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Estradiol replacement therapy regulates innate immune response in ovariectomized arthritic mice

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ABSTRACT

Neuroendocrine changes are essential factors contributing to the progression and development of rheumatoid arthritis. However, the role of estrogen in the innate immunity during arthritis development is still controversial. Here, we evaluated the effect of estrous cycle, ovariectomy, estradiol replacement therapy and treatment with estrogen receptor (ER)α and ERβ specific agonists on joint edema formation, neutrophil recruitment, and articular levels of cytokines/chemokines in murine zymosan-induced arthritis. Our results showed that articular inflammation of proestus/estrus was similar to metaestus/diestrus animals indicating that the inflammatory response in acute arthritis is not affected by the estrous cycle. However, ovariectomy increased joint swelling, neutrophil migration, and TNF-α level. Treatment for six consecutive days with estradiol cypionate re-established the acute inflammation in ovariectomized arthritic mice to responses similar to those in SHAM-proestrus/estrus or naive mice. Moreover, treatment with propylpyrazoletriol and diarylpropionitrile, two ERα and ERβ selective agonists, respectively, inhibited both edema and neutrophil recruitment. Finally, the non-genomic properties of estradiol were analyzed with an acute treatment with β-estradiol-water soluble, which reduced the edema only. In the present study, estradiol replacement therapy improves the innate immune responses in ovariectomized arthritic mice by activating nuclear estrogen receptors. These results suggest that estradiol can induce a protective anti-inflammatory effect in arthritis during ovaries failure, as observed in the menopause.

1. Introduction

Rheumatoid arthritis is an autoimmune disorder characterized by a chronic synovial inflammation, proliferation of synoviocytes, formation of pannus that leads to progressive cartilage destruction, bone erosion, and functional disability [1]. Neutrophils, key components of innate immunity, are effector cells in the onset and progression of arthritis [2]. In physiological conditions, these cells are responsible to eliminate microorganisms and help to clear debris in sterile inflammation by the production of cytotoxic mediators such as reactive oxygen species, neutrophil extracellular traps, and proteinases. However, neutrophils do not distinguish between microbial and host cells, and they can activate adaptive immunity inducing chronic inflammation and tissue destruction [3]. Thus, there is an intensive effort to search for neutrophil inhibitors for the treatment of arthritis and other inflammatory disorders [4].

The etiology of rheumatoid arthritis remains elusive and not well-understood. The incidence and severity of arthritis is affected by complex interactions between genetic, immune, environmental, and endocrine factors. For example, rheumatoid arthritis is two- to three-fold more frequent in women than in men [5]. The peak incidence of arthritis coincides with the age of menopause, implying that ovary failure can contribute to the disease [6]. Women with arthritis often experience clinical improvement during pregnancy, indicating that sex

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hormones might have a therapeutic effect in arthritis [7]. Many studies reported that ovariectomy increases the susceptibility and severity of chronic arthritis in female rodents and that estrogen can protect against arthritis progression; while others demonstrated positive properties of hormone replacement therapy in women with postmenopausal arthritis [8–11]. However, the role of nuclear and membrane receptors, which could be involved in the estrogen protection against the arthritis development in females, is not understood [10,12].

In the present study, we assessed the effects of ovariectomy in the knee joint edema, articular neutrophil migration and cytokine/chemokine production in murine zymosan-induced arthritis as well as the role of estrogen replacement therapy in ovariectomized arthritic animals. Additionally, the role of estrogen receptors (ER) α and β in these inflammatory parameters were also investigated by using selective agonists. Finally, we established a relationship between the genomic and non-genomic effects of estrogen in the inflammatory response of this acute arthritis model.

2. Material and methods

2.1. Animals

Adult female Swiss mice (30–35 g) were obtained from the breeding facility of the Federal Rural University of Rio de Janeiro. Animals were housed in a temperature-controlled (22 ± 1 °C) room and maintained on a 12-hour light/dark cycle with lights on at 7:00 a.m. The mice were housed in groups of ten per cage (40 cm × 33 cm × 18 cm) and given free access to food and water. All the experiments were conducted following the National Institute of Health guidelines for the welfare of experimental animals after approval by CEUA/ICBS from Federal Rural University of Rio de Janeiro (Protocol 019/2017).

2.2. Chemicals

Propylpyrazoletriol (PPT) and diarylpropionitrile (DPN) were purchased from Tocris Bioscience (Minneapolis, MN, USA). Zymosan, estradiol cypionate (EC) and β-estradiol-water soluble were purchased from Sigma Chemical Company (St. Louis, MO, USA). Veterinary pentabiotic was purchased from Fort Dodge - Zoetis (São Paulo, SP, Brazil). Estradiol cypionate, PPT and DPN were dissolved in corn oil while zymosan and β-estradiol-water soluble were suspended and dissolved in sterile saline (0.9%), respectively.

2.3. Ovariectomy

We use bilateral ovariectomy as an experimental model for menopause in mice [13]. Under anesthesia with ketamine (80 mg/kg) and xylazine (10 mg/kg), a small abdominal incision was made and the oviduct and ovarian blood vessels were tied and sectioned for ovariectomy. The muscle and skin walls were then sutured with silk thread. Sham mice underwent the same procedure except for the sectioning of the oviducts and the removal of the ovaries [14]. All animals received prophylactic veterinary pentabiotic (2000 U/mL, intra-muscular) following the surgical procedures. After the surgery, daily vaginal smears were collected from all mice to determine the estrous cycle phase and the efficiency of the ovariectomy, as previously described [15]. The efficiency of the ovariectomy procedure and estradiol treatment was also confirmed by estradiol plasma concentration after six days of treatment.

2.4. Zymosan-induced arthritis

We adapted the zymosan-induced arthritis model in mice for our study [16,17]. Briefly, 10 μL of zymosan suspension (150 μg/μL) in sterile saline (0.9% NaCl; vehicle) was injected into the femoral-tibial joint (intra-articular; i.a.) of both knees of mice under light isoflurane anesthesia (1.5%) 30 min after the treatments (vehicle, EC, PPT, DPN or β-Estradiol-Water Soluble). *Joint swelling* (*articular edema*): After six hours (h), the knee joint thicknesses were measured by caliper in millimeters (mm). Results were expressed as the mean ± SEM of the difference between the diameter before (basal) and after zymosan administration (Δmm). *Knee neutrophil recruitment*: After edema formation measurement, mice were euthanized by cervical displacement under isoflurane anesthesia and then the knee joint was opened and washed with saline solution containing EDTA (1 mM). Aliquots of the resulting washes were diluted in Turk solution. Leukocytes were counted with the aid of a Neubauer chamber and a light microscope. Differential cell counts were stained with hematoxylin–eosin and counted under a light microscope. Results were expressed as the mean ± SEM of the difference between the diameter before (baseline) and 6 h after zymosan administration (Δ mm). To investigate the non-genomic effects of estradiol, the articular edema and neutrophil recruitment were evaluate 1 h after i.a. zymosan administration. *Articular cytokines/chemokines determination*: The articular levels of TNF-α and MIP-2 were determined 1.5 h after injection of zymosan. In brief, joints were dissected out, frozen with liquid nitrogen, crushed in a mortar and pestle, and then solubilized in PBS containing anti-proteases. Then TNF-α and MIP-2 concentrations were evaluated using a commercially available enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Duo-Set kits; R&D Systems, Minneapolis, MN, USA). Results were expressed as the mean ± SEM of cytokine levels in pg/mg of joint tissues.

2.5. Plasma estradiol determination

For plasma estradiol measurements, the animals were submitted to blood collection (~0.5 mL) from the jugular vein at 6 h after the induction of arthritis with zymosan in chilled tubes containing heparin. Plasma was obtained after centrifugation (20 min; 3000 rpm, 4 °C) and stored at −80 °C until analysis. The plasmatic estradiol concentration was measured using an estradiol Express EIA Kit from R&D System® (Minneapolis, MN, USA) following the procedures detailed in the instructions.

2.6. Experimental protocols

In order to investigate the effect of ovariectomy, estradiol hormone replacement, and genomic effect of estradiol, naive (not submitted to surgery or anesthesia), sham and bilateral ovariectomized mice were randomly separated into groups subcutaneously (s.c.) treated with vehicle (corn oil, 10 mL/kg), EC (1.25, 5 or 20 μg/kg), PPT (ERα agonists, 300 μg/kg) or DPN (ERβ agonists, 300 μg/kg) starting 24 h after ovariectomy and conducted once a day for six consecutive days between 07:00 and 08:00 AM. Sham groups were treated with vehicle and divided into proestrus/estrus and metaestrus/diestro according epithelial cells in the vaginal smears. Zymosan was then injected 30 min after the treatments (i.a.). The articular edema and neutrophil recruitment were evaluated 6 h after zymosan administration.

To investigate the involvement of TNF-α and MIP-2 in the ovariectomy and estradiol effect on zymosan-induced arthritis, the mice were subcutaneously treated with vehicle (corn oil; 10 mL/kg) or EC (1.25 μg/kg) starting 24 h after ovariectomy surgery and conducted one time per day for six consecutive days between 07:00 and 08:00 AM. Sham groups were treated with vehicle. Naive mice were not submitted to surgery, anesthesia or treatment. Zymosan was then injected 30 min after the treatments (i.a.). The articular edema and cytokines determination were evaluated 1.5 h after zymosan administration.

Finally, to investigate the non-genomic effects of estradiol, the animals were subcutaneously treated with vehicle (saline 0.9%, 10 mL/kg) or β-estradiol-water soluble (1 mg/kg) only at the day seven after ovariectomy. SHAM groups were treated with vehicle. Zymosan was then injected 30 min after the treatments (i.a.). The articular edema and

neutrophil recruitment were evaluated 1 h after zymosan administration.

2.7. Data analysis

Neutrophil recruitment to the knee joint and cytokines measurement were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison post hoc test, with the aid of the GraphPad Prism 6 Software (San Diego, CA, USA). The joint diameter (edema) was analyzed with the two-way ANOVA for repeated measures followed by the Bonferroni's post hoc test when indicated. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Ovariectomy worsens the inflammatory response in zymosan-induced arthritis, which is reversed by estrogen therapy replacement

In order to investigate the role of female hormones in the zymosan-induced arthritis, we compared the knee joint inflammation of animals in different phases of estrous cycles and in ovariectomized mice. The experimental protocol is outlined in Fig. 1A. Articular inflammation (edema and neutrophils recruitment) is similar among naive, sham-proestrus/estrus, and sham-metaestrus/diestrus animals (Fig. 1B, C). However, ovariectomy significantly increases both edema formation and neutrophil recruitment as compared to naive or sham animals after i.a. zymosan injection (Fig. 1B, C). The ovarian failure induced by surgical ovariectomy was confirmed by daily analysis of the vaginal smear cytology (data not shown) and by the reduction in the blood estrogen levels in ovariectomized animals as compared to sham animals (Fig. 1C). We also observed that the plasma concentration of estradiol is significantly higher in the proestrus/estrus cycle than in the metaestrus/diestrus cycle or after ovariectomy (Fig. 1D). These results confirmed that the animals were properly separated according to their estrous cycle. However, although ovariectomy worsens the articular inflammation, estrogen variation in the proestrus/estrus and metaestrus/diestrus cycles do not affect joint swelling or neutrophil migration after i.a. zymosan administration (Fig. 1B, C).

We next analyzed the potential of hormone therapy replacement. Treatment with estradiol cypionate (s.c.; 1.25; 5 and 20 μg/kg) for six consecutive days markedly re-established the joint edema and neutrophil migration in ovariectomized arthritic animals to responses similar to those in Sham-proestrus/estrus or naive mice (Fig. 1B, C). Moreover, all animals treated with estradiol cypionate switch their estrous cycle phase from metaestrus/diestro to proestrus/estrus. The lowest dose was able to produce the maximum effect and raised the plasma estradiol concentrations to levels similar to that in sham-proestrus/estrus mice and significantly higher than those in ovariectomized animals (Fig. 1C). These data demonstrate that administration of estradiol cypionate (1.25 μg/kg) for six consecutive days promotes plasma estradiol concentrations similar to physiological conditions found in the proestrus/estrus cycle.

Next, we measured the levels of TNF-α and MIP-2, two important inflammatory mediators involved in arthritis development, in the articular tissue of ovariectomized mice after i.a. injection of zymosan. Mice were treated with vehicle (corn oil) or estradiol cypionate (s.c.; 1.25 μg/kg) for six consecutive days. The experimental protocol is outlined in Fig. 2A. The i.a. zymosan injection increased the local levels of TNF-α and MIP-2 in sham-mice compared with naive plus i.a. saline injection. However, after i.a. zymosan injection, ovariectomy only increased the local levels of TNF-α compared with sham-mice. Estradiol replacement therapy reduces the TNF-α level in arthritic ovariectomized mice similar to those in arthritic sham mice (Fig. 2B). By contrast, the estradiol treatment does not affect the knee joint MIP-2 levels of arthritic ovariectomized mice (Fig. 2C).

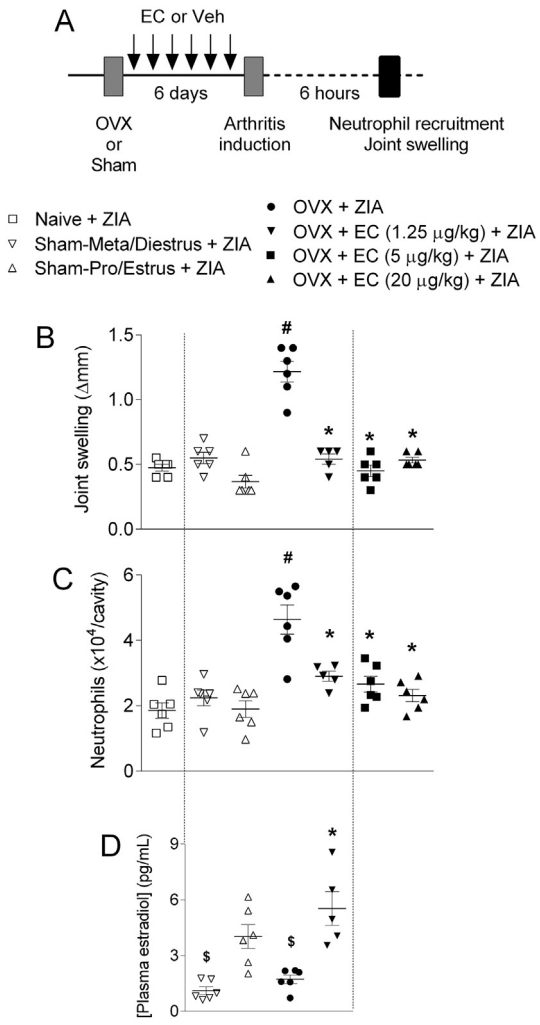

Fig. 1. Estrogen therapy replacement improved articular inflammation in ovariectomized arthritic mice. (A) Experimental protocol. (B and C) Effects of estrogen therapy replacement in the (B) edema formation and (C) neutrophil migration in zymosan-induced arthritis (ZIA). Ovariectomy (OVX) and Sham were performed seven days before zymosan administration (i.a.; 150 μg). Sham animals were divided into metaestrus/diestrus (Sham-Meta/Diestrus) or proestrus/estrus (Sham-Pro/Estrus) according to the analysis of its vaginal smear cytology. Mice were s.c. pre-treated with vehicle (Veh; corn oil) or estradiol cypionate (EC; 1.25; 5 and 20 μg/kg) for six days before zymosan administration. Joint swelling and neutrophil recruitment were measured 6 h after zymosan injection. (*D*) Plasmatic levels of estradiol in sham, OVX and EC-treated (s.c.; 1.25 μg/kg) mice. Estradiol was quantified by ELISA. Dashed lines highlight the same groups in the different graphics. Data are expressed as the mean ± SEM (n = 5–6 mice/group). (B and C) # and * $p < 0.05$ compared to naive + ZIA and OVX + ZIA groups respectively; (D) $ and * $p < 0.05$ compared to Sham-Proestrus/Estrus + ZIA and OVX + ZIA groups respectively (one-way ANOVA followed by Tukey's post hoc test and Student's *t*-test).

3.2. ER-agonists decreased inflammation in experimental arthritis

We analyzed the potential anti-inflammatory effects of ER-agonists in these experimental models of arthritis. The role of ERα and ERβ receptors on the edema formation and neutrophil migration was determined by administration of PPT (s.c.; 300 μg/kg) or DPN (s.c.; 300 μg/kg) for six days in ovariectomized arthritic mice (Fig. 3A). Both ER-agonists significantly inhibit both inflammation and neutrophil migration in ovariectomized mice and induce an effect similar to that of the estradiol replacement (1.25 μg/kg) (Fig. 3B, C). In addition, all animals treated with PPT or DPN switch their estrous cycle phase from metaestrus/diestro to proestrus/estrus.

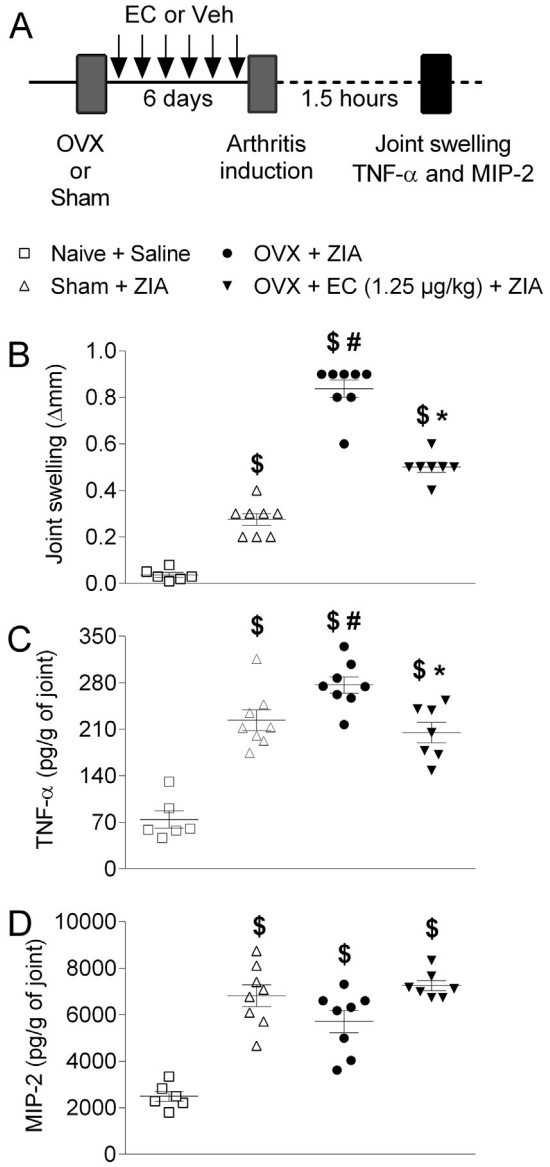

Fig. 2. Estrogen therapy replacement decreases articular TNF-α level in ovariectomized arthritic mice. (A) Experimental protocol. (B and C) Effects of estrogen therapy replacement in the (B) edema formation and knee joint (C) TNF-α and (D) MIP-2 levels in zymosan-induced arthritis (ZIA). Ovariectomy (OVX) and Sham were performed seven days before zymosan administration (i.a.; 150 μg). Mice were s.c. pre-treated with vehicle (Veh; corn oil) or estradiol cypionate (EC; 1.25 μg/kg) for six days before zymosan administration. Joint swelling and TNF-α/MIP-2 levels were measured 1.5 h after zymosan injection. TNF-α and MIP-2 were quantified by ELISA. Data are expressed as the mean ± SEM (n = 6–8 mice/group). $, # and * $p < 0.05$ compared to Naive + Saline, Sham + ZIA and OVX + ZIA groups respectively (one-way ANOVA followed by Tukey's post hoc test and Student's t-test).

3.3. Non-genomic estradiol effect decreased edema formation but not the neutrophil migration

Finally, in order to investigate whether estradiol induces its effects through a genomic mechanism, we analyzed the effects of β-estradiol-water soluble (s.c.; 1 mg/kg) administrated 30 min before the i.a. injection of zymosan only at day seven after ovariectomy. In these conditions, the joint swelling and neutrophil recruitment were evaluated 1 h after the zymosan injection (Fig. 4A). The β-estradiol-water soluble (s.c.; 1 mg/kg) treatment significantly decrease edema formation but not the neutrophil migration (Fig. 4B, C).

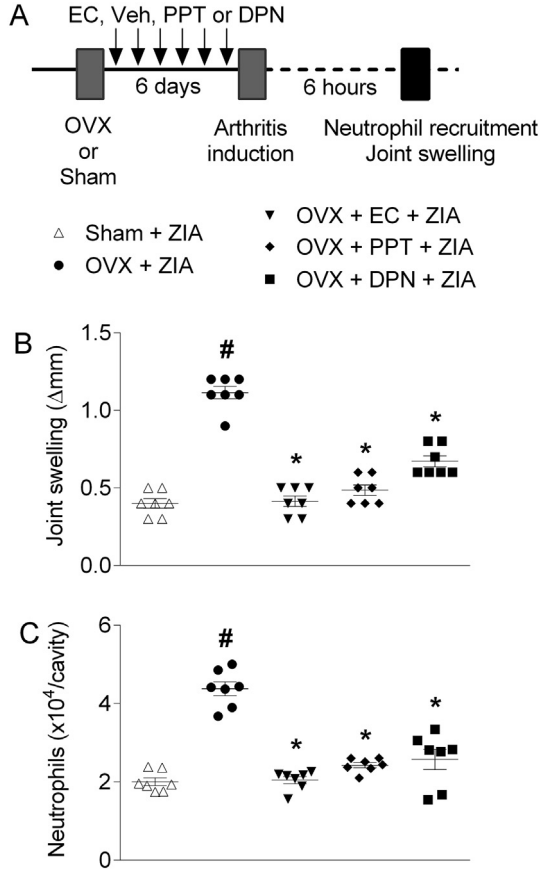

Fig. 3. Estrogen ERα and ERβ agonists improved experimental arthritis in ovariectomized mice. (A) Experimental protocol. (B and C) Effects of selective ERα and ERβ agonists in the (B) edema formation and (C) neutrophil migration in zymosan-induced arthritis (ZIA). Ovariectomy (OVX) and Sham were performed seven days before zymosan administration (i.a.; 150 μg). Mice were s.c. pre-treated with vehicle (Veh; corn oil) or estradiol cypionate (EC; 1.25 μg/kg), ERα agonists (PPT; 300 μg/kg) or ERβ agonists (DPN; 300 μg/kg) for six days before zymosan administration. Joint swelling and neutrophil migration were measured 6 h after zymosan injection. Data are expressed as the mean ± SEM (n = 7 mice/group). # and * $p < 0.05$ compared to Sham + ZIA and OVX + ZIA groups respectively (one-way ANOVA followed by Tukey's post hoc test and Student's t-test).

4. Discussion

Previous studies have shown that hormones such as progesterone [18,19] and estradiol [10,20–22] produce anti-inflammatory effects in several models of arthritis [23], but their modulatory properties are still contradictory. Our present results show that ovariectomy increase the articular inflammation in zymosan-induced arthritis. These results indicate that the ovaries play an important protective role in acute arthritis. Conversely, estradiol replacement treatment reverses the effects of ovariectomy as shown by analyzing knee joint swelling, neutrophil recruitment, and local cytokine production. As expected, estradiol plasma concentrations in hormone-treated ovariectomized animals were similar to that in sham-proestrus/estrus mice, and significantly higher than in sham-metaestrus/diestrus or ovariectomized animals. These results confirm the success of ovariectomy surgery and that the animals were properly separated according to their estrous cycle. Furthermore, ovariectomized animals treated with estradiol cypionate switch their estrous cycle phase from metaestrus/diestro to proestrus/estrus, indicating that the doses used were able to activate nuclear estrogen receptors.

Although ovariectomy worsens inflammation in zymosan-induced arthritis, the variation of estrogen release produced by the estrous cycle

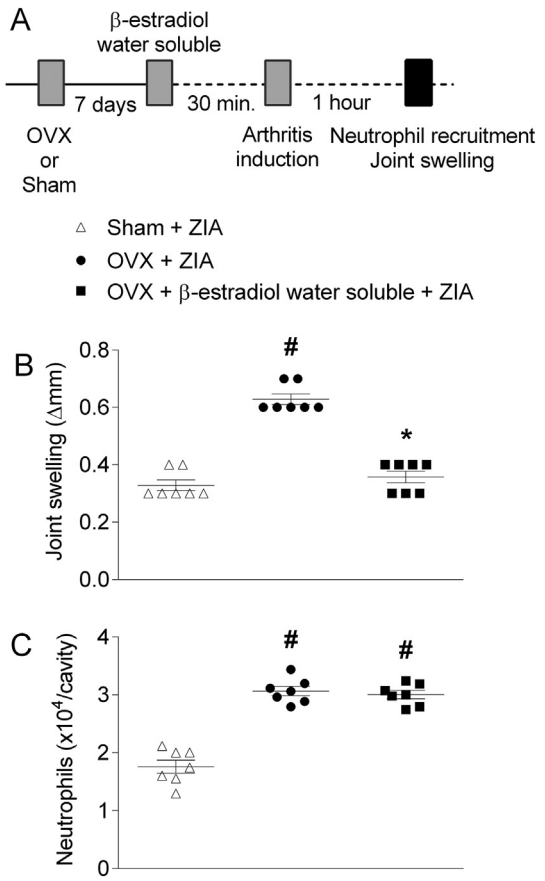

Fig. 4. Non-genomic effect of estradiol improved articular edema formation but not neutrophil recruitment in ovariectomized mice. (A) Experimental protocol. (B and C) Effect of β-estradiol-water soluble in the (B) edema formation and (C) neutrophil migration in zymosan-induced arthritis (ZIA). Ovariectomy (OVX) and were performed seven days before zymosan administration (i.a.; 150 μg). Mice were pre-treated with vehicle (Veh; saline) or β-estradiol-water soluble (s.c.; 1 mg/kg) 30 min before zymosan administration. Joint swelling and neutrophil migration were measured 1 h after zymosan injection. Data are expressed as the mean ± SEM (n = 7 mice/group). # and * $p < 0.05$ compared to Sham + ZIA and OVX + ZIA groups, respectively (one-way ANOVA followed by Tukey's post hoc test and Student's *t*-test).

do not alter edema or neutrophil recruitment as observed in sham-proestrus/estrus to metaestrus/diestrus animals in this arthritis model. Genomic effects of estradiol are delayed and prolonged, which may explain the non-worsening of arthritis in sham-metaestrus/diestrus animals [10]. In fact, the estrous cycle of mice is very short, lasting around 4–5 days and the mice remains with high estradiol levels for 37–41 h and low levels for 61–65 h [24], while ovariectomized animals remain without estradiol for seven days.

It is well known that zymosan induces MIP-2 release for neutrophils recruitment. Here, we relate the increase of local MIP-2 in this model of arthritis. These findings are in agreement with the chemokine increase reported in chronic arthritis models [25,26]. However, neither ovariectomy nor estradiol replacement prevent the increase in local MIP-2 induced by i.a. zymosan injection. Ovariectomy also significantly increases articular TNF-α concentration, the main pro-inflammatory cytokine involved in arthritis progression [27,28]. In addition, estradiol replacement therapy reduces articular TNF-α concentration to levels similar to those in sham arthritic mice. These results are concur with in vitro studies showing that estradiol decreases TNF-α production in cultured macrophages, considered the main source of TNF-α in zymosan-induced arthritis [29,30]. Together, these results indicate that TNF-α, but not MIP-2, is involved in the worsening of articular inflammation induced by ovariectomy as well as in the anti-inflammatory effects of exogenous estrogen administration.

We noticed that arthritic ovariectomized mice treated with low doses of estradiol cypionate shows joint swelling, neutrophil recruitment, and articular TNF-α concentration similar to that in sham-proestrus/estrus mice, which have equivalent plasma estradiol concentrations. However, the highest doses of estradiol cypionate do not produce additional effects, indicating that physiological release of estradiol produces the maximum effect and exogenous estradiol in the regular estrous cycle do not produce additional anti-inflammatory effects.

Recent studies showed the role of ERα in the anti-inflammatory effects of estradiol on arthritis [31–36]. By contrast, the potential involvement of ERβ in the anti-inflammatory activity of estradiol is controversial. Although some studies report the involvement of ERβ in the anti-inflammatory activity of estradiol in arthritis [37–40], others show the opposite effects [33–36]. In our study, both PPT (ERα-selective agonist) and DPN (ERβ-selective agonist) reduce edema formation and neutrophil recruitment in zymosan-induced arthritis suggesting that both nuclear receptors are involved in the local anti-inflammatory effects of estradiol. Moreover, ovariectomized animals treated with PPT and DPN switch their estrous cycle phase from metaestrus/diestro to proestrus/estrus, indicating that the doses used were able to activate nuclear estrogen receptors.

Finally, it is well known that the rapid and short non-genomic properties of estradiol may be mediated through the G protein-coupled receptor also known as GPR-30 receptor [10,41,42] as well as by the membrane-associated ERα [10,42]. To investigate the non-genomic effects of estradiol, we used a high dose of β-estradiol-water soluble administrated 30 min before the zymosan and only at day seven after ovariectomy, while edema formation and neutrophil recruitment were evaluated 1 h after i.a. zymosan injection. The changes of the pharmacological approach and the dose used in this experiment were designed to ensure a fast absorption and high bioavailability of estradiol while the reduction in the time of evaluation of the inflammatory parameters was aimed at reducing the genomic effects of estradiol. We observed that β-estradiol-water soluble inhibit the local edema formation, but not the neutrophil infiltration into the knee joint. These results suggest that the protective role of estrogen is exerted by non-genomic mechanisms. This specific effect on the reduction of edema could be explained by the fact that non-genomic GPR-30 and ERα activation elicit fast effects in the acute vascular responsiveness [38,41,43,44]. Moreover, treatment with GPR-30 agonist did not change the frequency and severity of inflammation in chronic arthritis models [35].

The present study shows two limitations that have to be pointed out. First, although we have modified our initial protocol to minimizing the genomic effects and increase the bioavailability of estradiol and we observed a reduction in the articular edema in β-estradiol-water soluble-treated ovariectomized animals, our experimental protocol does not confirm the direct activation of GPR-30 or membrane-associated ERα. Second, it is questionable whether estrogen agonists could show systemic anti-inflammatory effects that would inhibit the local inflammatory effect. However, we have demonstrated previously that the i.a. administration of zymosan in lower doses does not induce systemic inflammatory response [45]. In addition, we also reported that i.a. administration of modulatory drugs can inhibit the neutrophil migration toward the knee joint suggesting that zymosan-induced arthritis is a local and restricted inflammatory model [17]. Future studies are needed to decode the real importance of local and systemic anti-inflammatory effects of estrogen in the arthritis development.

The present study reports that estradiol replacement therapy provides anti-inflammatory effect, mainly by activating ERα and ERβ, in several local inflammatory parameters in our acute arthritic model. These results show the protective properties of this hormone against arthritis development and its potential clinical use in hormonal replacement to treat arthritis in menopausal women.

Conflict of interest

The authors indicated no potential conflicts of interest.

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