

RESEARCH ARTICLE

Exosomes derived from human dental stem cell enhance the viability of odontoblasts

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ABSTRACT

Objective(s): The purpose of this study was to assess the effects of exosomes derived from dental pulp mesenchymal stem cells (DPSCs) on the viability of odontoblast cells.

Methods: Exosomes were extracted from human DPSC cells via ultracentrifugation. Scanning electron microscopy and western blot analysis for CD9 and CD81 demonstrated characteristics of exosomes. Developing odontoblasts were exposed to increasing concentrations of exosomes (10-400 ng/ml) over time periods of 24, 48, 72, and 96 hours. Cell viability was evaluated using MTT assays. In addition, real-time PCR was used to assess expression levels of Wnt, beta-catenin, PI3K, Akt, Bcl-2, and Bax in cells after 24-96 hours of exposure to 100 and 400 ng/ml exosomes.

Results: Odontoblast viability was found to be enhanced by DPSC-derived exosomes, with greater impacts at higher concentrations and time periods of 72-96 hours. Expression of PI3K, AKT, Wnt, beta-catenin, and Bcl-2 were notably upregulated in odontoblasts following 96 hours of DPSC-exosome treatment. Conversely, Bax expression was significantly downregulated. These findings suggest DPSC-exosomes promote odontoblast survival through modulated expression of genes involved in key survival pathways over extended exposure periods.

Conclusions: Our findings demonstrated that exosomes derived from DPSC cells possess innate nanoparticle properties, leading to enhanced survival of odontoblast cells. This effect is achieved through the activation of the Wnt/beta-catenin and P3K/AKT signaling pathways, ultimately resulting in an increased Bcl-2/Bax ratio.

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INTRODUCTION

Numerous tissue and organ defects may be treated using regenerative medicine and stem cell-based tissue engineering. When it comes to flexible applications in translational medicine, the use of adult stem cells is particularly intriguing (1, 2). Adult humans' third molars have unveiled a newfound presence of DPSCs. This specialized connective tissue, known as the dental pulp, comprises four distinct regions: the pulp core, intermediate cell-rich zone, intermediate cell-free zone, and peripheral odontogenic zone (3). DPSCs exhibit substantial potential as a cell source

for various regenerative medicine endeavors, offering promising prospects in this field. Dental applications for the regeneration of tooth structures rely on their natural function in the generation of odontoblasts to produce reparative dentin (4, 5). DPSCs are distinguished from other adult stem cells by their high rate of recovery from the disposable dental pulp following occlusion management. Their isolation process uses non-invasive methods and has no significant ethical limitations. Significantly, after cryopreservation, DPSCs retain their stemness, viability, proliferation, and differentiating abilities (6).

Recent advances in translational research

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and nanomedicine have facilitated more rapid development of targeted drug delivery systems by the global scientific community (7). Exosomes, possessing inherent properties reflective of their source cell, show promise as vehicles for cell-free drug delivery analogous to established techniques, due to their native traits (8). Extracellular vesicles (EcVs), also called exosomes, are naturally occurring extracellular structures found within cells, tissues, and bodily fluids throughout the body. Exosomes play roles in maintaining tissue equilibrium and communication between cells, influencing disease processes. Studies have cataloged some of the constituents within exosomal cargo, including 9,769 types of proteins, 2,838 microRNAs, 3,408 messenger RNAs, 1,116 varieties of lipids, and 2,838 microRNAs (9,10). Exosomes can be isolated from cells, tissues, and biological fluids using different kinetic profiling methods. They interact and enter recipient cells through surface receptors and ligands, with internalization occurring via micropinocytosis and phagocytosis pathways. Advancing technologies in regenerative medicine have enabled researchers to isolate exosomes from mesenchymal stem cells (MSCs), capitalizing on the remarkable healing abilities resident within such exosome cargos to develop promising therapeutic applications for various medical conditions (11,12). Exosomal cargo is a crucial component in making a diagnosis and carrying out a therapeutic role while controlling a disease process. Exosomes have been shown to be safe, effective, and potentially therapeutic in a number of cancers, neurodegenerative, cardiovascular, and orthopedic diseases in various *in vitro* studies (13).

In the dental pulp's outermost region are odontoblasts, which are tall columnar cells (14, 15). Craniofacial ectomesenchymal cells that neural crest cells migrated into during the early developmental phases of the craniofacial region give rise to dental pulp stem cells. Odontoblasts lay down dentin, a mineralized connective tissue composed primarily of collagen, through secretion of collagenous and noncollagenous proteins that form the organic matrix and regulation of mineral ion deposition into the matrix (16). During tooth development and subsequent remodeling, odontoblasts extend cellular projections that penetrate deep into the mineralized dentin. Through this cellular process, odontoblasts are able to sequentially deposit additional layers of this mineralized tissue over the life of the tooth

(17). Odontoblasts have additionally been shown to synthesize a responding or restorative form of dentin in cases of dental caries or other extrinsic factors impacting teeth, representing an adaptive cellular function (17,18). The current research aimed to assess the possible effects of exosomes derived from dental pulp stem cells on odontoblast viability and multiplicity.

MATERIALS AND METHODS

Cell culture

DPSC cells of human origin were obtained from Royesh Stem Cell Biotechnology (Tehran, Iran) and maintained in culture. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator at 37°C under a 5% CO₂ atmosphere. DMEM containing 10% FBS and 1% pen/strep was used for culturing the DPSCs. The cells were then stored at 37°C in a humidified atmosphere with 5% CO₂.

Exosome isolations

DPSC-derived exosomes were obtained from serum-free conditioned media (CM) using the MagCapture™ Exosome Isolation Kit according to the manufacturer's instructions. Initially, the CM was subjected to ultracentrifugation at 100,000 × g for 90 minutes to pellet exosomes. Following this, the supernatant was removed and the resultant pellet containing the isolated exosomes was collected for further use.

MTT assay

The impact of DPSC-derived exosomes on odontoblast viability was evaluated using the MTT assay according to the kit protocol. Briefly, odontoblasts were seeded in a 96-well plate at 5×10⁴ cells per well and incubated for 24 hours. Following this, the cells were treated with varying concentrations of exosomes (0-1000 ng/ml) for 24, 48, 72, and 96 hours time periods. At the end of each treatment duration, MTT analysis was performed to assess cell viability effects imparted by the exosome treatments. For the MTT assay, 20 µL of 5 mg/mL MTT solution was added to each odontoblast-containing well after treatment durations. The plates were then incubated for 4 hours at 37°C, following which the optical density (OD) of each well was measured at 570 nm using an ELISA plate reader. Three independent experiments

with four technical replicates each were performed to obtain reliable cell viability readings based on MTT conversion by metabolically active cells over the treatment periods.

RNA Isolation

Total RNA was extracted from odontoblasts using an mRNA Isolation Kit according to the manufacturer's instructions. A Nanodrop-2000 instrument was then used to assess the quality, quantity, and purity of the isolated RNA samples. Absorbance ratios and concentrations measured by the Nanodrop provided indicators of sample yield, integrity, and purity achieved through the RNA extraction process from the odontoblasts.

Reverse transcription and cDNA synthesis

Total RNA extracted from the odontoblasts was used to synthesize cDNA through reverse transcription employing a High Capacity cDNA Reverse Transcription Kit. This was done according to the manufacturer's protocol. The resultant cDNA was then utilized for real-time PCR (qPCR) analysis to quantify gene expression levels in the odontoblast cells. In addition, RT-PCR was performed using specific primers to assess their efficiency prior to the qPCR reactions. Briefly, 1 µg of total RNA was combined with 1 µL of random hexamer primer in a reaction mixture containing nuclease-free water to achieve a final volume of 12 µL. The tubes were initially incubated at 65°C for 5 minutes followed by chilling on ice at 4°C. The remaining reagents from the cDNA synthesis kit were then added to the mixture. The samples were subjected to a programmed thermocycling for cDNA amplification consisting of an initial step at 25°C for 5 minutes, followed by 60 minutes at 42°C and a final step of 5 minutes at 70°C. This resulted in the synthesis of first-strand cDNA from the target RNA samples.

Real Time-PCR

Real-time PCR utilizing cDNA, forward and reverse primers, distilled water, and EvaGreen qPCR Mastermix was performed to quantify target gene mRNA expression levels according to the kit protocol. The Mastermix contained dNTPs, Hotstart Taq polymerase, MgCl₂, EvaGreen fluorescent dye, reference dye, and buffers. Reactions were subjected to thermocycling with EvaGreen utilized for quantitative fluorescence detection of amplicons, defining the relative abundance of transcripts in

cDNA samples. Real-time PCR was performed in triplicate reactions with a final volume of 20 µL using the Step One Plus system (Applied Biosystems, USA). EvaGreen dye was applied as it cannot enter live cells or bind DNA, allowing quantitative fluorescence detection of amplicons. Cycling proceeded as per the manufacturer's preset parameters. The $\Delta\Delta CT$ method was utilized to analyze expression ratios. Target mRNA levels were normalized to the reference β -actin gene. Primer sequences used are provided in Table 1. EvaGreen dissociates from double stranded DNA during the melting processes involved in PCR thermocycling, facilitating real-time fluorescence measurement of amplicon accumulation.

Western blotting

Total protein extraction from samples utilized RIPA lysis buffer according to the manufacturer's guidelines. Equivalent concentrations of approximately 50 µg were subjected to SDS-PAGE, after which a semi-dry transfer placed proteins onto PVDF membranes. Membranes were blocked by treatment with 0.5% Tween-20 in PBS for two hours prior to further processing. The membrane was then treated with primary (goat) monoclonal antibodies (Thermo Fisher Scientific Inc) directed against the target genes for 24 hours in a dark environment. The membrane was then treated for an additional hour with HRP-conjugated secondary antibodies (rabbit) from Thermo Fisher Scientific Inc. An enhanced chemiluminescence kit enabled the visualization of protein bands. An imaging instrument captured images of the bands, which were quantified by densitometry and normalized relative to the density of β -actin (ACTB/ACTB) loading control bands.

Statistical analysis

Analysis of results was performed using GraphPad Prism version 8.01 software. Data were expressed as mean \pm SEM of triplicate experiments. Student's t-test assessed statistical significance between groups, with $P < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Western blotting results and SEM image

Figure (1) compares the results of western blot analysis of CD9 and CD81 expression between DPSCs and their derived exosomes. A higher level of these exosomal marker proteins was observed in

the DPSC exosomes relative to the DPSCs.

Also, SEM images verified the efficient isolation of exosomes from DPSCs (Fig 1).

DPSCs-exosome promoted the viability of odontoblasts

Figure (2) depicts the results of MTT assays examining odontoblast viability following

treatment with 10-400 ng/ml concentrations of DPSC exosomes for 24-96 hours. DPSC exosomes significantly increased odontoblast proliferation in a time- and dose-dependent manner ($P < 0.05$), with the strongest effect observed after 96 hours of exposure to 400 ng/ml exosomes. Thus, DPSC exosomes supported odontoblast growth in a manner correlated to both treatment duration and

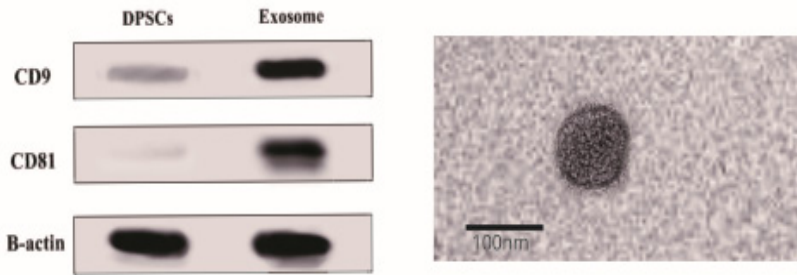
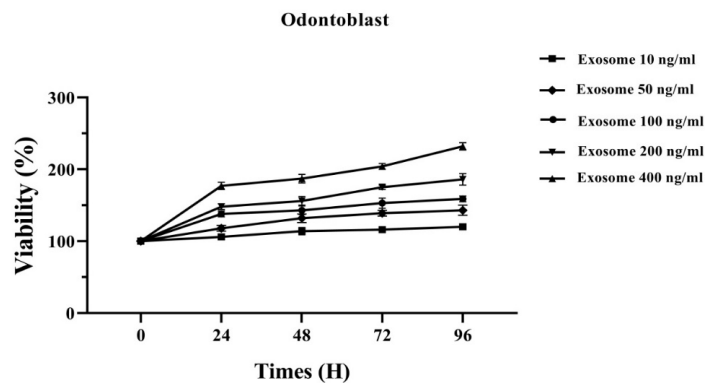


Fig 1. Western blot results (left) and SEM images (right)

A



B

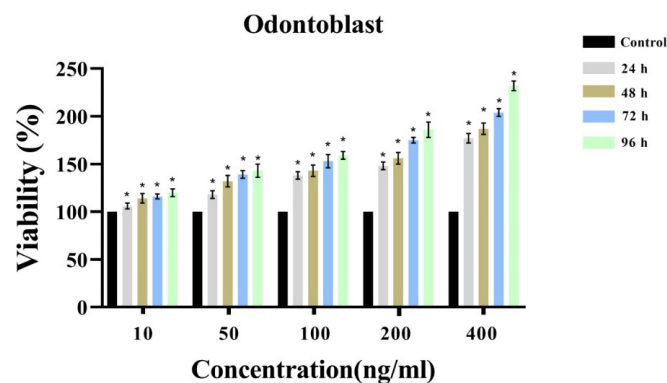


Fig 2. The results of MTT assay.

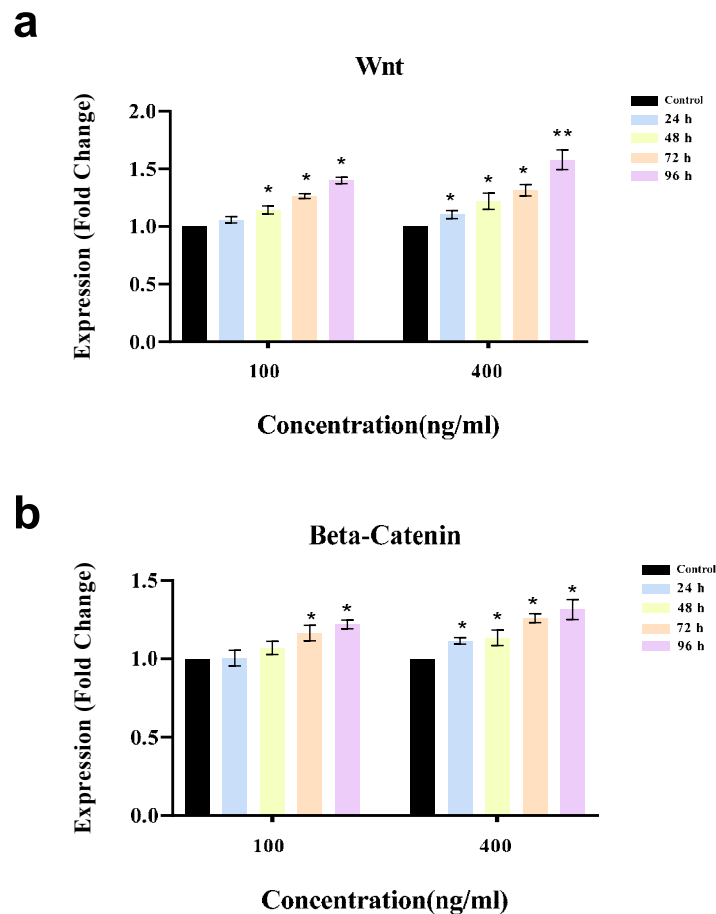


Fig 3. Wnt and Beta-catenin expression in odontoblasts.

exosome dosage.

Previous more extensive basic and preclinical research explored exosomes derived from dental pulp mesenchymal stem cells, finding they may serve as a promising novel therapeutic agent with potential clinical applications for treating various conditions, owing to their regenerative properties (8,19). Paracrine signaling mediated via secreted factors is a primary mechanism through which stem cells exert therapeutic effects. In this process, exosomes play an important role by facilitating intercellular communication involved in the paracrine actions of stem cells (7,20). Extracellular vesicles called exosomes that are secreted by mesenchymal stem cells through paracrine signaling carry proteins and nucleic acids shown to maintain tissue equilibrium and restore key cellular activities by activating repair and regeneration pathways. Research has demonstrated exosomal KLF3-AS1 enhances chondrocyte

proliferation and inhibits apoptosis via a miR-206/GIT1 axis, highlighting how these cargos modify recipient cell behaviors (21). Other studies have similarly indicated that exosome serves key roles in proliferation, migration and anti-senescence skin cells, making it an ideal option for wound healing and anti-ageing (22, 23, 24).

DPSC-exosome induced Wnt/Beta-catenin pathway

The effects of different concentrations of DPSCs-exosome (100 and 400 ng/ml) on the expression of Wnt and beta-catenin in odontoblast cells were evaluated over time periods of 24-96 hours according to Real-Time PCR results. The 100 ng/ml concentration of DPSCs-exosome did not impact Wnt expression within the first 24 hours compared to the control group, but increased its expression within 48, 72 and 96 hours of treatment (Fig. 3a) ($P < 0.05$). However, the 400 ng/ml concentration significantly boosted the expression of Wnt

throughout all the experimental periods (Fig. 3a) ($P < 0.05$). In addition, the 400 ng/ml concentration enhanced beta-catenin expression from 24 to 96 hours of incubation (Fig. 3b) ($P < 0.05$). Also, treatment with 100 ng/ml concentrations enhanced beta-catenin expression at 72 and 96 hours but not at 24 and 48 hours (Fig. 3b) ($P < 0.05$). The Wnt and beta-catenin expression in odontoblast cells were assessed upon treatment with 100 and 400 ng/ml concentrations of DPSCs-exosome within 24- 96 hours of treatment (Figs. 3a, b).

DPSCs-exosome induced PI3K/AKT pathway

Figure 4a shows real-time PCR analysis of PI3K expression in odontoblasts treated with 100 ng/ml DPSC exosomes from 24-96 hours. Based on the results, 100 ng/ml concentrations of DPSCs-exosome increased PI3K expression at 72 and 96 hours compared to control cells ($P < 0.05$), but

had no effect at 24 and 48 hours of incubation. Besides, Figure 4a shows 400 ng/ml DPSC exosome concentrations were able to up-regulate PI3K expression at 48, 72 and 96 hours of treatment ($P < 0.05$). Additionally, Figure 4b demonstrates 100 ng/ml DPSC exosome concentrations boosted Akt expression at 48, 72 and 96 hours but not at 24 hours ($P < 0.05$), while 400 ng/ml concentrations increased Akt expression from 24 to 96 hours of incubation ($P < 0.05$).

Recent studies demonstrated that exosomes derived from human embryonic stem cell-derived mesenchymal stem cells were able to reduce infarct size and improve cardiac function in mice with acute myocardial infarction by promoting cardiomyocyte proliferation (25). These effects are mediated by phosphorylation of Akt and GSK3 thus activating PI3K/AKT and GSK3 axes (25).

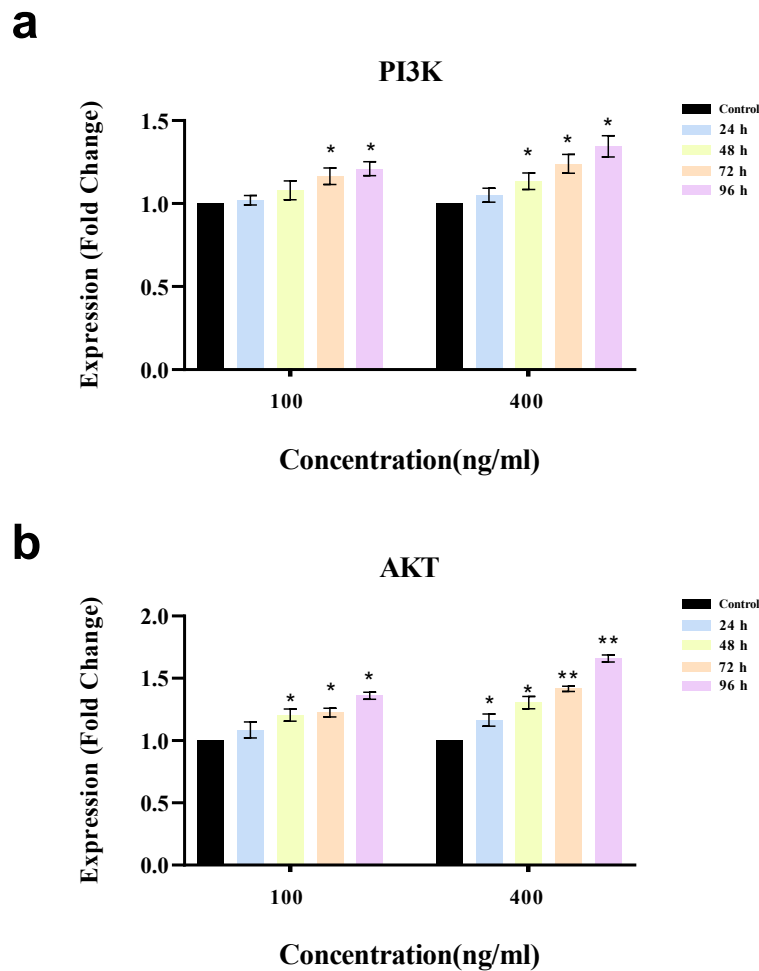


Fig 4. PI3K and AKT expression in odontoblasts.

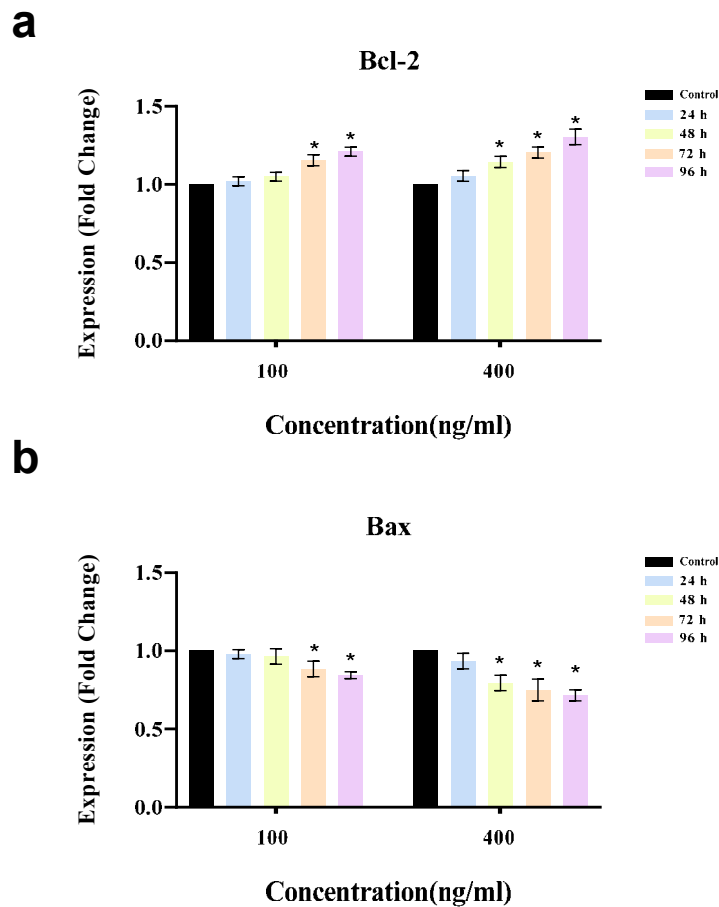


Fig 5. Bcl-2 and Bax expression in odontoblasts.

DPSCs-exosome increase BCL2/Bax ratio

Figures 5a and 5b show real-time PCR analysis of Bcl-2 and Bax expression in odontoblasts treated with 100 or 400 ng/ml DPSC exosomes from 24-96 hours. Figure 5a shows real-time PCR results of Bcl-2 expression in odontoblasts treated with 100 ng/ml DPSC exosomes from 24-96 hours. Based on these results, 100 ng/ml concentrations increased Bcl-2 expression at 72 and 96 hours compared to control cells ($P < 0.05$), but had no effect at 24 and 48 hours of incubation. Besides, Figure 5a shows that 400 ng/ml DPSC exosome concentrations up-regulated Bcl-2 expression at 48, 72 and 96 hours of treatment ($P < 0.05$). Additionally, Figure 5b demonstrates that 100 ng/ml DPSC exosome concentrations decreased Bax expression at 72 and 96 hours compared to controls ($P < 0.05$), but had no effect at 24 and 48 hours of incubation. Moreover, Figure 5b shows that 400 ng/ml concentrations of DPSC exosomes down-regulated Bax expression at

48, 72 and 96 hours of treatment ($P < 0.05$).

A previous study also demonstrated that exosomes could positively influence target cell survival and proliferation through downregulating PTEN, resulting in Akt activation and subsequent induction of Bcl-2 expression (26).

CONCLUSION

The current study revealed that DPSC-derived exosomes enhanced odontoblast viability potentially by activating Wnt/Beta-catenin and PI3K/AKT signaling, correlated with upregulating the Bcl-2/Bax ratio. These findings provided evidence supporting the notion that DPSC exosomes may be utilized as a treatment for dental tissue conditions linked to compromised or absent odontoblast function.

CONFLICT OF INTEREST

There is no conflict of interest in conducting or

reporting the results of this research work.

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