



Original Article

Cell-derived vesicles from adipose-derived mesenchymal stem cells ameliorate irradiation-induced salivary gland cell damage

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ABSTRACT

Introduction: Salivary gland (SG) damage is commonly caused by aging, irradiation, and some medications, and currently, no damage modifying agent is available. However, cell therapy based on mesenchymal stem cells (MSCs) has been proposed as a therapeutic modality for irradiated SGs. Therefore, we administered cell-derived vesicles (CDVs) of adipose-derived mesenchymal stem cells (ADMSCs) to irradiated SG cells to investigate their radioprotective effects *in vitro*.

Methods: The artificial CDVs were obtained from ADMSC by tangential flow filtration (TFF) purification and ultracentrifugation. Cultured human SG epithelial cells were exposed to 2, 5 or 15 Gy of 4 MV X-rays produced by a linear accelerator. The effects of ADMSC-CDVs on SG epithelial cells damaged by irradiation were tested by proliferation activity, transepithelial electrical resistance (TEER), and amylase activity.

Results: Exposure to penetrating radiation inhibited the proliferation of SG epithelial cells, but the radiation intensity required to reduce the proliferation of human submandibular gland epithelial cells (hSMGECs) was greater than required for other SG cells. ADMSC-CDVs restored the proliferative ability of SG epithelial cells reduced by irradiation, and the proliferation capacities of irradiated human parotid gland epithelial cells (hPGECS) and human sublingual gland epithelial cells (hSLGECs) were increased by administering ADMSC-CDVs to non-irradiated SG epithelial cells. Furthermore, amylase activity in irradiated hPGECS, hSMGECs, and hSLGECs was lower than in non-irradiated controls. However, amylase activity was restored in all by ADMSC-CDV treatment. Also, TEER was diminished by irradiation in hPGECS, hSMGECs, and hSLGECs and restored by ADMSC-CDV administration.

Conclusion: Overall, our findings demonstrate that ADMSC-CDVs have potent radioprotective effects on irradiated SG cells.

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Abbreviations: ADMSCs, Adipose-derived mesenchymal stem cells; CDVs, Cell-derived vesicles; hPGECS, human parotid gland epithelial cells; hPL, human platelet lysate; hSLGECs, human sublingual gland epithelial cells; hSMGECs, human submandibular gland epithelial cells; MSCs, Mesenchymal stem cells; SG, Salivary gland; TFF, Tangential flow filtration; TEER, Transepithelial electrical resistance.

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1. Introduction

Saliva plays an essential role in maintaining oral homeostasis, and salivary glands (SGs) are essential for the production of saliva. However, the functions of SGs are reduced by, for example, penetrating radiation, drugs, and aging. Various methods based on the use of growth factors, bioactive factors, scaffolds, stem cells, secretome, and others, have been studied to repair damaged SGs [1–7]. Kim et al. reported that adipose tissue-derived mesenchymal stem cells (ADMSCs) effectively regenerated damaged salivary

glands [8], and Choi et al. reported that this effect of ADMSCs was enhanced when scaffolds were added to ADMSCs [7]. In addition, Ahn et al. confirmed that the ADMSC secretome induced by hypoxia also promoted salivary gland regeneration [6].

Cell-derived vesicles (CDVs) are a new form of extracellular vesicles and like exosomes, can function as intercellular communicators and drug carriers [9]. Because exosomes are vesicles naturally secreted by cells, their production is limited. However, CDVs can be produced in large quantities within a short time by direct extrusion from various human cell types. CDVs share many similarities with exosomes, such as size, morphology, and membrane molecular composition [10], and can deliver various effector molecules and drugs that affect cellular functions. Some recent studies have demonstrated that CDVs derived from adipose tissue-derived mesenchymal stem cells (ADMSCs) promote the regeneration of damaged tissues, decrease the degradation of aged tissues, and contribute to the maintenance of tissue homeostasis [11,12]. However, little is known of the ability of CDVs to improve SG function. Here, we investigated the effect of ADMSC-derived CDVs on SG cells damaged by irradiation.

2. Methods

2.1. Production of cell-derived vesicles (CDVs)

Adipose-derived mesenchymal stem cells (ADMSCs) were purchased from Lonza (Basel, Switzerland, Material number: PT-5006). Cells were seeded into two layers of CellSTACKs® (Corning, NY, US) containing Dulbecco's modified Eagle's medium-low glucose (Gibco BRL, MA, US) supplemented with 4% human platelet lysate (hPL) (Helios, AventaCell Biomedica, GA, US) and 1% penicillin/1% streptomycin (Gibco BRL, MA, US). Cells were maintained at 37 °C in a 5% CO₂ atmosphere. After two passages, cells were harvested and resuspended at 5 × 10⁵ cells/mL in PBS.

The cell suspension was then serially extruded through a series of membrane filters of pore sizes 10, 3, and 1 μm (Whatman Inc., NJ, USA) using a prototype extruder ES50 (MDimune Inc., Seoul, Korea). The crude CDVs obtained were first treated with Benzonase® endonuclease (Millipore, MA, US) at 10 U/μg DNA for 90 min at 37 °C and centrifuged at 3000 g for 10 min at 18 °C. The supernatant was then subjected to tangential flow filtration (TFF) purification using a MidiKros 750 kDa MWCO hollow fiber (Repligen, CA, US). Finally, the purified CDVs were passed through a syringe filter of pore size 0.2 μm (Pall Incorporation, NY, US) and concentrated by ultracentrifugation (Beckman Coulter, CA, US) at 120,000 g for 2 h. The size of CDV (153.86 ± 3.68 nm) was checked by Zetasizer Nano (Malvern Panalytical, UK). CDVs were stored at –80 °C until required. A list of reagents and consumables is provided in Table 1.

2.2. Cell culture

For human primary salivary epithelial cell culture, a small portion of a non-tumor bearing parotid gland (PG), submandibular gland (SMG), and sublingual gland (SLG) were resected and washed with Hanks' Balanced Salt Solution (Invitrogen, USA) containing 1% antibiotics (Invitrogen, USA). Tissues were chopped with fine scissors for 7 min, centrifuged at 1500 rpm for 5 min, plated on a culture dish containing DMEM/F12 medium (Invitrogen, USA) containing 10% FBS (ATCC, USA) and 1% antibiotics (Invitrogen, USA), and incubated in a 5% CO₂ atmosphere at 37 °C. All specimens were collected after obtaining informed consent and institutional review board approval [NON2017-002].

2.3. Irradiation

Cultured human salivary epithelial cells from a parotid gland (human parotid gland epithelial cells, hPGECS), submandibular gland (human submandibular gland epithelial cells, hSMGECs), and sublingual gland (human sublingual gland epithelial cells, hSLGECs) were exposed to 2, 5 or 15 Gy of 4 MV X-rays produced by a linear accelerator (Mevatro MD, Siemens Medical Laboratories Inc., Germany).

2.4. Cell proliferation test

The proliferations of hPGECS, hSMGECs, and hSLGECs were evaluated using the CELLMAX™ assay (PreCareGene, Korea). Briefly, cells were seeded in 96-well plates (4 × 10³ cells/well) and irradiated with 0, 2, or 5 Gy in the presence or absence of 5 × 10⁷, 1 × 10⁸, 5 × 10⁸, or 1 × 10⁹ CDVs and then incubated for nine days in 5% CO₂ at 37 °C. On day 9, cells were treated with CELLMAX™ solution and incubated for 1 h at 37 °C in 5% CO₂. Absorbance was read at 450 nm with a microplate reader (Molecular Devices, USA).

2.5. Amylase activity assay

The amylase activities of hPGECS, hSMGECs, and hSLGECs were determined using an α-amylase assay kit (Abcam, USA). Cells were seeded in 96-well plates (4 × 10³ cells/well) and irradiated or not with 5 Gy in the presence of 5 × 10⁷, 1 × 10⁸, 5 × 10⁸, or 1 × 10⁹ CDVs and incubated for 6–9 days at 37 °C in a 5% CO₂ atmosphere. Cells were then homogenized with assay buffer, centrifuged at 13,000 rpm for 5 min at 4 °C, and supernatants were transferred to clean tubes. Reaction mixtures were added, and absorbances were measured at 405 nm using a microplate reader.

Table 1
Reagents and consumables.

Name	Catalog no	Manufacturer
Adipose-derived stem cell	PT-5006, batch no: 0000669,429	Lonza
CELLSTACK®	3296	Corning
Dulbecco's modified Eagle's medium low glucose	11,885,092	Gibco
Penicillin-streptomycin	15,140,163	Thermo Scientific
PBS	10,010,049	Gibco
hPL	HPCFDCRL50	AventaCell
Membrane filter 10 μm	111,115	Whatman
Membrane filter 3 μm	111,112	Whatman
Membrane filter 1 μm	111,110	Whatman
Benzonase® endonuclease	71,206–3	Millipore
MidiKros 750 kDa MWCO hollow fiber	D02-E750-05-N	Repligen
0.2 μm Filter	4612	Pall

2.6. Transepithelial electrical resistance (TEER) detection

hPGECs, hSMGECs, and hSLGECs were seeded in trans-well plates (Corning, USA) at 1×10^5 cells/well and cultured in a 5% CO₂ atmosphere at 37 °C until over-confluent. Cells were then irradiated or not with 5 Gy in the presence of 5×10^7 , 1×10^8 , 5×10^8 , or 1×10^9 CDVs and incubated for 6–9 days in 5% CO₂ at 37 °C. TEER (Ohm/cm²) values were measured using a Millicell ERS-2 (EMD Millipore, USA).

2.7. Western blot analysis

Western blotting was performed to assess the changes in apoptosis related protein expression in hPGECs treated with 1×10^9 CDV under IR exposure. Cells were homogenized in PRO-PREPTM protein extraction solution (iNtRON Biotechnology, Korea), incubated on ice for 30 min, centrifuged at 13,000 rpm for 10 min at 4 °C, and supernatants were collected. Primary antibodies for caspase-3, caspase-9, Bax, p21, p16, and GAPDH (all from Santa Cruz Biotechnology, 1:1000) were used. Goat anti-mouse IgG-HRP was used as the secondary antibody (Santa Cruz Biotechnology, 1:5000). Proteins were visualized using Super-Signal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA) using a ImageQuant™ LAS 4000 unit (GE Healthcare, USA). All blots derive from the same experiment and were processed in parallel. Quantitative analysis was performed using ImageJ software (version 1.49; Wayne Rasband, National Institutes of Health).

2.8. Statistical analysis

The analysis and graphical presentations of data were carried out in Graphpad Prism 8.2.1 (GraphPad Software Inc., USA). One- and two-way ANOVA were used to determine the significances of intergroup differences. Statistical significance was accepted for P values < 0.05.

3. Results

3.1. Irradiation inhibited the proliferation of SG epithelial cells

Irradiation dose-dependently reduced the proliferations of hPGECs, hSMGECs, and hSLGECs (Fig. 1, all $p < 0.05$), though human submandibular gland epithelial cells (hSMGECs) were more resistant than other SG cells.

3.2. ADMSC-CDVs restored irradiation-induced reductions in proliferative capacities

Irradiation-induced reductions in the proliferation capacities of hPGECs, hSMGECs, and hSLGECs were partially restored by ADMSC-CDVs, and these increases in proliferative capacities were significant when SGs were treated with 1×10^9 ADMSC-CDVs (Fig. 2, $p < 0.05$ for all). ADMSC-CDV administered SGs, irradiation-only treated SGs, and irradiation + ADMSC-CDV treated SGs were compared with non-treated controls. The proliferative ability of ADMSC-CDV administered hPGECs was greater than that of non-treated controls, but that of irradiated hPGECs was less. Proliferative ability was restored in the irradiation + ADMSC-CDV administered cells (Fig. 3, all $p < 0.05$). For hSMGECs, proliferative abilities of ADMSC-CDV treated SGs and controls were similar, but proliferative abilities of irradiated cells were less than controls, and irradiation-induced reduction in proliferative ability was recovered by ADMSC-CDV treatment ($p < 0.05$). Results for hSLGECs and hPGECs were similar ($p < 0.05$).

3.3. Effect of ADMSC-CDVs on irradiation-induced reductions in amylase activity

Amylase activity was reduced in hPGECs exposed to radiation (Fig. 4, $p < 0.05$). Amylase activity was higher in irradiation + ADMSC-CDV cells than irradiated cells, particularly in cells treated with 1×10^9 ADMSC-CDVs. Results for hSMGECs and hSLGECs were similar (Fig. 4, $p < 0.05$).

4. Effect of ADMSC-CDV on irradiation-induced reductions in transepithelial electrical resistance

Transepithelial resistance (TEER) was measured to evaluate the function of SG epithelial cells. In hPGECs, hSMGECs, and hSLGECs irradiation-induced reductions in TEER values were partially restored by ADMSC-CDV treatment (Fig. 5, $p < 0.05$).

4.1. ADMSC-CDV regulates apoptosis related protein expression in IR damaged hPGECs

We studied the expressions of the apoptosis related molecules caspase 3, caspase 9, Bax, p21, and p16 by Western blot and found the expressions of all tested molecules were significantly increased in IR treated hPGECs than in control hPGECs. And increased expression levels of caspase 3, caspase 9, Bax, p21 and p16 were significantly diminished after treatment of ADMSC-CDV (Fig. 6, $p < 0.05$).

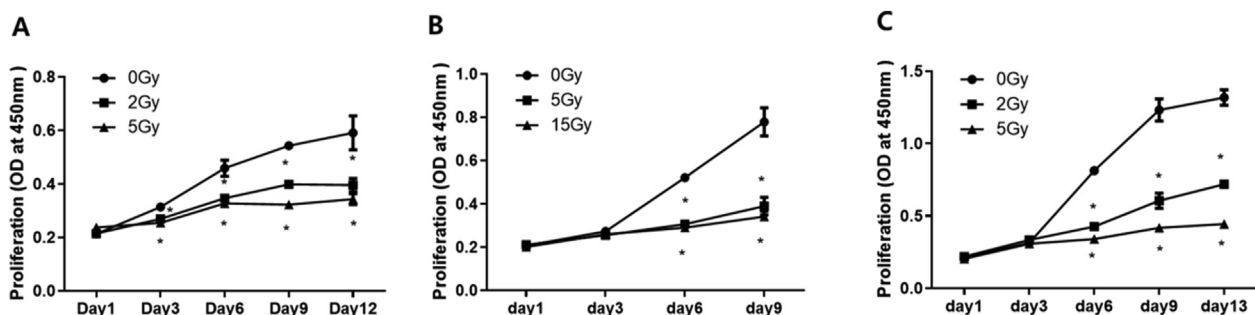


Fig. 1. Irradiation-induced reductions in the proliferation of SG cells. (A) Human parotid gland epithelial cells (hPGECs), (B) human submandibular gland epithelial cells (hSMGECs), (C) human sublingual gland epithelial cells (hSLGECs). Irradiation inhibited the proliferation of all three SG epithelial cells. Dots and error bars denote means \pm standard deviations. *, vs. non-treated controls. * $p < 0.05$.

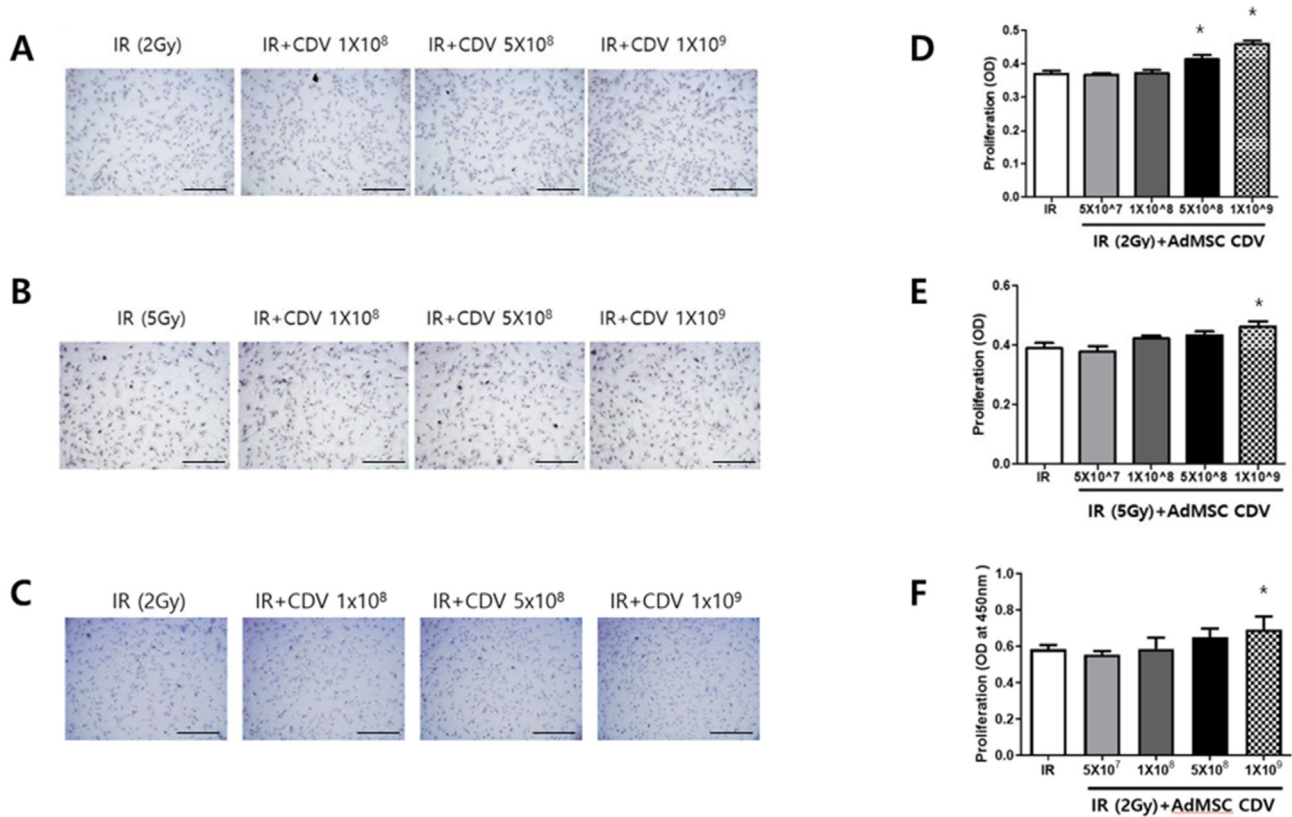


Fig. 2. The effects of ADMSC-CDVs on the proliferation of irradiated SG cells. (A) Human parotid gland epithelial cells (hPGEs), (B) human submandibular gland epithelial cells (hSMGECs), and (C) human sublingual gland epithelial cells (hSLGECs). Quantitative results showed ADMSC-CDVs restored the proliferative abilities of irradiated (D) hPGEs, (E) hSMGECs, and (F) hSLGECs. Scale bar: 50 μ m. Error bars represent standard deviations. *, compared to irradiated cells. *p < 0.05 (IR, Irradiation; CDV, Cell-derived vesicle).

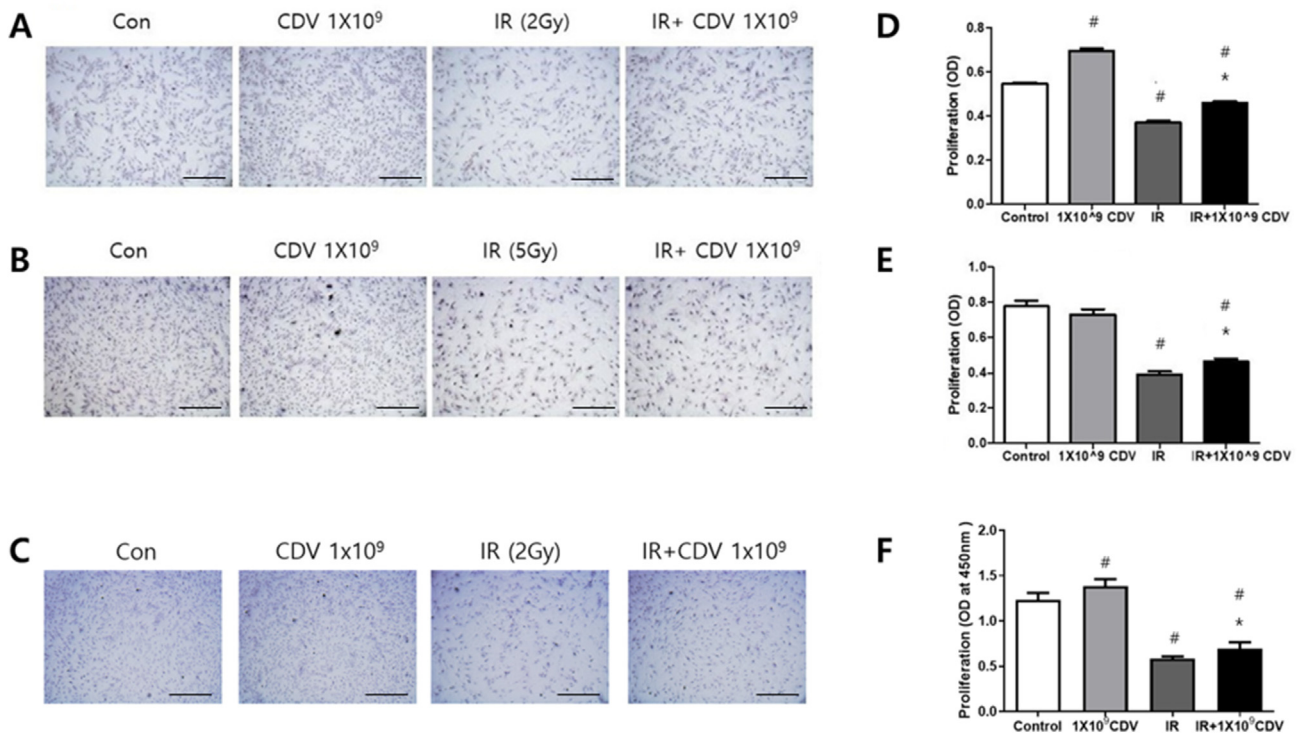


Fig. 3. The effects of ADMSC-CDVs on the proliferation of SG cells. (A) Human parotid gland epithelial cells (hPGEs), (B) human submandibular gland epithelial cells (hSMGECs), and (C) human sublingual gland epithelial cells (hSLGECs). Quantitative results showed ADMSC-CDVs restored the proliferative abilities of irradiation and non-irradiated (D) hPGEs, (E) hSMGECs, and (F) hSLGECs. Scale bar: 50 μ m. Error bars represent standard deviations. #, compared to non-treated controls; *, compared to irradiated cells. *p < 0.05, #p < 0.05 (CON, Control; IR, Irradiation; CDV, Cell derived vesicle).

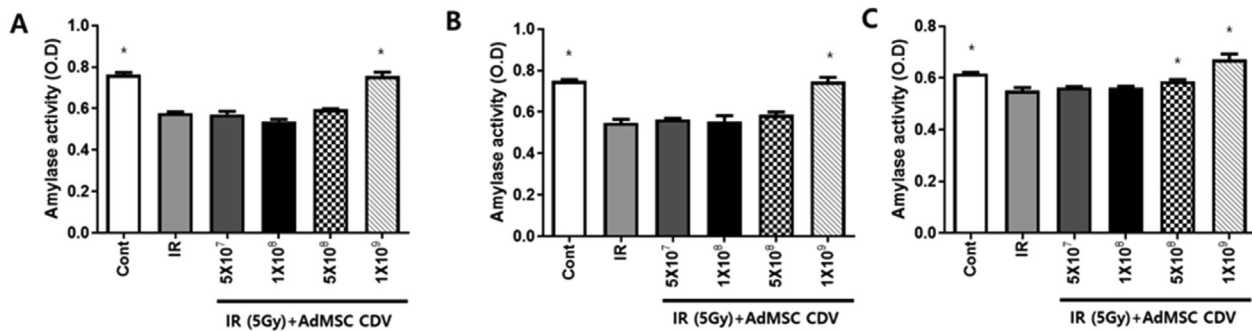


Fig. 4. The effects of ADMSC-CDVs on irradiation-induced changes in SG cell amylase activities. (A) Human parotid gland epithelial cells (hPGECS), (B) human submandibular gland epithelial cells (hSMGECs), (C) human sublingual gland epithelial cells (hSLGECs). The amylase activity was increased by ADMSC-CDV administration in all irradiated SG cells. Error bars denote standard deviations. **p* < 0.05, compared to IR cells (Cont, controls; IR, Irradiation; CDV, Cell derived vesicle).

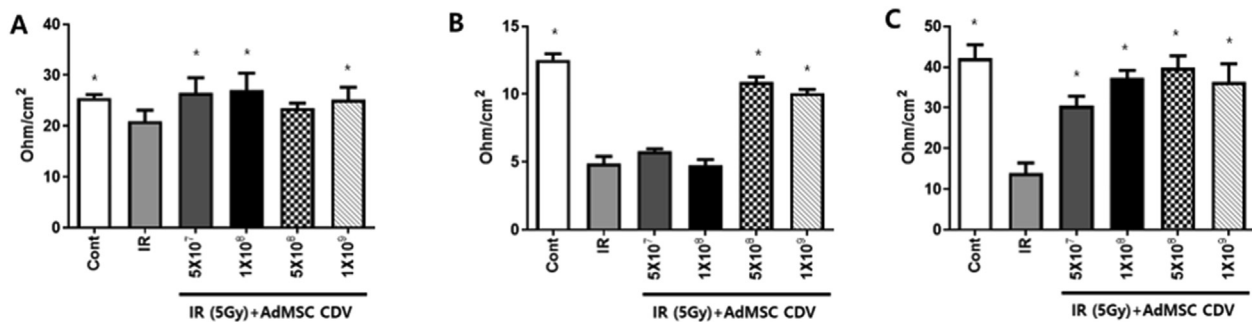


Fig. 5. The effects of ADMSC-CDVs on irradiation-induced changes in transepithelial electrical resistance (TEER) in SG cells. (A) Human parotid gland epithelial cells (hPGECS), (B) human submandibular gland epithelial cells (hSMGECs), (C) human sublingual gland epithelial cells (hSLGECs). ADMSC-CDV administration increased TEER values in all irradiated SG cells. Error bars denote standard deviations. **p* < 0.05, compared to IR cells (CON, controls; IR, Irradiation; CDV, Cell derived vesicle).

5. Discussion

The parotid, submandibular, and sublingual glands are the primary SGs, and it is well known that saliva maintains oral homeostasis, aids digestion due to amylase secretion, prevents tooth decay, and improves the sense of taste. However, SG function may decline due to aging or radiation exposure. To address SG dysfunctions, experiments have been conducted on SG regeneration using stem cells, and because ADMSCs are easily collected and abundant, they are commonly used to regenerate organs. Many studies have been conducted on the regeneration of SGs, and as we have reported, ADMSCs provide an effective means of salivary gland regeneration [6–8].

CDVs are exosome-mimicking nanovesicles and can be harvested in quantity at high yields. CDVs participate in cell-to-cell communication by transferring cellular information to neighboring cells. Furthermore, CDVs derived from MSCs contain various bioactive factors such as proteins, mRNAs, and microRNAs [13], and it has been shown that extracellular vesicles derived from ADMSCs contain functional components that can regenerate damaged tissues, including SGs, by mimicking the paracrine actions of stem cells [14–16]. Thus, we hypothesized that CDVs derived from ADMSC might also have a SG regenerating effect and initiated this study to determine whether ADMSC-CDV administration can reverse irradiation-induced damage in human SGs. To validate the contribution of ADMSC-CDV in salivary epithelial cell proliferation, ADMSCs were co-cultured with salivary epithelial cells, and the proliferation of salivary epithelial cells damaged by IR significantly increased after co-culture with ADMSCs.

In the present study, SG cells exposed to radiation exhibited a gradual decrease in proliferative ability. hSMGECs were found to be more resistant to the effects of radiation than hPGECS, which is consistent with previous reports that the parotid gland is more sensitive to radiation than the submandibular gland [17]. Nonetheless, damaged hPGECS, hSMGECs, and hSLGECs tended to recover their proliferative abilities in proportion to the amount of ADMSC-CDV administered, a similar effect was observed for the ADMSC-CDV-induced recovery of amylase activity. TEER values, which provide a measure of SG activity and are sensitive to culture environment and duration [18], also exhibited a weaker positive correlation between ADMSC-CDVs administered and the recovery of amylase activity.

The previous study showed that ADMSC protected the mice salivary gland against IR induced cell death by secreting paracrine factors. And also, ADMSC treatment exhibited a decreased TUNEL positive cells and the expressions of apoptosis related protein such as Bax, caspase-3 and caspase-9 in IR exposed mouse salivary gland tissue [7]. In this study, irradiation increases expression of pro-apoptotic proteins such as caspase 3, caspase 9 and Bax, and ADMSC-CDVs treatment significantly decreases the expression of pro-apoptotic proteins in salivary epithelial cells. These results could suggest the possibility that ADMSC-CDV as one of the paracrine bioactive factors of ADMSC exhibits the effect of restoring salivary epithelial cell functions through the anti-apoptosis mechanism.

Here, we report for the first time that ADMSC-CDVs can affect the SG cell proliferation. We chose to use irradiation to damage SGs because this technique is commonly used to investigate SGs with reduced functions and to study SG regeneration. The present

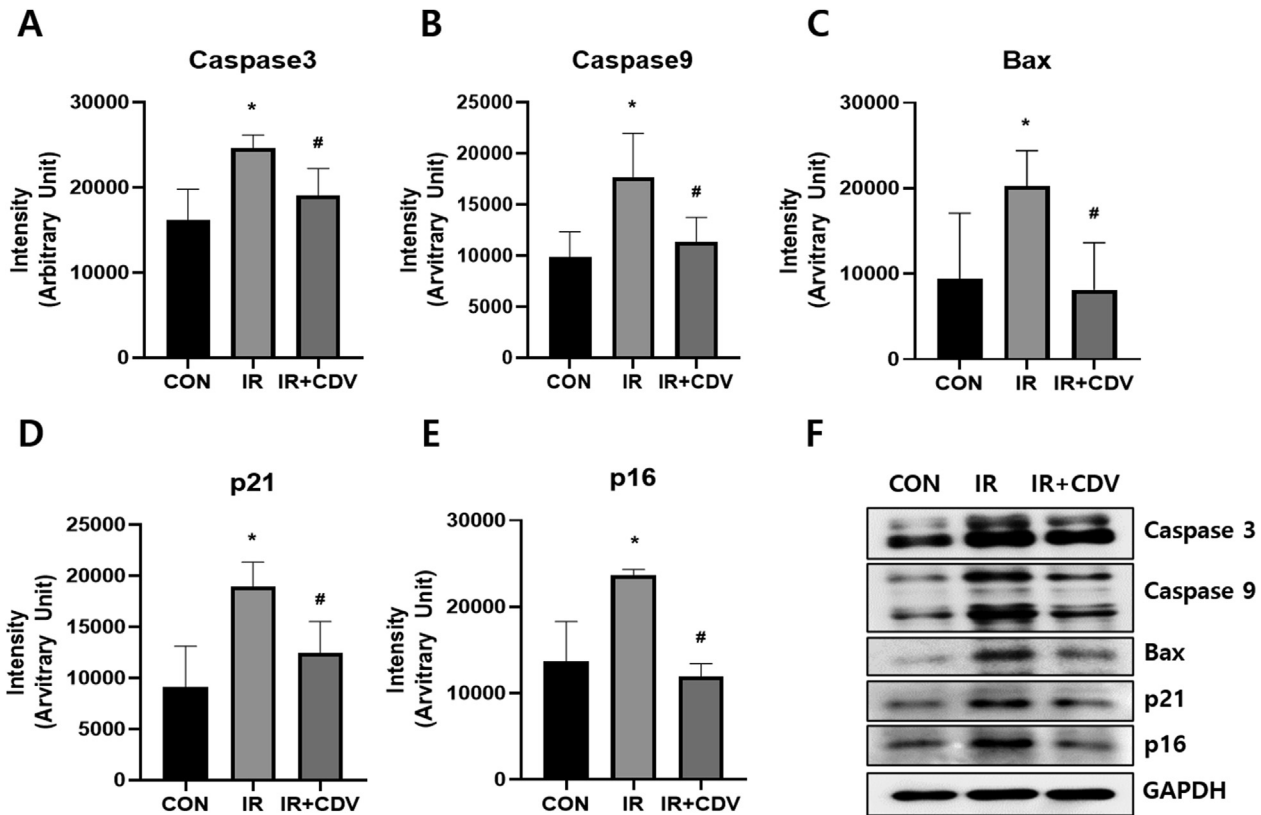


Fig. 6. Effect of ADMSC-CDV on apoptosis-related protein expression. (A–E) Quantitative analysis of Western blot image. (G) The representative image of Western blot analysis. Error bars denote standard deviations. *, compared to non-treated controls; #, compared to irradiated cells. *p < 0.05, #p < 0.05 (CON, Control; IR, Irradiation; CDV, Cell derived vesicle). (CON, controls; IR, Irradiation; CDV, Cell derived vesicle).

in vitro study confirms that ADMSC-CDVs have reparative effects on SGs. We suggest that further studies be conducted in animal models and that attempts be made to identify the active substances present in ADMSC-CDVs.

6. Conclusion

Summarizing, this study demonstrates that ADMSC-CDVs have the ability to restore the proliferative abilities of SG epithelial cells exposed to penetrating radiation. Our findings show ADMSC-CDVs should be considered a potential cell-free therapy in the regenerative field.

Author contributions

J.M.K., M.E.C., E.J.J., J-M.P and J.E.P conducted the experiments. S.W.O and J-S.C advised and critically revised the manuscript. J.M.K, J.E.P and J-S.C. wrote the manuscript. S.W.O and J-S.C. supervised the study. All authors contributed to manuscript revision and approved the final version of the manuscript.

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Declaration of competing interest

The authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reth.2022.09.007>.

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