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ORIGINAL RESEARCH

# Loss of Renewal of Extracellular Vesicles: Harmful Effects on Embryo Development in vitro

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**Background:** Extracellular vesicles (EVs), as a promising platform for drug delivery, have attracted much attention. Degradation and regeneration of EVs maintain their homeostasis in vivo, but this regeneration is missing in the in vitro culture (IVC) system, which is likely to lead to negative effects. It is particularly concerning that most studies involving the addition of EVs in IVC seem to overlook this point.

**Methods:** We used rabbit embryos and oviduct fluid EVs as a model of embryo development to examine the effect of loss or gain of EV functionality in an IVC system. Embryonic development ratios were determined in each group. Malondialdehyde and ammonium ions in the culture medium were measured. RNA-seq, reactive oxygen species (ROS) staining, immunofluorescence of LC3 and H3K36me3, and qPCR of oxidative stress-related genes and autophagy-related genes of blastocysts in the in vivo group, non-EVs group, con-EVs group, and R-EVsM group was implemented.

**Results:** Incubation of embryos with $9.1\times10^{11}$ EV particles/mL had a positive effect at 48 h and 72 h, which disappeared by 96 h, however. EVs at a concentration of $9.1\times10^{12}$ particles/mL even showed a negative effect at 96 h. As culture time in the IVC system was increased, the amount of malondialdehyde and ammonium ions in the culture medium was increased, and there was a decrease in embryonic development activity of EVs. Lack of EV renewal in the IVC system impaired embryonic development competence, while replacement of EVs and medium during IVC could sustain embryonic development. Loss or gain of renewal in the IVC system affected EVs' influence on embryo transcriptome, embryonic ROS, autophagy, epigenetic state and apoptosis.

**Conclusion:** Loss of renewal in the IVC system affected EVs' role in embryonic development by causing an imbalance in ROS, autophagy, abnormal H3K36me3 levels and apoptosis, while gain of renewal in the IVC system reduced these adverse effects and ensured the beneficial function of EVs.

**Keywords:** extracellular vesicles, hazardous effect, renewal, embryo, in vitro culture

## Introduction

Extracellular vesicles (EVs) are a heterogeneous group of cell-derived membrane structures, which originate from the endosomal system or plasma membrane.[1] EVs can carry cargos including proteins, lipids, genetic material, etc., which can be taken up by cells, and elicit a variety of effects on intercellular signaling and cell-to-cell communication during physiological and pathological processes.[2] For example, astrocyte-derived EVs contributed to neurodegeneration, and tumor-derived EVs contributed to cancer cell progression and metastasis.[3] EVs' tissue tropism, low immunogenicity, and ability to carry exogenous drugs, RNA or protein make them a promising vehicle for drug delivery in treatment of cancer, degenerative diseases, metabolic diseases, and cardiovascular disease.[4,5] EVs in both the male and female reproductive tracts participate in critical intercellular interactions involving gametogenesis, fertilization, implantation, and development from conception to birth.[6,7] EVs in the male reproductive tract help to program sperm function, establish maternal immune tolerance and transmit epigenetic information to offspring.[8] They are also implicated in genitalia-resident

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immunity and may block early protein transcriptional activator recruitment and subsequent transcription of HIV-1.[9] EVs in the female reproductive tract play important roles in oocyte maturation, embryo development, and implantation by preparing the endometrial vascular net and priming the endometrium for embryo implantation.[10] Given the importance of EVs during embryo-maternal crosstalk, they are now being considered as new diagnostic and therapeutic agents for assisted reproduction and prevention of genetic diseases.[5]

The development of the fertilized egg in the oviduct and its transport to the uterus is highly dynamic, involving sperm-egg fusion, nuclear reprogramming, first division, zygote gene activation, and differentiation.[11] Any subtle alteration in these events may have a critical effect on embryonic development and particularly on the regulatory factors present in oviduct fluid EVs.[12] Up to now, EVs have not been utilized in clinically assisted reproduction for human in a real sense and also have not been widely used for large-scale animal reproduction on farms and ranches. Previous studies found that there exists a dynamic mutual paracrine communication between the embryonic and the maternal environments, and signals can be transmitted via EVs.[13,14] The embryo-maternal crosstalk mediated by EVs has been missing during in vitro embryo culture, which may have contributed to the large gap between the superior development of in vivo embryos and the inferior development of in vitro embryos.[12,15] Some studies reported that the addition of EVs to IVC systems could be beneficial to embryonic development, while other studies found no significant effect.[16,17] The in vitro environment is significantly different from the in vivo, where the embryo is connected to a complex circulatory system, in which the forces of regeneration and degradation maintain homeostasis.[18] Degraded EVs could be absorbed by other cells of the body, and newly produced EVs are continuously resupplied in vivo.[5,19–21] However, in the in vitro system, this EV renewal mechanism is missing; degraded EVs and harmful substances may accumulate, which is likely to lead to negative effects. It is particularly concerning that most studies have ignored this point. Regardless of whether the in vitro experiments involve stem cells, organoids, embryos or somatic cells, little consideration has been given to this lack of renewal and its adverse effects on cells or tissues.[18] In this study, rabbit embryos cultured in vitro with oviduct fluid EVs were used as models to study this important interaction.

# Materials and Methods

## Animals and Reagents

Adult male and female white New Zealand rabbits were provided by the Laboratory Animal Centre of Xi'an Jiaotong University Health Science Centre (Xi'an, China). The rabbits were housed at 24°C with a 14:10 h light/dark cycle. The experiments were approved by the Laboratory Animal Care Committee of Xi'an Jiaotong University. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

## Collection of Oviduct Fluid and Embryos

Female rabbits were injected with 80 IU of PMSG (NSHF, Ningbo, China), and 72 h later, 100 IU of hCG (NSHF) was injected intravenously; the female rabbits were immediately mated with male rabbits. Nineteen hours after mating, the female rabbits were anesthetized, the abdominal cavity was opened and an incision was made in the oviduct. The oviduct fluid was collected using a plastic pipette (2 mm diameter) connected to a 2-mL syringe and transferred into a 35-mm dish as micro-drops under sterile conditions. Embryos were collected with a glass needle under a stereomicroscope, and placed in Dulbecco's phosphate-buffered saline (DPBS). The pooled oviduct fluid was then centrifuged for 10 minutes at 300 g and the supernatant was collected for EV isolation.

## Ultracentrifugation, Transmission Electron Microscopy (TEM), and Nanoparticle Tracking Analysis for EVs

EVs were isolated, purified, and identified as previously described.[12] A total of 396 rabbits were used for collecting oviduct fluid, and six biological replicates were used in isolating EVs. Collected oviduct fluid or embryo culture medium was centrifuged at 2000 g for 10 min at 4°C and this supernatant was then re-centrifuged at 10,000 g for 30 minutes, and at 100,000 g for 2 h. EVs were obtained as pellets, which were resuspended in 20 μL of phosphate-buffered saline (PBS) for TEM, nanoparticle tracking analysis, and Western blotting. EVs for embryo culture treatment were resuspended in

embryo culture medium. For TEM, the samples were loaded onto 300-mesh grids, and stained with 2% phosphotungstic acid and imaged by TEM (JEOL, Tokyo, Japan). For nanoparticle tracking analysis, particles were analyzed by ZetaView (Particle Metrix, Inning am Ammersee, Germany) for size, distribution, and number of EVs. For Western blot of EVs, particles were lysed in RIPA buffer and SDS-polyacrylamide gel electrophoresis was used to separate proteins. The proteins were transferred to a polyvinylidene difluoride membrane, blocked with blocking buffer for 1 h at RT and then incubated with CD9 antibody (Santa Cruz, CA, USA, 1:200 dilution) or CD63 antibody (Santa Cruz, 1:200 dilution), or Calnexin (Beyotime, Shanghai, China, 1:200 dilution) for 1 h at RT, followed by thorough washing and incubation with appropriate secondary antibodies (Beyotime) for 1 h at RT; the blots were immediately visualized using an enhanced chemiluminescence detection kit according to instruction (Millipore, Billerica, MA, USA).

## In vitro Culture of Embryos

Fertilized embryos were collected, treated with hyaluronidase for denudation of granulosa cells, washed three times with embryo culture medium, and transferred into a culture dish. Each embryo occupied 10 μL of culture medium and each culture drop contained 18–24 embryos, and was covered with mineral oil. A total of 4048 embryos were used in this study. The embryo culture medium contained: 110 mM NaCl, 7.17 mM KCl, 1.19 mM $KH_2PO_4$, 1.71 mM $CaCl_2{\cdot}2H_2O$, 0.49 mM $MgCl_2{\cdot}2H_2O$, 25.07 mM $NaHCO_3$, 0.30 mM Na pyruvate, 1.50 mM glucose, 3 mM myoinositol, 30 μL/100 mL Na-lactate, 2 mL/100 mL essential amino acids, 1 mL/100 mL non-essential amino acids, 1 mL/100 mL ITS, 10 ng/mL EGF, 2 mg/mL polyvinyl alcohol. The embryos were cultured at 38.5°C in an incubator with 100% humidity and 5% $CO_2$. EVs were then added according to the experimental plan, and EVs of the groups in the same experiment were from the same pool of rabbits.

For the experiments on the influence of EV concentration on IVC embryos, fertilized embryos were collected and divided into four groups for treatment: (1) control group without EVs, (2) EVs at $9.1\times10^{10}$ particles/mL, (3) EVs at $9.1\times10^{11}$ particles/mL, and (4) EVs at $9.1\times10^{12}$ particles/mL. For experiments testing whether the biological activity of EVs decreased during IVC, embryos were cultured without EVs for 48 h, then divided into four groups: (1) no-EVs control, (2) addition of fresh EVs, (3) recycled 48 h-EVs, and (4) recycled 96 h-EVs. For the no-EVs group 1, medium without EVs was treated as control group. For fresh EVs in group 2, the EVs ($9.1\times10^{11}$ particles/mL) derived from oviduct fluid were immediately added to embryo culture medium for treatment after isolation. For group 3, EVs ($9.1\times10^{11}$ particles/mL) were incubated in culture medium for 48 h at 37°C, then isolated by ultracentrifugation and immediately added into embryo culture medium for recycling treatment. For group 4, EVs ($9.1\times10^{11}$ particles/mL) were incubated in culture medium for 96 h at 37°C, isolated from the medium, and immediately added to embryo culture medium.

In the experiment examining the role of renewal in the IVC system on EVs' effectiveness, four groups were tested: (1) control-EVs group, (2) R-M, (3) R-EVs, and (4) R-EVsM. For those groups, embryos were cultured and treated with EVs ($9.1\times10^{11}$ particles/mL) for 48 hours, then ultracentrifuged to separate EVs from medium. For the con-EVs group 1, both recycled EVs and used culture medium were retained. For the R-M group 2, the used culture medium was replaced with fresh culture medium and recycled EVs were added into the fresh culture medium. For the R-EVs group 3, the recycled EVs were discarded and fresh EVs ($9.1\times10^{11}$ particles/mL) were added to the retained used culture medium. For the R-EVsM group 4, both the recycled EVs and the used culture medium were discarded and replaced with fresh EVs ($9.1\times10^{11}$ particles/mL) and fresh culture medium. For the experiment examining the role of loss or gain of renewal in the IVC system, four groups were tested: in vivo group, non-EVs group, con-EVs group, and R-EVsM group.

## Measurement of pH and Concentration of Malondialdehyde (MDA) and Ammonium Ions

Embryo culture medium or oviduct fluid was collected at each time point from each group and immediately assayed for pH, MDA and ammonium ion concentration. The pH was determined by pH meter (Mettler/Toledo, Zurich, Switzerland). MDA was assayed by reaction with thiobarbituric acid (TBA) to generate an MDA-TBA adduct, which was quantified colorimetrically. The MDA assay steps are as follows. Culture medium or oviduct fluid was mixed with 42 mM $H_2SO_4$ and incubated with phosphotungstic acid solution at room temperature for 5 min. The reactants were centrifuged at 13,000 × g for 3 min, supernatants were removed, and pellets were resuspended in $ddH_2O$. TBA reagent was added and suspensions were incubated at 95°C for 1 h, then chilled in an ice bath for 10 min. The absorbance at 532 nm was

measured immediately using a microplate reader (Tecan, Mannedorf, Switzerland). Ammonium in samples was determined based on the following reaction: glutamate dehydrogenase + α-ketoglutarate + NADH + $NH_4^+$ → glutamate + $NAD^+$ + $H_2O$, following the procedure previously reported.[22]

## RNA Isolation and Sequencing

Blastocysts (n = 12) at 96 h were collected from each group, treated with hyaluronidase at 37°C for 3 min, then the cells outside the zona pellucida were gently dislodged from the blastocysts using a 200-μm diameter pipette. The blastocysts were washed three times in PBS, and one blastocyst from each group was immediately lysed and RNA was isolated with the RNeasy Micro kit (QIAGEN, Duesseldorf, Germany). Concentration and purity of the total RNA was determined using a NanoDrop spectrophotometer and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). For cDNA synthesis, 1 μg of qualified RNA of each sample was reverse transcribed using random primers, DNA Polymerase I and RNase H (BGI, Shenzhen, China). The library construction and transcriptome sequencing were performed on the BGISEQ-500 (BGI). Low quality reads were removed, along with joint contamination and unknown bases. Clean reads were mapped to the reference genome sequence using HISAT, and aligned to the reference gene sequence with Bowtie2 (the reference genome version was GCF_000003625.3_OryCun2.0) to give calculated fragments per kilobase of exon per million mapped fragments (FPKM) of each gene. Screening for differentially expressed genes (DEGs) was adjusted for the false discovery rate (FDR) and log2 fold change (log2FC) with the DEseq2 method. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs were performed as previously reported.[23]

## Quantitative PCR

Blastocysts at 96 h from each group were collected, and freed from cells outside the zona pellucida as described above. Five blastocysts from each group were immediately lysed using the Cells-to-Signal Kit (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using the PrimeScriptTM RT reagent kit (TaKaRa, Tokyo, Japan). SYBR premix Ex Taq (Takara) was used to perform real-time PCR, and signals were detected on a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The relative expression of oxidative stress-related genes (GPX3, SOD2, and GSTM2) and autophagy-related genes (ATG5, BECN1, and MAP1LC3C) were detected. Primer sequences are listed in Table S1.

## Determination of Reactive Oxygen Species (ROS) Level

Blastocysts (n = 20 to 25 in each group) were analyzed with an ROS assay kit (Beyotime). Blastocysts were incubated in serum-free medium containing dichlorodihydrofluorescein diacetate (DCHF-DA) at 37°C for 20 min, then washed 3x with DPBS, imaged with a fluorescence microscope equipped with a digital camera (Nikon), and quantitated using Image-Pro Plus (v6.0, Media Cybernetics).

## Immunofluorescence Detection of H3K36me3 and LC3

Blastocysts (n = 20 to 25 per group) were incubated with pronase at 37°C for 5 minutes to remove the zona pellucida and mucin coat. For H3K36me3 immunostaining, blastocysts were fixed in 4% paraformaldehyde/PBS for 1 h at RT, then incubated in 3 M hydrochloric acid for 10 minutes. For LC3 immunostaining, blastocysts were fixed in 4% paraformaldehyde/PBS for 1 h at RT without hydrochloric acid treatment. Subsequently, the blastocysts were permeabilized with 0.1% Triton X-100 for 30 minutes at RT, blocked with blocking buffer, and incubated with primary antibodies, anti-H3K36me3 (ABclonal Technology, Wuhan, China, 1:200 dilution), anti-LC3 (Abcam, Cambridge, MA, USA, 1:500 dilution) for 2 h at RT, followed by an appropriate secondary antibody labeled with Alexa-Fluor488 (Abcam, 1:200 dilution) for 1 h at RT. After washing, the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Beyotime) for 10 min. The embryos were transferred to slides, sealed with neutral resin, cover-slipped, and imaged with a fluorescence microscope equipped with a digital camera (Nikon), followed by analysis using Image-Pro Plus (Media Cybernetics, Silver Springs, MD, USA).

## Apoptosis Assay

The DeadEnd Fluorometric TUNEL system (Promega, Madison, WI, USA) was used as previously described.[22] The zona pellucida and mucin coat were removed, and the blastocysts (n = 20 to 25 per group) were fixed with paraformaldehyde and permeabilized with Triton X-100. Blastocysts were treated with equilibration buffer at RT for 5 min, and after this step, all subsequent steps were performed in the dark. Blastocysts were incubated with reaction mixture containing 45 μL of equilibration buffer, 5 μL of nucleotide mix, and 1 μL of rTDT at 37°C for 5 min in the dark. The reaction was terminated with 2x SSC for 15 min. After washing, the nuclei were stained with DAPI, and blastocysts were mounted on slides, imaged and analyzed using Image-Pro Plus. The apoptotic index was defined as the ratio of the number of apoptotic cells (green) to the total number of cells in the blastocysts (blue).

## Statistical Analysis

Differences in embryonic development ratio were assessed using the Chi-squared test. Differences in ROS level, mRNA expression, H3K36me3 intensity, LC3 intensity, apoptosis index, and total cell number per blastocyst were analyzed using Student's *t*-test or one-way ANOVA with the least-significant difference (LSD) test using SPSS Statistics 20 software (IBM, Armonk, NY, USA). $P < 0.05$ were considered statistically significant.

# Results

## Effects of EVs Derived from Oviduct Fluid on Embryonic Development Ratio

EVs were isolated from oviduct fluid and characterized by TEM, Western blotting and nanoparticle tracking analysis. The particles exhibited typical vesicle structure (Figure 1A) and CD9 signals were detected in isolated EVs (Figure 1B). Nanoparticle tracking analysis showed that diameters of the particles ranged from 30 to 500 nm, with a median particle size of 125 nm, and concentration of $9.1\times10^{10}$ particles/mL (Figure 1C).

Fertilized embryos were collected and divided into four groups for in vitro culture: (1) control group without EVs, (2) EVs at $9.1\times10^{10}$ particles/mL, (3) EVs at $9.1\times10^{11}$ particles/mL, and (4) EVs at $9.1\times10^{12}$ particles/mL. The results showed that the 8-cell ratio was not significantly different between each group at 24 h (Figure 1D and H). At 48 h, groups 3 and 4 showed a significantly increased morula ratio compared with control group 1 ($P < 0.05$) (Figure 1E and H). At 72 h, group 3 had a significantly increased blastocyst ratio compared to control ($P < 0.05$), but there was no significant change in group 4 (Figure 1F and H). At 96 h, there was no significant difference in blastocyst ratio between group 3 and control; however, the ratio in group 4 blastocysts was significantly lower than the control group ($P < 0.05$) (Figure 1G and H). For group 2, the development rate showed no significant differences within the 4-day experimental period compared to the control group.

## Loss of IVC System Renewal Caused Accumulation of MDA and Ammonium Ions in the Embryo Culture Medium

The culture medium in each of the four groups was not renewed during the 96 h of culture and the pH value, MAD level and ammonium ion content in the culture medium of each group was measured at 24, 48, 72 and 96 h. The results showed that pH values ranged from 7.10 to 7.46 and there was no significant difference in pH value between the groups at any point (Figure 1I). Concentration of MDA in group 3 embryo culture medium was significantly higher than control at 48, 72 and 96 h ($P < 0.05$), and in group 4, the levels were significantly higher than the control group at 24, 48, 72 and 96 h ($P < 0.05$). MDA levels in group 3 and group 4 also increased gradually with culture time (Figure 1J). No significant difference was found between group 1 and group 2, although there was a trend towards higher MDA levels in group 2 at 48, 72 and 96 h compared to group 1 (Figure 1J). Similar to the tendency of MDA, the ammonium ion concentration in group 3 and group 4 was significantly higher than control at 24, 48, 72 and 96 h ($P < 0.05$), but no significant difference was found between group 1 and group 2 (Figure 1K). The ammonium level in each group increased gradually with culture time (Figure 1K).

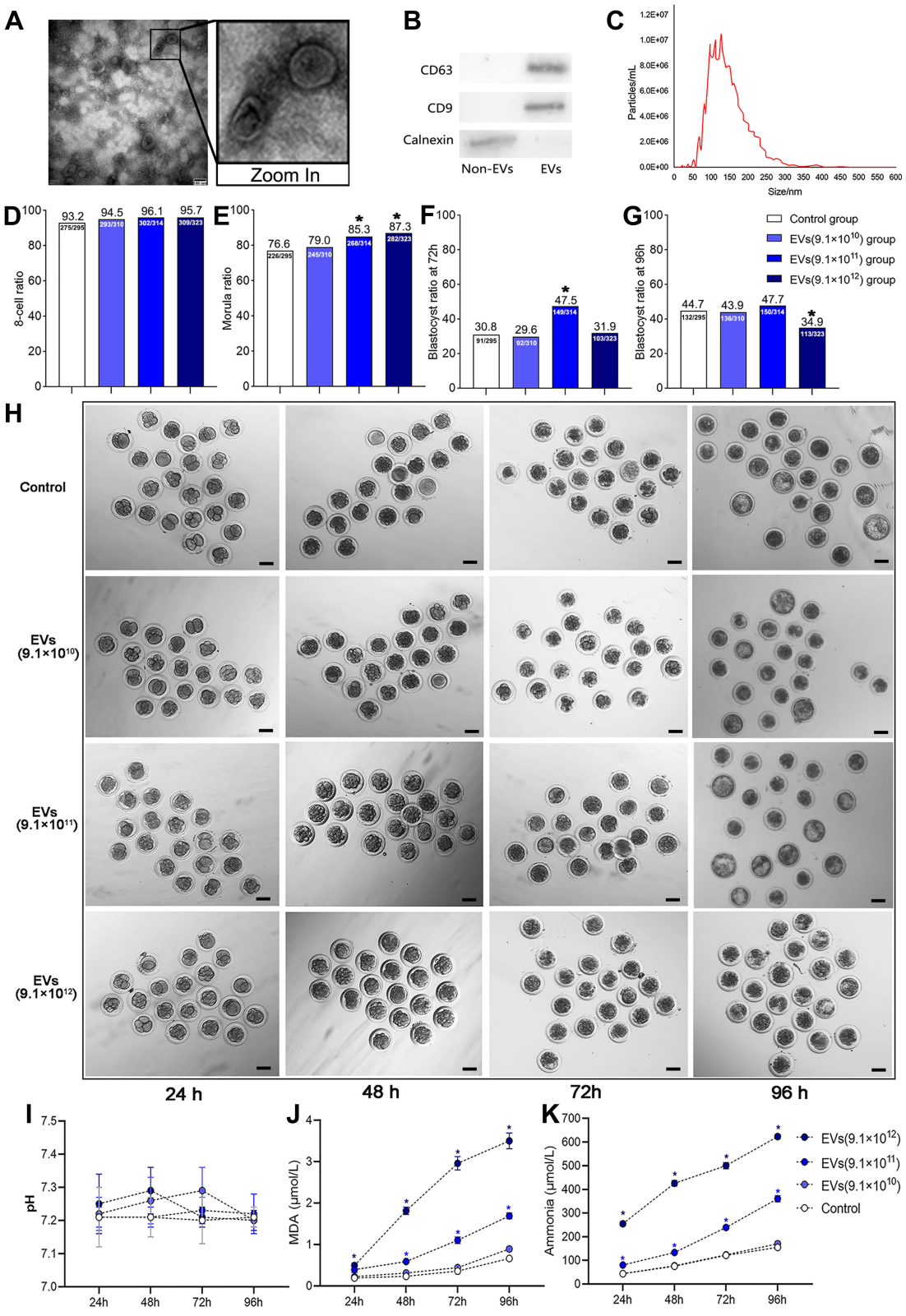


**Figure 1** Effects of different quantities of EVs derived from oviduct fluid on embryonic development ratio, pH, MDA and ammonium ion levels. (**A**) EV particles imaged using transmission electron microscopy (TEM), scale bar = 100 nm; (**B**) Western blotting for CD9; (**C**) quantification and size distribution of particles by nanoparticle tracking analysis; (**D**) 8-cell ratio at 24 h; (**E**) morula ratio at 48 h, (**F**) blastocyst ratio at 72 h and (**G**) blastocyst ratio at 96 h in groups: control, EVs at $9.1\times10^{10}$ particles/mL, EVs at $9.1\times10^{11}$ particles/mL, and EVs at $9.1\times10^{12}$ particles/mL; (**H**) representative images of embryos at 24, 48, 72 and 96 h of in vitro culture in the four groups, scale bar = 100 μm; (**I**) pH value; (**J**) MAD concentration; and (**K**) ammonium concentration at 24, 48, 72 and 96 h in culture medium of the four groups. The *above the bars indicates significant difference ($P < 0.05$) compared with control group.

## Loss of in vitro Culture System Renewal Impaired Embryo Development Competence

Based on the above results (Figure 1D and H), the lowest concentration of EVs ($9.1\times10^{10}$ particles/mL) had no effect on embryo development competence, while higher concentrations ($9.1\times10^{11}$ EVs/mL) had a positive impact on embryonic development competence at 48 h and 72 h; but this positive impact disappeared at 96 h. At the highest EV dose of $9.1\times10^{12}$ particles/mL, there was a negative impact at 96 h that seemed to indicate that EV's activity was time- and dose-dependent. We hypothesized that the biological activity of EVs might decrease or be lost with increasing culture time, because of toxic metabolites accumulating in the culture system.

To further test whether the biological activity of EVs decreased during IVC, we carried out the following experiments. Embryos were cultured without EVs for 48 h, then were divided into four groups: (1) no-EVs control, (2) addition of fresh EVs, (3) recycled 48 h-EVs, and (4) recycled 96 h-EVs (Figure 2A). After 24 h of culture, the blastocyst ratio (Figure 2B and D) and the total number of cells per blastocyst (Figure 2C and D) were determined. The results showed that adding fresh EVs to group 2 resulted in a higher blastocyst ratio and total number of cells per blastocyst than group 3 and group 4 ($P < 0.05$); and the ratio in group 4 was lower than in group 3 ($P < 0.05$). There was no significant difference between the no-EVs group 1 and group 4 (Figure 2B–D). The results indicated that the EV-induced stimulation of embryonic development decreased with time in culture.

In order to determine the role of EV regeneration in the IVC system on effectiveness of EVs, four groups were tested: (1) control-EVs group, (2) regenerated medium, R-M, (3) regenerated EVs, R-EVs, and (4) both regenerated, R-EVsM (Figure 2E). After culturing for 24 h, the blastocyst ratio (Figure 2F and H) and total number of cells per blastocyst (Figure 2G and H) were determined and both were higher in the R-M and R-EVs groups than the control group, and the values were higher in the R-EVsM group than in the other groups ($P < 0.05$) (Figure 2F–H). These results suggested that the absence of EV renewal in the traditional IVC system impaired embryonic development. Replacement of EVs and medium during IVC sustained embryonic development.

## Regeneration in the IVC System Affects EVs' Influence on Embryo Transcriptome

To determine the effects of EV or renewal on embryonic development, the relative gene expression in the various groups was analyzed by RNA-seq (Figure S1). Compared with the in vivo group, 2970 genes were down-regulated and 2921 genes were up-regulated ($\log_2|FC|>1$, FDR <0.05) in the non-EV group (Figure S2). The biological processes most enriched in terms of DEGs were protein phosphorylation, ATP synthesis coupled to proton transport, microtubule synthesis and activity, ATP hydrolysis coupled to proton transport, and cellular response to hydrogen peroxide (Figure S2). The most enriched KEGG pathways involved metabolism, oxidative phosphorylation, thermogenesis, cellular senescence, cell cycle, and lysosomal processing (Figure S2). These results indicated that oxidative stress, autophagy, and apoptosis might be causes of the inferior development of embryos in the non-EVs group, in which EVs were omitted.

To determine the effect of EVs on the embryonic transcriptome in the IVC system without renewal, the blastocyst gene expression in the con-EVs group was compared to the non-EVs group: the heatmap showed the differences in expression between the con-EVs group and the non-EVs group, including the in vivo group (Figure 3A). A total of 1046 genes were down-regulated and 1623 genes were up-regulated ($\log_2|FC|>1$, FDR <0.05) in the con-EVs group compared to the non-EVs group (Figure S3). The genes that consistently changed expression in the con-EVs group and in the in vivo group compared to the non-EVs group were defined as consistent genes, which may indicate a positive role of EVs; otherwise, they were referred to as inconsistent genes, which may indicate a negative influence of EVs. The ratio of inconsistent genes to the DEGs was 50.5% in the con-EVs group (Figure S3). A total of 779 genes were up-regulated ($\log_2|FC|>1$, FDR <0.05) and 523 genes were down-regulated ($\log_2|FC|>1$, FDR <0.05), in both the con-EVs and in vivo groups compared to the non-EVs group (Figure 3B). Moreover, 82 genes were up-regulated in the con-EVs group but down-regulated in the in vivo group ($\log_2|FC|>1$, FDR <0.05); 157 genes were down-regulated in the con-EVs group and also in the in vivo group ($\log_2|FC|>1$, FDR <0.05); and 1038 genes were up- or down-regulated ($\log_2|FC|>1$, FDR <0.05) in the con-EVs group, but not significantly changed (FDR >0.05) in the in vivo group (Figure 3B). The biological processes associated with the consistent genes of the con-EVs group were most enriched in microtubule-based operations, tRNA methylation, regulation of growth, tetrahydrobiopterin biosynthesis, and positive regulation of cell growth;

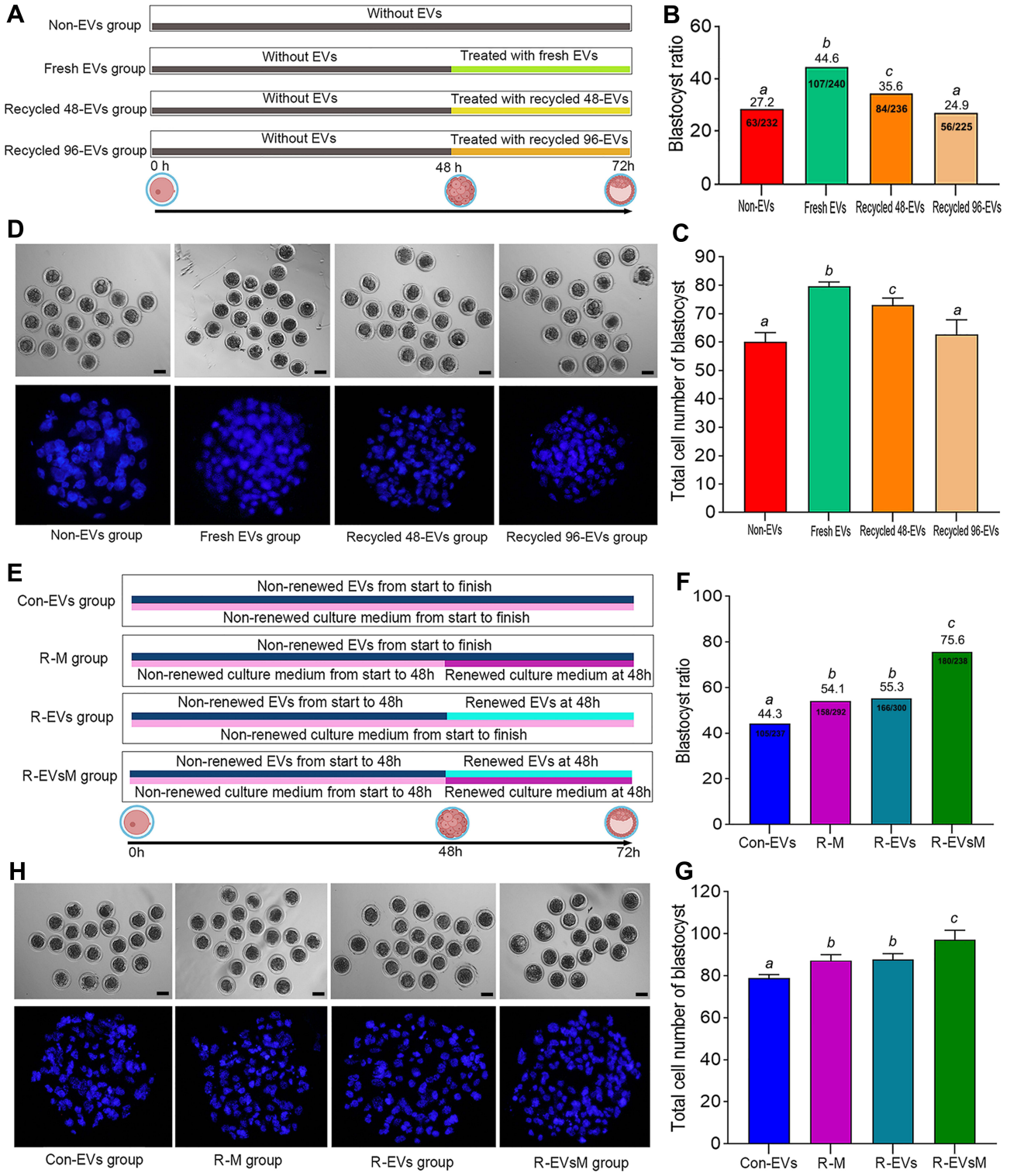


**Figure 2** Loss of renewal in the in vitro culture system impairs competence for embryo development. (**A**) Groups used: non-EVs, fresh EVs, recycled 48 h-EVs, and recycled 96 h-EVs; (**B**) blastocyst ratio and (**C**) numbers of cells per blastocyst at 72 h in the non-EVs, fresh EVs, recycled 48 h-EVs, and recycled 96 h-EVs group; (**D**) representative images of embryos at 72 h of the non-EVs, fresh EVs, recycled 48-EVs, and recycled 96-EVs group (upper panel, bright-field, scale bar = 100 μm; lower panel, DAPI staining in blue); (**E**) groups used: con-EVs, R-M, R-EVs, and R-EVsM group; (**F**) blastocyst ratio and (**G**) numbers of cells per blastocyst at 72 h in the con-EVs, R-M, R-EVs, and R-EVsM group; and (**H**) representative images of embryos at 72 h from the con-EVs, R-M, R-EVs, and R-EVsM group (upper panel, bright-field, scale bar = 100 μm; lower panel, representative blastocysts stained blue with DAPI). Different letters (the lowercase a, b and c) above the bars indicate significant differences at $P < 0.05$.


**Figure 3** Loss of renewal in the in vitro culture system affects EVs' role in regulating the embryo transcriptome (con-EVs vs non-EVs). (**A**) Heatmap showing differentially expressed genes in blastocysts between con-EVs group and non-EVs group, including the in vivo group; (**B**) consistent and inconsistent genes of the con-EVs group and in vivo group compared to the non-EVs group; (**C**) GO analysis and KEGG pathway enrichment analysis of the consistent genes; and (**D**) GO analysis and KEGG pathway enrichment analysis of the inconsistent genes.

but, only the FDR value of microtubule-based processes was <0.05 (Figure 3C). The most enriched KEGG pathways of consistent genes of the con-EVs group were folate biosynthesis, gap junction, and apoptosis pathways (Figure 3C). The biological processes of the inconsistent genes of the con-EVs group were mostly enriched in mitochondrial respiratory chain complex I assembly, and the most enriched KEGG pathways of the inconsistent genes of the con-EVs group were oxidative phosphorylation, thermogenesis, retrograde endocannabinoid signaling, metabolic pathways, and carbon metabolism (Figure 3D).

To further explore the effects of renewal on embryo development in the IVC system, we compared the transcriptome of the R-EVsM group and the non-EVs group. The heatmap showed the DEGs between the R-EVsM and the non-EVs group, including the in vivo group (Figure 4A). The results showed 555 genes were down-regulated and 969 genes were up-regulated (log2|FC|>1, FDR <0.05) in the R-EVsM group compared to the non-EVs group (Figure S3); 459 genes were up-regulated (log2|FC|>1, FDR <0.05), and 737 genes were downregulated (log2|FC|>1, FDR <0.05) in both the R-EVsM and in vivo group compared with non-EVs group (Figure 4B). Thirteen genes were up-regulated (log2|FC|>1, FDR <0.05) in the R-EVsM group but down-regulated (log2|FC|>1, FDR <0.05) in the in vivo group, six genes were down-regulated (log2|FC|>1, FDR <0.05) in the R-EVsM group, and up-regulated (log2|FC|>1, FDR <0.05) in the in vivo group, 171 genes were up- or down-regulated (log2|FC|>1, FDR <0.05) in the R-EVsM group, but not significantly changed (FDR >0.05) in the in vivo group (Figure 4B). The ratio of the number of inconsistent genes to the number of DEGs was 13.7% in the R-EVsM group, which was far below that of the con-EVs group (Figure S3). Molecular functions of consistent genes were most enriched in catalytic activity, cytoskeletal protein binding, and actin filament binding (Figure 4C). The most enriched KEGG pathways associated with the consistent genes were metabolic pathways, carbon metabolism, biosynthesis of amino acids, lysosomes, focal adhesion proteins, citrate cycle, pyruvate metabolism, cellular senescence, pentose phosphate pathway, biosynthesis of secondary metabolites, etc. (Figure 4C); no significant MF, BP, or KEGG pathways were found for the inconsistent genes of the R-EVsM group (Figure 4D).

## Loss or Gain of Renewal in the IVC System Affects EVs' Role in Embryonic ROS

The concentration of MDA and ammonium ion in the non-EVs group, con-EVs group, R-EVsM and in vivo group were measured at 96 h. The results showed that MDA and ammonium levels in the in vivo group were significantly lower than other groups ($P < 0.05$), and both values in the con-EVs group were significantly higher than in the non-EVs group ($P < 0.05$), while MDA and ammonium were significantly lower in the R-EVsM group than in the con-EVs group ($P < 0.05$) (Figure 5A and B). Expression of oxidative stress-related genes, GPX3, SOD2, and GSTM2, in blastocysts from the four groups at 96 h was determined by qPCR; the relative expression of GPX3 and SOD2 in the non-EVs group was significantly higher than that in the in vivo group ($P < 0.05$), while GPX3 and SOD2 expression level was higher in the con-EVs group than that of con-EVs group ($P < 0.05$), and gain of renewal IVC system (R-EVsM group) significantly reduced the expression of GPX3 and SOD2 compared with con-EVs group ($P < 0.05$) (Figure 5C–E). Relative expression of GSTM2 in the non-EVs and con-EVs groups was significantly lower than in the R-EVsM and in vivo groups ($P < 0.05$) (Figure 5C–E). The relative level of ROS in blastocysts at 96 h in the non-EVs group was significantly higher than in the in vivo group ($P < 0.05$), and the ROS level in the con-EVs group was significantly higher than in the non-EVs group ($P < 0.05$). EV renewal in the IVC system significantly reduced blastocyst ROS in the R-EVsM group, compared to con-EVs and non-EVs groups ($P < 0.05$) (Figure 5F and G).

## Loss or Gain of Renewal in IVC Affects EVs' Role in Embryonic Autophagy, Epigenetics and Apoptosis

Expression of the autophagy-related genes, *ATG5, BECN1*, and *MAP1LC3C*, in blastocysts at 96 h in the four groups was determined by qPCR, and the results showed that the relative expression of BECN1, BECN1, and MAP1LC3C was significantly higher in the non-EVs group than in the in vivo group, and expression in the con-EVs group was significantly higher than in the non-EVs group ($P < 0.05$) (Figure 6A–C). Expression of ATG5 and MAP1LC3C in the R-EVsM group was significantly lower compared to the non-EVs and con-EVs groups, and expression of BECN1 in the R-EVsM group was significantly lower than in the con-EVs group ($P < 0.05$) (Figure 6A–C). The level of LC3 in

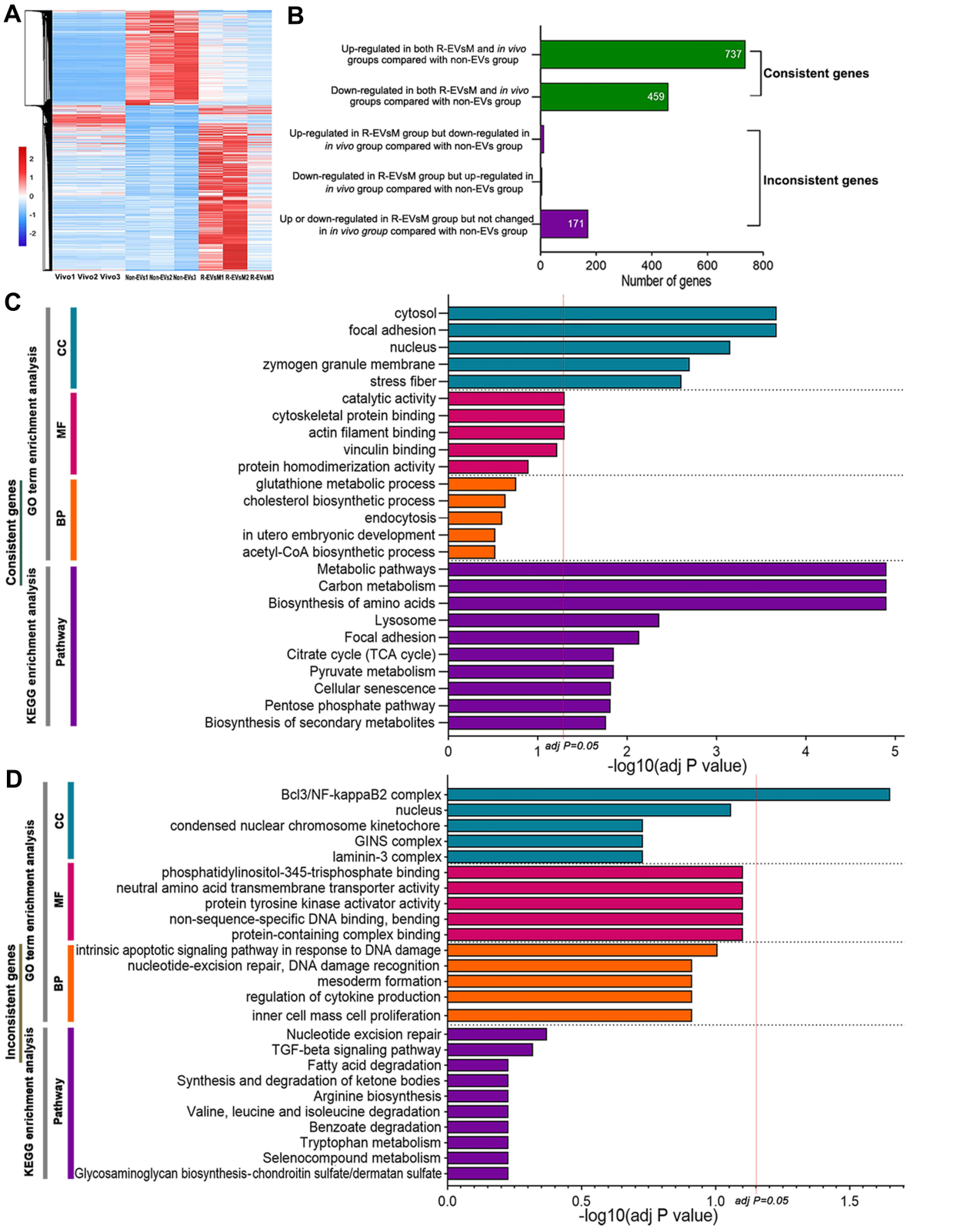


**Figure 4** Gain of renewal in the in vitro culture system affects EVs' role in regulating the embryo transcriptome (R-EVsM vs non-EVs). (**A**) Heatmap of differentially expressed genes in blastocysts between the R-EVsM group and non-EVs group, including the in vivo group; (**B**) consistent or inconsistent genes of the R-EVsM group and in vivo group compared to the non-EVs group; (**C**) GO analysis and KEGG pathway enrichment analysis of the consistent genes; and (**D**) GO analysis and KEGG pathway enrichment analysis of the inconsistent genes.

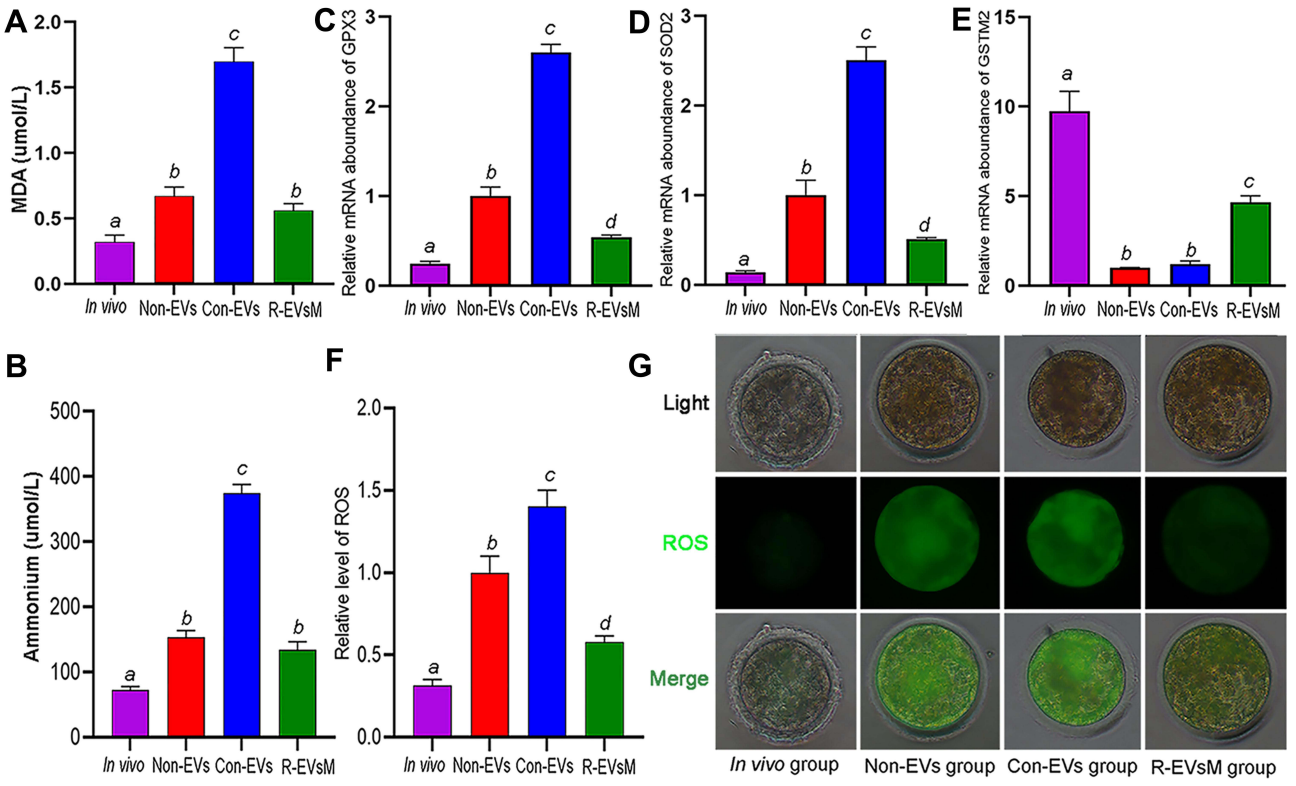


**Figure 5** Loss or gain of renewal in the in vitro culture system affects EVs' role in embryonic production of ROS. (**A**) MAD concentration and (**B**) ammonium concentration at 96 h in culture medium of the in vivo group, non-EVs group, con-EVs group, and R-EVsM group. Relative mRNA expression levels of GPX3 (**C**), SOD2 (**D**), and GSTM2 (**E**) in blastocysts at 96 h in the four groups; (**F**) quantification of ROS by fluorescence intensity in blastocysts at 96 h in the four groups; (**G**) representative images of ROS staining in blastocysts at 96 h in the four groups (upper panel, bright-field; middle panel, green fluorescence indicating ROS; lower panel merged bright-field and green fluorescence). Different letters (the lowercase a, b, c and d) above the bars indicate significant differences at $P < 0.05$.

blastocysts at 96 h in the four groups was determined by immunofluorescence staining, which showed that LC3 was significantly lower in the R-EVsM and in vivo groups compared to the non-EVs and con-EVs groups ($P < 0.05$), while it was higher in the con-EVs group than in the non-EVs group ($P < 0.05$) (Figure 6D and E).

The epigenetic marker, H3K36me3, was determined in blastocysts at 96 h in the four groups by immunofluorescence staining. The results revealed that the relative H3K36me3 level was significantly higher in the non-EVs and con-EVs groups than in the R-EVsM and in vivo groups ($P < 0.05$), while it was higher in the con-EVs group than in the non-EVs group ($P < 0.05$) (Figure 6F and G). No significant difference in H3K36me3 level was seen between the R-EVsM group and the in vivo group (Figure 6F and G). The apoptotic index of blastocysts at 96 h in the four groups was measured by TUNEL assay, and it was found to be significantly higher in the non-EVs group than in the in vivo group ($P < 0.05$), and significantly higher in the con-EVs group compared to the non-EVs group ($P < 0.05$) (Figure 6H and I). The apoptotic index in the R-EVsM group was significantly lower than in the con-EVs and non-EVs groups ($P < 0.05$) (Figure 6H and I).

## Discussion

Previous studies found that addition of oviduct EVs to IVC embryos improved the development and quality of the embryos produced.[12,24] However, some studies reported that oviduct EVs played only a limited role in embryonic development, and exposure to EVs from oviduct fluid did not affect blastocyst yield or cryotolerance.[16,17] Similarities were also found with in vitro experiments on other cells, such as endothelial cells, which synthesize EVs that act as ROS scavengers; but, it has also been suggested that EVs can produce ROS as part of the signaling processes in endothelial cells.[25,26] These controversial results with regard to the role of EVs might be related to the complexity of components in EVs or to stability problems from the extraction or preservation, which could inhibit biological activity.[18,27,28] What we think most likely is that EVs are in a dynamic balance of continuous production and clearance

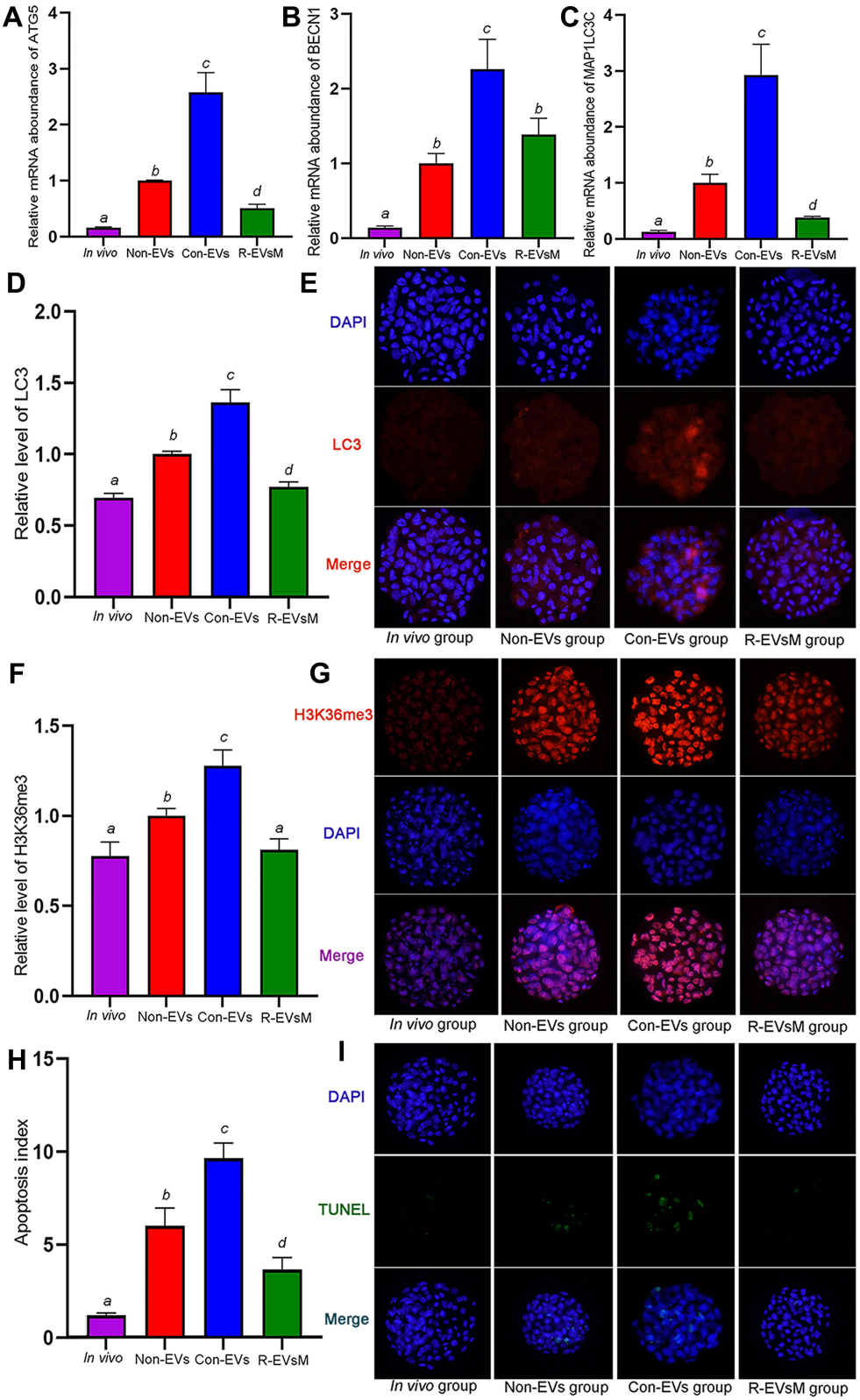


**Figure 6** Loss or gain of renewal in the in vitro culture system affects EVs' role in embryonic autophagy, epigenetic state and apoptosis. (**A**) Relative mRNA expression levels of ATG5, BECN1 (**B**), and MAP1LC3C (**C**) at 96 h in blastocysts from the in vivo group, non-EVs group, con-EVs group, and R-EVsM group; (**D**) relative fluorescence intensity of LC3 in blastocysts at 96 h from the four groups; (**E**) representative images showing immunofluorescence of LC3 in blastocysts from the four groups; (**F**) quantitation of fluorescence intensity of H3K36me3 (red) in blastocysts at 96 h from the four groups; (**G**) representative images showing immunofluorescence of H3K36me3 (red) in blastocysts from the four groups; (**H**) apoptotic index of blastocysts at 96 h from the four groups; (**I**) representative images of TUNEL assay of blastocysts, apoptotic blastomeres (red). Different letters (the lowercase a, b, c and d) above the bars indicate significant differences at $P < 0.05$.


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in vivo. Many kinds of cells secrete EVs, but others, like macrophages, ingest EVs; this dynamic, homeostatic condition of EVs is missing from the IVC system, and its absence adversely affects the function of EVs in vitro and may even lead to harmful effects.

To test this conjecture, we treated embryos cultured in vitro with different concentrations of EVs isolated from rabbit oviducts. The embryonic development ratio was improved on days 2 and 3 with an amount of EVs equivalent to ten times that of in vivo, but on day 4, this improvement was lost. An amount of EV particles equivalent to 100 times that of in vivo increased the development rate only on day 2, but reduced the development rate on 4 day. These results suggested that the in vitro effects of EVs occurred in a time- and dose-related manner. It is likely that the biological activity of EVs declines with time or that there is an increased production of toxic substances that suppress the beneficial effects of EVs. To further test this hypothesis, we measured the pH and the MDA and ammonium ion levels in the culture medium of blastocysts after treatment with different amounts of EVs. Our results showed that the concentration of MDA and ammonium increased with increases in EV particles/mL or culture time. MDA, as an end product of lipid peroxidation by free radicals, can cause cross-linking polymerization of macromolecules such as proteins and nucleic acids, which is cytotoxic. Despite the novel discovery in our study that MDA accumulated in EVs added to an IVC system, we were still unable to unequivocally explain how this MDA was produced. Based on previous studies on EVs metabolomics, we speculate that EVs contain a large number of lipids, which are likely to be the main source of MDA.[29] Also, EVs are enriched in proteins or peptides, which might be degraded to yield ammonium ions, which are toxic to cells and can inhibit embryonic development.[22,30] Very few cell culture experiments have considered the possibility of the accumulation of harmful substances in EVs. Here, we found that EVs were subject to an accumulation of toxic metabolites such as MDA and ammonium in the IVC system, which could have adverse consequences for experiments aimed at understanding EV biology and function.

Whether the biological activity of EVs is lost or there are dynamic changes in their activity during IVC are still not clear and there are insufficient data to support these conclusions. Previous studies have shown that EV size, particle number, and cargo was affected by storage temperature.[27] Treatment of human endothelial cells with EVs from KSHV-infected cells showed that EVs lost functionality after day 4 and that biological activity of EVs was affected by temperature.[28] Here, the development ratio was improved on days 2 and 3, but reduced on day 4 with an amount of EVs equivalent to ten times that in oviduct fluid; the result seemed to be related to the loss of bioactive functions of EVs during IVC. Embryos treated with fresh EVs showed higher quality than those incubated with recycled 48-h EVs or recycled 96-h EVs, which were ineffective. This indicated that the bioactive functions of EVs were diminished during the process of IVC. Embryo quality could be improved by adding fresh EVs, indicating that EV renewal is a viable strategy for dealing with the loss of EV function in vitro. The effect of renewing both the culture medium and the EVs was greater than that of either alone during IVC, indicating that renewal of the IVC system, establishing the in vivo situation of EV production and clearance in vitro, is an important strategy for removing the accumulation of toxic substances or countering the loss of biological activity. In most reported IVC studies, however, few measures for renewal of EVs were considered and followed. We think that the inconsistent results of different in vitro studies on EVs seem to be most likely a result of the lack of renewal (regeneration of the medium and the EVs).

The striking difference between the non-EVs group and the in vivo group is that EVs are completely missing in the non-EVs group. The KEGG pathways enrichment of DEGs suggested that the inferiority of in vitro embryos under EV deletion may be closely related to oxidative stress, autophagy and apoptosis. In vitro embryos treated with EVs but lacking renewal, showed a greater number of inconsistent genes, which were closely related to oxidative stress, imbalance of autophagy and apoptosis, suggesting negative effects of EVs in the absence of renewal. Embryos from IVC medium containing fresh EVs plus gain of renewal showed a smaller number of inconsistent genes. Transcriptomes of embryos from IVC medium containing EVs plus gain of renewal were more similar to the transcriptomes of in vivo embryos. The transcriptome results revealed a kind of crosstalk between embryo and oviduct, as previously described,[31] and also provided the molecular signature of oviduct EVs on IVC embryos, confirming that oviduct EVs play an important role in the regulation of embryonic gene expression. Loss of renewal tends to disrupt gene expression while gain of renewal normalizes gene expression.

ROS are a by-product of oxygen metabolism and some are essential in cell signal transduction and homeostasis.[32] ROS production and ROS elimination are balanced in embryos developing in vivo; however, IVC embryos showed an excess of ROS.[33] Excessively high ROS causes damage to lipids, proteins and DNA, abnormal transcription of genes, modulation of

autophagy, cellular growth, differentiation, proliferation, apoptosis, and impaired embryonic development.[34,35] Previous studies revealed that EVs and ROS are closely interrelated.[18] The cargos that EVs can introduce into cells may contain the enzymatic components necessary for regulating ROS content. The EV cargo can also incorporate signaling molecules that can modify gene expression involved in the regulation of redox processes, which affects the cellular ROS content.[18,36] EVs contain the proteins, GPX, GST, PRDX, SOD2, and CAT, as well as miRNAs, such as miR-126, miR-21, miR-128 and some antioxidant compounds, all involved in oxidative stress.[18] Here, IVC embryos without EVs showed higher levels of ROS than developing embryos in vivo suggesting that a lack of EVs in vitro was closely related to high ROS levels as previously reported.[12] Blastocysts that contained EVs and gained renewal had lower ROS levels than the con-EVs and non-EVs group, indicating that EVs have an antioxidant role. Blastocysts that contained EVs but lacked renewal (con-EVs group) showed a higher ROS level than the in vitro group without EVs (non-EVs group), which seemingly contradicts the antioxidant role of EVs. Some studies even reported that EVs produced by some pathological cells induced ROS.[26,37] As far as we know, the possibility that EVs can directly increase ROS in the extracellular compartment has not been demonstrated. Here, we found that MDA and $NH_4^+$, which are important products of embryonic ROS activity, accumulated in culture medium when treated with EVs during in vitro culture. Accumulation of hazardous compounds and lack of renewal of the IVC medium may be responsible for the higher levels of ROS in the con-EVs group. In view of our observation that the biological activity of EVs was lost with longer incubation, we offered the conjecture that the lack of renewal in the traditional IVC system caused accumulation of hazardous compounds and loss of biological activity of EVs, leading to loss of antioxidant activity of EVs and higher levels of embryonic ROS; And gain of renewal in the IVC system can be a useful strategy to counteract this loss of EV functionality.

The autophagy-lysosomal pathway existing in various cells continuously and stably maintains cellular homeostasis by degrading damaged organelles and misfolded proteins that are prejudicial to cells.[38] Under physiological conditions, autophagy results in the orderly degradation of excess or damaged components in the cytosol through the lysosomes.[38,39] Under pathological situations, however, inappropriate autophagy can damage normal cells and lead to aging and death.[38,39] Previous studies reported that excessive accumulation of ROS could degrade cellular homeostasis, resulting in oxidative stress, mitochondrial dysfunction, and autophagy.[40,41] Recent research studies have revealed the molecular mechanism underlying the interaction between EVs and autophagy signaling pathways, but the intracellular degradation pathway that occurs after EVs enter cells is still not fully understood.[42–44]

Autophagy has been found to regulate a number of important processes involved in embryonic development.[45] In this study, the mRNA expression of autophagy-related genes and LC3 protein levels were higher in the non-EVs group than in the in vivo group, and higher in the con-EVs group than the non-EVs group, suggesting that embryos that have lost EVs in vitro seem to be in a state of autophagy activation, and over-activation of autophagy may be exacerbated by treatment with EVs without replacement of the IVC medium. Selected genes were down-regulated when embryos were treated with both fresh EVs and medium, demonstrating that regenerative treatments in IVC systems can overcome excessive autophagy of embryos in vitro.

During early embryonic development of mammals, epigenetic modifications change dramatically.[46] H3K36me3 is an abundant, conserved epigenetic factor, which plays an important role in mediating developmental stress.[47,48] Embryos showing an abnormal H3K36me3 pattern have reduced viability and development competence, and even undergo lethality.[48] In this study, the H3K36me3 level in blastocysts in the in vitro group without EVs was higher than that in the in vivo group, and the level was higher in the group treated with EVs without renewal than the group without EVs, while gain of renewal significantly decreased the level of H3K36me3. The result indicated that lack of renewal may cause an abnormal epigenetic state, and treating embryos with EVs and gain of renewal in an IVC system can correct abnormal H3K36me3 modifications in vitro.

The apoptotic index is an important factor in embryonic development potential, and a lower index often corresponds with a higher development potential. A higher level of ROS is considered a crucial cause for apoptosis; autophagy imbalance and abnormal epigenetic modification can also cause apoptosis.[49,50] The apoptotic index was higher in the group treated with EVs without renewal than the group without EVs, indicating that the accumulation of toxic substances or the loss of biological activity of EVs can induce embryonic apoptosis which is hazardous to embryo development. The apoptotic index in the group treated with EVs plus gain of renewal was significantly lower than the non-EVs group and con-EVs group, suggesting that gain of renewal in the IVC system can be a useful strategy to deal with the apoptosis caused by accumulation of toxic substances or loss of biological activity of EVs.

## Conclusions

Our research demonstrates that lack of renewal in the IVC system leads to accumulation of harmful substances and attenuation of the biological activity of EVs, resulting in a series of adverse processes, such as oxidative stress, autophagy imbalance, abnormal epigenetic modification, and decreased embryonic development ability. Gain of renewal in the IVC system can reduce these adverse aspects and ensure the beneficial function of EVs. In this study, embryos sensitive to environmental substances were used as the model, to examine the role of oviduct fluid EVs on embryo development in vitro, and to some extent, our results can explain the controversial results that allow different conclusions from using similar EVs. Our findings not only have important value for long-term embryo culture in vitro, but also provide useful reference values for in vitro experiments with EVs on other cells. The data are also of great significance in complementing our understanding of EV biology in vitro and developing new procedures for safe and effective application of EVs.

## Data Sharing Statement

Data available upon request to the authors.

## Ethical Approval

The experimental protocol was carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and was approved by the Laboratory Animal Care Committee of Xi'an Jiaotong University.

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## Supplementary information

The authors confirm that the data supporting the findings of this study are available within the supplementary materials.

## Disclosure

The authors declare no conflicts of interest.

## References

1. Margolis L, Sadovsky Y. The biology of extracellular vesicles: the known unknowns. *PLoS Biol*. 2019;17:e3000363. doi:10.1371/journal.pbio.3000363
2. Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular vesicles: composition, biological relevance, and methods of study. *Bioscience*. 2015;65:783–797. doi:10.1093/biosci/biv084
3. Varcianna A, Myszczynska MA, Castelli LM, et al. Micro-RNAs secreted through astrocyte-derived extracellular vesicles cause neuronal network degeneration in C9orf72 ALS. *EBioMedicine*. 2019;40:626–635. doi:10.1016/j.ebiom.2018.11.067
4. Saleh AF, Lazaro-Ibanez E, Forsgard MA, et al. Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale*. 2019;11:6990–7001. doi:10.1039/C8NR08720B
5. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science*. 2020;367. doi:10.1126/science.abb5060
6. Gervasi MG, Soler AJ, Gonzalez-Fernandez L, Alves MG, Oliveira PF, Martin-Hidalgo D. Extracellular vesicles, the road toward the improvement of ART outcomes. *Animals*. 2020;10:2171.
7. Harris EA, Stephens KK, Winuthayanon W. Extracellular vesicles and the oviduct function. *Int J Mol Sci*. 2020;21:8280. doi:10.3390/ijms21218280
8. Chan JC, Morgan CP, Adrian Leu N, et al. Reproductive tract extracellular vesicles are sufficient to transmit intergenerational stress and program neurodevelopment. *Nat Commun*. 2020;11:1499. doi:10.1038/s41467-020-15305-w
9. Marques de Menezes EG, Jang K, George AF, et al. Roan, seminal plasma-derived extracellular-vesicle fractions from HIV-infected men exhibit unique MicroRNA signatures and induce a proinflammatory response in cells isolated from the female reproductive tract. *J Virol*. 2020;94:e00525–20.
10. Nguyen HP, Simpson RJ, Salamonsen LA, Greening DW. Extracellular vesicles in the intrauterine environment: challenges and potential functions. *Biol Reprod*. 2016;95:109. doi:10.1095/biolreprod.116.143503
11. Yuan S, Wang Z, Peng H, et al. Oviductal motile cilia are essential for oocyte pickup but dispensable for sperm and embryo transport. *Proc Natl Acad Sci U S A*. 2021;118:45.

12. Qu P, Luo S, Du Y, et al. Extracellular vesicles and melatonin benefit embryonic develop by regulating reactive oxygen species and 5-methylcytosine. *J Pineal Res*. 2020;68:e12635. doi:10.1111/jpi.12635
13. Saadeldin IM, Kim SJ, Choi YB, Lee BC. Improvement of cloned embryos development by co-culturing with parthenotes: a possible role of exosomes/microvesicles for embryos paracrine communication. *Cell Reprogram*. 2014;16:223–234. doi:10.1089/cell.2014.0003
14. Saadeldin IM, Oh HJ, Lee BC. Embryonic-maternal cross-talk via exosomes: potential implications. *Stem Cells Cloning*. 2015;8:103–107. doi:10.2147/SCCAA.S84991
15. Gangadaran P, Hong CM, Ahn BC. An update on in vivo imaging of extracellular vesicles as drug delivery vehicles. *Front Pharmacol*. 2018;9:169. doi:10.3389/fphar.2018.00169
16. Liu C, Yao W, Yao J, et al. Endometrial extracellular vesicles from women with recurrent implantation failure attenuate the growth and invasion of embryos. *Fertil Steril*. 2020;114:416–425. doi:10.1016/j.fertnstert.2020.04.005
17. Banliat C, Le Bourhis D, Bernardi O, et al. Oviduct fluid extracellular vesicles change the phospholipid composition of bovine embryos developed in vitro. *Int J Mol Sci*. 2020;21:5326. doi:10.3390/ijms21155326
18. Bodega G, Alique M, Puebla L, Carracedo J, Ramirez RM. Microvesicles: ROS scavengers and ROS producers. *J Extracell Vesicles*. 2019;8:1626654. doi:10.1080/20013078.2019.1626654
19. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;30:255–289. doi:10.1146/annurev-cellbio-101512-122326
20. Takahashi Y, Nishikawa M, Shinotsuka H, et al. Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. *J Biotechnol*. 2013;165:77–84. doi:10.1016/j.jbiotec.2013.03.013
21. Lazaro-Ibanez E, Faruqu FN, Saleh AF, et al. Selection of fluorescent, bioluminescent, and radioactive tracers to accurately reflect extracellular vesicle biodistribution in vivo. *ACS Nano*. 2021;15:3212–3227. doi:10.1021/acsnano.0c09873
22. Qu P, Zhao Y, Wang R, et al. Extracellular vesicles derived from donor oviduct fluid improved birth rates after embryo transfer in mice. *Reprod Fertil Dev*. 2019;31:324–332. doi:10.1071/RD18203
23. Zhang J, Qu P, Zhou C, et al. MicroRNA-125b is a key epigenetic regulatory factor that promotes nuclear transfer reprogramming. *J Biol Chem*. 2017;292:15916–15926. doi:10.1074/jbc.M117.796771
24. Lopera-Vasquez R, Hamdi M, Maillo V, et al. Effect of bovine oviductal extracellular vesicles on embryo development and quality in vitro. *Reproduction*. 2017;153:461–470. doi:10.1530/REP-16-0384
25. Bodega G, Alique M, Bohorquez L, Ciordia S, Mena MC, Ramirez MR. The antioxidant machinery of young and senescent human umbilical vein endothelial cells and their microvesicles. *Oxid Med Cell Longev*. 2017;2017:7094781. doi:10.1155/2017/7094781
26. Burger D, Turner M, Munkonda MN, Touyz RM. Endothelial microparticle-derived reactive oxygen species: role in endothelial signaling and vascular function. *Oxid Med Cell Longev*. 2016;2016:5047954. doi:10.1155/2016/5047954
27. Qin B, Zhang Q, Hu XM, et al. How does temperature play a role in the storage of extracellular vesicles?*J. Cell Physiol*. 2020;235:7663–7680. doi:10.1002/jcp.29700
28. Park SJ, Jeon H, Yoo S-M, Lee M-S. The effect of storage temperature on the biological activity of extracellular vesicles for the complement system. *In Vitro Cell Dev Biol Anim*. 2018;54(6):423–429. doi:10.1007/s11626-018-0261-7
29. Gatien J, Mermillod P, Tsikis G, et al. Metabolomic profile of oviductal extracellular vesicles across the estrous cycle in cattle. *Int J Mol Sci*. 2019;20:6339.
30. Lane M, Gardner DK. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biol Reprod*. 2003;69:1109–1117. doi:10.1095/biolreprod.103.018093
31. Alminana C, Corbin E, Tsikis G, et al. Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. *Reproduction*. 2017;154:153–168. doi:10.1530/REP-17-0054
32. Liu J, Zhao X, Nie W, et al. Tumor cell-activated "Sustainable ROS Generator" with homogeneous intratumoral distribution property for improved anti-tumor therapy. *Theranostics*. 2021;11:379–396. doi:10.7150/thno.50028
33. Jamil M, Debbarh H, Aboulmaouahib S, et al. Reactive oxygen species in reproduction: harmful, essential or both? *Zygote*. 2020;28:255–269. doi:10.1017/S0967199420000179
34. Luo Z, Xu X, Sho T, et al. ROS-induced autophagy regulates porcine trophectoderm cell apoptosis, proliferation, and differentiation. *Am J Physiol Cell Physiol*. 2019;316:C198–C209. doi:10.1152/ajpcell.00256.2018
35. Huang Y, Xia Y, Tao Y, et al. Protective effects of resveratrol against the cardiac developmental toxicity of trichloroethylene in zebrafish embryos. *Toxicology*. 2021;452:152697. doi:10.1016/j.tox.2021.152697
36. Gao Y, Huang X, Lin H, et al. Adipose mesenchymal stem cell-derived antioxidative extracellular vesicles exhibit anti-oxidative stress and immunomodulatory effects under PM2.5 exposure. *Toxicology*. 2021;447:152627. doi:10.1016/j.tox.2020.152627
37. Heinrich LF, Andersen DK, Cleasby ME, Lawson C. Long-term high fat feeding of rats results in increased numbers of circulating microvesicles with pro-inflammatory effects on endothelial cells. *Br J Nutr*. 2015;113:1704–1711.
38. Mizushima N, Levine B. Autophagy in human diseases. *N Engl J Med*. 2020;383:1564–1576. doi:10.1056/NEJMra2022774
39. Levine B, Kroemer G. Biological functions of autophagy genes: a disease perspective. *Cell*. 2019;176:11–42. doi:10.1016/j.cell.2018.09.048
40. Shen M, Jiang Y, Guan Z, et al. Protective mechanism of FSH against oxidative damage in mouse ovarian granulosa cells by repressing autophagy. *Autophagy*. 2017;13:1364–1385. doi:10.1080/15548627.2017.1327941
41. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem Sci*. 2011;36:30–38. doi:10.1016/j.tibs.2010.07.007
42. Kuang Y, Zheng X, Zhang L, et al. Adipose-derived mesenchymal stem cells reduce autophagy in stroke mice by extracellular vesicle transfer of miR-25. *J Extracell Vesicles*. 2020;10:e12024. doi:10.1002/jev2.12024
43. Zheng J, Tan J, Miao YY, Zhang Q. Extracellular vesicles degradation pathway based autophagy lysosome pathway. *Am J Transl Res*. 2019;11:1170–1183.
44. Leidal AM, Huang HH, Marsh T, et al. The LC3-conjugation machinery specifies the loading of RNA-binding proteins into extracellular vesicles. *Nat Cell Biol*. 2020;22:187–199. doi:10.1038/s41556-019-0450-y
45. Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N. Autophagy is essential for preimplantation development of mouse embryos. *Science*. 2008;321:117–120. doi:10.1126/science.1154822

46. Qu P, Wang Y, Zhang C, Liu E. Insights into the roles of sperm in animal cloning. *Stem Cell Res Ther*. 2020;11:65. doi:10.1186/s13287-020-01599-6
47. Wu SF, Zhang H, Cairns BR. Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. *Genome Res*. 2011;21:578–589. doi:10.1101/gr.113167.110
48. Xu Q, Xiang Y, Wang Q, et al. SETD2 regulates the maternal epigenome, genomic imprinting and embryonic development. *Nat Genet*. 2019;51:844–856. doi:10.1038/s41588-019-0398-7
49. Zhang L, Li L, Xu J, Pan MH, Sun SC. HT-2 toxin exposure induces mitochondria dysfunction and DNA damage during mouse early embryo development. *Reprod Toxicol*. 2019;85:104–109. doi:10.1016/j.reprotox.2019.02.011
50. Bazopoulou D, Knoefler D, Zheng Y, et al. Developmental ROS individualizes organismal stress resistance and lifespan. *Nature*. 2019;576:301–305.