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RESEARCH ARTICLE

Peripheral Anti-nociceptive and Anti-inflammatory Effect of Oleanolic Acid in a Rat Model of Osteoarthritis

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Abstract: ***Background*:** Oleanolic acid (OA) is a naturally occurring pentacyclic triterpenoid with multifarious actions. The anti-inflammatory effect it exerts when taken orally is the most important; however, the underpinning mechanisms of such effects have not yet been fully explored.

***Methods*:** In the present study, we evaluated the anti-inflammatory and anti-nociceptive effect of OA by injecting it directly into the knee joint using an animal model of osteoarthritis. Behavioral and electrophysiological studies were conducted to determine whether OA exerts a direct modulatory effect on primary sensory afferents that can lead to a decrease in pain-related behaviors and inflammatory responses. Rats were divided into two main groups: a pre- and a post-treatment group. Knee joint inflammation was induced by injecting a mixture of 3% kaolin and carrageenan (K/C). In the pre-treatment group, two different doses of OA [5 mg/ml (n=5) and 30 mg/ml (n=4); 0.1 ml per injection] were administered into the synovial cavity of the knee joint before induction of inflammation. In the post-treatment group, rats received only one dose [5 mg/ml (n=5)] of OA after induction of inflammation.

***Results*:** Results indicate that intra-articular injection of OA improves motor coordination and attenuates nociceptive behavior and inflammatory reactions. More importantly, we observed a direct depolarizing action of OA on articular sensory fibers, a crucial mechanism that activates descending inhibitory pathways and controls incoming nociceptive signals to the spinal cord.

***Conclusion*:** Overall, our findings suggest that OA can be used as a preventive and therapeutic approach for the management of osteoarthritis.

Keywords: Inflammation, Kaolin/carrageenan, motor coordination, nociception, oleanolic acid, osteoarthritis, pain.

1. INTRODUCTION

Osteoarthritis is a common degenerative joint disease and a major cause of pain. It is characterized by a change of healthy homeostatic state into a catabolic state by doing pathological changes in the joints such as thickening of the subchondral bone and continuous loss and destruction of the articular cartilage [1]. Patients with osteoarthritis suffer from chronic pain, joints stiffness and instability, narrowing of joint space, and hypertrophy of the joint capsule [2].

Epidemiological studies indicate that osteoarthritis is a large and growing problem for society, affecting more than 25% of the population with age greater than 18 years old [1]. In the United States alone, more than 46 million individuals have self-reported doctor-diagnosed arthritis [3]. Studies also showed that osteoarthritis is very common among individuals aged 60 years or older (affecting 27 to 44%) [4, 5] and its prevalence increases with age throughout the elderly years [4].

Although osteoarthritis is classified as an idiopathic disease, there are numerous factors that can lead to its development. Aging is one such factor. In particular, it has been shown that beyond the age of 56, people are more likely to have osteoarthritis [4] mainly due to changes in muscles, tissues, and subchondral bone, including the cartilage [1]. In addition to aging, obesity is one of the causes that lead to osteoarthritis [1]. Moreover, sports injury has been associated with increased risk of osteoarthritis; in particular, individuals that have experienced some kind of knee injury are four times more likely to develop osteoarthritis, and about 41 to 51% of patients of young ages show symptoms of osteoarthritis in the years following a knee injury [6]. Osteoarthritis can also occur due to trauma following an extreme load on the joint, which causes damage in the subchondral bone and rupture of blood vessels in the joint capsule [7]. Last but not least, genetic factors have been shown to play an important role in the underlying pathophysiology of osteoarthritis [1, 8].

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Despite significant progress in research, there is still no cure for osteoarthritis. Most of the available current treatments are only useful in helping patients cope with their symptoms. Common therapeutic strategies include the use of pharmacological interventions to decrease inflammation and related pain and surgical interventions to repair damaged joints [1]. In this study, we propose the use of intraarticular oleanolic acid (OA) as an alternative therapeutic approach to prevent or attenuate the inflammatory responses associated with osteoarthritis. OA is a pentacyclic triterpenoid acid extracted from numerous plants, including licorice, hawthorn fruit, basil, and brown mustard [9-11]. It can also be extracted from Lufla cylindrical seeds [12] and is found in olive leaf extract, olive pomace, mistletoe sprouts, and clove flowers [13]. Interestingly, OA can be found in the human diet and in more than 1620 plant species, including medicinal plants [14-17].

The overarching goal of the present study is to evaluate the use of OA in the prevention and treatment of inflammation associated with osteoarthritis in rats through the following three aims: (i) to investigate whether treatment of the knee joint with OA prior to induction of inflammation can prevent the development of nociceptive behaviors, knee joint swelling, and motor dysfunction, (ii) to examine whether treatment of the knee joint with OA after induction of inflammation can stop the development of nociceptive behaviors, knee joint swelling, and motor dysfunction, and (iii) to assess the direct effect of OA on the electrophysiological activity of articular nerve afferents before and after the induction of inflammation.

2. MATERIALS AND METHODS

2.1. Subjects

In this study, adult male Sprague-Dawley rats (n=28) with a weight ranging from 150 g to 300 g were used. All experimental procedures were approved by the institutional animal care and use committee at the American University of Beirut and followed the ethical guidelines for experimental pain on conscious animals. The animals were maintained on a 12:12 hr light/dark schedule and kept in cages at a controlled room temperature of 25 °C. They all received water and food ad libitum.

2.2. Experimental Groups

Rats were randomly divided into 3 pre-treatment groups and 2 post-treatment groups and were treated with a low (0.5 mg) and/or a high (3 mg) dose of OA. These doses were determined through a pilot study using a small group of animals. In the pre-treatment groups, intra-articular injection of 0.1 ml of a solution containing a low (5 mg/ml, 0.1ml/injection, n=5) or a high (30 mg/ml, 0.1ml/injection, n=4) dose of OA was performed before induction of inflammation. Control rats (n=4) received the equivalent volume of the vehicle (0.1 ml; 100% ethanol) in which OA was dissolved. In the post-treatment group, intra-articular injection of a low (5 mg/ml, 0.1ml/injection, n=5) dose of OA was administered after 30-60 min following induction of inflammation. Control rats (n=5) for this group also received the equivalent volume of the vehicle (100% ethanol).

2.3. Induction of Knee Joint Inflammation

The kaolin and carrageenan (K/C)-based osteoarthritis rat model was prepared as described earlier (Sur *et al*., 2019). Rats were briefly anesthetized using a mixture of isoflurane and oxygen. Osteoarthritis was induced by a single injection of 0.1 ml of a mixture of 3% K/C (Lambda carrageenan Type IV and kaolin dissolved in sterile saline, Sigma Chemical Company, St. Louis, USA) into the right knee joint cavity, followed by flexing and extending the joint for 5-10 min.

2.4. Nociceptive Behavioral Testing

Prior to testing, animals were left in the experimental room for 1 hr to acclimatize to the new testing environment. During the light phase of the cycle, all rats were tested for nociceptive behaviors prior to and at 4, 8, 24, 48 hrs, and 1 week following induction of inflammation. Three nociceptive behavioral tests were conducted: the mechanical allodynia and hyperalgesia tests, and the heat hyperalgesia test.

2.4.1. Mechanical Allodynia Test

This test measures paw withdrawal frequencies to an innocuous mechanical stimulus. Prior to testing, all rats were placed in a transparent chamber on a metal wire mesh floor and left for 30-min accommodation period. A von Frey filament with a bending force of 2 g was applied perpendicularly to the medial plantar surface from below the mesh grid until a paw withdrawal was observed. A single trial consisted of 5 applications of the filament within a 2-3 s period on each c; each test was repeated five times at approximately 5-min intervals.

Baseline values were obtained prior to OA or K/C injection into the knee joint. Testing was repeated at 4, 8, 24, 48 hrs, and 1 week following induction of inflammation. The five trial responses of both hind paws were recorded for each animal, and the measurements obtained for each rat were averaged. An increased measure of paw withdrawal frequency indicated the development of mechanical allodynia.

2.4.2. Mechanical Hyperalgesia Test

This test measures paw withdrawal responses to a noxious mechanical stimulus. Paw withdrawal frequency to the application of von Frey filament with a bending force of 15g was recorded. This force has previously been shown to activate mechanoreceptors and nociceptors. Testing started by poking the plantar surface for 5 consecutive times with the 15g filament until the animal elicited a behavioral response. Five trials separated by 5 min time intervals were conducted. Baseline values were obtained prior to any treatment. Testing was repeated at 4, 8, 24, 48 hrs, and 1 week following induction of inflammation. Responses of both paws to mechanical stimulation were recorded for all groups, and averaged measurements were taken for each animal and later analyzed.

2.4.3. Heat Hyperalgesia Test

The heat hyperalgesia test was assessed by measuring the paw withdrawal latency to radiant heat applied to the plantar surface of the hind paw. Approximately 30 min prior to testing, each rat was placed individually in a clear plastic cage atop an elevated 3-mm thick glass plate for accommodation. The intensity of the light source was adjusted so that the heat applied to the plantar surface of a naïve animal evokes a withdrawal response after approximately 10-12 s. An infrared heat source (Ugo Basile) was applied 3 times with a 5-min resting period between trials to avoid conditioning limb withdrawal. The time taken to withdraw the paw from the heat stimulus was recorded as the withdrawal latency. A cut off of 20 s was imposed to avoid tissue damage.

2.5. Motor Behavioral Test

Motor behavior was assessed using the Rotarod test. This test is used to assess animal's motor coordination. It provides a rapid and simple first estimation of whether an inflammatory agent or a treatment has any effect on neuromuscular coordination. The Rotarod apparatus (Ugo Basile) consists of a horizontal rod turning at a constant or accelerating speed, subdivided into four compartments by small disks. Animals placed on the rotating rod tried to balance and remain on it to avoid falling into a platform located 30 cm below. Each rat was tested 5 times, with each trial lasting 300 s and separated by at least a 10 min inter-trial period. Rats were placed on the rod, and the apparatus was set at a fixed speed of 5 rpm. The latency to fall off the rotating rod was recorded at the end of each test.

2.6. Joint Circumference Measurement

In order to evaluate the effect of OA on the severity of joint inflammation, the circumference of the rat's knee joint was measured before and at 4, 8, 24, 48 hrs, and 1 week following induction of inflammation. Joint circumference measurements for both the ipsilateral and contralateral knee joint were done using a flexible tape measure wrapped around the center of the knee joint.

2.7. *In vivo* Electrophysiology

Extracellular recording of electrical signals from the articular nerve was conducted to assess the direct effect of OA on the activity ofarticular sensory receptors. Rats were generally anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg), and the body temperature was maintained at 37 °C. Following isolation of the tibial nerve, the epineurium was removed, and a small pool filled with mineral oil was formed around the nerve by retracting the skin flaps. The tibial nerve was cut proximally, and fibers were teased apart under a dissecting microscope and placed on a fine silver electrode for multiunit fiber recording. Spontaneous activity and responses to OA injection were recorded. OA (3mg/injection) was injected in the knee joint, near the terminal ends of the nerve. The discharges were amplified, displayed on a digital oscilloscope, and collected *via* Spike 2 data-acquisition system for analysis.

2.8. Statistical Analysis

All data sets were checked for normality using the Shapiro-Wilkes test with $p<0.05$ accepted as a non-normal distribution. Two normally distributed groups were compared using student's t-test, while non-normally distributed groups were compared using the Mann-Whitney U test. Analysis of differences among groups, and within groups at different time points was done using two-way ANOVA with repeated measures. All data were expressed as mean ± standard error of the mean (SEM).

3. RESULTS

3.1. Effect of Oleanolic Acid Pre-treatment and Post-treatment on Heat Hyperalgesia

It is well established that K/C injection into the rat's knee joint induces the development of heat hyperalgesia. Ethanol-treated rats showed a statistically significant decrease in paw withdrawal latencies at every tested time point compared to their baseline values (Fig. **1**). Moreover, rats pre-treated with the low dose of OA (5 mg/ml) followed a similar pattern to that of control but with slightly higher latencies that were not statistically significant ($p > 0.05$) (Fig. **1A**). However, analysis of the average treatment effect post-injection yielded a significant difference between the OA- and ethanol-treated rats ($p < 0.05$) (Fig. **1A**). Conversely, rats pre-treated with the high dose of OA (30 mg/ml) showed a significant increase in paw withdrawal latency at every time point post-injection ($p < 0.05$) (Fig. **1B**), indicating a decrease in heat hyperalgesia. The running average of withdrawal latencies post-injection also yielded a significant effect between OA- and ethanol-treated rats ($p < 0.001$) (Fig. **1B**). Analysis of the post-treatment effect of OA (5 mg/ml) revealed a decrease in heat hyperalgesia that was statistically significant at 4 hrs and 24 hrs ($p < 0.05$) (Fig. **1C**); in the same way, the average effect of treatment post-injection showed a significant difference between the OA and the ethanol-treated groups ($p < 0.01$) (Fig. **1C**).

3.2. Effect of Oleanolic Acid Pre-treatment and Post-treatment on Mechanical Hyperalgesia

The effect of OA treatment at a low (5 mg/ml) and high (30 mg/ml) dose on mechanical hyperalgesia was tested prior to (pre-treatment; low and high dose) or following (post-treatment; low dose only) the induction of inflammation by measuring paw withdrawal frequency to a mechanical stimulus of 15N (Fig. **2**). Compared to control rats, rats pre-treated with the low dose of OA (5 mg/ml) showed a decrease in mechanical hyperalgesia at 8hrs and 1-week post-injection ($p < 0.05$) compared to control rats, but no changes at 4hrs, 24hrs, and 48hrs were observed($p > 0.05$) (Fig. **2A**). However, the average effect post-injection showed no significant differences between OA- and ethanol-treated rats ($p > 0.05$) (Fig. **2A**). On the other hand, rats pre-treated with the high dose of OA (30 mg/ml) showed a decrease in mechanical hyperalgesia at 4hrs post-injection by the decrease in paw withdrawal frequency ($p < 0.05$) (Fig. **2B**). No statistically significant changes in paw withdrawal frequency were noticed at

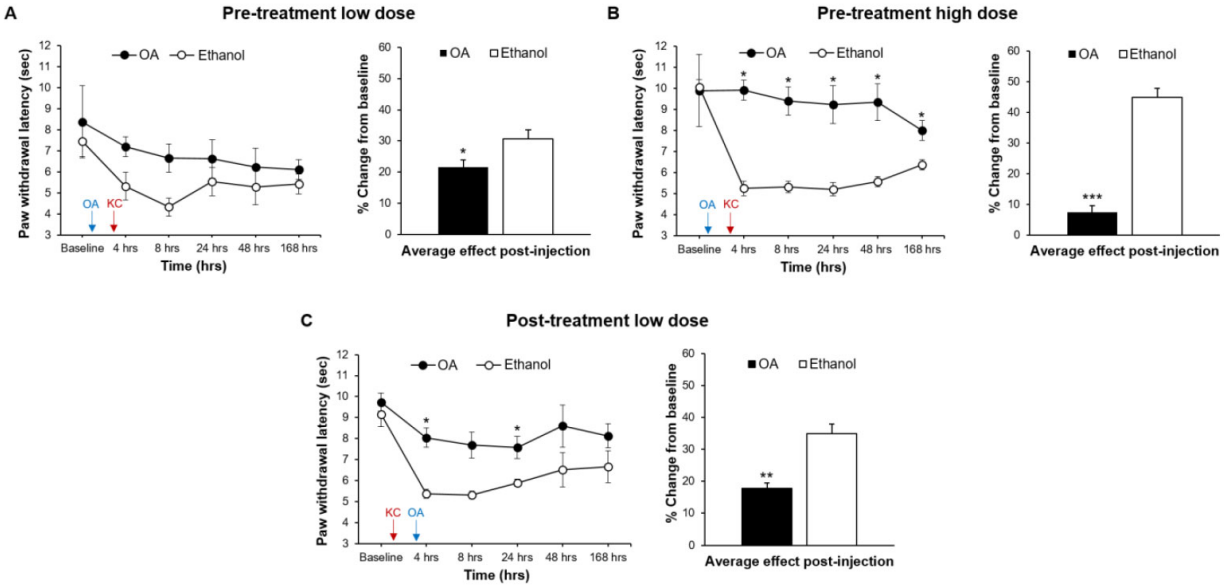

Fig. (1). Effect of OA pre-and post-treatment on heat hyperalgesia. (A) Changes in paw withdrawal latency following pre-treatment with OA at a low dose (5 mg/ml; n=5) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (B) Changes in paw withdrawal latency following pre-treatment with OA at a high dose (30 mg/ml; n=4) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (C) Changes in paw withdrawal latency following post-treatment with OA at a low dose (5 mg/ml; n=5) or post-treatment with the vehicle (100% ethanol; n=5) over a 168 hrs period. The blue and red arrows indicate injection of OA and K/C, respectively. Data are expressed as mean + SEM. Statistical significance with the control group is indicated by * for $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

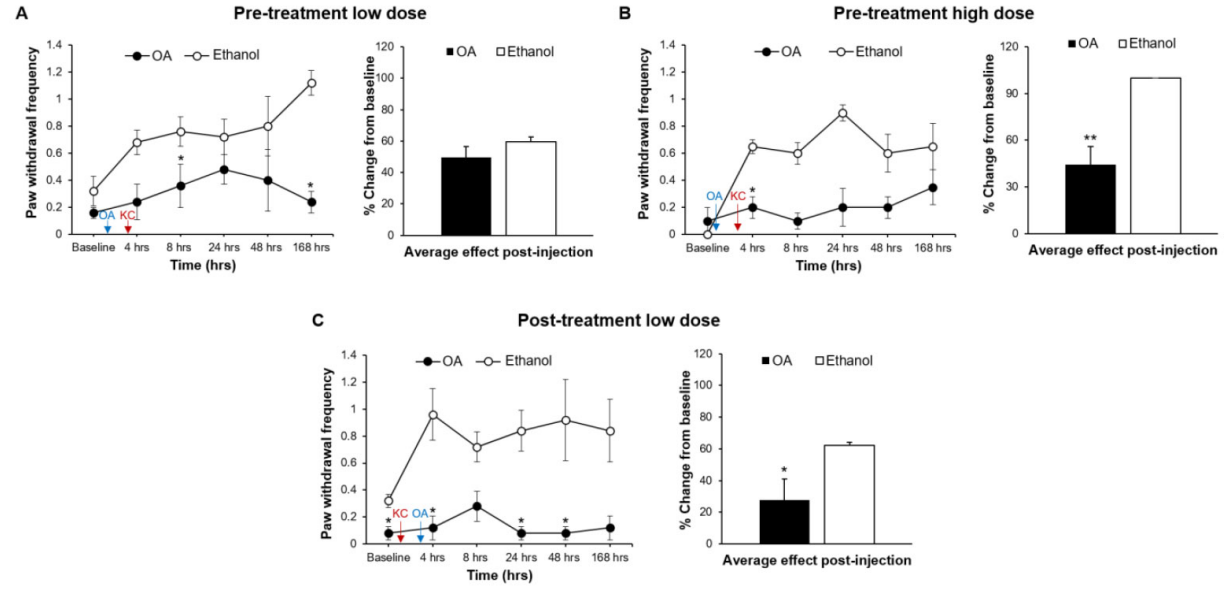

Fig. (2). Effect of OA pre-and post-treatment on mechanical hyperalgesia. (A) Changes in paw withdrawal frequency following pre-treatment with OA at a low dose (5 mg/ml; n=5) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (B) Changes in paw withdrawal frequency following pre-treatment with OA at a high dose (30 mg/ml; n=4) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (C) Changes in paw withdrawal frequency following post-treatment with OA at a low dose (5 mg/ml; n=5) or post-treatment with the vehicle (100% ethanol; n=5) over a 168 hrs period. The blue and red arrows indicate injection of OA and K/C, respectively. Data are expressed as mean + SEM. Statistical significance with the control group is indicated by * for $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

8hrs, 24hrs, 48hrs, and 1 week post-injection ($p > 0.05$) despite an observable decrease compared to the control group (Fig. **2B**), but analysis of the average effect post-injection yielded a significant effect between OA-treated rats and ethanol-treated rats ($p < 0.01$) (Fig. **2B**). Finally, rats post-treated with the low dose of OA (5 mg/ml) showed a decrease in mechanical hyperalgesia at 4hrs, 24hrs, and 48hrs post-injection ($p < 0.05$) but no changes at 8hrs and 1-week post-injection ($p > 0.05$) (Fig. **2C**). Analysis of the average effect post-injection yielded a significant effect between OA- and ethanol-treated rats ($p < 0.05$) (Fig. **2C**).

3.3. Effect of Oleanolic Acid Pre-treatment and Post-treatment on Mechanical Allodynia

The effect of OA treatment at a low (5 mg/ml) and high (30 mg/ml) dose on mechanical allodynia was tested prior to (pre-treatment; low and high dose) or following (post-treatment; low dose only) the induction of inflammation by measuring paw withdrawal frequency to a mechanical stimulus of 2N (Fig. **3**). Compared to the control, rats pre-treated with the low dose of OA (5 mg/ml) showed a statistically significant decrease in mechanical allodynia at 8hrs post-injection ($p < 0.05$) compared to control rats, but no changes at 4hrs, 24hrs, 48hrs, and 1-week post-injection were observed($p > 0.05$) (Fig. **3A**). Similarly, the running average of the responses post-injection showed no significant effect between OA- and ethanol-treated rats ($p > 0.05$) (Fig. **3A**). On the other hand, rats pre-treated with the high dose of OA (30 mg/ml) showed a significant decrease in mechanical allodynia at 4hrs and 1 week post-injection ($p < 0.05$) by the decrease in paw withdrawal frequency (Fig. **3B**). No change was noticed at 8hrs, 24hrs, and 48hrs post-injection ($p > 0.05$), and analysis of the average effect post-injection yielded no significant effect between OA- and ethanol-treated rats ($p > 0.05$) (Fig. **3B**). Finally, rats post-treated with the low dose of OA (5 mg/ml) showed a decrease in mechanical allodynia at 8hrs post-injection ($p < 0.05$) but no changes at 4hrs, 24hrs, 48hrs, and 1-week post-injection were observed($p > 0.05$) (Fig. **3B**). Analysis of the running average response post-injection yielded a significant effect between OA- and ethanol-treated rats ($p < 0.01$) (Fig. **3B**).

3.4. Effect of Oleanolic Acid Pre-treatment and Post-treatment on the Rotarod Test

The effect of OA treatment at a low (5 mg/ml) and high (30 mg/ml) dose on the rotarod test was assessed prior to (pre-treatment; low and high dose) or after (post-treatment; low dose only) induction of inflammation by measuring the duration until the rat falls from the rotating rod (Fig. **4**). Compared to control rats, rats pre-treated with the low dose of OA (5 mg/ml) showed no change in the duration to fall from the rotarod at all time points compared to control rats ($p > 0.05$) (Fig. **4B**). Similarly, analysis of the average effect post-injection revealed no difference between OA- and ethanol-treated rats ($p > 0.05$) (Fig. **4B**). On the other hand, rats pre-treated with the high dose of OA (30 mg/ml) showed an increase in the duration to fall from the rotarod at 4hrs post-injection ($p < 0.05$) (Fig. **4B**). No change was noticed at 8, 24, 48 hrs, and 1 week post-injection ($p > 0.05$), and analysis of the average effect post-injection yielded no significant difference between OA- and ethanol-treated rats ($p > 0.05$) (Fig. **4B**). Finally, rats post-treated with the low dose of OA (5 mg/ml) showed no change in the duration to fall from the rotarod at all time points compared to control rats ($p > 0.05$) (Fig. **4C**). However, analysis of the average effect post-injection demonstrated a significant effect of OA- on motor coordination as compared to the control ($p < 0.01$) (Fig. **4C**).

3.5. Effect of Oleanolic Acid Pre-treatment and Post-treatment on Joint Circumference

The effect of OA treatment at a low (5 mg/ml) and high (30 mg/ml) dose on joint circumference was assessed prior to (pre-treatment; low and high dose) or following (post-treatment; low dose only) the induction of inflammation (Fig. **5**). Compared to control rats, rats pre-treated with the low dose of OA (5 mg/ml) showed a decrease in joint circumference at 24hrs post-injection ($p < 0.05$) but no changes at 4, 8, 48 hrs, and 1-week post-injection were observed ($p > 0.05$) (Fig. **5A**). Nevertheless, analysis of the average effect post-injection yielded a significant difference between OA- and ethanol-treated rats ($p < 0.05$) (Fig. **5A**). On the other hand, rats pre-treated with the high dose of OA (30 mg/ml) showed a decrease in joint circumference at 4hrs and 24hrs post-injection ($p < 0.05$) (Fig. **5B**). No change was noticed at 8hrs, 48hrs, and 1-week post-injection ($p > 0.05$) (Fig. **5B**), but analysis of the average effect post-injection revealed that OA significantly reduced the knee joint swelling in comparison with the control group ($p < 0.05$) (Fig. **5B**). In the OA post-treatment group, rats showed no significant change in joint circumference at all time points compared to control rats ($p > 0.05$) (Fig. **5C**). However, analysis of the average effect post-injection demonstrated a marked difference between OA- and ethanol-treated rats ($p < 0.05$) (Fig. **5C**).

3.6. Effect of Oleanolic Acid on the Activity of Articular Fibers

The effect of OA on the activity of articular sensory fibers was assessed by doing *in vivo* electrophysiological recordings of a total of 12 sensory fibers before and after the induction of inflammation (Fig. **6A-C**). Sensory fibers of 2 rats were isolated using the spike 2 acquisition program and used for the current electrophysiological study. Fibers responding to mechanical stimulation of the skin around the joint or to knee joint flexing were selected. Prior to induction of inflammation, most fibers were silent except for 2 of them that showed spontaneous activity in a 5-min interval. Some fibers showed a short burst-type discharge but became quiescent afterward. OA injection elicited discharges in 3 out of 4 silent fibers, increasing activity by 38.5% over baseline (Fig. **6C**). The average latency for OA to evoke discharges in initially quiescent fibers was about 2 sec. This evoked response lasted for only 2 min. On the other hand, recordings from sensory fibers, four hours after induction of inflammation showed remarkable spontaneous activity with a

mean discharge rate of 0.06 spikes/sec. OA was administered after 4 hrs of K/C injection significantly increased the discharge rate by 344% within 10 sec of application; an effect that was considerably higher than what was observed before the induction of inflammation ($p < 0.05$) (Fig. **6C**). This effect lasted for 10 min, after which it gradually decreased to return to baseline values (Fig. **7**).

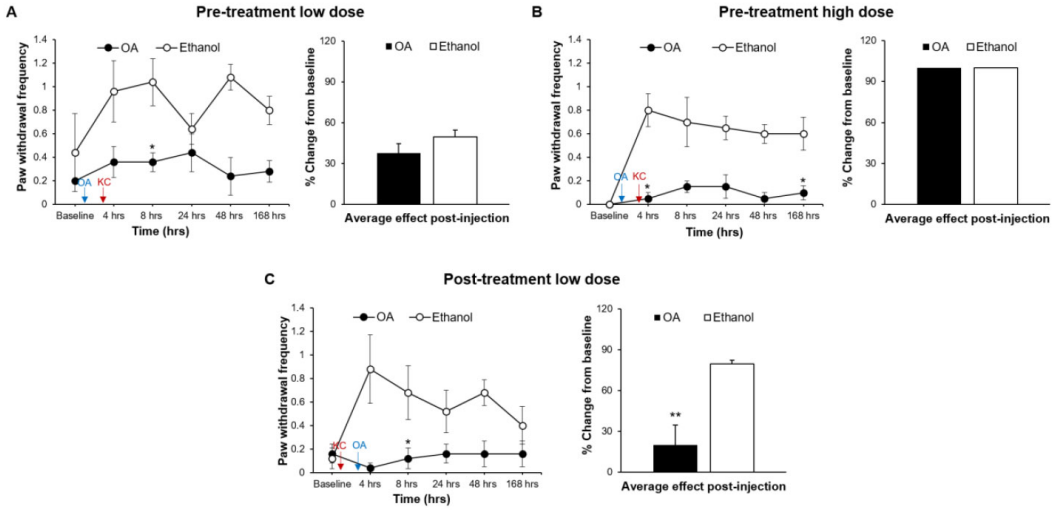

Fig. (3). Effect of OA pre-and post-treatment on mechanical allodynia. (A) Changes in paw withdrawal frequency following pre-treatment with OA at a low dose (5 mg/ml; n=5) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (B) Changes in paw withdrawal frequency following pre-treatment with OA at a high dose (30 mg/ml; n=4) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (C) Changes in paw withdrawal frequency following post-treatment with OA at a low dose (5 mg/ml; n=5) or post-treatment with the vehicle (100% ethanol; n=5) over a 168 hrs period. The blue and red arrows indicate injection of OA and K/C, respectively. Data are expressed as mean + SEM. Statistical significance with the control group is indicated by * for $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

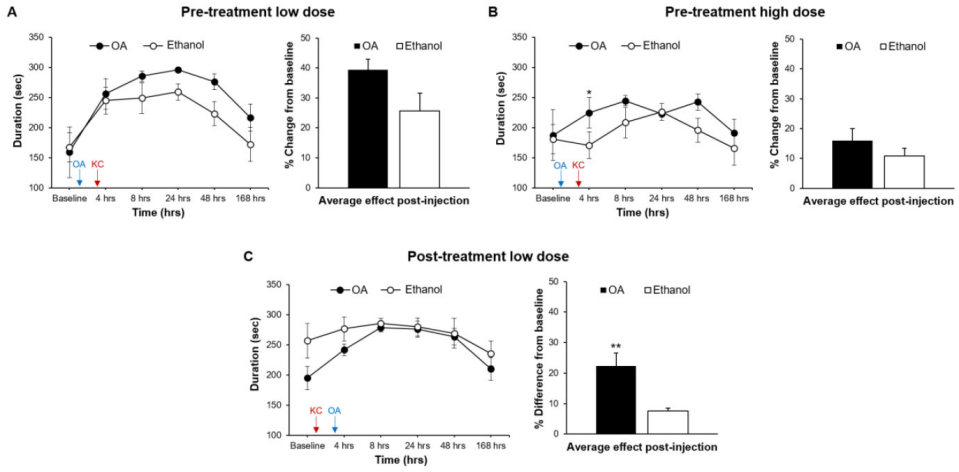

Fig. (4). Effect of OA pre-and post-treatment on motor activity using the rotarod test. (A) Changes in the duration (in sec) of the rat to fall from the rotarod wheel following pre-treatment with OA at a low dose (5 mg/ml; n=5) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (B) Changes in the duration (in sec) of the rat to fall from the rotarod wheel pre-treatment with OA at a high dose (30 mg/ml; n=4) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (C) Changes in the duration (in sec) of the rat to fall from the rotarod wheel following post-treatment with OA at a low dose (5 mg/ml; n=5) or post-treatment with the vehicle (100% ethanol; n=5) over a 168 hrs period. The blue and red arrows indicate injection of OA and K/C, respectively. Data are expressed as mean + SEM. Statistical significance with the control group is indicated by * for $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

In the present study, a K/C rat model of osteoarthritis was used to investigate the effect of OA on osteoarthritis-related symptoms of pain, inflammation, and motor coordination. OA was applied directly into the knee joint synovial cavity either prior to or post-induction of inflammation.

4.1. Effect of OA on Nociceptive Behaviors

Our results show that pre-treatment with OA at the high dose led to a significant decrease in heat hyperalgesia and mechanical hyperalgesia but no change in mechanical allodynia when assessing the average effect post-injection and normalizing with baseline values. On the other hand, pre-treatment with OA at the low dose led to a significant decrease in heat hyperalgesia but not in mechanical hyperalgesia or allodynia. These findings suggest that OA exerts a differential preventive effect on the development of inflammation-induced mechanical hyperalgesia and allodynia compared to heat hyperalgesia. On the other hand, the results of the post-treatment study show that injection of OA at a low dose resulted in decreased heat hyperalgesia, mechanical hyperalgesia, and mechanical allodynia, suggesting that OA exerts an inhibitory control on nociceptive behaviors induced by inflammation; this effect is presumably produced through modulation of the activity of nociceptors and mechanoreceptors at a peripheral or central level.

4.2. Comparison of the Anti-nociceptive Effects of OA with Previous Studies

The findings of the present study are consistent with previous reports confirming the anti-nociceptive effects of OA in different experimental models of inflammatory pain [18-20]. However, it should be noted that the route of administration of OA was different in our study. While these past studies have assessed the effect of OA through an oral or intraperitoneal route [18-20], ours have demonstrated that a direct local effect in the knee joint can cause symptomatic relief from osteoarthritis. In addition, our study employed a rat model of K/C-induced peripheral inflammation to evaluate the anti-nociceptive effect of OA, while past studies tested the anti-nociceptive properties of OA in ICR mice [18], in a zebrafish model of orofacial pain [19], or in a mouse model of colonic inflammation [20]. Also in line with our findings are studies in rodents showing that a mixture of OA and acetylsalicylic acid [21] and a derivative of OA [22] induce dose-dependent analgesic effect in a hot plate test. Although the present study did not directly evaluate the mechanisms by which OA exerts anti-nociceptive effects, we can speculate that these effects are most likely due to the recruitment of opioid and serotonin systems as pre-treatment with the opioid antagonist, naloxone, or the serotonin receptor antagonist, methysergide, was previously shown to attenuate the anti-nociceptive effects of OA in mice [18, 20, 23]. Another likely mediator of the anti-nociceptive effect of OA is the Transient Receptor Potential Vanilloid 1 (TRPV1) receptor, which is expressed by primary afferent sensory neurons of the pain pathway [19]. Evidence also suggests that OA exerts anti-nociceptive effects possibly due to mechanisms involving nitric oxide and ATP-sensitive potassium (KAT-P)-channels [23]. The adrenergic receptor is, on the other hand, not likely to mediate the anti-nociceptive effect of OA since pre-treatment with an adrenergic receptor antagonist in mice is shown to be ineffective in altering the anti-nociceptive effect of OA [18].

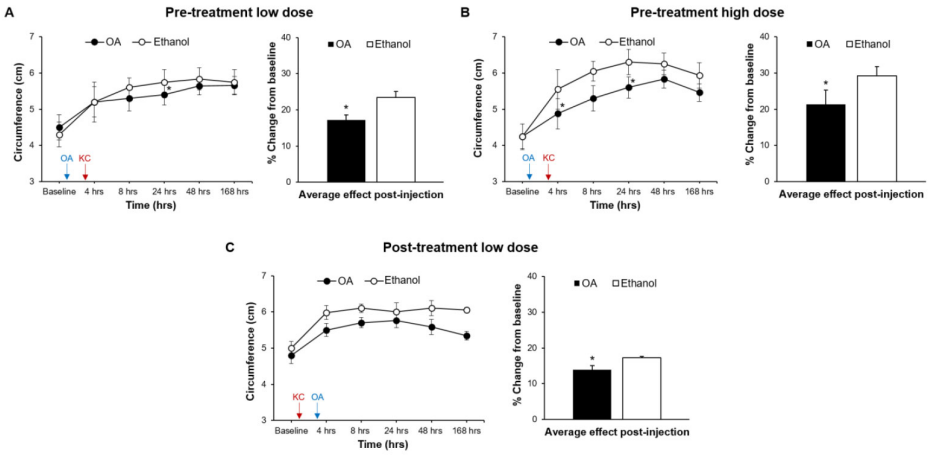

Fig. (5). Effect of OA pre-and post-treatment on joint circumference measure. (A) Changes in circumference measure (cm) of the knee joint following pre-treatment with OA at a low dose (5 mg/ml; n=5) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period (B) Changes in circumference measure (cm) of the knee joint following pre-treatment with OA at a high dose (30 mg/ml; n=4) or pre-treatment with the vehicle (100% ethanol; n=4) over a 168 hrs period. (C) Changes in circumference measure (cm) of the knee joint following post-treatment with OA at a low dose (5 mg/ml; n=5) or post-treatment with the vehicle (100% ethanol; n=5) over a 168 hrs period. The blue and red arrows indicate injection of OA and K/C, respectively. Data are expressed as mean + SEM. Statistical significance with the control group is indicated by * for $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

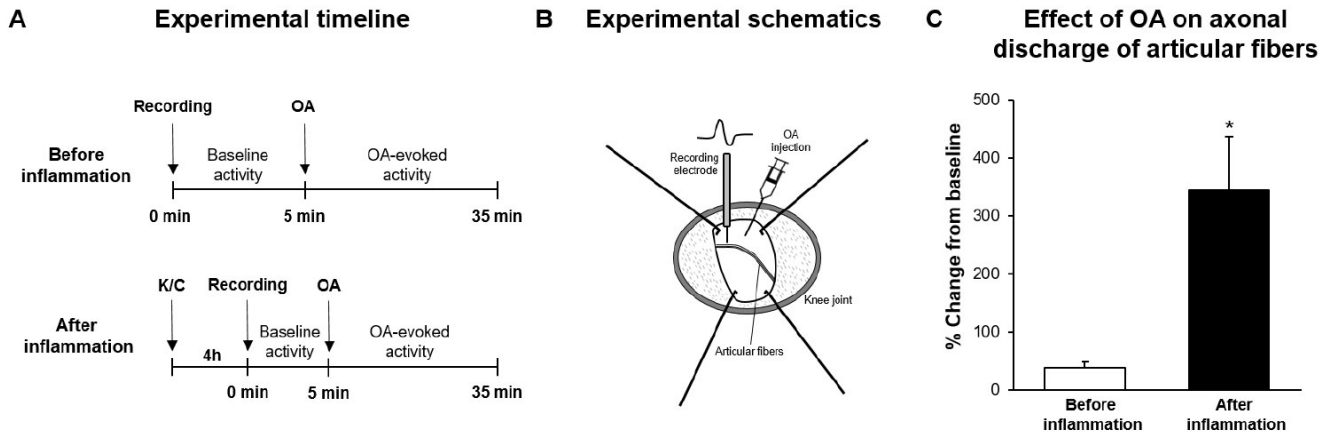

Fig. (6). Effect of OA injection at a high dose (30 mg/ml) on the axonal discharge rate of articular fibers. (A) Experimental timeline illustrating the time of recording and injections to measure OA-evoked activity before and after the induction of inflammation. For each experiment, a recording was made from 12 sensory fibers of a single rat. (B) Schematics illustrating the isolation of the articular fibers from the rat's knee joint. (C) Effect of OA on axonal discharge of articular fibers as illustrated by the percentage change from the baseline before and after the induction of inflammation. Data are expressed as mean + SEM. Statistical significance with the control group is indicated by * for $p < 0.05$.

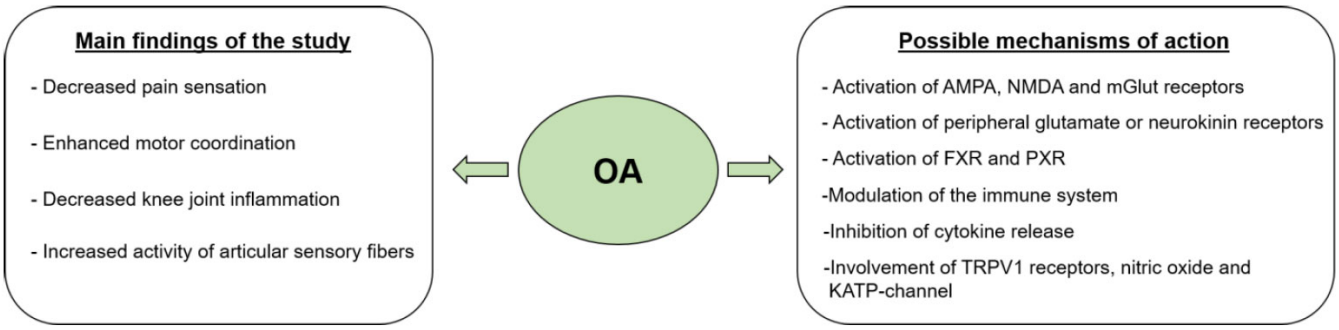

Fig. (7). Schematic illustration depicting the main findings of the present study and the possible mechanisms of action of OA. Abbreviatons: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA); Farnesoid X receptors (FXR); ATP-sensitive potassium (KATP); metabotropic glutamate (mGlut); N-methyl-D-aspartate (NMDA); Pregnane receptors (PXR); Transient Receptor Potential Vanilloid 1 (TRPV1). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4.3. Differential Anti-nociceptive Effects of OA at the Low Versus High Dose

Interestingly, when assessing heat and mechanical hyperalgesia, the results of the present study show differential anti-nociceptive effects of OA at the low versus high dose. While pre-treatment with OA at the high dose was effective in decreasing both heat and mechanical hyperalgesia, pre-treatment with the low dose was only effective in decreasing heat hyperalgesia. These results suggest that heat and mechanical hyperalgesia may be mediated by different mechanisms and may involve molecular players that show different affinity to OA. In support of this hypothesis, collective evidence indicates that coactivation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and the metabotropic glutamate (mGlut) receptors are both necessary and sufficient for mechanical hyperalgesia, while activation of N-methyl-D-aspartate (NMDA) receptors is necessary for heat hyperalgesia [24]. As such, our results indicate that OA indirectly causes activation of AMPA and mGlut receptors, which are involved in mechanical hyperalgesia, and less activation of NMDA receptors, which are involved in heat hyperalgesia. However, more work is needed to validate this hypothesis.

4.4. Effect of OA on Motor Coordination

Another interesting finding of the present study is that post-treatment with OA at the low dose resulted in improved motor coordination in rats as assessed by the rotarod test, while pre-treatment with OA at the low and high dose failed to affect motor coordination significantly. These findings suggest that OA shows more effectiveness in treating, rather than preventing, the impairment of motor coordination in-

duced by inflammation. Also, our findings suggest that the anti-nociceptive effects of OA that are observed with the pre-treatment at both the low and high dose are most likely not a consequence of motor abnormality. Lastly, the lack of effect on motor coordination observed with pre-treatment of OA at the low and high dose in the present study is in line with previous studies showing that administration of OA in experimental models of pain does not alter the locomotor activity in the open field test [19, 20, 23] nor the motor coordination in the rotarod test [23]. However, findings of Maia *et al*. [23] contradict our results, showing improved motor coordination following post-treatment with OA at the high dose. Such discrepancy in the results can likely be attributed to differences in the experimental conditions, animal models, doses, and route of administration of OA.

4.5. Effect of OA on knee Joint Inflammation

Results of the present study showing that pre-treatment with OA at both the low and high dose resulted in decreased joint inflammation are in line with previous findings showing that OA [25] or analogous compounds [26-28] exert anti-inflammatory effects. Also consistent with our findings are studies in rodents showing that a mixture of OA and acetylsalicylic acid [21] and a derivative of OA [22] yielded marked reductions in carrageenan-induced skin inflammation in rats, as demonstrated by the decrease in hind paw thickness. Finally, our results agree with studies showing that OA exerts an anti-inflammatory action in carrageenan and dextran-induced edema in rats [29], and also agree with *in vitro* studies showing that OA suppresses lipopolysaccharide (LPS)-mediated pro-inflammatory responses in human endothelial cells, including the production of TNF alpha and the activation of NFKB [30, 31]. Altogether, results of the present study showing that pre- and post-treatment with OA decrease inflammation as assessed by changes in joint circumference suggest that OA is highly effective in both preventing and managing the inflammatory response induced by K/C injection. In light of the pre-treatment results, it is presumed that a higher dose of OA will show visible efficacy in reducing the knee joint swelling once inflammation comes into effect.

4.6. Effect of OA on Sensory Articular Fibers Activity

More importantly, this preliminary study is the first to show a direct effect of OA on sensory articular fibers *in vivo*. Multiunit recordings from the articular nerve of an anesthetized rat after OA treatment suggest that OA exerts an excitatory effect on articular afferent activity. This increased articular nerve activity supports the results of a previous study showing that ursolic acid, the isomeric triterpenic acids of OA, promotes the neural regeneration of the sciatic nerve following injury [32]. We speculate that the anti-nociceptive effect of OA is due to the increased depolarization of the articular nerve leading to activation of descending inhibitory pathways and inhibition of incoming pain signals into the spinal cord. Although the mechanism of OA action on sensory neurons cannot be determined from the results of this study, one could speculate that this excitatory effect may be mediated by either a direct or indirect activation of peripheral glutamate or neurokinin receptors [33, 34]. More interestingly, the action of OA can also be mediated by activation of nuclear receptors such as farnesoid X receptors (FXR), or pregnane receptors (PXR) that are found in articular cartilage; the latter has been shown to predominantly have a protective and supporting effect on articular cartilage [35-37]. OA may also mediate its effects by reducing the expression of cytokines and by modulating helper T cell immune responses and matrix-degrading enzymes [38].

4.7. Limitation of the Present Study

A major limitation of the present study is that the anti-nociceptive and anti-inflammatory effect of post-treatment with OA was evaluated at the low dose only. However, considering the results of the pre-treatment group, we speculate that a higher dose of OA in the post-treatment group will result in more significant enhancement and attenuation of motor coordination and inflammatory manifestations, respectively. Future work assessing the effect of varying doses of post-inflammatory treatment with OA on nociceptive, inflammatory, and motor responses is nonetheless needed.

CONCLUSION

In conclusion, the results of the present study show that injection of OA into the synovial cavity prior to or post-induction of inflammation produces anti-nociceptive and anti-inflammatory effects and leads to a marked improvement in motor coordination in a K/C model of osteoarthritis. This protective effect can be the result of direct activation of articular sensory neurons that trigger central inhibition of nociceptive inputs. The present work provides clear and robust evidence of the anti-inflammatory and anti-nociceptive effect of OA in a K/C model of osteoarthritis and demonstrates that this compound can potentially be used for the prevention and treatment of this disease. This therapy may potentially be relevant for other forms of joint diseases, including rheumatoid arthritis and juvenile idiopathic arthritis, where most of the available treatments have a potential risk of side effects and poor tolerability [39, 40]. However, more work is needed prior to drawing any conclusion. Future research should also focus on investigating the safety and therapeutic efficacy of OA in patients with osteoarthritis or other joint diseases using controlled clinical trials.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures were approved by the institutional animal care and use committee at the American University of Beirut

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. All experimental procedures followed the ethical guidelines for experimental pain on conscious animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors have no conflicts of interest, financial or otherwise.

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