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Seminal exosomes induce interleukin-6 and interleukin-8 secretion by human endometrial stromal cells

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ABSTRACT

Objective: Exosomes are extracellular microvesicles that participate in intercellular communication. Seminal plasma (SP) contains very large amounts of exosomes which are deposited in female genital tract after insemination. Although the response of vaginal cells to seminal exosomes (SE) is recently being elucidated, the interaction of uterine cells with SE is still unknown. Here, we aimed to evaluate the effect of SE on cytokine secretion by human endometrial stromal cells (eSC).
Study design: Exosomes were isolated from the semen samples of healthy men with proven fertility and characterized using common exosome characterization methods. Human eSC were isolated from endometrial biopsies obtained from healthy premenopausal women. For exosome internalization analysis, SE were labeled with PKH67 green fluorescent dye and incubated with the cells. For investigating the effect of SE on cytokine secretion of eSC, we measured levels of interleukin (IL)-6, IL-8, IL-10, IL-1α, and leukemia inhibitory factor (LIF) in the culture supernatants of control and experimental groups by enzyme-linked immunosorbent assay (ELISA) after 24 h of incubation.
Results: Our results demonstrated that SE are internalized by eSC and subsequently induce them to produce IL-6 and IL-8, the cytokines which are involved in the immunology of embryo implantation.
Conclusion: The findings of the present study suggest that SE contribute to the immunoregulatory functions of SP in the uterus and may participate in embryo implantation process. Therefore dysfunction of intracellular machineries of SE biogenesis and secretion, inadequate production, defective transportation to the uterus and impaired communication with endometrium may play a distinct role in pathophysiology of embryo implantation failure.

Introduction

It has been well established that seminal plasma (SP) is more than a carrier and protective medium for sperm in mammalian species [1]. Recently, there has been a focus on the immunological roles of SP components and their participation in modulating female immune responses prior to embryo implantation [2,3]. Seminal exosomes (SE), highly abundant extracellular microvesicles secreted by different cellular sources in male reproductive tract, play distinct roles in SP physiology including, but not limited to, sperm maturation and immunomodulation [4,5]. Although it is now broadly accepted that SE affect the immune and mucosal cells residing in lower female genital tract [6,7], very little is known about direct effects of these structures on the upper tract, particularly the endometrial cells.

According to the literature, SE are enriched in bioactive molecules including proteins and small non-coding RNAs, including microRNA, Y RNAs, and tRNAs [8,9]. let-7 family, miR-148a, mir-375, and miR-22 are amongst the most abundant microRNAs inside SE which have proven regulatory functions and target immune-related mRNAs including interleukins [9]. This cargo could potentially impact signaling pathways inside recipient mucosal cells upon delivery [1]. In a study conducted on porcine uterus, it was found that SE induce immune and inflammatory gene expression in endometrial epithelial cells [10]. It is doubtful whether exosomes in human semen could exert such effects on immune regulation in the endometrium. We hypothesized that human SE may

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per se stimulate endometrium and provoke cytokine secretion by endometrial stromal cells (eSC), which are the most abundant cells that comprises the endometrium [11–13]. This stimulation may consequently act as a contributing factor for priming immune environment required during early embryo implantation.

The current study aimed at investigating cytokine secretion by cultured eSC after exposure to the exosomes isolated from SP. The studied cytokines, including interleukin (IL)-6, IL-8, IL-10, IL-1α, and leukemia inhibitory factor (LIF), were selected based on their crucial roles during implantation as a pro-inflammatory stage [14–17] and literature showing the expression and secretion of these cytokines by endometrial cells after exposure to SP [13,18].

Materials and methods

Ethical consideration

In this study, usage of human specimens was approved by the regional Ethics Committee at School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, under IR.SBMU.MSP.REC.1395.448 reference number. Written informed consent was obtained from all participants.

Endometrial biopsies

A total of 12 individuals were enrolled and assessed for eligibility to enter the study. Endometrial biopsies were obtained from healthy premenopausal women, and patients with endometriosis, hydrosalpinx, and other inflammatory conditions were excluded (n = 6). Selected women (n = 6) were 25–40 years of age with menstrual cycles of 25–30 days, proven fertility, and undergoing examination for benign gynecological conditions. Each biopsy of the functional layer of the endometrium was taken from several spots of the fundal part of the uterine cavity using a biopsy catheter (Pipelle® Endometrial Suction Curette, Cooper Surgical, USA). Samples were retrieved during the proliferative phase of the menstrual cycle based on the timing of the last menses.

Isolation of eSC

The samples were transferred to the laboratory in tissue culture media and immediately processed for isolation of eSC according to the published protocols with some modification [19,20]. Briefly, biopsies were cut into small fragments (1–2 mm) with a scalpel in fresh Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) and digested with collagenase type I (2.5 mg/ml) for 1 h at 37 °C. The dissociated cellular elements were then transferred through a 40 μm cell strainer to separate single stromal cells from segments of epithelial sheets and glands. The cells were passed through the strainer and cultivated in T-25 culture flasks for 12 h for purification. Afterwards, non-adherent cells were discarded and adherent stromal cells were allowed to propagate. The purity of the isolated eSC was established by immunofluorescence staining of vimentin.

Semen samples

The study was carried out on 10 healthy men referred to IVF clinics for preimplantation sex selection. Semen samples of individuals with proven fertility (men who fathered a child in the last 5 years before the study) and normal criteria according to the World Health Organization (WHO) 2010 guideline were used for exosome isolation (n = 6). Table 1 represents semen criteria of selected men.

The ejaculates were allowed to liquefy for 1 h at 37 °C and then centrifuged for 10 min at 300 g to pellet sperm. Then, the SP was transferred to a new tube and stored at −20 °C until exosome isolation.

Isolation of exosomes from SP

At the time of exosome isolation, the frozen samples were simultaneously thawed and combined. Isolation procedure was performed using 2.5 ml of the pooled sample. To remove cellular debris, the pooled sample was centrifuged at 3000 g for 15 min and filtered by 0.45 μm syringe filter. Then, the exosomes were isolated using ExoQuick exosome precipitation reagent (Exo-Quick-TC, System Biosciences, CA, USA) according to the manufacturer's instruction. Briefly, exosome precipitation solution was added at a ratio of 4:1 (SP/ExoQuick) each time. The mixture was then resuspended by inversion and incubated at 4 °C overnight. Next, exosomes were pelleted by centrifugation at 1500 g for 30 min and resuspended in appropriate volume of Dulbecco's Phosphate-Buffered Saline (DPBS) without ca^{2+} and mg^{+2}. Finally, the total protein content of exosomal fraction was quantified by the Bicinchoninic Acid (BCA) protein assay kit and the suspension was aliquoted and stored at −80 °C until use. A concentration of 2 mg/ml was utilized for characterizing exosomes.

Exosome size determination

Exosomes were diluted with DPBS and size measurement was performed by a Dynamic Light Scattering (DLS) Zetasizer (Malvern, Worcestershire, UK) in triplicate at room temperature immediately after isolation. Data were obtained and analyzed by the Malvern software (Zetasizer Ver. 7.11).

Scanning electron microscopy

A diluted aliquot of isolated exosomes was fixed in 2.5% glutaraldehyde, dried at room temperature on a glass substrate, sputter coated with gold, and then visualized under scanning electron microscope (KYKY-EM3200, Beijing, China).

Transmission electron microscopy

In order to simplify the embedding process, the purified exosomes were embedded in 2% agarose gel. The sample was then fixed in 2.5% glutaraldehyde for 12 h and post-fixed in 2% osmium tetroxide for 30 min. After dehydration in an ascending series of

Table 1
Characteristics of the semen samples obtained for this study.

Sample ID	Days of Abstinence (Days)	Sperm Concentration (10^6/ml)	Total Sperm Count (10^6/Ejaculate)	Progressive Motility (%)	Total Motility (%)	Normal Morphology (%)
1017	3	50	150	45	50	6
1074	3	49	98	60	67	7
1117	4	120	180	53	60	10
1145	3	82	164	58	70	6
1222	2	25	100	50	55	5
1290	5	40	40	61	71	7

ethanol, the specimen was finally embedded in 812 Resin (TAAB, UK) and polymerized for 24 h at 60 °C. Ultrathin sections (less than 100 nm-thick) were cut, picked up on copper grids and stained with 2% uranyl acetate for 3 min and 0.5% lead citrate for 5 min. Ultimately, the sections were documented with a transmission electron microscope (Zeiss EM900).

Western blot analysis of exosome markers

Exosomes were lysed by adding an equal volume of Radioimmunoprecipitation assay (RIPA) lysis buffer containing Phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. Soluble proteins (20–40 μg) were denatured by heating at 95 °C for 5 min, separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Hybond-C pure, Amersham) using a semi-dry transfer cell (Trans-Blot® SD, Bio-Rad), according to the manufacturer's protocol. Blocking was performed using 3% skim milk in Tris-buffered saline solution with tween 20 (TBST) for 3 h at room temperature. Then, the membrane was probed for two exosome markers using anti-human CD63 (1:1000) (LEAF™ Purified anti-human CD63, BioLegend) and anti-human CD81 (1:1000) (Purified anti-human CD81 (TAPA-1), BioLegend) as primary antibodies, followed by appropriate Horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, the signals were visualized using enhanced chemiluminescent (ECL) detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Cell lysate was used as a negative control.

Uptake of SE by eSC

For exosome internalization assay, SE were labeled with PKH67 green fluorescent dye following the manufacturer's instruction with some modifications. Briefly, exosomes (100 μg) were suspended in 500 μl of diluent C. Then, an equal volume of diluted dye (3 μl of PKH67 in 500 μl of diluent C) was added to the exosomes suspension and incubation was performed for 5 min at

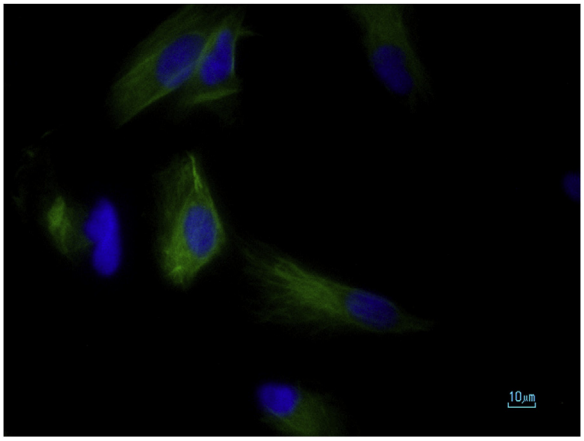

Fig. 1. Immunofluorescence staining of Vimentin in isolated eSC. Green fluorescence shows antibody reactivity against Vimentin and blue fluorescence shows nuclear DAPI reactivity (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

37 °C. The staining reaction was stopped with 250 μl of Fetal bovine serum (FBS) for 1 min, and the labeled exosomes were then separated from the unbound dye using ExoQuick exosome precipitation reagent. After incubation at 4 °C overnight, precipitated PKH67-labeled exosomes were resuspended in 50 μl of Phosphate-buffered saline (PBS) and added to subconfluent eSC cultured in glass bottom chamber slides. After 24 h of incubation, the cells were washed with PBS, fixed for 5 min with 4% paraformaldehyde at room temperature and immediately analyzed using a confocal laser scanning microscope (Leica TCS SPE).

Experimental groups and collection of cell culture supernatant

The experiment was performed on eSC seeded onto 6-well tissue culture plates (in duplicates) at a density of 3×10^5/well in

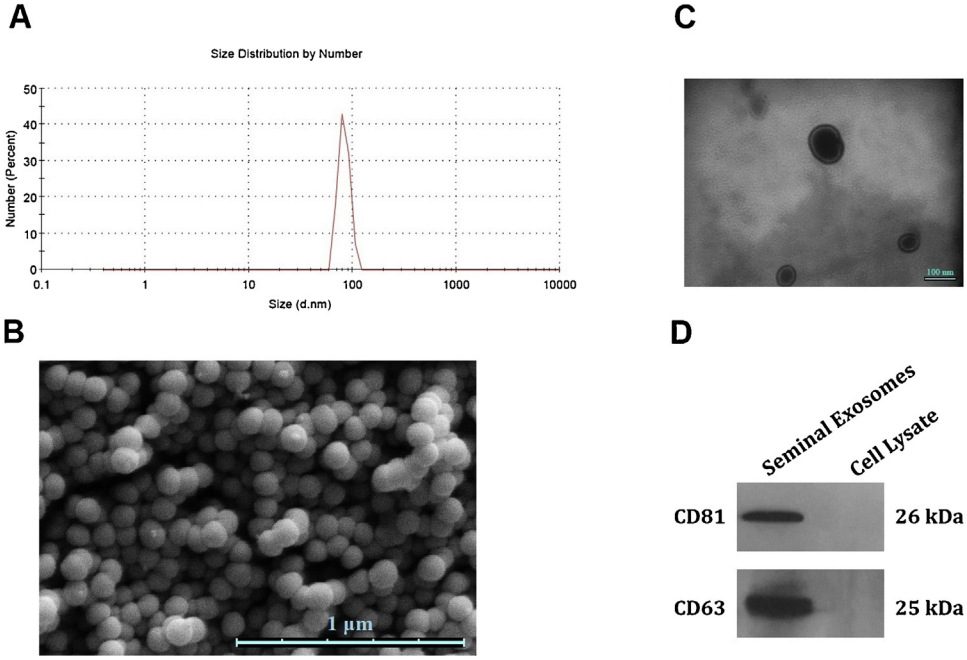

Fig. 2. Characterization of SE. (A) Representative dynamic light scattering (DLS) number distribution measurement of isolated SE showing a single peak at 84 nm. (B) Scanning electron micrograph of isolated SE depicting spherical membrane-encapsulated vesicles. (C) Transmission electron micrographs of SE showing individual vesicles of varying densities with intact lipid bilayers. (D) Western blot analysis on SE using antibodies against the common exosomal markers (CD63 & CD81). Lysates from MCF-7 cells were used as a negative control.

2 ml of culture media. Experimental groups consisted of cells exposed to SE (100 μg/ml in DMEM/F-12 supplemented with 10% FBS) or SP (1% in DMEM/F-12 supplemented with 10% FBS). The cells in control group were cultured in DMEM/F-12 supplemented with 10% FBS. After 24 h of culture, the supernatants were collected, centrifuged at 3000 g for 15 min, and stored at −80 °C until cytokine assay.

Cytokine quantitation

The concentrations of interleukin (IL)-6, IL-8, IL-10, IL-1α, and leukemia inhibitory factor (LIF) were quantified twice by using DuoSet® ELISA Development Systems (R&D Systems, Minneapolis, MN, USA) in the cell-free supernatants of cultured eSC according to manufacturers' instruction. The minimum detectable threshold for IL-6, IL-8, IL-10, IL-1α, and LIF were 9.4, 31.2, 31.2, 7.8, and 15.6 pg/ml, respectively. The results were calculated using standard curves fitted with sigmoidal four-parameter logistic (4 PL) regression for each cytokine. It should be noted that levels of the selected cytokines were also measured in 100 μg/ml solution of SE and 1% solution of SP for distinguishing the factors secreted by eSC in response to SE and SP from the factors endogenously present in SE suspension and 1% solution of SP.

Data analysis

Statistical analyses were performed with SPSS v.19 software (Chicago, IL, USA). Comparisons between the three groups were conducted using one-way ANOVA. LSD *Post-hoc* test was used for pairwise comparisons. A P value of <0.05 was considered statistically significant.

Results

eSC isolation and characterization

Endometrial biopsies of 4 women yielded a sufficient number of cells and were used for experiments in this study. Immunofluorescence staining of vimentin was used to confirm the stromal phenotype of the isolated cells which were 94% vimentin-positive (Fig. 1).

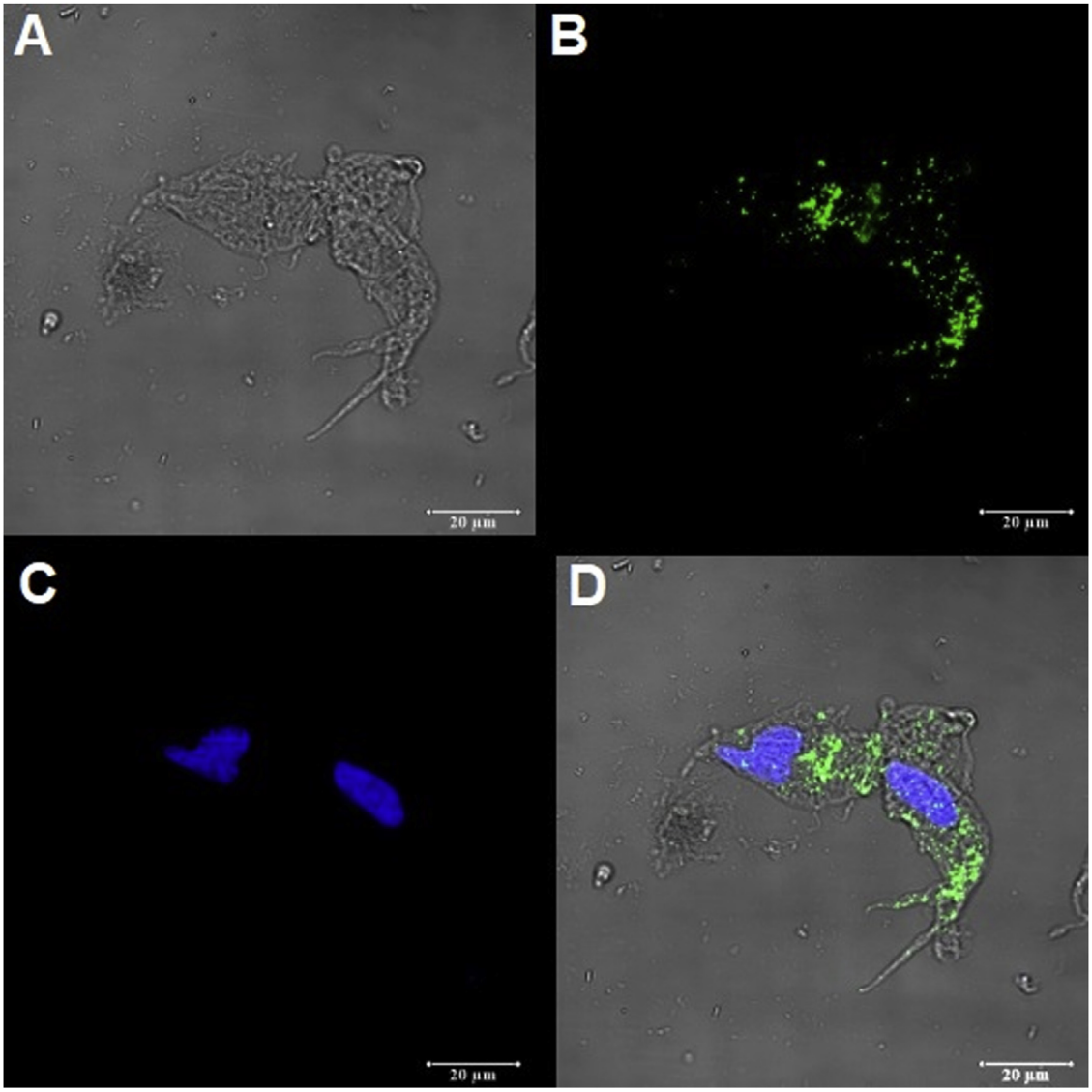

Fig. 3. Internalization of exosomes labeled with PKH67 (green) in eSC, illustrated by confocal laser scanning microscope after 24 h of incubation. (A) eSC, (B) PKH67 green labeled SE, (C) DAPI-stained nuclei, (D) Merge (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Characterization of SE

Exosome isolation from SP yielded a protein concentration of 20–30 mg/ml as determined by BCA assay. The size, morphology, and protein markers of typical exosomes were analyzed and all were indicative of successful exosome isolation. SE had an average diameter of 84 nm determined by dynamic light scattering (Fig. 2A). Scanning electron microscopic examination showed that exosomes had a spherical shape with a diameter of 60–100 nm (Fig. 2B). Subsequent transmission electron microscopy confirmed that the spherical particles are lipid bilayer-enclosed microvesicles in the expected size range of exosomes (Fig. 2C). Western blot analysis revealed presence of the universal exosome markers CD63 and CD81 in purified exosomes but not in the cell lysate as a negative control (Fig. 2D). Together, these data confirmed the identity of SE.

Internalization of SE by eSC

After incubation of PKH67-labeled SE with sub-confluent eSC for 24 h, cytoplasmic localization of the exosomes were detected by confocal microscope (Fig. 3). This observation is indicative of the capacity of SE to be internalized by eSC which is a prerequisite for subsequent signaling processes evoked by exosomes.

The effect of SE on cytokine production by eSC

Although relatively high levels of IL-6 and IL-8 were produced by the stromal cells, results showed that the stromal cells failed to produce detectable amounts of IL-10, IL-1α, and LIF in both experimental and control groups during 24 h, therefore no statistical analysis was performed for these cytokines. The levels of IL-6 and IL-8 in groups exposed to SE and SP were significantly higher compared with the control group ($P < .05$). Likewise, there were significant differences in the concentration of these two cytokines between the groups receiving SE and SP as the cytokine levels were higher in the group which received SP (Fig. 4). The levels of the cytokines were not in detectable ranges in the 100 μg/ml solution of SE and 1% solution of SP.

Comment

The study presented herein provides, for the first time to our knowledge, the data of direct effects of SE on human endometrial cells *in vitro*. We investigated internalization of the human SE by eSC and explored their consequent effect by quantifying the concentration of selected cytokines including IL-6, IL-8, IL-10, IL-1α, and LIF in the culture supernatants. The results demonstrated that SE were internalized by eSC and the cells were stimulated to produce IL-6 & IL-8 afterwards. The observed stimulatory effect is possibly due to the delivery of various bioactive components including a unique and selective profile of small RNAs enriched in these highly abundant microvesicles in seminal fluid [9,21]. Vojtech et al. reported that many of the small RNAs enriched in SE have immunoregulatory potential for target cells which uptake them [9]. It has also been showed that there are various population of proteins in exosomal fraction of SP which may serve as means of signal transduction to the endometrial cells upon delivery [8,22].

Chen et al. reported that eSC respond to SP at both transcriptional and cytokine/chemokine secretion levels which consequently results in recruiting certain leukocytes to the endometrium [13]. As demonstrated here, eSC secrete cytokines after exposure to SE as well, but to a lesser extent than the time they are exposed to SP. The observed difference is possibly due to several soluble factors which exist in SP but not in exosomal fraction of it.

Our results go in line with previous animal model study which showed that porcine SE induce endometrial immune and inflammatory responses [10]. Actually, human SE stimulated the secretion of pro-inflammatory factors including IL8 and IL6 from eSC, the induction which was previously attributed solely to the activity of soluble factors inside seminal fluid [18,23–25]. These cytokines are capable of recruiting immune cells to the uterus which then can differentiate into functional cells in response to cues from the microenvironment [26,27]. In this context, IL8 is chemotactic for peripheral monocytes that are capable of differentiating into dendritic cells (DC) which play a pivotal role during early embryo implantation process by providing an immune-tolerant condition for the embryo [17,28]. In addition, IL-6 plays a key role in immune adaptation for pregnancy [29] and

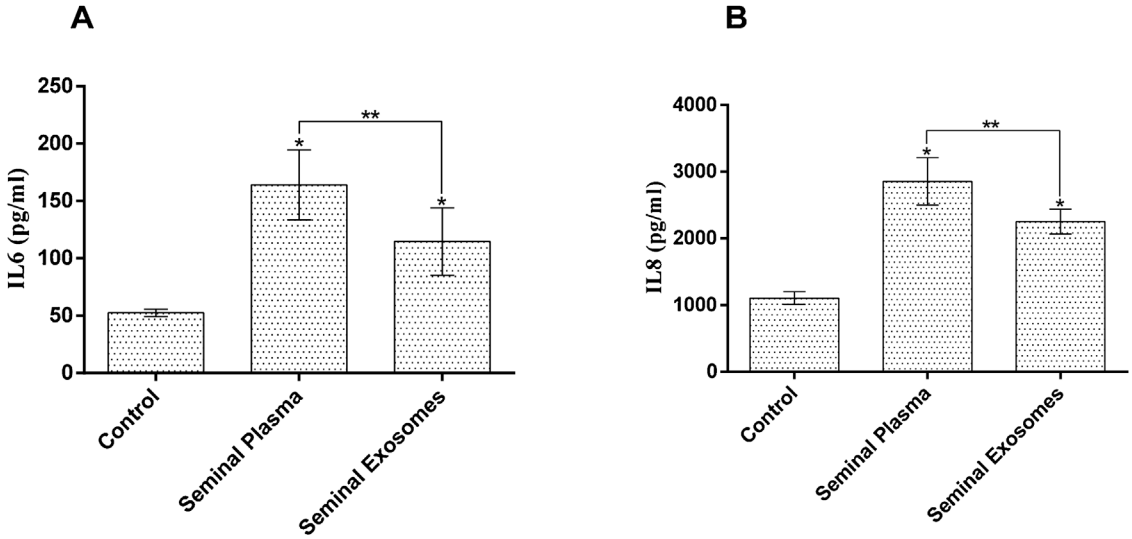

Fig. 4. SE stimulate eSC to produce cytokines after 24 h of incubation. Quantitative analysis of IL-6 (A) and IL-8 (B) secretion by eSC isolated from endometrial samples (n = 4) recovered on proliferative phase of the menstrual cycle after 24 h incubation with SP and SE. Control groups received no treatment. Error bars represent standard deviations (SD). *$P < 0.05$ vs. Control, **$P < 0.05$.

is directly involved in implantation process by attracting trophoblast cells to the implantation site [15].

Altogether, this observation supports the idea that exosomes contribute to the immune functions of SP in the endometrium and participate in modulating the immune milieu of upper female genital tract. Of note, our study was to test the initial hypothesis of stimulatory effect of exosomal fraction of SP on human eSC, the major cell types of endometrium, on an *in vitro* designed experiment. Studies using *in vivo* animal models or clinical human studies are required to investigate the response of other dominant cell types residing in endometrium i.e. epithelial and immune cells to SE, especially from the embryo implantation point of view. This may help to develop a potential application for SE as a mild stimulator for preparing endometrium prior to embryo transfer during ART cycles.

Declaration of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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