scientific reports

Check for updates

OPEN

Seminal plasma exosome derived miR-26-5p can regulate decidual macrophage polarization via PTEN / PI3K / AKT signaling pathway

Yan Zhang[1,3], Xiaolin Chen[2,3], Jie Li[1], Xin Chen[1], Jing Zhao[1], Qing Liu[1], Xiaoling Li[1], Xinyu Wang[1] & Zhuoni Xiao[1]✉

The immunomodulatory effects of seminal plasma (SP) on the maternal immune system play an important role in the implantation and development of the embryo. Decidual macrophages (dMΦs) are one of the major immune cells in the maternal–fetal immune microenvironment, and their M2-type polarization facilitates the establishment and maintenance of pregnancy. However, the role of SP on the polarization of dMΦs is unknown. In this study, we investigated the role and mechanism of SP on the polarization of dMΦs by gene chip sequencing as well as in vitro and in vivo experiments. The results revealed that SP promoted dMΦs M2-type polarization. Gene chip sequencing revealed that miR-26-5p was highly expressed in seminal exosomes (SEs) which could act on PTEN/PI3K/AKT signaling pathway and significantly promote MΦs M2 polarization. Moreover, SEs supplementation significantly reduced embryo resorption in spontaneously aborted mice. In conclusion, our study demonstrated that the SEs derived miR-26-5p in SP promoted the M2 polarization of dMΦs by targeting PTEN/PI3K/AKT signaling pathway, which created an immune-tolerant environment conducive to embryo implantation and development. This study provided new ideas for clinical SP-assisted therapy to improve pregnancy outcomes.

Keywords Seminal plasma, Seminal exosomes, Macrophage polarity, miR-26-5p, Embryo implantation and development

For the mother, the embryo is a semi-allograft with the paternal antigen. Therefore, the appropriate immune state at the maternal–fetal interface is very important for embryo implantation and early embryo development[1]. The maternal–fetal interface is composed of trophoblast cells, decidual stromal cells and decidual immune cells. Decidual immune cells mainly include natural killer cells (NKs), macrophages (MΦs), T cells and dendritic cells (DC) which secrete the corresponding cytokines involved in the regulation of immune homeostasis at the maternal–fetal interface.

Decidual macrophages (dMΦs), the second largest population in decidual immune cells, play a crucial role in creating a maternal tolerance environment and regulating tissue remodeling during blastocyst implantation and placental development[2]. MΦs exhibit a high degree of plasticity and can adapt their phenotype in response to environmental stimuli[3]. M1-dMΦs are potent effector cells that induce pro-infammatory T helper 1(Th1) cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). By contrast, M2-dMΦs attenuate these Th1 responses by producing anti- infammatory Th2 cytokines, such as interleukin-10 (IL-10)[4]. Furthermore, inducible nitric oxide synthase (iNOS) produced by M1-dMΦs can inhibit migration and cell trophoblast invasion by inducing higher levels of nitric oxide (NO) production, however recombinant human arginase-1 secreted by M2-dMΦ can inhibit immune cytotoxicity and promote endometrial decidualization and angiogenesis[5]. Thus, it is now widely accepted that M2-type polarization of dMΦs enriched at the maternal–fetal interface would be conducive to promoting the immune tolerance of semi-allograft embryos, remodeling local tissues and blood vessels, and benefiting other pregnancy-related physiological processes[6].

Seminal plasma (SP) has previously been considered as a carrier only to transport sperm, but researches now suggest that SP also has an auxiliary role in embryo implantation and early embryonic development[7–9]. SP has been viewed as simply a vehicle to carry sperm to fertilize the oocyte before, but evidence is now building that

[1]Reproductive Medical Center, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei, China. [2]Department of Endocrinology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei, China. [3]Yan Zhang and Xiaolin Chen contributed equally to this work. ✉email: RM001111@whu.edu.cn

attributes of SP other than sperm fertilizing capability influence reproductive outcomes in mammals[10,11]. Recent studies on SP have focused on regulation of the reproductive immune status and SP is thought to promote immunological tolerance at the maternal–fetal interface. Jena et al. demonstrated that impaired modulation of immune response and improper placental development due to altered cytokine levels in seminal components may be the contributing paternal factors in recurrent pregnancy loss (RPL)[12]. In another study, they showed low expression of Growth Differentiation Factor 15(GDF-15) and overexpressed C3 in the SEs may distort maternal immune response to paternal antigens[13]. Endometrial stromal fibroblasts (eSFs) are a key cell type in the endometrial, belong to the fibroblast family and are mainly distributed in the stromal layer of the endometrial lining, play a vital role in the menstrual cycle, including responding to hormones, promoting angiogenesis, immune regulation, and providing essential support during embryo implantation. George et al. found that SP promotes decidualization by acting on eSFs through the immunomodulatory factors—IL-11[14]. Assisted reproductive technologies (ARTs) have been extensively employed in the treatment of infertility. Clinical meta-studies find low quality evidence that the application of SP as a therapeutic approach to improve the clinical pregnancy rate in cycles of ART[15].

There have been some experimental studies showing that human SP can stimulate the female immune response to provoke a controlled inflammatory response that facilitates embryo implantation, and promotes generation of immune tolerance for pregnancy, such as regulating the levels of dendritic cell (DC) or uterine natural killer cells (uNK) cells related cytokines and chemokines[16,17]. Animal experiments also confirm that cytokines synthesized in the male accessory glands are transferred to the female at insemination, activating changes in gene expression that lead to modifications in structure and function of the female tissues. The consequences are increased fertilization rates, conditioning of the female immune response to tolerate the conceptus, and changes in the endometrium that facilitate embryo development and implantation[18]. However, whether SP can regulate dMΦs polarity has not yet been reported.

Seminal exosomes (SEs) are one of the main mediators of SP to perform their reproductive regulatory functions and play regulatory functions mainly through the DNA, RNA, and proteins they contain[19]. For examples: SEs can be internalized by human endometrial stromal cells (eSCs) and subsequently induce them to produce interleukin-6 (IL-6) and interleukin-8 (IL-8) which are involved in the immunoregulation of embryo implantation[20]; age-related alterations of SEs may be partially responsible for lower implantation rates in the aged-SP group compared with those in the young-SP group, which were mediated by uterine immunomodulation[21].

Phosphatase and tensin homolog (PTEN), a tumor suppressor gene, regulates many biological processes, including proliferation, survival, cellular architecture, motility, energy metabolism, and genomic stability[22]. PTEN can also participate in regulating embryo implantation and development by regulating trophoblast invasion, promoting endometrial epithelium cells (EECs) proliferation, and inducing endometrial stromal cells (ESCs) apoptosis at the maternal–fetal interface[23–25]. However, few articles has reported its regulation on MΦs in female reproductive system.

This topic aims to explore the regulation function and mechanism of SP on dMΦs, which is conducive to the development of new targets for intervention to improve reproductive outcomes and may also provide new ideas for SP-assisted treatment of clinical infertility.

Results

Characterization of seminal plasma exosomes

Electron microscopy and Nanoparticle Tracking Analysis (NTA) revealed that particles isolated from SP contained exosomes (SEs) with diameters ranging from 30–150nm.And the particle concentration of SEs from 6.3×10^7/ml (Fig. 1A,B).The proteins concentration of SEs above were 1.0ug/ul.The Western Blot (WB) experiment confirmed that SEs expressed exosome-specific-markers CD9, CD63 and HSP70 (Fig. 1C). SEs were stained with green fluorescent dye PKH67, and then co-cultured with THP-1cells for 24 h. The green fluorescent was exhibited in the cytoplasm and diffused as small spots around the cell nucleus by confocal microscopy (Fig. 1D) confirming that SEs could be uptaken by THP-1 cells.

SEs promote the polarization of macrophages towards M2 phenotype

To investigate the potential regulation of SEs on MΦs polarization, SEs and controls were separately co-cultured with THP-1 cells. Flow cytometry results showed that the ratio of M2 (CD206+)/M1 (CD86+) was increased after THP-1 cells treated with SEs (Fig. 2A), implying that SEs may induce M2–MΦs polarization. Subsequently, detection of protein markers associated with MΦs polarization revealed that co-culture with SEs increased the expression of the M2–MΦs-related proteins (Arginase-1 and IL-10), consistent with a decrease in the M1–MΦs-related proteins (iNOS and IFN-γ) (Fig. 2B). The above results comprehensively indicated that SEs could promote the differentiation of MΦs to M2 type, induce the secretion of anti-inflammatory factors and inhibit the expression of inflammatory factors, thus creating an immune tolerance environment conducive to embryo implantation.

SEs promote M2 polarization through PTEN/PI3K/AKT signaling pathway

In the study, WB experiment discovered that the expression of PTEN/PI3K/AKT signaling pathway was altered after MΦs co-cultured with SEs (Fig. 3A), which suggested that SEs promoted MΦs to M2 polarization through PTEN/PI3K/AKT signaling pathway.

The similar results were found in human decidual tissue. During in vitro Fertilization (IVF) assisted pregnancy process, barrier contraception was usually used to avoid unintended pregnancy, thus lacking the direct contact regulation effect of SP on the uterus. We collected decidual tissues from patients with spontaneous abortion after natural pregnancy (NP group) and IVF assisted pregnancy (IVF group), determined the PTEN expression using immunohistochemistry (IHC) assay. The PTEN expression in the IVF group was significantly

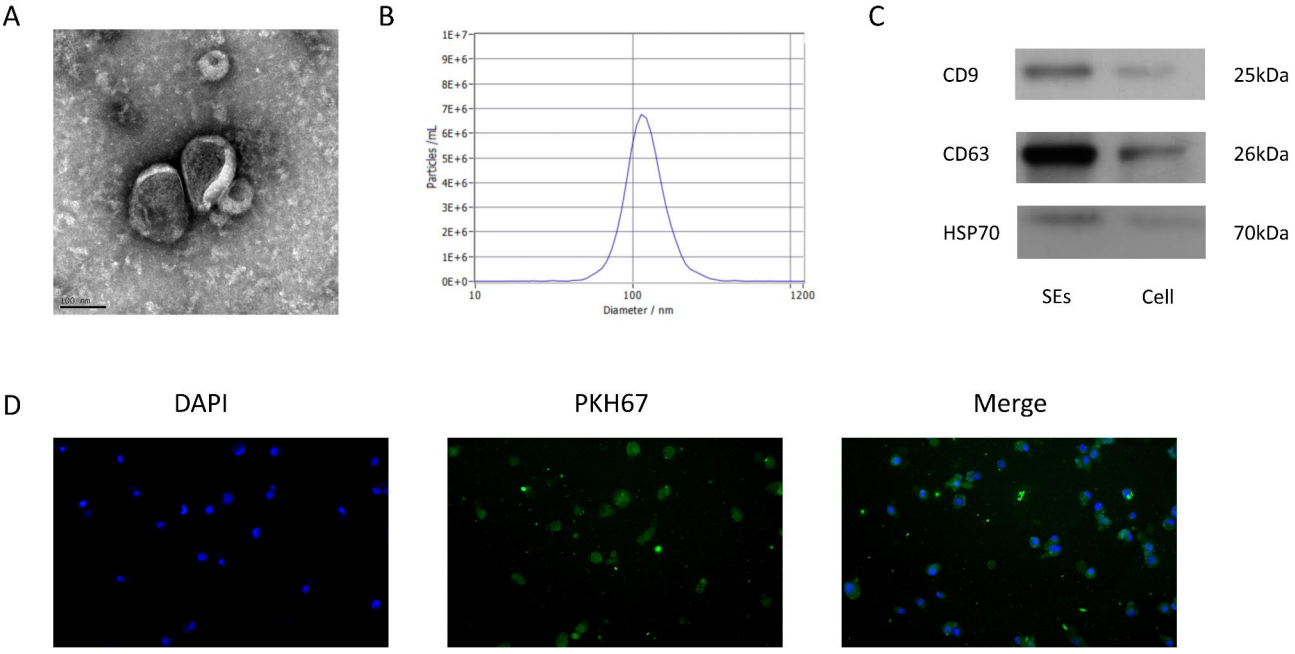

Fig. 1. Cellular internalization of Exosomes and its characteristics. (**A**,**B**) The shape and diameter of SE were observed by transmission electron microscopy and Nanoparticle Tracking Analysis (NTA) (Scale bar, 100 nm). (**C**) Western blot analysis revealed the expression of exosome-specific-markers CD9, CD63 and HSP70 in SEs. (**D**) SEs were labeled with PKH67 green fluorescent dye, and then co-cultured with THP-1 cells for 24 h.

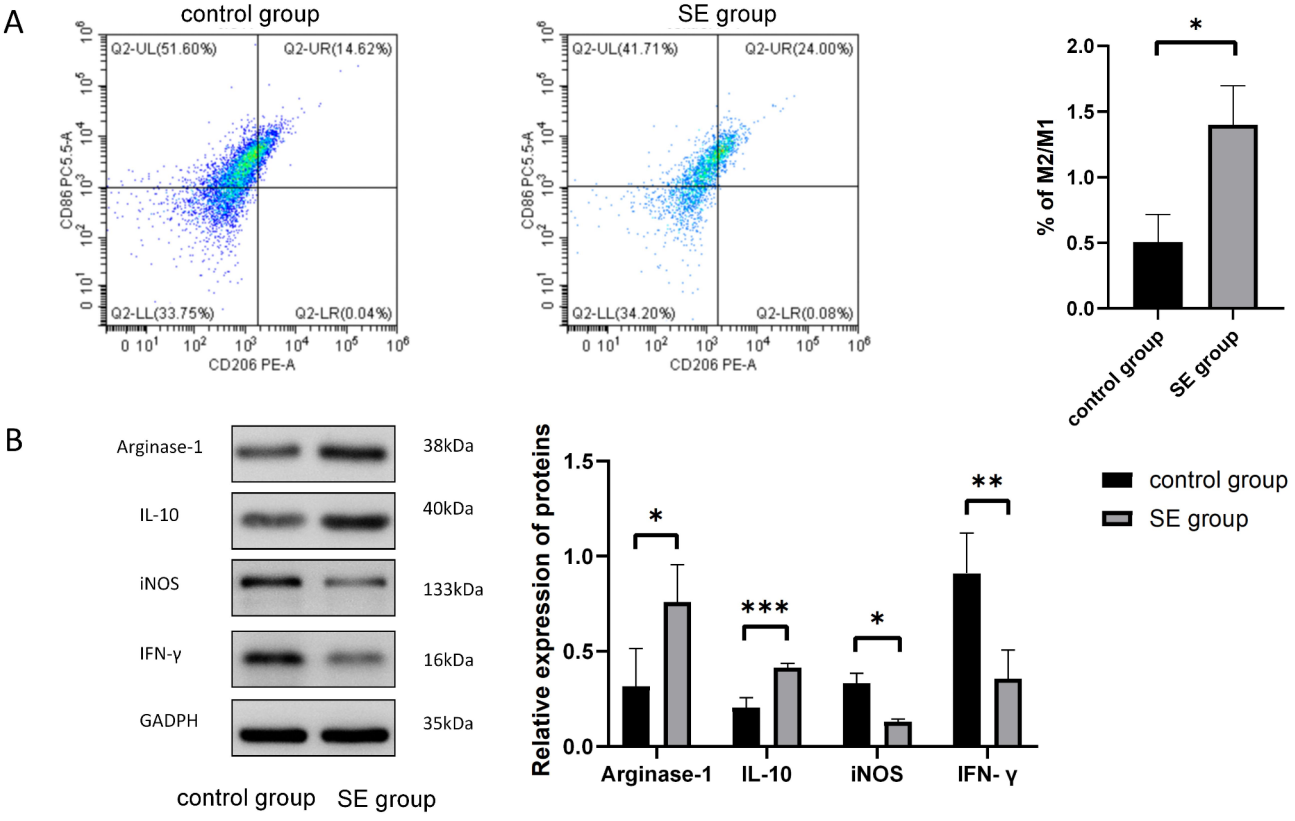

Fig. 2. SEs promote the polarization of macrophages towards M2 phenotype. (**A**) The percentages of M1(CD86+) and M2(CD206+) were detected by flow cytometry. (**B**) Western blot analysis was used to examine the expression of M1/M2–MΦs-related proteins (iNOS/IFN-γ and Arginase-1/IL-10). Values were listed as the mean ± SD,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

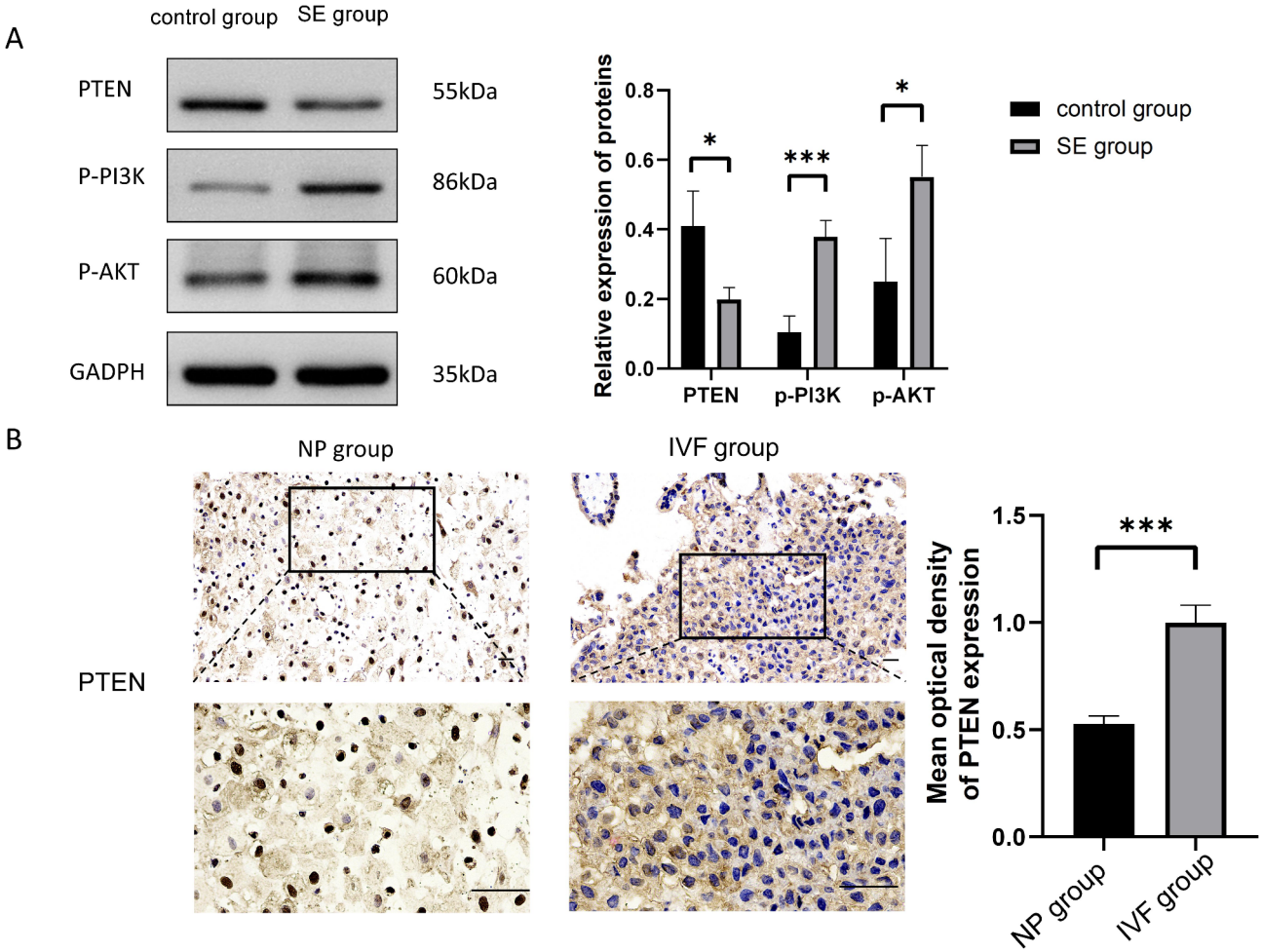

Fig. 3. SEs promote M2 polarization through PTEN/PI3K/AKT signaling pathway and the PTEN expression in decidual tissues. (**A**) Western blot analysis was used to examine the expression of PTEN/phosphorylated PI3K/AKT (p-PI3K and p-AKT). Values were listed as the mean SD, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**B**) The decidual tissues from patients with spontaneous abortion after natural pregnancy (NP group) or IVF assisted pregnancy (IVF group). The expression of PTEN was detected by IHC and compared by Image J. Graphs, mean ± SD. Magnification, 100× and 400×. Scale bars = 50 μm; $^{***}P<0.001$. (The original blot/gel is shown in "Supplementary Figure").

higher than that in the NP group ($P<0.05$) (Fig. 3B). From this result, it could be speculated that SP mainly the SEs has some regulatory effects on the expression of PTEN in the decidua.

miR-26a-5p is critical for SEs to promote macrophage M2 polarization

As SEs primarily manifest their biological influence by delivering bioactive compounds, including miRNAs, we sought to investigate the plausible ingredients of SEs for macrophage polarization. The GeneChip™ sequencing results showed that miR-26a-5p, which had a targeted modulation effect with PTEN, was abundant in the SEs (Fig. 4). The expression of miR-26a-5p increased in THP-1 cells after co-culture with SEs confirming that miR-26a-5p was highly expressed in SEs and can be transported into THP-1 cells (Fig. 5A). The increase of miR-26a-5p expression in THP-1 cells by the transfection with miR-26a-5p mimics laid the foundation for subsequent experiments (Fig. S1).

Flow cytometry assay showed that THP-1 cells were polarized to M2 and increased the M2/M1 ratio after transfection of miR-26a-5p mimic compared with the control group and miR-26a-5p inhibitor group (Fig. 5B). Meanwhile, M2–MΦs-related proteins Arginase-1 and IL-10 were increased, while M1–MΦs-related protein iNOS and IFN-γ were decreased (Fig. 5C). WB experiment further showed that SEs transported the loaded miR-26a-5p to THP-1 cells by acting on PTEN/PI3K/AKT signaling pathway (Fig. 5C).

SEs help to improve pregnancy outcome in spontaneously aborted mice.

The procedures of animal experiment were shown as Fig. 6A. SEs labeled with red fluorescent PKH26 were detected at the maternal–fetal interface of mice after administered by transvaginal injection which confirmed

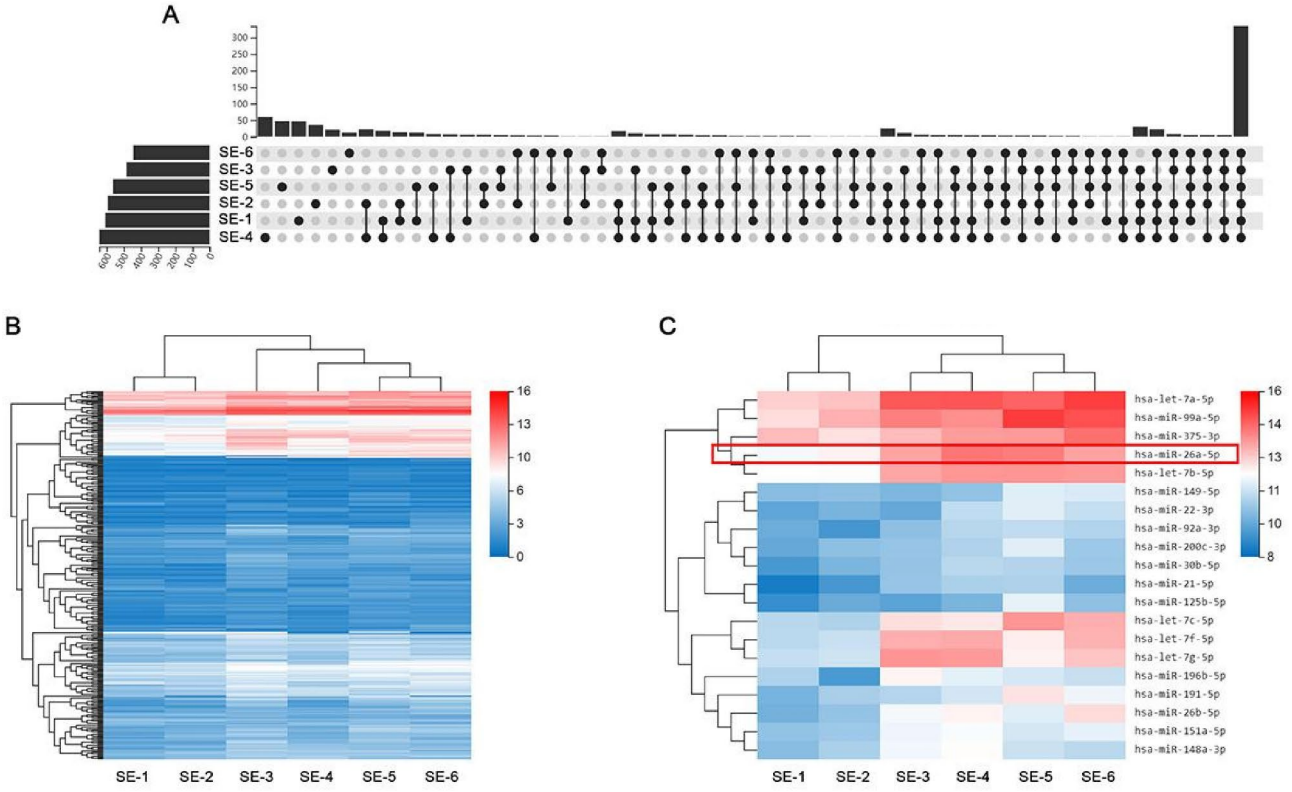

Fig. 4. The expression of miR-26a-5p of SE was abundant the GeneChipTM sequencing. (**A**) The GeneChipTM sequencing obtained the gene number of miRNAs in SEs samples. (**B**) Clustering heat map of 335 miRNAs expressed in SEs. (**C**) The levels of miR-26a-5p were abundant in the SEs.

that SEs could reach the maternal uterus through sexual intercourse and directly regulated the cell function at the maternal–fetal interface (Fig. 6B).

Then, we used spontaneous abortion mouse model to test the protective effect of SEs on pregnancy. There was a significant decrease in embryo resorption rate after vaginal injection of SEs to spontaneous abortion mice which proved that SEs can ameliorate embryo loss (Fig. 6C, Table 1).

Mechanically, in the SE group, miR-26a-5p expression was significantly increased, accompanying with a significantly decrease in PTEN expression at the maternal–fetal interface compared with the control group (Fig. 6D,E), which was very similar to the results of human decidua.

Altered PTEN/PI3K/AKT and M1/M2–MΦs-related proteins level indicated that SEs regulated PTEN/PI3K/AKT signaling pathway by targeted transmission of their endogenous miR-26a-5p to MΦs, accordingly promoted MΦs polarization to M2, thereby shaped an immune tolerance state to alleviate abortion (Fig. 6F).

Discussion

The success of pregnancy mainly depends on immune tolerance of the mother for the semi-allogeneic fetus. Therefore, immunological status at the maternal–fetal interface is critical for the establishment and maintenance of pregnancy[26]. M2-type MΦs contribute to embryo cultivation and development[27–29]. In this study, we identified, for the first time, the essential roles of SP on MΦs at the maternal–fetal interface. Mechanistically, SP, namely SEs promote the polarization of MΦs towards M2 phenotype through PTEN/PI3K/AKT signaling pathway. miR-26a-5p is the key regulatory molecules in SEs playing the regulatory role (Fig. 7). Therefore, SEs, especially miR-26a-5p, can have a potential regulatory role in improving the immune imbalance at the maternal–fetal interface and thus promoting embryonic development.

MΦs are the second largest community at the maternal–fetal interface. M1/M2 polarity balance is proposed to be required for a successful pregnancy. Compared to M1 phenotype, M2-type MΦs are alternatively activated which have immunosuppressive capacities[30]. MΦs exhibit a high degree of plasticity and can adapt their phenotype in response to the signals present in the surrounding environment, such as trophoblast-derived microRNAs[31]. It has been unknown whether SP, as a component which can directly contact with MΦs, can induce M2 polarization. In this study, the direct regulation effect of SP on MΦs polarization was determined by co-culture of SEs with THP-1. SEs were found to be phagocytosed by THP-1 cells and induced their transition to M2-type macrophages, accompanied by increased expression of M2-associated proteins, such as arginase-1 and IL-10. This experiment confirmed for the first time that SP, particularly SEs, has a direct regulation of macrophage polarization except for decidual DC maturation[16], CD11c + antigen presenting cells (APC) and paternal antigen-specific regulatory T cells (Tregs) development[32,33].

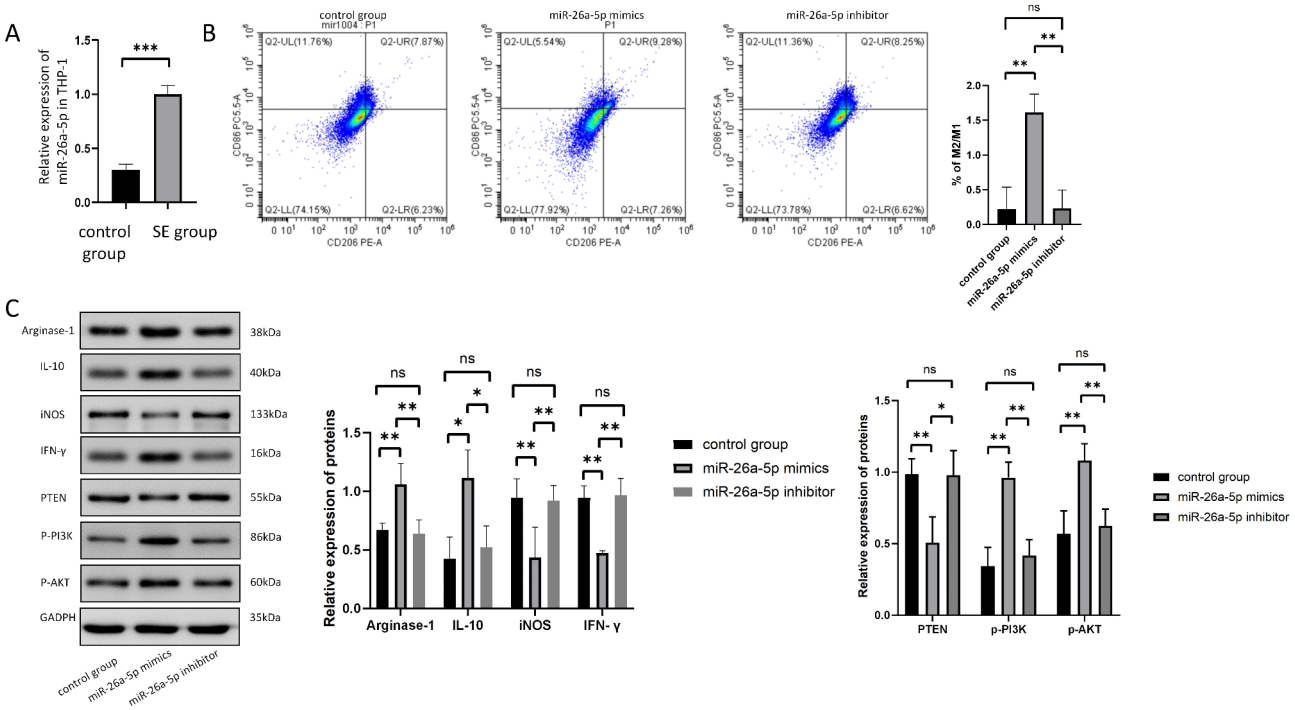

Fig. 5. miR-26a-5p is critical for SEs to promote macrophage M2 polarization. (**A**) RT-PCR assays measured the levels of miR-26a-5p in THP-1 cells treated with SE and control group. (**B**) The percentages of M1(CD86+) and M2(CD206+) were detected by flow cytometry after THP-1 cells transfection with controls/miR-26a-5p mimics/miR-26a-5p inhibitor. (C)Western blot analysis was used to examine the expression of M1/M2–MΦs-related proteins (iNOS/IFN-γ and Arginase-1/IL-10) and PTEN/phosphorylated PI3K/AKT (p-PI3K and p-AKT). Values were listed as the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (The original blot/gel is shown in "Supplementary Figure").

PTEN/PI3K/AKT signaling pathway is one of the highly conserved important molecules in regulating cell biological function at the maternal–fetal interface[34]. As human specimens and animal experiments indicate that SP or SEs may favor embryo implantation by altering certain signaling pathways[10,35], the IHC experiments of human specimens demonstrated that SP has regulatory effects on decidual PTEN expression. In vitro experiments also further cleared that SEs promote M2 polarization toward through PTEN/PI3K/AKT signaling pathway. Although cell experiments could confirm that SEs regulate macrophage polarization through PTEN/PI3K/AKT signaling pathway, we did not perform colocalized staining for macrophages and PTEN/PI3K/AKT in human specimens, which is insufficient in this experiment.

miRNAs were identifed as crucial constituents of exosomes, signifcantly determining the impact of exosomes on target cells. The GeneChip™ sequencing was applied to reveal the abundance of different miRNAs present in the SEs. miR-26a-5p, which has been recognized having the targeted modulation effect on PTEN, was abundant in the SEs[36,37]. miR-26a-5p promoting M2 polarization by regulating PTEN/PI3K/AKT signaling pathway prove that SEs play the regulatory role mainly via contained miR-26a-5p.

Native or engineered exosomes are low toxicity and low immunogenicity, therefore they are effective and biocompatible biological carriers for research and therapeutic applications[38,39]. Current studies have applied human derived exosomes for animal models to elucidate the mechanism of exosomes action[40,41]. CBA/J females paired with DBA/2 males can build the mouse model of recurrent spontanous abortion. Increased embryo absorption rate and exorbitant proinflammatory cytokines were found in these mice[42]. In this research, exosomes derived from human seminal plasma can improve pregnancy outcomes by targeting PTEN/PI3K/AKT signaling pathway through miR-26a-5p. We unfurled the remarkable therapeutic ability of SEs in abortion-prone mice, and this was achieved by targeting MΦs-M2 polarity thus favoring immune tolerance at the maternal–fetal interface.

Materials and methods

SEs isolation

All procedures (n = 6) involving participants in this study were approved by the Clinical Trial Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China, CTEC number: WDRY2021-K044). The clinical characteristics of the participants are in S Table 1. To isolate SEs, seminal fluid was centrifuged at 800 g for 15 min at 25 °C to separate spermatozoa, and then centrifuged at 10,000 g for 30 min at 4 °C to remove cell debris and other impurities. The supernatant was in turn centrifuged at 10,000 g for 90 min to pellet microvesicles (MVs). Remaining supernatant was ultracentrifuged at 100,000 g for 70 min and the exosome-containing pellets

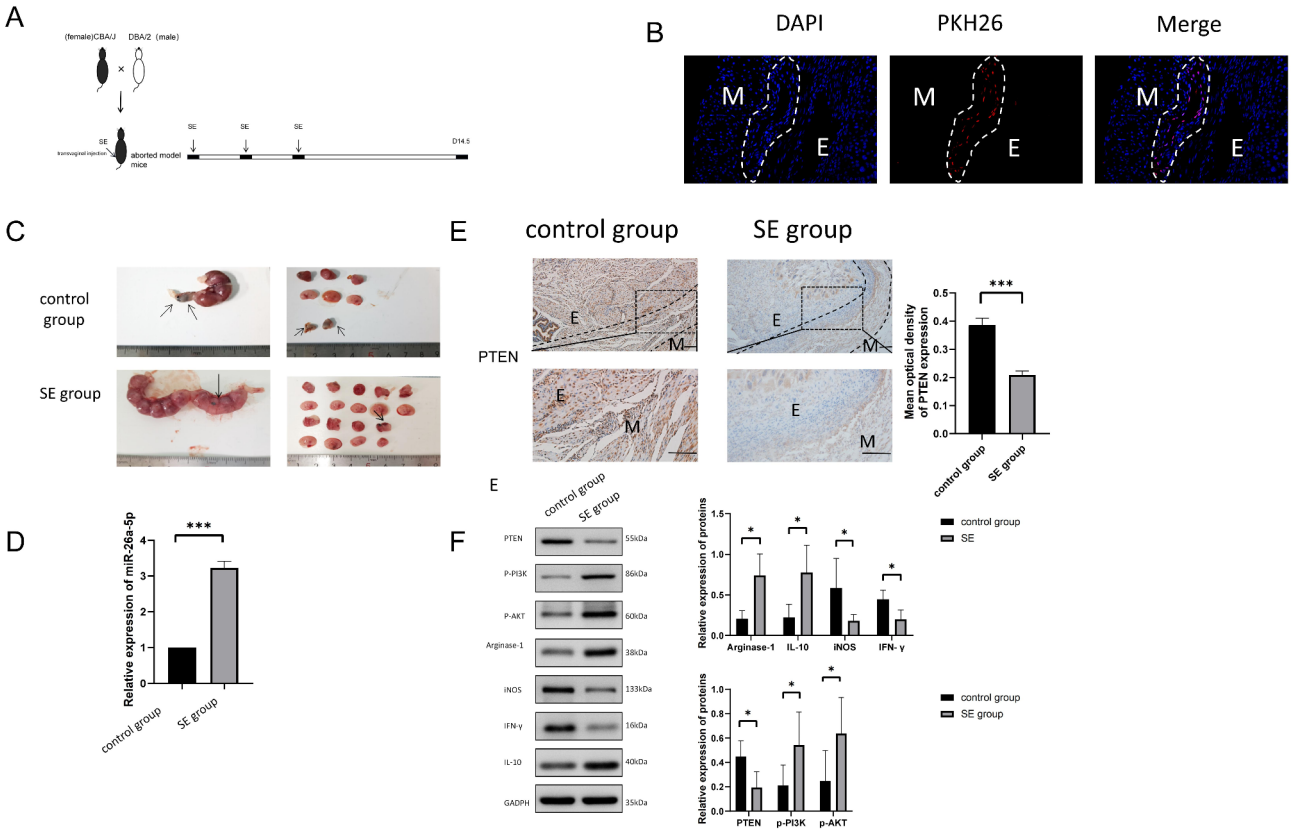

Fig. 6. SEs help to improve pregnancy outcome in spontaneously aborted mice. (**A**) The procedures of animal experiment. (**B**) SEs labeled with red fluorescent PKH26 could be observed at the maternal–fetal interface. (**C**) Compared the embryonic absorption rate of SE group and control group in spontaneously aborted mice. Abortion sites were the necrotic appearance. The embryonic absorption rate was calculated as the ratio of resorption sites to total implantation sites. (**D**,**E**) RT-PCR assays measured the levels of miR-26a-5p and IHC assays were performed to detect the expression of PTEN at the maternal–fetal interface in SE group and control group. (**F**) Western blot analysis was used to examine the expression of M1/M2–MΦs-related proteins (iNOS/IFN-γ and Arginase-1/IL-10) and PTEN/phosphorylated PI3K/AKT (p-PI3K and p-AKT). Values were listed as the mean ± SD, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (The original blot/gel is shown in "Supplementary Figure").

	Control group (n = 4)	SE group (n = 4)	*P* value
Surviving fetuses	20	32	
Resorbed fetuses	9	4	
Resorption rate (%)	31.03	11.11*	$P<0.05$

Table 1. The embryo resorption rate after vaginal injection of SEs in mice with spontaneous abortions. $^{*}P<0.05$.

were washed with PBS for two times. SEs ultimately resuspended in PBS and stored at − 80 °C for the following steps. In the cell and animal experiment, exosomes with a concentration of 1ug/uL were used.The antibodies used were as follows: anti-CD9 (1:1000, ab92726, Abcam, USA), anti-CD63 (1:1000, ab216130, Abcam, USA), anti-HSP70 (1:2000, ab181606, Abcam, USA). The size and purity of the isolated SEs were determined using nanoparticle tracking analyzer (NTA, ZetaView 7PMX 120, Particle Metrix, Germany), transmission electron microscope (TEM, JEOL, Tokyo, Japan), and Western blot analysis.

Isolated exosomes were treated with a 1% glutaraldehyde solution for 10 min and then cleaned with deionized water. A 10 μL aliquot of the exosome mixture was deposited onto formvar and carbon-covered 300-mesh copper grids (Agar Scientific Ltd., Stansted, UK), and left to rest for 5 min at room temperature. The exosomes were subsequently stained with a 2% solution of uranyl oxalate for a minute at room temperature. The grids underwent three PBS washes and were left to dry in the air for 5 min. The samples were then examined and imaged using a TEM (JEM-2100, Jeol, Japan).

NTA was conducted following the protocol provided by the manufacturer (NTA, ZetaView 7PMX 120, Particle Metrix, Germany), as detailed in our previous studies[43].

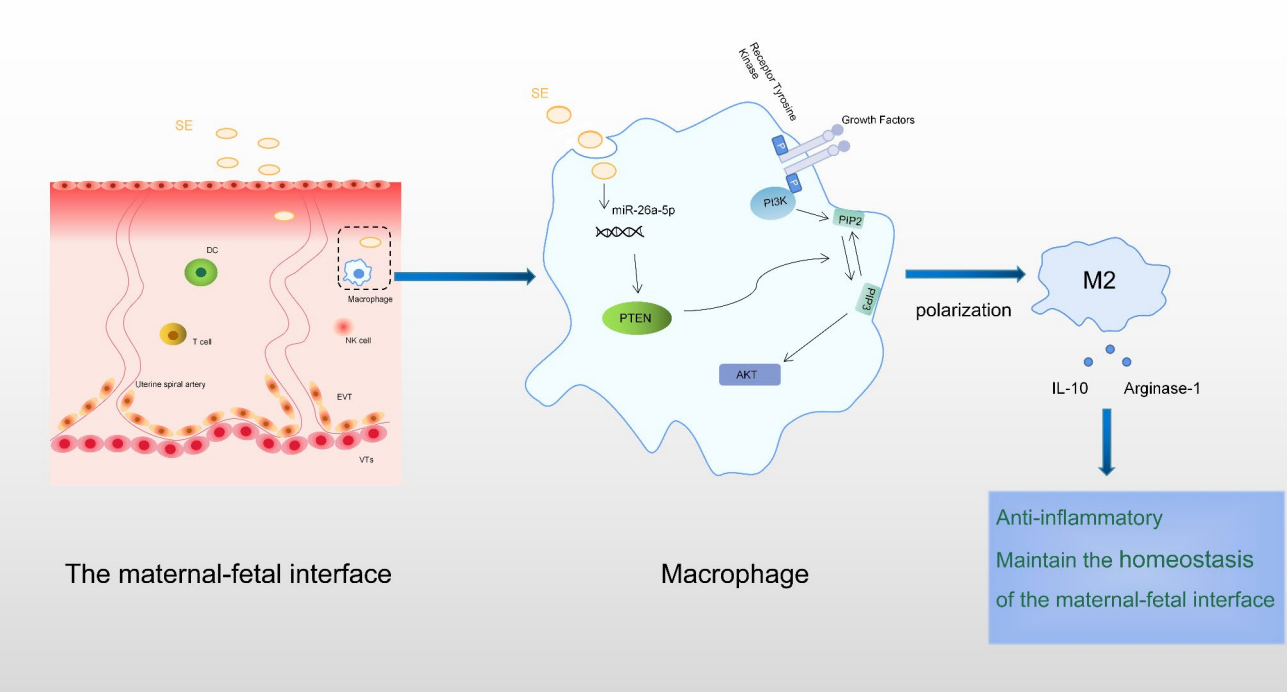

Fig. 7. Schematic illustration of SEs-derived miR-26a-5p regulate the polarization of decidual MΦs through the PTEN/PI3K/AKT pathway in maternal–fetal interface. SEs release the contained miR-26a-5p, target the PTEN/PI3K/AKT signaling pathway, promote macrophage polarization toward M2 phenotype, and release inflammatory inhibitors Arginase-1 and IL-10, thus shaping an immune tolerance microenvironment. NK cell, natural killer cell; DC, dendritic cell; VTs, villous trophoblasts; EVT, extracellular villous trophoblast.

Cell culture

The THP-1 cell line serves as an invaluable model for investigating a spectrum of monocyte and macrophage-related activities. Monocyte THP1 was purchased from Wuhan Procell Life Science and Technology Co., Ltd., China, and cultured in RPMI-1640 medium (Gibco, USA) which contained 10% FBS (Gibco, USA) and 1% penicillin–streptomycin (Servicebio, Wuhan) at 37 °C in a humidified incubator under 5% CO_2. THP1 monocytes were matured into M0 macrophages with 100ng/mL of phospholipid 12-myristic acid 13-acetate (PMA, Sigma-Aldrich, USA) for 48 h to macrophage polarization.

Cellular internalization of SEs

SEs were labelled with PKH67 according to the manufacturer's instructions (Sigma-Aldrich, USA). Shortly, SEs were added to diluent C, and PKH67 were added to diluent C. The mixture was incubated at room temperature. An equal volume of FBS was added to stop the dyeing reaction and bind the excess dye. Excess dye was removed by centrifuging the SEs at 400 g at 25 °C for 10 min. The nuclei were stained with DAPI (0.5 μg/mL, Invitrogen, USA). The labelled SEs were incubated with THP-1 cells for 48 h. The internalization of SEs by THP-1 cells was observed using the fluorescence microscope (BX53, Olympus, Japan). The protein concentration of SEs was measured using the BCA (Bicinchoninic Acid) assay with the BeyoBCA Protein Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, samples and bovine serum albumin (BSA) standards (Sigma-Aldrich, USA) were prepared in duplicate and loaded into a 96-well plate. The BCA working solution was added to each well, and the plate was incubated at 37 °C for 30 min. The optical density (OD) was measured at 562 nm using a microplate reader. A standard curve was generated from the BSA standards, and the protein concentration of the samples was calculated based on this curve.

Western Blot (WB) assay

Proteins from cell lysates were separated by Radioimmunoprecipitation assay (RIPA) (Servicebio, Wuhan) lysis buffer and were resolved by SDS-PAGE, subsequently transferred to polyvinylidene fluoride (PVDF, Millipore, USA) membranes. The PVDF membranes were blocked with 5% skimmed milk and incubated with appropriate primary antibodies at 4 °C overnight. The membranes were rinsed with TBST on three occasions, with a duration of 10 min for each wash. Thereafter, the PVDF membranes were incubated with horseradish peroxidase conjugated secondary antibodies. Subsequently, the membranes underwent three rounds of washing with TBST. Finally, Protein blots were visualized by using the ECL-Plus western blotting detection system (Thermo Fisher Scientific, USA). The antibodies used were as follows: rabbit anti-CD9 (1:1000, ab92726, Abcam, USA), rabbit anti-CD63 (1:1000, ab216130, Abcam, USA), rabbit anti-HSP70 (1:1000, ab181606, Abcam, USA), rabbit anti-PTEN (1:1000, ab267787, Abcam, USA), rat anti-p-AKT (1:1000, ab38449, Abcam, USA), rat anti-p-PI3K (1:500, ab182651, Abcam, USA), rabbit anti-Arginase-1 (1:1000, ab133543, Abcam, USA), rabbit anti-iNOS

(1:1000, ab178945, Abcam, USA), rabbit anti-IL-10 (1:1000, ab133575, Abcam, USA), rabbit anti-IFN-γ (1:1000, ab267369, Abcam, USA).

Flow cytometry

Cells were suspended in the staining buffer (PBS + 3% BSA). Phycoerythrin (PE)-conjugated anti-human CD68 antibody (eBioscience, USA), PE-Cy7-conjugated anti-human CD86 and CD206 antibody were used for staining. All the data were acquired using FACS Canto II, BD Company, USA, and processed using FlowJo (Tree Star, Ashland, OR, USA).

Clinical sample collection

All participants were recruited from the Reproductive Medical Center at the Renmin Hospital of Wuhan University from June, 2021 to June, 2022. First-trimester human decidual tissues were obtained from patients with spontaneous abortion after natural pregnancy (NP group, n = 6) or IVF assisted pregnancy (IVF group, n = 6) Spontaneous abortion caused by fetal chromosome abnormalities, endocrine disorders or metabolic abnormalities, uterine malformations, autoimmune diseases were excluded. All patients undergoing IVF treatment were required to use barrier contraception to reduce unplanned pregnancy. Characteristics of the patients in the groups in S Table 2.

Immunohistochemistry (IHC)

Decidual tissues were collected from spontaneous abortion after natural pregnancy and IVF assisted pregnancy. These tissues were fixed in 10% formaldehyde at 37 °C for 6h, followed by routine deparaffinization and dehydration for immunohistochemistry. Formalin-fixed paraffin-embedded tissues were cut into 4 μm sections. 3% hydrogen peroxide was used to block the endogenous peroxidase activity, and the non-specific binding was blocked with 5% bovine serum albumin for 20 min. The sections were incubated at 37 °C with rabbit anti-PTEN antibody (1:100, ab267787, abcam, USA) for 4 °C overnight. After washed in PBS, the sections were then incubated with HRP-labelled goat anti-rabbit IgG (1:500, AS1107, ASPEN, USA) secondary antibodies for 30 min at room temperature, followed by wash with PBS three times for 5 min. The color was developed with a DAB kit (Dako Cytomation, Glostrup, Denmark). Positive signals were visualized as brown. Mean fuorescence intensity (IntDen/Area) was quantitatively analyzed using Image J software.

Quantitative real-time PCR (qRT-PCR)

The total RNA in the tissues was isolated using TRIzol Reagent (ELK Biotechnology, China), according to the manufacturer's instructions. The cDNA was performed on the ABI7900 system (Applied Biosystems, USA) for mRNA expression. The reverse transcription kit (ELK Biotechnology, China) was used to reverse-transcribe the RNA. The relative RNA quantification was performed using the comparative $2-\Delta\Delta Ct$ method.

Cell transfection

In all cell transfections, 5×10^5 cells were plated on 35 mm plates. 5 μl oligonucleotides including miR-26a-5p mimics or miR-26a-5p inhibitor were diluted in 250 μl Opti-Medium (Life Technology, USA) and mixed with 5 μl Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instructions. These mixture was then added with 500 μl dye solution and 1500 μl basal medium. All of the culture media were completely replaced after 6 h. Each experiment was repeated three times.

Animal experiments

An abortive mouse model was constructed as previously reported[44]. All animal operations were approved by the ethics committee for laboratory animal welfare (IACUC) of Renmin Hospital of Wuhan University (Approval number: No. WDRM animal (f) No. 20201207). 6–8 weeks of age ICR mice, CBA/J female mice and DBA/2 male mice were obtained from the Animal Experiment Center of Wuhan University. ICR female mice mated ICR male mice at 1:1 for normal pregnant group (n = 6); CBA/J female mice mated with DBA/2 male mice at 1:1 for spontaneous abortion group. Overall, 8 spontaneous abortion mice were randomly divided into two groups as follows: naive-control (n = 4) and SEs treatment (n = 4). Pregnant mare serum gonadotropin (PMSG, China) 10IU was given at 8 pm on the first day, and human chorionic gonadotropin (HCG, China) 10IU was given at 8 pm on the third day to induce superovulation. The day on which the vaginal plug appeared in female mice was recorded as the 0.5 day (D0.5) of pregnancy. For the SE groups, three shots of 100 μl PHK26 dye labeled SEs (1 ug/ul in PBS) were injected by vaginal on D0.5, D3.5 and D6.5. The naive-control group received a vaginal injection of PBS. On D14.5, the mice were euthanized via CO_2 inhalation. The placental tissues, including decidual tissues and the fetus, were collected for the following experiments. Each experiment was repeated three times.

Ethics statement: All research animals in this study were handled, monitored and euthanised in accordance with Regulations of Hubei Province on the Administration of experimental animals. All rodents were raised and maintained in the Animal Facility of the Renmin Hospital of Wuhan University Animal House, with free access to food and water. The animals were kept under a controlled environment with a 12-h light/12-h dark cycle at a stable temperature of 21–22 °C. Prior to experimentation, the animals were humanely euthanized using carbon dioxide (CO_2) asphyxiation, ensuring minimal distress and in accordance with the ethical guidelines for the care and use of laboratory animals. All methods are reported in accordance with ARRIVE guidelines. All methods were carried out in accordance with relevant guidelines and regulations.

Statistical analysis

All experiments were repeated at least three times. SPSS software (22.0, USA) was used to calculate and assess statistical differences. Groups of discrete variables were compared by Kruskal–Wallis nonparametric analysis of variance or Mann–Whitney U test. $P < 0.05$ was considered statistically significant.

Conclusions

After coitus, SP has a direct contact regulation effect on the female reproductive tract, and the SEs are one of the main media playing the SP regulation function. In this study, we found that SP, namely SEs promote the polarization of MΦs towards M2 phenotype through PTEN/PI3K/AKT signaling pathway. miR-26a-5p is the key regulatory molecules in SEs playing the regulatory role. The regulatory effect of SEs on MΦs helps to construct an immune-tolerant environment that promotes embryo cultivation and development (Fig. 7). This protective effect was confirmed in abortive mouse model trials. Thus, SEs are therefore expected as a novel treatment to improve pregnancy outcomes.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

Received: 19 August 2024; Accepted: 3 March 2025
Published online: 17 March 2025

References

1. Yang, F., Zheng, Q. & Jin, L. Dynamic function and composition changes of immune cells during normal and pathological pregnancy at the maternal–fetal interface. *Front. Immunol.* **10**, 2317 (2019).
2. Locati, M., Curtale, G. & Mantovani, A. Diversity, mechanisms, and significance of macrophage plasticity. *Annu. Rev. Pathol.* **15**, 123–147 (2020).
3. Wang, L. L. et al. Decorin promotes decidual M1-like macrophage polarization via mitochondrial dysfunction resulting in recurrent pregnancy loss. *Theranostics* **12**(17), 7216–7236 (2022).
4. Hu, J. J. et al. *Lactobacillus murinus* alleviate intestinal ischemia/reperfusion injury through promoting the release of interleukin-10 from M2 macrophages via Toll-like receptor 2 signaling. *Microbiome* **10**(1), 38 (2022).
5. Kieler, M., Hofmann, M. & Schabbauer, G. More than just protein building blocks: How amino acids and related metabolic pathways fuel macrophage polarization. *FEBS J.* **288**(12), 3694–3714 (2021).
6. Zhang, Y. H., He, M., Wang, Y. & Liao, A. H. Modulators of the balance between M1 and M2 macrophages during pregnancy. *Front. Immunol.* **8**, 120 (2017).
7. Shen, Q. et al. Immune regulation of seminal plasma on the endometrial microenvironment: Physiological and pathological conditions. *Int. J. Mol. Sci.* **24**(19), 14639 (2023).
8. Ahmadi, H. et al. Composition and effects of seminal plasma in the female reproductive tracts on implantation of human embryos. *Biomed. Pharmacother.* **151**, 113065 (2022).
9. Schjenken, J. E. & Robertson, S. A. The female response to seminal fluid. *Physiol. Rev.* **100**(3), 1077–1117 (2020).
10. Roertson, S. A. et al. Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. *J. Reprod. Fertil.* **107**(2), 265–277 (1996).
11. Rodriguez-Martinez, H. et al. Seminal plasma: Relevant for fertility?. *Int. J. Mol. Sci.* **22**(9), 4368 (2021).
12. Jena, S. R. et al. Comparative proteome profiling of seminal components reveal impaired immune cell signalling as paternal contributors in recurrent pregnancy loss patients. *Am. J. Reprod. Immunol.* **89**(2), e13613 (2023).
13. Jena, S. R. et al. Paternal contributors in recurrent pregnancy loss: Cues from comparative proteome profiling of seminal extracellular vesicles. *Mol. Reprod. Dev.* **88**(1), 96–112 (2021).
14. George, A. F. et al. Seminal plasma promotes decidualization of endometrial stromal fibroblasts in vitro from women with and without inflammatory disorders in a manner dependent on Interleukin-11 signaling. *Hum. Reprod.* **35**(3), 617–640 (2020).
15. Ata, B. et al. Application of seminal plasma to female genital tract prior to embryo transfer in assisted reproductive technology cycles (IVF, ICSI and frozen embryo transfer). *Cochrane Database Syst. Rev.* **2**(2), CD11809 (2018).
16. Craenmehr, M. H. C. et al. Effect of seminal plasma on dendritic cell differentiation in vitro depends on the serum source in the culture medium. *J. Reprod. Immunol.* **137**, 103076. https://doi.org/10.1016/j.jri.2019.103076 (2020).
17. Taima, A. et al. A semen-based stimulation method to analyze cytokine production by uterine CD56bright natural killer cells in women with recurrent pregnancy loss. *J. Reprod. Immunol.* **142**, 103206 (2020).
18. Robertson, S. A. Seminal fluid signaling in the female reproductive tract: Lessons from rodents and pigs. *J. Anim. Sci.* **85**(13 Suppl), E36-44 (2007).
19. Machtinger, R., Laurent, L. C. & Baccarelli, A. A. Extracellular vesicles: Roles in gamete maturation, fertilization and embryo implantation. *Hum. Reprod. Update* **22**(2), 182–193 (2016).
20. Paktinat, S. et al. Seminal exosomes induce interleukin-6 and interleukin-8 secretion by human endometrial stromal cells. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **235**, 71–76 (2019).
21. Wang, D. et al. Seminal plasma and seminal plasma exosomes of aged male mice affect early embryo implantation via immunomodulation. *Front. Immunol.* **12**, 723409 (2021).
22. Chen, C. Y. et al. PTEN: Tumor suppressor and metabolic regulator. *Front. Endocrinol.* **9**, 338 (2018).
23. Chen, L. et al. CircSTAM inhibits migration and invasion of trophoblast cells by regulating miR-148a-5p/PTEN axis. *J. Assist. Reprod. Genet.* **40**(1), 201–210 (2023).
24. Zhang, L. et al. miR-26a promoted endometrial epithelium cells (EECs) proliferation and induced stromal cells (ESCs) apoptosis via the PTEN-PI3K/AKT pathway in dairy goats. *J. Cell Physiol.* **233**(6), 4688–4706 (2018).
25. Terakawa, J. et al. Ovarian insufficiency and CTNNB1 mutations drive malignant transformation of endometrial hyperplasia with altered PTEN/PI3K activities. *Proc. Natl. Acad. Sci. USA* **116**(10), 4528–4537 (2019).
26. Zhang, Y., Liu, Z. & Sun, H. Fetal–maternal interactions during pregnancy: A 'three-in-one' perspective. *Front. Immunol.* **14**, 1198430 (2023).
27. PrabhuDas, M. et al. Immune mechanisms at the maternal–fetal interface: Perspectives and challenges. *Nat. Immunol.* **16**(4), 328–334 (2015).
28. Ding, J. et al. M2 macrophage-derived G-CSF promotes trophoblasts EMT, invasion and migration via activating PI3K/Akt/Erk1/2 pathway to mediate normal pregnancy. *J. Cell Mol. Med.* **25**(4), 2136–2147 (2021).
29. Ding, J. et al. Granulocyte colony-stimulating factor in reproductive-related disease: Function, regulation and therapeutic effect. *Biomed. Pharmacother.* **150**, 112903 (2022).

30. Wang, N., Liang, H. & Zen, K. Molecular mechanisms that influence the macrophage m1–m2 polarization balance. *Front. Immunol.* **5**, 614 (2014).
31. Yang, J. et al. Trophoblast-derived miR-410-5p induces M2 macrophage polarization and mediates immunotolerance at the fetal–maternal interface by targeting the STAT1 signaling pathway. *J. Transl. Med.* **22**(1), 19 (2024).
32. Shima, T. et al. Uterine CD11c+ cells induce the development of paternal antigen-specific Tregs via seminal plasma priming. *J. Reprod. Immunol.* **141**, 103165 (2020).
33. Robertson, S. A. et al. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. *Biol. Reprod.* **80**(5), 1036–1045 (2009).
34. Liu, J. et al. IFN-τ mediated miR-26a targeting PTEN to activate PI3K/AKT signalling to alleviate the inflammatory damage of bEECs. *Sci. Rep.* **12**(1), 9410 (2022).
35. Schjenken, J. E. & Robertson, S. A. Seminal fluid and immune adaptation for pregnancy–comparative biology in mammalian species. *Reprod. Domest. Anim.* **49**(Suppl 3), 27–36 (2014).
36. Gao, P. et al. Long noncoding RNA LINC-PINT retards the abnormal growth of airway smooth muscle cells via regulating the microRNA-26a-5p/PTEN axis in asthma. *Int. Immunopharmacol.* **99**, 107997 (2021).
37. Wang, R. Q. et al. Lnc-GAN1 expression is associated with good survival and suppresses tumor progression by sponging mir-26a-5p to activate PTEN signaling in non-small cell lung cancer. *J. Exp. Clin. Cancer Res.* **40**(1), 9 (2021).
38. Zhang, B. et al. Engineered EVs with pathogen proteins: Promising vaccine alternatives to LNP-mRNA vaccines. *J. Biomed. Sci.* **31**(1), 9 (2024).
39. Lokumcu, T. et al. Proteomic, metabolomic, and fatty acid profiling of small extracellular vesicles from glioblastoma stem-like cells and their role in tumor heterogeneity. *ACS Nano* **18**, 2500 (2024).
40. Huang, B. X. et al. Exosomes derived from human adipose mesenchymal stem cells improve ovary function of premature ovarian insufficiency by targeting SMAD. *Stem Cell Res. Ther.* **9**(1), 216 (2018).
41. Sun, B. et al. miR-644-5p carried by bone mesenchymal stem cell-derived exosomes targets regulation of p53 to inhibit ovarian granulosa cell apoptosis. *Stem Cell Res. Ther.* **10**(1), 360 (2019).
42. Liu, Z. et al. Bushen Antai recipe alleviates embryo absorption by enhancing immune tolerance and angiogenesis at the maternal–fetal interface via mobilizing MDSCs in abortion-prone mice. *Phytomedicine* **123**, 155164 (2024).
43. Sausset, R. et al. Comparison of interferometric light microscopy with nanoparticle tracking analysis for the study of extracellular vesicles and bacteriophages. *J. Extracell. Biol.* **2**(2), e75 (2023).
44. Zarnani, K. et al. In-utero transfer of decidualized endometrial stromal cells increases the frequency of regulatory T cells and normalizes the abortion rate in the CBA/J x DBA/2 abortion model. *Front. Immunol.* **15**, 1440388 (2024).

Author contributions
Z.N.X. and X.L.C.; methodology: Y.Z., X.C., Q.L., X.Y.W. and X.L.L.; validation: Q.L., X.Y.W. and X.L.L.; investigation: J.L. and J.Z.; formal analysis: Y.Z., X.L.C.; writing—original draft preparation: Y.Z. and X.L.C.; writing—review and editing: Z.N.X.; visualization: J.L. and J.Z.; project administration: Y.Z. and X.L.C.; funding acquisition: Z.N.X.; All authors have read and agreed to the published version of the manuscript.

Funding
This work was supported by The Interdisciplinary Innovative Talents Foundation from Renmin Hospital of Wuhan University (JCRCGW-2022-003).

Declarations

Competing interests
The authors declare no competing interests.

Informed consent
Informed consent was obtained from all subjects involved in the study.

Institutional Review Board Statement
The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Clinical Trial Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China, CTEC number: WDRY2021-K044) and the ethics committee for laboratory animal welfare (IACUC) of Renmin Hospital of Wuhan University (Approval number: No. WDRM animal (f) No. 20201207).

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-92880-2.

Correspondence and requests for materials should be addressed to Z.X.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

© The Author(s) 2025