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Obacunone alleviates chronic pelvic pain and pro-inflammatory depolarization of macrophage induced by experimental autoimmune prostatitis in mice

Yadong Wang [a,b], Zhaohui Dang [c], Xu Wang [c], Yuanyuan Chen [c], Peng Dong [d], Gang Liu [d], Weibin Tan [d], Zhong Gui [d], Fan Bu [c,*], Feng Lin [b,**], Chaozhao Liang [a,***]

[a] Department of Urology, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, 230022, China
[b] Department of Urology, The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, Shenzhen, Guangdong, 518033, China
[c] Department of Neurology & Psychology, The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, Shenzhen, Guangdong, 518033, China
[d] Department of Neurosurgery, The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, Shenzhen, Guangdong, 518033, China

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ABSTRACT

Chronic pelvic pain syndrome (CPPS) is a common complication of prostatitis, which was associated with the pathological depolarization of macrophage and the neuroinflammation. However, its underlying reason is far from clear and few effective treatments is applicable. In this study, we tested the effect of obacunone (Oba), a highly oxygenated triterpenoid, on CPPS. The experimental autoimmune prostatitis (EAP) was induced by subcutaneous injection of heterologous prostate homogenate in mice. We found that EAP led to prostatodynia, neuronal activation of spinal dorsal horn, and the pro-inflammatory depolarization of macrophage within prostate, which was significantly alleviated by oral administration of Oba in a dose-dependent manner. Mechanistically, EAP-induced production of IL-6 on prostatic macrophage was suppressed by Oba. Moreover, co-administration of Oba and MIF inhibitor ISO-1 did not lead to additive effect when compared with either alone. In summary, we conclude that Oba prevents the production of macrophage-derived pro-inflammatory factors by inhibiting MIF, which eventually alleviates CPPS after prostatitis.

1. Introduction

Prostatitis is one a common urologic disease accounting for 8 % of urologist visits [1–3]. Chronic pelvic pain syndrome (CPPS) is a major complication of prostatitis, which is characterized as a chronic pain in the region of the pelvis [3]. Although amounts of studies focused on CPPS in the past years, its underlying reason is far from clear, and pharmacological treatment is limited [4]. Mice induced with experimental autoimmune prostatitis (EAP) perform similar pathological feature with the clinical population of CPPS, which is popularly utilized for the fundamental study [5,6]. In which, the production of cytokines and chemokines by prostatic resident cells and infiltrated leukocytes, mainly including macrophages, T cells ($CD4^+$, $CD8^+$) and dendritic cell are considered as the major reasons causing obstinate pain [6,7]. Intraprostatic cytokines and chemokines, including interleukin (IL)-6 and cyclooxygenase (COX)-2 et al., can activate nociceptors within prostate. Once nociceptors activated, the nociceptive afferent pathway within spinal dorsal horn of lumbar vertebra 6 (L6) and sacral vertebra 2 (S2) and supraspinal connections will be facilitated, which eventually leads to persistent pain [8–10]. In this case, targeting infiltrated leukocytes and suppressing inflammatory response might be an effective strategy in the treatment of CPPS.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine expressed in multifarious cell types, including macrophages/microglia, astrocytes and neurons [11]. In particular, secretory MIF can recruit macrophages/microglia and promote their inflammatory depolarization [12,13]. It is known that MIF contributes to hypersensitivity in rodent pain models. For instance, people living with spinal cord injury exhibit acute and chronic elevation of circulating levels of MIF [14]. MIF inhibitor, ISO-1, reversed spinal cord injury-induced ongoing pain

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: fanbu123@outlook.com (F. Bu), lf0237@gzucm.edu.cn (F. Lin), liang_chaozhao@163.com (C. Liang).

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behavior [15]. Under the condition of CPPS, the clinical and basic studies have shown an increase of MIF in prostate and plasma, accompanied with the infiltration of macrophages into prostate [9,16,17]. The pathological expression of MIF was considered as one of the inducers in the genesis and development of prostatic hyperplasia and prostatitis [18], although no direct evidence showing the anti-CPPS and anti-prostatitis effects by targeting MIF yet.

Obacunone (Oba) is an oxygenated triterpenoid with known structure (Fig. 1A), and commonly found in citrus and other plants of the Rutaceae family, e.g. Phellodendron chinense, Dictamnus dasycarpus and Euodia rutaecarpa [19]. Oba exhibits various pharmacological activities, including anti-oxidative, anti-hyperglycemic, neuro-protective and anti-tumor [20–23]. In terms of mechanism, computational target-fishing technology indicated that MIF is the possible target of Oba [20]. *In vitro* study further demonstrated that Oba performed an anti-inflammatory effect on cultured macrophage, which was evidenced by that perfusion of Oba inhibited the production of nitric oxide, IL-6 and monocyte chemotactic protein-1 induced by lipopolysaccharide [20]. It raises the possibility that Oba may also protect against prostatitis and CPPS via the similar approach. In the present study, we would mainly investigate the effect of Oba on CPPS and inflammatory infiltration of prostate in animal models. In addition, the potential target of Oba relevant to MIF would be tentatively explored under EAP condition.

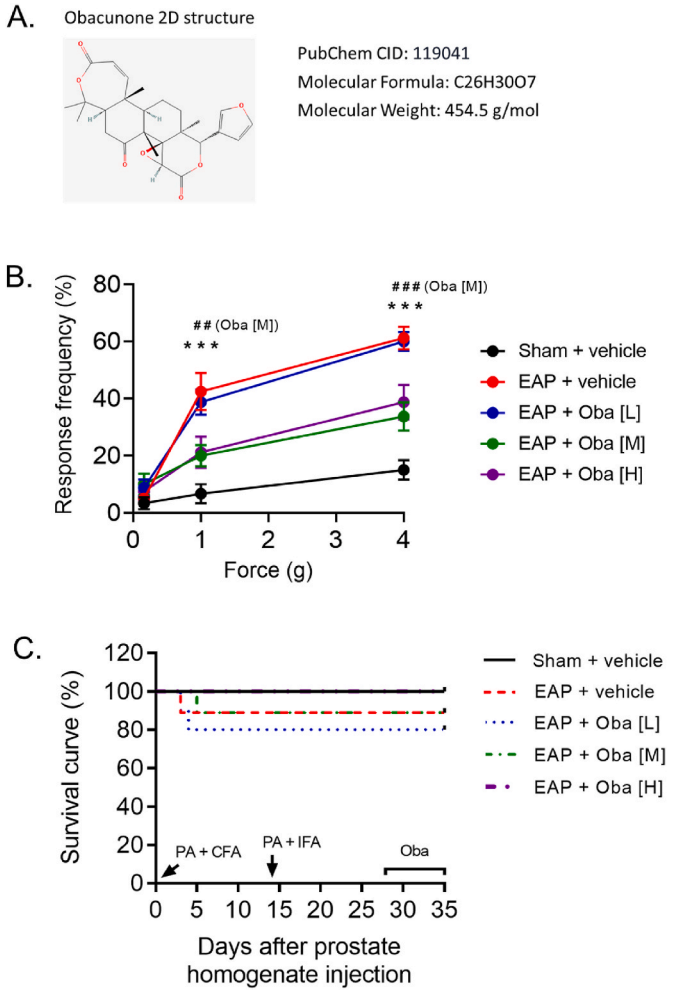

Fig. 1. The effect of Oba to EAP-induced CPPS. (A) The 2D structure of Oba. (B) Von Frey behavior assaying prostatodynia. In order to establish EAP model, subcutaneous injection of prostate antigens and complete Freund's adjuvant was initially performed, followed by prostate antigens and incomplete Freund's adjuvant 14 days later in mice. Saline was injected in the sham group. Fourteen days after heterologous prostate homogenate injection, Oba (10, 25, 50 mg/kg/daily) and vehicle (0.5 % methylcellulose) gavage was performed for 7 continuative days. Prostatodynia in the pelvic area was tested at the 7th day of Oba gavage. (C) Survival rate. N = 6–10/group. All the values are shown as means ± SEM. Two-way ANOVA followed by Bonferroni multiple comparisons test. Sham + vehicle group vs. EAP + vehicle group, $^{***}p < 0.001$ at 1 g and 4 g. EAP + vehicle group vs. EAP + Oba [M] group, $^{\#\#}p < 0.01$ at 1 g, $^{\#\#\#}p < 0.001$ at 4 g. Significant difference was considered when $P < 0.05$.

2. Results

2.1. Oba alleviated CPPS in a dose-dependent manner

We first induced the CPPS model as described previously, in which chronic prostatodynia was allowed to establish 14 days after the injection of heterologous prostate homogenate [9]. After that, Oba gavage (10 [L], 25 [M], 50 [H] mg/kg/day) was performed for continuative 7 days in order to investigate its effect to CPPS. The same volume of methylcellulose (0.5 %) was applied as vehicle control. We found that EAP induced prostatodynia, compared to the sham group ($^{***}p < 0.001$, Fig. 1B), which was significantly suppressed by Oba in a dose-dependent manner (EAP + vehicle vs. EAP + Oba [M], $^{\#\#}p < 0.01$ at 1 g, $^{\#\#\#}p < 0.001$ at 4 g). Oba did not affect the survival rate even at maximal dose (Fig. 1C), suggesting no toxicity of Oba in our preparation.

2.2. Oba alleviated EAP-induced neuronal activity and the production of pain-associated factors within spinal cord

It is known that EAP leads to neuronal activation within spinal dorsal horn of L6 and S2, which conducts afferent nociceptive signals [8]. In order to further clarify the effect of Oba to prostatodynia, the neuronal activities within the dorsal horn of L6-S2 was further investigated. We found that the localization of $NeuN^{+}$/$C\text{-}fos^{+}$ was induced after EAP (Fig. 2A), accompanied with the production of pain-associated factors, C–C motif chemokine ligand (CCL)-3 and brain-derived neurotrophic factor (BDNF) (Fig. 2B), which is in line with the previous study [24]. Importantly, Oba gavage [M] significantly suppressed EAP-induced activation of neuron and production of CCL-3 and BDNF, which further supports the inhibitory effect of Oba to prostatodynia.

2.3. Oba reduced tissue inflammation within prostate after EAP

In order to investigate whether Oba could alleviate EAP-induced infiltration of inflammatory cells and tissue injury into prostate, histopathology was examined once 7-days Oba [M] administration completed. We found that prostate mucosal cells showed degeneration and shedding, and that inflammatory cells infiltrated into prostatic tissue in the EAP group, which was ameliorated when application of Oba (Fig. 3). Quantitatively, the increase of histopathological scores induced by EAP was significantly reversed by Oba.

2.4. Oba reduced EAP-induced pro-inflammatory depolarization of macrophage within prostate

It is known that EAP-induced elevation of pro-inflammatory factors, e.g. IL-6 and COX-2, within prostate could excite nociceptors and facilitate pain signal ascending pathway [9,25]. In order to further understand the underlying reason of Oba in mediating CPPS, the content of IL-6 and COX-2 within prostate was assayed. We found that the level of IL-6 and COX-2 increased after EAP, which was in line with previous studies (Fig. 4A). Importantly, the elevation of IL-6 was significantly suppressed by Oba [M], suggesting an anti-inflammatory effect of Oba in EAP.

The previous study has shown that Oba is the small molecule inhibitor of MIF, a pleiotropic protein that involves in macrophage activation and migration [26]. The elevation of macrophage and MIF within prostate might be associated with the severity of inflammation and CPPS

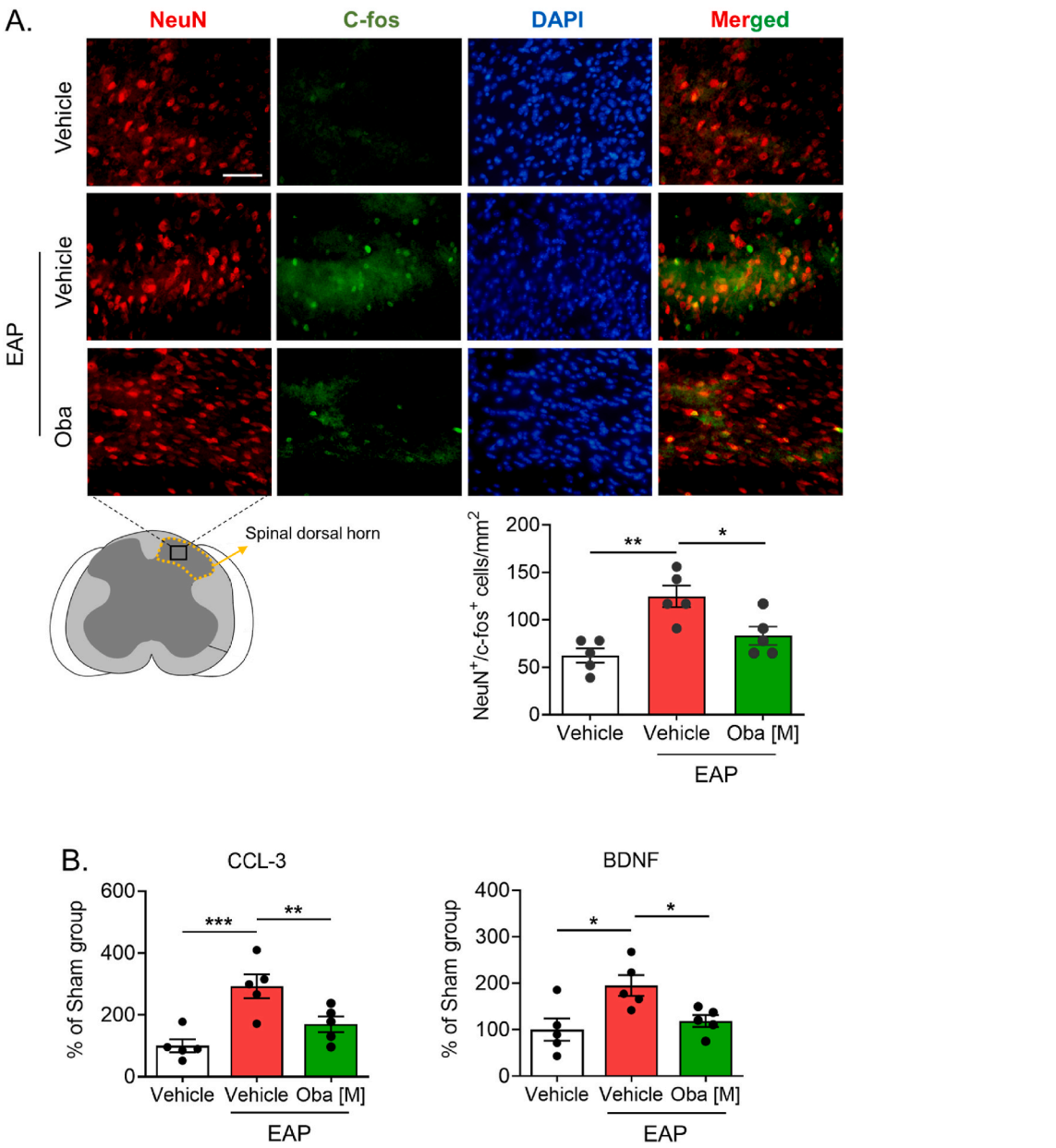

Fig. 2. The effect of Oba to EAP-induced neuronal activity and the production of pain-associated factors within spinal cord. (A) Neuronal activity ($NeuN^+/c\text{-}fos^+$) in the dorsal horn of L6-S2 section was assayed by immuno-staining. Scale bar: 100 μm. (B) The production of CCL-3 and BDNF in the dorsal horn of L6-S2 section was assayed by ELISA. N = 5/group. All the values are shown as means ± SEM. One-way ANOVA followed by Bonferroni multiple comparisons test. Significant difference was considered when $P < 0.05$.

[6,16,27]. Herein, we further investigated the effect of Oba to the inflammatory depolarization of macrophage after EAP. Our data showed that EAP induced the activation of macrophage ($Iba1^+$) in prostatic stroma, which was accompanied with a higher expression of IL-6 on macrophage (Fig. 4B). As expected, application of Oba significantly suppressed Iba1 and IL-6 intensity. In addition, the expression of Arginase (Arg) 1, the marker of anti-inflammatory subtype of macrophage/microglia was also investigated. We found that EAP increased the expression of either Iba1 or Arg1 within prostate (Fig. 4C). However, different to the performance of Iba1, the expression of Arg1 was not altered by the treatment of Oba after EAP. These data suggested that the inhibitory effect of Oba to the pro-inflammatory depolarization of macrophage might be the underlying reason in mediating CPPS.

2.5. Oba played no addictive effect on EAP-induced CPPS in the presence of MIF inhibitor

It is known that MIF elevated in the serum of chronic pelvic pain syndrome patients [16], raising the possibility that MIF might be contribute to CPPS progress. Herein, we first detected the expression of MIF in EAP mice. We found that the protein level of MIF significantly increased within serum and prostate, compared to the sham group (Fig. 5A and B). In addition, MIF-selective inhibitor ISO-1 significantly suppressed EAP-induced prostatodynia in a dose-dependent manner (5, 10, 20 mg/kg; Sham + vehicle vs. EAP + vehicle, $^{***}p < 0.001$; EAP + vehicle vs. EAP + ISO-1 [M], $^{\#}p < 0.05$; Fig. 5C). Importantly, Oba played no addictive effect on CPPS in the presence of ISO-1 (EAP + vehicle vs. EAP + ISO-1 [M], $^{\#}p < 0.05$; EAP + vehicle vs. EAP + Oba [M], $^{*}p < 0.05$, $^{**}p < 0.01$; Fig. 5D), suggesting that Oba alleviates CPPS via inhibiting the activity of MIF.

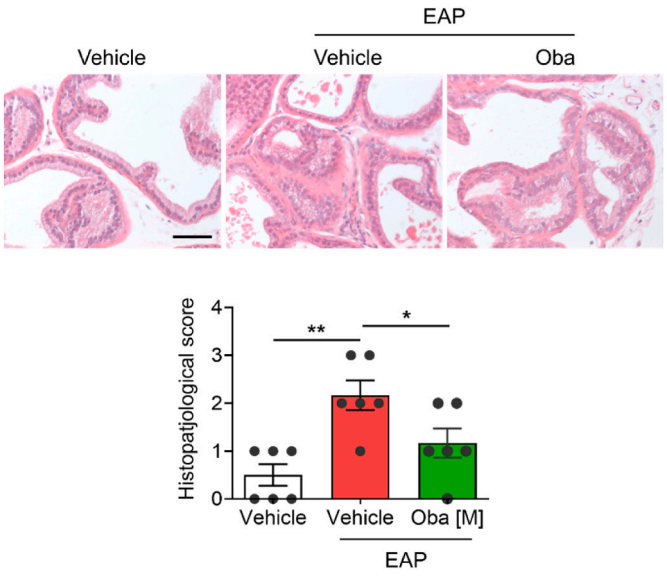

Fig. 3. The effect of Oba to EAP severity. Histological evaluation for the degree of inflammation within prostate. Scale bar: 100 μm. N = 6/group. All the values are shown as means ± SEM. One-way ANOVA followed by Bonferroni multiple comparisons test. Significant difference was considered when $P < 0.05$.

3. Discussion

The present study identified that Oba protected against CPPS and pro-inflammatory depolarization of macrophage within prostate in mice, which might be attributed to its inhibitory effect to MIF. They were supported by the significant findings: (i) Oba alleviated chronic prostatitis-induced CPPS in a dose-dependent manner; (ii) Oba inhibited macrophage activation and the elevation of IL-6 within prostate; (iii) Oba has no addictive effect to CPPS in the presence of MIF inhibitor.

To our knowledge, the present study is the first to report Oba in mediating chronic pelvic pain and prostatitis. In order to investigate the therapeutic effect of Oba, chemicals were delayed applied after EAP-induced CPPS model established [9]. Our data showed that Oba significantly alleviated mechanical allodynia of the pelvic area in a dose-dependent manner without affecting survival rate. As the conduction hub of the nociceptive signal projected from prostatic area, neurons within the L6 – S2 sections of spinal dorsal horn tend to be active when EAP occurs in mice [9,28]. Herein, we found that EAP-induced activation of neurons ($NeuN^+$/$c\text{-}fos^+$) in these areas was reversed by Oba. In addition, the elevation of pain-associated mediators, including CCL-3 and BDNF [24,28], within L6 – S2 sections were inhibited as well, which confirming the analgesic effect of Oba to CPPS.

The suppressive effect of Oba to the activity of MIF within prostate might be one of the reasons in mediating CPPS. The previous computational target-fishing technology indicated that Oba can bind to the hydrophobic pocket of MIF [20]. It is known that the serum level of MIF was higher in CPPS patients, in which MIF might make a significant contribution to the development of CPPS [16]. In the mice model of protease-activated receptors-induced bladder pain, people found that inhibition of MIF using its small molecular inhibitor ISO-1 significantly eliminated pain hypersensitivity [29]. In the present study, we found that the protein level of MIF was significantly increased within serum and prostate in EAP mice, which is in line with the clinical study [16]. Importantly, administration of either Oba or ISO-1 eliminated CPPS, and that administration of Oba and ISO-1 together has no addictive effect to CPPS compared to that applying either alone, suggesting that Oba performs analgesic effect via deactivating MIF within prostate of EAP mice.

The promotion of MIF to the production of pro-inflammatory factors by macrophage may contribute to the development of CPPS. MIF is mainly synthesized and stored in immune cells, such as macrophages, T cells and mast cells, in response to multiple stimuli and immune response [30]. It is known that the accumulation of inflammatory cells within prostate, such as T-cell, macrophages and granulocytes, and the production of pro-inflammatory cytokines were observed after prostatitis, which was positively related with higher inflammation score and CPPS evidenced by clinical and fundamental studies [31,32]. In the present study, we found that EAP led to the distribution of macrophage (labeling by macrophage/microglial marker Iba1) and the co-localization of IL-6 and Iba1 within prostatic interstitium and epithelium accompanied with the development of prostatodynia. Importantly, these were significantly suppressed by Oba, which rises the likelihood that inhibition of MIF can prevent the pro-inflammatory activation of macrophage within prostate and further ameliorate CPPS.

How MIF mediating pro-inflammatory depolarization of macrophage after EAP was not well addressed here, but we assumed it could be attributed to, at least partially, the receptors of MIF and the intracellular signal pathways. For instance, Hickey's lab revealed that MIF-CD74 axis contribute to macrophage adhesion and migration [33]. Deletion of either MIF or its receptor, CD74, significantly reduced chemokine (CCL-2)-induced macrophage recruitment *in vivo* [33]. Mechanically, the author suggested that MIF (−/−) macrophages exhibited an elevation of mitogen-activated protein kinase (MAPK) phosphatase-1, which was associated with attenuated MAPK phosphorylation. In addition, MIF can contribute to the production of pro-inflammatory factors via activating MAPK signaling [13]. Roger and co-workers demonstrated that MIF (−/−) macrophages failed to secrete IL-6 due to profound suppression of inhibitor of NF-kB kinase-β/NF-kB activity [34]. Alternatively, MIF may play as kinase in mediating inflammatory factor production. In this study, application of lipopolysaccharide onto cultured macrophage led to the reduction of MAPK phosphatase-1 and phosphorylated activation of MAPK/P38, as well as the release of IL-6 [20]. These was reversed by Oba but not MIF neutralizing antibody, suggesting an intracellular mechanism of MIF. Nevertheless, under the condition of EAP, the downstream signaling of MIF in mediating pro-inflammatory factors production from macrophage requires further investigation.

It was worth noting that MIF level is altered in multiple tissues under prostatitis condition, including prostate, bladder and spinal cord [27]. In addition, MIF could contribute to pain development via hyper-exciting neurons within either dorsal root ganglion or spinal cord under multiple diseases [15,35,36]. In this case, the possibility cannot be ruled out that systematically application of either Oba (gavage) or ISO-1 (intraperitoneal injection) may perform a direct neuro-modulation, in addition to affecting macrophage. Silencing the expression of MIF or its receptor within macrophage might be required for investigating the pharmacological mechanism of Oba in mediating CPPS in future.

One limitation in this study is that we only explored the effect of Oba in male mice. It is known that sex difference was exhibited in multiple diseases [37–40], including CPPS [41–43]. In the animal models of neurogenic cystitis and overactive bladder, pelvic pain was observed in both male and female rodents [44,45]. Estrogens can modulate the function of the nervous and immune systems, which can consequently lead to a diverse response to nociceptive stimulus in women [46]. In this case, it is worthy to investigate the effect of Oba to CPPS in the female animal models. Even so, the possibility cannot be ruled out that the efficiency of Oba to alleviate CPPS in females may be same as what is seen in males.

In summary, this study utilizes multiple approaches to reveal the therapeutic effect of Oba in CPPS in animal models. We conclude that Oba alleviate EAP-induced CPPS and pro-inflammatory depolarization of macrophage within prostate via, at least partially, deactivating MIF (Fig. 6). Targeting MIF by Oba may offer a potential therapeutic strategy in the treatment of CPPS.

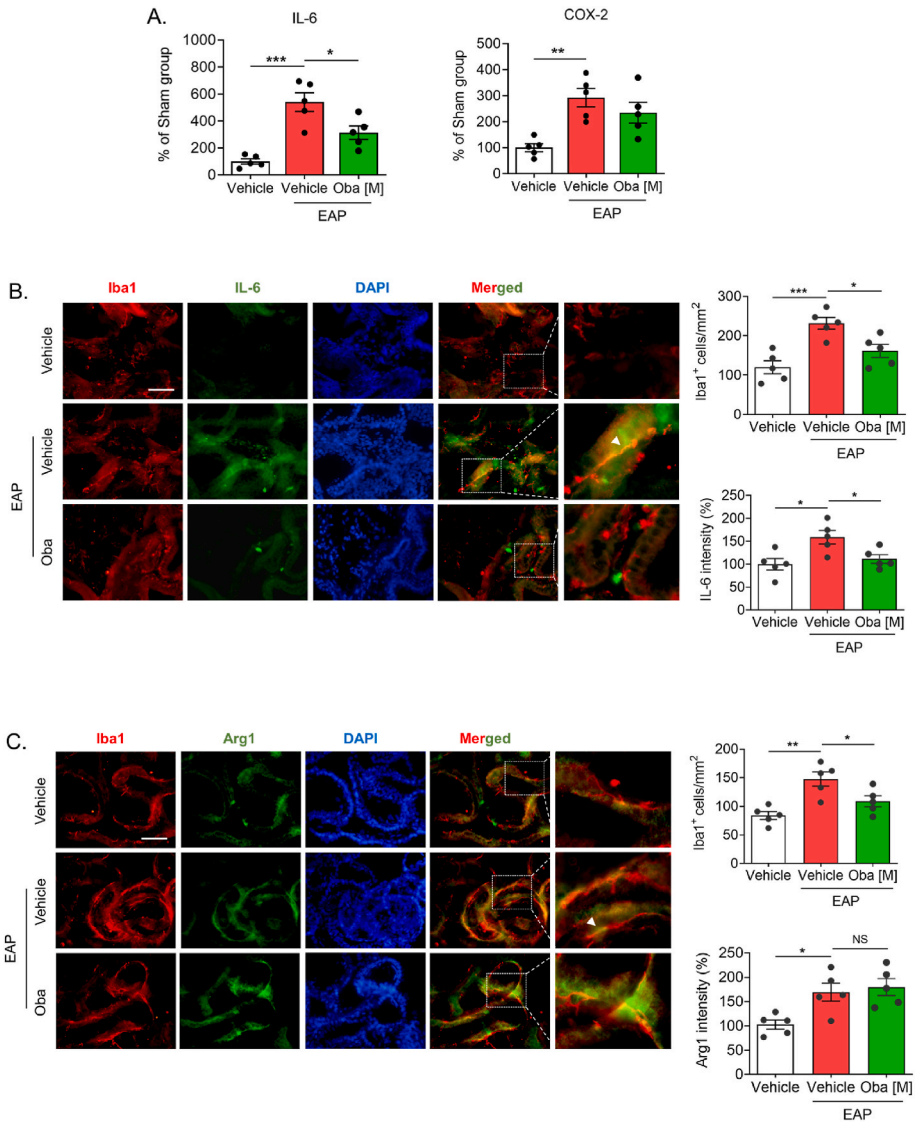

Fig. 4. The effect of Oba to the expression of inflammatory factors and macrophage activation within prostate. (A) The level of IL-6 and COX-2 within prostate assayed by ELISA. (B and C) The distribution of macrophage (Iba1^{+}), IL-6 and Arg1 within prostatic stroma were assayed by immuno-staining. Scale bar: 100 μm. N = 5/group. One-way ANOVA followed by Bonferroni multiple comparisons test. All the values are shown as means ± SEM. Significant difference was considered when $P < 0.05$.

4. Materials and methods

4.1. Animals and study design

All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Animal protocol was approved by the Animal Center of Luohu Hospital. Mice were housed in independent ventilation cages under specific pathogen-free condition. Maximal 5 mice were allowed to place per cage with free access to food and water. Total of 133 young (7–8 week) male C57BL/6J wide-type mice were purchased from GemPharmatech Co., Ltd. (China), in which 15 mice was eliminated due to death after inoculation of prostate homogenate or failure to complete behavior evaluation. The animals were randomly allotted into groups, which was blinded to the examiners and data analysts. An experimental flowchart was presented in Fig. 7.

4.2. CPPS model

The EAP mouse model was successfully induced as previously described with minor modification. Briefly, the prostate homogenate was obtained from the prostate of a Sprague–Dawley rat. The supernatants were collected and emulsified by complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) or incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) respectively. A total of 0.1 mL mixed emulsion was subcutaneously injected into the posterior neck, the lower back, and the base of the tail. In detail, the emulsion of prostate with complete Freund's adjuvant was injected initially. Fourteen days later, animals were further immunized by the prostate with incomplete Freund's adjuvant. At the 28th day after the initial injection of emulsion, a typical behavior of prostatodynia can be observed as previously described [8].

4.3. Chemicals administration

Oba, a limonoid (Mw. 454.51) with identified inhibitory effect to MIF, was administrated once EAP model established. Briefly, Oba was dissolved into 0.5 % methylcellulose and daily gavaged for continuative

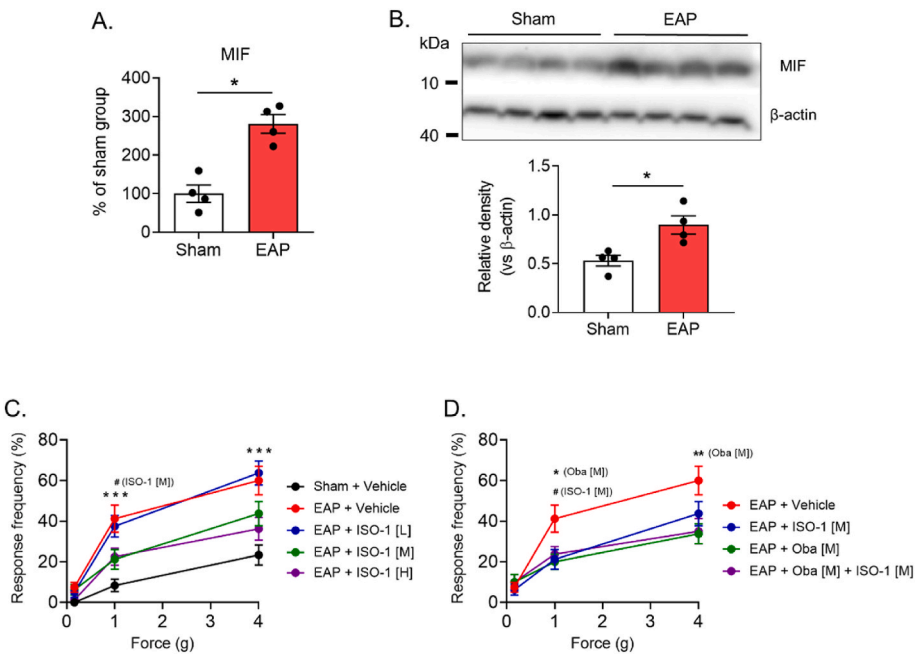

Fig. 5. The effect of Oba and MIF inhibitor ISO-1 on CPPS. (A) The protein level of MIF in (A) serum and (B) prostate of EAP mice. N = 4/group. Unpaired *t*-test. (C) The prostatodynia was assayed 7 days after MIF inhibitor application (Sham + vehicle group vs. EAP + vehicle group, $^{***}p < 0.001$ at 1 g and 4 g; EAP + vehicle group vs. EAP + ISO-1 [M] group, $^{\#}p < 0.05$ at 1 g). (D) The effect of Oba [M] on prostatodynia in the presence or absent of ISO-1 [M] (EAP + vehicle vs. EAP + ISO-1 [M], $^{\#}p < 0.05$ at 1 g; EAP + vehicle vs. EAP + Oba [M], $^{*}p < 0.05$ at 1 g, $^{**}p < 0.01$ at 4 g). N = 8–10/group. Two-way ANOVA followed by Bonferroni multiple comparisons test. All the values are shown as means ± SEM. Significant difference was considered when $P < 0.05$.

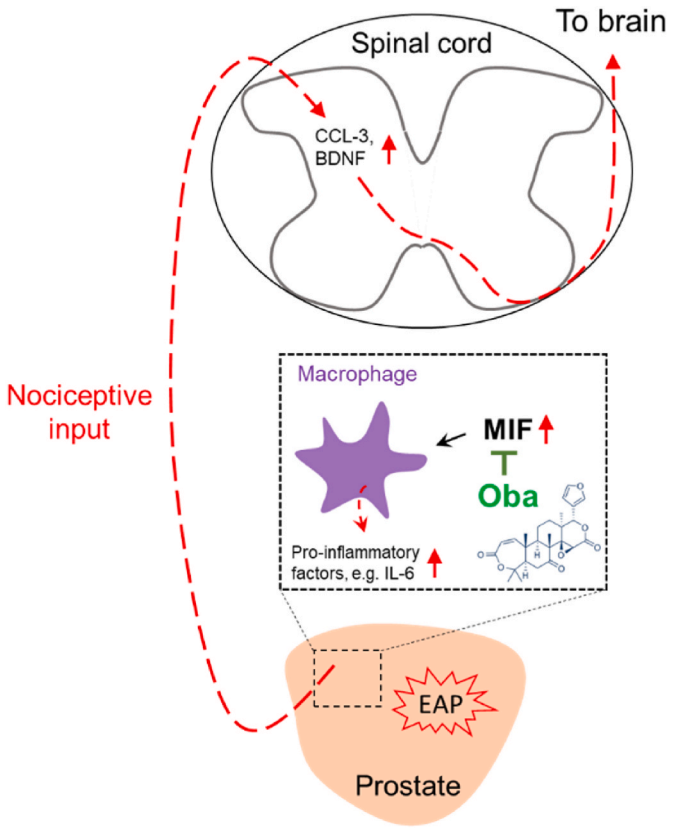

Fig. 6. Schematic illustration of main findings. We proposed that deactivation of MIF by Oba prevents macrophage-derived inflammatory infiltration into prostate, which further alleviates EAP-induced central sensitization and CPPS.

7 days (10, 25, 50 mg/kg/day, 200 μL each time). The same volume of 0.5 % methylcellulose was used in the vehicle control group. ISO-1, a known MIF inhibitor, was administrated through intraperitoneal injection for continuative 7 days once EAP model established (5, 10, 20 mg/kg/day, 200 μL each time). The same volume of 5 % DMSO was used for vehicle control.

4.4. Behavioral tests

All behavior procedures were carried out during the daytime (light cycle). Von Frey force filaments test was performed for measuring tactile allodynia 7 days after EAP model completion. Briefly, mice were habituated to a plastic box with a perforated metal floor for 30 min before test. Monofilaments (Aesthesio Precision Tactile Sensory Evaluator, DanMic Global, USA) with the force of 0.16 g, 1 g and 4 g were individually applied to the pelvic area for 1–2 s with an interval of 5 s. Ten times were attempted, and three types of behaviors were considered as positive responses to stimulation: (i) sharp retraction of the abdomen, (ii) immediate licking or scratching of the area of filament stimulation, (iii) jumping. The response rate was calculated according to the following equation (number of response/number of attempts × 100).

4.5. Histochemistry

Mice were deeply anesthetized with 5 % isoflurane and immediately perfused with phosphate buffer saline followed by 10 % formalin. The isolated prostate was post-fixed in 10 % formalin for 48 h, and prepared for slicing as previously described [47]. Briefly, fixed prostates were gently rinse with running water, followed by dehydrated in a graded alcohol series (75 %, 85 %, 95 % and 100 %), substituted with xylene, and embedded into paraffin using tissue processor (Cat# HP300, Dakewe, China). Embedded tissue was cut into 5 μm thick sections by microtome (Cat# RM2245, Leica, USA). Mounted sections were rehydrated and submerged in hematoxylin (Cat# BL735B, Biosharp) for 10 min, and then submerged in eosin for 30 s at 25 °C. After that, sections were dehydrated followed by immersion in xylene.

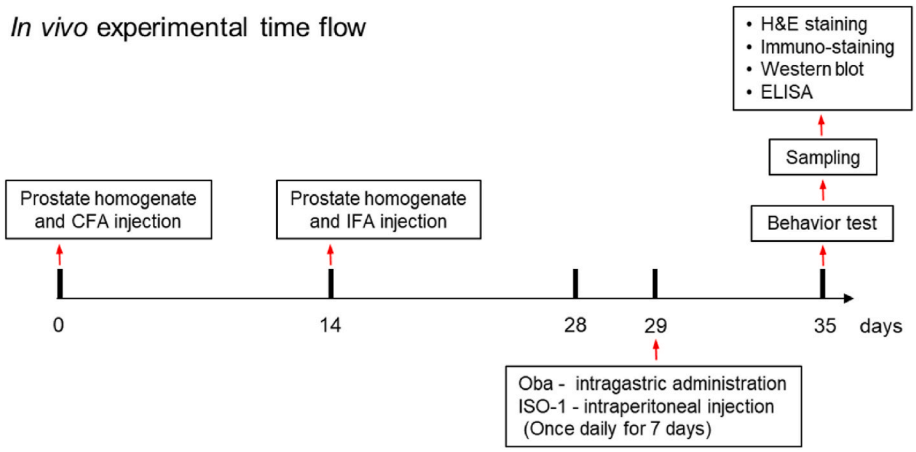

Fig. 7. Schematic representation of the study design.

4.6. Immunofluorescence

The formaldehyde-fixed tissue was graded dehydrated using sucrose (15 % and 30 %) for 48 h 30 μm thick section was cut by cryostat microtome (Cat# POLAR-D-JC, Tissue-Tek Polar, Japan). The sections were then incubated in 0.1 % Triton X-100 (15 min) for permeabilization, followed by 1-h antigen blocking in 1 % bovine serum albumin at 25 °C. Next, sections were covered by the primary antibodies overnight at 4 °C: goat anti-NeuN (1:1000, Cat# NBP3-05554, Novus Biologicals), rabbit anti-c-fos (1:1000, Cat# 2250, Cell Signaling Technology), goat anti-Iba1 (1:100, Cat# NB100-1028, Novus Biologicals), rabbit anti-Arg1 (Alexa Fluor 488 conjugate, 1:400, Cat# 66297S, Cell Signaling Technology) and rabbit anti-IL-6 (1:200, Cat# 12912, Cell Signaling Technology). Once excess primary antibodies were washed off by phosphate buffer saline, the secondary antibodies including anti-goat (Alexa Fluor 594, Cat# ab150136, Abcam) and anti-rabbit (Alexa Fluor 488, Cat# ab150073, Abcam) at 1:500 were added for 1 h at 25 °C. Last, slices were covered by anti-fade solution with cell nucleus colorant 350 nm 4', 6-diamidino-2-phenylindole (Cat# ab104139, Abcam). Regions of interest were randomly acquired from the similar area among groups by microscopy, and quantified using computer software (ImageJ V1.52e).

4.7. Western blot analysis

Prostate was shattered in 1 × NP-40 buffer containing 1 mM phenylmethanesulfonyl fluoride, protease inhibitor (Cat# HY-K0010, MedChemExpress) and phosphatase inhibitor (Cat# HY-K0021, MedChemExpress). Proteins were then harvested from supernatant after centrifuging tissue lysate at 20,000 g for 20 min at 4 °C. Protein concentration was measured by bicinchoninic acid assay (Cat# P0012, Beyotime Biotechnology). 30 μg protein per well were loaded onto 12 % precast protein gels (Cat# P0456 M, Beyotime Biotechnology) for electrophoresis. After that, proteins were transblotted to polyvinylidene fluoride membrane and blocked with 5 % skim milk for 1 h at 25 °C. The membranes were then exposed to primary antibodies rabbit anti-MIF (1:1000, Cat# 3436, Cell Signaling Technology) and rabbit anti-β-actin (1:1000, Cat# 4970S, Cell Signaling Technology) for 1 h at 25 °C. Once excess primary antibody was washed off by tris-buffered saline with tween 20, membranes were incubated with anti-rabbit HRP-linked secondary antibody (1:4000, Cat# 7074S, Cell Signaling Technology) for 1 h at 25 °C. The gray value of regions of interest were quantified by ImageJ (V1.52e).

4.8. ELISA

Prostatic and spinal cord homogenate was prepared as described previously [9]. The level of cytokines, including IL-6 (Cat# PI326, Beyotime), BDNF (Cat# PB070, Beyotime), COX-2 (Cat# ab210574, Abcam), CCL-3 (Cat# ab100726, Abcam) and MIF (Cat# ab209885, Abcam) were quantified by commercially sandwich-type ELISA kits according to manufacturer's instruction.

4.9. Statistical analyses

The results are presented as means ± SEM. Data was analyzed by GraphPad Prism (version 9.0.0 for Windows, GraphPad Software, San Diego, California USA). Normal distribution and homogeneity of variance were examined by Kolmogorov–Smirnov test. Unpaired *t*-test was applied for comparison between two groups. One-way or two-way ANOVA followed by Bonferroni test were applied for multiple comparison among groups. Significant differences were set when $P < 0.05$.

Author contributions

Conceptualization, F.B., F.L., C.L.; Formal analysis, Y.W., X.W., Y.C., P.D., G.L.; Funding acquisition, F.B., C.L.; Investigation, Y.W., Z.D., X. W.; Methodology, Y.W., W.T., Z.G.; Supervision, F.B., F.L., C.L.; Writing – original draft, Y.W., F.B.; Writing – review & editing, F.L., C.L.

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Institutional review board statement

The animal study protocol was approved by the Experimental Animal Ethics Committee of Luohu Hospital (Approved number: No. 20210901-SZY), and the approval date was 1 September 2021.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101565.

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