this study.

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