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ORIGINAL ARTICLE

Dental Pulp-Derived Mesenchymal Stem Cells Increase Axon Numbers in Mental Nerve Repair

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Abstract

Aim The mental nerve, the extended part of the inferior alveolar nerve, is often injured during dentoalveolar, orthognathic, or tumor surgery. Numerous therapeutic interventions, including surgery and pharmacotherapy, have been used to enhance the recovery of nerve injuries. Dental pulp stem cells (DPSCs) represent an easily accessible source of adult stem cells that can be isolated from the pulp of extracted teeth. This study evaluated the effect of DPSCs on the regeneration of the mental nerve injury model of rabbits.

Methods In this presented study, DPSCs were cultured and cell characterizations were performed by using flow cytometry and immunostainings. Bilateral mental nerve injury models of rabbits were created. In the control group (n = 10), saline was applied, and in the study group (n = 10), 2 × 106 DPSCs were applied to the repaired nerve areas. After 3 weeks, animals were killed and histological examination was obtained by using Masson's trichrome staining. An unpaired Student's t test was used when comparing the groups. Differences were considered to be statistically significant at P values of less than 0.05.

Results The DPSCs demonstrated a homogeneous population of mesenchymal stromal cells which expressed cluster of differentiation CD44, CD73, CD90, and CD105 and lack of CD34, CD45, and HLA-DR. Our finding clearly demonstrated that a lower number of cross-sectioned axons were founded in the control group (60.18 ± 2.52) compared to the study group (72.96 ± 2.43) (p = 0.00).

Conclusions DPSCs promote mental nerve axonal regeneration. These results suggest that DPSCs provide an important accessible source of adult stem cells for mental nerve regeneration.

Keywords Inferior alveolar nerve · Mental nerve · Peripheral nerve regeneration · Mesenchymal stem cells

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Introduction

Peripheral nerves may be damaged related to performing various procedures such as injection trauma or any other trauma to the nerve fiber that may cause temporary or permanent damage to the peripheral nerve. The mental nerve, the extended part of inferior alveolar nerve (IAN), is often injured during dentoalveolar, orthognathic, or tumor surgery [1]. Numerous therapeutic interventions, including surgery and pharmacotherapy, have been used to enhance the functional recovery of nerve injuries. The surgical interventions using microsurgery techniques have the advantage of considering improving the management of peripheral nerve injuries. Additionally, autografting of an intact nerve remains the gold standard for bridging a nerve gap defect for the peripheral nerve lesion [2]. However, there are some limitations of the autologous nerve grafting technique, including the limited number of donor nerves available, unesthetic scarring, wound infection, wound pain, relatively long surgical time, a learning curve for the success of nerve grafts, and poor regeneration. [2].

Currently, novel approaches are described as stem cell-based therapies, and their combination with tissue engineering strategies has the potential for addressing nerve regeneration and repair [3]. There are many sources of adult stem cells to use in clinical and experimental investigations [4] in peripheral nerve regeneration. Previous reports showed that bone marrow-derived stem cells (BMSCs) and adipose-derived stem cells (ADSCs) have positive effects on nerve healing [5, 6]. However, the invasive collections of these stem cell sources are an uncomfortable process for tissue donation.

Dental pulp-derived mesenchymal stromal cells (DPSCs) represent an easily accessible source of adult stem cells as they can be isolated from the pulp of extracted teeth [7]. DPSCs have the capacity to differentiate into neural lineages under controlled in vitro conditions [3, 8]. The proliferative and immunomodulatory properties of DPSCs are more pronounced compared with bone marrow-derived mesenchymal stem cells [9], which enforce their therapeutic potential. Furthermore, their neural crest origin has triggered extensive research on the neurogenic differentiation potential of DPSCs [10]. Specifically, DPSCs can express neural markers, generate neurotrophic factors, and axon guidance, and differentiate into functionally active neurons [11]. Additionally, DPSCs secrete various trophic factors that enhance peripheral nerve regeneration [12]. Therefore, recent reports showed the regenerative potential of DPSCs on facial peripheral nerve regeneration in the oral and maxillofacial regions [13].

Considering the regenerative potential of dental pulp-derived mesenchymal stem/stromal cells for peripheral nerve injuries, this study evaluated the regeneration of the mental nerve of rabbits after the transection injury model.

Materials and Methods

All experimental procedures were conducted in accordance with ARRIVE Guideline, the ethical guidelines of the International Association for Study of Pain in Animals, and were approved by the ethical committee of the Erciyes University Kayseri, Turkey. The groups were considered *control* and *study* groups.

Culture and Characterization of DPSCs

Human DPSCs were obtained from Erciyes University, Genome, Stem Cell Center and were cultured according to the previously described method [14]. Briefly, cells were thawed rapidly in a 37 °C water bath and seeded into culture plates under a 5% CO_2 humidified atmosphere. The complete cell culture media of the DPSCs was minimum essential media-alpha (α-MEM, Biological Industries, Israel), 20% Fetal Bovine Serum (FBS), 2 mM L-glutamine, and 100 U/ml antibiotics. Sub-cultured cells in the third passage were used in vivo experiments. Phenotyping was carried out by flow cytometry-specific antibodies for the cluster of differentiation (CD) markers including CD34, CD45, HLA-DR, CD73, CD90, CD44, and CD105. Immunocytochemistry was performed against CD44, CD105, and CD34. The data were analyzed with KALUZA software (Beckman Coulter, the USA). More than 80% of staining was regarded as positive.

Animals and Surgical Procedure

10 male, New Zealand White rabbits (3.1–3.3 kg) were anesthetized with intramuscular xylazine 1 mg/kg (Ketalar 50 mg/mL; Pfizer Inc, New York, NY) and 0.5 mg/kg (Rompun 23.32 mg/mL; Bayer, Mefar Ilac¸ San AS, Istanbul, Turkey). Articaine solution of 0.2 mL was injected subcutaneously as a local anesthetic agent. The mental nerve was exposed following a longitudinal 1-cm dermal incision made through extra-oral submandibular incitation and performing careful dissections. After the mental nerve was exposed (Fig. 1), the nerve was transected in order to mimic an injury bilaterally. A microsurgical repair was then immediately achieved. In the *control group*, 200 µl sterile saline was, and in the *study group,* 2×10^6 DPSCs/ 200 µl was applied by using a 33-gauge blunt tip needle along the proximal and distal nerve stumps immediately to the repaired nerve area. Then, the skin was closed primarily. All animals were maintained in individual cages under 12 dark/light cycles and fed ad libitum. Three weeks after the operation, all animals

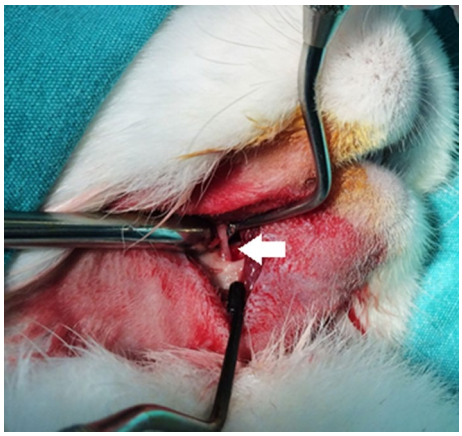

Fig. 1 White arrow indicates *N. mentalis* in rabbit animal model

were killed. Nerve samples were harvested and fixed in 10% formalin for histological analysis.

Histopathologic Evaluation

The study included formalin-fixed, paraffin-embedded nerve samples from the control and experimental groups. For histopathologic evaluation, routine paraffin embedding procedures were used. In brief, the tissue samples were fixed in %10 formalin solution, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin wax and 5-µm-thick sections were cut. The sections are stained with Masson's trichrome. Photographs were taken with an Olympus BX51 light microscope (Olympus BX51, Tokyo, Japan) photomicroscope to evaluate a morphological overview of the nerve tissues.

Quantitative Histomorphometry

For evaluation to associate with a number of cross-section nerve fibers in experimental groups, we captured random fields of least five in areas of each experimental group in the same high magnification (×100 magnification). To quantitative histomorphometry of carefully defined reference areas counted the number of total axons with the Image J software program [15]. All the preparations were blindly appraised by two histologists.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.00 for windows (GraphPad Software, San Diego California, the USA). An unpaired Student's t test was used when comparing the groups. Differences were considered to be statistically significant at *P* values of less than 0.05.

Results

All 10 rabbits tolerated the surgical procedure well. There was no complication or adverse reaction to human DPSCs.

Isolation and Characterization of Stem Cells

Human DPSCs, which were isolated from the dental pulp of a wisdom tooth, were easily cultured in α-MEM supplemented with 20% FBS. At the end of passage 3, DPSCs showed spindle-like morphology and phenotype characterization of the DPSCs demonstrated a homogeneous population of cells that expressed CD 90 (99.94%); CD44 (99.52%); CD73 (98.10%) and CD105 (99.87%). DPSCs were found negative for CD34 (1.17%); CD45(1.80%) and HLA-DR (0.12%) (Fig. 2). In addition, the results of immunofluorescent staining showed CD44 and CD105 positive cells and negative for CD45 (Fig. 2). These results confirmed the cells prevent their stem cell properties before the in vivo application.

Histologic Findings

In order to investigate the overall structure of the nerve, Masson's trichrome-stained histologic sections were assessed. Nerve tissue consisted of a vast number of axons held together by connective tissue in the study group. In the control groups, the well structure of the nerve was not observed. Microscopic examination of the nerve showed degenerated axons and axonal connective tissue loss between axons in the control group. Furthermore, we investigated the influence of DPSCs on the number of total axons. Our finding clearly demonstrated that a lower number of cross-sectioned axons were founded in the control group (60.18 ± 2.52) compared to the study group (72.96 ± 2.43) ($p = 0.00$). But there were also fewer fibers in the control group (Fig. 3).

Discussion

The etiology of nerve injuries that develop after operations performed under peripheral nerve block includes direct needle trauma; and intra-neural injection of local anesthetic neurotoxicity [16]. IAN or the branch *N. mentalis* injuries generally occur during mandibular fractures or oral surgical procedures such as third molar extractions or dental implant placements, and complications may occur including dysesthesia, allodynia, and hyperalgesia [17]. There are several attempts to enhance peripheral nerve regeneration; however, the repair and healing results of these approaches still remain insufficient [18].

There are many stem cell sources for the repair and regeneration of injured peripheral nerves [10]. The dental

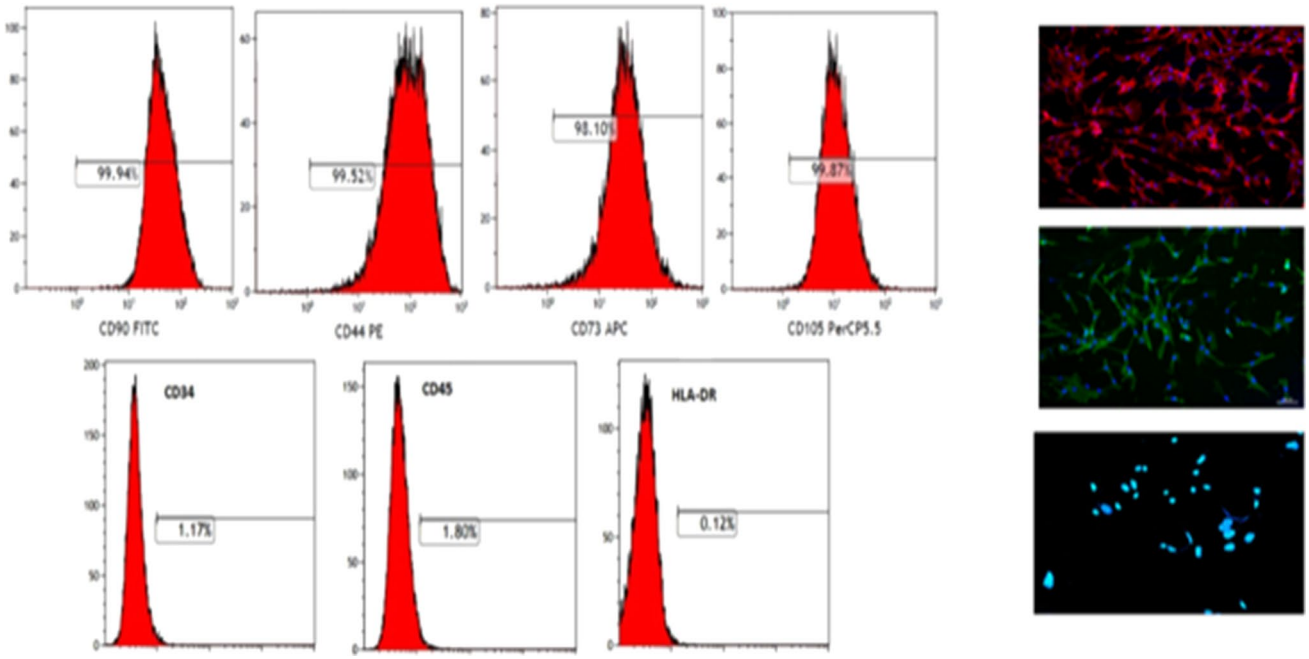

Fig. 2 DPSCs were positive expressed CD90, CD105, CD44, CD73 however negative for CD34, CD 45 and HLA-DR according to Flow cytometry analyses; CD44 positive Texas Red (red), CD105 positive FITC (green), CD45 negative FITC according to Immunocytochemistry

pulp is derived from the neural crest, and DPSCs are easily isolated from discarded teeth following extraction without any further surgical necessity. The promoting effects of DPSCs on nerve regeneration such as the replacement of lost cells by differentiating into mature oligodendrocytes, antiapoptotic effect on neurons, astrocytes, and glial cells, and acceleration of migration and differentiation of neuronal progenitor cells have been demonstrated in the central nerve lesion (spinal cord injury model and focal cerebral ischemia model) [19]; moreover, transplantation of DPSCs has been reported to promote peripheral nerve regeneration such as facial nerve regeneration [17]. However, the in vivo neurogenic property of hDPSCs has not been studied yet on mental nerve regeneration. Considering their origin, dental stem cells inherit some typical traits of neural cells. It has been reported that dental stem cells can express specific neural markers, such as nestin, s100-beta, and GFAP [19]. Ullah et al. (2017) reported that stem cells from three different dental tissues including dental follicle, pulp, and root apical papilla showed excellent in vitro neurogenic differentiation with positive nerve conduction ability. In addition, some other researchers have reported the positive nerve regeneration activities of hDPSCs [20]. According to our study results, DPSCs may have a significant effect on axon number in regeneration and a higher number of cross-sectioned axons were found in a study group ($p = 0.00$). Allogenic DPSCs were transplanted to the nerve repair area without any neuronal differentiation in vitro. It may provide an economically and biologically feasible method for urgent clinic usage. Further studies may be planned on the usage of DPSCs in different dosages.

The mental nerve is easy to locate and expose in rabbits. Most of the sensory neurons of the inferior nerve are localized in the trigeminal ganglion, and 65–70% of the axons are distributed to the mental nerve. The nerve transection model of *N. mentalis* was created to simulate surgical trauma, and DPSCs were applied to the nerve at the time of repair. When the stem cells are transplanted, they can differentiate and support regeneration [21]. Moreover, during axonal growth, neovascularization is an important prerequisite step. Schwann cells and axons extend together with angiogenesis, which precedes axonal regeneration. Blood vessel regeneration increases axonal regrowth and Schwann cell proliferation in the presence of VEGF [22]. Yamamoto et al. reported that DPSCs may support angiogenesis by secreting VEGF [23].

Li et al. reported human periodontal ligament stem cells showed a potential value in the repair of mental nerve injury [21]. However, DPSCs have some advantages over nerve regeneration compared to other stem cell sources because dental pulp tissue includes neuronal tissues that originated from the neural crest, which may make DPSCs have higher neurogenic differentiation potential than other MSCs [24]. Neural crest easily facilitates differentiation of DPSCs into neuronal cells as compared with other cell lines, and DPSCs can activate microglia/astroglia in the host microenvironment, which in turn enhances Wallerian degeneration and

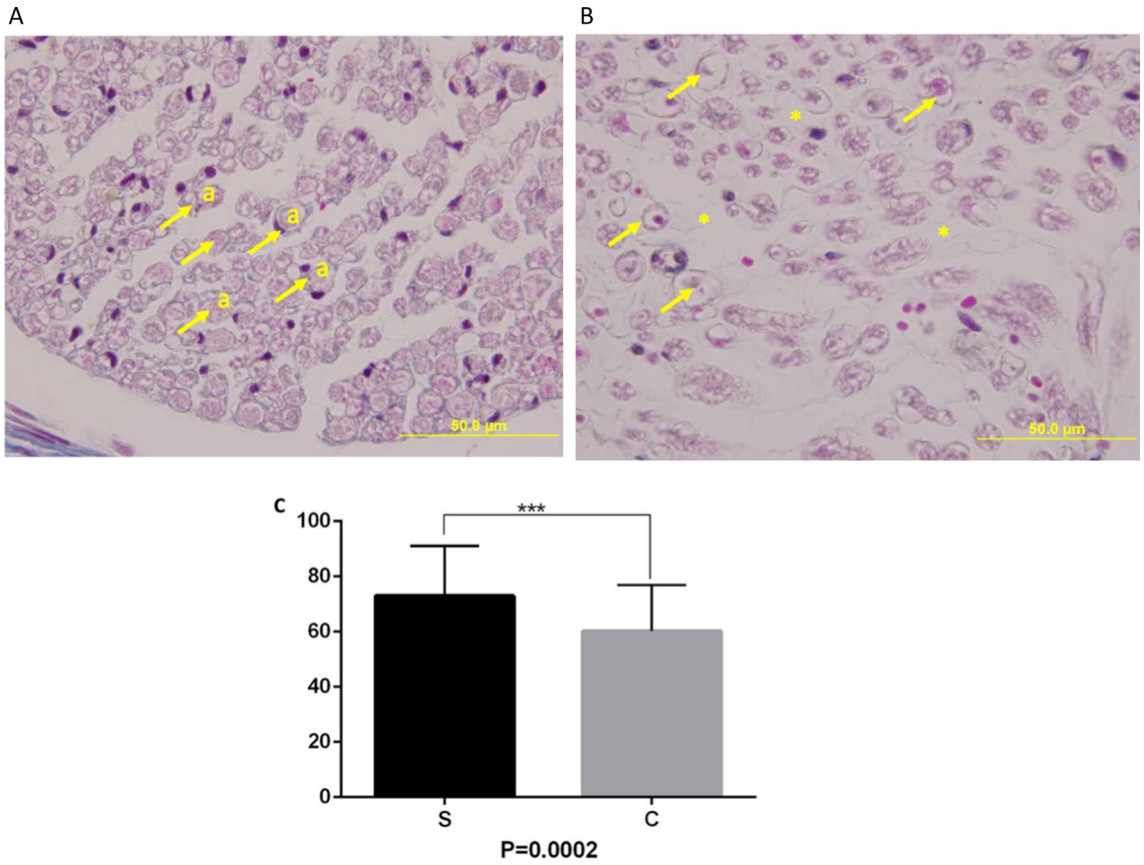

Fig. 3 Light microscopic appearance of sections of nerve in experimental groups **A** High power photomicrograph from study group. The axons (**a**) appear clear and the myelin is represented by surrounding the axons (**arrow**; myelin sheath). The connective tissue has been stained blue. It forms a network around the myelinated axons. **B** In the control group showed degenerated axons (**arrow**) and axonal connective tissue loosing (**star**) between axons. Masson's trichrome, original magnification × 100. **C** Graph showing quantification of experimental groups. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

macrophage invasion, thereby accelerating peripheral nerve regeneration [20]. Farther, a recent report from Omi et al. (2017) highlighted the contribution of DPSCs in ameliorating long-term diabetic polyneuropathy in rats; indeed, injected hDPSCs were able to improve the impaired sciatic nerve blood flow, to increase the sciatic motor-sensory nerve conduction speed and to increase the capillary number-to-muscle and intra-epidermal nerve fiber density ratio [25].

There are several limitations in this study; firstly, we did not evaluate the influence of DPSCs at different time points on the recovery of nerve injuries. Second, we did not use different doses of DPSCs. Third, we chose only Masson trichrome technique to analyze the axon numbers. Specific histological and immunohistological techniques are still needed to test their effects on myelination and fascicular organization of the mental nerve. Neurophysiological and behavioral studies may be further to test the effects of DPSCs on neurosensory function.

Translation of stem cell studies to clinical settings has been challenging. Although, there has been the necessity of a special laboratory environment such as the Good Manufacturing Practices (GMP) Laboratory for the clinical translation of MSCs, recently, more than 47,548 patients have been enrolled in clinical trials in different indications including nerve injuries [26].

Based on this present study, DPSCs increase mental nerve axon counts following transection and immediate repair of the mental nerve in rabbits. It may represent a novel therapeutic target to support regeneration after *N. mentalis* peripheral nerve injury and repair.

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Declarations

Conflict of interest Authors declare that there is no conflict of interest.

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