

Embryonic stem cell-derived small extracellular vesicles alleviate lead-induced cochlear spiral ganglion neurons injury by activating PI3 K/AKT signaling pathway

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Abstract

Objective: Embryonic stem cell (ESC)-derived small extracellular vesicles (sEVs) exhibit considerable potential as an innovative therapeutic approach in the fields of regenerative medicine and disease management. This in vitro study aimed to evaluate the protective efficacy of ESC-sEVs against lead acetate (PbAc)-induced damage in cochlear spiral ganglion neurons (SGNs).

Methods: Cochlear SGNs of neonatal rats were primarily cultured and administrated with (i) serum-free medium (control); (ii) 25 μM PbAc; (iii) 0.05 $\mu\text{g}/\mu\text{l}$ ESC-sEVs (ESC-sEVs); (iv) 25 μM PbAc + 0.05 $\mu\text{g}/\mu\text{l}$ ESC-sEVs (PbAc + ESC-sEVs). After treatment, cell viability of SGNs was assessed by CCK-8 assay. The changes in levels of reactive oxygen species, lipid peroxides, and apoptosis were detected by immunofluorescence and flow cytometry. The protective mechanisms of ESC-sEVs against lead-induced SGNs damage were elucidated by RNA sequencing and western blot analysis.

Results: The findings revealed that ESC-sEVs significantly increased the viability of SGNs subjected to PbAc exposure. Immunofluorescence and flow cytometry revealed that ESC-sEVs effectively attenuated oxidative stress, lipid peroxidation, and apoptotic processes in PbAc-exposed SGNs. Furthermore, RNA sequencing and western blot analysis demonstrated that ESC-sEVs activated the PI3 K/AKT signaling pathway, which plays a pivotal role in alleviating lead-induced neuronal injury.

Conclusion: In conclusion, this study provides the first evidence supporting the therapeutic potential of ESC-sEVs in addressing lead-induced ototoxicity.

Keywords

Lead acetate, spiral ganglion neurons, embryonic stem cell, small extracellular vesicles, PI3 K/AKT signaling pathway

Introduction

Heavy metal lead (Pb^{2+}) has consistently ranked among the top two substances on the ATSDR Substance Priority List for decades.¹ Owing to its pervasive environmental contamination, lead exposure poses significant public health challenges.² Lead poisoning has a broad spectrum of detrimental effects on the nervous system, including developmental neurotoxicity, which disrupts neuronal proliferation and migration, as well as cognitive and behavioral impairments, such as attention deficits and learning disabilities.³ Given the irreversible nature of these effects, lead-induced neurotoxicity remains a critical and unresolved issue in environmental medicine, with its ototoxic manifestations presenting particular diagnostic and therapeutic challenges. We previously reported that injury to cochlear spiral ganglion neurons (SGNs) is a primary manifestation of lead-induced ototoxicity.^{4–6} Although the precise mechanisms underlying lead-induced ototoxicity are not fully understood, lead exposure has been shown to disrupt neurotransmitter systems, induce oxidative stress, and trigger apoptosis. These pathophysiological cascades ultimately result in SGNs death and subsequent sensorineural hearing loss.^{7,8}

The management of lead exposure involves several strategies aimed at reducing the body's burden of lead and mitigating its toxic effects. Chelation therapy has been proposed as a potential intervention to counteract lead neurotoxicity; however, it can also result in the loss of essential metals and the redistribution of lead.⁹ Currently, there are no effective treatments available for lead-induced ototoxicity in preclinical and clinical practice. Therefore, the development of novel therapeutic strategies is urgently needed to address this unmet clinical need.

Small extracellular vesicles (sEVs) can facilitate intracellular communication by transferring bioactive molecules, including proteins, nucleic acids, and lipids. In recent years, sEVs derived from stem cells have shown considerable potential in modulating cellular responses and promoting tissue repair, making them promising therapeutic approaches for various diseases.^{10–12} Studies have reported that stem cells and their derived sEVs have significant protective effects against inner ear injury caused by noise, aminoglycosides, cisplatin, and other ototoxic agents.^{13–16} However, to date, no studies have investigated the capacity of embryonic stem cell-derived EVs (ESC-sEVs) to counteract lead-induced SGNs injury, nor have they elucidated the molecular mechanisms underlying this protection. This knowledge gap significantly impedes the development of targeted therapies for lead-induced ototoxicity. In this study, we investigated for the first time the protective effect of ESC-sEVs on lead acetate (PbAc)-induced injury in cochlear SGNs *in vitro*.

Materials and methods

Animals and ethics statement

A total of 360 Sprague–Dawley rats, aged 3–4 days, of mixed sex, and weighing approximately 6 grams, were purchased from Hunan SJA Laboratory Animal Company (Hunan, China). All the animal experiments were conducted in compliance with the 8th edition of the Guide for the Care and Use of Laboratory Animals,¹⁷ were approved by the Animal Ethics Committee of Xiangya Hospital, Central South University (approval number: 202402035), and were conducted in accordance with the ARRIVE 2.0 guidelines.¹⁸ We made efforts to minimize the number of animals used and to decrease their suffering. Appropriate anesthetics and analgesics were used to ensure animal welfare, and all procedures were conducted to minimize distress.

Isolation and identification of embryonic stem cell-small extracellular vesicles

The ES-E14TG2a ESCs (ATCC, USA) were used to prepare ESC-sEVs. For ESC-sEVs isolation, ESCs were cultured with sEVs-free serum. Then, the culture medium was collected and processed through differential centrifugation (500 g for 10 min, followed by 3000 g for 30 min, and then 10,000 g for 30 min at 4°C) to remove dead cells and debris. The supernatant was then filtered through 0.22 µm sterile filters (Millipore, USA) and ultracentrifuged at 100,000 g for 70 min at 4°C. The resulting pellets, containing the sEVs, were resuspended in PBS and ultracentrifuged again to wash and obtain high-

purity EVs. Different batches of ESC-sEVs, obtained from the ES-E14TG2a ESCs through this consistent culture and extraction process, were used. For each experiment, ESC-sEVs from the same batch were utilized to ensure consistency and reproducibility.

The nanoparticle tracking analysis (Particle Mtx, Germany) was performed to measure the diameter, size distribution, and particle concentration of the ESC-sEVs. In addition, the morphology of ESC-sEVs was observed using a transmission electron microscopy (TEM) (HITACHI, H600, JAPAN). The exosomal-positive marker TSG101, CD81 and the exosomal-negative marker CALNEXIN were detected by western blotting.

Primary culture and verification of spiral ganglion neurons

SGNs were isolated and cultured as previously described.⁴ Briefly, after anesthesia, post-natal day 3–4 Sprague–Dawley rat pups (n = 40 per experimental repeat) were sacrificed by rapid decapitation. The cochleae were microdissected from the temporal bones under stereomicroscopic guidance and maintained in ice-cold Hanks' balanced salt solution (Biosharp, CHINA). Through meticulous subdissection of the osseous spiral lamina, SGN clusters were mechanically separated from the organ of Corti using 25-gauge spinal needles and then enzymatically dissociated with 0.25% trypsin-EDTA (Gibco, USA) for 15 min at 37°C with gentle agitation. Cell suspensions were plated at 5×10^4 cells/cm² in poly-L-lysine-coated 24-well plates using DMEM/F-12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Procel, CHINA) and 1% penicillin-streptomycin. After 12 h adhesion, cultures underwent two-stage purification to eliminate proliferating fibroblasts. Four experimental groups were established in triplicate: Control: Serum-free medium; PbAc group: culture medium + 25 μ M PbAc (467863, Sigma-Aldrich, USA); ESC-sEV group: culture medium + 0.05 μ g/ μ L ESC-sEVs; PbAc + ESC-sEV group: combined PbAc and ESC-sEVs treatment. Following 36 h treatments at 37°C/5% CO₂, cells were fixed in 4% paraformaldehyde for 30 min at 4°C. Immunofluorescence verification utilized a mouse anti-neurofilament 200 kDa (NF200) (BioLegend, 846002, 1:200, USA) with a goat anti-mouse IgG-Alexa Fluor 594 secondary antibody (Invitrogen, A11005, 1:500, USA). Nuclei were counterstained with DAPI (Thermo Fisher, D1306, 1:5000, USA). Fluorescence images were acquired using a Leica confocal microscope (Leica, STELLARIS 8, Germany).

Cell counting kit (CCK-8) assay

Cell viability of the SGNs was measured by CCK-8 assay. Following treatment, the medium in the culture dishes was replaced with culture medium containing 10% CCK-8 solution and then incubated at 37°C for 2 h. The optical density of each well was measured at 450 nm using a microplate reader (BioTek, CYTATION 1, USA).

Immunofluorescence and flow cytometry

To detect the production of reactive oxygen species (ROS) and lipid peroxides, cells were stained with 1 μ mol/L dihydroethidium (DHE) (MedChemExpress, HY-D0079, 1:10000,

USA) or 1 $\mu\text{mol/L}$ Liperfluo (Dojindo Laboratory, L248, 1:1000, JAPAN) for 30 min at 37°C. A subset of these cells was subsequently immunostained with NF200 and imaged, while the rest were subjected to flow cytometry analysis. To detect apoptosis in SGNs, a portion of the cells was subjected to TUNEL staining in accordance with the manufacturer's protocol using a One Step TUNEL Apoptosis Assay Kit (Beyotime, C1086, CHINA), and the TUNEL signals were visualized using a confocal microscope. The remaining cells were subjected to Annexin V/PI double staining using an Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN, 40302ES, CHINA), followed by flow cytometry analysis (BD Biosciences, LSRFortessa, USA).

RNA sequencing and bioinformatic analysis

Total RNA from the control, PbAc, and PbAc + ESC-sEV groups were extracted from the cells via TRIzol reagent. For each sample, 1 μg of total RNA with an RNA quality number greater than 6 was used for sequencing with the Illumina NovaSeq 6000. The differentially expressed genes (DEGs) between the PbAc group and the control group, as well as between the PbAc + ESC-sEVs group and the PbAc group, were analyzed using the DESeq2 R package (version 1.46.0). DEGs with an adjusted p-value less than 0.05 and a log2 (fold-change) greater than 1 or less than -1 were considered significant. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs were conducted using the online tool DAVID (2021 Update).¹⁹

Western blot

The cells and ESC-sEVs were lysed in RIPA lysis buffer supplemented with a protease inhibitor cocktail (APExBIO, K1010, 1:100, USA) and a phosphatase inhibitor cocktail (APExBIO, K1015, 1:100, USA) for 10 min at 4°C. Following centrifugation, the total protein extracts were obtained by collecting the supernatant and mixed with protein loading buffer. Subsequently, 10 μg of total protein extract was separated on SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with TBST containing 5% bovine serum albumin for 2 h and then incubated with TSG101 polyclonal antibody (Proteintech, 282283, 1:1000, CHINA), CD81 monoclonal antibody (Proteintech, 66866, 1:1000, CHINA), CALNEXIN polyclonal antibody (Proteintech, 10427, 1:1000, CHINA), PI3 kinase p110 alpha monoclonal antibody (Proteintech, 670071, 1:1000, CHINA), AKT rabbit polyclonal antibody (Proteintech, 10176, 1:1000, CHINA), phospho-AKT rabbit monoclonal antibody (Proteintech, 66444, 1:1000, CHINA), mTOR rabbit polyclonal antibody (Cell Signaling Technology, 2972S, 1:1000, USA), p-mTOR rabbit polyclonal antibody (Cell Signaling Technology, 2974 T, 1:1000, USA), Bcl-2 rabbit polyclonal antibody (Proteintech, 26593, 1:1000, CHINA), BAX rabbit polyclonal antibody (Proteintech, 50599, 1:1000, CHINA), cleaved caspase-3 rabbit monoclonal antibody (Cell Signaling Technology, 9664 T, 1:1000, USA), or ACTIN mouse monoclonal antibody (Zen Bioscience, 200068, 1:10,000, CHINA) at 4°C overnight. Thereafter, the membranes were incubated with goat anti-rabbit IgG (H + L)-HRP (1:10,000, Abcam, USA) or goat anti-mouse IgG (H + L)-HRP (1:10,000,

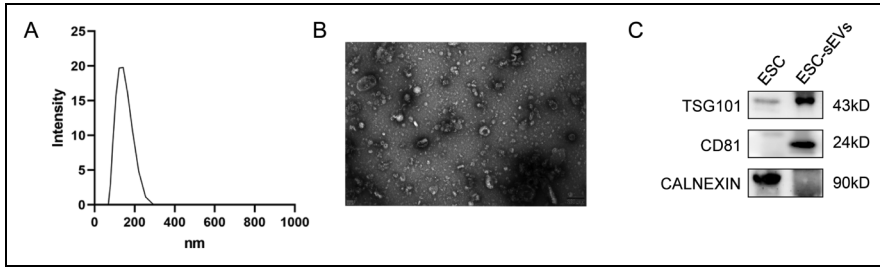


Figure 1. Characterization of ESC-sEVs. A, particle size distribution of ESC-sEVs. B, morphology of USC-Exos under transmission electron microscopy. Scale bar, 200 nm. C, western blots showing the expression of TSG101, CD81, and CALNEXIN in ESC and ESC-sEVs. sEV: small extracellular vesicle; ESC: embryonic stem cell.

Abcam, USA) for 1 h. Finally, the protein bands were visualized with ECL reagent using the Azure Biosystems C300 (Azure Biotech, C300, USA).

Statistical analysis

All the quantitative experimental procedures were independently repeated three times. Statistical analyses were conducted using GraphPad Prism 6 software. Differences were analyzed using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons. All statistical analysis results are presented as the mean $\pm$ SD, and a p -value < 0.05 was considered statistically significant.

Results

Characterization of embryonic stem cell-small extracellular vesicles

Malvern particle size analysis indicated that the size of these ESC-sEVs ranged from 70 to 160 nm (Figure 1A). TEM imaging confirmed their characteristic cup-shaped or spherical morphology, with an average diameter of approximately 100 nm (Figure 1B), which is consistent with the reported shape and size distribution of sEVs. Western blotting further validated the presence of EV-specific markers, including CD81 and TSG101, while confirming the absence of the negative marker CALNEXIN (Figure 1C). These findings suggested that the ESC-sEVs were successfully enriched, rendering them suitable for subsequent experimental applications.

Embryonic stem cell-small extracellular vesicles attenuate lead-induced cytomorphological alterations in spiral ganglion neurons

Primary cultures of SGNs displayed characteristic oval somata with extended bipolar neurites, which progressively arborized to form intricate intercellular networks

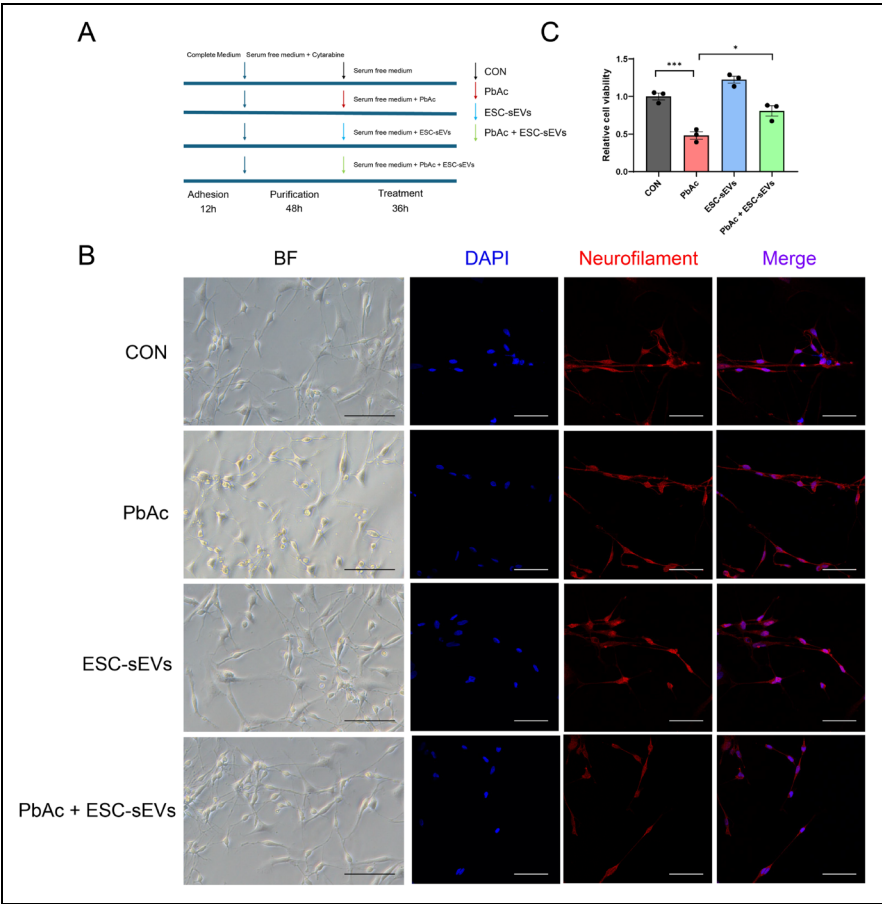


Figure 2. ESC-sEVs mitigated PbAc-induced SGNs injury. A, schematic diagram of the treatment procedures for different groups. B, morphological changes of SGNs in control, PbAc, ESC-sEVs, and PbAc + ESC-sEVs group. Magnification: 400×, scale bar: 50 μ m. C, Relative cell viabilities of different treatment groups. (CON: 1.00 ± 0.08 ; PbAc: 0.48 ± 0.08 ; ESC-sEVs: 1.23 ± 0.08 ; PbAc + ESC-sEVs: 0.81 ± 0.11) * $P < 0.05$; *** $P < 0.001$. All quantitative experimental procedures were independently replicated three times. sEV: small extracellular vesicle; ESC: embryonic stem cell; PbAc: lead acetate; SGN: spiral ganglion neuron.

(Figure 2B). Exposure to PbAc induced significant morphological deterioration, characterized by somatic rounding, neurite retraction and fragmentation. Quantitative analysis revealed a $48 \pm 8\%$ reduction in cellular viability in PbAc group ($p < 0.001$ vs control) (Figure 2C). Notably, treatment with ESC-sEVs preserved neuronal architecture, with SGNs maintaining near-normal soma morphology and neurite complexity. The protective efficacy was particularly evident in PbAc + ESC-sEVs group, which exhibited $81 \pm 12\%$ cellular viability, representing a 1.68-fold improvement over the PbAc-alone group ($p <$

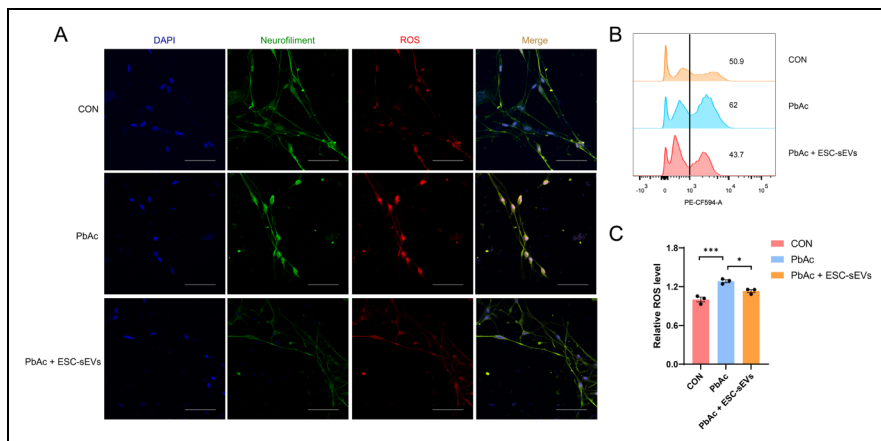


Figure 3. ESC-sEVs suppressed PbAc-induced oxidative stress in SGNs. A, fluorescence images showed ROS signals in control, PbAc, and PbAc + ESC-sEVs group. Magnification: 400×, scale bar: 50 μm. B, flow cytometry analysis of ROS signals in control, PbAc, and PbAc + ESC-sEVs group. C, statistical analysis of ROS levels among control, PbAc, and PbAc + ESC-sEVs group. (CON: 1.00 ± 0.06 ; PbAc: 1.28 ± 0.04 ; PbAc + ESC-sEVs: 1.13 ± 0.04) * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. All quantitative experimental procedures were independently replicated three times. sEV: small extracellular vesicle; ESC: embryonic stem cell; PbAc: lead acetate; SGN: spiral ganglion neuron; ROS: reactive oxygen species; ROS: reactive oxygen species.

0.05). These findings demonstrated that ESC-sEVs effectively mitigate lead-induced neurocytotoxicity and preserve neuronal cytoarchitecture and membrane integrity. The observed neuroprotective effects suggested that ESC-sEVs may have therapeutic potential against PbAc-induced ototoxicity.

Embryonic stem cell-small extracellular vesicles attenuated lead acetate-induced oxidative injury in spiral ganglion neurons

Consistent with our previous findings,⁴ PbAc exposure resulted in significant oxidative injury in SGNs. After PbAc exposure, DHE fluorescence signal was significantly increased in SGNs (Figure 3A). The quantitative analysis of DHE fluorescence intensity revealed a 1.28-fold increase in ROS production in SGNs PbAc-exposed SGNs compared with the control ($p < 0.001$) (Figure 3B,C). This oxidative stress response was markedly attenuated by ESC-sEVs, with 12% lower ROS levels in the PbAc + ESC-sEVs group than in the PbAc group ($p < 0.05$). These findings collectively suggest that ESC-sEVs exert protective effects against lead-induced ototoxicity via ROS scavenging mechanisms.

Embryonic stem cell-small extracellular vesicles attenuated lead acetate-induced lipid peroxidation injury in spiral ganglion neurons

Lipid peroxidation is a major contributor to cellular damage under oxidative stress conditions. We used Liperfluo fluorescence probe to detect PbAc-induced lipid peroxidation.

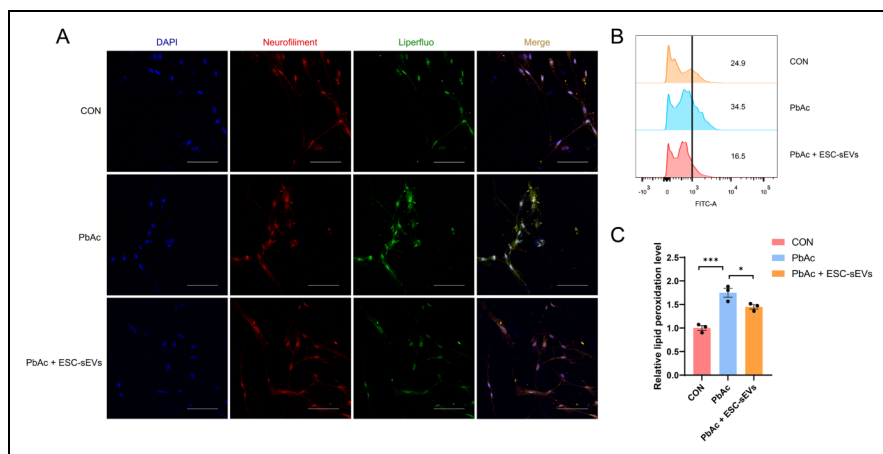


Figure 4. ESC-sEVs alleviated PbAc-induced lipid peroxides production in SGNs. A, fluorescence images showed Liperfluo signals in control, PbAc, and PbAc + ESC-sEVs group. Magnification: 400×, scale bar: 50 μ m. B, flow cytometry analysis of Liperfluo signals in control, PbAc, and PbAc + ESC-sEVs group. C, statistical analysis of lipid peroxidation levels among control, PbAc, and PbAc + ESC-sEVs group. (CON: 1.00 ± 0.09 ; PbAc: 1.75 ± 0.16 ; PbAc + ESC-sEVs: 1.45 ± 0.08) *P < 0.05; **P < 0.01, ***P < 0.001. All quantitative experimental procedures were independently replicated three times. sEV: small extracellular vesicle; ESC: embryonic stem cell; PbAc: lead acetate; SGN: spiral ganglion neuron.

As is shown in Figure 4A, the level of lipid peroxidation was significantly elevated in PbAc group. Flow cytometry quantification revealed a 1.75-fold increase in lipid peroxidation in PbAc-exposed SGNs compared with the control ($p < 0.001$). ESC-sEVs treatment attenuated this response, reducing lipid peroxidation levels to 145% of the baseline level ($p < 0.05$ vs. 175% in PbAc group) (Figure 4B,C). These findings indicate that ESC-sEVs mitigate lead-induced ototoxicity through neutralization of lipid peroxidation cascades.

Embryonic stem cell-small extracellular vesicles attenuated lead acetate-induced apoptosis injury in spiral ganglion neurons

Apoptotic cascades in SGNs were detected using the TUNEL assay. SGNs exposed to PbAc presented a greater density of apoptotic nuclei than the controls. Treatment with ESC-sEVs significantly reduced apoptotic signals induced by PbAc in SGNs. Flow cytometric analyses corroborated these findings, showing that ESC-sEVs decreased the percentage of late apoptotic cells from $19 \pm 1\%$ to $14 \pm 2\%$ (Figure 5B,C). These data mechanistically demonstrated that ESC-sEVs mitigate lead-induced ototoxicity by modulating the apoptosis pathway.

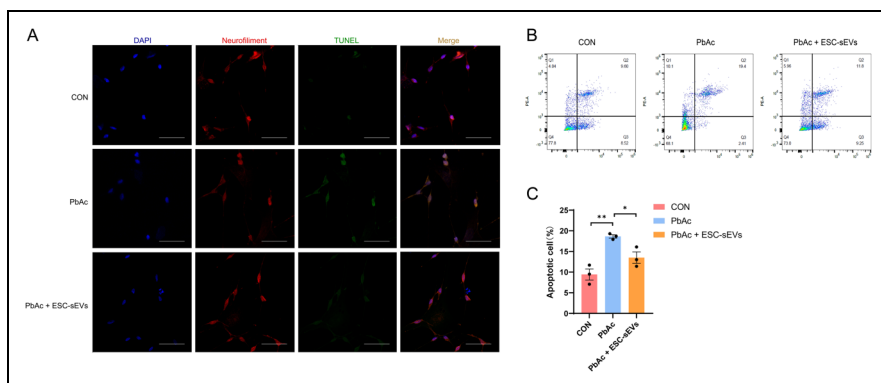


Figure 5. ESC-sEVs inhibited PbAc-induced apoptosis in SGNs. A, fluorescence images showed TUNEL signals in control, PbAc, and PbAc + ESC-sEVs group. Magnification: 400 $\times$, scale bar: 50 μ m. B, flow cytometry analysis of Annexin V/PI signals in control, PbAc, and PbAc + ESC-sEVs group. C, statistical analysis of apoptotic levels among control, PbAc, and PbAc + ESC-sEVs group. (CON: 9.43 ± 2.32 ; PbAc: 18.63 ± 0.7 ; PbAc + ESC-sEVs: 13.5 ± 2.39) * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. All quantitative experimental procedures were independently replicated three times. sEV: small extracellular vesicle; ESC: embryonic stem cell; PbAc: lead acetate; SGN: spiral ganglion neuron.

RNA sequencing revealed gene expression changes and pathway enrichment through pairwise comparisons

RNA sequencing of SGNs exposed to PbAc, with or without ESC-sEVs intervention, unveiled distinct transcriptomic remodeling. Principal component analysis revealed three-cluster segregation among the control, PbAc, and PbAc + ESC-sEVs group (Figure 6A). A whole-transcriptome heatmap confirmed that the PbAc-induced decrease in expression was partially reversed by ESC-sEVs (Figure 6B). A total of 2574 genes were found to be differentially expressed between the PbAc group and the control group. Among the DEGs, there were 1639 genes presented significantly increased expression in the PbAc group, whereas 935 genes exhibited significantly decreased expression (Figure 6C). Furthermore, compared with those in PbAc group, there were 526 DEGs were identified following ESC-sEVs intervention, among which 270 genes were upregulated and 256 genes were downregulated (Figure 6D).

To investigate the protective mechanisms of ESC-sEVs in PbAc-induced SGNs injury, the DEGs that exhibited opposing trends between the PbAc group and the control group, as well as between the PbAc + ESC-sEVs group and the PbAc group, were subjected to KEGG pathway enrichment analyses. Notably, nine pathways were significantly enriched in both the downregulated DEGs in the PbAc vs. control group (Figure 6E) and the upregulated DEGs in the PbAc + ESC-sEVs vs. PbAc group (Figure 6F). Among these significantly enriched pathways, the PI3 K/AKT signaling pathway has emerged as a critical mediator of cell survival in response to external stimuli.

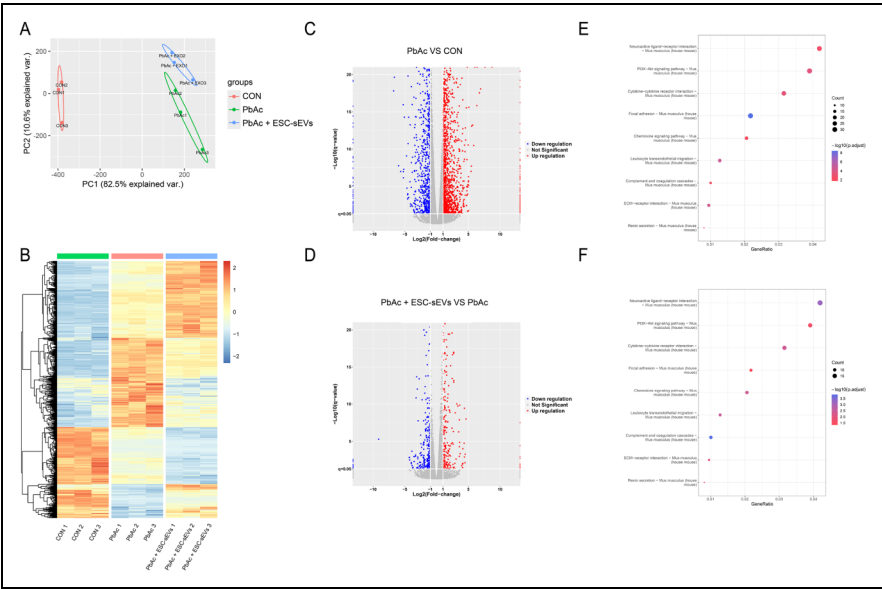


Figure 6. RNA sequencing for control, PbAc, and PbAc + ESC-sEVs group of SGNs. A, PCA analysis of RNA sequencing data. B, the heatmap showed the hierarchical clustering analysis of DEGs compared across the control, PbAc, and PbAc + ESC-sEVs groups. C, volcano plot showed the comparative expression of genes in PbAc and control group. D, volcano plot showed the comparative expression of genes in PbAc + ESC-sEVs and PbAc group. E, KEGG pathway enrichment analysis of DEGs in PbAc vs control group. F, KEGG pathway enrichment analysis of DEGs in PbAc + ESC-sEVs vs PbAc group. sEV: small extracellular vesicle; ESC: embryonic stem cell; PbAc: lead acetate; SGN: spiral ganglion neuron; PCA: principal component analysis; DEG: differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Embryonic stem cell-small extracellular vesicles mitigate lead-induced spiral ganglion neurons injury by activating the PI3 K/AKT signaling pathway

Western blot analyses were conducted to further assess the effects of ESC-sEVs on the PI3 K/AKT signaling pathway in SGNs exposed to PbAc (Figure 7A). Compared with the control group, the expression of PI3 K, the ratio of p-AKT/AKT, and the ratio of p-mTOR/mTOR were significantly decreased in PbAc group, while the ratio of BAX/Bcl-2 and cleaved caspase-3 were significantly increased (Figure 7B–E). Following ESC-sEVs treatment, the levels of PI3 K, p-AKT/AKT and p-mTOR/mTOR were significantly greater than those in the PbAc group (Figure 7A–C). Additionally, ESC-sEVs treatment suppressed the levels of BAX/Bcl-2 and cleaved caspase-3 (Figure 7A, D, E). These results indicated that ESC-sEVs could rescue lead-induced apoptosis in SGNs by activating the PI3 K/AKT signaling pathway.

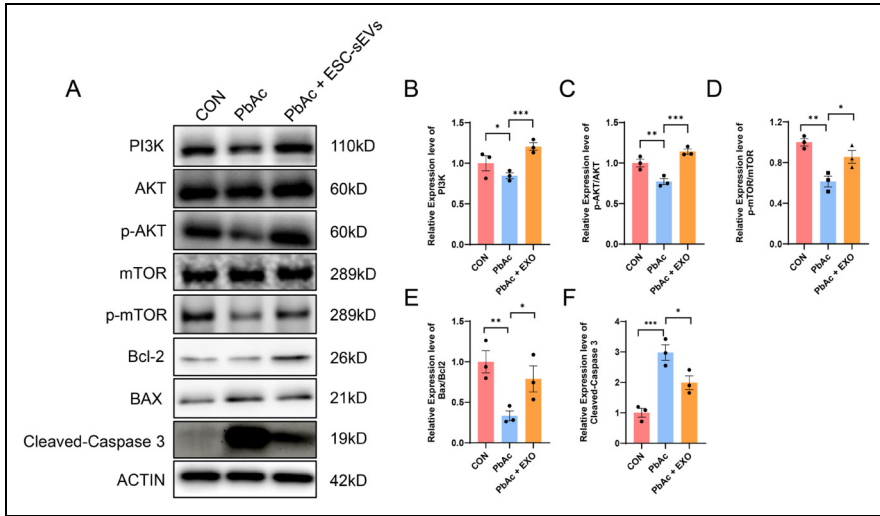


Figure 7. ESC-sEVs inhibited PbAc-induced SGNs damage by activating PI3 K/AKT signaling pathway. A, representative western blot bands showed the expression of PI3 K, AKT, p-AKT, mTOR, p-mTOR, Bcl-2, BAX, Cleaved caspase-3, and ACTIN in the control, PbAc, and PbAc + ESC-sEVs groups. B–F, statistical analysis showed the relative expression levels of PI3 K (CON: 1 ± 0.16 ; PbAc: 1.30 ± 0.07 ; PbAc + ESC-sEVs: 1.06 ± 0.04), p-AKT/AKT (CON: 1 ± 0.03 ; PbAc: 1.51 ± 0.08 ; PbAc + ESC-sEVs: 1.23 ± 0.11), p-mTOR/mTOR (CON: 1 ± 0.06 ; PbAc: 0.61 ± 0.08 ; PbAc + ESC-sEVs: 0.86 ± 0.09), BAX/Bcl-2 (CON: 1 ± 0.31 ; PbAc: 1.55 ± 0.19 ; PbAc + ESC-sEVs: 1.32 ± 0.14), and Cleaved caspase-3 (CON: 1 ± 0.26 ; PbAc: 3.03 ± 0.37 ; PbAc + ESC-sEVs: 2.02 ± 0.33). ns, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All quantitative experimental procedures were independently replicated three times. sEV: small extracellular vesicle; ESC: embryonic stem cell; PbAc: lead acetate; SGN: spiral ganglion neuron.

Discussion

Exposure to heavy metal lead has been associated with a series of severe health issues. The toxic effects of lead and its potential mechanisms have been extensively documented. A growing body of evidence indicates a significant correlation between lead exposure and hearing impairment.^{20–22} In the cochlea, it has been demonstrated that SGNs are the primary targets of lead-induced ototoxicity.⁵ Animal studies have further demonstrated that lead exposure predominantly results in delayed-onset injury to cochlear SGNs.⁶ Moreover, lead exposure has been shown to potentiate the effects of noise-induced and age-related hearing loss, as these physical and chemical hazards often coexist in certain occupational environments.^{20,23} In this study, SGNs were primarily cultured and treated with PbAc. Consistent with our previous findings,⁴ ROS, lipid peroxides, and apoptosis were markedly increased, and the levels of BAX/Bcl-2, and cleaved caspase-3 were correspondingly elevated in PbAc-exposed SGNs. These findings support the notion that oxidative stress and apoptosis are the primary causes of lead-induced injury in SGNs.

Currently, there are no effective therapeutic interventions available for lead-induced ototoxicity. Exosomes are nanoscale extracellular vesicles with diameters ranging from 30 to 150 nanometers. They are released from multivesicular bodies after fusing with the cell membrane and carry active components such as proteins, miRNAs, mRNAs, and lipids, which facilitate cell-free therapy by regulating gene expression, remodeling the microenvironment, and promoting intercellular communication. Numerous studies have highlighted the therapeutic potential of exosomes in the inner ear.^{24,25} Breglio et al. reported that exosomes carrying heat shock protein 70 can protect auditory hair cells from aminoglycoside-induced injury by binding to Toll-like receptor 4 on the hair cells.²⁶ Chen et al. revealed that mesenchymal stem cell (MSC)-derived sEVs, which are enriched with a variety of neurotrophic proteins, can promote the development of SGNs and protect against ouabain-induced SGNs injury.²⁷ Furthermore, exosomes have been utilized as nanocarriers for targeted drug delivery to the inner ear. Exosomes derived from BDNF-overexpressing MSCs can serve as a delivery carriers for BDNF, thereby providing protection against noise-induced hearing loss.²⁸ ESC-sEVs have also demonstrated significant potential in modulating cellular responses and promoting tissue repair.^{29–31} However, to date, no studies have investigated the capacity of ESC-sEVs to counteract lead-induced SGNs injury. In this study, we found that ESC-sEVs significantly attenuated ROS and lipid peroxide production and reduced apoptosis in SGNs. It indicated that ESC-sEVs may serve as a potential therapeutic agent to mitigate lead-induced injury in SGNs.

The PI3 K/AKT signaling pathway exerts its protective effects against oxidative stress through multiple downstream pathways, with mTOR, BAD, and Nrf2 collectively contributing to the regulation of antioxidant gene expression, inhibition of apoptosis, and enhancement of cell survival under oxidative stress conditions.³² Various studies have demonstrated that modulating of this pathway through either specific activators or natural compounds, can suppress ROS production, preserve mitochondrial function, and inhibit apoptosis.^{33–35} Liu et al. reported that puerarin can protect against lead-induced hepatotoxicity and nephrotoxicity by modulating PI3 K/AKT signaling pathway.^{36,37} In the inner ear, it has been demonstrated that the PI3 K/AKT signaling activation can protect against hearing loss induced by various ototoxic agents.^{38,39} In alignment with these findings, our RNA sequencing data and western blot analysis revealed that PI3 K/AKT signaling pathway was significantly downregulated in SGNs exposed to PbAC, whereas it was markedly activated in the PbAc + ESC-sEVs group. These results suggest that ESC-sEVs may confer protection against lead-induced SGNs injury by activating PI3 K/AKT signaling pathway.

In addition to the PI3 K/AKT pathway, which we have found to be significantly activated by ESC-sEVs, other signaling pathways enriched in our RNA sequencing data may also play a role in mediating the neuroprotective effects. For instance, pathways involved in immune responses, such as cytokine–cytokine receptor interactions, chemokine signaling, leukocyte transendothelial migration, and complement and coagulation cascades, may modulate inflammatory processes and promote tissue repair. Additionally, pathways related to cell adhesion and migration, including focal adhesion and ECM–receptor interactions, are crucial for maintaining tissue integrity and facilitating repair mechanisms.

Neuroactive ligand–receptor interactions and renin secretion pathways may further contribute to neuronal function and homeostasis. Further experiments are needed to validate the roles of these pathways by which ESC-sEVs exert their neuroprotective effects.

One limitation of our study is that we did not perform a sample size calculation prior to the study. This decision was based on preliminary data and feasibility considerations. However, we recognize that the limited number of samples may affect the statistical significance of the results. Future studies should consider performing a sample size calculation to ensure adequate power and more reliable statistical outcomes.⁴⁰ In addition, further research is needed to validate the protective effects of ESC-sEVs against lead-induced ototoxicity *in vivo*. Moreover, the precise mechanisms by which ESC-sEVs exert their protective effects, as well as their regulatory roles in the PI3 K/AKT signaling pathway, need further elucidation.

In summary, ESC-sEVs effectively alleviated the oxidative and apoptotic injury induced by PbAc in primary cultured SGNs through activation of the PI3 K/AKT signaling pathway. This finding highlights the potential of ESC-sEVs as a novel therapeutic strategy for the treatment of lead-induced SGNs injury.

Conclusion

This study demonstrated that ESC-sEVs effectively mitigate lead-induced damage in cochlear SGNs by activating the PI3 K/AKT signaling pathway. These findings highlight the therapeutic potential of ESC-sEVs for lead-induced ototoxicity and warrant further investigation.

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Ethical considerations

The experimental protocol was established, according to the ethical guidelines and was approved by the Animal Ethics Committee of Xiangya Hospital, Central South University (approval number: 202402035).

Author contributions

LW, YB, and JL did conceptualization, data curation, formal analysis, investigation, methodology, resources, visualization, and writing—original draft. LL, YH, and GL did investigation and visualization. DL, ZH, JS, and HW did validation and writing—review and editing. HL and XW did conceptualization, funding acquisition, project administration, supervision, and writing—review and editing.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability statement

Data will be made available on request.

Supplemental material

Supplemental material for this article is available online.

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