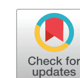
Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Full length article

Conditioned medium derived from seminal extracellular vesicles-exposed endometrial stromal cells induces inflammatory cytokine secretion by macrophages



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ARTICLE INFO

Article history:
Received 25 January 2020
Received in revised form 6 March 2021
Accepted 8 May 2021
Available online xxx

Keywords:
Semen
Extracellular vesicles
Endometrium
Stromal cells
Macrophages

ABSTRACT

Objective: Seminal plasma (SP) contains large numbers of sub-cellular structures called extracellular vesicles (EV) which have been postulated to have immunological functions due to their bioactive contents including proteins and small non-coding RNAs. Although the response of endometrial cells to seminal EV (SEV) is recently being elucidated, the impact of these signaling vesicles on stroma-immune crosstalk is still unknown. Herein, we aimed to investigate the effect of conditioned medium (CM) derived from SEV-exposed endometrial stromal cells (eSC) on cytokine secretion by macrophages.
Study design: SEV were isolated from SP samples of healthy donors and characterized by common methods needed for EV characterization, including size determination by dynamic light scattering (DLS), transmission electron microscopy (TEM), and western blot analysis of EV markers. Endometrial biopsies were obtained from healthy individuals and eSC were isolated and characterized. EV internalization assay was performed by labeling the SEV with PKH67 green fluorescent dye. Then, the eSC were exposed to SEV and the CM was collected. Finally, the CM from SEV-exposed eSC was added to the macrophage culture and the level of inflammatory (interleukin (IL)-1α and IL-6) and anti-inflammatory (IL-10) cytokines were measured in the culture supernatant of macrophages.
Results: The results demonstrated that the CM derived from SEV-exposed eSC induce IL-1α and IL-6 secretion by the macrophages, while the secretion of IL-10 was reduced.
Conclusion: Our results support the idea that the stroma-immune interaction is affected by SEV. This effect may be a part of immunoregulatory function of SP inside upper female genital tract and have an obvious impact during peri-implantation period.

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Introduction

Seminal fluid induces immune responses upon delivery to the female genital tract and contributes in preparation of the female immune system for pregnancy [1,2]. The most popular components which are known to mediate the immunological effects of seminal fluid are transforming growth factor beta (TGF-β) and

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https://doi.org/10.1016/j.ejogrb.2021.05.019
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prostaglandins [3,4]. Anyhow, semen contains vast quantities of specific sub-cellular structures called seminal extracellular vesicles (SEV) which are enriched in bioactive molecules with potential immunological activities [5,6]. Although there are studies which show the effects of SEV on immune and epithelial cells of female lower genitalia [7,8], possible immunological impacts of these extracellular vesicles (EV) inside endometrial microenvironment still remain rudimentary.

Recently, we have demonstrated that SEV induce cytokine, interleukin (IL)-6 and IL-8, secretion by endometrial stromal cells (eSC) in an in-vitro culture condition [9]. These cytokines are among pro-inflammatory factors which play pivotal roles during early implantation by recruiting immune cells to the maternal-fetal interface [10,11]. Despite this stimulatory effect on inflammatory cytokine secretion, the potential effect of the overall factors derived from SEV-exposed eSC on immune cells are completely unknown. As we know, there is a complex interaction between stroma-derived factors and immune system [12,13]. For instance, the stroma-derived factors contribute to differentiation and activation of the immune cells upon recruitment to the endometrium [13].

Macrophages are the second largest group of immune cells at the implantation site [14]. These cells display crucial roles in embryo implantation and development, placentation, and parturition [15,16]. It has been revealed that the main source of decidual macrophages is peripheral blood monocytes [17]. Upon delivery to the endometrium, these monocytes differentiate into specific subsets of macrophages due to local concentrations of certain factors [18]. According to a simplified classification, there are two major subtypes of tissue-specific macrophages based on their activation states, M1 with pro-inflammatory and M2 with anti-inflammatory actions [19,20], both of which play fundamental roles throughout pregnancy [16]. The number and proportion of these two subtypes change during different stages of pregnancy at the maternal-fetal interface so that the balance of polarization between them at each stage is essential for the maintenance of pregnancy [16]. Although the importance of macrophage activation and polarization in pregnancy is well understood, the possible involved mechanisms are not fully elucidated [17].

To the best of our knowledge, there have been no investigations into the direct or indirect influence of seminal plasma (SP) components on macrophage polarization. Determining whether SEV stimulate eSC, as the most abundant cells of the endometrium, to educate monocytes during preimplantation period will further our understanding of SP contribution in embryo implantation process. In this study, we hypothesized that conditioned medium (CM) derived from SEV-exposed eSC might directly induce inflammatory cytokine secretion by macrophages and may contribute to their polarization.

Materials and methods

Ethics statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the regional ethics committee (at School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, with reference number: IR.SBMU.MSP.REC.1395.448) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants gave written informed consent.

Isolation and culture of eSC

A total of 12 healthy premenopausal women undergoing examination for benign gynecological conditions accepted to enter the study. Patients with inflammatory conditions such as endometriosis, hydrosalpinx, *etc.* were excluded from the study (n = 6). Primary eSC were isolated from endometrial biopsies obtained from individuals with proven fertility (one live birth in the last five years prior to the study), aged 25–40 years with normal menstrual cycles (25–30 days) (number of selected women = 6). Each biopsy was taken from several regions of functional layer of fundal part of endometrium using a biopsy catheter (Pipelle® Endometrial Suction Curette, Cooper Surgical, USA). Based on the timing of last menses, all samples were obtained during proliferative phase of menstrual cycle.

eSC isolation was performed according to the previous studies [21,22] with little modifications. Briefly, the biopsies were mechanically minced into small fragments and incubated with 2.5 mg/mL of collagenase type I (Sigma-Aldrich, USA) for 1 h at 37 °C. After enzymatic digestion, the dissociated cellular fragments were passed through a 40 μm cell strainer (SPL Life Sciences, South Korea) to isolate single stromal cells from aggregates of epithelial cells. After two times washing by centrifugation at 300× g for 2 min, the cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Sigma-Aldrich, USA), supplemented with 10 % fetal bovine serum (FBS) and penicillin-streptomycin (100 IU/mL and 100 μg/mL, respectively) (Gibco, USA) for 12 h at 37 °C and 5 % CO_2. The next day, non-adhered cells were washed out and adhered cells were allowed to proliferate. Medium change was performed every 2 days and the cells were passaged at ~80 % of confluence. To establish the purity of isolated eSC, immunofluorescence staining was performed to detect vimentin intermediate filament protein.

Semen sample collection and SEV isolation

A total of 6 healthy men undergoing preimplantation sex selection procedure were enrolled to enter the study. All the individuals had proven fertility (fathered at least one child in the last five years prior to the study) and normal sperm characteristics based on the World Health Organization (WHO) 2010 guideline (Table 1). After seminal fluid sample collection and liquefaction for 1 h at 37 °C, the cellular fraction was extracted by centrifugation for 10 min at 300× g, then the SP fraction was stored at −20 °C until SEV isolation.

For SEV isolation, the frozen SP samples were thawed and pooled together. EV isolation was performed by ExoQuick-TC exosome precipitation reagent (System Biosciences, USA) according to the manufacturer's instruction. This reagent isolates small

Table 1
Characteristics of the semen specimens obtained from healthy fertile donors.

Sample ID	Abstinence Days	Total Sperm Count (million/Ejaculate)	Total Motility (%)	Normal Morphology (%)
TH2514	2	260	44	5
TH8252	6	152	67	6
MH7709	5	100	60	4
SC4676	3	410	75	7
MC5418	2	98	55	5
AH9246	2	49	63	4

EV, mostly exosomes. First, the remaining sperm and cellular debris were removed by centrifugation at 3000× *g* for 15 min and passing through a 0.45 μm syringe filter. Next, the precipitation solution was added to the filtered SP at a ratio of 4:1 (SP/precipitant). Afterwards, the solution was resuspended by inverting and flicking, then incubated at 4 °C overnight. For pelleting the EV at the final step, the suspension was centrifuged at 1500× g for 30 min. The resulting pellet was resuspended in ca^{2+}/mg^{+2} free Dulbecco's Phosphate- Buffered Saline (DPBS) and stored at −80 °C until use.

SEV characterization

The protein content of SEV suspension was quantified by Bicinchoninic Acid (BCA) protein quantification kit (Parstous Biotechnology, Iran). For characterizing the isolated SEV, a concentration of 2 mg/mL was used.

Size determination was performed using a Zetasizer (Malvern, UK) by dynamic light scattering (DLS) in triplicate at room temperature (RT). The obtained data were analyzed by the Malvern software (Zetasizer Ver. 7.11).

For morphology observation *via* transmission electron microscopy (TEM), first the SEV were embedded in 2 % agarose gel and the fixation process was performed in 2.5 % glutaraldehyde (for 12 h) and 2 % osmium tetroxide (for 30 min). Next, the dehydration procedure was conducted in ascending series of ethanol. Finally, the specimen was embedded in 812 Resin (TAAB, UK) and polymerization was performed at 60 °C for 24 h. After sectioning by an ultramicrotome, the ultrathin sections were collected on formvar-carbon coated copper grids and contrasted with 2 % uranyl acetate and 0.5 % lead citrate (TAAB, UK). Ultimately, the documentation was carried out by Zeiss EM900 transmission electron microscope.

For western blot analysis of EV markers, the SEV were lysed by radioimmunoprecipitation assay (RIPA) buffer which contained phenylmethylsulfonyl fluoride (PMSF) as a serine protease inhibitor. Protein denaturation was conducted by heating the soluble proteins at 95 °C for 5 min. Then, the proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on a nitrocellulose membrane (GE healthcare, Amersham, UK) by a semi-dry transfer cell (Trans-Blot® SD, Bio-Rad, USA). Blocking of non-specific bindings was performed by placing the membrane in a 3 % solution of non-fat dry milk in tris-buffered saline (TBS) at RT for 3 h. After washing in tris-buffered saline solution containing tween 20 detergent (TBST), the membrane was incubated in primary antibodies including anti-human CD63 and anti-human CD81 (BioLegend, USA) as markers that are enriched in EV. In addition, primary antibody against Argonaute family proteins (Ago1/2/3) (Active Motif, USA), which are absent or under-represented in EV, was utilized to show that the purified EV are devoid of cell debris. Subsequently, the membrane was incubated in appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and the signals were detected by enhanced chemiluminescent (ECL) detection system (Amersham Pharmacia Biotech, UK). Lysate of MCF-7 cell line was utilized in order to show that the SEV preparation have limited or no contamination with cell debris.

SEV internalization by eSC

The green fluorescent dye PKH67 (Sigma-Aldrich, USA) was used to label SEV and track their uptake by eSC. First, 100 mg of SEV were suspended in 500 mL of diluent C according to the manufacturer's instructions. Next, 3 mL of PKH67 dye was diluted in 500 mL of diluent C and added to the first suspension. Incubation was performed at 37 °C for 5 min. Then, the staining was stopped by adding 250 mL of FBS and the labeled SEV were separated from the dye-containing suspension by ExoQuick-TC reagent as described previously. The precipitated PKH67-labeled SEV were resuspended in phosphate-buffered saline (PBS) and added to eSC culture. After an overnight incubation, the washing was performed with PBS and the cells were fixed with 4 % paraformaldehyde at RT for 5 min. Finally, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, USA) and the cells were analyzed by Leica TCS SPE confocal laser scanning microscope.

eSC treatment with SEV

eSC were seeded in 6-well cell culture plates (SPL Life Sciences, South Korea) at a density of 3×10^5 per each well. After an overnight incubation, treatment was conducted in three groups including: 1- eSC exposed to SEV (100 μg/mL SEV in DMEM/F-12 with 10 % FBS), 2- eSC exposed to SP (1 % in DMEM/F-12 with 10 % FBS), and 3- eSC cultured in DMEM/F-12 with 10 % FBS as control group. The culture continued for 24 h at 37 °C and 5 % CO_2. This experiment was fully repeated two times. The number of replicates of each group in each of these experiments was two.

Harvesting CM form eSC culture

To obtain the CM of the above-mentioned groups, the culture supernatants were harvested after finishing the incubation period and centrifuged at 300× *g* for 5 min to discard suspended cells. Then, the supernatants were filtered through a 0.22 μm syringe filter to remove cellular debris and stored at −80 °C until use.

Monocyte isolation and characterization

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor's blood sample by Ficoll hypaque density gradient centrifugation method (Lymphosep®, BioWest, France) according to manufacturer's instructions. The isolated cells were seeded onto T25 cell culture flask (SPL Life Sciences, South Korea). After 2 h incubation in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) supplemented with FBS, non-adhered mononuclear cells were discarded and attached macrophages were washed with PBS and then detached using Accutase® (Sigma Aldrich, USA) for further use in experiments. The purity of the isolated macrophages was confirmed by flow cytometric analysis of CD14 surface marker. The purity was >95 %.

Macrophage treatment with CM derived from eSC culture

To study the effect of the factors derived from SP or SEV-exposed eSC on macrophages, freshly isolated macrophages (1×10^5 cells per each well of 24-well plate in duplicates) were incubated with CM from SP or SEV-treated and untreated eSC. After 48 h of incubation, the supernatants were discarded and the cells were washed with PBS twice. Then, culture continued for an extra 24 h in RPMI-1640 supplemented with 5 % FBS to allow cytokine secretion. Ultimately, supernatants were harvested and centrifuged at 3000× g for 15 min to remove cells and debris, and then stored at −80 °C until cytokine assay. This experiment was fully repeated two times. The number of replicates of each group in each of these experiments was two.

Measurement of cytokines in supernatant of macrophages stimulated by CM derived from eSC culture

To determine the stimulated cytokines, the levels of inflammatory (IL-1α and IL-6) and anti-inflammatory (IL-10) cytokines secreted by the macrophages to their culture medium were

measured in duplicate using DuoSet® ELISA Development Systems (R&D Systems, USA) according to manufacturers' instruction. The minimum detectable threshold for IL-1α, IL-6, and IL-10 were 7.8, 9.4, and 31.2 pg/ ml, respectively. The results were finally quantified utilizing standard curves fitted with sigmoidal four-parameter logistic (4 PL) regression.

Statistics

The ELISA data was analyzed by SPSS version 19 software (SPSS, USA) and described as means ± standard deviation. The means of data in the groups were compared by one-way ANOVA using LSD *post-hoc* test. Significance was established at P value<0.05 (*), P value<0.01 (**) or P value<0.001 (***) levels.

Results

eSC isolation and characterization

Out of 6 biopsy samples obtained from selected women, 4 yielded a sufficient number of eSC and were used in the study. Based on the immunofluorescence staining, 94 % of the isolated cells expressed vimentin protein, so the stromal phenotype of the cells was confirmed (Fig. 1).

SEV characterization

The BCA assay showed a protein content of 25 mg/mL in the isolated SEV suspension. Isolated SEV from 6 different donors had an average diameter of ~84 nm determined by DLS, which is within the characteristic size range of the small EV, namely exosomes (Fig. 2a). Furthermore, size distribution by intensity graphs was indicative of the absence of larger EV (mostly microvesicles and apoptotic bodies) which validated the purity of the isolated small EV. TEM analysis showed particles with classic morphology of EV, *i.e.* lipid bilayer-enclosed single particles within the expected size range of the small EV (Fig. 2b). Western blot analysis revealed that the tetraspanins (CD63 and CD81), which are commonly used for EV identification [23], were enriched in SEV preparation but not in the cell lysate (Fig. 2c). In addition, the SEV preparation was negative for Ago1/2/3, unlike the cell lysate, which indicates that the preparation had limited or no cell debris. Together, these obtained data confirmed the identity of isolated SEV and were consistent with published studies for EV characteristics in general.

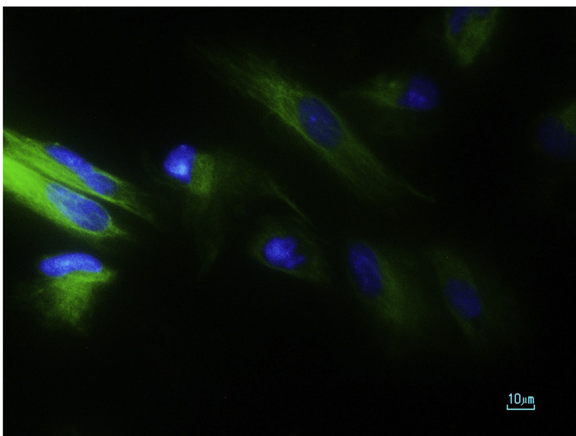

Fig. 1. Representative immunofluorescence image of vimentin positive eSC. Antibody reactivity against vimentin is shown in green fluorescence. The Nuclei are stained with DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

SEV uptake by eSC

For tracking the uptake of SEV by eSC, the vesicles were labeled with green fluorescent dye and then added to the eSC culture. Cytoplasmic localization of the PKH67-labeled SEV within the eSC (Fig. 3) is indicative of the capacity of eSC to uptake SEV which is the initial step for subsequent signal transduction process conducted by the contents of the SEV.

CM derived from SEV-exposed eSC induces inflammatory cytokine secretion by macrophage

To investigate the impact of the factors derived from SP or SEV-exposed eSC on macrophages, we measured the level of selected cytokines produced by macrophages after exposure to the CM from SP or SEV-treated and untreated eSC. The results demonstrated that the macrophages treated with CM derived from SEV-exposed eSC release significantly higher amounts of IL-1α in comparison to the macrophages treated with CM from SP-exposed eSC ($P < 0.01$) and the macrophages treated with CM from untreated eSC ($P < 0.001$) (Fig. 4a). A same pattern was observed in the amounts of secreted IL-6, but to a lesser extend (Fig. 4b). In contrast, the results were indicative of lower production of IL-10 from the macrophages treated with CM derived from SEV-exposed eSC than the other groups (Fig. 4c), even so this lower production was significant compared to the macrophages treated with CM from SP-exposed eSC ($P < 0.01$) but not significant from the macrophages treated with CM from untreated eSC. This cytokine secretion profile may indicate inflammatory macrophage polarization following stimulation by CM derived from SEV-exposed eSC.

Discussion

To the best of our knowledge, this is the first study which investigated the effect of SEV on the interaction between eSC and macrophages. We investigated the effect of CM derived from SEV-exposed eSC on macrophage cytokine secretion by quantifying the levels of inflammatory (IL-1α and IL-6) and anti-inflammatory (IL-10) cytokines released by macrophages after exposure to the CM. The results showed a significant increase in IL-1α and IL-6 production, while the production of IL-10 was reduced. This cytokine secretion profile may be indicative of inflammatory subtype of macrophages, namely M1 phenotype; however, it is too preliminary to draw a conclusion on macrophage polarization due to the complex secretory and gene expression profiles of different subtypes of macrophages [16].

According to the study conducted by Chen et al., SP induced global transcriptomic changes in eSC which consequently stimulated monocyte chemotaxis [24]. However, the subsequent effect of CM from SP-treated eSC on monocyte cytokine secretion and polarization was not explored. Our results showed that CM from SP-exposed eSC induces IL-1α, IL-6 and IL-10 secretion by macrophages. This observed cytokine pattern was different from the pattern induced by CM from SEV-exposed eSC, which may be due to the soluble components of SP that are not packaged in SEV. A comprehensive explanation for the disparate effect of CM from SP-exposed eSC *versus* CM from SEV-exposed eSC on IL-10 secretion by macrophages should be searched in the secretome of eSC following exposure to SP and SEV. As indicated by Chen et al. [24], SP induced the production of multiple cytokines in eSC, including monocyte chemoattractant protein-1 (MCP-1/CCL2), MCP-3 (CCL7), growth-regulated alpha protein (GROa/CXCL1), fractalkine (CX3CL1), fibroblast growth factor 2 (FGF2), IL-6, IL-8, and vascular

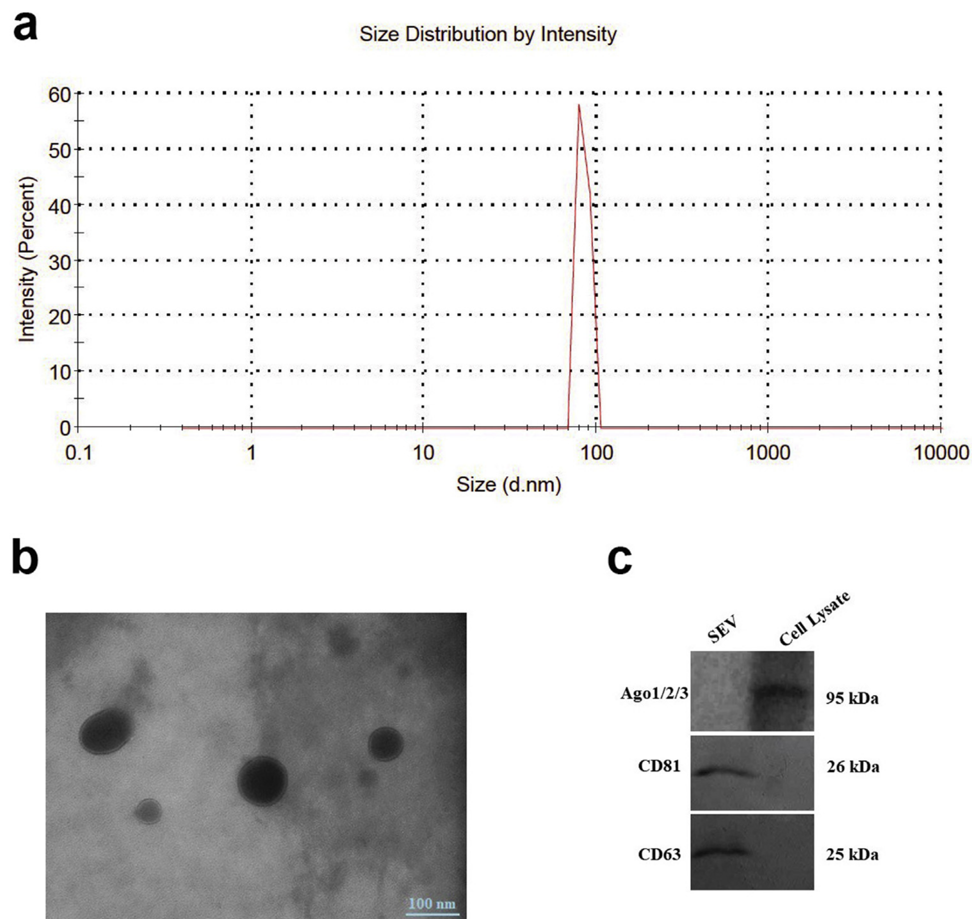

Fig. 2. Characterization of isolated SEV. (a) Suspension of isolated SEV was analyzed using dynamic light scattering (DLS) method to determine the size distribution of isolated EV. The intensity graph is indicative of the absence of large EV (microvesicles and apoptotic bodies). (b) Transmission electron micrograph of isolated SEV shows lipid bilayer-enclosed single vesicles of varying densities. (c) Western blot analysis was positive for common EV markers, CD81 and CD63, and negative for intracellular Argonaute family proteins (Ago1/2/3), in SEV suspension. MCF-7 cells lysate showed positive reaction with antibody against Ago1/2/3; however, CD81 and CD63 were not detected in the lysate.

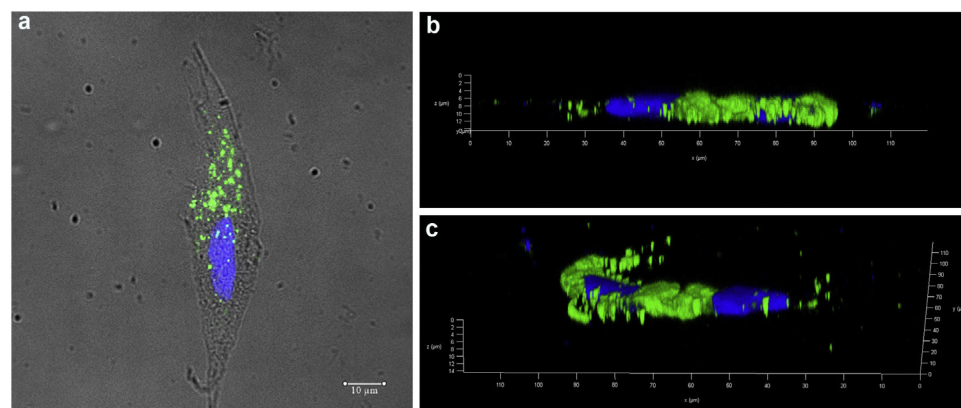

Fig. 3. Confocal microscopy of eSC exposed to PKH67-labeled SEV for 12 h shows cytoplasmic localization of SEV within eSC. (a) 2D view of PKH67-labeled SEV (green) in the cytoplasm and around cell nucleus (blue). (b and c) 3D views showing that the labeled SEV have been taken up by the eSC and exist in the cytoplasm and around cell nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

endothelial growth factor A (VEGFA). These cytokines have different effects on macrophage polarization. For instance, it has been demonstrated that co-treatment of anti-inflammatory subset of macrophages with IL-6 resulted in spontaneous release of IL-10, despite the fact that IL-6 is commonly being associated with pro-inflammatory functions [25]. As another example, it has been revealed that CX3CL1 can induce the expression of IL-10 in tissue-specific macrophages [26]. Although the effect of SP on eSC secretions is frequently studied, the effect of SEV on eSC secretion is rarely investigated. Studying the secretomic changes of eSC after exposure to SP and SEV will help in unveiling the subsequent differences in the induced immunological responses. Besides, SEV is one fraction of SP, and the overall impact of SP in modulating immune response may be the result of cumulative effects of both

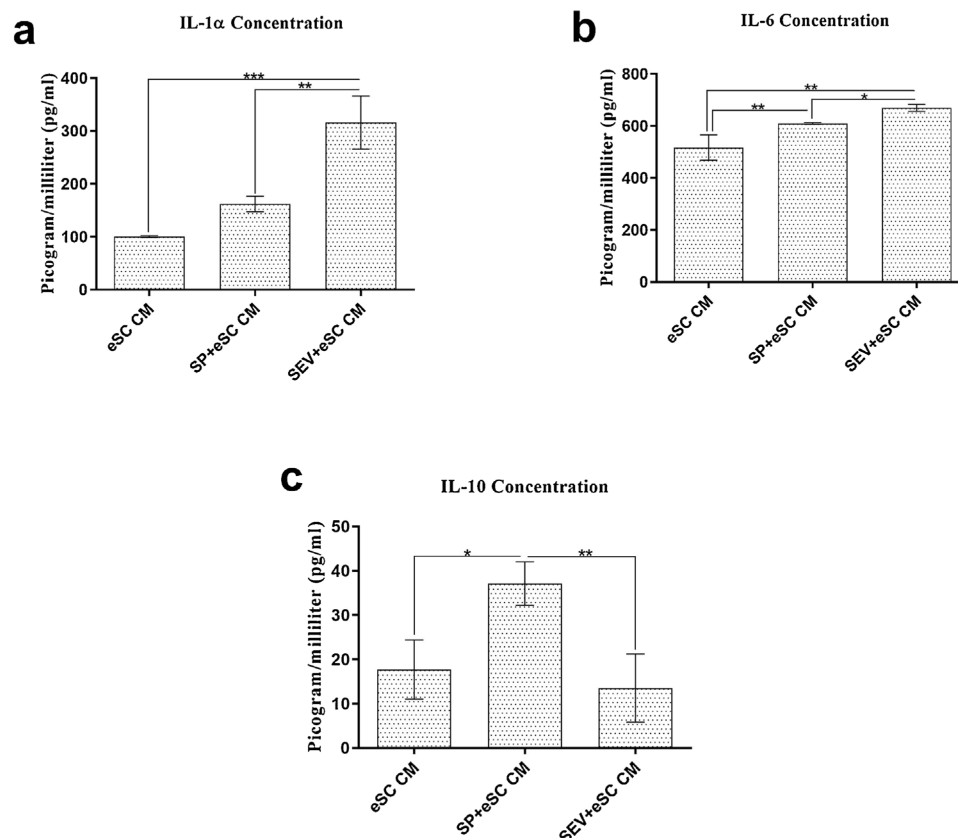

Fig. 4. Level of inflammatory and anti-inflammatory cytokines secreted by macrophages after exposure to CM derived from SP and SEV-exposed eSC. CM from SEV-exposed eSC significantly induced IL-1α (a) and IL-6 (b) secretion by macrophages in comparison to the CM from SP-exposed eSC and CM from untreated eSC. While the production of IL-10 (c) by macrophages treated with CM from SEV-exposed eSC revealed no significant difference from macrophages treated with CM from untreated eSC. Error bars represent standard deviations (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

SEV and other components of SP. This is more similar to what happens *in vivo* where whole semen reaches the endometrial environment. Collectively, the observed difference in subsequent effects of SP and SEV makes it essential for considering all components of SP in studying the immunological impacts of semen on the endometrium. More transferable results will be obtained from *in vitro* studies if the whole semen, containing spermatozoa, is considered.

Recently, Vojtech et al. studied the effect of SEV on adaptive cellular immune responses [27]. They revealed that SEV alter the function of antigen-presenting cells (APC) directly and subsequently induce a shift toward immunosuppressive phenotype of T cells which is essential to promote successful pregnancy [28]. By the way, there are other components that participate in the process of immune regulation at the maternal-fetal interface including trophoblasts and eSC [12]. The eSC comprise the majority of the cellular volume of the endometrium [24,29,30] and are located under the epithelial cells so that the SP contents can gain direct access to them following coitus [24]. The access of SP to the underlying stroma may seem impossible at first glance due to the presence of epithelial barrier composed of tight junctions; however, there are some studies which have provided evidence of the permeability across tight junctions in some situations. Someya et al. showed that progesterone can induce permeability, by downregulating the barrier function of tight junctions, in primary polarized endometrial epithelial cells [31]. In the same study, it was demonstrated that the progesterone increased the formation of stress fibers and this modification of the actin organization resulted in a reduction of the barrier function. Collectively, these data suggest that the passage of luminal content (such as semen) through the polarized epithelial cells to the underlying stroma could be possible during the progesterone-dominated secretory phase. It has been also demonstrated that tight junctions in the luminal epithelial cells of the human endometrium show a reduction in junctional complexity from the late proliferative/early luteal phase to the late luteal phase of the menstrual cycle [32]. As it has been shown by previous studies, seminal plasma has the ability to induce inflammation in the uterus [33]. Chen et al. showed that seminal plasma can induce the secretion of TNF-α by endometrial epithelial cells [24]. It has been shown that TNF-α secreted by epithelial cells contributes significantly to the disruption of barrier function in epithelial cells [34]. Altogether, these data suggest that seminal plasma itself may increase the access of endometrial stromal cells to the luminal contents. This evidence can be validated by the studies which have revealed that the inflammation can influence production or arrangement of tight junction components leading to elevated permeability and exposure of tissues to antigens [35]. Recently, some studies have stated that exosomes, known as small EV, have the ability to cross biological barriers in both directions [36]. For instance, Pieragostino et al. revealed the presence of exosomes with central nervous system (CNS) origin in tears possibly due to the elevated permeability of blood–brain barrier (BBB) in some conditions [37]. It has been also demonstrated that the uptake of microvesicles, which are larger EV than exosomes, can induce dysregulation of the genes related to tight junctions which finally leads to a significant elevation of permeability across BBB [38]. These data raise the possibility that SEV may have a similar ability in crossing the epithelial cells lining the endometrium to access the stromal cells. More studies are needed to evaluate the impacts of

SP and SEV on tight junction structure and their ability to pass the epithelial barrier to access stromal cells. In any case, reciprocal communication exists between eSC and macrophages, as classic APC [39–41]. According to our results, this crosstalk is influenced by SEV, a primary data which needs to be examined extensively to obtain new hints of the potential roles which SEV play in context of immune regulation inside the endometrium.

In conclusion, this observation supports the notion that the stroma-immune crosstalk is affected by SEV and these highly abundant extracellular structures take part in immunoregulatory functions of SP inside the endometrial microenvironment. It is important to mention that our study was conducted on a simply designed *in vitro* experiment. As discussed before, there is a complex communication between stromal cells, embryonic cells and different types of immune cells inside the endometrium [12]. Additionally, ovarian hormones affect the biological activity of these cells [24,42]. Therefore, studies should be considered to investigate the effects of SEV on the endometrial environment in the presence of all the involved cell types and the ovarian hormones, for instance through the use of endometrial organoids containing immune cells. The output may be development of a clinical method to take advantage of SEV as immunoregulatory components for preparation of the endometrium prior to embryo transfer during assisted reproduction cycles.

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.

Acknowledgments

This paper has been extracted from the PhD thesis written by Mr. S Paktinat in School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Registration No:151) and was funded by the university under project No: 11071.

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