



Plasma-derived extracellular vesicles improve mice embryo development

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Abstract

Background To investigate the effect of plasma-derived extracellular vesicles (EVs) or conventional medium in fertilization and early embryo development rate in mice.

Methods and results MII oocytes (matured in vivo or in vitro conditions) were obtained from female mice. The extracellular vesicles were isolated by ultracentrifugation of plasma and were analyzed and measured for size and morphology by dynamic light scattering (DLS) and transmission electron microscopy (TEM). By western blotting analysis, the EVs proteins markers such as CD82 protein and heat shock protein 90 (HSP90) were investigated. Incorporating DiI-labeled EVs within the oocyte cytoplasm was visible at 23 h in oocyte cytoplasm. Also, the effective proteins in the early reproductive process were determined in isolated EVs by western blotting. These EVs had a positive effect on the fertilization rate ($P < 0.05$). The early embryo development (8 cell, morula and blastocyst stages) was higher in groups supplemented with EVs ($P < 0.01$).

Conclusion Our findings showed that supplementing in vitro maturation media with EVs derived- plasma was beneficial for mice's embryo development.

Keywords Oocyte maturation · Plasma-derived EVs · Fertilization · Electronic microscopy

Introduction

For the first time, Extracellular Vesicles (EVs) were detected 56 years ago in plasma [1]. Other studies found that other biological fluids, such as milk, urine, semen, and follicular fluid, possess these vital and effective particles [2]. The general definition of EVs represents a heterogeneous population of nanovesicles (microvesicles) with < 1000 nm diameter. The EVs are divided into at least three categories: As first, exosomes (EXOs) with a 30–100 nm diameter are released from the fusion of multicellular bodies with the plasma membrane. The second category consist of microvesicles released from a small section of the plasma membrane. While the third category includes apoptotic bodies released from dying cells [3]. Because there are overlaps in the density, cargo, and size of these different nanoparticles, determining the precise type of nanoparticles extracted from different cell types or body fluids is difficult [4]. The typical EVs contain proteins, genetic materials (such as mRNA and DNA), and lipids enclosed by lipid membrane bilayers [5]. Among these vesicles, exosomes can transport molecular cargo and other biological particles to the recipient cells [6, 7]. These vesicles have recently been recognized

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as a novel intercellular communication system and play key roles in cellular functions [8]. Today, EVs' protein particles have been extensively evaluated for their role in triggering signaling cascades in target cells. In recent decades, human and animal infertility has been treated by Assisted Reproductive Technologies (ARTs). ART techniques such as In Vitro Fertilization (IVF) could be an efficient tool for understanding early embryonic development [9]. Despite tremendous improvements in ARTs in recent years, the case of in vitro mammal embryo production development differs from in vivo conditions in the physiologic process [10]. The competence of the female gamete plays a key role in determining the stage of embryonic development [11]. This event can be appraised by its ability to arrive at cytoplasmic maturation together with nuclear maturation [12]. In Vitro Maturation (IVM) of immature oocytes is a necessary technique used in developmental biology and ART research. It is also an impressive treatment that has reported significant results in pregnancy rates [13].

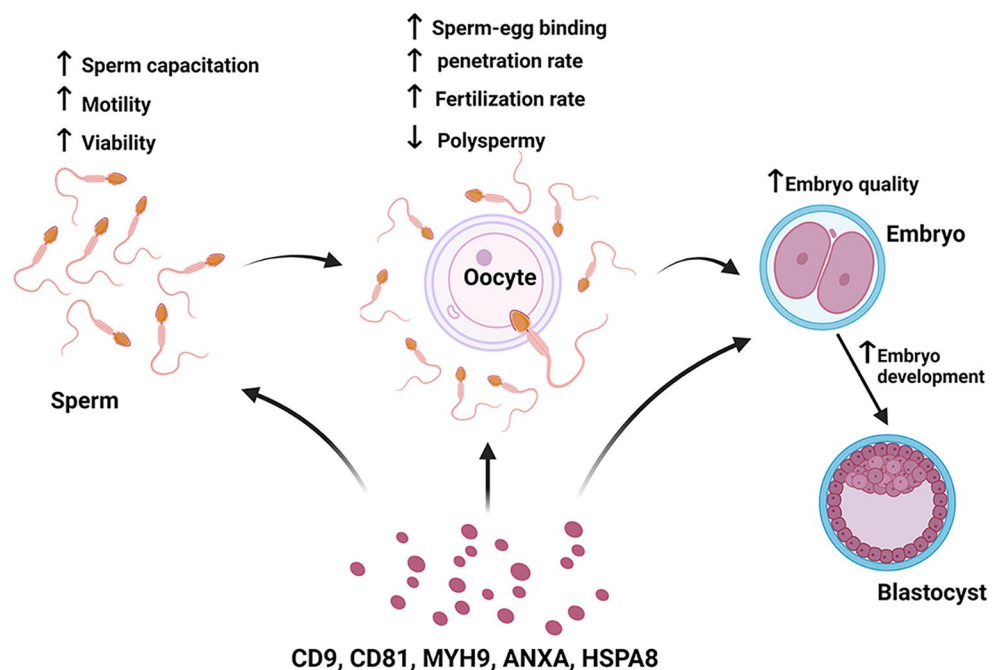
In most mammals and in the IVM process, immature oocytes are collected in an immature Germinal Vesicle (GV) stage from the ovaries in a certain condition. This process allows the re-initiation of the meiotic resumption of oocytes and makes them achieve the meiotic competence stage of Metaphase II (MII). This current IVM media is an unappropriated oocyte maturation in mammal species because, in these conditions, the oocytes stay away from their specific physiological environment, including follicular fluid secretions [14]. Therefore and overall, in vitro development (IVD) after IVF has lower embryonic development than in vivo-derived embryos [15]. Improving embryo culture media is needed to overcome present differences in

developmental and quality potential differences between *Vitro*- and *Vivo*-derived embryos [16].

Many reproductive proteins (Fig. 1) are effective in fertilization, including various proteins needed to attach to the transparent layer and regulate its glycoprotein structure [17]. The completion of fertilization in mammals requires different membrane fusion in which other membrane proteins are involved. Reproductive physiology-related proteins include myosin 9 (MYH9), annexin A (ANXA) protein family, the tetraspanin membrane protein family (CD9 and CD81), and heat shock protein 8 (HSPA8). These proteins play a vital role (Fig. 1) in sperm and oocyte fertilization [18]. According to recent investigations, EVs have been vital components in regulating gamete or embryo interactions in the oviduct [19]. Emerging pieces of evidence show that EVs-mediated cell-to-cell communication can help oocyte maturation [20], sperm penetration into the oocyte, and embryo development. However, in numerous reports, EVs' functions in reproductive events have remained unexplored so far [21]. Recent studies imply EVs derived from reproductive tracts play a critical role in gamete maturation and fertilization. Also these vesicles help achieve in vitro embryos with similar development stage levels to in vivo embryos [16].

Blood plasma contains a large amount of protein and essential substances and high concentrations of EVs, such as exosomes [22]. The components of EVs' protein have been investigated in detail because of their role in signaling cascades. Transporting proteins *via* EVs may form the bioactivity of target cells in tissues [23]. In recent years, the therapeutic effects of plasma-derived EVs have been observed in various diseases, including cardiac and

Fig. 1 The schematic diagram of the proposed biological functions of reproductive proteins. These proteins can bind to gametes. Following binding to sperm, they enhance sperm capacitation, motility, and viability. Also, these can increase sperm-egg binding, decrease polyspermy, increase penetration and fertilization rates of sperm, and enhance embryo quality



autoimmune diseases (e.g., rheumatoid arthritis and diabetes) [24]. EV-based treatment significantly reduces inflammation and compared to other treatments, is non-toxic with fewer side effects [25]. Also, some observe that in cardiac disorder, the cardioprotective components could be transferred by these vesicles to cardiomyocyte [24]. In addition, in the previous study, the present authors demonstrated the beneficial role of these EVs in improving oocyte maturation [20]. Because of the similarity of plasma and follicular fluid [26], we hypothesized these EVs may be utilized as a supplement to culture media during the IVM of mice oocytes and IVD stages of mice embryos.

Methods

Study design and embryo culture

Oocytes were obtained from BALB/c mice (8- to 10-week-old). All mice ($n=7$) used in this experiment were purchased from the animal house of the Tabriz University of Medical Sciences. All animals were housed in standard laboratory mice cages and were given food and water ad libitum. Mice were maintained under 12 h light: dark cycle. The implemented methods in the present study were approved by the Tabriz University of Medical Sciences' Committee on Animal Experimentation (ethics number: IR.TBZMED.VCR.REC.1398.289) and in accordance

with the National Institute of Health's Laboratory Animals Guidelines (8th edition, 2011). After collecting oocytes by rupturing the follicles ($n=440$), the following three types of MII stage oocytes of the mouse (Fig. 2) were used: (1) MII stage oocytes matured in vivo (*MIIOocytes*); (2) GV stage oocytes that matured in vitro by EVs supplementation (*IVM₁oocytes*); (3) GV stage oocytes that matured in vitro without EVs supplementation (*IVM₂oocytes*). For *MIIOocytes* retrieval, female mice were superovulated (intra-peritoneal) with 10 IU of human menopausal gonadotropin (HMG; NV Organon, The Netherlands), followed by an injection of 10 IU of human chorionic gonadotropin (hCG; NV Organon, The Netherlands) 48 h later. The ovulated oocytes were recovered from oviducts 24 h post-hCG. Then, COCs were washed with PBS. Only mature oocytes (MII stage) were selected under a stereomicroscope (Olympus-Japan). The COCs were washed twice with PBS and then placed into microdrops (25mL) immersed in mineral oil (Sigma Aldrich, USA). The microdrops contained conventional medium (MEM alpha medium) with 75 mg/ml penicillin (Sigma-Aldrich, USA) and 50 mg/ml streptomycin sulfate (Sigma-Aldrich, USA). The *IVM₁* and *IVM₂* oocytes were retrieved following in vitro maturation of GV oocytes under different culture medium conditions, as mentioned in the previous study [20]. Briefly, COCs were released from female mice, and COCs in an immature oocyte were selected for culture. COCs were washed and cultured in microdrop of MEM alpha medium (with penicillin and streptomycin

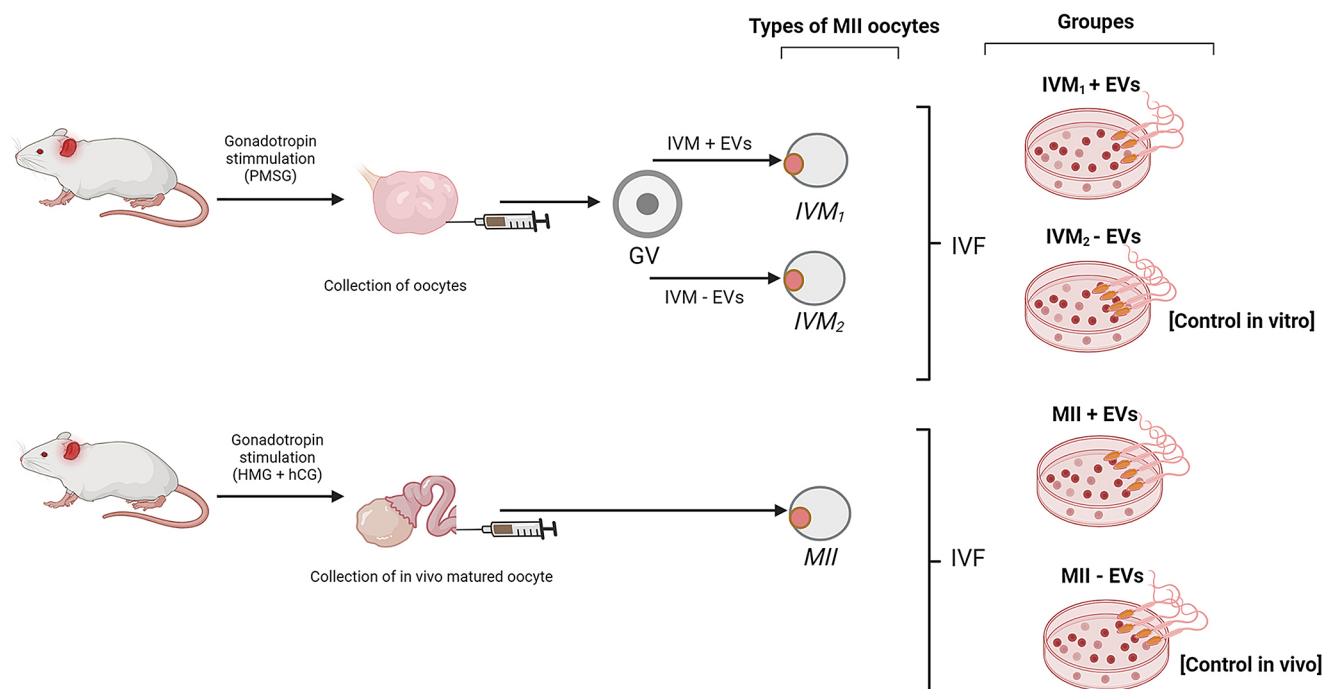


Fig. 2 The schematic representation overview of the experimental design provides the in vitro matured oocytes (IVM1 and IVM2 oocytes) and in vivo matured oocytes (MII oocytes). The matured oocytes were

distributed in four groups for In Vitro Fertilization (IVF) supplemented by 10 μ g protein/ml plasma-derived extracellular vesicles or without

sulfate). The mouse oocytes matured *in vitro* in the presence (*IVM₁oocytes*) or absence (*IVM₂oocytes*) of EVs.

MII oocytes were fertilized using motile spermatozoa from BALBC mice. Initially, sperm were collected from the cauda epididymis of the male BALBC mice (≥ 9 weeks old) and were selected by swim-up processing. The pellet of sperm cells was re-suspended at fertilization medium (Ham's F10 medium [Irvine Scientific, Santa Ana, CA]). Then pellets supplemented with 4 mg/ml BSA for capacitation) for 60 min at 37 °C with 5% CO₂ humidified atmosphere. After capacitation, sperm was added to a 50 μ l microdrop of fertilization medium (Ham's F10 medium) supplemented with 1 mM pyruvate and 100 μ g/ml streptomycin and penicillin. The medium was supplemented with or without EVs considering differential groups for fertilization.

The co-incubation of sets of 20–25 oocytes (total oocytes = 110 per group) with 1.5×10^5 spermatozoa cell mL⁻¹ was performed under equilibrated mineral oil for 20 h at 37 °C with a 5% CO₂ humidified atmosphere. **IVF was performed in four groups:** **MII+EVs** group: for *MII oocytes* by EVs supplementation; **IVM₁+EVs** group: for *IVM₁oocytes* by EVs supplementation; **In vivo control** group: for *MII oocytes* without EVs supplementation (only supplemented by conventional medium); **In vitro control** group: for *IVM₂oocytes* without EVs supplementation (only

supplemented by conventional medium) (Fig. 2). Condition medium in IVM₁+EVs and MII+EVs groups was supplemented with EVs (10 μ g protein/ml). 24 h after IVF (day 2), the fertilization rate was evaluated based on the number of MII oocytes reaching the 2-cell stage. The early embryo developmental rate was investigated by quantifying the percentages of 8-cell, morula, and blastocyst stages. Developmental rates of the embryo were observed at 44 h (day 3), 74 h (day 4), and day 5 of culture. The development rate was defined as the percentage of embryos that achieved the development scale in relation to the initial count of MII oocytes. 440 oocytes in three IVF replicates were evaluated.

Blood samples collection and EVs extraction

According to the previous methods, small EVs with a size of 30 to 150 nm were collected from plasma [27]. Whole blood samples of the hearts of 3 months old female mice (in proestrus cycle; $n = 5$) were collected in EDTA Vacutainer tubes (FLmedical, Italy) (Fig. 3A). They were centrifuged (Olympus, Tokyo, Japan) at $1,800 \times g$ for 15 min at 4 °C, and the collected plasma was immediately stored at -80 °C. The standard procedure of the differential ultracentrifugation method was utilized for EVs extraction. Then, the plasma was diluted (1:4 with PBS) and centrifuged again

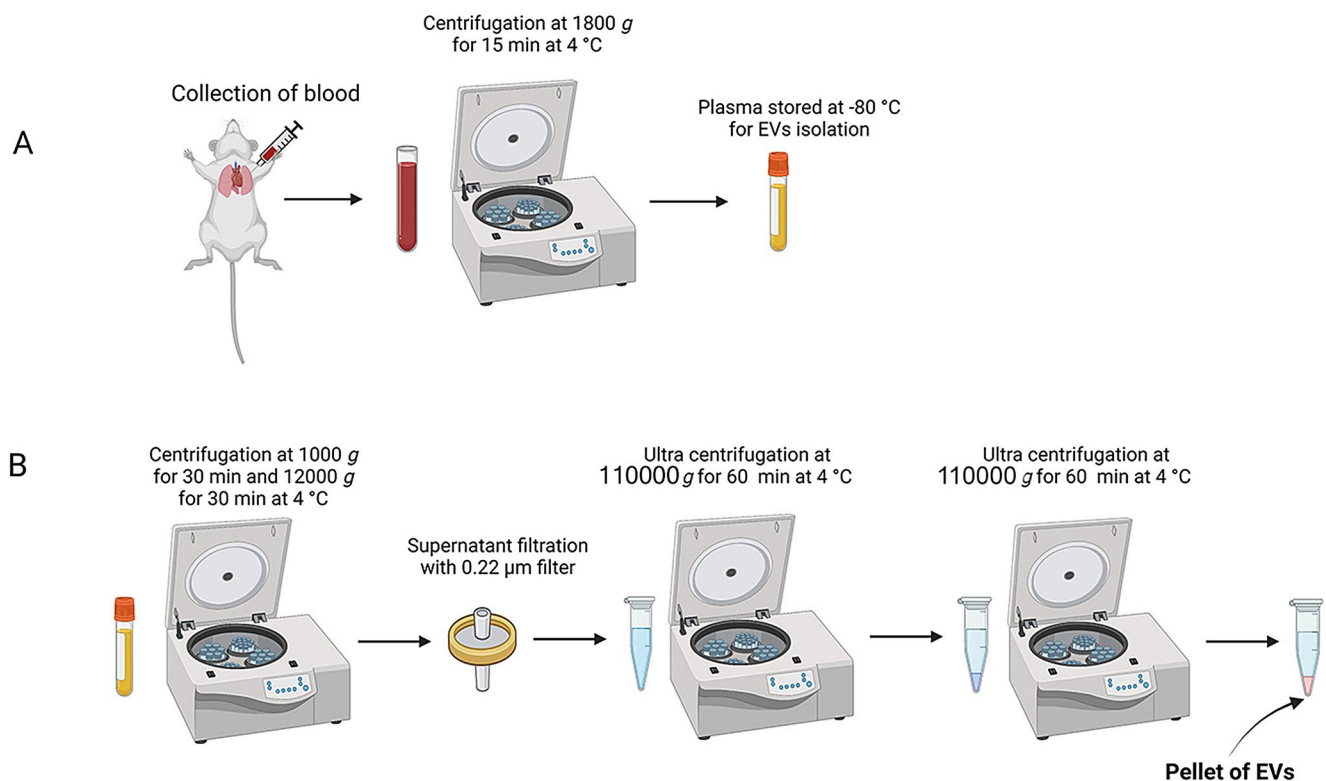


Fig. 3 The schematic overview of blood collection (A) and isolation of plasma-derived EVs (B). (A) Whole blood was isolated from the heart of the female mouse and stored at -80 °C. (B) Plasma sample

was subjected to the EVs isolation method standard procedure of the differential ultracentrifugation

(Olympus, Tokyo, Japan) ($1,000 \times g$) for 30 min at 4°C to remove residual cells and then centrifuged ($12,000 \times g$) for 30 min. Afterward, a supernatant was filtered (using a $0.22\text{-}\mu\text{m}$ filter) and ultracentrifuged (Beckman Optima TLX ultracentrifuge, Ramsey, US) ($110,000 \times g$) for 1 h at 4°C to obtain EVs' pellets (Fig. 3B). Afterward, the pellets were washed and centrifuged ($110,000 \times g$) for 1 h and then re-suspended in PBS. The EVs' protein level was evaluated using a bicinchoninic acid protein assay kit (BCA) (Thermo Fisher Scientific, MA, USA).

EVs analysis

Transmission electron microscopy

The rounded shape and diameter range of the EVs were identified using a Transmission Electron Microscope (TEM, Zeiss, Germany). The 300-mesh grids coated with formvar were exposed to EVs droplets for 12 min at room temperature (RT). The samples were negatively stained by Uranyl Acetate (2%) (UA 2%) and allowed to dry at RT. Then, the grids were observed on a Zeiss 100 kV.

Western blot analysis

Plasma-derived EVs were examined by western blot analysis to investigate the expression of EV-specific protein markers (CD82 and HSP90) and one negative protein marker (Grp94). These EVs' samples were investigated for proteins with practical function during fertilization, such as MYH9, CD9, HSPA8, and ANXA5.

Santa Cruz Biotechnology (CA, USA) supplied the primary antibodies (anti-CD82, anti-HSP90, anti-MYH9, anti-HSPA8, anti-CD9 and anti-ANXA5 antibodies), and secondary antibodies (mouse anti-rabbit IgG-HRP) for western blotting analysis. The EVs' samples were lysed for 50 min in the lysis buffer. The supernatants were centrifuged for 10 min. The protein concentration was determined using the BCA protein assay kit. Then, the proteins ($25\ \mu\text{g}$) were loaded into SDS-polyacrylamide gels (10%), electrophoresed, and transferred to polyvinyl difluoride membranes. The membranes were blocked with Bovine Serum Albumin (BSA [3%]) in PBS and incubated with primary antibodies (mentioned above) overnight. After a day, these membranes were washed and then incubated with a secondary antibody for an hour. The blots were developed with a chemiluminescence detection system (Pierce ECL, Thermo Fisher Scientific). Ultimately, immunoreactive bands were scanned utilizing the image j software (Fiji).

Dynamic light scattering size analysis

The size distribution by number, zeta potential, and polydispersity index (PDI) of these vesicles was investigated by Dynamic Light Scattering (DLS) measurements on a Nano Zetasizer ZEN 3600 (Malvern, UK). Dispersion technology software (version 6.01) (Malvern Instruments, UK) was used for analysis. In this method of nanoparticle examination, no sample preparation is required, and the sample is easily measured in the solution. The evaluation of the device is based on the interaction of light with particles, which is the light scattered by the nanoparticles in the suspension that specifies the particle's diameter. Before measurement, each EV' sample was sonicated at 4°C for 15 min to dissolve the EV aggregates. The samples were diluted in filtered PBS solution a (1: 5 diluted) and then loaded into the cuvette of Nano ZS.

Plasma-derived EVs uptake testing

Plasma-derived EVs were stained by Dil dye (Invitrogen, Paisley, UK). First, $5\ \mu\text{l}$ Dil ($1\ \mu\text{M}$ Dil) was incubated with $60\ \mu\text{l}$ of total EVs (rehydrated in PBS) for 15 min at 37°C . The excess dye was removed by centrifugation of labeled EVs at $110,000\ g$ for 60 min. Then, the pellets were washed (twice in PBS). Freshly collected mice COCs were co-incubated in $25\ \mu\text{l}$ droplets of the medium, supplemented by labeled EVs. The oocytes were imaged under a fluorescence microscope (Labomed, NY, USA) after 16, 23, and 30 h of co-incubation at 37°C .

Statistical analysis

The effect of EV supplementation during fertilization and early embryonic development stages was analyzed by one-way analysis of variance (ANOVA) using version 9 GraphPad analysis software. The assumptions of normality of the residual variances were evaluated using the Shapiro–Wilk test. Variances homogeneity was assessed by the Levene test. The means of the groups were compared at a significance level of $p < 0.05$. These data came from three replicate experiments.

Results

EVs characterization

Using ultracentrifugation, EVs were isolated from the plasma of female mice. EVs characterizations such as size, morphology, and protein markers were analyzed respectively by Dynamic Light Scattering (DLS), Transmission

Electron Microscopy (TEM), and western blotting. TEM results showed EVs in plasma and the round membrane populations and heterogeneous nanovesicles (30–150 nm in diameter) that were determined in the plasma-derived EVs (Fig. 4A). Western blotting was performed in plasma-derived EVs for protein markers (Fig. 4B). Plasma-derived EVs were positive for CD82 and HSP90 proteins recognized exosomal protein markers, and Grp78, an endoplasmic reticulum protein (negative marker). DLS measurements showed an average exosome diameter of 30 and 73 nm. A peak corresponding to diameter was noted at 46.32 nm, and the associated PDI was 0.251. DLS results also revealed a negative zeta potential of -7.86 mV for plasma-derived

nanovesicles, indicating the exosome particles' electric charge (Fig. 4C).

EVs internalization

EVs' internalization was evaluated by fluorescence microscopy, and Dil-labeled EVs were incorporated by cumulus cells after 16 h and as mentioned in the previous study [20]. However, EVs' uptake in the oocyte cytoplasm was not detected after 16 h of culture (Fig. 5A). Incorporating EVs within the oocyte cytoplasm was visible after 23 h incubation (Fig. 5B). Moreover, 30 h after the initiation of IVM, Dil-labeled EVs internalization was detected to be abundant

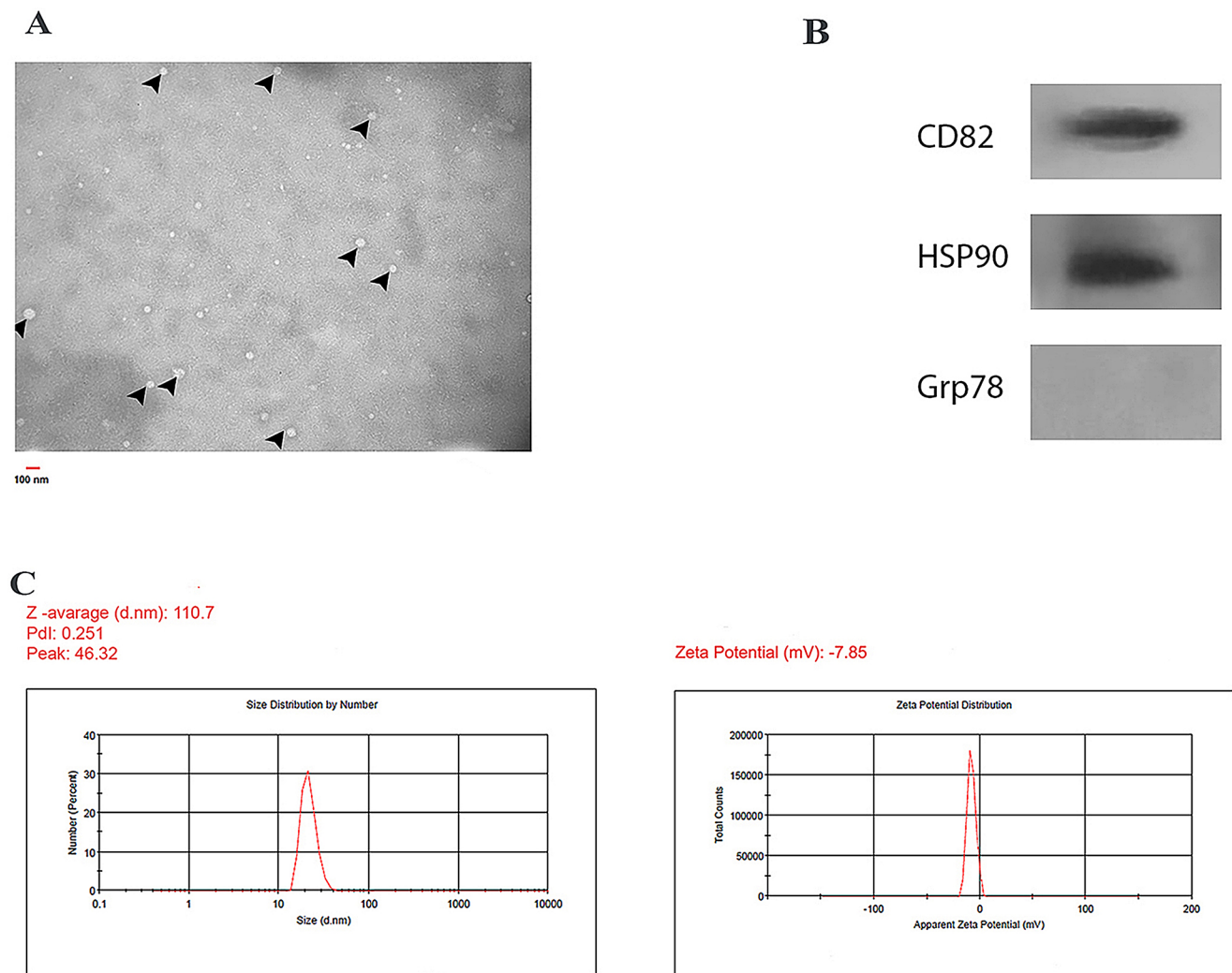


Fig. 4 The characterization of plasma-derived extracellular vesicles. (A). Transmission Electron Microscopy (TEM). Rounded nanovesicles were isolated from mice plasma. TEM observations of EVs represented the bilipid layer structures by a single membrane with a 30–100 nm size resembling exosomes. The Arrowheads detect the exosomes. Scale bar = 100 nm. (B). Western blotting analysis. The Western blot assay of EVs protein markers (CD82 and HSP90 anti-

bodies) and negative protein markers (Grp78) in small EVs. The CD82 and HSP90 protein expressions in EVs were indicated by western blotting. (C). Dynamic light scattering measurements. Size-determining results of these nanovesicles represented a peak of heterogeneous populations. Also, the particle size was approximately 39.8 nm (number: 96.8%), and the zeta potential was -7.85 mV, corresponding to plasma EVs

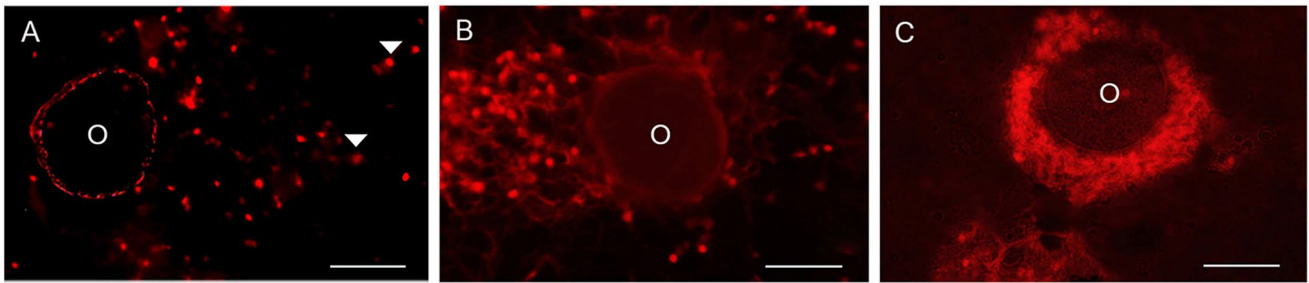


Fig. 5 Incorporation of Dil-labeled EVs (red spots) by oocytes (O). (A). EVs, labeled with Dil dye, are seen within the cumulus cells under fluorescent microscopy after 16 h of IVM but not in the oocyte cytoplasm. (B). EVs are incorporated within the cumulus cell mass and

into oocyte cytoplasm after 23 h of culture. (C). Oocyte with abundant incorporation of EVs in its cytoplasm and the cells of corona radiata since 30 h of incubation. Arrowheads indicate some aggregation of EVs (A). Scale bar = 50 μ m

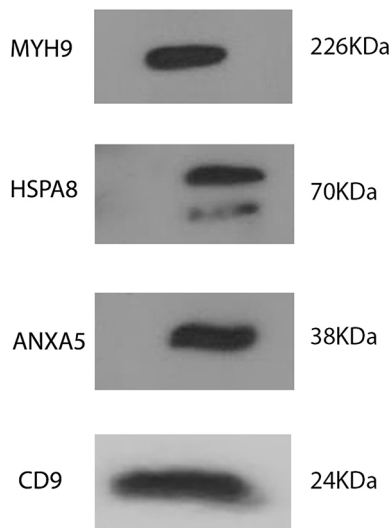


Fig. 6 Western blotting assay of plasma-derived EVs for proteins with established reproductive roles. EVs expressed heat-shock protein A8 (HSPA8), annexin A5 (ANXA5), myosin 9 (MYH9), and a member of the tetraspan membrane protein family (CD9)

within cells next to the zona pellucida and oocyte cytoplasm (Fig. 5C).

Differential reproductive protein profile in plasma-derived EVs

Western blotting analysis showed that EVs also expressed reproductive physiology protein (Fig. 6), myosin 9 (MYH9),

membrane trafficking process protein (ANXA5), CD9 and Heat Shock Protein 8 (HSPA8). Also, the western blotting EVs protein markers results indicated expression of CD81 and HSP70 proteins (the effective proteins in the reproduction process) in previous study [20].

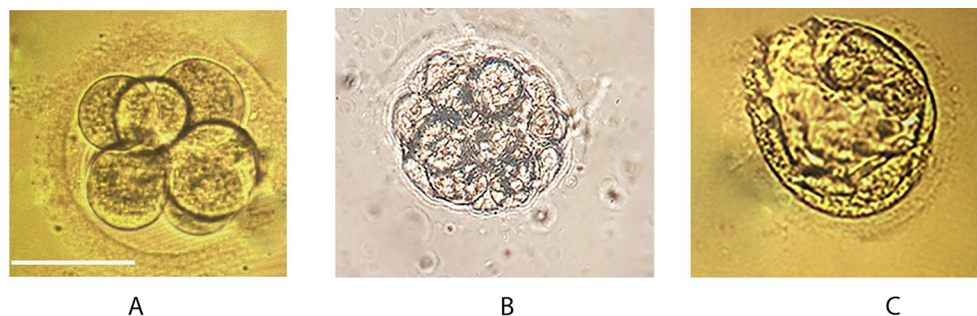
Developmental competence of MII oocytes

Effects on preimplantation embryos' developmental stages were identified at the two-cell, eight-cell (Fig. 7A), morula (Fig. 7B) and blastocyst stages (Fig. 7C). A significant increase was observed in the percentage of embryos' developmental stage using plasma-derived EVs.

The role of treatment conditions (+ EVs or -EVs) in fertilization of mature oocyte (in vivo or in vitro)

Fertilization can be simulated by both types of MII oocyte (matured in vivo or in vitro). Thus, the effects of treatment conditions in the fertilization rate, by quantifying the frequency of 2-cell embryos (among the total MII-stage oocytes) in each group was determined. To evaluate the treatment contribution during insemination, 2-cell-stage embryos (the percentage) related to the total MII-stage oocytes were quantified. It was found that treatment with plasma-derived EVs in the insemination media enhanced the fertilization percentages. As a result, in the IVM₁ + EVs and MII + EVs groups, supplementing insemination media

Fig. 7 Representative photographs of mouse embryo development: (A) The 8-cell stage, (B) The morula stage and (C) The blastocyst stage. Images obtained using an inverted microscopy (Olympus-Japan). Scale bar = 50 μ m. The scale bar in (A) applies also to (B) – (C)



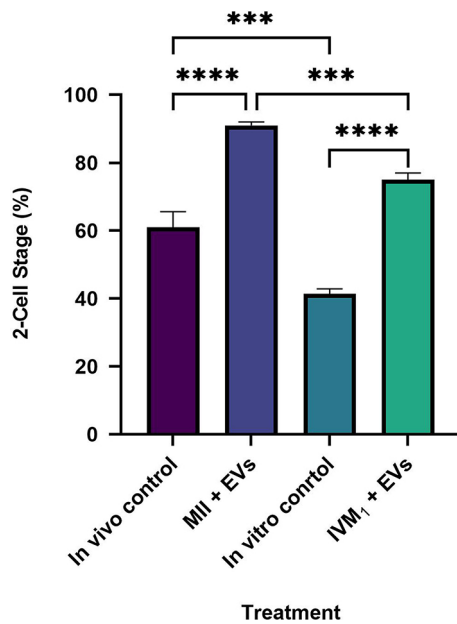


Fig. 8 Preimplantation development of matured oocytes in the presence (+) or absence (-) of Extracellular Vesicles (EVs). The two-cell stage embryos' percentage (number of embryos at the two-cell stage/number of activated MII oocytes) 24 h after activation. Data indicated the mean \pm SEM of at least three independent experiments. The asterisk represents significance ($p < 0.05$). *** significant at ($p < 0.001$); **** significant at ($p < 0.0001$)

Table 1 Effects of the experimental treatments on the early embryo developmental stages

| Groups | 2 cell rate % | 8 cell rate % | Morula rate % | Blastocyte rate % |
|------------------------|------------------|--------------------------------|------------------|-------------------|
| MIII + EVs | 91 \pm 0.57 | 80 \pm 1.15 | 61.13 \pm 2.99 | 50.65 \pm 0.93 |
| In vivo control | 61 \pm 2.64 | 30.29 \pm 1.41 | 23.29 \pm 1.75 | 20 \pm 1.15 |
| IVM ₁ + EVs | 75 \pm 1.15 | 60 \pm 2.88 20 \pm 1.15 | 53.36 \pm 1.26 | 46.11 \pm 1.11 |
| In vitro control | 41.33 \pm 0.88 | | 10.33 \pm 0.81 | 9.18 \pm 2.40 |

Values are mean \pm SEM of percentage of embryos from three independent repeats

with plasma-derived EVs caused a higher fertilization percentage than in their control groups (Fig. 8). Also in similar treatment conditions, the data suggested that the in vivo-matured oocytes reached a higher fertilization outcome than in vitro-matured oocytes. After insemination with spermatozoa, the MII + EVs group showed the highest fertilization percentage than IVM₁ + EVs group. The lowest result of fertilization percentages ($P < 0.01$) was detected in the IVM oocytes of the In vitro control group (Fig. 8).

The role of treatment conditions (+ EVs or -EVs) in embryo development

To find the effect of treatment conditions methods to embryonic development, the embryo percentages were quantified from each stage (Table 1). The highest embryo development percentage belonged to MII + EVs oocytes in the 8-cell, morula and blastocyst stages (80 \pm 1.15%, 61.13 \pm 2.99% and 50.65 \pm 0.93% respectively). In the IVM₁ + EVs group, the 8-cell stage (60 \pm 2.88%) was significantly lower than the MII + EVs group. But in the morula and blastocyst stages (53.36 \pm 1.26% and 46.11 \pm 1.11% respectively) that no difference was noted. The lowest embryo development percentage was observed in the IVM oocytes in the In vitro control group ($P < 0.01$) (Fig. 9A, B and C).

In other words, in the MII oocytes (MIII + EVs compared with In vivo control groups), the treatment by **plasma-derived EVs** during insemination significantly affected embryo development ($P < 0.01$) (Table 1). In IVM oocytes (IVM₁ + EVs compared with In vitro control groups), similar results were achieved for the 8-cell stage, morula and blastocyst stages (Fig. 9). As a result, the absence of plasma-derived EVs treatment decreased embryo development.

In totally, the treatment by plasma-derived EVs effectively increased the percentage of embryo development. It indicated, adding plasma-derived EVs to the IVF media caused the embryos to have more and better development. Despite the difference in MII oocytes no significant difference was observed in the morula and blastocyst stages between the IVM₁ + EVs and MII + EVs groups ($p > 0.05$). The blastocyst stage was reached by 9.18 \pm 2.40% of embryos in the In vitro control group and 20 \pm 1.15% of those in the In vivo control group (Fig. 9C). Overall, adding plasma-derived exosomes significantly affected fertilization and embryo development percentages after IVF compared to the untreated controls (Table 1).

Discussion

Recently, EVs have been reported as key elements in body fluids, which play a critical role in modulating different signaling pathways and communication among multiple target cells [28]. There is evidence that plasma-derived EVs are strongly involved in treating diseases such as heart failure, hepatic disorder, etc [29]. The results were indicated that the treatment by EVs in IVF protocols can be a valid strategy for improving mouse in vitro embryo production. Following IVF, the percentage of 2-cell embryos was higher IVM₁ + EVs group in the compared to the two control groups but lower when compared to the MII + EVs group. According to the earlier study, the percentages of

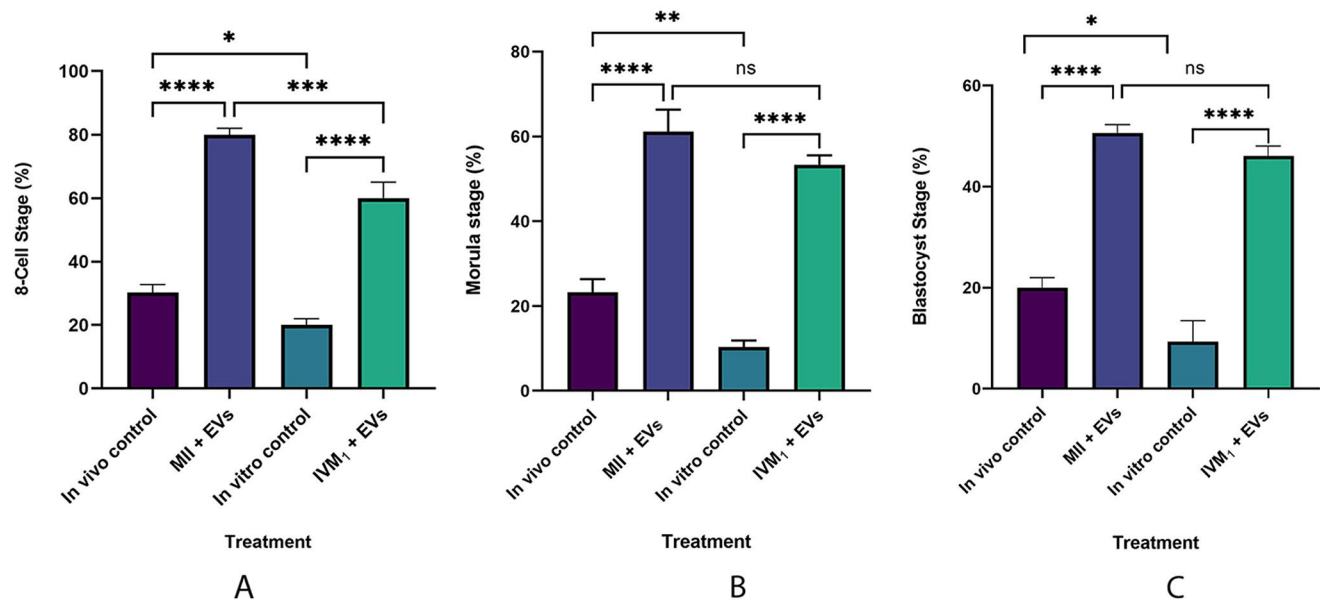


Fig. 9 Supplemented groups by plasma-derived EVs enhanced the developmental potential of embryos (MII+EVs and IVM1+EVs groups). (A). The embryos' percentage attaining the eight-cell stage (number of embryos at the eight-cell stage/number of 2-cell stage embryos) 44 h after activation; (B). The embryos' percentage reaching to the morula stage (number of embryos at the morula stage/number of

2-cell stage embryos) after 74 h of culture. (C). The embryos' percentage reaching to the blastocyst stage (number of embryos at the blastocyst stage/number of 2-cell stage embryos) after 5 days of culture. * Significant at ($p < 0.05$); ** significant at ($p < 0.01$); *** significant at ($p < 0.001$); **** significant at ($p < 0.0001$); ns = non-significant

metaphase II oocytes in the IVM condition were significantly higher for EVs' supplemented group when compared to conventionally matured oocytes. In vitro, EVs' treatment in embryo culture improves embryo quality in cattle [30]. The previous study demonstrated that sperm-oocyte binding and recognition proteins increased fertilization rate, including CD9 and CD81 [31]. EVs were analyzed for different reproductive proteins, such as reproductive physiology protein (MYH9), membrane trafficking process protein (ANXA5), and a member of the tetraspan membrane proteins family (CD9). Analysis by western blotting showed the presence of MYH9, CD9, HSPA8, and ANXA5 in EVs; which all of these proteins are involved in interactions between gamete-oviduct. OVGPI and MYH9 in EVs, as critical reproductive proteins, are necessary for both gamete interactions [32]. It was hypothesized that MYH9 could perform these functional results via EVs, possibly by binding to the ZP, and in this way, EVs increase monospermic fertilization. Further experiments are required to detect the vital roles (polyspermy reduction and ZP hardening) of the MYH9-OVGPI couple. It has been reported that the EVs supplementation (derived-oviduct fluid) of embryo culture improves the blastocyst rate in bovine and murine [33]. Annexins are sperm-binding proteins; in different species responsible for fertilization and arrangement of sperm storage and with this sperm selection, it improves ART [34]. CD9 and CD81 belong to the tetraspanin family and play a vital role in sperm-oocyte interaction and fusion in mice.

The CD9-containing EVs are released from the oocyte and facilitate sperm-oocyte fusion by transferring them to the spermatozoa head [35]. The CD81 protein is especially localized in the ZP zone, and the inhibition of CD81 protein expression on the oocyte surface in mice causes a 40% female fertility reduction. According to the studies, HSPA8 increases in vitro sperm viability in bulls and sperm of other animals and improves sperm quality and IVF efficiency [36]. This study hypothesizes that these proteins presented in the plasma-derived EVs could improve sperm-oocyte fusion and with ANXA5 protein, they prevent incomplete interactions between sperm and oocyte.

An interesting point is that despite different maturation methods, the percentage of the morula and blastocyst stages did not show any difference between the IVM₁+EVs and MII+EVs groups. IVF with in vivo-matured oocytes has shown an excellent embryo stage, which might be illustrated by the higher quality of embryos obtained from the in vivo maturation of oocytes. Oocyte meiotic maturation was considered as an oocyte quality control marker. This vital process involves several processes, such as stages of the germinal vesicle, germinal vesicle breakdown, metaphase I, and then metaphase II arrest until fertilization [37]. According to the findings, the percentage of different embryo levels was significantly higher in MII oocytes in the same treatment condition than in IVM oocytes. IVM of oocytes deprives them of the conditions of the normal follicular environment and subsequently reduces developmental

levels compared to the *in vivo* maturation of oocytes [38]. The *in vitro* spontaneous nuclear resumption maturation prevents oocytes from achieving synchronous nuclear and cytoplasmic maturation [39]. Several differences have been noted in regulating genes between *in vivo* or *in vitro* culturing settings of oocytes. *In vitro* maturation conditions could disturb protein synthesis, increasing gene expression and compensation [40]. Preventing a spontaneous resumption of oocyte nuclear maturation (*in vitro*) synchronously permits obtaining cytoplasmic and nuclear maturation, which results in increased oocyte developmental competence [39]. Comparing the MII oocyte obtained following superovulation (*in vivo*) and the IVM oocyte shows that many genes are highly expressed in the IVM oocyte [41]. Failing to acquire the follicular environment probably causes inadequate *in vitro* maturation conditions. This incompetence has been clarified by poor cytoplasmic maturation of oocytes [42]. Adding EVs in the IVM medium for 24 h changed the resumption of oocyte meiosis more effectively. Also synchronization of oocytes' nuclear and cytoplasmic maturation consequently occurred and embryonic development increased [43]. The plasma-derived EVs created the typical levels of cytoplasmic and nuclear maturation in oocytes. These particles can mimic the *in vivo* condition of ovulation and significantly impact on embryo development. The results showed that supplementing IVF condition media with plasma-derived EVs benefited the embryo development of mice.

Conclusion

The results suggested the potential of plasma-derived EVs to be added to IVF media. Further studies on the EVs' contents could help identify more factors in the EVs that indicate their potential aspects of oocytes, spermatozoa, and embryos.

Author contributions Maryam Javadi and Leila Roshangar designed this study. Maryam Javadi and Mohammad Sadegh Gholami Farashah wrote the main manuscript text. Maryam Javadi prepared figures and analyzed the collected data. Jafar Soleimani Rad and Leila Roshangar edited the main text. All authors reviewed the manuscript and replied to revise.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval All procedures performed in present study were approved by Tabriz University of Medical Sciences' Committee on Ani-

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Conflict of interest The authors declare no conflict of interest.

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