Placental mesenchymal stem cells improve ovarian function in a rat model of premature ovarian insufficiency by inhibiting NLRP3 inflammasome activity

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Research Article

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Abstract

Background

Premature ovarian insufficiency (POI) is a common clinical problem but there are currently no effective therapies. Pyroptosis induced by the NLRP3 inflammasome is considered a possible mechanism of POI. Placental mesenchymal stem cells (PMSCs) have excellent immunomodulatory potential and offer a promising method for treating POI.

Methods

Female Sprague–Dawley rats were randomly divided into four treatment groups: control (no POI), POI with no PMSCs, POI with PMSCs transplant, and POI with hormones (estrogen + progesterone) as positive control. POI was induced by exposure to 4-vinylcyclohexene diepoxide (VCD) for 15 days. After four weeks, all animals were euthanized and examined for pathology. Hormone levels were measured and ovarian function was evaluated in relation to the estrous cycle. Levels of NLRP3 inflammasome pathway proteins were determined by immunohistochemistry and western blot.

Results

VCD significantly damaged the rat follicles at different estrous stages. Injection of human PMSCs improved ovarian function and reproductive ability of POI rats compared to the sham and hormone groups. Our data also showed that PMSCs could remarkably suppress cell pyroptosis via downregulation of the NLRP3 inflammasome, caspase-1, IL-1β and IL-18 compared to the other two groups. The human PMSCs increased the expression of IL-4 and IL-10 and decreased pro-inflammatory factors.

Conclusions

Our findings revealed a novel mechanism of follicular dysfunction and ovarian fibrosis via activation of the NLRP3 inflammasome followed by secretion of pro-inflammatory factors. Transplantation of PMSCs into POI rats suppressed pro-inflammatory factor production, NLRP3 inflammasome formation and pyroptosis, and improved ovarian function.

Background

The low estrogen levels and loss of female fertility caused by premature ovarian insufficiency (POI) can lead to serious disease. The pathogenesis of POI is currently unclear, and there is no effective method to restore ovarian function. Women with POI develop amenorrhea before the age of 40, accompanied by endocrine symptoms such as increased Follicle stimulating hormone (FSH) levels (FSH ≥ 25 U/L) and decreased estrogen levels [1]. Clinical manifestations vary in severity, but include night sweats, poor sleep,

mood changes, inattention, osteoporosis, fluctuations in blood lipids and blood pressure, and cardiovascular system diseases. Hormone replacement therapy, as a first-line clinical treatment, can reduce the symptoms caused by estrogen deficiency, but there is still no method for restoring follicular development in failed ovaries [2]. In recent years, many studies have suggested that transplantation of stem cells may be able to restore ovarian function [3]. When mesenchymal stem cells (MSCs) were used to treat POI, there was a decrease in apoptosis of ovarian granulosa cells (GCs), recovery of ovarian function, and increased levels of sex hormones [4–7]; however, the underlying mechanism remained elusive. Human placenta is a valuable biological resource and a promising source of stem cells. Recent reports suggested that human placental mesenchymal stem cells (hPMSCs) can restore ovarian function by exerting anti-inflammatory and tissue regenerative effects, the mechanisms of which mainly focus on improving the ovarian microenvironment [8–11]. We previously found that a CD200 + subset within hPMSCs presented a stronger immunomodulatory potential [12]. Inflammatory cell death (pyroptosis) induced by NLRP3 inflammasomes is considered a possible mechanism of POI [13]. Whether hPMSCs can reduce pyroptosis in ovarian tissue induced by inflammasomes, protect ovarian tissue from inflammatory damage, and reduce the occurrence of POI remains to be determined.

Inflammasomes are cytoplasmic polyprotein complexes, which mediate the host's immune response to microbial infection and cell damage. The aggregation of inflammasomes causes proteolytic cleavage of procaspase-1 to generate activated caspase-1, which can induce a pro-inflammatory form of cell death, known as pyroptosis. So far, the receptor proteins include NLRP1, NLRP3, NLRC4 and other members of the NLR family and Absent in Melanoma 2 (AIM2) from the HIN200 family. According to different receptor proteins, inflammasomes mainly contain NLRP3, NLRC4, AIM2, and NLRP1. NLRP3 is relatively nonspecific as it responds to a large number of agonists that are unrelated in origin, chemical composition, and structural properties. The secretion of proinflammatory cytokines IL-1β and IL-18 and pyroptosis caused by the activated NLRP3 inflammasome help to resist exogenous microbial infection and endogenous cell damage, and to maintain the internal environment in a stable state [14]. Studies have shown that human MSCs increased cell viability and proliferation and alleviated tissue damage by reducing NLRP3 inflammasome formation, caspase-1 activation and Interleukin-1β (IL-1β) maturation [15, 16]; but, whether there is such a mechanism for the alleviation of POI by MSC treatment remains unclear.

POI induced by 4-vinylcyclohexene diepoxide (VCD) has been increasingly used in recent years as a model for testing various therapies. In previous research, it was found that long-term, high-dose VCD not only kills small follicles as part of the pathogenesis of premature ovarian failure, but also accelerates the development and discharge of follicles in the growth phase [17]. Combined exposure to VCD and phthalates significantly reduced the numbers of primary follicles and consequently increased the risk of premature menopause; combined exposure to phthalates and VCD in early menopausal women is likely to aggravate POI [18]. We found that VCD had the advantages of safety, strong alignment, and good success rate in producing a POI model.

Materials And Methods

Laboratory animals

Female Sprague–Dawley (SD) rats were supplied by the Laboratory Animal Center of Ningxia Medical University and housed in a specific pathogen free (SPF) area. The rats were caged individually and kept at a temperature of 23 ± 2°C with a 12 h/12 h light/dark cycle, and food and water provided ad libitum. The rats were acclimatized for one week before starting the experiment. All procedures were implemented in accordance with the criteria for the care and use of laboratory animals of Ningxia Medical University. The protocol was approved by the Academic Committee on the Ethics of Animal Experiments of Ningxia Medical University (Permit Number: SCXK(Ning)2015-0001).

Premature ovarian insufficiency (POI) model establishment

Female SD rats (n = 60, eight-weeks-old; weight 230–255 g) were divided randomly into four groups of 15 animals each. The control received equal volume of castor oil, while the other three groups received daily intraperitoneal injections of 80 mg/kg of VCD (Sigma-Aldrich, Germany)that soluble in castor oil(MCE, China)for 15 days. The sex hormone levels and ovarian histomorphology were used to measure the success rate for creation of the POI model.

Research Design

The VCD-treated rats were randomly assigned to three groups: control with no PMSCs (saline injection only), injection of PMSCs, and hormone treatment (estrogen + progesterone) with no PMSCs. To test the effects of PMSC transplantation, rats were intravenously injected with 1×10^7cells/rat on the first and 7th days. The hormone treatment group rats received no PMSCs, but were injected with estrogen (1 mg/kg) and progesterone (2.5 mg/kg) (Abbott, Netherlands) every 4 days for 28 days. All animals were continuously observed for 28 days [17]. At the end of the experiment, the rats were fasted overnight and anesthetized by i.p. injection of 0.4 mL/kg sodium pentobarbitone (100 mg/mL). Blood samples were taken from the heart, allowed to clot at room temperature and centrifuged at 4℃ to obtain serum. The rats were euthanized with CO_2 and bilateral ovaries were removed. One ovary was fixed with 4% paraformaldehyde for histology analysis, and the other was stored at -80℃ for protein immunoblotting.

Estrous cycle characterization

Vaginal exfoliation cytology smears were obtained from the experimental rats. Animals were immobilized, and 0.1ml of normal saline in a Pasteur pipet was gently inserted 5–6 mm into the vagina, aspirated 2–3 times, and examined under a microscope. The estrous cycle was categorized as follows: (1) pre-estrus with large numbers of small nucleated epithelial cells, individual or in clusters; (2) estrus with large numbers of irregular keratinocytes and small numbers of nucleated epithelial cells; (3) post-estrus with keratinocytes, nucleated epithelial cells and leukocytes in equal proportions; and (4) inter-estrus with large numbers of leukocytes.

Isolation and culture of human placental MSCs

Human placental tissue was obtained following informed consent from healthy volunteers who tested negative for HIV-I, hepatitis B, hepatitis C, cytomegalovirus, rubella virus and herpes simplex virus. The acquisition protocol and the informed consent document were approved by the Institutional Ethics Committee of the general hospital of Ningxia medical university. Placental chorionic membranes were mechanically separated from the fetal side, cut into 1 mm3 pieces, and washed with phosphate buffered saline (PBS). The tissue pieces were incubated for 60 min at 37°C with MSC-ACF tissue digestion mix (VivaCell Biosciences, Shanghai, China). The program of tissue dissociation was executed on the GentleMACS Octo-dissociator with heaters (Miltenyi Biotec, Germany). The digested tissue was washed twice with PBS, sequentially filtered through a 70 μm filter and centrifuged at 300 g for 5 min at room temperature. The pelleted cells were seeded in 75 cm^2 culture flasks containing UltraCulture™ medium (Lonza, Grand Island, NY, USA) supplemented with Ultroser G serum substitute (Pall, USA) and 2 mM GlutaMAX™(Gibco) and incubated at 37°C in 5% CO^2. After 4–5 passages, the cells were tested for MSC surface markers by flow cytometry and used for transplantation in POI rats.

Flow cytometry assay

A flow cytometry assay for MSC markers was performed on PMSCs from passage three (P3) cultures. The cells were harvested and washed, then incubated with fluor-conjugated (PE or FITC) antibodies to CD105, CD90, CD73, CD45, CD14 and CD34 (BioLegend, San Diego, USA). The flow cytometry analysis was performed on a BD FACSCalibur™.

In vitro analysis of differentiation capacity

PMSCs were cultured with induction medium kits (ScienCell Research Laboratories, Carlsbad, USA) for adipogenesis, chondrogenesis and osteogenesis. For adipogenic and osteogenic differentiation, PMSCs were expanded in CellBind-treated culture dishes (Corning, CellBIND Surface). The medium was replaced after 24 h. Cells were allowed to differentiate for three weeks then fixed and stained with oil red O (ScienCell Research Laboratories, Carlsbad, USA) or alizarin red solution (ScienCell Research Laboratories, Carlsbad, USA). For chondrogenic differentiation, MSCs were cultured as pellets in complete MSC chondrogenic differentiation medium (ScienCell Research Laboratories, Carlsbad, USA), with weekly medium replacement, for four weeks. At the end of the incubation, the cell aggregates were fixed in 4% paraformaldehyde for 30 min and dehydrated with 30% sucrose solution overnight at 4°C. The spherules were frozen within embedding agent (Tissue-Tek O.C.T. compound; Sakura, Japan) and sectioned at -20°C using a cryostat (Leica, Germany) followed by staining with an Alcian blue kit, according to the instructions and digitally imaged under a microscope (Olympus BX51, Japan)

Mixed lymphocyte culture (MLC) to identify immune characteristics of PMSCs

Healthy human peripheral blood (20 mL) was collected in heparinized tubes and cells were separate by centrifugation on Histopaque-1077 to obtain human peripheral blood monocytes (PBMCs), which are reactive cells. The PMSCs were pretreated with mitomycin C (2 μg/ml, Sigma, Germany) and seeded in 6-well plates as stimulated cells. Response cells (10:1 ratio to stimulated cells) were added to the upper

chamber of a 24 mm transwell in a 6-well plate, and agent I and II from the CIK activated agent kit (CIK-CYT, DaKeWei, China) were added to each well according to the instructions. After five days of co-culture, the distribution of lymphocyte subtypes was determined by flow cytometry. In another experiments, after co-cultivation with PMSCs for 48 hours, the PBMCs were removed and replaced with fresh medium. The culture medium was collected after 24 hours, and the levels of interferon gamma (IFN-γ), interleukin-2 (IL-2) and interleukin-10 (IL-10) were measured by ELISA.

ELISA assay

Serum anti-müllerian hormone (AMH), follicle-stimulating hormone (FSH), and estradiol (E2) concentration were determined using ELISA kits according to instructions (Shanghai JiangLai Biotechnology Co., Ltd). Ninety-six-well plates coated with antibodies were incubated with serum samples (1:10 dilution, n = 8) at RT for 2 h. Absorbance was measured with a microplate reader, and compared to standard curves to determine hormone concentrations.

Histological evaluation

Tissues were fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 24 h, then dehydrated, cleared and embedded in paraffin. The tissue blocks were sectioned at 5 μm using a microtome (Leica, Germany). The sections on slides were treated with 3% hydrogen peroxide for 20 min, blocked with normal goat serum for 1 h at RT and then incubated overnight at 4°C with primary antibodies against NLRP3, NFκB, ASC(apoptosis-associated speck-like protein containing CARD), Caspase-1, TLR4, TNF-α and IL-1β, diluted 1:500-1:1000. After washing, the sections were incubated with secondary antibody, visualized using diaminobenzidine substrate, and counterstained with hematoxylin, or hematoxylin and eosin (H&E). Images were captured by TissueFAXS CHROMA and analyzed by TissueFAXS imaging software, 7.0.

Van Gieson (VG) staining for ovarian fibers

Dewaxed ovarian tissue sections were first stained with celestin blue (nuclei) for 5 mins, washed with DIW and stained with hematoxylin for 5 mins. After washing well in running tap water for 5 mins, the slides were flooded with Curtis stain (saturated aqueous picric acid, 1% ponceau S, glacial acetic acid at a 9:1:1 ratio) for 5 mins, dehydrate rapidly in ethanol series, cleared and mounted. The elastic fibers were stained blue-black and background was stained yellow.

Western immunoblotting

Ovarian tissue samples were placed in RIPA (Radio Immunoprecipitation Assay) lysis buffer with a cocktail of protease and phosphatase inhibitors and homogenized using a frozen tissue grinder. BCA assay was used to quantify the amount of total protein in each sample. Equal amounts (20 μg) of protein were loaded into the wells of a 10% SDS-PAGE gel and run for 2 h at 150 V. The proteins were transferred from the gel to a PVDF (Polyvinylidene Fluoride) membrane for 1 h at 300 mA. The membrane was blocked for 1 h at RT with TBST + 5% nonfat dry milk, then with appropriate dilutions of primary antibody in blocking buffer overnight at 4°C. The membrane was washed three times with TBST, then incubated

with the recommended dilution of HRP-conjugated secondary antibody in blocking buffer at RT for 1 h. The membrane was washed three times with TBST (Tris Buffered Saline with Tween® 20), 5 min each, and incubated in enhanced chemiluminescence (ECL) reagent according to manufacturer's directions. Images were acquired using darkroom development techniques for chemiluminescence with the GE-Amersham Imager 600. Intensities of protein bands were measured using Image J. Relative protein levels were normalized to expression of GAPDH.

Statistical analysis

SPSS 23.0 (IBM, Armonk, NY, U.S.A.) was used for statistical analyses. The Shapiro-Wilk (S-W) test was used to determine normality and lognormality of the data. Data that were normally distributed were analyzed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Data are expressed as mean ± standard deviation. Non-normally distributed continuous variables are expressed as medians and were compared using a nonparametric test. The Wilcoxon-test was used to determine whether differences were statistically significant by comparing the paired samples before and after treatment, and the Mann-Whitney test was used to determine significance by comparing the unpaired samples with the control group, before or after treatment. A p-value < 0.05 was considered statistically significant.

Results

VCD-induced rat model presents a POI phenotype

The experimental design and progression of steps are shown in Figure 1A. After 15 days of daily i.p. injections with VCD (80 mg/kg), the body weights in each group were measured and vaginal smears were obtained every morning at 8 am to confirm establishment of the rat model of POI. The estrous cycle showed a regular progression from pre-estrus, to estrus, then post-estrus and inter-estrus in 5-6 days. About 20 days after the last injection of VCD, the rats' estrous cycles were disrupted, and this resulted in a longer period of estrus (**Fig. 1C**). At 28 days after the last injection, H&E staining was used to visualize changes in ovarian morphology. Compared with the control group, H&E staining of ovarian sections revealed that the number of early follicles in the ovaries of the rats, including primordial follicles and primary follicles were significantly decreased, the arrangement of GCs was disordered, and interstitial fibrosis was more severe in the POI group (**Fig. 1B, 3C**). Body weight, ovarian weight and ovarian size were significantly reduced in the POI group (**Fig. 1D-F**). Collectively, these findings demonstrate that VCD significantly damaged the follicles at different stages. Thus, the POI model was successfully established.

Characterization of human PMSCs

Placenta-derived MSCs show typical MSC phenotype and morphology, with a characteristic spindle-like shape (**Fig. 2A**). Oil red O staining showed accumulated triacylglycerols indicative of adipogenesis, alizarin red S staining showed mineral deposition from osteogenesis, and Alcian blue staining showed proteoglycan from chondrogenesis in the cells. Flow cytometry analysis demonstrated that over 95% of

the PMSCs retained their ability to express MSC surface immunophenotypic markers, such as CD105 (Clusters of Differentiation 105), CD73 and CD90, but lacked expression of hematopoietic markers CD34, CD14, CD45, and the MHC class II molecule, HLA-DR (**Fig. 2B**).

PMSCs preserve ovarian function in VCD-induced rat POI model

Animal experiments were performed according to the experimental timeline (**Fig. 1A**). PMSC transplantation was done after establishment of the POI model and the effects of PMSCs on ovarian function were determined. At ten days after the first injection of PMSCs, we observed a partial restoration of the estrous cycle in the MSCs group and hormone therapy group (**Fig. 3A**) compared to the saline control group. At 28 days after the first PMSC injection, the ovarian tissue structure in the control group was disordered, there were multiple atresia follicles, and the GC layer significantly decreased (p<0.01). Compared to control, H&E staining of ovarian sections revealed that the numbers of follicles in all stages were significantly increased in the PMSC group. The overall ovarian tissue morphology was improved along with numbers of primary and secondary follicles, while the number of atresia follicles had decreased (p<0.01) (**Fig. 3B, C**). H&E staining showed good follicle formation at all stages in the control group, and mature follicles were overdeveloped in the hormone therapy group. Ovarian function was evaluated in terms of follicle-stimulating hormone (FSH), estrogen (17β-E_2), luteinizing hormone (LH), anti-Müllerian hormone (AMH) and 17-hydroxyprogesterone (17-OHP). In the POI model rats, loss of ovarian function resulted in abnormally low levels of estrogen, and AMH, but high levels of FSH. There were significant increases in serum levels of sex hormones E2 (p<0.01) and AMH (p<0.01) after injection of PMSCs, and FSH was effectively reduced to close to normal levels (**Fig. 3D**). Fertility results of the four groups showed that the transplantation of PMSCs significantly improved the reproductive functions of POI rats (**Fig. 3E**). The number of offspring was significantly suppressed in the POI group compared with the normal group (p<0.001). In contrast, treatment with hPMSCs significantly increased the number of offspring compared with the POI group without stem cells (p<0.05) (**Fig. 3F**). However, after four weeks of hormone therapy, the first litter was similar to those of the normal group, but the number of offspring decreased in the latter two litters. The time-to-birth in the POI group was significantly prolonged, but this effect was reversed by hPMSCs transplantation. Taken together, these findings showed that administering hPMSCs can greatly improve ovarian function and reproductive ability in POI rats.

PMSCs alleviate VCD-induced rat ovarian fibrosis by down-regulating pro-inflammatory factors

PMSCs have a significant inhibitory effect on the proliferation of T lymphocytes stimulated by CD3 and CD28. When activated T lymphocytes were co-cultured with PMSCs at a ratio of 10:1, the proliferation of $CD4^+$T lymphocytes was inhibited (p<0.01); however, the proportion of $CD4^+CD25^+foxp3^+$ regulatory T cells was significantly increased after co-culture with PMSCs (**Fig. 4A**). When activated T lymphocytes

and PMSCs were co-cultured at a ratio of 10:1 for 48 hours, PMSCs significantly down-regulated the level of IFN-γ, IL-2 and TNF-α secreted by activated T lymphocytes (p<0.05). The level of IL-4, IL-10 and TGF-β secretion by regulatory T lymphocytes was significantly up-regulated by PMSCs (p<0.05) (**Fig. 4B**). Thus, PMSCs have a significant inhibitory effect on the proliferation of TH1 lymphocytes and their level of cytokine secretion.

VG staining results are shown in figure 4C. The control group ovarian tissue showed significantly greater numbers of red and yellow collagen fibers, and the degree of fibrosis was higher than that of the normal group and hPMSCs transplanted group (p<0.01). The difference between control and the hormone group was not statistically significant (p >0.05).

PMSCs regulate the expression of the NLRP3 inflammasome pathway and related inflammatory factors in ovarian tissue

The results of immunohistochemistry on ovary sections are shown in figure 5A. The staining for NALP3 in POI rats was primarily positive and IL-1β was up-regulated compared with the normal group. In the hPMSCs transplant group, the ovaries were minimally stained for NALP3 and IL-1β.
NFκB nuclear staining was observed in the POI saline group but not in the other groups, indicating that NFκB pathway activity was significantly restored. The hPMSC transplant group had significantly lower NALP3 inflammasome expression compared to the control and hormone groups.

The extracted ovarian proteins were subjected to immunoblotting and quantitation (**Fig. 5B, C**). Compared to the normal group, the POI rat ovaries showed increased expression of NALP3, ASC, and IL-1β. The expression of NALP3 in the hormone group and the PMSC group decreased significantly. The PMSC treatment group also had lower expression of ASC and caspase-1 (both precursor and cleaved). Compared to control, expression of the TLR4/NFκB signaling pathway proteins TLR4, IL-1β, TNF-α and NFκB (p<0.05) was decreased in the PMSC group (**Fig. 5B, C**).

Discussion

Countries around the world are making efforts to restore the birth population. However, in addition to facing the choice of "to give birth or not to give birth", those women who "want to give birth but cannot" still encounter the difficulties of childbearing. From a clinical point of view, the ovarian aging process that results in impaired female fertility is currently pharmacologically uncontrollable and presents health threats to perimenopausal women such as osteoporosis and cardiovascular disease [2, 19].

It has been found that exposure to environmental toxins like pesticides, insecticides, exhaust gas, food additives, and second-hand smoke, can cause reproductive toxicity leading to reduction in the number of

oocytes and their quality, menstrual disorders, decreased follicular reserve, and infertility [20]. Estrogen replacement therapy (HRT) is very important for young patients with POI because it can relieve symptoms of low estrogen and can prevent long-term complications. But long-term HRT also has certain risks, such as the occurrence of endometrial cancer and breast cancer [1]. Mesenchymal stem cell transplantation is considered to be a very promising treatment strategy for reversing the negative effects of POI [3]. One of the key events that contribute to ovarian aging includes follicular atresia as it is associated with the pyroptosis and necrosis of granulosa cells and thecal cells in aging ovaries. Follicular atresia shows many pathophysiological alterations associated with physiological aging such as mitochondrial dysfunction, oxidative stress, and inflammation.

The NLRP3 inflammasome recognizes a large and highly diverse set of agonists that respond to pathogen invasion, environmental stress, and tissue pathology. Studies have evaluated the role of the NLRP3 inflammasome in ovarian aging and female fertility [13, 21, 22]. Age-dependent increased expression of NLRP3 in the ovary was observed in WT mice during reproductive aging [13]. As an important part of the inflammatory response, the inflammasome was discovered by the group of Jürg Tschopp in 2002 [23]. The inflammasome is a protein complex several microns in diameter that mainly includes receptor protein (receptor), adaptor protein (adaptor), ASC and the downstream caspase-1. After the receptor protein is activated by an agonist, it will attract ASC and caspase-1 to assemble into inflammasomes, thereby inducing self-cleavage and activation of caspase-1. Active caspase-1promotes the maturation and secretion of pro-inflammatory cytokines including IL-1β and IL-18, but it also triggers pyroptosis, which disposes of damaged cells and pathogens [24]. Multiple molecular or cellular events, including changes in ion flux, mitochondrial dysfunction, reactive oxygen species (ROS) generation, and lysosomal damage, have been shown to activate the NLRP3 inflammasome [14, 25]. The active NLRP3 inflammasome causes follicular dysfunction and turns on the ovarian fibrosis signaling pathway [21]. There are currently few reports confirming that PMSCs protect ovaries against the damaging effects of POI, and it is unclear whether the mechanism involves suppression of the NLRP3 inflammasome by PMSCs. In this study, we clearly demonstrated that pyroptotic factors, such as NLRP3, ASC, caspase-1and IL-1β in the ovary, were also activated by VCD. Aged ovarian tissue is exposed to priming stimuli, such as ligands for toll-like receptors (TLRs), NLRs (e.g. NOD1 and NOD2), ROS or inflammatory cytokines, which activate the transcription factor NF-κB. NF-κB upregulates the expression of NLRP3 and promotes inflammasome formation and conversion of pro-IL-1β. Downstream maturation and release of IL-1and IL-18 continues to be involved in the feedback activation of the NLRP3 inflammasome via the TLR4/NFκB pathway, further exacerbating inflammation. In our experiments, immunohistochemistry and western blot were used to quantitate NLRP3 inflammasome protein levels in the ovarian tissues of rats in each group. The expression of NLRP3, caspase-1, IL-1β, and IL-18 were measured to indirectly detect the level of inflammasome activity. At the same time, the nuclear translocation of NFκB and the NFκB signaling pathway activity were detected in ovarian cells. The results of that experiment showed that the expression levels of NLRP3, ASC, caspase-1, IL-1β and IL-18 decreased significantly after the injection of PMSCs into POI rats. There were a large number of NFκB nuclear translocations in the ovarian tissue of

the rats in the POI group, but the level of NFκB was significantly reduced by PMSCs transplantation. These results suggest that the activity of the NFκB inflammatory pathway was significantly decreased, which should inhibit activation of NLRP3 inflammasomes in damaged ovaries. The inhibition of inflammatory pathways may be caused by a reduction in the secretion of inflammatory factors in the ovarian microenvironment, and thus play a role in preventing reproductive harm from VCD-induced POI. In contrast, PMSC transplantation restored the hormone secretion function of granulosa cells and theca cells by inhibiting the expression of pyroptosis proteins such as NLRP3, ASC, caspase-1and IL-1β. In fact, the anti-inflammatory activity of PMSCs has already been demonstrated by modification of the type of paracrine inflammatory factors caused by proliferation and differentiation of lymphocytes. Thus, the results of this experiment show that PMSCs can inhibit the activation of ovarian NLRP3 inflammasomes and decrease the degree of inflammation and pyroptotic death of ovarian GCs in POI rats.

During the development of POI, follicular dysfunction and anovulation are closely related to ovarian fibrosis. Numerous studies suggest that persistent inflammation contributes to ovarian injury. The findings of the present study indicate that activation of the NLRP3 inflammasome accelerates ovarian fibrosis in POI rats. Thus, the NLRP3 inflammasome is implicated as a potential target in the prevention of ovarian fibrosis progression. In this study, our findings revealed a novel mechanism by which VCD activated the NLRP3 inflammasome causing pro-inflammatory factor secretion, and driving follicular dysfunction and ovarian fibrosis; however, administering hPMSCs significantly improved ovarian function by blocking this positive feedback loop.

Conclusions

Our findings revealed a novel mechanism of follicular dysfunction and ovarian fibrosis via activation of the NLRP3 inflammasome followed by secretion of pro-inflammatory factors. Transplantation of hPMSCs into POI rats suppressed pro-inflammatory factor production, NLRP3 inflammasome formation and pyroptosis, and improved ovarian function. POI has become a disease that seriously endangers women’s reproduction and health. In the future, the mechanism of the pathogenesis of POI, the protection of ovarian function in groups at high risk of POI, and the development of new POI therapies should be the focus and direction of research to lay the foundation for the early diagnosis and mitigation of POI.

Abbreviations

PMSCs
Placental mesenchymal stem cells
POI
Premature ovarian insufficiency
VCD
4-vinylcyclohexene diepoxide
FSH
Follicle stimulating hormone

GCs
granulosa cells
E2
Estradiol
P4
progesterone
AMH
Serum anti-müllerian hormone
17αOHP
17-α-hydoxy progesterone
LH
Luteinizing hormone
IL-1β
Interleukin-1β
NLRP3
NOD-like receptor thermal protein domain associated protein 3
HE
Hematoxylin and Eosin
SPF
Specific Pathogen Free
SD
Sprague–Dawley
MLC
mixed-lymphocyte culture
PBMCs
peripheral blood monocytes
IFN-γ
interferon gamma
IL-2
interleukin-2
IL-10
interleukin-10
NF-κB
Nuclear Factor Kappa Beta
PFA
paraformaldehyde
VG
Van Gieson
RIPA
Radio Immunoprecipitation Assay Lysis

TBST (Tris Buffered Saline with Tween® 20)
PVDF
Polyvinylidene Fluoride
GAPDH
Glyceraldehyde-3-phosphate dehydrogenase
PBS
Phosphate-buffered saline
NC
Negative control
HRT
Estrogen replacement therapy
ROS
reactive oxygen species
TLRs
toll-like receptors
ASC
apoptosis-associated speck-like protein containing CARD

Declarations

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Authors' contributions

HM Ma, XY Liang, and DM Chen conceived and designed the experiments; DM Chen and HM Ma analyzed the data and drafted the manuscript; MH Ma, N Hu, SL Guo, FY Zhang, and Xiaona Ma and SS Xin performed experiments and acquired data; SD Liu and XN Ma collected samples; XY Liang interpreted data and critically revised the manuscript. All authors read and approved the final version of the manuscript.

Availability of data and material

Data sharing not applicable to this article as no datasets were generated or analysed during the current study. The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Human placental tissue was collected with a protocol approved by the Ethics Committee for the Conduct of Human Research at the General Hospital of Ningxia Medical University. Written consent was obtained

from every individual according to the Ethics Committee for the Conduct of Human Research protocol. All participants provided written informed consent for the publication of the data. The Human Research Ethics Committee at the General Hospital of Ningxia Medical University approved this study.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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References

1. Chon SJ, Umair Z, Yoon MS. Premature Ovarian Insufficiency: Past, Present, and Future. Front Cell Dev Biol. 2021;9:672890.
2. Nguyen HH, Milat F, Vincent AJ. New insights into the diagnosis and management of bone health in premature ovarian insufficiency. Climacteric. 2021;24:481–90.
3. Ulin M, Cetin E, Hobeika E, Chugh RM, Park HS, Esfandyari S. A. Al-Hendy, Human Mesenchymal Stem Cell Therapy and Other Novel Treatment Approaches for Premature Ovarian Insufficiency. Reprod Sci. 2021;28:1688–96.
4. Tian C, He J, An Y, Yang Z, Yan D, Pan H, Lv G, Li Y, Wang Y, Yang Y, Zhu G, He Z, Zhu X, Pan X. Bone marrow mesenchymal stem cells derived from juvenile macaques reversed ovarian ageing in elderly macaques. Stem Cell Res Ther. 2021;12:460.
5. Shareghi-Oskoue O, Aghebati-Maleki L, Yousefi M. Transplantation of human umbilical cord mesenchymal stem cells to treat premature ovarian failure. Stem Cell Res Ther. 2021;12:454.
6. Salvatore G, De Felici M, Dolci S, Tudisco C, Cicconi R, Campagnolo L, Camaioni A, Klinger FG. Human adipose-derived stromal cells transplantation prolongs reproductive lifespan on mouse models of mild and severe premature ovarian insufficiency. Stem Cell Res Ther. 2021;12:537.
7. Li Z, Zhang M, Zheng J, Tian Y, Zhang H, Tan Y, Li Q, Zhang J, Huang X. Human Umbilical Cord Mesenchymal Stem Cell-Derived Exosomes Improve Ovarian Function and Proliferation of Premature Ovarian Insufficiency by Regulating the Hippo Signaling Pathway. Front Endocrinol (Lausanne). 2021;12:711902.

8. Cho J, Kim TH, Seok J, Jun JH, Park H, Kweon M, Lim JY, Kim GJ. Vascular remodeling by placenta-derived mesenchymal stem cells restores ovarian function in ovariectomized rat model via the VEGF pathway. Lab Invest. 2021;101:304–17.
9. Kim KH, Kim EY, Kim GJ, Ko JJ, Cha KY, Koong MK, Lee KA. Human placenta-derived mesenchymal stem cells stimulate ovarian function via miR-145 and bone morphogenetic protein signaling in aged rats. Stem Cell Res Ther. 2020;11:472.
10. Choi JH, Seok J, Lim SM, Kim TH, Kim GJ. Microenvironmental changes induced by placenta-derived mesenchymal stem cells restore ovarian function in ovariectomized rats via activation of the PI3K-FOXO3 pathway. Stem Cell Res Ther. 2020;11:486.
11. Yin N, Zhao W, Luo Q, Yuan W, Luan X, Zhang H. Restoring Ovarian Function With Human Placenta-Derived Mesenchymal Stem Cells in Autoimmune-Induced Premature Ovarian Failure Mice Mediated by Treg Cells and Associated Cytokines. Reprod Sci. 2018;25:1073–82.
12. Zhu Y, Yang Y, Zhang Y, Hao G, Liu T, Wang L, Yang T, Wang Q, Zhang G, Wei J, Li Y. Placental mesenchymal stem cells of fetal and maternal origins demonstrate different therapeutic potentials. Stem Cell Res Ther. 2014;5:48.
13. Navarro-Pando JM, Alcocer-Gomez E, Castejon-Vega B, Navarro-Villaran E, Condes-Hervas M, Mundi-Roldan M, Muntane J, Perez-Pulido AJ, Bullon P, Wang C, Hoffman HM, Sanz A, Mbalaviele G, Ryffel B, Cordero MD. Inhibition of the NLRP3 inflammasome prevents ovarian aging, Sci Adv, 7 (2021).
14. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. Cell Mol Immunol. 2016;13:148–59.
15. Yuan X, Li T, Shi L, Miao J, Guo Y, Chen Y. Human umbilical cord mesenchymal stem cells deliver exogenous miR-26a-5p via exosomes to inhibit nucleus pulposus cell pyroptosis through METTL14/NLRP3. Mol Med. 2021;27:91.
16. Na L, Wang S, Liu T, Zhang L, Ultrashort Wave Combined with Human Umbilical Cord Mesenchymal Stem Cell (HUC-MSC) Transplantation Inhibits NLRP3 Inflammasome and Improves Spinal Cord Injury via MK2/TTP Signalling Pathway, Biomed Res Int, 2020 (2020) 3021750.
17. Cao LB, Leung CK, Law PW, Lv Y, Ng CH, Liu HB, Lu G, Ma JL, Chan WY. Systemic changes in a mouse model of VCD-induced premature ovarian failure. Life Sci. 2020;262:118543.
18. Tran DN, Jung EM, Yoo YM, Ahn C, Kang HY, Choi KC, Hyun SH, Dang VH, Pham TN, Jeung EB. Depletion of follicles accelerated by combined exposure to phthalates and 4-vinylcyclohexene diepoxide, leading to premature ovarian failure in rats. Reprod Toxicol. 2018;80:60–7.
19. Stevenson JC, Collins P, Hamoda H, Lambrinoudaki I, Maas A, Maclaran K, Panay N. Cardiometabolic health in premature ovarian insufficiency. Climacteric. 2021;24:474–80.
20. Vabre P, Gatimel N, Moreau J, Gayrard V, Picard-Hagen N, Parinaud J, Leandri RD. Environmental pollutants, a possible etiology for premature ovarian insufficiency: a narrative review of animal and human data. Environ Health. 2017;16:37.
21. Wang D, Weng Y, Zhang Y, Wang R, Wang T, Zhou J, Shen S, Wang H, Wang Y. Exposure to hyperandrogen drives ovarian dysfunction and fibrosis by activating the NLRP3 inflammasome in

mice. Sci Total Environ. 2020;745:141049.
22. Lliberos C, Liew SH, Mansell A, Hutt KJ. The Inflammasome Contributes to Depletion of the Ovarian Reserve During Aging in Mice. Front Cell Dev Biol. 2020;8:628473.
23. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002;10:417–26.
24. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol. 2010;10:210–5.
25. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature. 2011;469:221–5.

Figures

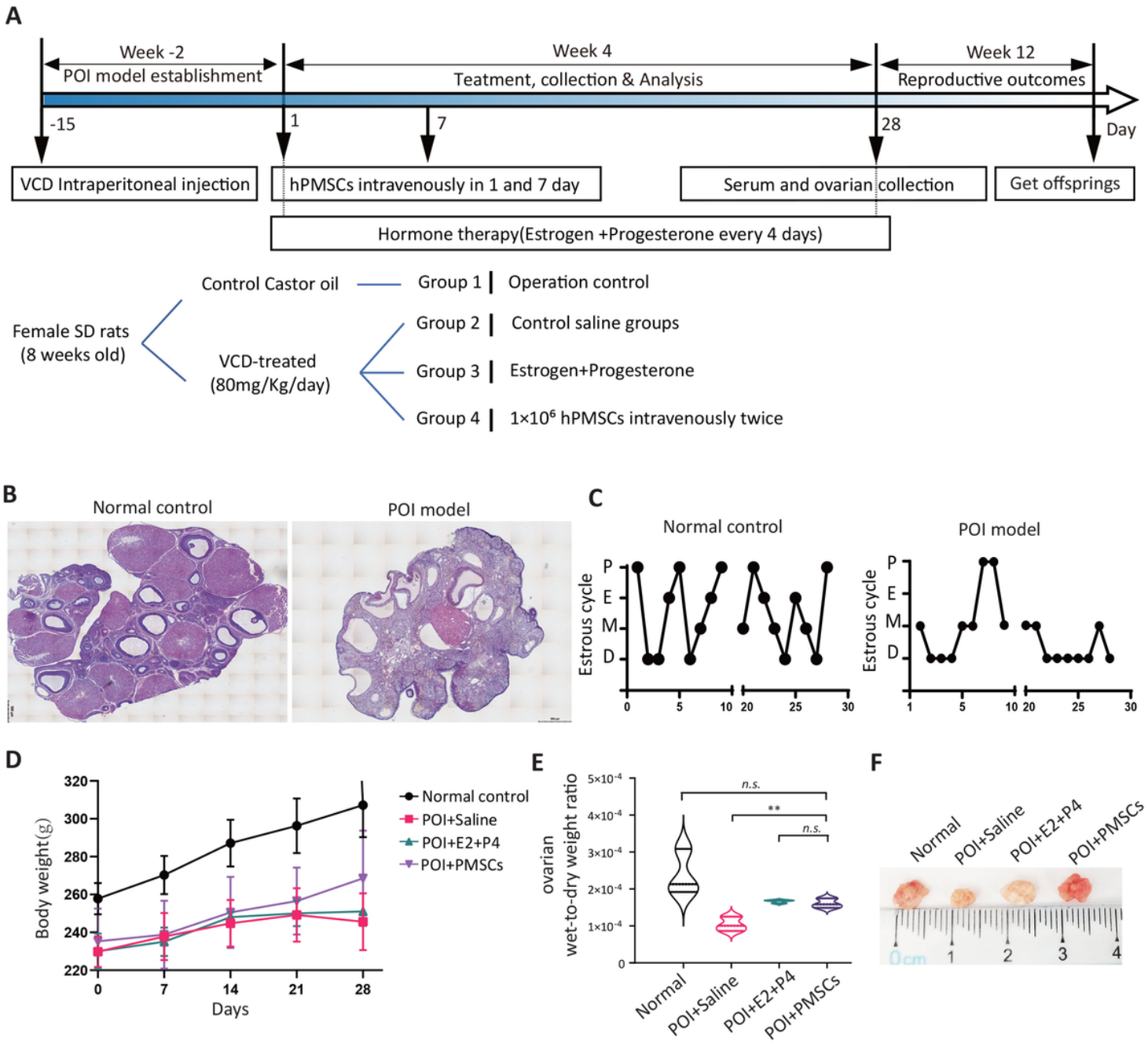

Figure 1

Experimental timeline and characterization of POI in rat model.

(A) Experimental timeline. After the POI model was established by VCD injection, seven days after the first treatment, MSC transplantation was performed by two injections of PMSCs. Estrogen and progesterone treatment was used as a positive control. (B) H&E staining of ovaries in the normal and POI group. Scale bar: 200 μm. (C) Estrous cycle was measured by exfoliated vaginal cell staining in the normal and POI group. (D) Changes in weight in the three groups. (E), (F) Ovarian weight and size in each group.

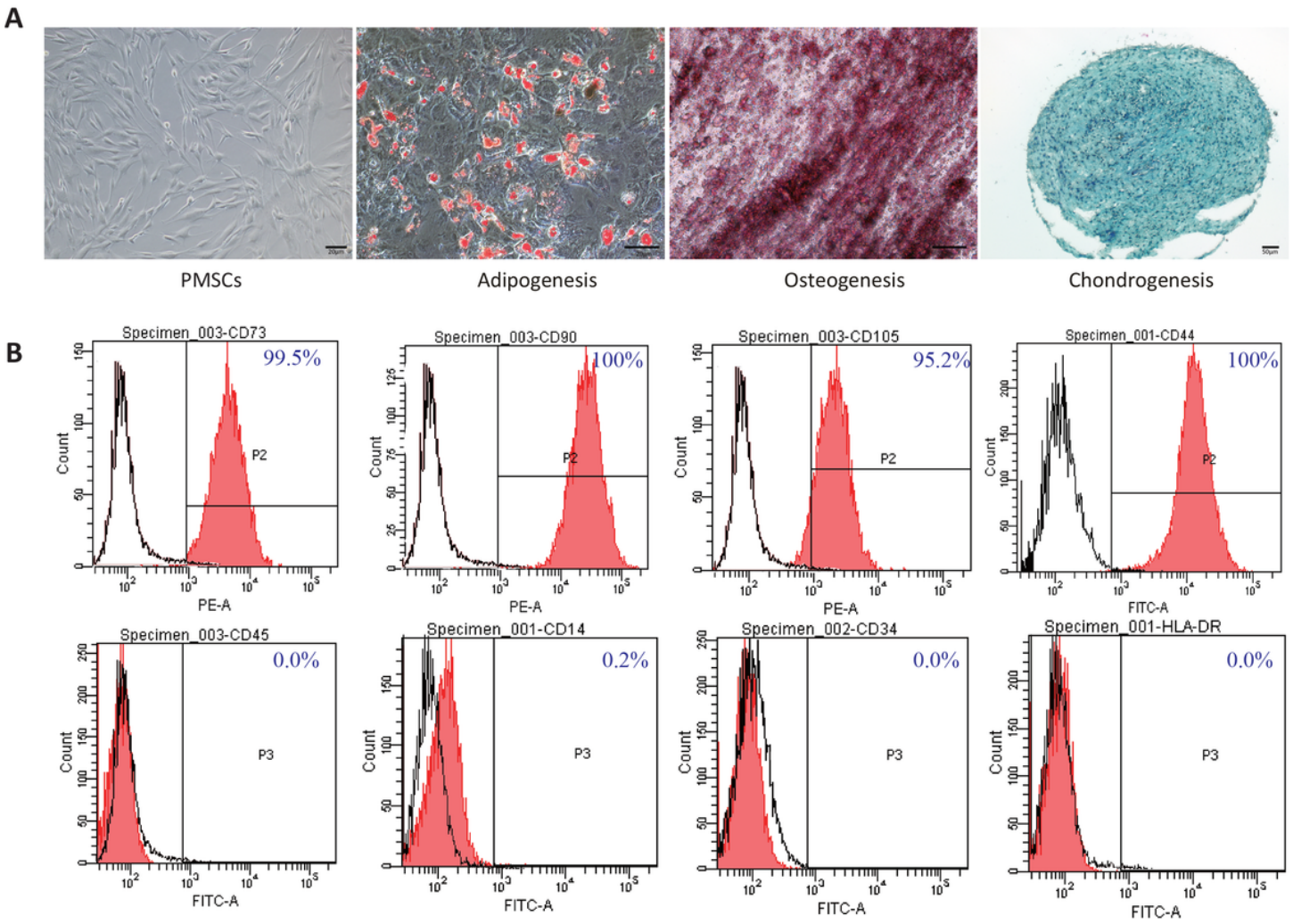

Figure 2

Experimental timeline and characterization of POI in rat model.

(A) Experimental timeline. After the POI model was established by VCD injection, seven days after the first treatment, MSC transplantation was performed by two injections of PMSCs. Estrogen and progesterone treatment was used as a positive control. (B) H&E staining of ovaries in the normal and POI group. Scale bar: 200 μm. (C) Estrous cycle was measured by exfoliated vaginal cell staining in the normal and POI group. (D) Changes in weight in the three groups. (E), (F) Ovarian weight and size in each group.

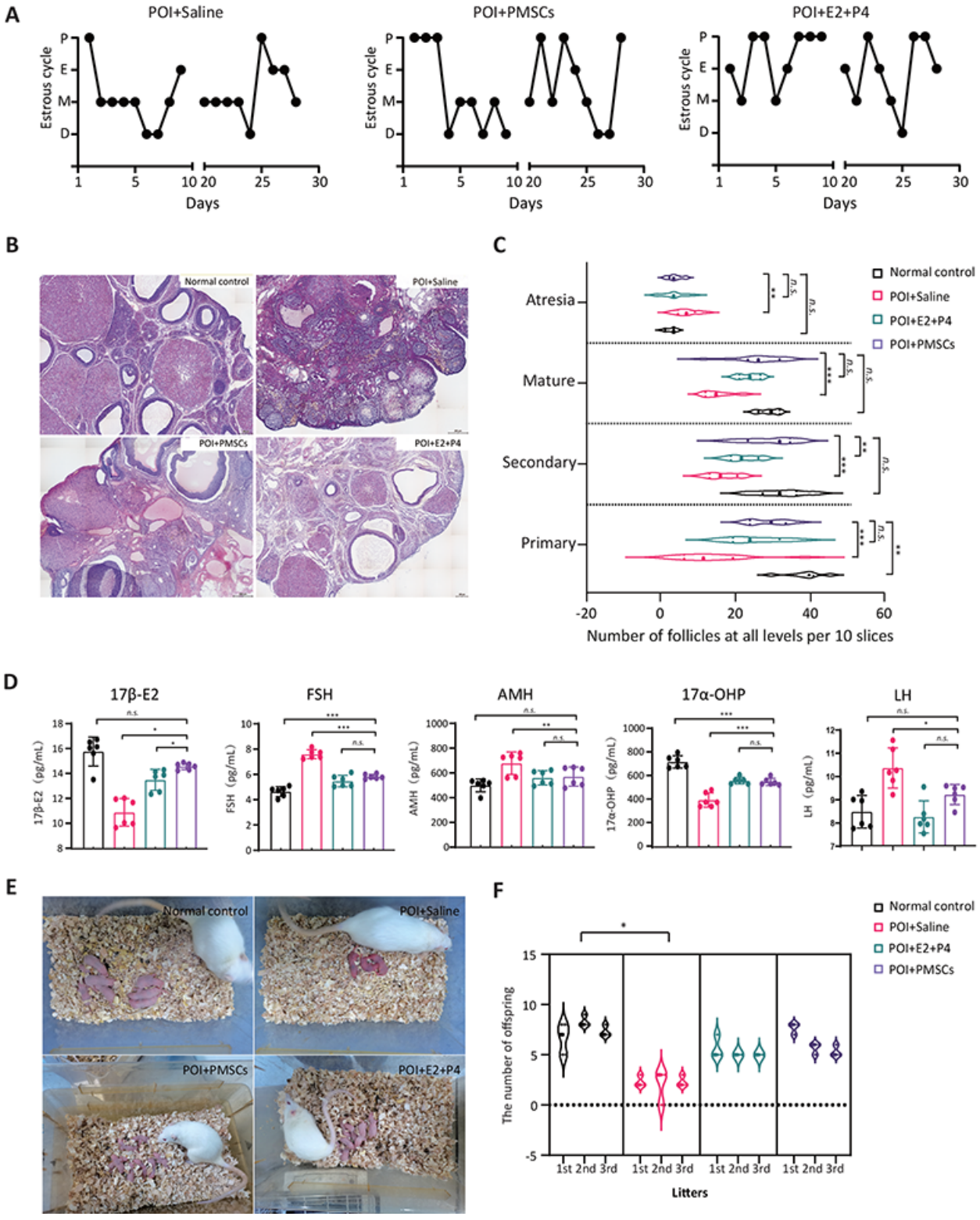

Figure 3

Therapeutic effects of PMSCs in a rat model of POI.

(A) Stage of estrous cycle in the four groups was determined by assessment of stained vaginal exfoliated cells. (B) H&E staining of ovaries in the control and POI groups. Scale bar: 50 μm . (C) The number of follicles at different stages in each group. (D) AMH, E2, LH and FSH levels were significantly elevated in

four groups (E) (F) The number of offspring. Data are presented as the mean ± SD. *p< 0.05, **p< 0.01, ***p< 0.001, *ns*, not significant. Data are means of three independent experiments in each group.

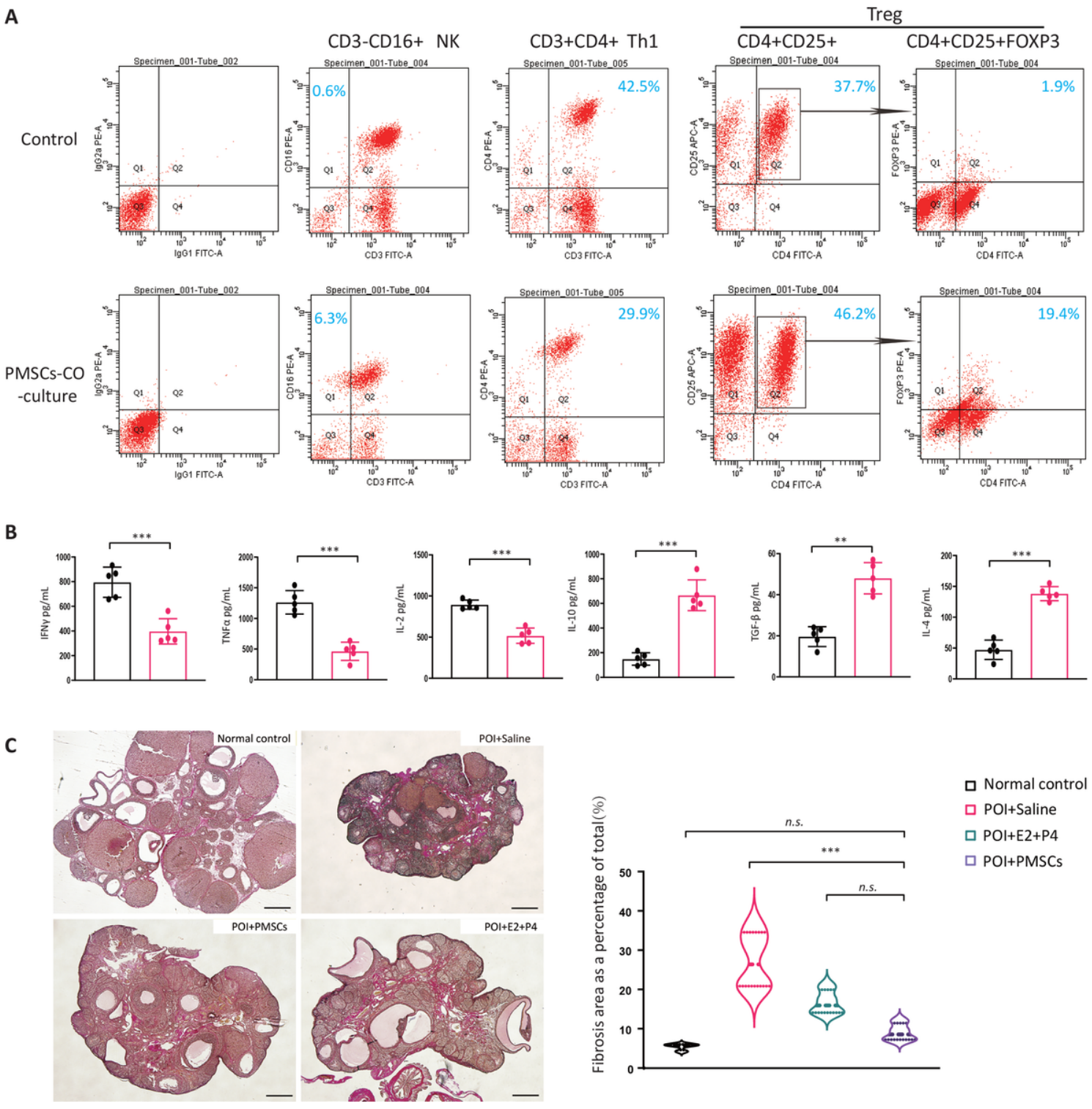

Figure 4

Anti-inflammatory and anti-fibrotic function of PMSCs in vitro and in vivo.

(A) Flow cytometry analysis of immune cell subsets after co-culture of peripheral mononuclear cells and PMSCs. (B) Inflammation-related cytokine expression detection by ELISA (C) The VG staining in each group. Scale bar: 200 μm. (D) Fibrosis levels were significantly elevated in four groups compared. Data are presented as the mean ± SD. *p< 0.05, **p< 0.01, ***p<0.001, *ns*, not significant. Data are means of three independent experiments in each group.

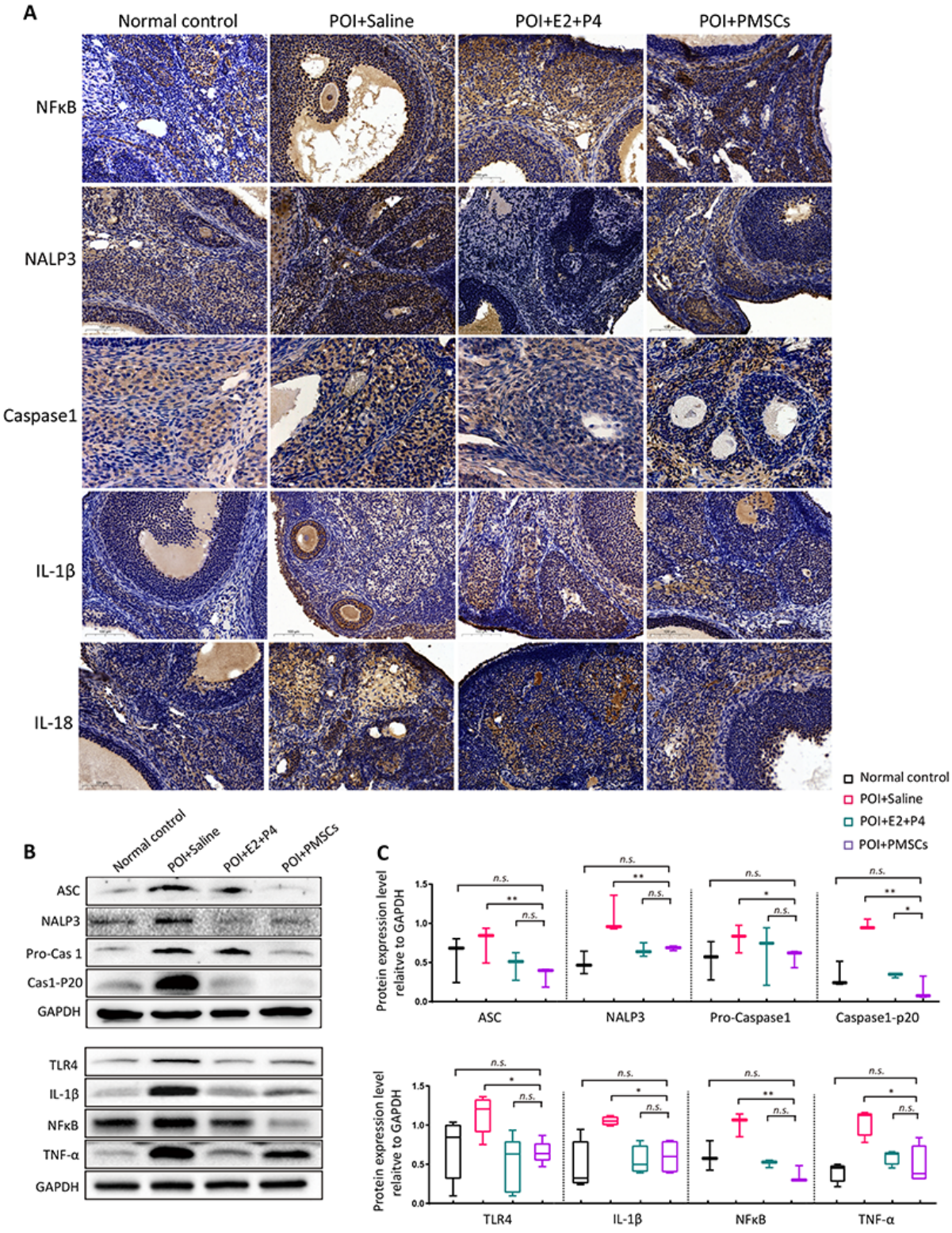

Figure 5

Expression NLRP3 inflammasome pathway and related inflammatory factors. (A) Immunohistochemistry assay for NLRP3, NFκB, ASC, caspase-1, IL-18 and IL-1β. Scale bar: 100 μm (B) Immunoblot analysis of NLRP3 inflammasome pathway and related inflammatory factors. Data are mean ± SD. *p< 0.05, **p< 0.01, ***p< 0.001, ns, no significance. Data are means of three independent experiments in each group.