Bone mesenchymal stem cell-derived exosomes prevent hyperoxia-induced apoptosis of primary type II alveolar epithelial cells *in vitro*

Wei Yang[1*], Chao Huang[2], Wenjian Wang[3], Baozhu Zhang[4], Yunbin Chen[5], Xinlin Xie[1]

[1] Department of Pediatrics, The Second Affiliated Hospital of Shenzhen University (The People's Hospital of Baoan Shenzhen), Shenzhen, China, 518101.
[2] Department of Traditional Chinese Medicine, The Second Affiliated Hospital of Shenzhen University (The People's Hospital of Baoan Shenzhen), Shenzhen, China, 518101.
[3] Department of Respiratory Medicine, Shenzhen Children's Hospital, Shenzhen, China, 518026.
[4] Department of Oncology, The Second Affiliated Hospital of Shenzhen University (The People's Hospital of Baoan Shenzhen), Shenzhen, China, 518101.
[5] Department of Pediatrics, Guangdong Women's and Children's Hospital, Guangzhou, China, 510630.

Corresponding Author:
Wei Yang[1]
No.118.Longjing Road,2nd Baoan District, Shenzhen, 518101, China
Email address: robertyung@163.com

Abstract

Background: The presence of alveolar epithelial type II cells (AECIIs) is one of the most important causes of bronchopulmonary dysplasia (BPD). Exosomes from bone mesenchymal stem cells (BMSCs) can reduce hyperoxia-induced damage and provide better results in terms of alveolar and pulmonary vascularization parameters than BMSCs. Currently, intervention studies using BMSC-derived exosomes on the signaling pathways regulating proliferation and apoptosis of alveolar epithelial cells under the condition of BPD have not been reported. This study investigated the effects of rat BMSC-derived exosomes on the proliferation and apoptosis of hyperoxia-induced primary AECIIs *in vitro*.

Methods: The isolated AECIIs were grouped as follows: normal control (21% oxygen), hyperoxia (85% oxygen), hyperoxia+exosome (20 μg/mL), hyperoxia+exosome+LY294002 (PI3K/Akt inhibitor, 20 μM), and hyperoxia+exosome+rapamycin (mTOR inhibitor, 5 nM). We used the PI3K/Akt inhibitor LY294002 and the mTOR inhibitor rapamycin to determine the

70   regenerative capabilities oftype II alveolar epithelial cells (AECIIs) underlie recovery after

71   alveolar epithelial injury (Hou et al. 2015; Wu et al. 2018). Thus, aberrant proliferation and

72   differentiation of AECIIs in damaged lung tissues affect normal repair and recovery of the

73   alveolar structure and function (Wu et al. 2018; Yang et al. 2014).

74   Exosomes are the smallest identified extracellular vesicles that contain proteins, enzymes, or

75   growth factors, with diameters ranging between 40 and 150 nm. They can be released by several

76   cell types *in vitro* and have been detected in biological fluids *in vivo* (Braun et al. 2018; Lesage

77   & Thébaud 2018). Exosomes mediate intercellular communication and have potential

78   applications in clinical therapy (Collins 2020; Gortner et al. 2012; Li et al. 2020b). Of note,

79   treatment with exosomes has shown significant reductions in oxidative damage and apoptosis of

80   AECIIs (Braun et al. 2018).

81   Given the known role of PI3K/Akt/mTOR/Ki67 signaling in regulating proliferation and

82   apoptosis (Feng et al. 2019; Rahmani et al. 2020; Samakova et al. 2019; Zhang et al. 2020a;

83   Zhang et al. 2021; Zhang et al. 2020b; Zhang et al. 2020c), in this study, we investigated its role

84   in exosome-mediated regulation of AECII proliferation and apoptosis during hyperoxia-induced

85   BPD using hyperoxia rat models. We examined the effect of bone mesenchymal stem cell

86   (BMSC)-derived exosomes on the proliferation and apoptosis of hyperoxia-induced primary

87   AECIIs using a cell viability assay, annexin V-FITC staining, and western blot analysis.

88   Furthermore, we used PI3K/Akt and mTOR inhibitors to confirm the protective effects of

89   exosomes on AECIIs under hyperoxia conditions. Our findings provide preliminary insights into

90   the underlying mechanism of BMSC-derived exosomes on hyperoxia-induced AECII

91   proliferation. The knowledge gained from this study could have potentialclinical therapeutic

92   applicationsin the future.

93

94   ## Materials & Methods

95   **BMSC isolation and culture**

96   Animal experiments were performed with approvals from the Laboratory Animal Welfare Ethics

97   Committee of the Medicine Center at Shenzhen Peking University and Hong Kong University of

98   Science and Technology, China [approval number: 2021-147]. For the experiments, sixteen 6-

99   week-old Sprague Dawley rats weighing about 200 g (Experimental Animal Center of Southern

100 Medical University, Guangzhou, China) were housed in steel cages at18–22°C under 50–60%

101 relative humidity. Astandard pellet diet and water were provided *ad libitum*. The rats

102 weresacrificed by cervical dislocation after 1 week, and the femur and tibia were collected under

103 aseptic conditions. The bone marrow cavity was washed with Dulbecco's modified Eagle

104 medium (DMEM; Gibco, Lifetech, China), filtered,and centrifuged at 1,000 rpm for 5 min. Next,

105   the BMSCs were resuspended in DMEM containing 10% fetal bovine serum (FBS; Gibco),

106   seeded (1×10^6/mL) in 10-cm culture plates, and cultured for 3 days before refreshing half of the

107   medium with freshculture medium. At approximately 80–90% confluency (after ~10 days of cell

108   culture), the BMSCs were trypsinized (0.25% trypsin, purchased from Gibco). BMSCs from

109   passages 4 to 8 were used for subsequent experiments. The osteogenic and adipogenic potentials

110   of the stem cells were determined by Alizarin red staining and oil red staining, respectively, as

111   described previously (Futrega et al. 2018). The same differentiation medium and staining

112   solution were used for skin fibroblasts as the negative control group.

113

114   **Isolation of AECIIs from pregnant rats and culture grouping**

115   The AECIIs were isolated from six 4-week-old pregnant Sprague Dawley rats weighing

116   approximately 80–100 g (Experimental Animal Center of Southern Medical University,

117   Guangzhou, China). Primary AECIIs were isolated as previously described (Gonzalez & Dobbs

118   2013), with modifications. Briefly, pregnant rats at 20 days of gestation were first

119   anesthetizedintraperitoneally with 3% pentobarbital sodium (50 mg/kg; Shanghai Sangong

120   Bioengineering, China) and then subjected to uterine-incision delivery to induce premature labor.

121   Fetal lungs were removed from the thoracic cavity, rinsed with precooled phosphate-buffered

122   saline (PBS), diced into small pieces, and trypsinized(0.25% trypsin) for 25 min at 37℃. DMEM

123   containing 10% FBS was added to the cell suspension to stop the digestion, and then the cells

124   were filtered and centrifuged at 1,000 rpm for 5 min. The cell pellets obtained were incubated

125   with collagenase IV (10 μg/mL) for 15 min at 37°C. DMEM containing 10% FBS wasthen added

126   to stop the reaction. Next, the cell suspension was centrifuged, resuspended in DMEM, and then

127   inoculatedin a 10-cm culture plate. After the second passage, the primary AECIIs were incubated

128   with the AECII-specific surface marker surfactant protein C (SP-C) antibody followed by the

129   corresponding FITC-conjugated secondary antibody for detection by confocal microscopy

130   (Leica). We also detected lamellar bodies in ACEIIs using a JEOL 1400 transmission electron

131   microscope. The isolated AECIIs were divided into five culture conditions: normal control (NC;

132   21% oxygen), hyperoxia (85% oxygen), hyperoxia+exosome (20 μg/mL),

133   hyperoxia+exosome+LY294002 (a PI3K/Akt inhibitor, Sigma, Shanghai, China; 20 μM), and

134   hyperoxia+exosome+rapamycin (an mTOR inhibitor, Sigma, Shanghai, China; 5 nM).

135

136   **Isolation and characterization of BMSC-derived exosomes**

137   BMSC-derived exosomes cultured at 37°C under 5% CO_2 for 12 hwere harvested from FBS-free

138   culture medium using Total Exosome Isolation Reagent (Life Technology, Shanghai, China),

139   according to the manufacturer's instructions. The isolated BMSC-derived exosomes were then

140   washed three times with PBS. As described previously (Zhang & Chan 2010), the BMSC-

141   derived exosomes were deposited on Formvar/carbon-coated electron microscopy grids and

142   allowed to air dry. Subsequently, the BMSC-derived exosomes were fixed with 2%

143   glutaraldehyde, counterstained with 4% uranyl acetate, and then scanned using a JEOL 1400

144   transmission electron microscope (JEOL USA). The BMSC-derived exosomes were assessed by

145   detection of two well-characterized exosome surface protein markers, CD9 and CD63, through

146   nanoparticle tracking analysis with a ZetaView® Nanoparticle Tracking Analyzer (Particle

147   Metrix, Meerbusch, Germany) and western blot analysis.

148

149   **Cell counting kit-8 (CCK8) cell viability assay**

150   AECIIs (5×10^3 cells/well) were seeded in 96-well plates and cultured in DMEM containing

151   10% FBS for 6 h under 85% oxygen before exosomes and/or LY294002 or rapamycin inhibitor

152   were added. After culturing for 24, 48, and 72 h, respectively, the AECII cell viability was

153   assessed at 490 nm by the CCK8 assay (Beyotime Biotech, Shanghai, China), according to the

154   manufacturer's instructions. Briefly, AECII cultures were incubated with 0.5 mg/mL CCK8 for

155   20 min before measurements were taken.

156

157   **Annexin V-FITC apoptosis assay**

158   AECII apoptosis was assessed using an annexin V-FITC apoptosis detection kit (Beyotime

159   Biotech). After treatment with exosomes and/or LY294002 or rapamycin inhibitor for 24 h,

160   AECIIs were spun down and incubated for 15 min at room temperature in the dark. Next, the cell

161   pellets were resuspended in 500 μL of binding buffer containing 5 μL of annexin V-FITC and 5

162   μL of propidium iodide. The AECIIs were prepared using a flow cytometry assay kit (Abcam,

163   Cambridge, UK) and were then counted using a BD Accuri C6 Plus flow cytometer (BD

164   Biosciences).

165

166   **Western blotting**

167   Total protein was extracted from AECIIs treated with BMSC-derived exosomes and/or inhibitors

168   using radioimmunoprecipitation assay lysis buffer (Beyotime). The protein concentration was

169   determined using a bicinchoninic acid kit (Beyotime). Proteins were separated by gel

170   electrophoresis, and the bands were transferred onto polyvinylidene difluoride membranes

171   (Millipore, MA, USA). The membranes were first incubated with mouse anti-human AKT, p-

172   AKT, p-mTOR, mTOR, Bax, Bcl-2, or Ki67 primary antibodies (Santa Cruz, USA) at 4°C

173   overnight, followed by the corresponding anti-rabbit and anti-mouse secondary antibodies

174   (BOSTER Technology, Wuhan, China) at room temperature for 1 h. The bands were visualized

175   using the enhanced chemiluminescence substrate and were then analyzed using ImageJ software

176   (NIH, USA). GAPDH (Sanjian Biology, Tianjin, China) was used as the loading control.

177

178 **Statistical analysis**

179 Statistical analysis was performed using SPSS v20 software. Data from three independent

180 experiments were expressed as the mean ± standard deviation (SD). Comparison among groups

181 was performed by one-way analysis of variance, while the two-tailed Student's t-test was used to

182 compare groups. Values of *P*<0.05 were considered statistically significant.

183

184 ## Results

185 **Morphology and stem cell characteristics of BMSCs**

186 Unlike the BMSCs seeded at day1 (Fig. 1A), the cells appeared as fibroblast-like mononuclear

187 cells and underwent logarithmic growth in the second passage (Fig. 1B). The adipogenic and

188 osteogenic potential of the BMSCs were confirmed by oil red staining (Fig. 1C, D) and Alizarin

189 red staining (Fig. 1E, F), respectively, indicating successful isolation of rat BMSCs. Fibroblasts

190 were not positively stained by either oil red or Alizarin red (Fig. 1D, F).

191

192 **Characterization of BMSC-derived exosomes**

193 From transmission electron microscopy,we observed particles with diameters of 40–100 nm,

194 which is characteristic of exosomes (Fig. 2A). Vesicles with an average particle diameter size of

195 100–200 nm and a maximum diameter of 600 nm were detected from nanoparticle tracking

196 analysis (Fig. 2B). Western blot analysis also confirmed successful isolation of BMSC-derived

197 exosomes (Fig. 2C).

198

199 **Morphology and characterization of ACEIIs**

200 Typical lamellar bodies were detected by transmission electron microscopy (Fig. 2D). AECIIs

201 formed colonies and exhibited a cuboidal cobblestone morphology under a light microscope

202 (Fig. 3A). Detection of the commonly expressed AECII marker SP-C by immunofluorescence

203 also confirmed successful AECII isolation (Fig. 3B, C, D).

204

205 **BMSC-derived exosomes protect AECIIs from hyperoxia-induced apoptosis**

206 Next, we determined the effect of exosomes on AECII cell viability and apoptosis under

207 hyperoxia. The CCK8 assay showed no significant changes in cell viability for all groups at 24h,

208 but the cell viability was significantly decreased in the hyperoxia group at 48h (t=6.3246,

209 *P*=0.0032) and 72 h (t=16.5132, *P*=0.0001) compared with the normal control group (Fig. 3E).

210 Of note, hyperoxia-mediated growth inhibition was significantly reduced in the

211 hyperoxia+exosome group at 48h (t=7.8558, *P*=0.0014) and 72h (t=10.4151, *P*=0.0005),

212   compared with the hyperoxia group (Fig. 3E). The hyperoxia+exosome+LY294002 and
213   hyperoxia+exosome+rapamycin groups showed significantly lower cell viabilities, compared
214   with the hyperoxia+exosome group at 48h (Fig. 3E; hyperoxia+exosome+LY294002
215   vs.hyperoxia+exosome, t=8.8822,P=0.0009; hyperoxia+exosome+rapamycin
216   vs.hyperoxia+exosome, t=9.8590,P=0.0006) and 72 h (Fig. 3E, hyperoxia+exosome+LY294002
217   vs.hyperoxia+exosome, t=11.4614,P=0.0003; hyperoxia+exosome+rapamycin
218   vs.hyperoxia+exosome, t=9.3915, P=0.0007).
219   There were significant differences of early and late apoptosis or necrosis of AECIIs between the
220   normal control group (Fig. 4A) and the hyperoxia group (Fig. 4B, t=27.3758, P=0.0000). In
221   addition, a marked decrease in apoptotic and necrotic AECIIs was observed for the
222   hyperoxia+exosome group (Fig. 4C, t=22.8889, P=0.0000); however, the effects were reversed
223   for the hyperoxia+exosome+LY294002 group (Fig. 4D, t=15.6880, P=0.0001) and the
224   hyperoxia+exosome+rapamycin group (Fig. 4E, t=27.0877, P=0.0000).
225
226   **Exosomes affect the levels of apoptosis-related proteins and the phosphorylation of key**
227   **factors of the PI3K/Akt/mTOR signaling pathway**
228   The effect of BMSC-derived exosomes on hyperoxia-induced apoptosis of AECIIs was assessed
229   by western blot analysis (Fig. 5A, B). The Bax/Bcl-2level was significantly increased, while the
230   Ki67 level was decreased in the hyperoxia group, compared with the normal control group (Fig.
231   5A, B; t=6.1929, P =0.0035; t=3.3567, P=0.0284, respectively). In addition, Akt, PI3K, and
232   mTOR phosphorylation was inhibited in the hyperoxia group but was rescued in the
233   hyperoxia+exosome group (Fig. 5A, B; t=25.5119, P=0.0000; t=9.2418, P=0.0008; t=9.0355,
234   P=0.0008, respectively). Furthermore, decreased p-Akt and Ki67 protein levels as well as mTOR
235   phosphorylation were noted in the hyperoxia+exosome+LY294002 group, compared with the
236   hyperoxia+exosome group (Fig. 5A, C; t=21.4586, P=0.0000; t=6.5485, P=0.0028; t=14.1400,
237   P=0.0001, respectively).
238

Discussion

240   Hyperoxia induces the generation of oxidative free radicals that promote AECII apoptosis. In
241   addition, treatment with exosomes can improve lung tissue histology and promote macrophage
242   M2 polarization, thereby reducing inflammation in a hyperoxia-induced BPD mouse model
243   (Willis et al. 2018a). Moreover, exosomes have been reported to increase pulmonary vascularity,
244   promote alveolar formation, and restore interrupted alveolar growth during tissue hyperoxia
245   (Collins 2020; Lesage & Thébaud 2018; Willis et al. 2018b; Willis et al. 2020). Exosomes
246   possess the mesenchymal stem cell markers CD29, CD90, CD73, and CD44 as well as the

247 exosome-specific markers CD9, CD63, and CD81 (Klymiuk et al. 2019). Thus, BMSC-derived

248 exosomes possess stem cell-like therapeutic properties and were studied for their potential

249 application in clinical therapy. In this study, we demonstrated the stem cell characteristics of rat

250 BMSCs by confirming the adipogenetic and osteogenic potential using oil red O and Alizarin red

251 staining.

252 In this study, we explored the efficacy of BMSC-derived exosomes in alleviating hyperoxia-

253 induced AECII damage in Sprague Dawley rats. We examined the morphology of BMSC-

254 derived exosomes using transmission electron microscopy and confirmed the successful isolation

255 of BMSC-derived exosomes from rat AECIIs by western blotting. Several signaling pathways

256 like the PI3K/Akt/Foxo3a (Wu et al. 2018) and PI3K/Akt/mTOR (Gu et al. 2020) pathways have

257 been implicated in exosome-mediated inhibition of hyperoxia-induced AECII

258 apoptosis.Recently, it has been reported that BMSC-derived exosomes rich in miR-30B-3p can

259 effectively inhibit AECII apoptosis and promote its proliferation in lipopolysaccharide-induced

260 acute lung injury by downregulating the target gene *SAA3* (Yi et al. 2019). It has also been

261 shown that BMSC-derived exosomes can reverse the apoptosis of AECIIs in acute lung injury by

262 inhibiting the NRF-2/ARE and NF-κB signaling pathways (Li et al. 2020a). In this study, we

263 noted decreased Akt and mTOR phosphorylation and Ki67 levels, decreased AECII

264 proliferation, but increased apoptosis in the hyperoxia group. Of note, treatment with exosomes

265 appeared to reverse the effects seen in the hyperoxia rats. However, application of the PI3K/Akt

266 blocker LY294002 and the mTOR inhibitor rapamycin reversed the protective effects of BMSC-

267 derived exosomes on AECII proliferation in rats under hyperoxia. Thus, treatment with

268 exosomes likely reversed the effects in the hyperoxia+exosome group, compared with that for

269 the hyperoxia group.

270 A limitation of this study is that it was conducted *in vitro* and *in-vivo* data are lacking. Therefore,

271 we will aim to confirm our findings using animal models in a follow-up study. Elucidation of the

272 critical component of exosomes that confers protective effects to AECIIs via the

273 PI3K/Akt/mTOR/Ki67 pathway will be the focus of future research, which could potentially

274 advance BPD clinical therapy.

275

Conclusions

277 Our results suggest the potential role of the PI3K/Akt/mTOR/Ki67 pathway in regulating

278 exosome-mediated inhibition of hyperoxia-induced AECII apoptosis (Fig. 6). In particular, the

279 expression of antiapoptotic proteins was elevated while that of proapoptotic proteins was

280 downregulated in the hyperoxia+exosome group. Despite the promise of stem cell therapy,

281 challenges like cell heterogeneity, tumorigenicity, immune regulation, and secretion of

282 inflammatory factors (Riester et al. 2020) must be overcome to realize the full potential of
283 effective applications of stem cells in clinical treatment.
284

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410 **Figure Legends**

411 **Figure 1.** Light microscopy images of BMSC morphology and stem cell characteristics. A:
412 BMSC suspension at day 1 of culture. B: Adherent BMSCs at day 12 of culture. C: Oil red
413 staining of BMSCs demonstrate their adipogenicity. D: Oil red staining of fibroblasts as a
414 negative control. E: Alizarin red staining of BMSCs demonstrate their osteogenecity. F: Alizarin
415 red staining of fibroblasts as a negative control.

416 **Figure 2.** Identification of BMSC-derived exosomes and lamellar bodies of AECIIs. A:
417 Transmission electron microscopy image showing exosomes (scale bar=100 nm). B: Profile
418 showing the diameters of isolated BMSC-derived exosomes from BMSCs. C: Western blot
419 detection of exosomes by their characteristic CD9 and CD63 markers. D: Transmission electron
420 microscopy image of lamellar bodies (scale bar=200 nm).

421 **Figure 3.** Isolation, identification, and activity of rat AECIIs in the five experimental groups:
422 normal control (NC), hyperoxia, hyperoxia+exosome, hyperoxia+exosome+LY294002, and
423 hyperoxia+exosome+rapamycin. A: Representative confocal microscopy image of adherent
424 AECIIs. Representative immunofluorescence images of B: DAPI-stained AECIIs, C: Surfactant
425 protein C (SP-C)/FITC-labeled AECIIs, and D: merged images of B and C. E: CCK8 cell
426 viability assay profile of AECIIs. $^{\#}P<0.05$, $^{*}P<0.01$, $^{**}P<0.001$, ns, not statistically significant.

427 **Figure 4.** Flow cytometry and semi-quantitative profiles of AECII apoptosis. A–E: Flow
428 cytometry profiles of AECIIs of the five experimental groups. F: Plot of the percentage of
429 AECIIs in the five experimental groups. Data are shown as the mean±SD; n=3. $^{**}P<0.05$ vs.
430 hyperoxia; $^{\#}P<0.01$ vs. hyperoxia.

431 **Figure 5.** Effect of BMSC-derived exosomes on the expression of apoptosis-related proteins in
432 AECIIs. A: Western blot analysis of apoptosis-related proteins and key proteins of the
433 PI3K/Akt/mTOR signaling pathway. Histograms of B: The phosphorylation levels of p-PI3K, p-
434 Akt, and p-mTOR and the Bax/Bcl2 ratio. C: The Ki67 level in the five experimental groups.
435 Data are shown as the mean±SD from three independent experiments (n=3). $^{**}P<0.01$ and
436 $^{*}P<0.05$; normal control (NC) vs. hyperoxia. $^{\#\#}P<0.01$ and $^{\#}P<0.05$; Exosome+hyperoxia vs.
437 hyperoxia.

438 **Figure 6.** Schematic illustration of the protective effect of exosomes on AECII proliferation
439 under hyperoxia.

440

Figure 1

Light microscopy images of BMSC morphology and stem cell characteristics.

A: BMSC suspension at day 1 of culture. B: Adherent BMSCs at day 12 of culture. C: Oil red staining of BMSCs demonstrate their adipogenicity. D: Oil red staining of fibroblasts as a negative control. E: Alizarin red staining of BMSCs demonstrate their osteogenecity. F: Alizarin red staining of fibroblasts as a negative control.

Figure 2

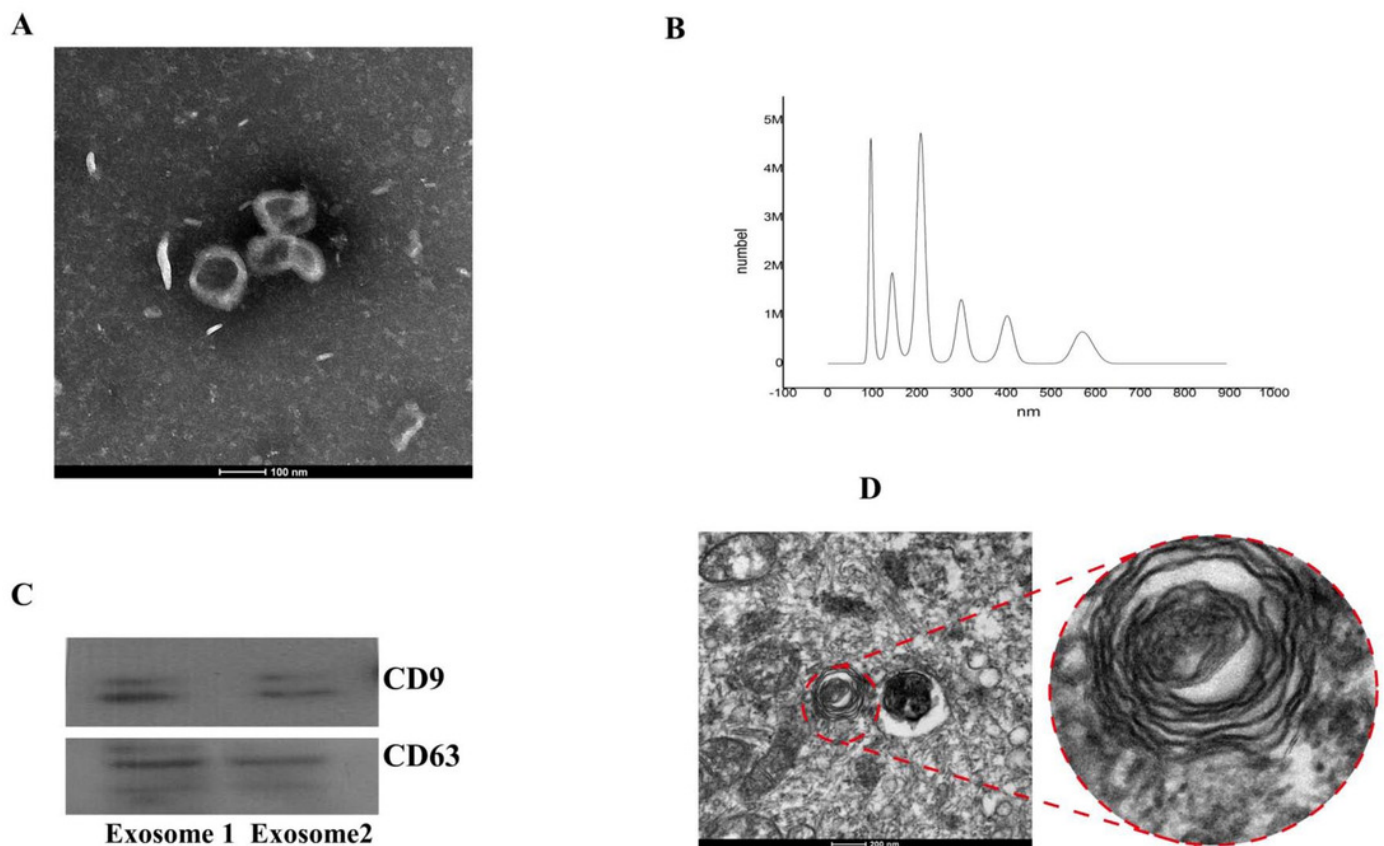
Identification of BMSC-derived exosomes and lamellar bodies of AECIIs.

A: Transmission electron microscopy image showing exosomes (scale bar=100 nm). B: Profile showing the diameters of isolated BMSC-derived exosomes from BMSCs. C: Western blot detection of exosomes by their characteristic CD9 and CD63 markers. D: Transmission electron microscopy image of lamellar bodies (scale bar=200 nm).

A

B

C

D

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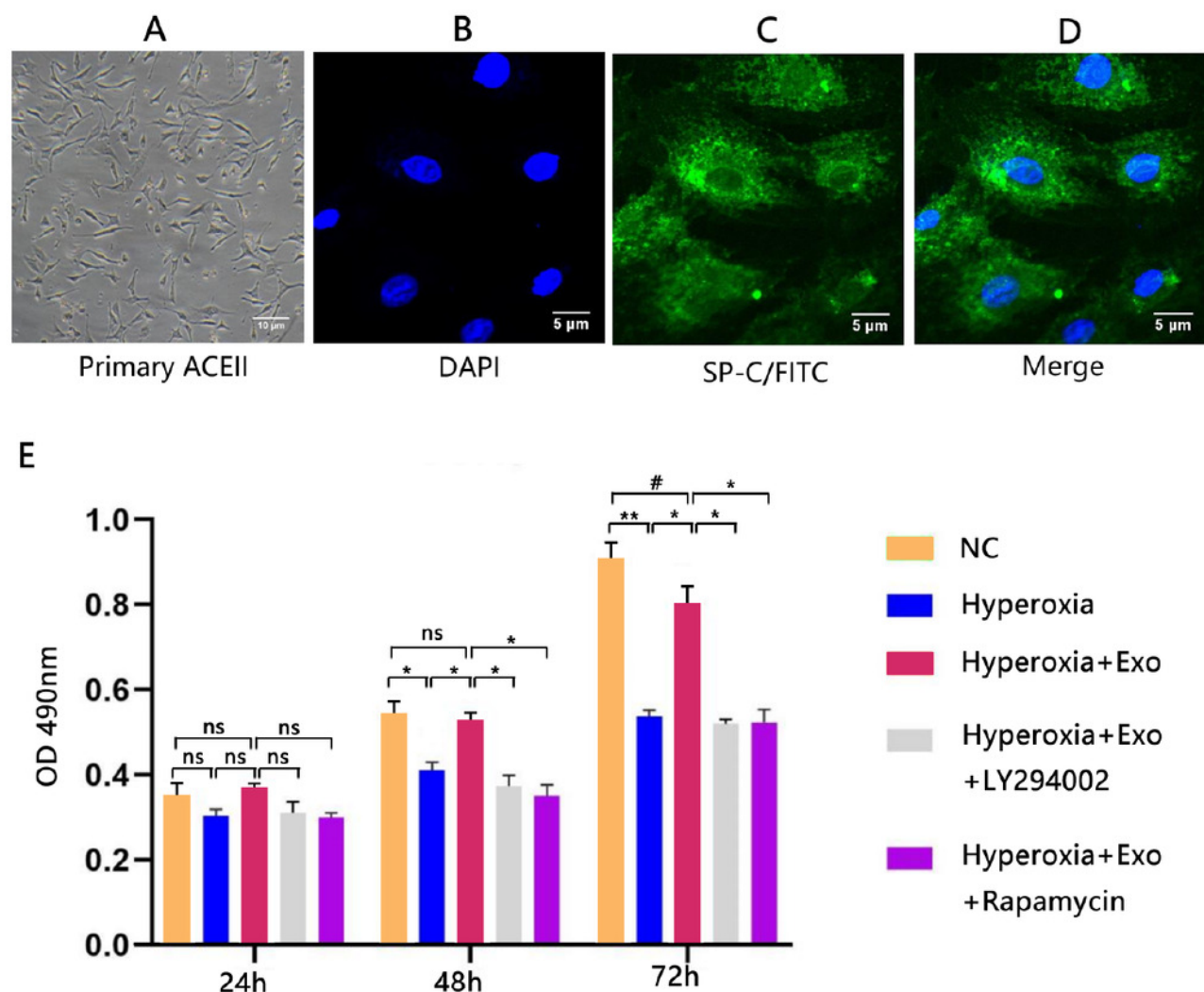
Isolation, identification, and activity of rat AECIIs in the five experimental groups: normal control (NC), hyperoxia, hyperoxia+exosome, hyperoxia+exosome+LY294002, and hyperoxia+exosome+rapamycin.

A: Representative confocal microscopy image of adherent AECIIs. Representative immunofluorescence images of B: DAPI-stained AECIIs, C: Surfactant protein C (SP-C)/FITC-labeled AECIIs, and D: merged images of B and C. E: CCK8 cell viability assay profile of AECIIs. #*P*<0.05, *P*<0.01, **P*<0.001, ns, not statistically significant.

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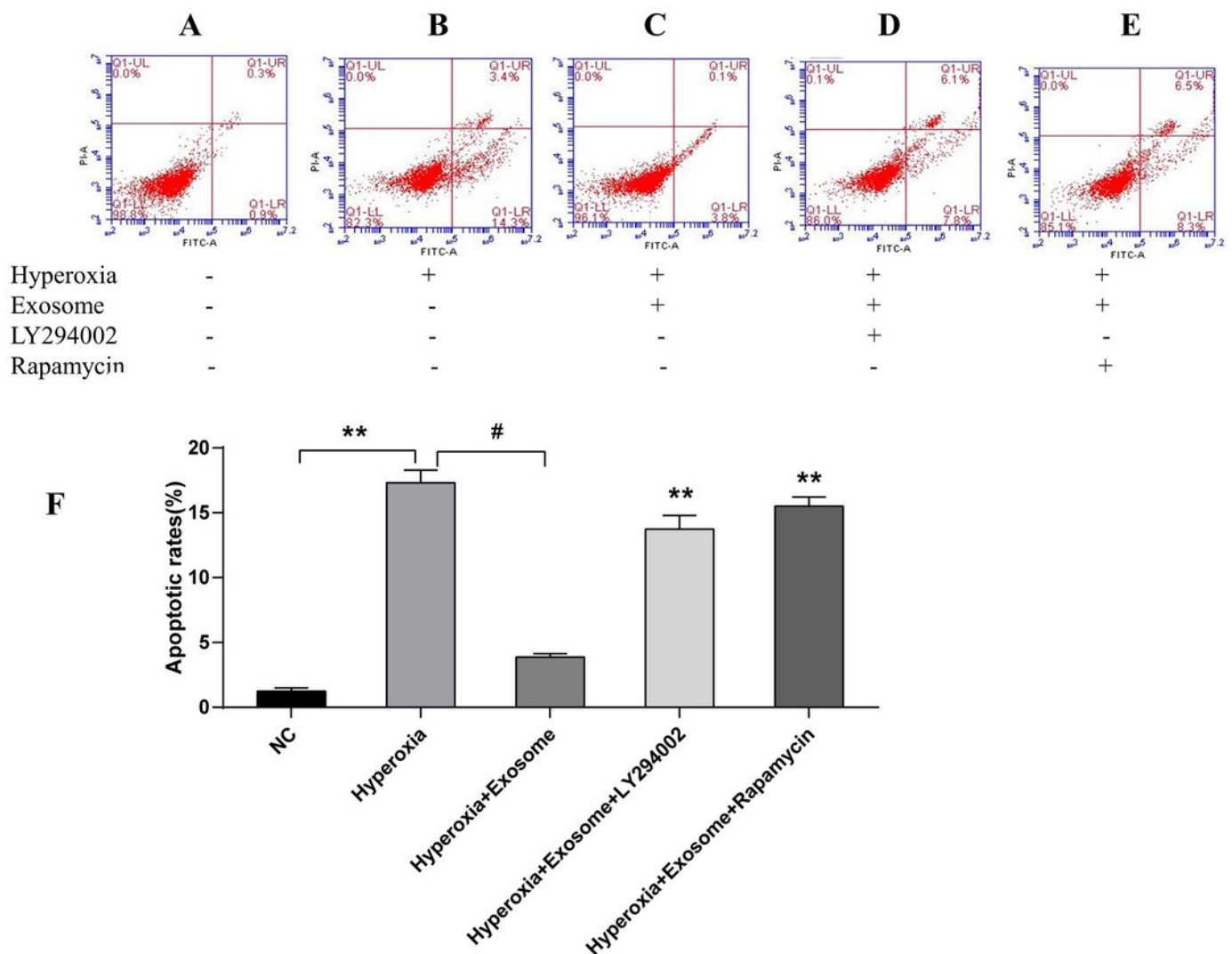
Flow cytometry and semi-quantitative profiles of AECII apoptosis.

A–E: Flow cytometry profiles of AECIIs of the five experimental groups. F: Plot of the

percentage of AECIIs in the five experimental groups. Data are shown as the mean±SD;*n*=3.

**P<0.05 vs. hyperoxia; #P<0.01 vs. hyperoxia.

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Effect of BMSC-derived exosomes on the expression of apoptosis-related proteins in AECIIs.

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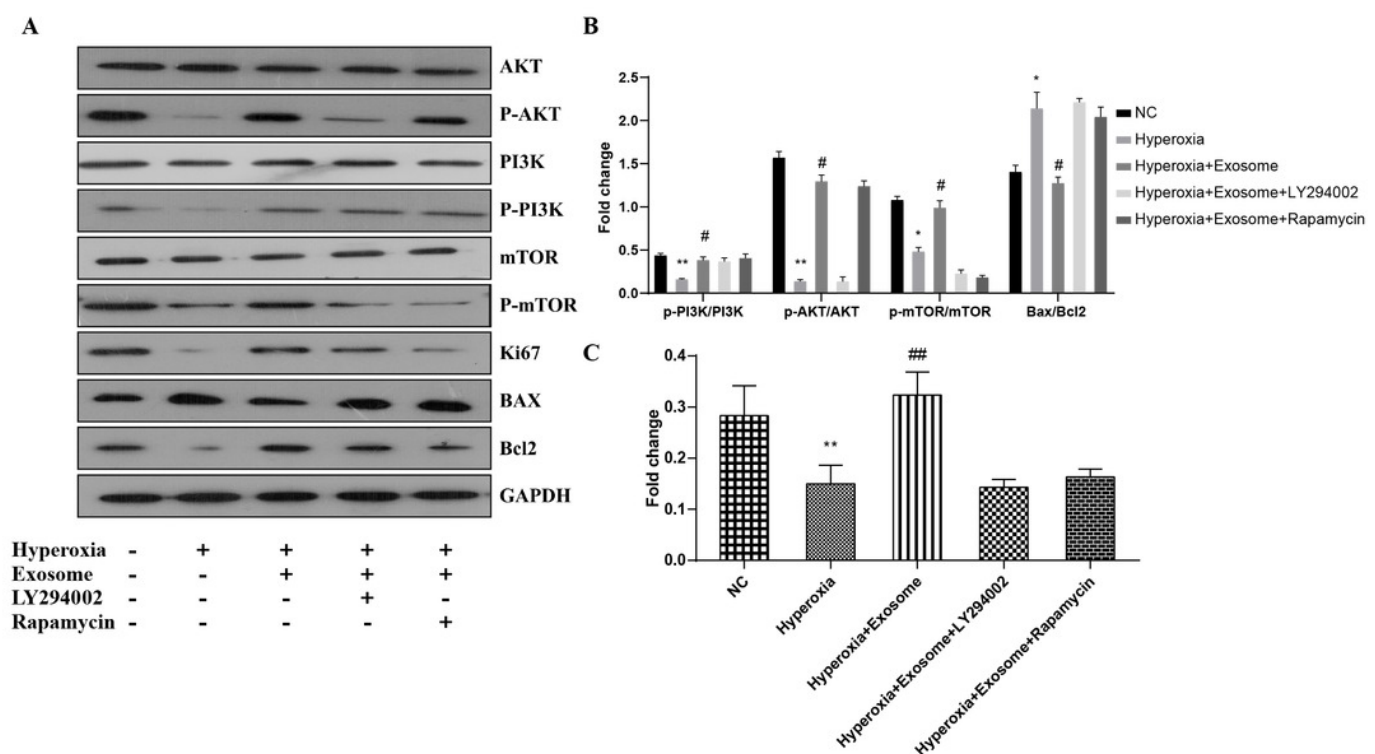
Figure 6

Schematic illustration of the protective effect of exosomes on AECII proliferation under hyperoxia.