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ORIGINAL ARTICLE

Estrous cycle modulates the acute and persistent inflammatory muscle hyperalgesia in sedentary but not in exercised female mice

O ciclo estral modula a hiperalgesia muscular inflamatória aguda e persistente em fêmeas sedentárias, mas não exercitadas

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ABSTRACT

BACKGROUND AND OBJECTIVES: Persistent pain is a significant public health issue, profoundly impacting quality of life. Women experience persistent pain more frequently and with greater intensity than men, which is attributed to sexual hormonal fluctuations. This study investigated the influence of the estrous cycle on the development and maintenance of acute and persistent inflammatory muscle hyperalgesia in sedentary and exercised female mice.

METHODS: The study analyzed mechanical muscle hyperalgesia in female Swiss mice that underwent a model of acute and persistent inflammatory muscle hyperalgesia, during each of the four estrous cycle phases (proestrus, estrus, metestrus or diestrus). Swimming exercises were performed before the induction of acute and persistent inflammatory muscle hyperalgesia.

RESULTS: The inflammatory stimulus of carrageenan during the proestrus phase induced the most intense acute muscle hyperalgesia. Conversely, in the estrus phase, the inflammatory stimulus induced the most intense persistent muscle hyperalgesia. The maintenance of persistent muscle hyperalgesia was not modulated by estrous cycle. Regular swimming exercise prevented the acute and persistent muscle hyperalgesia, regardless of the estrous cycle.

CONCLUSION: These findings suggest that the estrous cycle phase during which an inflammatory insult occurs is critical for the development of acute inflammatory muscle hyperalgesia and its transition to the persistent phase. This effect was observed in sedentary, but not in exercised female mice, suggesting that regular physical activity may provide analgesic benefits regardless estrous cycle phase or the associated fluctuations in gender hormones. Further research is necessary to elucidate the mechanisms underlying the development of inflammatory muscle pain in females.

KEYWORDS: Estrous cycle, Female mice, Persistent hyperalgesia, Physical exercise, Muscle.

RESUMO

JUSTIFICATIVA E OBJETIVOS: Dor persistente é um problema de saúde pública que impacta significativamente a qualidade de vida. Mulheres relatam sentir dores persistentes com maior intensidade e frequência do que homens, o que é atribuído às flutuações dos hormônios sexuais. Neste estudo, investigou-se a influência do ciclo estral no desenvolvimento e manutenção da hiperalgesia muscular inflamatória aguda e persistente em fêmeas sedentárias e exercitadas.

MÉTODOS: A hiperalgesia muscular mecânica foi avaliada em camundongos fêmeas, da linhagem Swiss, submetidos ao modelo de hiperalgesia muscular inflamatória aguda e persistente, durante as quatro fases do ciclo estral (proestro, estro, metaestro ou diestro). O exercício foi realizado através da natação previamente à indução da hiperalgesia muscular.

RESULTADOS: Quando o estímulo inflamatório da carragenina ocorre na fase proestro, desencadeia-se a hiperalgesia muscular aguda de maior intensidade. Por outro lado, durante a fase estro, o estímulo inflamatório desencadeia a hiperalgesia muscular persistente de maior intensidade. A duração da hiperalgesia muscular persistente não é modulada pelos ciclos estrais. Exercícios de natação regulares preveniram a hiperalgesia muscular aguda e persistente independentemente do ciclo estral.

CONCLUSÃO: A fase do ciclo estral em que ocorreu o estímulo inflamatório é determinante para o desenvolvimento da hiperalgesia muscular aguda e sua transição para a fase persistente. Esse efeito foi observado em fêmeas sedentárias, mas não em exercitadas, sugerindo que a atividade física regular pode promover benefícios analgésicos independentemente da fase do ciclo estral ou das flutuações hormonais associadas. Pesquisas adicionais são necessárias para elucidar os mecanismos subjacentes ao desenvolvimento da hiperalgesia muscular inflamatória em fêmeas.

DESCRITORES: Camundongos fêmeas, Ciclo estral, Exercício físico, Hiperalgesia persistente, Músculo.

HIGHLIGHTS

- The estrous cycle, in which an inflammatory insult occurred, was determinant in the development of acute and persistent inflammatory muscle hyperalgesia
- The inflammatory insult to the musculoskeletal muscle during the proestrus phase triggered the highest acute muscle hyperalgesia
- The inflammatory insult to the musculoskeletal muscle during the estrus phase triggered the highest persistent muscle hyperalgesia
- Regular swimming exercise prevented the acute and persistent muscle hyperalgesia, regardless of the estrous cycle phase

INTRODUCTION

Chronic pain is a significant global health issue with a high socioeconomic impact, affecting millions worldwide[1-3]. It's more common in women, typically increases after puberty, and fluctuates throughout menopause[4-6]. For instance, the prevalence of back pain, headache, stomach pain and temporomandibular joint pain increases with pubertal development in girls[7,8] and musculoskeletal pain, headaches or migraines, and vulvovaginal pain have a high incidence in the menopause period[6]. This strongly suggests that ovarian hormones have a significant influence on pain sensitivity.

While the impact of ovarian hormones on sensitivity to various pain conditions is clear, their role is remarkably complex. The two most important ovarian hormones are estrogen and progesterone. Several studies agree that fluctuations in estrogen levels significantly impact pain perception. These hormonal shifts lead to increased pain, while stable hormone levels offer a protective mechanism against nociception in females[9-12]. Progesterone, on the other hand, seems to have a pain-reducing effect[13-15]. Despite this complexity, most preclinical studies are performed using male subjects, which can lead to inefficient strategies to control chronic pain in women.

Regular physical exercise is a well-known strategy to reduce many chronic pain conditions[16-18]. Moreover, physically inactive individuals are more likely to develop chronic pain throughout their lives[19-21]. It was recently demonstrated that regular swimming exercise prevents the acute and persistent inflammatory muscle hyperalgesia in male mice by a mechanism dependent on macrophages[22]. However, little is known about the preventive effect of exercise on transition to chronic muscle hyperalgesia in female mice.

Given the significant implications of gender-related differences in pain processing, this study investigated the influence of estrous cycle phases on the development and maintenance of acute and persistent inflammatory muscle hyperalgesia in female mice. To this end, the authors used a model of acute and persistent inflammatory muscle hyperalgesia to analyze the behavioral nociceptive responses within each of the four estrous cycle phases in both sedentary and exercised female mice.

METHODS

Ethics and study design

A total of 100 female Swiss mice (Mus musculus), 60 days old, provided by Multidisciplinary Center for Biological Research (CEMIB) from UNICAMP, were used. The experimental procedure was approved by the local institutional Ethics Committee for animal's use (CEUA-UNICAMP, protocol number 5234-1/2019), following the guidelines of the National Council for the Control of Animal Experimentation (CONCEA, Brazil) research, Brazilian Federal Law 11,794/2008 (Arouca Law) and ethics committee of the International Association for the Study of Pain in Conscious Animals. The animals were kept in plastic cages (five per cage) containing wood shavings and plastic tubes, under light/dark cycles of 12 h (light switched on at 06:00 a.m.) with water and food ad libitum, except during muscle injections, exercise sessions, and mechanical muscle hyperalgesia tests. Experimental sessions were conducted during the light phase, from 7:00 a.m., in a quiet room with a controlled temperature (±23 °C). The researcher responsible for assigning the animals to groups was aware of the group allocations. All behavioral experiments were conducted with women who were blinded to group allocations. After the behavioral testing period, all animals were euthanized, following the approved ethical protocol.

This study was planned based on previously published experiments. A detailed protocol, including the research question, key design features, and analysis plan, was prepared before the study.

Determining the stage of the estrous cycle

The estrous cycle was determined indirectly by using the non-invasive vaginal lavage method[23]. Initially, to check whether the female mice were cycling regularly, 4 days before carrageenan injection or exercise, estrous cycle was analyzed. This procedure was always performed at 7:30 h a.m. The vaginal cells were flushed by introducing 15μL of saline through a pipette and placing a few drops of cell suspension on a glass slide for microscopic examination. The visual analysis of the cells was used to determine the estrous cycle as follows: proestrus (predominance of nucleated epithelial cells), estrus (predominance of cornified cells), metestrus (predominance of leukocytes and presence of some cornified cells) and diestrus (leukocyte predominance)[23]. Female animals were selected for each experiment based on the stage of the estrous cycle. Factors that could confound the results were controlled: females were grouped by estrous cycle phase and housed in separate cages during each experiment.

The model of acute and persistent inflammatory muscle hyperalgesia

In this study, a model of persistent inflammatory muscle hyperalgesia[24] was used, standardized in mice[22,25]. Immediately after determining the estrous cycle, λ – carrageenan (Cg, 100 μg/muscle) was injected into the belly of the gastrocnemius muscle to induce the acute inflammatory muscle hyperalgesia, after 10 days, when the nociceptive threshold was at baseline levels, Prostaglandin E_2 (PGE_2; 1 μg/muscle) was injected at the same site to trigger the persistent muscle hyperalgesia. The measurement of the hyperalgesic threshold was performed at the following time-points (Figure 1): 1) Immediately before the muscle injection (baseline); 2) after 1h, 3h and 6h of the injection at day 0; 3) daily until the 10 th day; 4) immediately before the injection of PGE_2 (10 th day); 5) after 1 h and 4 h of the injection of PGE_2 (10 th day); 6) at days 11, 12 and 17[22,25]. In control groups, isotonic saline (0.9% NaCl) was administered instead of carrageenan. Swimming exercise was performed before the first injection, and the estrous cycle was determined before carrageenan or PGE_2 (Figure 1). All animals assigned to each experimental group were included in the analysis.

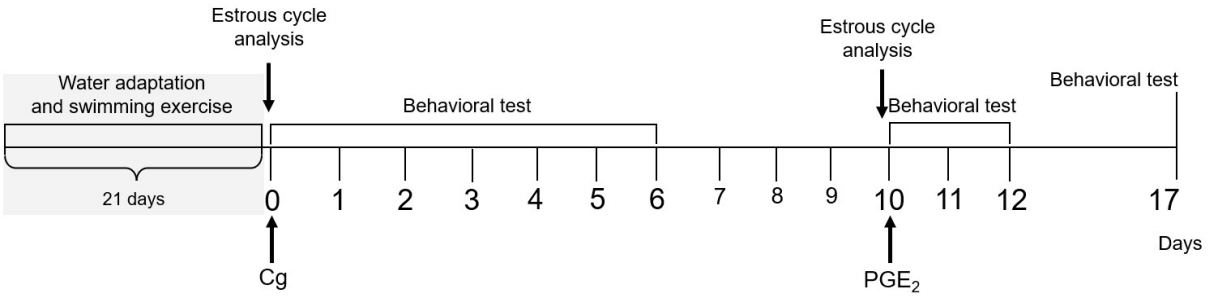

Figure 1. Experimental procedures in the model of persistent muscle hyperalgesia. Carrageenan (Cg) or saline (Sal) was administered into the gastrocnemius muscle. Behavioral test was applied from day 0 to 6 after the injection. At the 10th day, PGE_2 was administered and behavioral test was applied at days 10, 11, 12 and 17. Estrous cycle analysis was determined before Cg or PGE_2 injection. Swimming exercise procedures (gray square) were performed before Cg/Sal injections, with an interval of 48 h before the baseline behavioral test at day 0. Adapted from a reference study from 2021[22].

Mechanical muscle nociceptive threshold test

The mechanical muscle nociceptive threshold in mice was assayed through the Randall-Selitto digital algesimeter (Insight, Brazil)[22,25]. The device applies linear mechanical pressure to the gastrocnemius muscle through a rounded tip with 2 mm to evoke the nociceptive threshold of deep tissues[26]. Three measurements, at intervals of 5 minutes each, were performed to get the nociceptive threshold[22,25]. Baseline levels were measured two days after the end of the physical exercise period or equivalent to the sedentary group[22]. Mechanical muscle hyperalgesia was calculated as the difference (in grams) between the baseline measurement and the values obtained at each time point following carrageenan (or saline) and PGE_2 injections. Mechanical muscle hyperalgesia was represented in the y-axis by increasing values[22,25,27].

Intramuscular injection, drugs and doses

The drugs or their vehicles were injected into the mice's gastrocnemius muscle using a 30-gauge needle[28], connected to a polyethylene catheter and a Hamilton syringe (50µL). The final volume was 20µl. The following drugs were used: λ – carrageenan (100 µg/muscle) and prostaglandin E_2 (PGE_2, 1µg/muscle) [22,24,25]. All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA), and they were dissolved in isotonic saline (0.9% NaCl) to obtain the working solution. The stock solution of PGE_2 was dissolved in ethanol, according to the datasheet, and re-suspended in saline.

Swimming exercise protocol

Swimming was performed as regular physical exercise. The water temperature was maintained at 31 ± 1 °C and all procedures were carried out in individual cylindrical tanks with a smooth surface[22,29]. To minimize stress, mice were progressively adapted to the liquid environment, with a progressive increase in water depth, for 6 days[22,30]. Before starting the swimming exercise protocol, the animals remained at rest for 24 hours. The swimming protocol was performed for 15 days, split into three periods of 5 days each, with daily sessions of 50 minutes. To allow physiological adaptation and minimize the risk of fatigue or injury, a protocol with progressive increase in intensity was employed[23]. In the first period, mice performed swimming sessions with 4-minute passive pauses. In the second period, interval sessions of a 3-minute pause were used, and in the last period, mice swam 50 minutes without pauses. Mice were withdrawn from the water when inappropriate movements, such as floating, climbing, diving and bobbing, were observed[31]. For control groups, mice were maintained in individual tanks with shallow water for 10 minutes[22]. All animals from each experimental group were considered in the analysis.

Measurement of serum corticosterone levels

Before blood sampling, animals were habituated for one hour in a quiet room. Blood collection was carried out without the use of anesthesia or restraint. Blood samples were collected 24 hours before starting the exercise (baseline) and 24 hours after the end of the exercise protocol[22]. A small cut at the distal end of the animal's tail was made and 25 µL of blood was collected using non-heparinized glass capillaries. After 30 min, the samples were centrifuged, and the supernatant was collected and stored at -20 ∘ C until analysis. Corticosterone was measured using the Enzyme-Linked ImmunoSorbent Assay (ELISA) test, according to the manufacturer's instructions (Kit DetectX - Arbor Assays).

Statistical analysis

According to the Kolmogorov-Smirnov test, all data followed a normal Gaussian's distribution and allowed application of parametric tests. Quantitative data were analyzed by one-way ANOVA, two-way ANOVA, Mixed-effects Analysis or Student's t test (unpaired). Following one-way and two-way ANOVA, post hoc contrasts were performed using the Tukey test. Area Under the Curve (AUC) analysis was performed for behavioral experiments to evaluate general effects of the interventions in acute and chronic hyperalgesia. AUC from acute period was considered from day 0 to day 4 after carrageenan injection

(AUC Δ, g × 4 days) and AUC from chronic period was considered from day 10 to 17 (AUC Δ, g × 10–17 days). The sample size for continuous variables was calculated based on the estimated population standard deviation and the expected mean difference between groups, using the formula "$n=1+[2C^*(s/d)^2]$", where "$C=(z\alpha+z\beta)^2$". Parameters were set at α=0.05 (two-sided), power = 80%, s=0.2, and d=0.5[32]. Final values are reported in the figure legends. These values are expressed as means ± standard error of mean (SEM). All data were analyzed by GraphPad Prism 7.0 software. The outlier calculator tool from GraphPad website was applied to each group. For all tests, significance was set at p<0.05.

RESULTS

First, the authors analyzed the development of acute and persistent inflammatory muscle hyperalgesia in female mice independent of their estrous cycle. Administration of carrageenan into gastrocnemius muscle induced acute muscle hyperalgesia (n=20, p<0.05, Two Way ANOVA, Tukey post-test Figure 2A, AUC, Student *t* test, Figure 2B) when compared to saline control group (n=20). Administration of PGE_2 into gastrocnemius muscle previously sensitized by carrageenan showed the persistent muscle hyperalgesia (n=20, p<0.05, Two Way ANOVA, Tukey post-test Figure 2A; AUC, Student T test, Figure 2C) when compared to saline control group (n=20).

To investigate the influence of the estrous cycle on the development of acute and persistent inflammatory muscle hyperalgesia, carrageenan was administered at each of the four-cycle phases (proestrus, estrus, metestrus or diestrus). The estrous cycle phase in which PGE_2 was injected was not considered. When carrageenan was injected during the proestrus (n=5), estrus (n=5), metestrus (n=5) or diestrus (n=5) phases, there were development of acute and persistent muscle hyperalgesia when compared to the respective saline control groups (p<0.05, Mixed-effects Analysis test, Tukey post-test, Figure 3A; AUC, One Way ANOVA, Tukey post-test, Figure 3B).

A comparison across the different estrous cycle phases revealed that the carrageenan injection during the proestrus phase elicited a greater acute muscle hyperalgesia than that observed in the other phases (p<0.05, Mixed-effects Analysis test, Tukey post-test, Figure 3A; AUC, One Way ANOVA, Tukey post-test, Figure 3C). In addition, the carrageenan injection during the estrus phase elicited a greater persistent muscle hyperalgesia than that observed in the

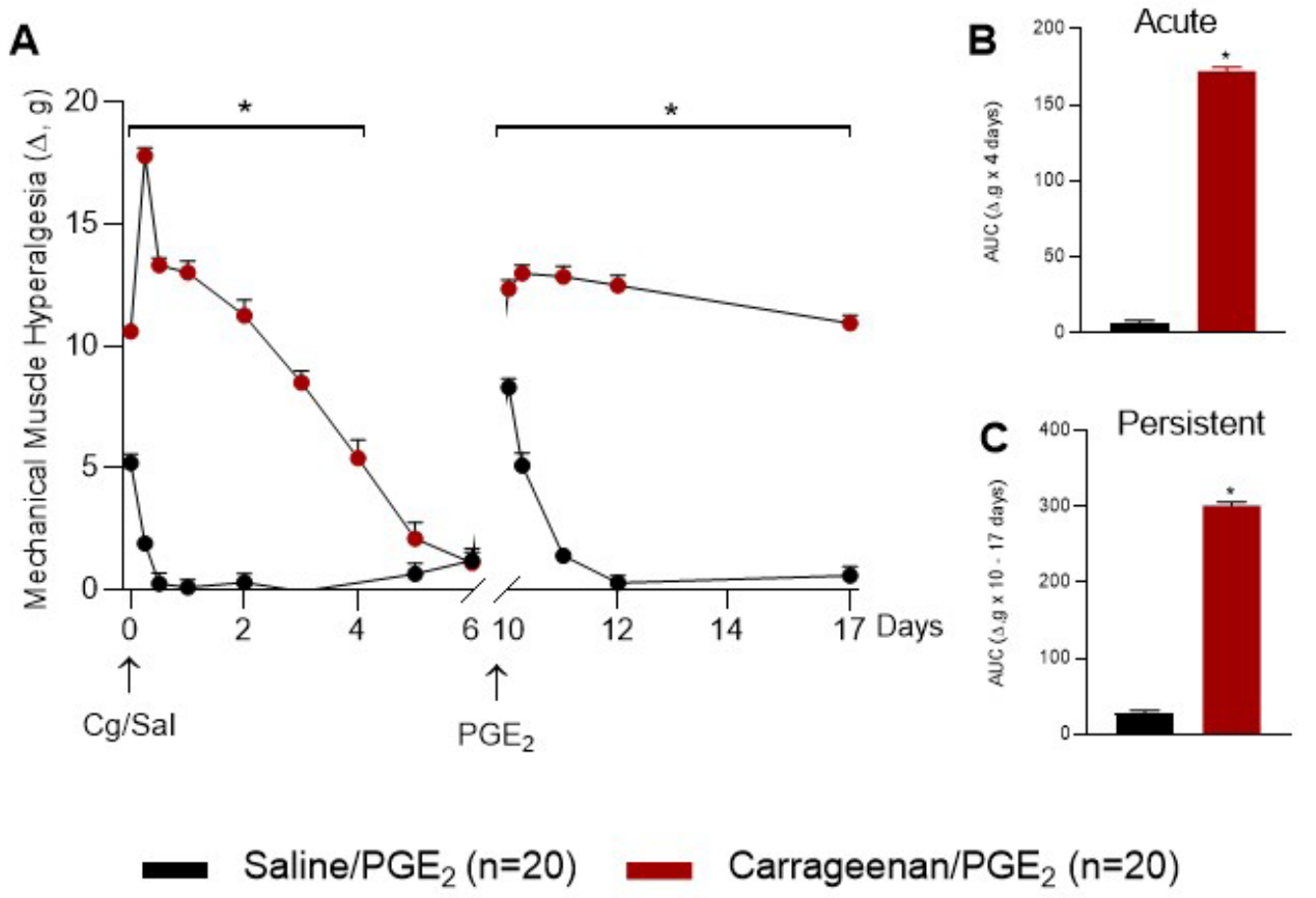

Figure 2. Sexual dimorphism in acute and persistent inflammatory muscle hyperalgesia. (A) Behavioral nociceptive responses at different time points. AUC of the acute (B) and persistent (C) muscle hyperalgesia. The symbol "*" indicates differences for the saline control group.

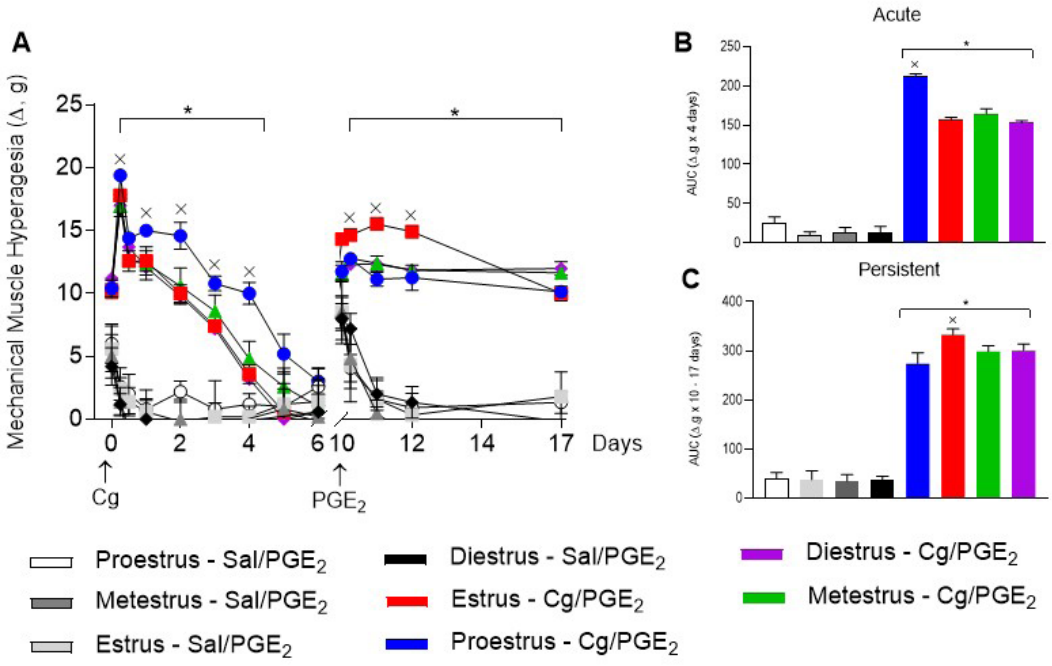

Figure 3. Estrous cycle modulates both acute and persistent muscle hyperalgesia. (A) Behavioral nociceptive responses in proestrus, estrus, metestrus and diestrus phases. AUC of the acute (B) and persistent (C) muscle hyperalgesia. The symbol "*" indicates differences for their respective saline control groups, "x" indicates differences for other groups in the acute or persistent phase.

other phases (p<0.05, Mixed-effects Analysis, Tukey post-test, Figure 3A; AUC, One Way ANOVA, Tukey post-test, Figure 3C).

The estrous cycle phase at the time of PGE_2 injection did not modulate the maintenance of persistent muscle hyperalgesia

At this moment an analysis was performed to determine whether the estrous cycle phase, at the time of PGE_2 injection in mice previously sensitized by carrageenan, would modulate the maintenance of the persistent muscle hyperalgesia. It was observed that the maintenance of this phase was not different among the estrous cycle phases (n=5, p>0.05, Mixed-effects Analysis, Figure 4A; AUC, One Way ANOVA, Figure 4B).

The estrous cycle did not modulate the regular physical exercise-prevented acute and persistent muscle hyperalgesia

It was previously shown that regular physical exercise by swimming prevents acute and persistent inflammatory muscle hyperalgesia in male mice[22]. After that, the authors analyzed whether the estrous cycle would modulate this prevention in female mice. First, they observed that swimming was effective in preventing acute and persistent muscle hyperalgesia in females (n=20, p<0.05, Mixed-effects Analysis, Tukey post-test, Figure 5A; AUC, One Way ANOVA, Tukey post-test, Figure 5A). When the different estrous cycle phases were compared, there were no differences in behavioral responses (n=5, p>0.05, Mixed-effects Analysis, Figure 5B; AUC, One Way ANOVA, Figure 5B).

To analyze whether stress modulated the exercise-induced prevention of muscle hyperalgesia, the plasma levels of corticosterone before the start and 24 h after the end of the swimming protocol were quantified. There was no increase in plasma levels of corticosterone in exercised female mice (n=16, p>0.05, Two Way ANOVA, Figure 5C).

DISCUSSION

This study demonstrated that the estrous cycle, in which an inflammatory insult occurred, is determinant in the development of acute inflammatory muscle hyperalgesia and the consequent transition to the persistent stage, but not in its maintenance. In addition, in animals previously exercised, the estrous cycle, in which an inflammatory insult occurred, did not modulate the exercise-prevention of muscle hyperalgesia.

The study showed that intramuscular injection of carrageenan into the gastrocnemius muscle of female mice induced acute and persistent inflammatory muscle hyperalgesia throughout all phases of the estrous cycle. Interestingly, this inflammatory insult differentially affected the intensity of acute and persistent muscle hyperalgesia across various phases of the estrous cycle. Specifically, in the proestrus phase, carrageenan induced the most intense acute inflammatory muscle hyperalgesia, while in the estrus phase, the most intense persistent muscle hyperalgesia. These data highlight the impact of the estrous cycle on the outcome of acute and persistent muscle hyperalgesia triggered by an inflammatory insult.

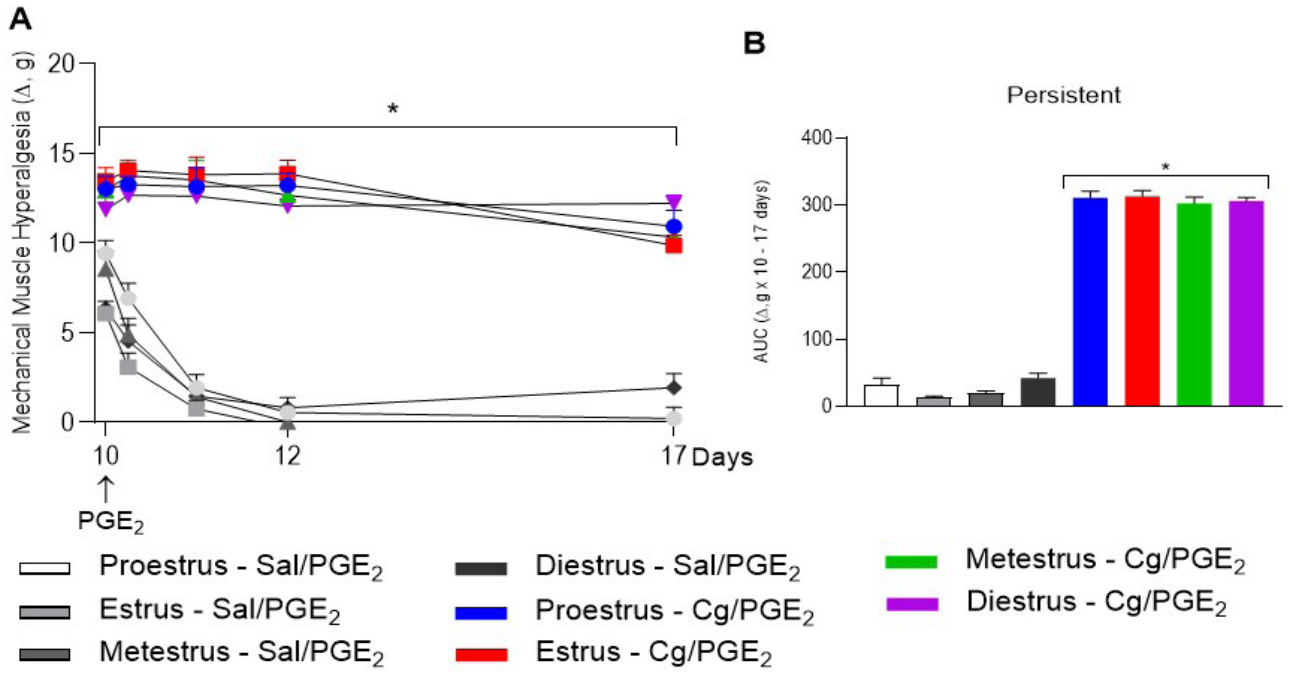

Figure 4 - Estrous cycle did not modulate persistent muscle hyperalgesia. (A) Behavioral nociceptive responses in proestrus, estrus, metestrus and diestrus phases. B) AUC of the persistent muscle hyperalgesia. The symbol "*" indicates differences for their respective saline control groups.

Usually, women or female rodents show more pain than men across several noxious modalities, including mechanical-, electrical-, thermal- and chemical-induced pain[33]. In addition, women are more likely than men to report different pain conditions[34,35]. This evidence is, in part, attributed to sexual hormonal fluctuations[36-40].

Estrogen[41] and progesterone are steroid hormones secreted from the ovaries that play important roles in numerous tissues[42], including in the metabolism of the musculoskeletal system[43-45]. Although there is a robust number of preclinical and clinical studies on the role of sex hormones in pain, the results are still unclear. There seem to be differences in the influence of female hormones based on the stimulus that triggered the pain condition[43,46-50]. Nevertheless, a consensus in the literature suggests a significant influence of estrogen fluctuations on pain sensitivity. Specifically, variations in hormone levels are associated with an increase in pain sensitivity, whereas stable hormonal profiles appear to confer a protective effect against nociception in females[36-40]. Progesterone has also been shown to be involved in pain processing, showing a protective role against pain[39]. Interestingly, a clinical pain model showed that increased pain associated with high progesterone levels was reduced by the increase in estradiol levels, suggesting an interaction between both hormones in pain perception[51].

It is well known that in female mice, estradiol levels begin to increase at metestrus, reaching peak levels during proestrus and returning to baseline at estrus. Progesterone secretion also increases during metestrus and diestrus, with a decrease afterwards. Then the progesterone value rises to reach its second peak towards the end of proestrus[52,53]. Considering these oscillations, it is possible to hypothesize that the higher progesterone levels and the lower estradiol levels at the end of proestrus phase may be related to the highest acute muscle hyperalgesia induced by the inflammatory insult in the proestrus phase. In another way, the lower levels of estrogens and progesterone may have contributed to the highest persistent muscle hyperalgesia induced by the inflammatory insult in the estrus phase. Finally, the protective role of the increasing levels of estrogens and progesterone may have contributed to the lower intensity of persistent muscle hyperalgesia induced by the inflammatory insult in the proestrus and metestrus phases, respectively. The mechanisms underlying the protective role of estrogen in inflammatory muscle hyperalgesia are still under investigation. However, evidence shows that estrogens downregulate proinflammatory cytokines[54-57], interact with adrenergic and serotoninergic systems[58], and inhibit the signaling of NFkappaB[59].

It was previously shown that regular swimming prevents the onset of acute inflammatory muscle hyperalgesia and its transition to the persistent stage in male mice[22]. Here, the authors demonstrated that females exhibit similar exercise-induced behavioral responses. Interestingly, the phase of the estrous cycle during the inflammatory insult did not affect the exercise-induced prevention of acute or persistent muscle hyperalgesia. Regular physical exercise of low or moderate intensity modulates the peripheral and central neuroimmune systems related to signaling pain pathways[60] Therefore, they are efficient non-drug strategies for the treatment and prevention of chronic painful conditions[61,62]. Although the study has not evaluated the mechanisms underlying the exercise-prevention of acute and persistent muscle hyperalgesia in female mice, it showed that the exercise protocol is also effective in females, independent of the estrous cycle phase.

A limitation of this study is that sex hormones were not directly measured; instead, estrous cycle phases were determined using

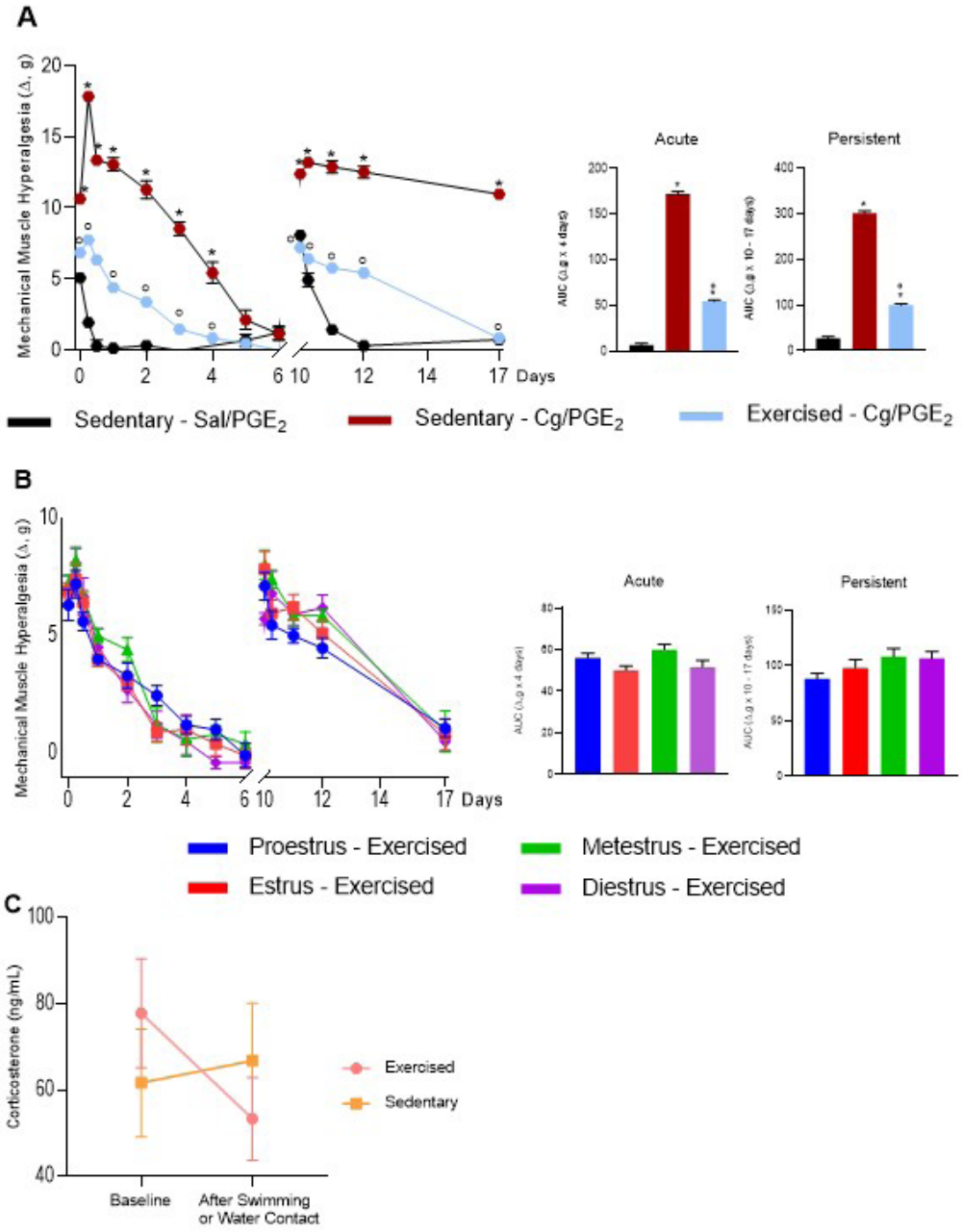

Figure 5 - Physical exercise-induced prevention of acute and persistent muscle hyperalgesia is independent of the estrous cycle. Behavioral nociceptive responses independent of the estrous cycle. (A) and in different phases of estrous cycle (B). Analysis of corticosterone levels (C). The symbol "*" indicates differences for their respective saline control groups and "°" indicates differences for the sedentary Cg/PGE_2 group.

the non-invasive vaginal lavage method. Although this technique does not provide exact hormone levels, it is widely used and reliable for identifying cycle stages. Therefore, the interpretations regarding hormonal influences were based on indirect evidence.

CONCLUSION

The estrous cycle phase during which an inflammatory insult occurs is critical for the development of acute inflammatory muscle hyperalgesia and its transition to the persistent phase. Therefore, they should be taken into account in the clinical management of such conditions. Although changes in sex hormones throughout the cycle are known to influence pain sensitivity, the present findings suggest that well-designed exercise interventions can effectively prevent the progression from acute to chronic muscle pain. These findings underscore the potential of integrating individualized exercise programs into pain management strategies for women, suggesting that regular physical activity

may provide analgesic benefits regardless menstrual cycle phase or the associated fluctuations in sex hormones.

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