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A Preclinical Study on the Applicability of Human Dental Pulp Stem Cells

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1 Chapter 1: Introduction

1.1 Overview of stem cells

Stem cells are specialized cells that are characterized by their long-term persistence within the body. They possess the capacity for self-renewal and rapid proliferation, along with the capability to differentiate into mature progeny cells under certain conditions. Multiple sources classify stem cells into two categories: embryonic stem cells and adult stem cells. Ethical and practical operational challenges are inherent in the study of embryonic stem cells. In contrast, adult stem cells possess characteristics such as a diverse array of origins, strong plasticity, and less immunological rejection. Furthermore, in recent years, they have garnered growing scholarly attention. Numerous studies have detected adult stem cells in several tissues of the human body, such as the brain, bone marrow, blood vessels, bone marrow muscle, skin, and liver. Could adult stem cells perhaps be present in the oral cavity as well? e.g. teeth. In theory, odontogenic stem cells are present during every stage of tooth development, and these stem cells adhere to specific developmental programs and processes in order to undergo differentiation into teeth. Human Dental pulp stem cells (hDPSCs) are found in the pulp tissue of adult teeth. These cells possess not only equivalent capacity for proliferation and differentiation as all other adult stem cells, but also provide the benefits of abundant sources, easy sampling, and less immunological rejection. Owing to these benefits, hDPSCs have emerged as a kind of seed cells with significant study potential[1] and have garnered increasing interest from tissue engineering experts.

1.1.1 The history and definition of dental pulp stem cells

Abundant sources of stem cells in the oral cavity include mucosal soft tissues, periodontal ligaments, and the tissue innervated by the blood vessels and nerves inside each tooth, known as dental pulp. During craniofacial development, ectodermal mesenchymal cells are the cells of origin of dental pulp tissue. Human dental pulp stem cells (hDPSCs) have similar plasticity to neural crest-derived stem cells and are capable of undergoing differentiation into neural and ectodermal mesenchymal progenitor cells. The initial isolation of stem cells from dental pulp tissue by Gronthos et al.[2] in 2000 marked a significant milestone in the field of hDPSCs research. At present, it is considered that hDPSCs are fibroblast-like cells that are uniformly present in dental pulp tissue and have the biological characteristics of stem cells. hDPSCs have many benefits in comparison to others[3]: 1) The presence of numerous sources and convenient collection methods can minimize the possibility of complications. 2) It possesses

immune and anti-inflammatory properties, which make it suitable for the study and implementation of allogeneic transplantation. 3) Under specific circumstances, it has a significant capacity to promote the formation of enamel and dentin cells. 4) hDPSCs obtained from impacted teeth can be isolated and expanded *in vitro*, and simultaneously exhibit robust proliferation and differentiation.

1.1.2 Culture and molecular markers of hDPSCs

1.1.2.1 hDPSCs culture

The hDPSCs content in dental pulp tissue is extremely low. To get an adequate number of cells for application requirements, *in vitro* expansion techniques must be employed to address this issue[4]. The most common dissociation methods of hDPSCs include enzymatic digestion, tissue explant outgrowth, and modified tissue explant enzyme digestion based on the two methods. Fetal bovine serum (FBS) is the most commonly used in cell culture medium that supplies essential constituents like nutrients, growth factors, and hormones for cell proliferation, migration, and differentiation. However, the application of FBS may give rise to ethical, scientific, and safety concerns. Recently, several studies have substituted FBS with concentrated platelet lysate and platelet-rich plasma for the purpose of expansion of stem cells *in vitro*[5, 6]. However, the lack of clarity in the chemical composition and the need for a substantial quantity of peripheral blood in the procedure prevents the fulfillment of the desire for large-scale expansion. Utilizing their comprehensive knowledge of hDPSCs and the advancements in serum-free culture technology, several studies have embraced this method to cultivate hDPSCs *in vitro*. Comparing with the conventional culture using serum, the hDPSCs cultivated without serum had a fairly comparable shape, but with a slenderer and three-dimensional appearance. The cultivated cells possess the capacity to generate clones, which aligns with the characteristics of hDPSCs[7].

1.1.2.2 Molecular markers of hDPSCs

The hDPSCs closely resemble fibroblasts in cell culture, characterized by a small number of cells and lack of clear surface molecular markers. Generally, hDPSCs are identified by analyzing the cell surface markers before and after directional differentiation into odontoblasts. Today, the study on surface markers of hDPSCs is in its early stages, mostly depending on conventional mesenchymal stem cell markers like CD13, CD44, CD90, and CD105. hDPSCs are mostly recognized by using Stro-1 and CD146. Multiple studies have demonstrated that Stro-1 is effective in the identification of hDPSCs, and the involvement of

bone morphogenetic protein-2 (BMP2) enhances this mechanism [8]. In other studies, it has been demonstrated that CD16, an antigen found on the surface of peritubular endothelial cells, is also expressed on the surface of hDPSCs[2]. In the future, it is imperative to conduct further research on the molecular markers of hDPSCs.

1.1.3 Biological characteristics of hDPSCs

1.1.3.1 The high proliferation and self-renewal ability of hDPSCs

hDPSCs possess a strong ability to self-proliferate as adult stem cells. An *in vitro* comparative study was conducted on the proliferation of the two (hDPSCs and BMSCs) after bromodeoxyuridine was added to the culture medium of the first-generation hDPSCs and bone marrow mesenchymal stem cells. It was found that the proliferation rate of hDPSCs was significantly higher than that of bone marrow stromal cells (BMSCs). Moreover, it was shown that hDPSCs retained a significant capacity for proliferation in following passages, therefore confirming the high proliferation potential of hDPSCs[2]. Guo et al. [9] used the method of single-cell cloning to isolate and culture rat hDPSCs. Following co-culturing hDPSCs with hydroxyapatite (HA)-tricalcium phosphate (TCP) cell scaffold to a certain extent, they were then transplanted under the skin of nude mice and histological examination was performed after a period of time. The tissue slice of the graft revealed the presence of a dentin-pulp complex-like structure, which further confirms the proliferation and differentiation capacity of hDPSCs. Gronthos et al.[10] implanted hDPSCs subcutaneously into immunodeficient mice aged 10 weeks by using HA/TCP ceramic particles as carriers. Expression of dentin-pulp-like complex and dentin sialophosphoprotein (DSPP) was seen after a period of 6 weeks. Upon detecting the isolated bone marrow stromal cells (MSCs) using a human aluDNA probe, it was determined that the majority of the MSCs showed positive results. Following the in vitro expansion of MSCs and subsequent retransplantation into nude mice, the resulting odontoblasts also expressed human-specific Alu-inserted genes. The positive reactivity for human dentin sialin confirmed the self-renewal capacity of hDPSCs.

1.1.3.2 Evaluation of pluripotency in hDPSCs

hDPSCs are a type of pluripotent stem cells that have the remarkable ability to differentiate into different types of cells under specific conditions. Current studies have confirmed that hDPSCs have the ability of differentiation into odontoblasts, osteoblasts, chondrocytes, adipocytes, nerve cells, vascular endothelial cells, and so on. hDPSCs differentiated into

odontoblasts, osteoblasts and adipocytes under certain induction conditions, and expressed the corresponding markers. For example, after 5 weeks of adipogenic induction of hDPSCs, positive lipid droplet aggregation was observed through Oil Red O staining, indicating the presence of adipocytes. In a study conducted by Zhang et al.[11], it was found that after 4 weeks of co-culturing human dental pulp cells with porcine dental epithelial cells, the expressions of type IV collagen, amelogenin, dentin sialophosphoprotein, and laminin 5 were observed. Additionally, the formation of irregular mineralized tissues was observed. Ishkitiev et al. [12] reported that hDPSCs possess the capacity of differentiation into hepatocytes. They cultured hDPSCs *in vitro* by using the cell culture medium containing hepatocyte growth factor, dexamethasone, insulin-transferrin-selene-X and onostatin and observed that the cells expressed hepatocyte-specific molecular markers. Additional studies are necessary to ascertain the potential of hDPSCs for differentiation into other cell types and to identify the specific induction conditions.

1.1.3.3 Paracrine effect

hDPSCs have been observed to secrete a series of cytokines through paracrine effects *in vivo[13-19]*, such as chemokine stromal cell-derived factor-1 (SDF-1), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), granulocyte-colony stimulation factor (G-CSF) and stem cell factor (SCF). These cytokines have been found to promote angiogenesis, inhibit apoptosis, and protect regenerated tissues. Song et al.[20] demonstrated that hDPSCs exhibited superior cytoprotection to astrocytes in an ischemic animal model. Implanting hDPSCs into the rodent brain within 24 h after focal cerebral ischemia resulted in a significant improvement in the neurological behavior and sensorimotor function of the contralateral forelimb after 4 weeks. Despite a low survival rate of approximately 2.3%, the implanted hDPSCs demonstrated the ability to migrate towards the ischemic area of the brain and undergo differentiation into astrocytes or nerve cells. They proposed that this improvement in function was not due to neural replacement, but rather relied on the paracrine effect of hDPSCs [21]. hDPSCs can also induce functional neovascularization in hindlimb ischemia in mouse models [22].

1.1.4 Application of hDPSCs in tissue regeneration

1.1.4.1 Repairing damaged dental pulp

Studies have found that although hDPSCs can form a dental pulp-dentin complex, they lack

dentinal tubules [2, 10]. To induce differentiation of hDPSCs into odontoblast-like cells and the formation of mineralized hard tissue, several circumstances must be met, including the existence of healthy and undamaged dentin [23]. Iohara et al. [24] co-cultured canine hDPSCs and adipocytes *in vitro* with cell culture medium containing with IGF and EGF, and mixed with collagen containing SDF-1. They then transplanted these cells into the incisors, where the dental pulp tissue had been excised. Following a period of 2 weeks, the presence of pulp-dentin-like complexes associated with the development of blood vessels and nerves was detected. According to Akai et al. [25], the artificial dental pulp regenerated using a poly-L-lactic acid scaffold for hDPSCs growing exhibited the formation of new dentin. In the future, it is anticipated that using hDPSCs to regenerate the dental pulp-dentin complex for the purpose of repairing injured dental pulp would become a stable and efficient therapeutic approach.

1.1.4.2 Reconstruction of Bone Defects

The differentiation of hDPSCs into osteoblasts can be achieved by introducing an osteogenic induction medium that includes dexamethasone, β-glycerophosphorus sodium, ascorbic acid, and DMEM during the culture procedure, which is a potential source of osteocytes in bone regeneration and repair. A combination of platelet-rich plasma and hDPSCs was implanted into the maxillary defect of dogs by Ito et al. [26]. Following an 8-week period, implants were placed and subsequently cultivated for a further 8 weeks. The findings indicated that there was not only the development of bone tissue around the implant, but also an enhancement in the binding competence with the adjacent bone tissue. By co-culturing human hDPSCs with silk fibroin scaffolds, Riccio et al.[27] effectively restored significant parietal bone defects in rats. This presents a highly promising reconstruction model for the future restoration of diverse forms of bone tissue defects.

1.1.4.3 Construction of tissue-engineered teeth

The rapid development of stem cell and tissue engineering technology in recent years has fostered optimism for tooth regeneration. The currently established experimental methods for tooth regeneration are as following[28]: 1) The tooth germ or cells are initially implanted into the living tissue. Once the tooth crown is formed, it is then implanted into the jaw bone to complete the tooth formation process. 2) After hDPSCs are first cultured, induced and expanded *in vitro* for a period of time, these cells are then implanted into the alveolar bone or other areas of the body to facilitate the growth of a complete tooth. Ibarretxe et al.[28]

conducted a study where they chose mouse teeth in the developmental cap stage. They isolated odontogenic epithelial cells from these teeth and subsequently co-cultured them with hDPSCs *in vitro*. The tooth germs were subsequently implanted into the mouse jaw. Upon further investigation, it was discovered that they seamlessly integrated with the alveolar bone and functioned like natural teeth. As research progresses, the development of *in vitro* tissue-engineered teeth has emerged as a promising field. This advancement holds immense importance in enhancing the quality of life for individuals with tooth loss.

1.1.4.4 Treatment of neurodegenerative diseases

Ibarretxe et al.[29] reported the advantages of hDPSCs in the treatment of Parkinson's disease: hDPSCs are easy to obtain and are autologous cells, thus minimizing the risk of immune rejection and inflammatory responses when utilized; hDPSCs exhibit the expression of glial cell and nerve cell markers; hDPSCs demonstrate electrical activities akin to neurons, such as expressing receptors of nerve cells and generating action potentials; hDPSCs have the ability to grow and survive in the host's neural tissue; hDPSCs can play an immunomodulatory role in the survival of adjacent cells by secreting cytokines. However, the extent to which hDPSCs can differentiate into neural stem cells and establish functional synaptic connections with host neurons when transplanted into neural tissue requires further investigation.

1.1.4.5 Application in muscle repair and reconstruction

Duchenne muscular dystrophy (DMD) is a severe muscular atrophy caused by the lack of functional structural protein dystrophin, resulting in restricted mobility. Typically, individuals succumb to myocardial and pulmonary muscle weakness at approximately 20 years of age, resulting in fatality. The study conducted by Chen et al.[30] demonstrated that the suppression of miR-143 expression greatly increased the expression of muscle differentiation factors and fast myosin heavy chain genes, and deduced that miRNAs play a role in controlling the expression of various muscle type functions in vertebrates. Li et al.[31] observed that the expression of miR-135 and miR-143 in hDPSCs was dramatically suppressed after hDPSCs were treated with 5-aza-2'-deoxycytidine. Additionally, hDPSCs exhibited notable myogenic properties and the development of myotubes. It is concluded that miRNAs play a decisive role in the process of inducing myogenic differentiation of hDPSCs.

1.1.4.6 Application in corneal repair and reconstruction

Corneal blindness is a severe ocular condition wholly causing vision loss. Currently, the

primary therapeutic approach is allogeneic corneal transplantation. The limited availability of donor sources and the manifestation of immunological rejection reactions restrict its clinical use.

Tissue-engineered corneal construction currently stands as a prominent research focus within the field of ophthalmology, with the selection of ideal seed cells being essential to the endeavor. Monteiro et al.[32] reported that human dental pulp stem cells (hDPSCs) can sustainably express limbal stem cell (LSC) specific markers in vitro. Subsequently, their team implanted tissue-engineered immature hDPSCs grafts directly onto the exposed stromal matrix of the cornea in rabbits with alkali burns. Over 1 to 12 weeks, the corneal transparency of the experimental animals gradually improved. Histological analysis revealed well-formed stromal layer structures and multi-layered epithelial formation at the site of the corneal burns, demonstrating that the engraftment of immature hDPSCs could promote the functional restoration of the corneal epithelial tissue in alkali-burned animals[33]. Kushnerev et al. [34] successfully transplanted hDPSCs onto the corneal surface using a corneal contact lens as a carrier and detected the expression of corneal epithelial markers CK3 and CK12. This further confirms the potential of hDPSCs to differentiate into corneal epithelial cells. Additional research[35] suggests that hDPSCs can promote the growth and regeneration of corneal epithelial cells and possess capabilities to prevent corneal conjunctivalization and maintain corneal transparency.

1.2.1 Cryopreservation of Human Pluripotent Stem Cells

1.2.1.1 Principles of Cryopreservation

The process by which cells, tissues, or organs are frozen at temperatures below -80°C, especially below -140°C, while ensuring their viability is referred to as cryopreservation[36]. Lowering the temperature affects the biochemical processes of cells. When cells are preserved long-term in liquid nitrogen (-196°C), the metabolism of cellular enzymes is inhibited, almost completely halting. Upon rewarming, cells regain their metabolic capabilities and continue their biochemical processes.

1.2.1.2 Common Cryopreservation Methods

One of the primary threats to cells during cryopreservation is damage to organelles and cell membranes caused by ice crystal formation at low temperatures. To prevent internal and external ice crystal formation, commonly used methods for the cryopreservation of human pluripotent stem cells include slow-freezing and vitrification.

Slow-freezing is a classical method of cryopreservation[37]. In slow-freezing, water outside the proximity of the specimen crystallizes slowly and orderly, gradually extracting water from within the cells and subjecting the specimen to extremely high osmotic pressures to prevent ice formation. The main steps of the slow-freezing method are[38]: ①collection of cells, ② addition of cryoprotectant, and ③ gradual solidification of the cell suspension at a controlled cooling rate (from -1 to -10°C/min). (④Long-term low-temperature storage of cells (typically in liquid nitrogen). ⑤Rapid thawing, with the cell suspension in a 37-40°C water bath. ⑥ Removal of the cryoprotectant by centrifugation. ⑦ Inoculation and cultivation of cells under appropriate conditions. Although the slow-freezing method is effective for various types of adult cells, hematopoietic cells, human mesenchymal stem cells, and even mouse embryonic stem cells, it is not suitable for the cryopreservation of human pluripotent stem cells due to its low recovery rates and high differentiation rates[39-41].

Vitrification was first used for cryopreserving bovine oocytes and embryos[42], and later applied to human pluripotent stem cells. This method involves sequentially immersing cell clusters in two cryoprotectant solutions with gradually increasing concentrations, where cells undergo brief and continuous exposures (37°C or room temperature for 60 seconds and 26 seconds, respectively). The primary components of the vitrification solution are dimethyl sulfoxide (DMSO) and ethylene glycol, with sucrose concentration varying according to the medium. For example, Reubinof et al[40] used a vitrification solution consisting of 20% DMSO, 20% ethylene glycol, and 0.5 mol/L sucrose for human pluripotent stem cells.

In vitrification, a high concentration vitrification solution and a rapid cooling rate prevent the formation of ice crystals, instead forming a glass-like state. The formation of this glassy state requires a sufficiently rapid cooling rate. To achieve a sufficiently rapid cooling rate, droplets of the cryoprotectant solution containing clusters (fewer than 10) are placed on the open tip of a micropipette and then directly immersed into liquid nitrogen[43, 44]. During the thawing process, to prevent the reformation of ice crystals, the warming rate must also be as rapid as possible. This can be accomplished by immersing the frozen clusters directly into a pre-warmed medium containing sucrose, using sucrose as an osmotic buffer to gradually wash away the cryoprotectant[45].

Comparison of Slow-Freezing and Vitrification Methods for Human Pluripotent Stem Cells: Some studies have reported that the recovery rate of vitrified human pluripotent stem cell clusters exceeds 75%, whereas programmed freezing yields only a 5%-10% recovery rate, leading many laboratories to adopt vitrification as their preferred method for cryopreserving human pluripotent stem cells[46, 47]. However, vitrification has its limitations: it requires

significant manpower and high technical skill from operators; it is not suitable for the large-scale cryopreservation of cells; and direct contact of cells with liquid nitrogen also poses a risk of contamination[48]. Many researchers are continually attempting to overcome these limitations.

The studies by Li et al[46] compared three cryopreservation protocols for human pluripotent stem cells, including slow-freezing using cryovials, and vitrification using both plastic straws and slow-freezing methods. The efficiency of the three cryopreservation protocols was evaluated by assessing the number of adherent undifferentiated clones on days one to two and seven to eight post-thaw. The findings indicated that vitrification had the highest efficiency (recovery rates of 80%-90%), while both slow-freezing methods were less efficient and not suitable for the cryopreservation of human pluripotent stem cells. Using a programmable freezer significantly improved the efficiency of the slow-freezing method (the recovery rate increased to 50% from 5%). Aside from slow-freezing using cryovials, the other two methods did not affect the pluripotency or the karyotype normality of the cells post-thaw. Another study from the same period reported that although using a programmable freezer improved the efficiency of slow-freezing (from 4%-8% to 10%-20%), it was still insufficient for the cryopreservation of human pluripotent stem cells. This study identified optimal cryopreservation conditions: gradually increasing concentrations of dimethyl sulfoxide and Serum Replacement in a series of cryoprotectant solutions, with human pluripotent stem cell clusters progressively transferred to these solutions prior to freezing; a reverse process was used for thawing to gradually replace the cryoprotectant. This approach, combining a programmable freezer and a stepwise method, yielded a cryopreservation efficiency of 30%-50%[49].

1.2.1.3 Cryogenic Damage

During cryopreservation, exposure to low temperatures can cause cellular damage or even death. Cryogenic damage can occur singly or in combination at one or more of the following stages: ①cytotoxic damage from cryoprotectants[50, 51], ②osmotic damage from cryoprotectants during the freezing and thawing process [52], ③mechanical damage from intracellular ice crystal formation[53], and ④ mechanical damage from recrystallization within cells during thawing [54]. In programmed freezing, rapid phase transitions between solid and liquid phases in extracellular solutions are primarily used to reduce such damage, whereas vitrification prevents ice crystal formation through the use of high concentrations of cryoprotectants, thus protecting cells from damage.

Cooling rate is another variable in the cryopreservation process that is associated with cellular damage. In vitrification, the cooling rate is very fast, leading to the glass-like solidification of both intracellular and extracellular environments without ice crystal formation, or only very small crystals form, which do not cause significant damage to cell membranes and organelles. Cells are also not exposed to high solute concentrations for extended periods, avoiding damage. In slow-freezing, if the cooling rate is too fast, ice crystals form before complete cellular dehydration, destroying cell organelles and membranes and causing cell death. Conversely, if the cooling rate is too slow, cells dehydrate completely due to osmotic forces, leading to cellular shrinkage and death. When the cooling rate is set within a range that both prevents the formation of intracellular ice crystals and mitigates severe cellular dehydration, cell damage can be avoided. This optimal cooling rate is referred to as the "recovery range" or "recovery window." Most eukaryotes, when not treated with cryoprotectants, either do not exhibit a recovery window or it is difficult to observe. Cryoprotectants minimally prevent the formation of intracellular ice crystals; rather, their primary function is to prevent or reduce dehydration and shrinkage during the slow freezing process[55]. Hence, regardless of the use of cryoprotectants, strict control of the cooling rate is crucial to reduce cellular damage during slow freezing. The use of a programmable cooling device allows for controlled cooling rates, which is more reliable and repeatable technologically. Some experiments have investigated the correlation between programmable cooling devices and the cryopreservation of pluripotent stem cells. According to Ware et al.,[56] using a programmable cooling device with dimethyl sulfoxide as the cryoprotectant and wheat straw as the carrier, the freezing efficiency was between 60% and 70%, with no increase in differentiation rates. This study identified three key factors for successful cryopreservation of human pluripotent stem cells:①maintaining the temperature above the point where intracellular ice formation occurs but below the extracellular freezing point (-7°C to -12°C); ②an appropriate cooling rate (0.3°C to 1.8°C per minute).③Rapid thawing (at temperatures between 25-37°C). Another study described an improved protocol: samples were cooled from 0°C to -35°C at a rate of 0.5°C per minute, with the temperature maintained below -10°C before immersion in liquid nitrogen or rapid thawing. This approach achieved a recovery rate of 80%[57].

1.2.2 Improvements in Human Pluripotent Stem Cell Cryopreservation Techniques

Over the past few decades, numerous laboratories have dedicated efforts to enhance the cryopreservation efficiency of human pluripotent stem cells, including using various cryoprotectants or molecular substances, employing animal-free cryoprotective solutions, and

freezing either adherent clusters, suspended cell clumps, or individual cell suspensions.

1.2.2.1 Selection and Optimization of Cryoprotectants

Cryoprotectants are compounds that can reduce damage to cells during the freezing and thawing process by diluting the solute concentration in the solution[58]. The mechanism of action involves the cryoprotectant permeating the cell membrane during cooling, thereby reducing cellular shrinkage[59]. Dimethyl sulfoxide (DMSO) is a non-electrolyte with a small molecular weight and high solubility, capable of penetrating cell membranes effectively. It lowers the freezing point, thereby reducing its cytotoxic effects on cells. Cryoprotectants are broadly classified into two types: ① permeating agents such as DMSO and ethylene glycol, and ② non-permeating agents such as sucrose and trehalose. They function differently: permeating agents can penetrate the cell membrane, entering the cell to create a certain molar concentration that reduces the concentration of electrolytes in the unfrozen solution both inside and outside the cell. This protects the cell from damage due to high concentrations of electrolytes and prevents excessive efflux of water, thus avoiding excessive cellular dehydration and shrinkage. Non-permeating agents, on the other hand, protect cells by forming a viscous, transparent shell on the exterior surface of the cell. Choosing the appropriate cryoprotectant or combination of protectants can improve the cell freezing rate. Studies have shown that DMSO itself is a toxic chemical agent, and at high concentrations, it can be toxic to cells [60]. However, pre-cooling DMSO in a refrigerator at 4°C can reduce its cytotoxicity[61]. Katkov[62] conducted a cytotoxicity comparison of four different cryoprotectants used for the freezing of induced pluripotent stem cells (dimethyl sulfoxide, ethylene glycol, propylene glycol, and glycerol). The cells were exposed to a solution containing 10% cryoprotectant at 37°C for 30 minutes. The results showed that DMSO had the strongest toxicity, while glycerol exhibited the least toxicity. DMSO is a small-molecule, highly permeable chemical and is currently the most commonly used cryoprotectant for cell preservation[63]. Its mechanism of action involves lowering the freezing point of the culture medium and preventing the aggregation of free proteins, thus enhancing the permeability of the cell membrane to water. When DMSO permeates into cells before freezing, it reduces the concentration of electrolytes in the unfrozen intracellular and extracellular solutions, thereby protecting cells from damage due to high concentrations of electrolytes, while also preventing excessive water efflux, which helps avoid excessive dehydration and shrinkage of cells[64]. DMSO is toxic to cells, and high concentrations can adversely affect the survival and recovery of cell activity after thawing. Fetal bovine serum inherently provides cellular

protection and can neutralize the toxic effects of DMSO; appropriately increasing its concentration can benefit cell survival after recovery[60]. Interestingly, the same cryoprotectant shows opposite results in slow freezing methods, where dimethyl sulfoxide exhibits the least toxicity and glycerol the most. This indicates that temperature significantly impacts the effectiveness of cryoprotectants. Additionally, the study clarifies that the low recovery efficiency of human pluripotent stem cells is primarily caused by the freeze-thaw process, rather than the addition and removal of cryoprotectants[62].

1.2.2.2 Cryopreservation of Adherent Human Pluripotent Stem Cell Clones, Suspension Clones, and Isolated Single Cells

Due to the low recovery rate of human pluripotent stem cells (hPSCs) frozen in suspension using the slow-freezing method, many studies have attempted cryopreservation of adherent clones using vitrification. Ji et al[65]. cryopreserved adherent hPSCs clones in a 24-well plate using 10% DMSO and 30% (v/v) FBS. Their study confirmed that the recovery rate of adherent clones was five times higher than that of cell suspensions. Katkov et al.[62] reported a multistep cryopreservation protocol for adherent induced pluripotent stem cell (iPSC) clones using ethylene glycol as a cryoprotectant and a programmable cooler, which achieved a sixfold higher efficiency than the slow-freezing method. Two reasons explain the higher efficiency of cryopreserving adherent clones: ① Suspension clones experience significant cell death or damage within the clone during freezing as cells are not adhered, which is a key factor leading to the low recovery rate of suspension clones. ② Continuous extracellular signaling among cells during the cryopreservation of adherent clones plays a crucial role in enhancing cell viability and reducing differentiation. The main disadvantage of this method is that it does not allow for the mass storage of cells. Additionally, the culture dishes cannot be sealed as tightly as cryovials, which increases the risk of cross-contamination during storage in liquid nitrogen. To address this, Nie et al[66]. attempted to cryopreserve adherent human pluripotent stem cells using microcarriers (Cytodex3), enabling the cryopreservation of large quantities of adherent cells. These microcarriers consist of a thin layer of collagen covalently bonded to a crosslinked dextran matrix, which, along with a MatrigelTM substrate layer or irradiated mouse embryonic fibroblasts (MEF), enhances the adhesion of human pluripotent stem cell clones. This study was the first to report the effective proliferation of human pluripotent stem cell clones on two types of microcarriers (coated with MatrigelTM and MEF), and it compared this cryopreservation method with the slow-freezing technique. The human pluripotent stem cell microcarriers were suspended in a cryoprotectant solution containing

10% DMSO and 30% (v/v) FBS, with a cell density of 1X10^6 cells/mL in a 10 cm dish. The cell suspension was transferred to cryovials and placed in a programmable cooling box (cooling rate of 1°C/min) before storage in liquid nitrogen. Cell recovery rates were assessed by cell counting seven days post-thaw and compared to the slow-freezing method. The study observed that the efficiency of cryopreservation on microcarriers was 1.7 times higher than that without microcarriers. Although the increase in recovery rate is not significant, further optimization of this method could potentially enable the mass cryopreservation of cells in the future. Human pluripotent stem cells tend to grow in clonal clusters, and when dispersed into single cells, they are prone to damage and apoptosis, which is why most cryopreservation protocols focus on freezing clonal clusters of human pluripotent stem cells to enhance efficiency[67]. Additionally, because accurate counts of viable cells post-thaw are challenging to calculate, freezing clonal clusters simplifies the assessment of recovery rates. However, freezing clonal clusters limits the exposure of internal cells within the clone to the cryoprotectant. Consequently, T'Joen[68] has improved the efficiency of freezing by dispersing cells into a mixture of single cells and microcellular clusters before cryopreservation.

1.3 Human Dental Pulp Stem Cells-inflamed Pulps (hDPSC-IPs)

Current research primarily utilizes dental pulp stem cells harvested from third molars and orthodontically extracted teeth, presenting limitations in both donor and source availability. Root canal therapy, the most prevalent dental pulp treatment, involves the crucial step of removing inflamed or necrotic dental pulp. The potential to isolate seed cells from the extracted pulp for use in tissue engineering has emerged as a research focus in recent years. In 2010, Wang et al.[69] isolated dental pulp stem cells from permanent teeth clinically diagnosed with irreversible pulpitis. By conducting comparative experiments with stem cells from healthy dental pulp and those from teeth diagnosed with irreversible pulpitis, they demonstrated that stem cells capable of proliferation still exist in inflamed pulp tissues. These cells also retain the ability to differentiate into odontogenic and osteogenic lines, albeit in reduced numbers.

Recent studies have confirmed the clinical applicability of dental pulp stem cells in regenerative medicine fields such as bone regeneration, neural regeneration, and dentin-pulp complex regeneration[70]. As a variant of dental pulp stem cells, hDPSC-IPs possess robust self-renewal capabilities and multidirectional differentiation potential, similar to normal dental pulp stem cells. These qualities form the basis and premise for the clinical applications

of hDPSC-IPs. With further research, the clinical use of inflamed dental pulp stem cells is poised to expand significantly.

1.4 Keloid Scars: Pathogenesis, Clinical Features, and Challenges in Treatment

Scars, medically referred to as scar tissue, are abnormal connective tissues that form on the skin or other tissues after an injury, through a process of repair and remodeling[71]. The formation of this tissue is a physiological response of the body to damage, intended to repair and replace the original tissue structure. The scar formation process involves complex biochemical and cellular dynamics, including inflammatory responses, neovascularization, migration and proliferation of fibroblasts, and the deposition of collagen and other matrix proteins. The nature of the scar is influenced by factors such as the nature of the trauma, the site and depth of the injury, the healing method, and individual differences. Scars can be classified based on their morphology, function, and pathology, including hypertrophic scars, atrophic scars, and keloids[72]. While hypertrophic and atrophic scars are more common, keloids represent a severe form of scarring disease, often exhibiting invasive characteristics, such as growth into surrounding tissues and a tendency to recur, behaving similarly to tumors [73]. Keloids arise from abnormal fibroblast proliferation and excessive collagen deposition during the skin's repair process following an injury, presenting as a benign tumor-like proliferation[74]. They not only affect the appearance and psychological well-being of patients but also cause symptoms such as pain, itching, contracture, and functional impairment, significantly impacting the quality of life. The pathogenesis of keloids is not fully understood. Current theories suggest it may be related to genetic factors, ethnicity, age, sex, hormones, inflammation, immunity, and oxidative stress[75, 76], Keloids commonly occur on the chest, shoulders, earlobes, neck, and facial areas [77], and are more likely to develop in areas of high skin tension[78]. The incidence of keloids varies among different populations and regions. Compared to Western countries, the incidence is higher in Asia, reaching up to 0.15%[79]. Furthermore, women are more prone to keloids than men, which may be related to hormonal levels, skin structure, and physiological characteristics specific to females[80]. Keloids are diverse in morphology, presenting as raised, nodular, cord-like, or patchy structures, with surfaces that are either smooth or rough. Their color can range from red to purple or brown, and they are typically hard in texture with unclear edges. Commonly, they are accompanied by symptoms such as itching, pain, and a sensation of tightness[81]. Various treatment methods for keloids include surgical excision, laser therapy, pharmacological treatments, physical therapies, and compression therapy. However, the outcomes are often

suboptimal with high recurrence rates, making this a continuing focus and challenge in medical research [82, 83].

1.5 Sjögren's Syndrome: Pathogenesis, Clinical Manifestations, and Challenges in Treatment

Dysfunction of the lacrimal and salivary glands and reduced glandular secretion are commonly caused by Sjögren's Syndrome (SS), radiation therapy, cancer, or aging. Permanent damage to secretory acini or the loss of supportive microenvironmental signals can lead to dry eye or dry mouth. SS is the primary cause of glandular dysfunction and currently has no cure, ultimately having a severe impact on the health and well-being of patients[84]. Regenerative strategies could significantly improve the quality of life for millions of people. In 1935, Swedish ophthalmologist Henrik Sjögren identified a disease characterized mainly by dryness symptoms, accompanied by muscle and joint pain[85], which was later named SS. This autoimmune disorder is caused by extensive lymphocytic infiltration into the exocrine glands, with pathological features including destruction of the glandular secretory units (acini and ducts). SS is categorized into primary and secondary types: primary SS is not associated with other autoimmune diseases, whereas secondary SS typically arises from other conditions such as rheumatoid arthritis and systemic sclerosis. Primary Sjögren's Syndrome (PSS) is currently the second most prevalent autoimmune disease after rheumatoid arthritis, with the incidence in females typically 10 to 15 times higher than in males. The peak age of onset for females is around 56 years, while for males, it usually occurs after 65 [86]. Exocrine gland dysfunction is the most common manifestation of PSS, with 95% of patients experiencing severe dry mouth, dry eyes, dental caries, oral ulcers, conjunctivitis, scleritis, and retinal vasculitis. Additionally, 33% of patients also develop extraglandular diseases, including neurological disorders (such as cranial nerve paralysis, transverse myelitis, and dorsal root ganglionopathy), reproductive system disorders (such as genital dryness and reduced libido), dermatological conditions (such as xerosis, skin itching, scaling, erythema annulare, atypical folliculitis, and Raynaud's phenomenon), sensory system disorders (such as olfactory and taste disturbances), and psychological illnesses (such as sleep disorders, anxiety, and depression)[87].

The specific pathogenesis of Primary PSS is still under investigation, but it is confirmed that, like other autoimmune diseases, PSS is associated with genetics, immune deficiencies, viral infections (such as cytomegalovirus, human immunodeficiency virus, human T-cell leukemia virus, and hepatitis virus), and environmental factors. These pathogenic factors induce abnormal activation and widespread infiltration of immune cells (especially B lymphocytes

and T lymphocytes) into the glands. The glands are primarily composed of ducts, acini, and epithelial cells. The extensive infiltration of immune cells and inflammatory factors leads to the destruction of glandular structure and function, thereby contributing to the onset of PSS. The pathomechanism of glandular inflammatory responses in PSS patients is extremely complex, involving multiple immune cells, inflammatory mediators, and signaling pathways, making it challenging to develop effective diagnostic and treatment strategies for this condition.

1.5.1 Current Treatment Status for Sjögren's Syndrome (SS)

1.5.1.1 Multidisciplinary Management Approach

Due to the heterogeneity in clinical presentations of SS, experts currently recommend specialized diagnosis and treatment for SS. Additionally, at the time of diagnosis, it is important not only to screen for non-autoimmune diseases but also to assess the extent of organ involvement in patients. Based on clinical symptoms and serological indicators, a personalized treatment plan should be developed[88]. This process requires collaboration across multiple disciplines, involving a medical team centered around rheumatology specialists and including various health professionals.

1.5.1.2 Local Treatment for Glandular Symptoms

Over 95% of SS patients experience dryness symptoms. Studies by Haldorsen et al.[89] indicate that, except in the early stages of the disease, the degree of glandular dysfunction in SS does not progressively worsen but rather maintains stability over a long period. Currently, there are no medications reported that can reverse glandular damage and cure dryness symptoms. Therefore, to minimize adverse reactions, current guidelines recommend prioritizing local treatments. Additionally, numerous studies have shown that initiating local treatment early in the onset of glandular dysfunction has a definite effect in alleviating dryness symptoms with minimal adverse reactions[90]. For dry mouth, treatment plans are tailored based on the degree of glandular dysfunction. Assessment methods include measuring salivary flow or conducting salivary gland scintigraphy[91]. For mild glandular dysfunction, non-pharmacological stimulation is preferred, such as taste stimulants (sugar-free acidic candies, xylitol, etc.). For moderate dysfunction, muscarinic agonists such as pilocarpine and cevimeline, with anethole trithione or bromhexine as alternatives for non-responders, are used. In cases of severe glandular dysfunction, where the salivary glands have atrophied and neither drugs nor non-drug treatments can stimulate saliva production, saliva substitutes are the first

choice. These include oral sprays, gels, and rinses.

For dry eyes, the first-line treatments include artificial tears and ocular gels. The current guidelines recommend using artificial tears containing methylcellulose or hyaluronate at least twice daily to increase tear volume and reduce friction between the eyelids and the eyeball [8]. Since ocular gels can cause blurred vision, they are recommended for nighttime use. In cases of refractory or severe dry eye, treatment should be guided by an ophthalmologist and may include medications such as topical cyclosporine, lifitegrast (inhibiting LFA-1), 1% dipyridamole, or diquafosol (a P2Y2 receptor agonist)[92-94]. Diluted autologous serum may be used as a local teardrop for refractory cases[95], although its efficacy remains controversial.

1.5.1.3 Systemic Whole-body Treatments Remain Controversial

Joint or muscle pain, fatigue, and weakness are common symptoms of Sjögren's Syndrome (SS). For acute pain, nonsteroidal anti-inflammatory drugs (NSAIDs) can be used; for recurrent musculoskeletal pain, hydroxychloroquine may be added. For chronic non-inflammatory pain, personalized treatment should be administered, avoiding repeated use of NSAIDs or glucocorticoids (GCs). Pregabalin can be used to improve fibromyalgia-like symptoms[96]. Additionally, some non-pharmacological treatments, such as aerobic exercise, can also help alleviate pain.

1.6 Clinical Manifestations and Treatment Challenges of Neurofibromatosis

Neurofibromatosis (NF) is a group of autosomal dominant genetic disorders, including three subtypes: Type 1 NF, Type 2 NF, and Schwannomatosis. These diseases show considerable phenotypic variability, primarily characterized by tumors in the nervous system, skin lesions, and peripheral nervous system abnormalities, causing multiple, progressive damages that pose significant treatment challenges. Current reasonable approaches include screening at-risk populations, early diagnosis, close follow-up, and establishing a multidisciplinary treatment framework based on the natural progression characteristics of NF.

1.6.1 Neurofibromatosis Type 1 (NF1)

Neurofibromatosis Type 1 (NF1), also known as Von Recklinghausen disease, is an autosomal dominant genetic disorder caused by mutations in the NF1 gene, with a global incidence of approximately 1 in 3,000 newborns [97, 98], about 50% of which are sporadic mutations. Patients often begin showing symptoms in childhood, with diverse clinical manifestations

such as café-au-lait spots, axillary or inguinal freckling, and multiple neurofibromas. NF1 frequently involves multiple systems, including the development of various benign and malignant tumors, skeletal abnormalities, cardiovascular and cerebrovascular diseases, and cognitive and psychological abnormalities[99-102]. Neurofibromas are characteristic findings of NF1, classified into cutaneous neurofibromas (cNF) and plexiform neurofibromas (pNF). Plexiform neurofibromas often exhibit invasive growth, damaging surrounding normal tissues and organs, with 8% to 13% of pNF cases progressing to malignant peripheral nerve sheath tumors[103-105]. Currently, treatment for NF1 primarily involves symptomatic management through surgery and medication. Although these approaches can alleviate symptoms, they do not cure the disease.

1.6.2 NF2-associated schwannomas (NF2)

NF2-associated schwannomas (NF2) is an autosomal dominant genetic disorder caused by mutations in the NF2 gene. It is clinically characterized by bilateral vestibular schwannomas (VSs) and is often accompanied by multiple tumors in the nervous system as well as related ocular and skin lesions. Common nervous system lesions include bilateral vestibular schwannomas, other cranial nerve schwannomas, intracranial meningiomas, spinal tumors, and peripheral neuropathies[106]. Typical ocular lesions include cataracts, preretinal membranes, and retinal hamartomas[107]. Common skin manifestations include skin tumors, skin patches, and subcutaneous tumors[108, 109].

Patients with NF2 face numerous challenges, such as hearing loss, the need for repeated surgeries to remove intracranial and spinal cord tumors, loss of critical neurological functions, and a shortened life expectancy[110, 111]. Given the variety of lesions, choosing a treatment strategy is highly complex. For newly diagnosed patients, disease assessment based on phenotype predictions from similar genotypes is beneficial. Treatment strategies differ significantly between patients at high risk of early bilateral hearing loss and those at lower risk[112].

2 Chapter 2: Publication in *In Vivo*, November 2020

Expansion of Human Dental Pulp Cells In Vitro Under Different Cryopreservation Conditions

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Personal contribution:

The following research article has been published in ***In Vivo***. I am the first author of the paper. I conducted the experiments and data collection and analyzed the results (raw data analysis and statistical analysis). I contributed to writing the manuscript draft and editing during the revisions.

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Expansion of Human Dental Pulp Cells *In Vitro* Under Different Cryopreservation Conditions

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Abstract. *Background/Aim: To optimize the expansion of human dental pulp cells in vitro by exploring several cryopreservation methodologies. Materials and Methods: The intra-dental pulp tissues from healthy subjects were extracted and divided into three separate tissue segments, which were randomly divided into the three following groups; the fresh group, the 5% DMSO group, and the 10% DMSO group. In the fresh group, dental pulp cells were directly cultivated as primary cultures, whereas in the DMSO groups, the dental pulp cells were cultivated from cryopreserved pulp tissue segments one month later. Results: The cell yield and the time it took for the cells to grow out of the pulp tissue and attach to the culture plate varied among the three groups; the 5% DMSO group was inferior to the fresh group but superior to the 10% DMSO group ($p<0.05$). Moreover, no differences were found at the 1st passage amongst the three groups regarding the following aspects ($p>0.05$); colony formation rate and cell survival rate. Furthermore, no differences were noted at the 3rd passage regarding the following aspects ($p>0.05$); proliferation ability, cell growth curve and surface marker expression of dental pulp cells. Conclusion: Five percent DMSO may be the most optimal condition for tissue storage to preserve stem cells in situ.*

Dental pulp cells are regarded as a convenient source of cells with stem cell characteristics that can be employed for regenerative therapies (1, 2). Not only they are a convenient source of cells because of their availabilty from readily available extracted teeth such as wisdom teeth and decidous teeth, but because they are also not associated with any ethical controversies due to either being routinetly prophylactically extracted for the former or naturally exfoliated for the latter (3). Moreover, dental pulp cells are a potent source due to their multi-differentiation potential and high proliferative potential (4-7). Therefore, successful preservation of intra-dental pulp tissue is a prime advantage for future regenerative therapies. Tissue preservation at ultra-low temperatures can be one of the most reliable methods for tissue storage. In the current study, after primary tissue culture, primary cells were stored in cryopreservative medium containing dimethyl sulfoxide DMSO (8, 9). Therefore, graded concentrations of DMSO were examined in this study. It has been shown that the addition of an appropriate volume ratio of DMSO to cryopreservative medium can prevent physical damage of cells caused by ice crystals during cryopreservation and thawing. Nevertheless, DMSO does possess cytotoxic effects. Moreover, besides being non-environmentally friendly, it can also directly affect the health of the operator during handling (10-12). Therefore, if the concentration of DMSO used can be reduced, the risk of cytotoxic effects can be reduced to some extent for the sake of more successful cell transplantation therapy applications. This study aimed to explore variations that could possibly occur to stem cell cultivation time, cell yield, as well as cellular activities when stem cells are cultivated from both fresh pulp tissue segments or frozen pulp tissue segments that have been preserved using different cryopreservation conditions. Thereby, coming a step closer to finding the most suitable scheme for storing pulp tissue samples as a convenient and readily available source for cells with stem cell characteristics and hence, lay down the foundation for a reasonable development of a sustainable clinical sample harvesting system that can be used for future cell transplantation applications.

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Key Words: Stem cells, stem cell culture and differentiation, dimethyl sulfoxide, cryopreservation, cell proliferation; osteogenic induction, adipogenic induction.

Materials and Methods

Extraction and cryopreservation of dental pulp tissue: Ten supernumerary and deciduous teeth of healthy children aged 10-15 years were collected from December of 2018 to May of 2019 at the department of Oral and Maxillofacial Surgery of our hospital. Before the surgery, all the patients and/or their respective guardians signed the informed consent, and were instructed to gargle with 1% hydrogen peroxide solution. The surgery also followed routine intra

and extra- oral disinfection measures. The teeth were then extracted and placed in culture medium-complete DMEM [(Dulbecco's Modified Eagle Media (cat. no. 11965084, ThermoFisher Scientific, Waltham, MA, USA)] with 10% fetal bovine serum (FBS, ThermoFisher Scientific, cat. no. 10438034) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (cat. no. 15140-148, Gibco BRL, Carlsbad, CA, USA) 4˚C. Experiments were conducted within 4 h thereafter. Teeth were rinsed with 4˚C PBS (Gibco, cat. 18912-014) solution (including 2×10^5 U/l penicillin and streptomycin) from the crown to the root repeatedly for a duration of 5-10 min. Teeth were then cut open to expose the pulp using a hammer. Thereafter, the intra-dental pulp tissues were immersed in culture medium and were then cut into 0.5 mm^3 tissue pieces.

The pulp tissue pieces were randomly divided into a fresh group and two frozen groups. For the frozen groups, 5% and 10% DMSO (cat. no. D8418, Sigma-Aldrich, St. Louis, MO, USA) medium were used to suspend the pulp tissue pieces to be stored in the –196˚C liquid nitrogen for 1 month after having been initially stored in the refrigerator at 4˚C for 1 h. Whereas, for the fresh group, the dental pulp cells were immediately cultivated.

Culture of human dental pulp cells: At the time of cultivation, tissues from each group were evenly distributed and spread in three 3.5 cm diameter culture dishes. Each tissue piece was dropped with 100 μl DMEM medium to ensure its attachment to the culture surface. The culture dishes were incubated at 37˚C in a 5% CO_2 humidified incubator. After 2 days, cell attachment and growth were observed under an inverted microscope, followed by the gradual formation of colonies until the desired level of confluency was reached. Then, the cells were harvested from the culture surface using 0.05% Trypsin-EDTA (ThermoFisher; cat. no. 25300-054) and were counted using cell-count boards. The cells were passaged at a density of $5\times10^3/cm^2$.

Observation of cell morphology: Primary cells were observed under an inverted microscope, and the time elapsed from the placement of tissue pieces to cellular outgrowth and attachment was noted for each of the three groups.

Calculation of cell yield/harvest: Upon reaching a state of confluency, primary cells of each group were subpassaged with 0.05% trypsin at 37˚C for 1-2 min. The process of trypsinization was stopped when cellular attachment was seen to be clearly disrupted by the appearance of floating cells in the medium. After centrifugation at 400×g for 8 min at room temperature, the primary dental pulp cells were resuspended in complete DMEM culture medium and were counted as a cell suspension under the microscope using cell counting plates/hemocytometer. The primary cell yield was calculated using the following equation.

Primary cell yield=Total cell counted/4×dilution factor× 10^4×volume of cell suspension (13).

Colony forming efficiency: The first generation of cells (2×10^3 cells) of each group was cultured into a 10 cm diameter culture dish for 14 days. After which the culture medium was discarded and the newly formed dental pulp colonies were fixed with 95% methanol for 15 min, followed by staining with 3% crystal violet dyes for 2 min, which was then rapidly decolorized using methanol, followed by PBS washes to remove residual crystal violet dye solution. Single colonies with a cell count greater than 50, were recorded as successfully formed colonies. Three parallel samples were set up in each group and counted twice. The Colony forming efficiency % was calculated using the following equation:

Colony forming efficiency (%)=number of successfully formed colonies/number of seeded cells×100 (14).

Cell survival rate: The 1st generation of cells from each group was observed under an inverted microscope after being stained with 0.4% Trypan Blue (Sigma-Aldrich). Dead cells were stained blue whereas surviving cells were left unstained. The survival cell rate of 500 cells was rapidly counted within 5 min and the cell survival rate was calculated using the following equation:

Cell survival rate (%)=number of unstained surviving cell/ 500×100 (15).

Evaluation of cell proliferation using the MTS assay: Dental pulp cells which were passaged for 3 generations were made into cell suspensions per generation and their proliferation was measured using the MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (cat. no. G3582, Promega, Madison, WI, USA). Cell were seeded at concentrations of 5×10^9/l into 96-well plates for 8 days and incubated at 37˚C and 5% CO_2. Cells from 5 wells were subjected to MTS colorimetric analysis in each group every day for 8 consecutive days. The proliferative readings were determined using UV spectrophotometry at a wavelength of 490 nm using a nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Determination of the immunophenotype of cells using specific surface markers: After the 3rd generation cells were filtered using a 70 μm filter, the cells were re-suspended in cold PBS at a concentration of 1×10^9/l. Surface markers of 3rd generation cells were identified by flow cytometry using antibodies against STRO-1 (cat. no. 14-6688-82; Thermo Fisher Scientific, Inc.), CD105 (cat. no. ab156756; Abcam, Cambridge, UK), CD90 (cat. no. ab139364; Abcam), CD34 (cat. no. 560941; BD Biosciences, San Jose, CA, USA), and CD45 (cat. no. MCA2035S; Bio-Rad Laboratories, Inc, Hercules, CA, USA) and antibodies (all 1:500) was employed to detect the phenotype of cells.

Multidifferentiation potential

Adipogenic differentiation: Third generation dental pulp cells were seeded at a density of $8\times10^3/cm^2$ onto 3.5 cm culture dishes. As cells reached 70%-80% confluency, growth culture medium was replaced by adipogenic inductive medium for a duration of 21 days. Medium was changed regularly 2-3 times per week. As the induction period advanced, lipid droplets were observed in the cytoplasm of cells. Following the 21 day period, inductive medium was discarded and cells were washed twice with PBS and then fixed by 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Fort Washington, PA, USA) for 15 min. Following fixation, cells were washed twice with PBS and then stained with 0.5% oil red O working solution (Sigma, cat. no. 00625, ~0.4%) at room temperature for 5-10 min. Stained lipid droplets in the cytoplasm were then observed under the microscope and photographed.

Quantitative analysis of adipogenic differentiation: Cultures stained with oil red O stain were carefully washed 3 times with distilled water. To dissolve the lipid droplets, 500 μl isopropanol (Sigma-

Table I. *Primer sequences and reaction conditions to measure adipogenic and osteogenic induced gene expression.*

Primer	Sequence of upstream and downstream	Ta (˚C)	Length product (bp)
Adipogenic induced gene			
Lipoprotein lipase	Upstream: 5'-GAC TGA GAG TGA AAC CCA TAC-3'		
	Downstream: 5'-CCC TAG AAC AGA AG ATC ACC-3'	54	290
Peroxisome Proliferator-Activated	Upstream: 5'-GCT TCT GGA TTT CAC TAT GG-3'		
Receptor Coactivator γ	Downstream: 5'-AAA CCT GAT GGC ATT ATG AG-3'	54	195
Osteogenic induced gene			
Type I collagen	Upstream: 5'-GGA CAC AAT GGA TTG CAA GG-3'		
	Downstream: 5'-TAA CCA CTG CTC CAC TCT GG-3'	58	461
Osteocalcin	Upstream: 5'-ATG AGA GCC CTC ACA CTC CTC-3'		
	Downstream: 5'-GCC GTA GAA GCG CCG ATA GGC-3'	57	293
Internal reference			
GAPDH	Upstream: 5'-ATC ACC ATC TCC AGG AGC G-3'		
	Downstream: 5'-GTT CTT CCA CCA CTT CGT CC -3	55	573

Aldrich, cat. no. 278475) was added into each well. The dissolved stain solution was then mixed and evenly distributed in 96-well plate. Finally, the absorbance value was read at a wavelength of 540 nm on the spectrophotometer.

Osteogenic differentiation: Third generation dental pulp cells at a density of $8\times10^3/cm^2$ were seeded onto 3.5 cm culture dishes. As cells reached 70%-80% confluency, growth culture medium was replaced by osteogenic inductive medium for a duration of 21 days. Medium was changed regularly 2-3 times per week. As the induction period advanced, calcified nodules were observed in the cytoplasm of cells. Following the 21 day period, inductive medium was discarded and cells were washed twice using DPBS and then fixed by 90% ethanol (EtOH; Sigma-Aldrich, cat. no. 270741) for 1 h. Following fixation, cells were washed twice with PBS and then stained with 2% Alizarin red S (cat. no. 130-22-3; Sigma-Aldrich) stain at room temperature for 5-10 min. Stained calcified nodules in the cytoplasm were then observed under the microscope and photographed.

Quantitative analysis of osteogenic differentiation: Cultures stained with Alizarin red S were carefully washed 3 times with distilled water. To dissolve the calcified nodules, 500 µl of 10% acetic acid (Sigma-Aldrich, cat. no. 33209) were added into each well. Equal volume of 10% ammonium hydroxide was then added to neutralize the acetic acid. The resultant solution was mixed and evenly distributed in the 96-well plate. Finally, the absorbance value was read at a wavelength of 405 nm using a spectrophotometer.

In the aforementioned differentiation experiments, 3 parallel samples were set up for each group, and 5 well replicates were analyzed for each sample. The whole experiment was repeated twice.

Reverse transcription PCR to measure adipogenic and osteogenic induced gene expression: To extract total RNA from cells, 1ml Trizol reagent (Invitrogen, cat no. 15596-018) was added to every 1×10^6 second-generation cells. The expression of the adipogenic specific genes Lipoprotein Lipase and Peroxisome proliferator-activated receptor- (PPAR-γ) and the osteogenic specific genes type I collagen and osteocalcin was measured by reverse transcription PCR. GAPDH was used as a reference gene. Primer sequences and reaction products are depicted in Table I.

The conditions of the PCR reaction were: 94˚C for 4 min; 94˚C for 30 s, 54-58˚C for 30 s, 72˚C for 1 min; 35-40 cycles; 72˚C for 7 min, 4˚C. Amplified PCR products were then evaluated by 2% agarose gel electrophoresis and 2% ethidium bromide staining. Finally, gel imaging was obtained under UV light. The relative expression of adipogenic and osteogenic gene mRNAs was measured by reverse transcription PCR. (Adipogenic specific genes: Lipoprotein Lipase and Peroxisome proliferator-activated receptor-(PPAR-γ); Osteogenic specific genes: type I collagen and osteocalcin).

Ethics approval and consent to participate: Administrative permissions were acquired by our team to access the data used in our research. The study protocol was approved by the Hamburg University ethics committee that approved the study. No: REC 1712/5/2008. Accordingly, all teeth were coded with number and all personal identification of the patients were removed. All parent or guardian of participants provided written informed consent for using their teeth, which otherwise would have been discarded as waste.

Statistical analysis: Student *t*-test was used to analyze differences in mean values between the three groups. *p*-Values less than 0.05 were considered statistically significant. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Effects of different cryopreservation conditions on primary cell cultivation and expansion. Morphologically cultured dental pulp cells, observed under the inverted microscope, appeared as either spindle-shaped, triangular or long spindle-shaped, with an abundant cytoplasm and a clear nucleus. No clear difference was noted between the two groups, as shown in Figure 1A. However, the time elapsed for cellular outgrowth and attachment was significantly shorter in the fresh group when compared to the frozen group ($p<0.05$), as shown in Figure 1B.

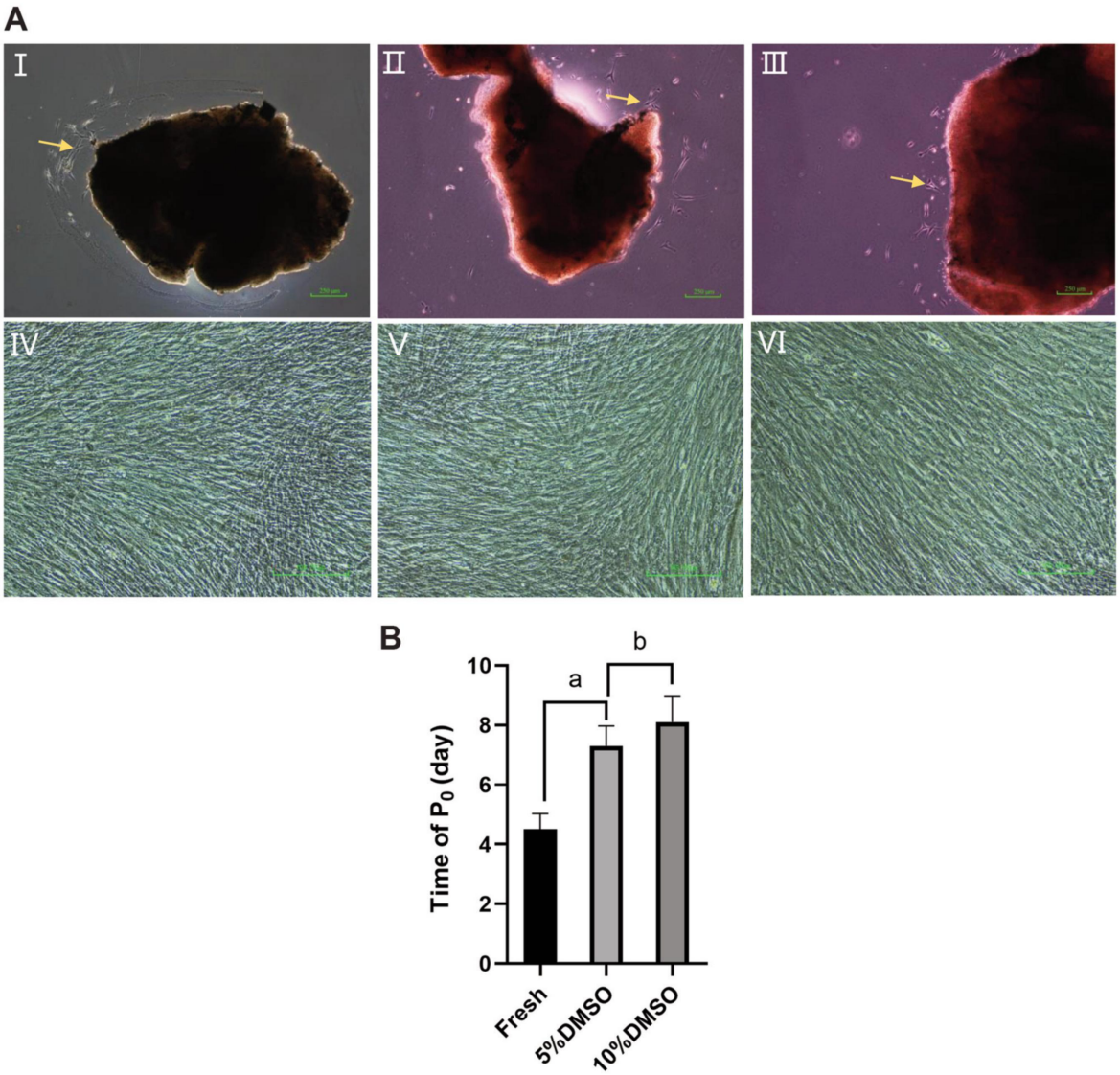

Figure 1. *Morphology of pulp cells under different cryopreservation conditions. A: Morphology of primary and 3rd generation/passage dental pulp cells. I-III: Primary cells from different groups grew out of the tissue mass (arrow, ×40); IV-VI: Dental pulp cells morphology at the 3rd passage/generation – depicting both fresh and frozen groups. B: Time of appearance of primary cells: Time of appearance of primary cells in the fresh group was significantly lower than that in frozen groups ($^{a}p<0.05$); Time of appearance of primary cells in the 5% DMSO group was significantly lower than that in the 10% DMSO group ($^{ab}p<0.05$). DMSO cryopreservation of primary pulp cells resulted in increased time to grow out of the tissue.*

Cryopreservation effects on the primary dental pulp cell yield/cell harvest. The number of primary dental pulp cells in both frozen groups was significantly lower than that in the fresh group ($^{a}p<0.05$). Furthermore, the lowest dental pulp cell yield number was observed in the 10% DMSO group only $(3.625\pm0.375)\times10^{5}$ ($^{b}p<0.05$) (Figure 2).

Cryopreservation effects on stem cell characteristics of primary dental pulp cells. Flow cytometric analysis was used to examine the expression of stem cell markers in the three groups. All groups showed greater than 70% positive expression of CD105 and CD90, and 8%-16% positive expression of STRO-1. The expression of CD34 and CD45

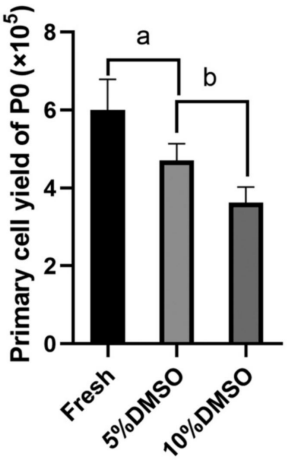

Figure 2. *Cryopreservation effects on primary dental pulp cell yield. A Dose-dependent inverse association between DMSO and dental pulp cell yield is observed.*

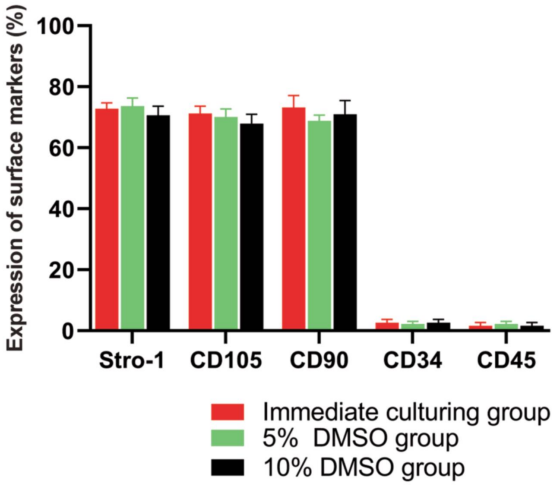

Figure 3. *Expression of dental pulp cell surface markers in each group.*

was less than 5% in all groups. Nevertheless, there was no significant difference in the positive expression rates of CD105, CD90, CD34, CD45 and STRO-1 between the three groups ($p<0.05$) (Figure 3).

Cryopreservation effects on primary dental pulp cell proliferation and renewal. Compared to the fresh group, no significant differences were noted regarding the colony forming efficiency, the survival rate of first generation dental pulp cells, and the growth curve third generation dental pulp cells ($p>0.05$)(Figure 4).

Cryopreservation effects on the multidifferentiation potential of dental pulp cells. Cellular products in the form of oil droplets and calcific deposits resulting from adipogenic and osteogenic induction, respectively, were subjected to staining protocols followed by spectrophotometric quantification. Moreover, expression of adipogenic and osteogenic specific genes was also measured. The results showed that regardless of the group, dental pulp cells possess an inherent ability for both adipogenic and osteogenic differentiation ($p>0.05$) (Figure 5).

Discussion

Stem cells are undifferentiated cells with a potent self-renewal ability and a multidifferentiation potential. Stem cells exist not only in embryos but also in adult tissues as well. Accordingly, they can be categorized into embryonic stem cells and adult stem cells. Dental pulp stem cells (DPSCs) are a type of adult stem cells derived from the dental pulp tissue. They, therefore possess a self-renewal ability and a multi-differentiation potential. Depending on the regulation of their microenvironment, they can differentiate into osteoblasts, odontoblasts, adipocytes, muscle cells and nerve cells. DPSCs play an important role in tooth regeneration, nerve repair, and bone tissue engineering. Therefore, dental pulp is regarded as a convenient and widely available source of stem cells for tissue regeneration and thereby, translational medicine.

Due to the convenience of their isolation from exfoliated primary or routinely extracted permanent teeth, they can be produced in sufficient quantities for various applications (16). Therefore, a problem to be solved at the present time is to establish an efficient and reliable banking system for this readily available and valuable tissue. Studies have shown that cryopreservation is an effective method for long-term preservation of biological tissue samples (17). However, in order to extract biologically active stem cells from frozen tissues, tissue samples cannot be simply placed in liquid nitrogen. A tissue cryopreservation should be developed and adjusted accordingly to ensure successful future stem cell cultivation and application (18).

DMSO is the main chemical reagent used for cell cryopreservation due to its protective effects against osmotic cell rupture. At severely low temperatures, as low as –200˚C, DMSO can prevent damage caused by ice crystal formation and osmotic pressure changes. However, although DMSO is currently the best cell cryoprotectant, it is also a toxic chemical reagent, mainly manifested during the process of cell recovery and culture (19). Studies have shown that when

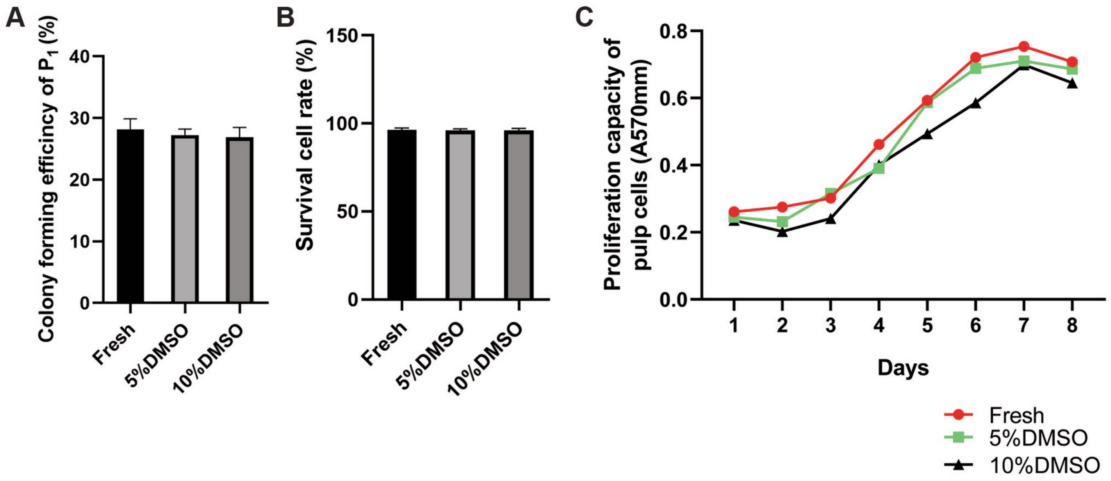

Figure 4. *Self-renewal and proliferation capacity of dental pulp cells in each group. A: Colony forming efficiency of primary dental pulp cells in each group; B: Survival rate of primary dental pulp cells in each group; C: Cell growth curve of primary dental pulp cells in each group (MTS).*

the concentration of DMSO in the culture solution is 10%, cell growth inhibition rate is nearly 100%. Whereas when its concentration is 0.1%, the inhibition rate drops to 35%. Furthermore, even when the concentration of DMSO is 0.004%, it continues to inhibit cellular growth (20). Moreover, as a "universal solvent" DMSO has also potent skin permeation and volatilization characteristics, making it a hazard for the researcher. Therefore, if the dosage and frequencies of DMSO usage in cell freezing and thawing procedures can be reduced, the operation safety factor can be also improved, in addition to reducing risks associated with stem cell transplantation therapies.

Therefore, there is a shift to switch from cryopreservation at a cellular level to cryopreservation at the tissue level. While doing so, the traditional 10% DMSO addition ratio will also be reduced to 5%, in an attempt to optimize cryopreservation and obtain the lowest risk: benefit ratio possible.

Our results showed that the time elapsed for primary cells to grow out of tissue pieces was significantly longer in both cryopreserved groups when compared to the positive control group. However, when comparing data among the 2 cryopreserved groups, the 5% DMSO group displayed significantly shorter time lapse for cellular outgrowth in comparison to the 10% DMSO group ($p<0.05$).

The aforementioned results are based on primary cells and do show the influence of cryopreservation and thawing on the initial stage of *in vitro* cell cultivation, which cause a prolongation in the time needed for cellular outgrowth and cultivation, and also a reduction in the total cell harvest or yield.

However, a series of experiments in the later period adopt the third generation of cells as the research object, because the cells can be naturally purified after passage, which is the suitable cell generation for clinical treatment recognized by the research. Therefore, the experimental cells can not only ensure the number of experimental cells, but also are very representative once it is proved that their biological activity will not be changed due to the freezing of donor tissue samples, which can lay a theoretical foundation for future clinical transplantation treatment and improve the safety of treatment.

Our findings confirmed the lack of significant differences in basic properties and stem cells characteristics amongst the cryopreservation conditions and the freshly extracted dental pulp cells ($p>0.05$). Our results are consistent with those of previous research (21).

Conclusion

In conclusion, cryopreservation can have a hazardous effect on the extraction and harvest of primary dental pulp cells, but an appropriate reduction in the ratio of DMSO can attenuate this effect. However, cryopreservation does not affect the biological activity of the cultured dental pulp stem cells and can therefore be safely applied to cellular transplantation therapies. In conclusion, judging from the total cell yield and the time required for successful *in vitro* cultures, the 5% DMSO was found to be superior to 10% DMSO and can thereby be regarded as an optimal option for dental pulp tissue storage. Moreover, considering the total

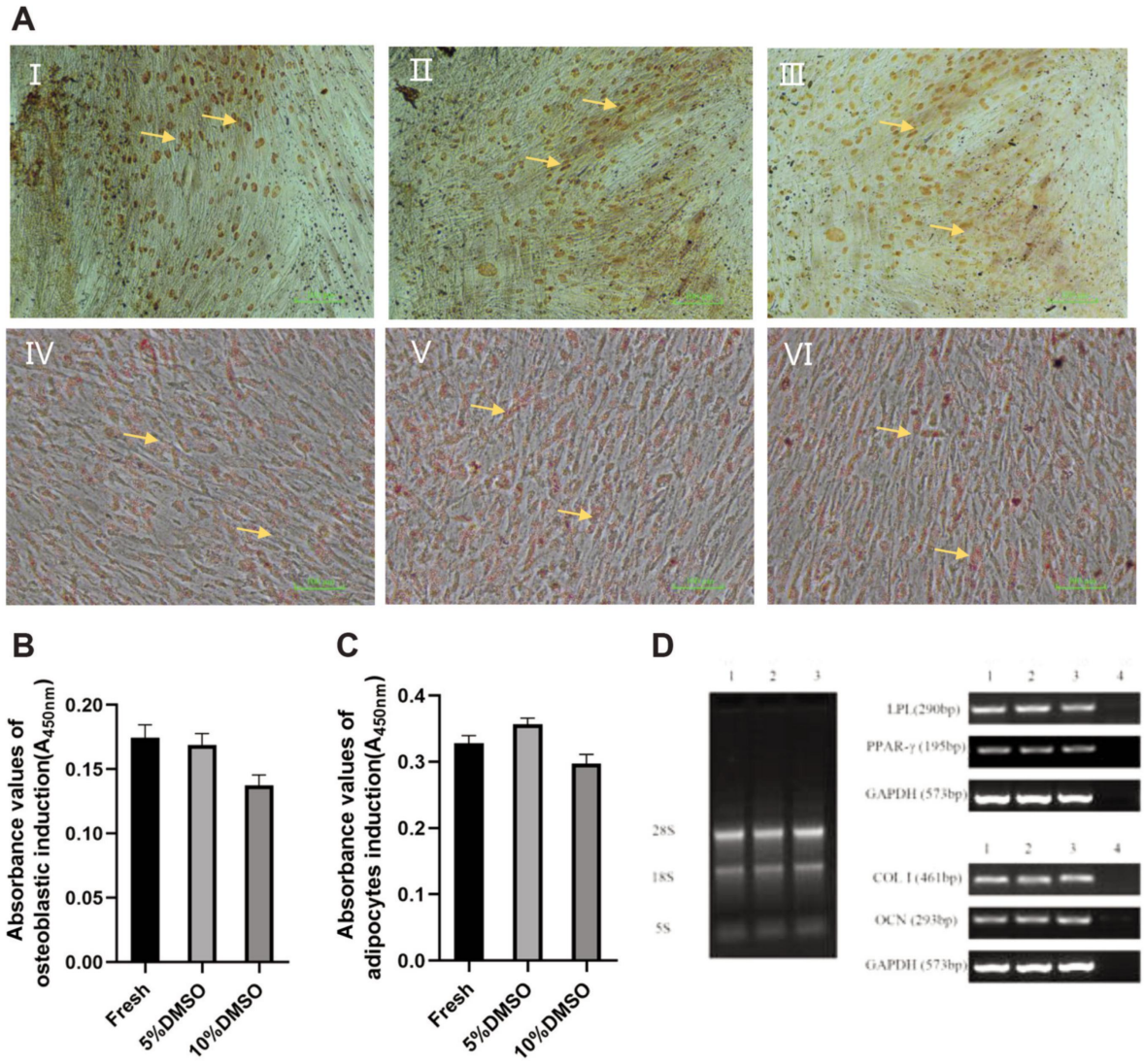

Figure 5. *Multi-differentiation potential of dental pulp cells from each group. A: Adipogenic and osteogenic differentiation potential of 3rd generation dental pulp cells in each group - Oil Red O and Alizarin Red S staining results, respectively. I-III: Osteogenic differentiation (2% Alizarin red stain, ×100). I: Fresh group; II: 5% DMSO group; III: 10% DMSO group. IV-VI: Adipogenic differentiation (0.5% Oil red o stain, ×100): IV: Fresh group; V: 5% DMSO group; VI: 10% DMSO group. B: The absorbance values (A values) after osteogenic differentiation by Alizarin Red S (ARS) staining of 3rd generation dental pulp cells in each group. C: The absorbance values (A values) after adipogenic differentiation by Oil Red O staining of 3rd generation dental pulp cells in each group. I: Electrophoresis of RNA: 1:Fresh group; 2:5% DMSO group; 3:10% DMSO group. II: Electrophoresis of adipogenic specific gene expression in dental pulp cells from each group:1:Fresh group; 2:5% DMSO group; 3:10% DMSO group; 4:Negative control. III: Electrophoresis of osteogenic specific gene expression of dental pulp cells in each group:1:Fresh group; 2:5% DMSO group; 3:10% DMSO group; 4:Negative control. D: The expression of specific adipogenic and osteogenic genes as a result of the respective differentiation by RT-PCR. Note: Dental pulp cells cultivated and expanded from the three groups demonstrated stem (progenitor) cell characteristics of proliferation, self-renewal and multipotentiality.*

volume of DMSO, it is suggested to opt for tissue cryopreservation as a whole rather than cellular cryopreservation in efforts to reduce the direct toxicity effects of DMSO to cells, thereby decreasing possible cell losses in order to maximize cell yield for cell transplantation therapies.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

MY: conceived the study, supervised the experiments and drafted the manuscript. LK: performed data collection and drafted the manuscript. REF: designed the experiments and drafted the manuscript. ON: data evaluation, medical writing and editorial assistance in preparing this manuscript for publication. RS: performed the data collection. MG: analyzed the data and revised the manuscript. All Authors have read and approved the manuscript.

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3 Chapter 3: Publication in *Biomedical Papers*, November 2021

Compare features of human dental pulp cells cultured from pulp tissues with and without cryopreservation

Ming Yan, Ola A Nada, Ralf Smeets, Martin Gosau, Reinhard E Friedrich, Lan Kluwe

Personal contribution:

The following research article has been published in ***Biomedical Papers***. I am the first author of the paper. I conducted the experiments and data collection and analyzed the results (raw data analysis and statistical analysis). I contributed to writing the manuscript draft and editing during the revisions.

Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2021 Dec; 165(4):445-451.

Compare features of human dental pulp cells cultured from pulp tissues with and without cryopreservation

Ming Yan, Ola A. Nada, Ralf Smeets, Martin Gosau, Reinhard E. Friedrich, Lan Kluwe

Background and Aims. Teeth extracted are usually disposed as bio-waste whereas they could serve as an autologous tissue for culturing multipotent dental pulp cells which have application potential in regenerative medicine. This study aimed to examine the feasibility of cryopreserving dental pulp tissue at teeth extraction for later culturing of cells.
Methods. The pulp tissue from each of a total of 10 teeth was cut into small fragments which were then divided into two portions. One portion was directly used for culturing pulp cells using the explant method. The other portion was cryopreserved with 10% DMSO in liquid nitrogen for at least one month and then thawed for culturing pulp cells.
Results. Vital cells were obtained from all the 10 pulp fragment suspensions which went through cryopreservation. The cell outgrowth from the explants of cryopreserved pulp fragments was two days later than that of corresponding fresh pulp tissue. Otherwise, no difference was observed in proliferation, expression of stem cell markers and differentiation into adipose cells and osteoblasts between the two groups of cells cultured from the fresh or the cryopreserved pulp fragments.
Conclusions. Cryopreserving fragmented dental pulp tissue provides a feasible option for saving pulp tissues as autologous cell sources for possible later application.

Key words: dental pulp cells, differentiation, dental pulp tissue, cell culture, tissue cryopreservation, osteogenic differentiation, adipogenic differentiation

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BACKGROUND

Dental pulp cells exhibit features of stem cells and therefore provide a valuable cell source for tissue engineering and regeneration medicine[1].These cells can be obtained from wisdom teeth and any other teeth which are extracted for medical indications[2,3]. For the patients, cells derived from their own teeth are autologous which will not cause host rejection in potential later application[4]. Dental pulp cells are vital, grow fast, are easy to handle in culture and exhibit high potential in multiple lineage differentiation especially into the osteogenic, adipogenic and condrogenic lineages[5]. With their neural crest origin, dental pulp cells exhibit higher neurogenic differentiation potential than bone marrow mesenchymal stem cells and therefore may be especially valuable for neuro-regeneration[6].

Cultured dental pulp cells can be cryopreserved for the long term and used for studies and applications at a later time[7]. However, conventional cryopreservation with 10% dimethyl sulfoxide (DMSO) is usually applied for single cells cultured in laboratories[8]. To obtain such single cells, the resources required are demanding and only available at limited hospitals and clinics. Though many people will likely have some of their teeth extracted during their life, teeth are disposed of as waste whereas they could provide a valuable autologous source for these patients for possible future application.

To solve this problem and to enable culturing pulp cells for more patients, cryopreservation of dental pulp tissues at surgery may provide a strategy. Indeed, cryopreservation has been shown to be feasible for storing adipose tissues for more than 6 weeks from which vital cells can be successfully cultured later[9] . Because the pulp tissues contain hardly any connecting tissues and the cells are loosely packed, we hypothesized that cryopreservation may be applied to dental pulp tissues without damaging large numbers of cells. When the pulp tissue is cut into small fragments, the cryo-protecting agent DMSO penetrates the tissues well and enters the cells as is the case of a single cell suspension. The present study is designed to test our hypothesis and to examine the feasibility of cryopreserving pulp tissues at the site of teeth extraction. Paired fresh pulp tissue and cryopreserved ones were used for culturing pulp cells in an identical experimental setting. Outgrowth from the tissue explants, morphology, proliferation, expression of stem cell markers and differentiation potential of the cells derived from the two difference sources were compared.

METHODS

Specimen

A total of 10 wisdom teeth of healthy donors were collected. According to the local regulation for privacy

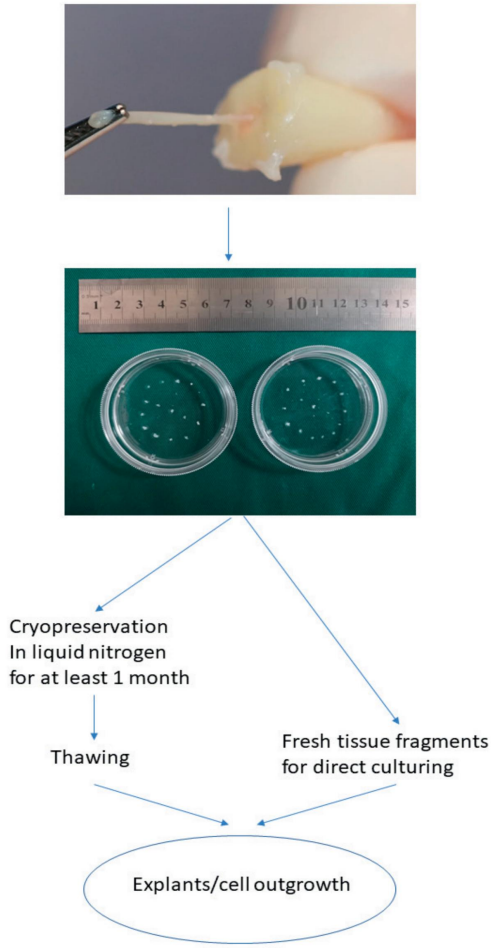

Fig. 1. An illustration of the procedure. A dental pulp pulled out from a tooth was cut into small fragments in medium. The fragment suspension was divided into two parts, one as fresh tissue for immediate culturing and the other for cryopreservation which was thawed later and used for culturing.

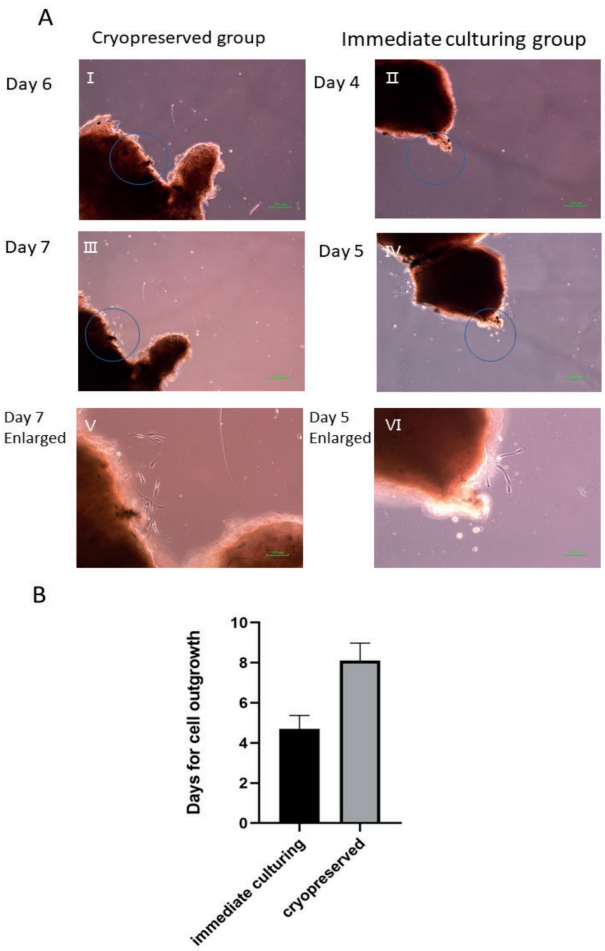

Fig. 2. A. Cell outgrowth from paired cryopreserved and immediate culturing groups. For cryopreserved group, cell growing out of the explants were visible in the circled area on day 7 (A-III). Note that no cells were visible one day before day 6 (A-I) in the same circled area. For immediate culturing group, cell outgrowth started two-day earlier, on day 5 (A-IV). **B.** Cell outgrowth for the 10 paired cryopreserved and fresh pulp fragments in mean and standard deviation.

protection, all specimens used for the study were anonymized. Patients signed the informed consent for the scientific use of teeth. Patients were instructed to gargle with a 1% hydrogen peroxide solution before the tooth extraction. The surgeon conducted a routine disinfection around the mouth. After the extraction, the teeth were kept in a Dulbecco's phosphate-buffered saline (DPBS) solution supplemented with 2 x 10^5 U/L penicillin and streptomycin at 4°C medium for up to 4 hours.

Cryopreservation of the pulp tissue fragments

Each tooth was rinsed with DPBS solution several times before it was broken with a hammer. The pulp tissue was pulled out with a tweezer and immersed into DMEM in culture dish where it was cut into small pieces of approximately 0.5 mm^3 (Fig. 1). The pulp tissue fragments were divided into 2 parts: one as fresh pulp tissue for immediate culturing and the other part for cryopreservation (Fig. 1). Cryopreservation was carried out by adding (DMSO) to a final concentration of 10%, cooling down the 1.6 mL suspension in a 4°C refrigerator for 1 hour, slowly freezing to -80°C overnight in a freezing container and finally storing in liquid nitrogen for at least six weeks.

Thawing the cryo-preserved tissue fragments and culturing cells

The tube containing the 1.6ml cryopreserved suspension of the pulp tissue fragments was removed from the liquid nitrogen and immediately placed in a water bath of 37°C. The cells were thawed within one minute and

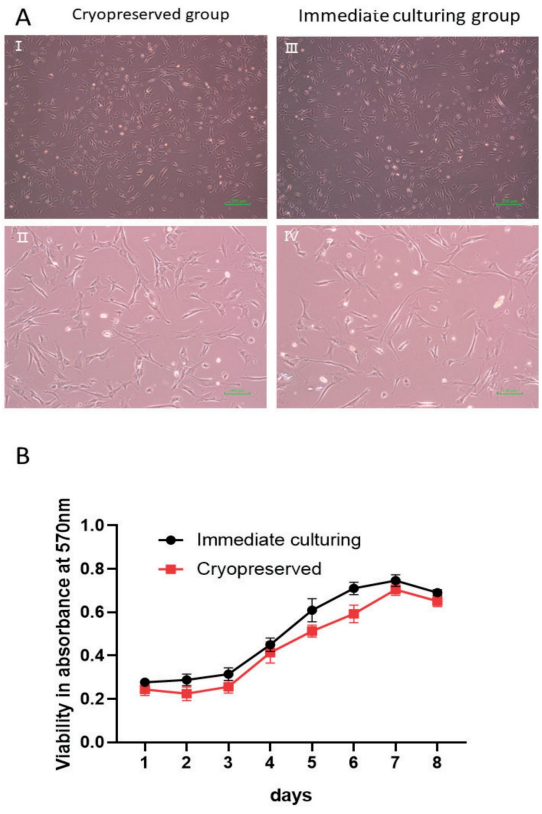

Fig. 3. A. Cells in monolayer in the 2nd passage. No difference in morphology was visible between the immediate culturing group (A-I, III) and cryopreserved group (A-II, IV). **B.** Growth curve of cells from the 10 pairs of immediate culturing/cryopreserved pulp tissue fragments (in mean and standard deviation).

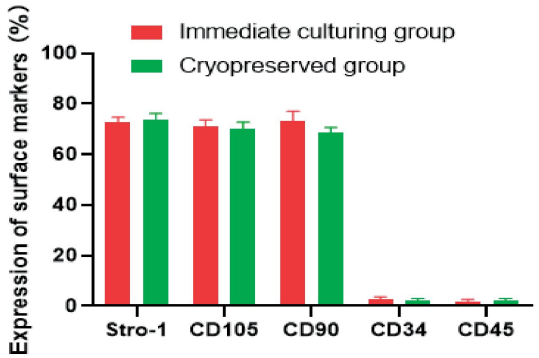

Fig. 4. Nearly identical expression of stem cell markers in cells from the paired immediate culturing/cryopreserved pulp tissue fragments.

then slowly transferred to a culture dish with 15 mL pre-warmed medium. Fresh pulp tissue fragments without cryopreservation was used for culturing cells in the same way (Fig. 1). The medium was changed on the next day. Then the dishes were kept in the incubator for two days. It was important not to move the dishes during these first two days to enable the pulp tissue explants to attach to the culture surface. From day 3, the dishes were checked daily under a microscope to follow the outgrowth of the cells from the explants. The day for first cell outgrowth was recorded. By confluence of 70-80%, the cells in monolayer were harvested with 0.125% trypsin, counted and passaged.

Characterization of the pulp cells

Cell viability was measured using a MTS assay (Promega) following the instruction of the supplier.

Cells in passage 3 were used for labelling with antibodies for surface markers of cells including STRO-1 (cat. no. 14-6688-82; thermo Fisher Scientific, Inc.), CD105 (cat. no. ab156756; Abcam), CD90 (cat. no. ab139364; Abcam), CD34 (cat. no. 560941; BD Biosciences) and CD45 (cat. no. MCA2035S; Bio-Rad Laboratories, Inc), all diluted at 1:500. The cell suspension was adjusted to a density of 1×10^9/L and passed through a 70 µm filter. After 30 min incubation at 4°C in darkness, the labelled cells were analyzed on a flow-cytometry device.

Differentiation

Cells in the 3rd passage were seeded at a density of 8×10^3/mL and grew to 70-80% confluence. Then the media were changed to adipogenic (with additives of 10 mg/mL insulin, 0.5 mM methylxanthine, 1mM dexamethasone, 0.1mM indomethacin) or osteogenic (with additives of 10mM β-glycerophosphate, 0.05mM vitamin C, 0.1mM dexamethasone) inducing media and the differentiation was continued for 3 weeks. At the end of the differentiation, the cells were stained with 0.5% oil red O (Sigma-Aldrich, Saint Louis, MI, USA cat no 00625) or 2% Alizarin red S (Sigma-Aldrich, Saint Louis, MI, USA cat. no. 130-22-3) to visualize oil droplets in adipose cells and mineral sediments produced in osteoblasts, respectively. To quantify lipid droplets, the oil red O in each well was dissolved in 500µL isopropanol and the absorption was read at 540 nm. Quantitative analysis of osteogenic differentiation was carried out by dissolving Alizarin red S in 500µL of 10% acetic acid and a subsequent neutralization with an equal volume of 10% ammonium hydroxide. The absorbance value was read at 405 nm.

RESULTS

Cell outgrowth and proliferation

For all the 10 cryopreserved dental pulp tissue fragments included in this study, the time to cell outgrowth (vital cells migrated from the tissues) was around 7 days. In the immediate culturing group, the cell was grown out from the tissue at day 5 whereas it was on day 7 in the

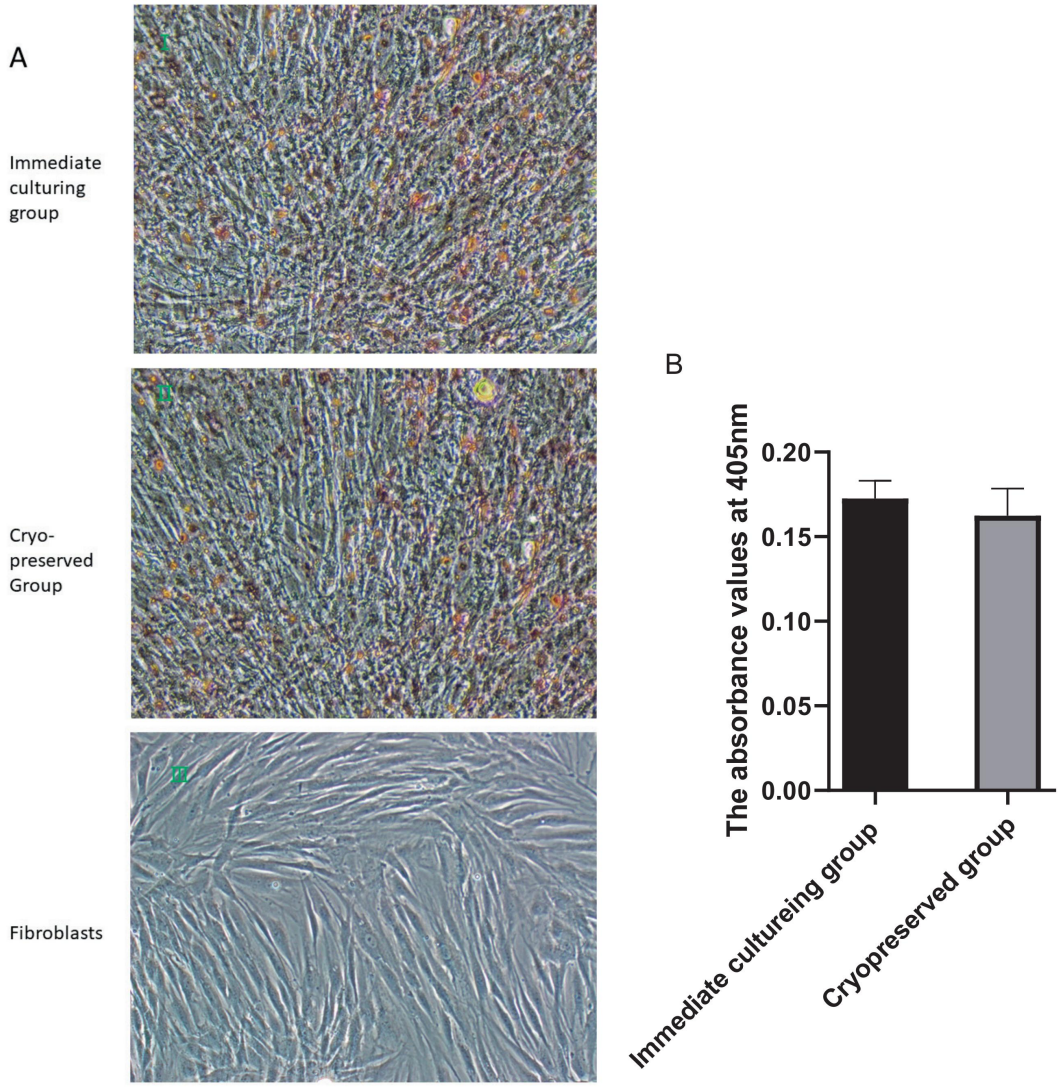

Fig. 5. A. Osteogenic differentiation of cells from paired immediate culturing/cryopreserved pulp fragments. Mineral sediments stained with Alzerin were clearly visible. Human gingival fibroblasts in primary culture were included as a negative control. **B.** quantiviation of dissolved alzerin did not detect significant difference between the immediate culturing/cryopreserved group (P>0.05).

cryopreserved group. (Fig. 2A). The mean time to cell outgrowth in immediate culturing group was significantly shorter than the cryopreserved group (P<0.05). For fresh pulp tissues, cell outgrowth was approximately 2-day earlier (Fig. 2B). However, once the cells migrated out and grew in monolayer, the same spindle, triangle and long spindle morphologies were observed for tissue fragments with and without cryopreservation (Fig. 3A). Also the growth curves of the cells from the two groups of tissue fragments were comparable (Fig. 3B).

Expression of stem cell markers

Pulps are made up of mesenchymal stem cells mostly[10], and markers that are more consistently reported are STRO-1, CD73, CD90, CD105, CD146, Oct4, Nanog, beta2 integrin positive, and CD14, CD34, CD45, and HLA-DR negative expression in mesenchymal stem cells[11-13]. In our study, flow cytometry analysis revealed compatible expression of 5 markers [(Stro-1, CD105, CD90 are positive), (CD45, CD34 are negative)] in cells from the pulp tissue fragments with and without cryopreservation. In both groups, approximately 70% of the cells were positive for Stro-1, CD105 and CD90. However, less than 10% of the cells were positive for CD34 and CD45 (Fig. 4).

Multipotential in differentiation

Dental pulp cells all exhibited the potential of adipogenic and osteogenic differentiation regardless of cryopreservation. Mineral sediment in cells under osteogenic

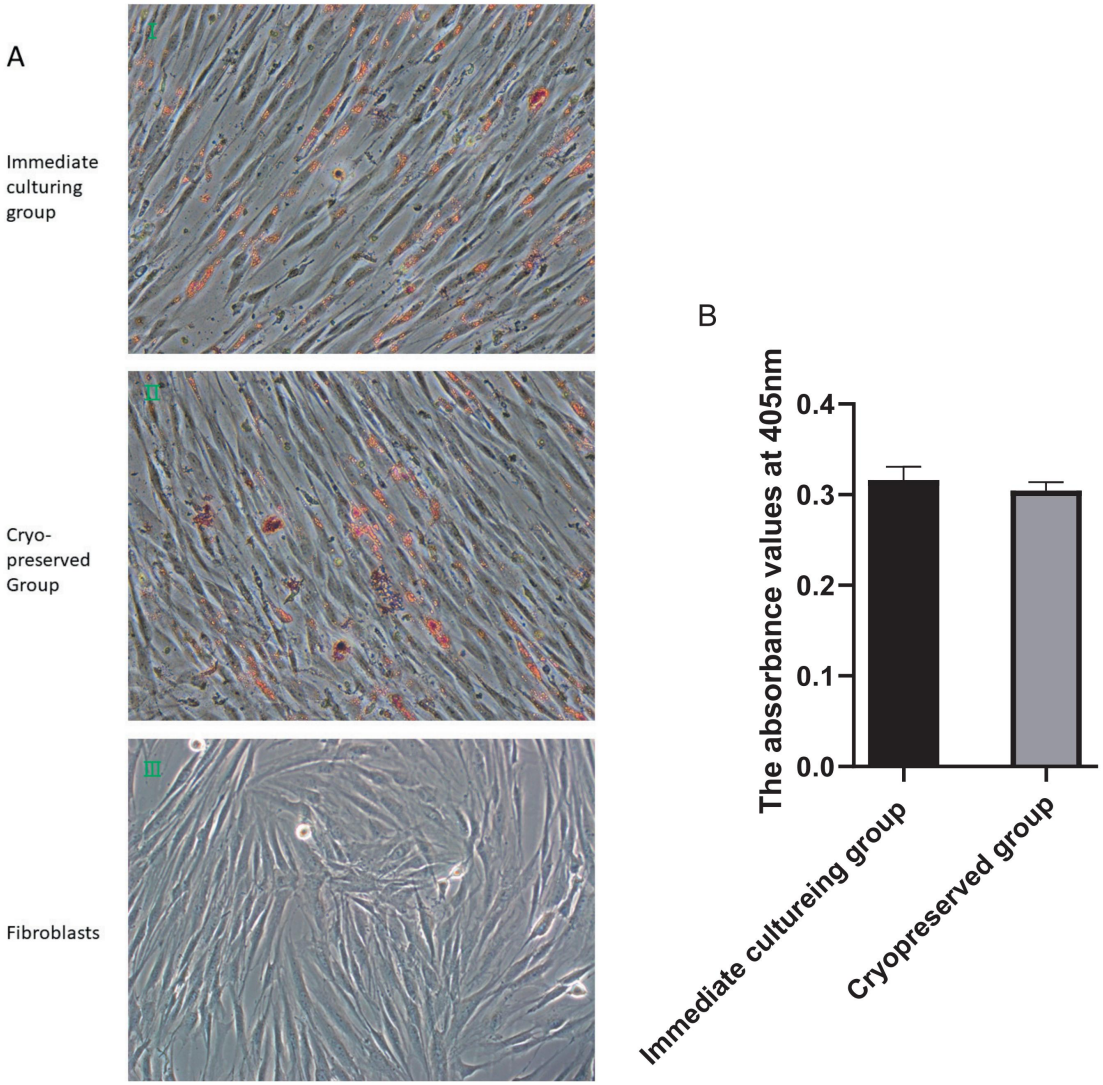

Fig. 6A. Adipogenic differentiation of cells from paired immediate culturing/cryopreserved pulp tissue fragments. Oil droplets stained with 0.5% Oil red O were visible in red-orange color. Human gingival fibroblasts in primary culture were included as a negative control. **B.** quantification of Oil red O did not revealed significant difference between the immediate culturing/cryopreserved group ($P>0.05$).

induction (Fig. 5) and oil-droplets in cells under adipogenic induction were clearly visible (Fig. 6). No difference was visible between the two groups of cells cultured using the fresh and cryopreserved tissue fragments. By contrast, no oil droplets nor mineral sediments were seen in the control primary gingival fibroblasts under the same inducing conditions, suggesting that the differentiation is a specific feature of the pulp cells, again, regardless of cryopreservation (Fig. 5A, 6A).

DISCUSSION

The choice of optimal seed cells is crucial during tissue engineering. Pulp cells which are one of the most studied stem cells, meet the rigorous criteria of an ideal source of seed cells for use in tissue engineering and cell therapy[14,15]. In pulp cell culture, the liquid nitrogen cryopreservation method is used as a standard method to store and bank[16,17]. Furthermore, methods have been updated continuously with the developments inbiotechnology[18,19]. The viability and differentiation capacity of dental pulp stem cells have been reported after two years of cryopreservation[20]. For the pulp tissue cryopreservation, Perry et al. showed that the tissues can be recovered from cryopreserved intact tooth at 4 °C refrigerator for up to 5 days[21] . In order to solve this problem, long-term cryopreserved dental pulp tissues, in our study, we demonstrated the feasibility of cryopreserving human dental pulp tissue fragments from which vital cells can be cultured later for long-term cryo-

preservation using our method. The cells cultured from the cryopreserved tissue fragments exhibited compatible proliferation and differentiation potential as cells cultured from their fresh counterparts. The difference between the two groups was the two-days delay in cell-outgrowth for the cryopreserved explants. This result is consistent with those of previous research that the growth of primary pulp cells requires an adaptation period after long-term cryopreservation[22].

Since the cultures are still in their 3-5 passages, we do not have the data at present for the total yield of cells. However, we expect that some cells will be damaged in the cryopreservation process and consequently, the total yield will not be as good as in the case of fresh tissues. Further studies need to address this issue.

A possible explanation for our success in obtaining vital cells from the cryopreserved pulp tissue fragments may be the loose packing structure of the pulp cells in the pulp tissue which allows sufficient penetration of the cryoprotecting agent DMSO as in the case of a single cell suspension. The other possible reason may be associated with the small volume of sample (<0.5 cm^3). As highlighted above, we propose minimizing the fragments volume as much as possible. For other tissues, this strategy may not be feasible. In the present study, our aim was to obtain single cells for culture. Therefore, it was not essential to maintain the original structure and cell organization of the tissue. However, the structure and cell organization may have been altered in the cryopreservation process and that may one reason for the delayed cell outgrowth from the one cryopreserved explants.

Despite the slightly delayed outgrowth, once vital cells were obtained from cryopreserved tissue fragments, similar features were expected for them as for the cells from fresh tissue fragments. We indeed observed compatible features in the paired fresh/cryopreserved tissue fragments as regarding proliferation, and adipogenic and osteogenic differentiation. Importantly, such differentiation was completely absent in the fibroblasts which were included as a negative control under the identical differentiation conditions, providing strong supporting evidence that the observed differentiation is specific for the human dental pulp cells. Our results are in agreement with published reports that cryopreservation does not cause changes in biological characteristics, structural, mechanical or biological properties[23].

Since the primary aim of the present study was to test the feasibility of cryopreservation of fragmented pulp tissues for later culturing vital cells, cutting the pulp tissues into fragments were carried out in the laboratory on a sterile culture bench to prevent contamination. In an ongoing study, we are testing the feasibility of preparing pulp tissues under standard clinical setting without a laboratory sterile bench. Should at least some of the pulp tissues survive the potential infection, preservation will be feasible at teeth extraction in any dental or oral clinic. The only necessary device is a home-hold -20°C freezer for temporary storage of tissue fragments which can be transported to a -80°C freezer later at any time of convenience and finally into the liquid nitrogen for long term preservation.

CONCLUSION

In summary, our study demonstrated that vital cells can be cultured from cryopreserved dental pulp tissue when cut into small fragments. Proliferation and differentiation potentials of these cells were sustained. Using this strategy, substantially more patients will have the option of saving their pulp tissues as an autologous cell sources for possible later application.

ABBREVIATIONS

DMSO, dimethylsulfoxide; CD105, Endoglin; CD90, Cluster differentiation 90/Thy-1 antigen; CD34, Hematopoietic progenitor cell antigen 1, and CD45, Leukocyte common antigen.

Ethics approval and consent to participate: Administrative permissions were acquired by our team to access the data used in our research. The study protocol was approved by the University Medical Center Hamburg-Eppendorf that approved the study. Accordingly, all teeth were coded with a number and all personal identification of the patients was removed. All parent or guardians of participants provided written informed consent for using the teeth which otherwise would have been discarded as waste.

Author contributions: MY: conceived the study, supervised the experiments and drafted the manuscript; RF: designed the experiments and drafted the manuscript; ON: data evaluation, medical writing and editorial assistance in preparing this manuscript for publication; RS: performed the data collection; MG: analyzed the data and revised the manuscript; LK: conceived the study together with All authors have read and approved the manuscript.

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4 Chapter 4: Publication in *International Journal of Molecular Sciences*, September 2022

Cultivation of Cryopreserved Human Dental Pulp Stem Cells—A New Approach to Maintaining Dental Pulp Tissue

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† These authors contributed equally to this work

Personal contribution:
The following research article has been published in ***International Journal of Molecular Sciences***. I am the shared first author of the paper. I conducted the experiments and data collection and analyzed the results (raw data analysis and statistical analysis). I contributed to writing the manuscript draft and editing during the revisions.

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Article

Cultivation of Cryopreserved Human Dental Pulp Stem Cells—A New Approach to Maintaining Dental Pulp Tissue

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Abstract: Human dental pulp stem cells (hDPSCs) are multipotent mesenchymal stem cells (MSCs) that are capable of self-renewal with multilineage differentiation potential. After being cryopreserved, hDPSCs were reported to maintain a high level of proliferation and multi-differentiation abilities. In order to optimize cryopreservation techniques, decrease storage requirements and lower contamination risks, the feasibility of new whole-tooth cryopreservation and its effects on hDPSCs were tested. The survival rates, morphology, proliferation rates, cell activity, surface antigens and differentiation abilities of hDPSCs isolated from fresh teeth were compared with those of one-month cryopreserved teeth in 5% and 10% DMSO. The data of the present study indicated that the new cryopreservation approach did not reduce the capabilities or stemness of hDPSCs, with the exception that it extended the first appearance time of hDPSCs in the teeth that were cryopreserved in 10% DMSO, and reduced their recovery rate. With the novel strategy of freezing, the hDPSCs still expressed the typical surface markers of MSCs and maintained excellent proliferation capacity. Three consecutive weeks of osteogenic and adipogenic induction also showed that the expression of the key genes in hDPSCs, including lipoprotein lipase (LPL), peroxisome proliferator-activated receptor-γ (PPAR-γ), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), type I collagen (COL I) and osteocalcin (OSC) was not affected, indicating that their differentiation abilities remained intact, which are crucial parameters for hDPSCs as cell-therapy candidates. These results demonstrated that the new cryopreservation method is low-cost and effective for the good preservation of hDPSCs without compromising cell performance, and can provide ideas and evidence for the future application of stem-cell therapies and the establishment of dental banks.

Keywords: human dental pulp stem cells; methodology; dimethyl sulfoxide; cryopreservation; cellular differentiation; teeth

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1. Introduction

In recent years, as research on stem-cell therapy and tissue engineering has intensified, the preservation and application of mesenchymal stem cells (MSCs) has become a hot research topic. MSCs are adult stem cells with self-renewal, a high proliferating ability and multi-differentiation potential, and they have a wide range of applications in the fields of stem-cell biology, vascular tissue engineering, and other areas of regenerative medicine [1]. Among them, human dental pulp stem cells (hDPSCs) are regarded as a reliable source due to their excellent self-renewal, proliferation capacity, and multipotent differentiation ability [2]. hDPSCs were first identified by Gronthos in the year 2000 [3]. They express the specific surface biomarkers of mesenchymal stem-cell antigens such as STRO-1, CD29,

CD44, CD59, CD73 and CD90, while CD19, CD24, CD34 and CD45 are not expressed [4,5]. It is reported that hDPSCs can be differentiated into various cell types in vitro, including adipocytes, chondrocytes, neurons, myocytes, and the most important for bone reconstruction in maxillofacial surgeries, osteoblasts [6–10]. Moreover, hDPSCs have a high differentiation potential in odontogenic lineages, and compared with bone marrow cells, a stronger ability to differentiate and proliferate [11,12]. Govindasamy et al. found that hDPSCs can even be differentiated into islet-like cell aggregates [13]. Dasari et al. found that DPSCs might be an appropriate source for therapeutic uses in neurological pathologies such as spinal cord injuries [14]. The repair and rebuilding of neuronal tissue [15–18], osseous tissue [19], hepatic tissue [16], muscle tissue [20] as well as salivary glands [21] are among the possible medicinal applications depending on DPSCs. Another significant benefit of hDPSCs is their easy accessibility: dental pulp cells can be conveniently isolated from extracted teeth, which might frequently be regarded as biological trash [22]. According to statistics, pulp tissue from interrupted third molars was most frequently used as a source of DPSCs [23]. In transplantation medicine, employing autologous cells can protect the regenerated tissue of donors from being rejected by the immune system by establishing histocompatibility banks [24]. Hence, if hDPSCs can be preserved in cell banks throughout therapy when their donors are both young and healthy, then there will be a stable supply of stem cells with excellent biological activities if their donors require them for regenerative therapies in the future.

The conventional cryopreservation process of dental pulp mesenchymal cells includes tooth disinfection, pulp extraction, cell isolation, cell proliferation, and cryopreservation [25]. However, dental clinics are not usually equipped with a research laboratory that ensures the sterile handling and storage of freshly extracted teeth. Before the hDPSC isolation, it is advantageous if the entire tooth is frozen and dental pulp is kept in an aseptic environment, namely pulp cavities, so that the possibility of sample contamination is minimized. Furthermore, in most cases it is a logistical and thus time-consuming effort to transport the extracted teeth immediately following extraction to a laboratory that is able to establish primary cell cultures.

So, we propose the immediate cryopreservation of whole teeth at −80 °C following extraction. Compared with the conventional cryopreservation procedure, this strategy can notably reduce the costs of cryopreservation—because the cell isolation step is omitted before clinical application—and storage in liquid nitrogen is unnecessary. Such limited processing might be more suitable for storing samples that do not have any imminent intentions for growth or usage. In addition, the majority of the cryopreservation methods can only be carried out in laboratories. However, the hDPSCs can only remain active in vitro for up to five days after teeth extraction [26]. The extra time needed to transport the tissue can cause damage to the cells and thus reduce the quality of the hDPSCs. To overcome this difficulty, the application of this novel method may extend the feasible period of hDPSCs in the event that samples cannot be handled in time. Therefore, the present study aimed to develop and evaluate a procedure that simplifies the storage of freshly extracted teeth intended to be used for hDPSCs. Dental pulp cells can be freshly preserved when they are most active, which may be more beneficial to the future use of these cells than long-term transportation.

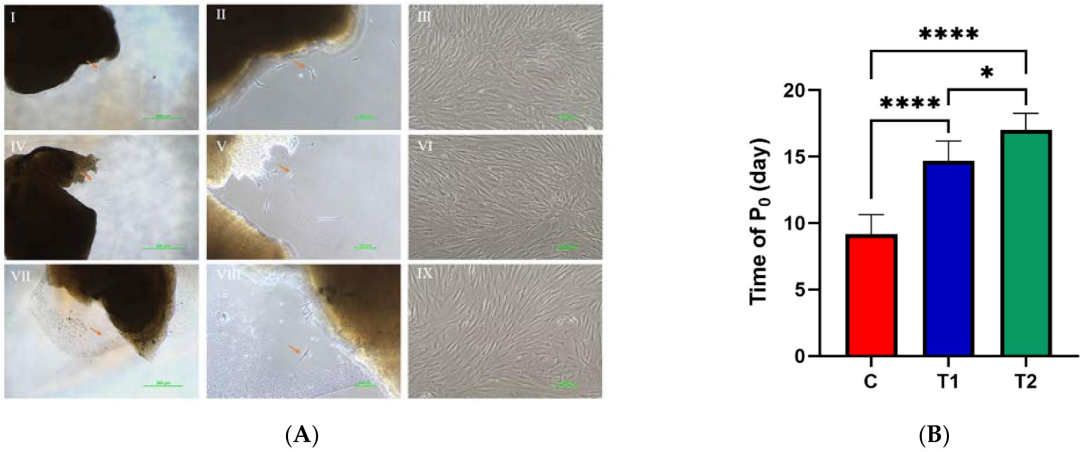
2. Results

2.1. Analysis of Results

2.1.1. The Impact of the Novel Cryopreservation Method on Cell Multiplication and Expansion

Dental pulp cells were inspected under a microscope every day to observe the cell morphology. The healthy cells resembled triangular or spindle shapes, with dark cytoplasm and a distinct nucleus. After the assessment of the morphology of the DPSCs by two trained examiners, Cohen's kappa coefficients of the T1 and T2 groups were κ = 88.9% and 83.3%, respectively, indicating almost perfect inter-observer agreement on the consistency of the

cellular morphology compared with the C group (Figure 1A). The dental pulp cells in the T2 frozen group took the longest time (16.830 ± 1.472 days) until the cells were initially observed to grow and attach. A significantly longer outgrowth time of the DPSCs in T1 group (14.670 ± 1.506 days) was observed compared to the C group (9.170 ± 1.472 days) (* $p < 0.01$) (Figure 1B).



(**A**) (**B**)

Figure 1. Morphology of dental pulp cells in different preservation strategy groups. (**A**) Morphology of dental pulp tissue and cells at the primary and the third generation. I–III: C group—Primary cells emerged from tissue blocks (×40; ×100) and the cells from the third generation after passage (×100). IV–VI: T1 group—Primary cells emerged from tissue blocks (×40; ×100) and the cells from the third generation after passage (×100). VI–IX: T2 group—Primary cells emerged from tissue blocks (×40; ×100) and the cells from the third generation after passage (×100). (**B**) The first appearance time of dental pulp cells in different groups: The first appearance time of dental pulp cells in the frozen groups was significantly longer than in the C group, and the first appearance time in the T2 frozen group was significantly longer than that in T1 group. The cryopreservation approach led to a longer period for cells to grow out of the tissue blocks. * $p < 0.05$, **** $p < 0.0001$.

2.1.2. Effects of New Cryopreservation Strategy on the Primary Cell Yield

A significantly larger number of DPSCs was collected in the C group ($(9.725 \pm 1.601) \times 10^5$) compared with the other two groups. The number of primary dental pulp cells harvested in T1 ($(6.333 \pm 1.341) \times 10^5$) and T2 ($(6.658 \pm 1.229) \times 10^5$) frozen group was smaller compared with the C group (* $p < 0.05$) (Figure 2).

2.1.3. Identification of Specific Stem-Cell Markers

CD34, CD45, CD73, CD90 and CD105 were detected through flow cytometry in the three groups: CD73, CD90 and CD105 were abundantly expressed, while CD34 and CD45 were not strongly expressed. (Figure 3).

2.1.4. Effects of New Cryopreservation Strategy on the Cell Survival Rate after Trypan Blue and Live–Dead Staining

There was no significant difference in the cell survival rate among the three groups ($p > 0.05$) (Figure 4B,C).

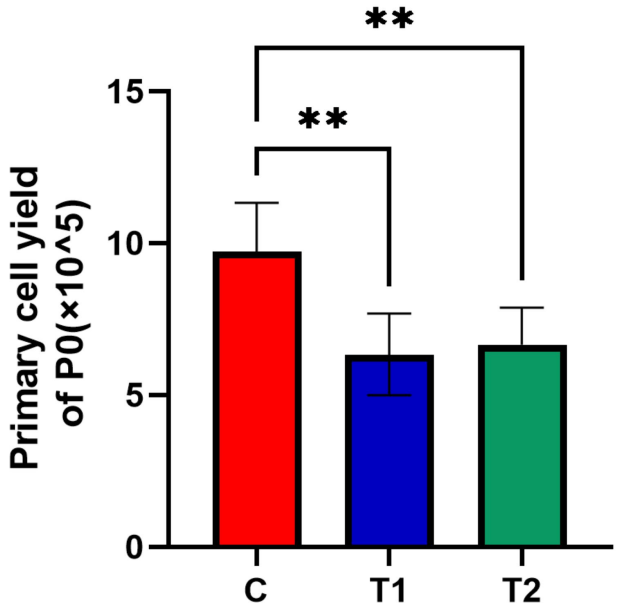


Figure 2. Effects of new cryopreservation strategy on the primary cell yield. The new cryopreservation strategy dramatically decreased the number of harvested primary cells compared with the C group (** $p < 0.01$).

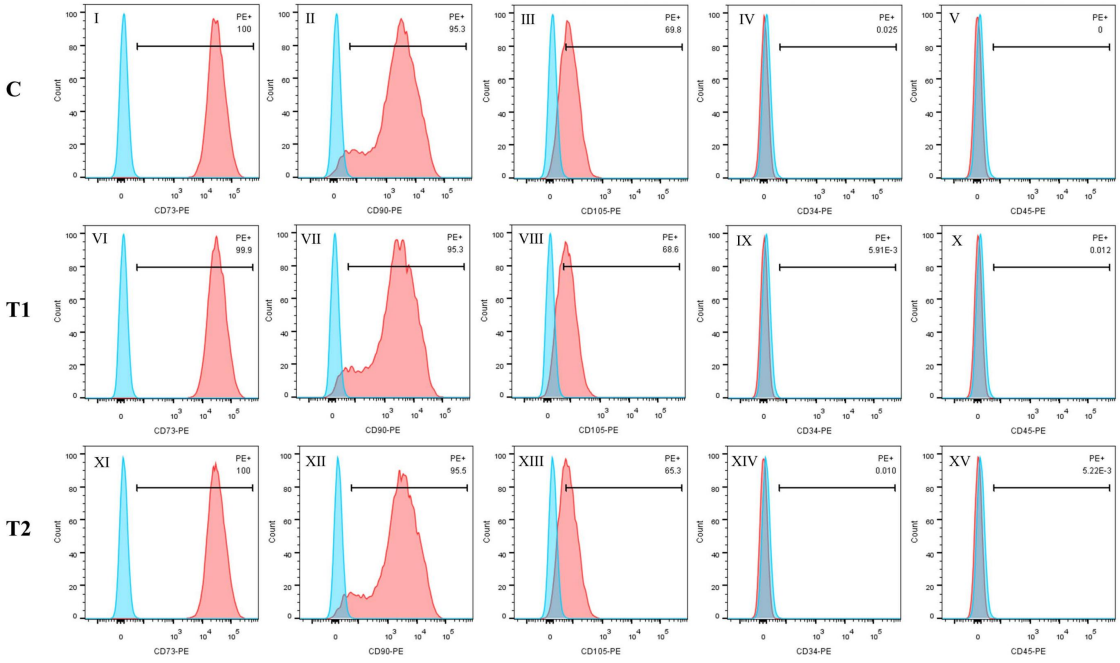


Figure 3. Identification of MSCs by flow cytometry in each group. I–V: C group; VI–X: T1 group; XI–XV: T2 group. The expression of CD73, CD90 and CD105 was positive; otherwise, CD45 and CD34 expression was negative in the three groups.

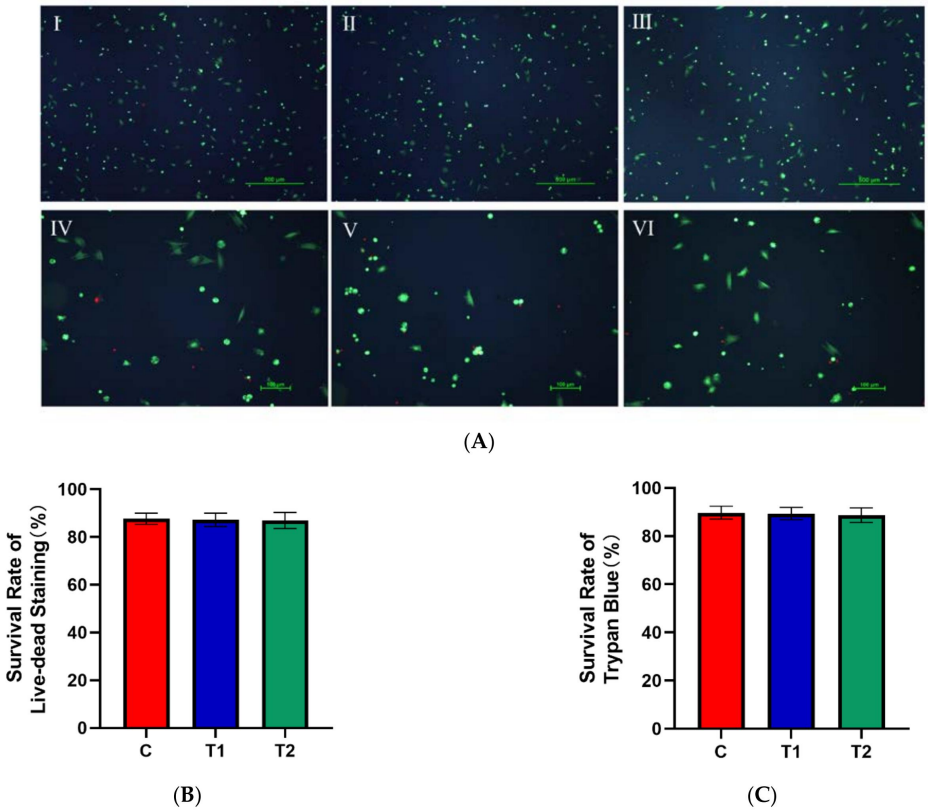


Figure 4. Effects of new cryopreservation strategy on the cell survival rate. (**A**) Live–dead staining results of dental pulp cells. I: C group (×40); II: T1 group (×40); III: T2 group (×40); IV: C group (×100); V: T1 group (×100); VI: T2 group (×100). (**B**) The survival rate of live–dead staining in each group. (**C**) The survival rate of Trypan Blue staining in each group.

2.1.5. Effects of New Cryopreservation Strategy on the Primary Cell Proliferation

There was no significant difference in the cell proliferation ability, including cell colony-forming efficiency as well as the growth curve among the three groups ($p > 0.05$) (Figure 5B,C).

2.1.6. Effects of New Cryopreservation Strategy on the Differentiation Potential of Dental Pulp Cells

The cells from the third generation were stained after osteogenic and adipogenic induction for three weeks following the respective protocols (Figure 6A) and the corresponding absorbance was detected by a spectrophotometer at different wavelengths. The results illustrated that there was no difference among the three groups in terms of the ability of osteogenic and adipogenic differentiation ($p > 0.05$) (Figure 6B,C).

2.1.7. Representative Gene Expression Profile of LPL, PPARG, ALP, RUNX2, COL I and OSC in Each Group

The relative expressions of two adipogenic and four osteogenic genes were not significantly different at the same time point among the three groups ($p > 0.05$). However, the relative expression of osteogenic genes changed with the time of osteogenic induction. (Figure 7A–C).

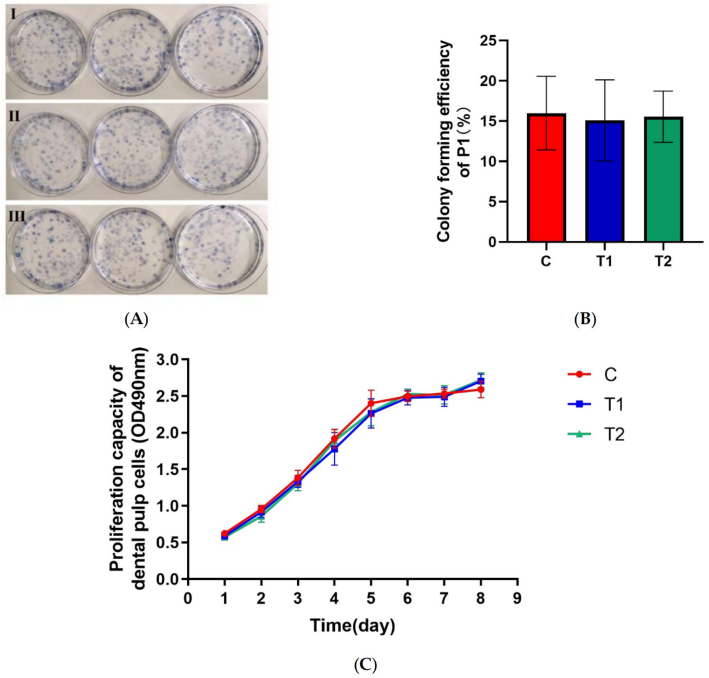


Figure 5. Proliferation ability of primary dental pulp cells in each group. (**A**) Giemsa staining results of dental pulp cells. I: C group; II: T1 group; III: T2 group. (**B**) Colony-forming efficiency. (**C**) Cell growth curve (MTS assay).

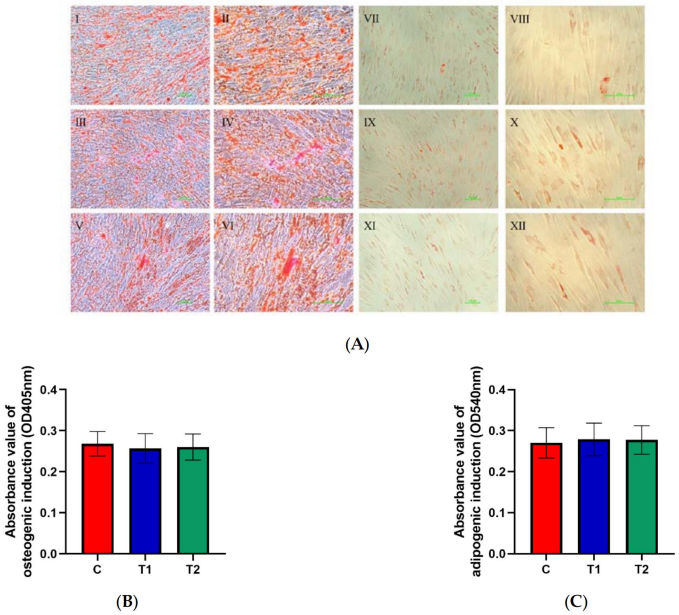


Figure 6. Differentiation potential of dental pulp cells. (**A**) Osteogenic and adipogenic differentiation results of dental pulp cells. I–VI: Osteogenic differentiation results. I: C group (×40); II: C group (×100);

III: T1 group (×40); IV: T1 group (×100); V: T2 group (×40); VI: T2 group (×100). VII–XII: Adipogenic differentiation results. VII: C group (×40); VIII: C group (×100); IX: T1 group (×40); X: T1 group (×100); XI: T2 group (×40); XII: T2 group (×100). (**B**) The absorbance value at 450 nm of dissolved solution after Alizarin Red S staining in the three groups. (**C**) The absorbance value at 540 nm of dissolved solution after Oil Red O staining in the three groups.

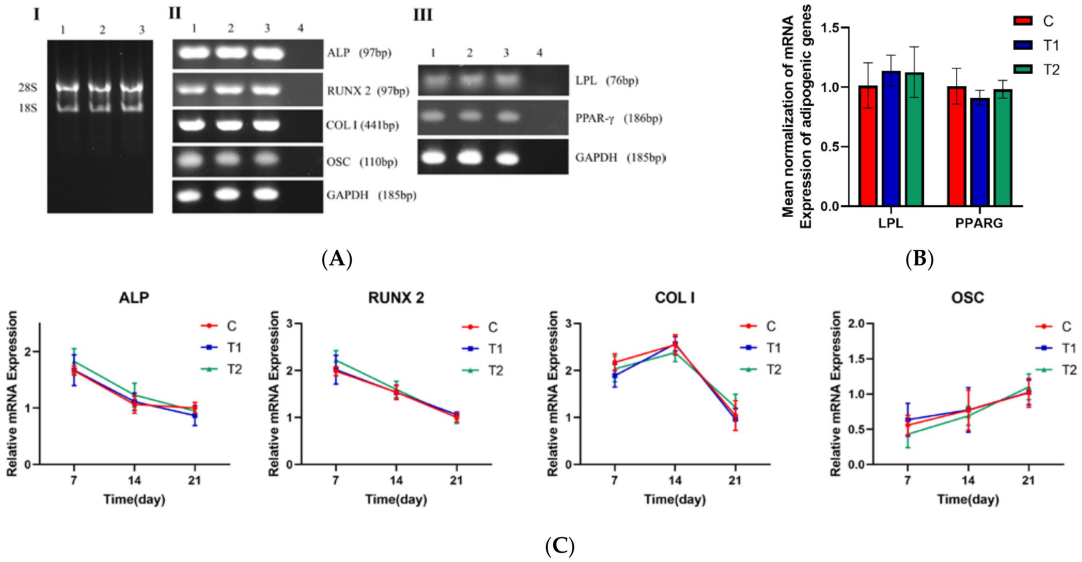


Figure 7. Representative gene expression profile in different groups. (**A**) Gel electrophoresis results. I: RNA electrophoresis of three groups. 1: C group; 2: T1 group; 3: T2 group. II: Electrophoresis results of osteogenic expression and reference genes in each group. 1: C group; 2: T1 group; 3: T2 group; 4: Negative control group. III: Electrophoresis results of adipogenic expression and reference genes in each group. 1: C group; 2: T1 group; 3: T2 group; 4: Negative control group. (**B**) Quantitative real-time PCR results of adipogenic genes. (**C**) Quantitative real-time PCR results of osteogenic genes.

2.1.8. ALP Assay Test to Identify the Osteogenic Activity of hDPSCs

There was no significant difference in the ALP activity among the three groups at the same time point. As the duration of osteogenic induction increased, ALP activity gradually decreased ($p > 0.05$, Figure 8).

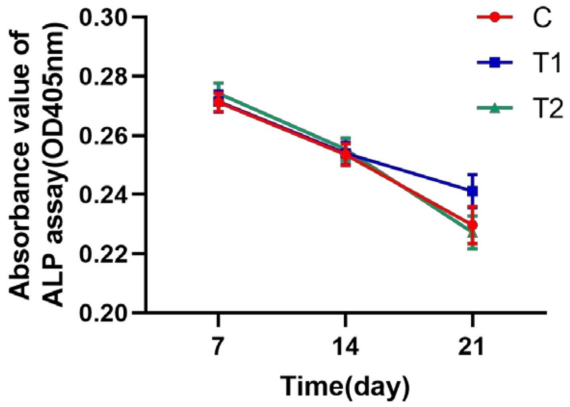


Figure 8. ALP assay results of dental pulp cells in each group.

3. Discussion

In this study, we devised a more accessible novel cryopreservation approach using varying concentrations of DMSO to preserve the third molars, which can be a source of hDPSCs for the donor. To evaluate the effect of the new cryopreservation strategy, 18 third molars were extracted and cell lineages were separated, then the difference between the frozen and unfrozen teeth was examined. It was demonstrated that the T1 and T2 DMSO frozen groups showed significantly longer times for the outgrowth of cells than the fresh dental pulp tissue, indicating that a higher dose of DMSO will extend the growth time of primary cells. Last but not least, the total number of primary cells yielded in the T1 and the T2 frozen groups was considerably lower than in the C group. After the first passage, however, the cryopreservation at −80 °C for one month showed no influence on the morphology of the dental pulp cells, including their cell size, cytoplasmic density and nucleus. The data of colony-forming efficiency, cell survival rate and the MTS test illustrated that the new cryopreservation method had no impact on dental pulp cell activity. In accordance with the International Cell Therapy Society's (ISCT) guidelines, we identified the surface antigens of the hDPSCs cultured from the teeth that were frozen using novel cryopreservation techniques and discovered that CD45 and CD34 expression was negative in the three groups, while CD73, CD90 and CD105 expression was positive, which is similar to a previous study [5]. Since these cells' surface antigens biologically match those of MSCs, cryopreservation techniques would not have a negative impact on the stemness of dental pulp cells. In addition, we noticed that the ability of dental pulp cells to differentiate into adipogenic and osteogenic tissues in cryopreservation groups was not significantly affected according to the detection of calcium and lipid deposits after 21 days of differentiation induction, and the relevant mRNAs—especially relevant osteogenic genes including ALP, RUNX2, COL I and OSC—were not observed to be downregulated at the same time point (day 7, day 14 and day 21). The ALP assay results among the three groups showed no significant difference, but the relative expression of osteogenic genes changed with the time of osteogenic induction, demonstrating that dental pulp cells in the DMSO frozen groups retained their differentiation capacity after the passage.

The present study by our group showed that the new cryopreservation strategy inevitably reduced the harvest of primary pulp cells and increased their initial appearance time, which may be due to the cryopreservation approach reducing the contact between the pulp tissue and the cryoprotectants that ensure the integrity of the teeth, resulting in an insufficient number of cells being protected. Furthermore, being one of the most widely used cryoprotectants, DMSO concentrations below 10% can be mildly hazardous, despite its ability to decelerate the ice-crystal-formation rate in cells and preserve the structural and functional integrity of cells after unfreezing [22,27]. The passaged pulp cells' growth proliferation and differentiating capacities, on the other hand, were not considerably harmed by freezing. This might be due to a month-long freezing period not being too lengthy; therefore, cell damage was not obvious. In addition, the dental tissue of the donors selected by this experiment had a high cell volatility, allowing the cells to withstand physical and chemical stimulation.

There is a significant demand for bone-regeneration therapy in individuals over 40 years of age due to dental implant treatment, tumor surgery, trauma, and periodontitis-induced alveolar bone loss [28]. In this regard, hDPSCs are considered a promising source of cells for regenerative medicine and tissue engineering [29]. However, research has shown that the quantity of MSCs and their ability of self-renewal are reduced as age increases [30]. The proliferation and differentiation ability of MSCs also decreases with age due to telomere shortening [31], DNA damage and epigenetic changes in transcriptional regulation [32,33]. In addition, increased secondary dentinogenesis and root-canal mineralization in older individuals resulted in the significant shrinkage of pulp tissue, which further increased the difficulty of collecting hDPSCs [34]. Therefore, preserving patients' hDPSCs in advance at a young age is one of the keys to ensuring their stem-cell function (including proliferation and differentiation ability). With the preservation method used in our experiment, we

can cryopreserve and store as many hDPSCs with the best stemness and the highest proliferative capacity as possible.

A previous study by our lab has shown that dental pulp tissue can be cryopreserved in a 5% and a 10% DMSO culture media at −196 °C for one month, with no significant difference in the proliferation, cell growth, and differentiation capacity of hDPSCs between the frozen and unfrozen groups [35]. This conclusion serves as a useful guide for the large-scale and long-term preservation of dental pulp tissue. However, there is still a risk of tissue contamination due to the constrained settings, primarily the lack of a sterile atmosphere in the operation blocks of clinics and hospitals. Moreover, most dental offices lack liquid nitrogen cryopreservation chambers at −196 °C to preserve tissue and cells. Our new approach supports evidence from prior studies, which noted that hDPSCs stored at −80 °C in 10% DMSO for 1–5 years still retained very high capabilities [36,37]. This is also partly consistent with a study showing that hDPSCs can be preserved at −85 °C for six months without a loss of function [38]. Ginani F et al. also discovered that after six-month storage at −80 °C, cells from human exfoliated deciduous teeth could retain similar properties in terms of the cell viability and proliferation rate in 10% DMSO for up to six months [39], suggesting that the method of the cryopreservation of whole teeth may still be suitable for the preservation of deciduous teeth.

The ability of stem cells to multi-differentiate is a prerequisite for their therapeutic use. Because of their ability to develop into endoderm, mesoderm, and ectoderm lineages, dental mesenchymal stem cells are considered an ideal source of stem cells in cell engineering and tissue regeneration. Yanasse et al. mixed hDPSCs with platelet-rich plasma (PRP) to form a stem-cell scaffold and found a significant improvement in articular cartilage repair in a rabbit model [40]; Wang et al. injected the cultured secretome or vehicle (DMEM) of hDPSCs into a mouse model of amyotrophic lateral sclerosis (ALS) and found a significant increase in the number of days of survival after the onset of the disease and in the total life span of the mice [41]; Li et al. cultured and intravenously transplanted hepatocyte growth factor (HGF)-transformed hDPSCs into a rat model of ulcerative colitis (UC) and found that HGF–DPSCs could inhibit inflammatory responses by transdifferentiating into intestinal stem cell (ISC)-like cells, promoting ISC-like cell proliferation, inhibiting inflammatory responses and reducing oxidative stress injury [42]; Hata et al. injected hDPSCs into diabetic polyneuropathic nude mice after culture and found that they significantly improved delayed nerve-conduction velocity, reduced blood flow and increased the sensory perception threshold [43]. Apart from that, hDPSCs have a significant advantage over other stem-cell sources in terms of accessibility. Third molars are the most common source of teeth for use in stem-cell cryopreservation compared to other teeth. It was reported by Carter et al. that the prevalence of wisdom teeth ranges from 18–68%, with 41% of those being diagnosed as mesioangular impaction [44], which were frequently suggested for prophylactic extraction by dentists. In this study, the teeth selected were incompletely erupted, impacted third molars, which do not affect the oral chewing function when extracted. Impacted third molars can cause swelling and ulceration of the surrounding gingival area, root damage to the second molars, decay of the second molars, and gingival and skeletal disease around the second molars. Impacted third molars are also related to pathological changes such as pericoronitis, root resorption, periodontal disease, caries, and the development of cysts or tumors [45]. In the long term, the retention of impacted wisdom teeth may enhance the risk of pathology in the surrounding structures, and their removal at later ages may result in more frequent and serious complications [46]. Therefore, the preventive extraction of asymptomatic healthy wisdom teeth, whether impacted or fully erupted, has long been regarded as appropriate care [47,48]. In addition to impacted third molars, the second common source of hDPSCs is premolars [23], especially those extracted during orthodontic treatment for severe crowding and Class II malocclusions. The age of these patients is usually around 10 to 16 years [49]. Multiple teeth are the third most common source of hDPSCs [23]. Therefore, from a biological point of view, any other healthy tooth, including the above three categories, may be used as a source of hDPSCs. Additionally, the premolars

extracted during orthodontic treatment are another frequent source of dental pulp cells [23]. Rather than being regarded as biological waste, viable dental pulp cells could be a dependable and universal source of stem cells that can be broadly applied to organ reconstruction and tissue regeneration if the teeth can be preserved appropriately following extraction surgeries. Amongst the conventional cryopreservation processes, the most critical step can be the extraction of the dental pulp, in which an improper technique can easily lead to dental tissue contamination. Moreover, as storage time rises, the amount of pulp stem cells that can be separated from removed teeth decreases [50]. Studies have shown that dental pulp stem cells can remain active in vitro for up to one night or 12 h [51]. To put it another way, in a sterile operating environment, this is the shortest possible exposure time in vitro. Our study has proved that whole-tooth cryopreservation at −80 °C is achievable, which would boost the success rate of obtaining healthy dental pulp stem cells while minimizing the risk of contamination during operation after tooth extraction and maintaining the phenotype of primary cells. Furthermore, through this method, we were able to lower the requirements as well as the expense of hDPSC preservation, which creates better conditions for the establishment of dental stem-cell banks and future clinical applications.

However, due to the limited time and samples, additional research into the longest period of cryopreservation of complete teeth is problematic. If we figure out this problem, then the whole-tooth cryopreservation schedule would be more flexible, ensuring that they are therapeutically deployed at the optimal period for cell function. Our future research may be conducted on this topic to provide a deeper understanding of the preservation of hDPSCs.

4. Materials and Methods

4.1. Collection of Samples

Teeth extraction and cryopreservation: Between November 2019 and June 2020, 18 impacted third molars of healthy teenagers aged 15–19 years old were gathered in the Department of Oral and Maxillofacial Surgery of University Medical Center Hamburg-Eppendorf. The experimental protocol was authorized by the institutional review board of the medical chamber of Hamburg (IRB-vote # REC 1712/5/2008). The patients and their guardians completed the informed permission forms prior to surgeries and were taught to gargle with 1% H_2O_2 solution for 3 min. Furthermore, the standard sterilization procedures were followed during the whole process of operations. The extracted third molars were then removed and immediately placed in DMEM (Cat. NO. 41965-049, Gibco, Loughborough, UK) with 10% FBS (Cat. NO. 10500-064, Gibco, Paisley, UK) and penicillin (100 U/mL)/streptomycin (Cat. NO. 15140-148, Gibco, Paisley, UK) at 4 °C. Teeth were then soaked in 4 °C DPBS (Cat. NO. 14190-094, Gibco, Paisley, UK) solution (containing 3×10^5 U/mL penicillin/streptomycin) for 30–60 min followed by experiments within 2–4 h (Figure 9). The whole progress of this experiments is depicted in a flow diagram (Figure 10).

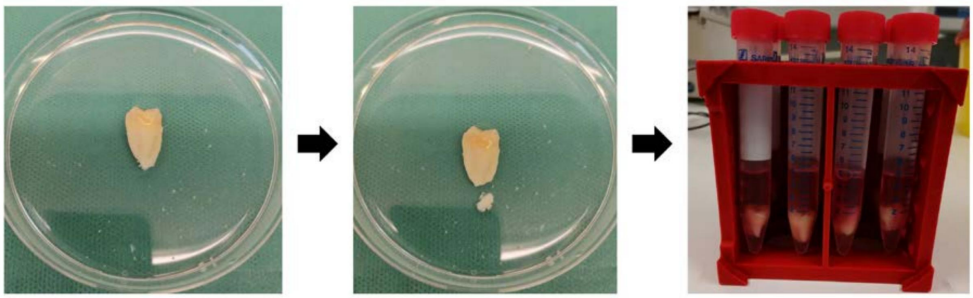


Figure 9. The procedure of teeth treatment in T1 and T2 frozen groups: 1/3 of the apical was removed and stored in the freezing medium containing DMSO.

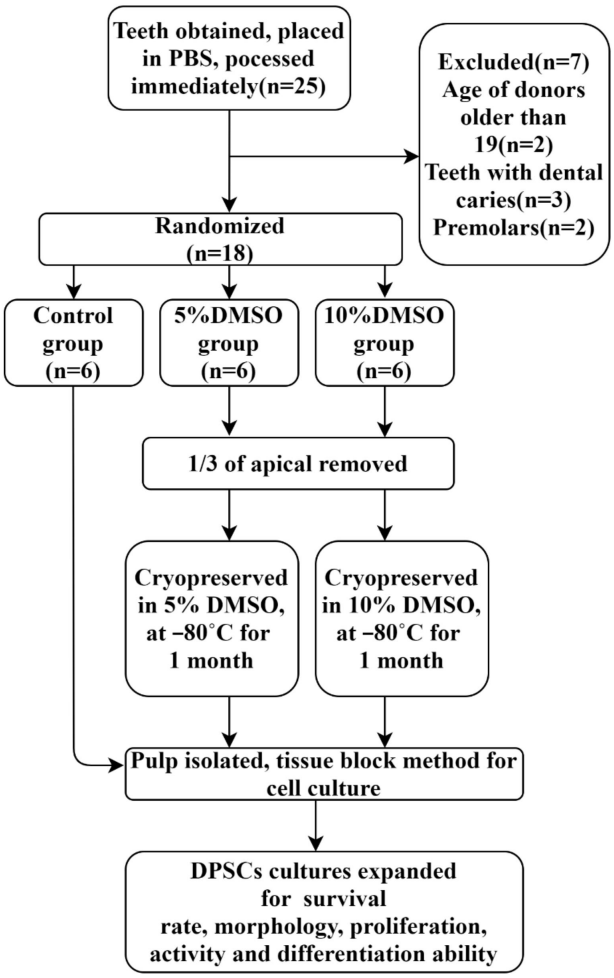


Figure 10. Flow diagram for teeth processing. DPSCs in six teeth were immediately cultured for cellular characterization experiments. After a month of cryopreservation, others were cultivated and examined.

4.2. Cryopreservation

The third molars were then allocated into 3 groups at random: a control group (C), a 5% DMSO group (T1), and a 10% DMSO group (T2). For the T1 and T2 groups, the corresponding concentration of DMSO (Cat. NO. 2308.0100, Geyer GmbH, Germany) medium was used to immerse the teeth. Previously, sterilized high-speed turbine drills were used to remove a section of the root from 1/3 of the apical in order for the DMSO cell-cryopreservation solution to have greater contact with the pulp tissue (Figure 8). After being stored in the refrigerator at 4 °C for 30 min and −20 °C for 1 h, the teeth in the T1 and T2 groups were then preserved in the −80 °C refrigerator for 1 month. The teeth in the C group, on the other hand, were split open using a sterilized high-speed turbine drill to expose the pulp. Thereafter, the intra-dental pulp tissues were cut into 0.5 mm^3 tissue blocks and placed in cell culture plates.

4.3. Culture of hDPSCs

Tissues of each group were dispersed in 24-well plates. Each tissue block was positioned in its own well and dipped in 600 uL DMEM culture medium (with 10% FBS and 100 U/mL penicillin/streptomycin) after it affixed to the bottom. The plates with tissue blocks were then incubated at 37 °C in 5% CO_2 incubators. Every day, an inverted microscope was used to evaluate cell adhesion and cultivation conditions. When confluency of colonies reached the optimal level, the cells were isolated with 0.05% Trypsin (Cat. NO. 25300-054, Gibco, Paisley, UK) and counted with cell-counting boards. The cells were passaged when a density of roughly $8 \times 10^3/cm^2$ was reached.

4.4. Assessment of Primary Cellular Morphology and Recording of Primary Cell Growth Time

Two trained and calibrated experimenters examined the pulp tissue daily with an inverted microscope, assessed the morphology of the primary cells and recorded the time from tissue block implantation to cell adhesion and expansion for each group. When the cells reached 80–90% confluency, the primary cells were digested and passaged.

4.5. Cell Yield Computation

After the primary cells reached 80–90% confluency, they were digested using 0.05% trypsin for 3 min. The trypsinization process was halted with an equivalent volume of DMEM culture medium as soon as the cellular morphology was observed to be spherical and floating. Afterward, the hDPSCs were transferred into 50 mL tubes and centrifuged at 1000 rpm for 10 min at room temperature. The supernatant was discarded and the cells were suspended again in DMEM culture medium. The number of cells was calculated with cell-counting plates. Primary cell quantity = Total cell number/4 × dilution factor × 104 × volume of cell suspension

4.6. Flow Cytometry

Dental pulp cells were collected for flow cytometry. Specific PE-conjugated antibodies including CD34 (Cat. NO. 343505, Biolegend, San Diego, CA, USA), CD45 (Cat. NO. 304007, Biolegend, San Diego, CA, USA), CD73 (Cat. NO. 344003, Biolegend, San Diego, CA, USA), CD90 (Cat. NO. 328109, Biolegend, San Diego, CA, USA) and CD105 (Cat. NO. 323205, Biolegend, San Diego, CA, USA) were chosen to test the cells following the protocol from the manufacturer. Each tube containing 2×10^5 cells received 100 µL of dye liquid containing 1:1000 live/dead dye and 1:100 antibody, which was then incubated for 20 min at room temperature. After being washed with PBS, cells were detected with BD LSRFortessa cell analyzer (Becton Dickinson Bioscience, Becton, USA) and BD FACSDiva software V6.1.3 (Becton Dickinson Bioscience, USA). Data were further analyzed with FlowJo software V10.0.7 (Treestar Inc., Ashland, OR, USA).

4.7. Colony-Forming Efficiency

A total of 1×10^3 cells of each group from the first generation were seeded on 10 cm-diameter culture dishes. The culture medium was changed every 3 days. The medium was removed two weeks later, and the cellular colonies were fixed in pure methanol for 10–15 min, rinsed three times with DPBS, and stained with Giemsa Solution (Cat. NO. 48900, Sigma-Aldrich, Schaffhausen, Switzerland) for 10 min. The colonies of dental pulp cells with a number greater than 50 were recorded as being available. Three parallel dishes were set for 1 sample. Colony-forming efficiency = number of available colonies/number of seeded cells × 100%

4.8. Cell Survival Rate

Trypan Blue staining: Cells from the first generation in the three groups were stained with 0.4% Trypan Blue (Cat. NO. 15250-061, Gibco, Paisley, UK) for 2 min and then observed using the inverted microscope. Within 500 dental pulp cells, the number of unstained living

cells was recorded and the cell survival rates were computed. Cell survival rate of Trypan Blue = number of unstained living cells/500 × 100%

Live–dead staining: Cells from the first generation of each group were seeded on TCC (tissue culture coverslips, Cat. NO. 83.1840.002, Sarstedt, Nümbrecht, Germany) at a density of 8×10^4/mL in 12-well plates, corresponding to 8×10^4 cells per well, and were incubated at 37 °C in 5% CO_2 incubators for 4 h. Next, 60 μL of propidium iodide (PI) (50 μg/mL in PBS) and 500 μL fluorescein diacetate (FDA) working solution (20 μg/mL) were added to each well. After 3 min of incubation at room temperature and rinsing with DPBS, samples were observed with a fluorescence microscope (Nikon ECLIPSE Ti-S/L100, Düsseldorf, Germany). The number of live cells, which were stained in green, were counted. The cell survival rate was calculated. Cell survival rate of live–dead staining = number of green-stained cells/number of total cells × 100%

4.9. Proliferation Testing with MTS Assay

Cells from the third generation were seeded in 96-well plates at a density of 2×10^4/mL, equating to 2×10^3 cells per well, and were incubated at 37 °C in 5% CO2 incubators. The proliferation of cells was measured with the MTS assay (Cat. NO. G1111, Promega, Madison, USA) for 8 continuous days in total. Cells from 3 wells of each sample in every group were subjected to MTS colorimetric analysis. Next, 20 μL of MTS reagent was added to each well and the absorbance was detected after 3 h of incubation using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at the wavelength of 490 nm.

4.10. Differentiation Potential Assessment

Adipogenic differentiation: Cells from the third generation were cultured at a density of 2×10^4/mL, corresponding to 4×10^4 per well in 6-well plates. EVE Automatic Cell Counter (NanoEntek, Seoul, Korea) was used to calculate the concentration of the cell suspension in each group 3 times. Then, the cell suspension was plated at the same volume into 6-well plates after normalizing the concentration of cells in each group to ensure that the number of cells added to each well and the volume of culture medium were the same. The cells were grown in adipogenesis-induction medium (DMEM containing 10% FCS, 5 μg/mL insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, and 10 μmol/L dexamethasone) for 3 weeks after reaching 60–70 percent confluency, and the medium in the wells was replaced every 3 days. On the 22nd day, the cells were fixed with paraformaldehyde (PFA; Electron Microscopy Sciences, Fort Washington, PA, USA) for 20 min and rinsed 3 times with DPBS. The stimulated cells were then stained with 0.5% Oil Red O solution (Cat. NO. O1391-250ML, Sigma-Aldrich, St. Louis, MS, USA) for 10–15 min. After staining, cells were rinsed 3 times with DPBS and the stained lipid droplets in the cytoplasm were seen and photographed using an inverted microscope.

Adipogenic differentiation quantitative analysis: To eliminate the remaining staining solution, stained cells in plates were washed three times with DPBS. Thereafter, 1 mL isopropanol (Cat. NO. 34965-1L, Honeywell, North Carolina, Charlotte, US) was added to each well to dissolve the lipid droplets, and the plates were gently shaken until the solution was equally colored. Then, the solution was transferred into 96-well plates at 100 μL well and detected using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 540 nm. Three parallel groups were set for 1 sample.

Osteogenic differentiation: Cells from the third generation were cultured at a density of 2×10^4/mL, corresponding to 4×10^4 per well in 6-well plates. EVE Automatic Cell Counterwas used to calculate the concentration of cell suspension in each group 3 times. Then, the cell suspension was plated at the same volume into 6-well plates after normalizing the concentration of cells in each group to ensure that the number of cells added in each well and the volume of culture medium were the same. After attaining 60–70 percent confluency, the cells were cultured for 3 weeks in osteogenic-induction medium (DMEM with 10% FCS, 10 mmol/L glycerophosphate, 5 mmol/mL ascorbic acid, and 1 mol/L dexamethasone). The medium was changed every 3 days. On the 22nd day, the cells were

fixed with paraformaldehyde for 20 min and washed 3 times with DPBS. The stimulated cells were then stained for 10–15 min with 0.1% Alizarin red S (Cat. NO. GT6383, Glentham, Germany) solution. After staining, cells were rinsed 3 times with DPBS and the inverted microscope was used to observe and take pictures of the stained calcium nodules.

Osteogenic differentiation quantitative analysis: To remove leftover staining solution, stained cells in plates were washed three times with DPBS. Following that, 750 µL of 10% acetic acid (Cat. NO. 2289.1000, Geyer GmbH, Stuttgart, Germany) was transferred into each well and the plates were gently shaken to completely dissolve the stained calcium nodules. To neutralize the acetic acid, an equivalent amount (750 µL) of 10% ammonium hydroxide was applied. The solution was transferred into 96-well plates at 100 µL/well and detected using a microplate reader at a wavelength of 405 nm. Three parallel groups were set for 1 sample.

4.11. Osteogenic Activity with ALP Assay

Following the ALP assay kit instructions (ab83369 Alkaline Phosphatase Assay Kit, Cambridge, UK), 80 µL of the supernatant of each group after osteogenic induction was added to 96-well plates for the ALP assay. Then, 20 µL of stop solution was added to the sample background control wells to terminate ALP activity in these samples. The samples were mixed well by pipetting up and down. Next, 50 µL of 5 mM pNPP solution was added to each well containing the sample and background sample controls. Then, 10 µL of ALP enzyme solution was added to each pNPP standard well. Plates were incubated at 25 °C for 60 min while protected from light. The reaction was ceased in sample wells and standard wells by adding 20 µL of stop solution. The output at OD 405 nm was measured on a microplate reader. The ALP activities of each group were detected at day 7, day 14 and day 21, respectively.

4.12. Gene Expression Detection

TRIzol reagent (Cat. NO. 15596026, Ambion, Austin, TX, USA) was used to extract total RNA from differentiated dental pulp cells, which was subsequently quantified using a spectrophotometer (Cat. NO. 51119700DP, Thermo Fisher, Singapore) and 1 percent agarose gel electrophoresis. The isolated total RNA was reverse transcribed to cDNA for the reverse-transcription PCR as well as real-time quantitative PCR analysis using GoScriptTM RT reagent Kit (Cat. NO. A5001, Promega, Madison, WI, USA) and Luna® Universal One-Step RT-qPCR Kit (Cat. NO. E3005, New England biolabs INC, Ipswich, MA, USA) according to the manufacturer's protocol. Primers were designed on Primerbank. Lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor-γ (PPAR-γ) were selected as adipogenic-specific genes; otherwise, alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), type I collagen (COL I) and osteocalcin (OSC) were selected as osteogenic genes and their expressions were detected at day 7, day 14 and day 21. GAPDH was selected as the reference housekeeping gene of each sample. The relative quantity of mRNAs was calculated with the $2^{-\Delta\Delta Ct}$ method after normalization. Table 1 shows the primer sequences as well as the length of the products.

Table 1. Primer sequences of adipogenic- and osteogenic-induced gene expression.

Primer	Direction	Sequence	Length of Products (bp)
LPL	Forward	ACAAGAGAGAACCAGACTCCAA	76
	Reverse	GCGGACACTGGGTAATGCT	
PPAR-γ	Forward	GGGATCAGCTCCGTGGATCT	186
	Reverse	TGCACTTTGGTACTCTTGAAGTT	
ALP	Forward	ACTGGTACTCAGACAACGAGAT	97
	Reverse	ACGTCAATGTCCCTGATGTTATG	

Table 1. *Cont.*

Primer	Direction	Sequence	Length of Products (bp)
RUNX 2	Forward	TGGTTACTGTCATGGCGGGTA	97
	Reverse	TCTCAGATCGTTGAACCTTGCTA	
Type I collagen	Forward	GGACACAATGGATTGCAAGG	441
	Reverse	AACCACTGCTCCACTCTGG	
Osteocalcin	Forward	GGCGCTACCTGTATCAATGG	110
	Reverse	GTGGTCAGCCAACTCGTCA	
GAPDH	Forward	GAGTCAACGGATTTGGTCGT	185
	Reverse	GACAAGCTTCCCGTTCTCAG	

4.13. Statistical Analysis

Variance in the mean values across the three groups was analyzed using a student *t*-test. Statistical significance was defined as a *p*-value of less than 0.05. For statistical analysis, SPSS 25.0 software (SPSS Inc., Chicago, IL, USA) and Graphpad Prism V9.0 (GraphPad Software, San Diego, CA, USA) was utilized.

5. Conclusions

Our research aimed to examine the effect of cryopreserving whole teeth with 5% and 10% DMSO on hDPSCs. The results of our study indicated that the new strategy of sample freezing and unfreezing had a negative effect on the initial culture stage of dental pulp cells, which prolonged the growth and culture time of dental pulp cells. However, the results of the cell experiments on the third generation showed that the biological activity of the experimental dental pulp stem cells was almost not affected. The hDPSCs maintained a high proliferation and differentiation ability. The new strategy, on the other hand, effectively reduced the exposure time of the samples to aseptic conditions, which can reduce the chance of infection. More importantly, the new cryopreserved method effectively lowers the tissue-preservation requirements, so that the common dental practice can preserve the samples in time, too. This maximizes the freshness of the pulp tissue and the biological activities of the dental pulp stem cells. Although the small sample size did not allow us to further investigate the maximum duration of the cryopreservation of whole teeth, our study shows the feasibility of the new cryopreservation method, which can provide a theoretical basis for the establishment of a dental stem-cell bank and create better conditions for future stem-cell therapy.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. All teeth were coded with number and all personal identification of the patients were removed. All parent or guardian of participants provided written informed consent for using their teeth which otherwise would have been discarded as waste.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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5 Chapter 5: Publication in *Biomedical Papers*, June 2022

A comparative study on the secretion of various cytokines by pulp stem cells at different passages and their neurogenic potential

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Personal contribution:

The following research article has been published in ***Biomedical Papers***. I am the first author of the paper. I conducted the experiments and data collection and analyzed the results (raw data analysis and statistical analysis). I contributed to writing the manuscript draft and editing during the revisions.

A comparative study on the secretion of various cytokines by pulp stem cells at different passages and their neurogenic potential

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Aims. By measuring the extent of cytokines secreted by human dental pulp stem cells (hDPSCs) from passages 2 through 10, the optimal passage of hDPSCs was determined. This offers a potential theoretical basis for the treatment of neurological disorders.
Method. After isolation and culture of hDPSCs from human teeth, the morphological features of the cells were observed under an inverted microscope. hDPSCs were identified by their immunophenotypes and their multiple differentiation capability. Cytokine concentrations secreted in the supernatants at passages 2-10 were detected by ELISA.
Results. hDPSCs were viewed as fusiform or polygonal in shape, with a bulging cell body, homogenized cytoplasm, and a clear nucleus. Moreover, they could differentiate into neuroblasts in vitro. hDPSCs at passage 3 were positive for CD29 (91.5%), CD73 (94.8%) and CD90 (96.7%), but negative for the hematopoietic markers CD34 (0.13%). ELISA results showed that hDPSCs at passage 3 had the highest secretion levels of vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), with the highest secretion level of Neurotrophin-3 (NT-3) being at passage 2.
Conclusion. hDPSCs have steady biological features of stem cells and exhibit optimal proliferation potential. hDPSCs at different passages have different capacities in the secretion of VEGF, BDNF, NGF, and NT-3. In conclusion cytokines secreted by hDPSCs may prove to be appropriate in the treatment of neurological diseases.

Key words: dental pulp stem cell, vascular endothelial growth factor, brain-derived neurotrophic factor, nerve growth factor, neurotrophin-3. immunophenotype, multidirectional differentiation, cytokines

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INTRODUCTION

Trauma, iatrogenic injuries, and neurodegenerative diseases can lead to neurite degeneration, synapse loss, or even neuronal loss. This is mainly due to the limited ability of the mature nervous system to differentiate into functional neuronal and glial cells especially after nerve damage[1]. The human body can maintain the genetic stabilization of the genome in many DNA repair pathways under normal physiological conditions. However, there are often cases, where dysregulation of replication and metabolism of DNA may influence cell proliferation, differentiation, migration, and consequently, the formation of synaptic connections. The treatment of irreversible diseases of the nervous system caused by neuronal loss, necrotic and apoptotic cell deaths represents a significant and common clinical burden worldwide. Nevertheless, the development of tissue engineering in recent years has demonstrated that stem cells can serve as a source for the replacement of damaged cells, thereby offering new therapeutic strategies to repair neurological tissue defects[2-4].

Mesenchymal stem cells (MSCs) for instance have a neural differentiation potential and are present in several tissues of mesenchymal origin, such as bone marrow, teeth, and adipose tissue[5-7]. Several articles have reported that Adipose-derived stem cells (ADSCs) tend to secrete glial cell line-derived neurotrophic factor (GDNF), where its overexpression has been used to satisfactorily treat sciatic nerve injuries in rats[8].

Since dental pulp stem cells (DPSCs) originate from neural crest cells, they thereby share with neural cells similar phenotypical characteristics and gene expression patterns[9], Mead et al.[10] found that DPSCs especially, secrete more neurotrophic factors (NTF) than bone marrow-derived mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ADSCs). Where the expression of neurotrophic growth factors (NGF), brain-derived neurotrophic factor (BDNF), and nerve growth factor-3 (NT-3) from DPSCs can be up to 2–3 times higher than the BMSCs. Likewise, neuroprotective effects are also often superior to BMSCs and ADSCs. When compared to mesenchymal elements of the dental papilla, dental follicle,

and periodontal membrane from the same teeth, Ullah et al. found that DPSCs exhibited a more neurotrophic character[11]. Therefore, and due to further advantages, such as their pluripotent differentiation ability, their low risk of rejection, minimal ethical controversies, and being operative friendly, DPSCs are regarded as a promising source of stem cells for the treatment of neurological diseases[12].

Changes in cytokine levels secreted from the pulp stem cells in relation to the cell passage number varies in vitro[13,14]. Therefore, it is of great practical significance to explore the cytokine levels from different passages of dental pulp stem cells to determine the optimal passage of DPSCs that can serve as an initial theoretical basis for the treatment of neurological disorders.

MATERIALS AND METHODS

Wisdom teeth were obtained from the biological waste of teeth extracted in the outpatient clinic of the Department of Oral and Maxillofacial Surgery under approved guidelines set by the Ethics Committee of our hospital. All patients provided their informed written consent. Teeth were anonymized and immersed in Phosphate Buffered Saline (Cat. no 14190094, Gibco) followed by immediate transportation to the laboratory.

Culturing human dental pulp cells

Every tooth used in the experiment was immersed entirely in sterile DPBS containing 500U/ml penicillin streptomycin (cat. no 15140122, ThermoFisher Scientific) and 500 µg/mL amphotericin-8 (cat. no 15290026, ThermoFisher Scientific) for 15 min. Soon after this step and under aseptic conditions, the dental crown was separated from the root, exposing the pulp. To isolate DPSCs, the outgrowth methodology was employed, where the pulp tissue was chopped into small fragments, which were then cultured onto a tissue culture plate. Cells were then observed to slowly grow out of the tissue fragments in the medium (cat. no 31095029, ThermoFisher Scientific) containing 15% heat-inactivated FBS (cat. no 10082147, ThermoFisher Scientific), 1% penicillin-streptomycin (cat. no 15140122, ThermoFisher Scientific), 1% amphotericin-B (cat. no 15290026, ThermoFisher Scientific) at 37 °C with 5% CO_2 and 95% humidity.

The adherent cells were defined as passage zero. When the cells grew to 70% confluency, they were passaged at 1/5 dilution after digestion by 0.05% trypsin-EDTA (cat. no 25300054, Thermo Fisher Scientific) and then passaged for further experimentation. Media was replaced every 3 days until the cells grew to the desired confluency. The images of cell morphology were captured under an inverted microscope.

Pulp stem cells were obtained by the limited dilution method

The cells in suspension were serially diluted to 10 cells/mL with a limiting dilution assay. The diluted cell suspension was distributed at 100 µL per well in a 96-well plate (Corning Life Sciences). Wells containing no cell or more than one single cell were excluded. The wells with a single cell were marked and inspected daily microscopically to count the number of cell clones. DPSCs were then stained with antibodies against STR0-1 (cat. no 398401, ThermoFisher Scientific) with a dilution of 1:500 - a stem cell surface marker. After STR0-1 was incubated with cell samples overnight at 4 °C followed by incubation with the secondary antibody, goat anti-mouse lgG-HRP (cat. no sc2371, Santa Cruz Biotechnology) with a dilution of 1:500 for 1 h at room temperature in the dark. After being rinsed thoroughly with PBS for 5 min three times, the samples were assessed using fluorescence microscopy.

Cell growth curves

Cells were identified according to the number of passages (P), starting with passage 1 (P1). DPSCs that were passaged for 3 generations were made into cell suspensions. Suspensions with a cell concentration of 4×10^3/mL were cultured in 24-well plates for 8 days. Cells from 3 wells were counted using MTT colorimetric analysis in each group every day. Numerical values were obtained by UV spectrophotometry at 490 nm using a Nanodrop spectrophotometer (NanoDrop™ 2000 or 2000c Spectrophotometer, ThermoFisher).

Immunophenotypic analysis was performed using flow cytometry

By passaging, cells were harvested using trypsin at 0.25%. Cell suspensions of 2×10^6 cells at P3 were collected and washed twice with PBS. Surface markers of 3rd generation cells were identified by flow cytometry using antibodies against CD29 (cat. no. 11-0299-42; Thermo Fisher Scientific), CD73 (cat. no. ab239246; Abcam), CD90 (cat. no. ab139364; Abcam), CD34 (cat. no. 560941; BD Biosciences). All antibodies were used at a dilution of 1:500. The antibodies were gently mixed and incubated for 30 min at 4 °C, then identified through surface antibody: antigen labeling by the use of flow cytometry (BD).

Neural differentiation

DPSCs at a density of 2×10^4 cells/well were seeded into 24-well plates and cultured in a standard culture medium. Upon confluency, neural differentiation was started by exchanging the standard medium for neural induction medium [Neurobasal Medium (cat. no. 21103049, Gibco); fortified by the following ingredients: 1x8-27™ Supplement (50x: cat. no. 17504044, Gibco); 2 nM human heregulin (rHRG-$\beta1_{177-244}$; provided by Dr. Steven Carrol & Dr. Jody Longo, University of South California); 0.5 nM 3-lsobutyl-1-methylxanthine (IBMX) (cat. no.17018, Sigma-Aldrich); 5 µM Forskolin (cat. no. F6886, Sigma-Aldrich); 10 ng/mL basic fibroblast growth factor (bFGF) (Cat No. F0291; Sigma Aldrich). The media was changed every 3 days and the neural differentiation period was continued for 21 days.

To stain the neural differentiation results, β-tubulin and MAP-2 were employed. First, the induction media was removed and cells were washed with PBS. Cells were

then fixed in 4% paraformaldehyde (Cat No. 30525894, Santa Cruz Biotechnology) (pH7.4) at room temperature for 10 min, followed by a triple rinse using ice-cold PBS. The fixation procedure was then completed by adding 60% isopropanol (Cat No. 19516, Merck) for 5 min. Subsequently, the cells were permeabilized by incubating them in 0.15% Triton X-100 (Cat No. X100, Sigma-Aldrich) for 10 minutes, and then washed 3 times with PBS for a duration of 5 min each time. Specimens were then incubated for 30 min with serum blocking solution, which is composed of PBS containing 1% bovine serum albumin (BSA) (Cat No. A7906, Sigma- Aldrich), 22.52 mg/mL glycine (Cat No. G8898, Sigma-Aldrich) and 0.1% Tween 20 (Cat No. P1379, Sigma-Aldrich), to suppress the nonspecific binding of the antibodies. Afterward, cells were labeled with primary antibodies overnight at 4 °C: β-tubulin (Cat No. H0417, Santa Cruz Biotechnology) and MAP-2 (Cat No. 3173528, Merck) at a dilution of 1:500. For immunofluorescence staining, cells were stained with the following fluorochrome-conjugated secondary antibodies, Alexa Fluor 488 goat anti-mouse (cat. no A11029, ThermoFisher Scientific) and Alexa Fluor 633 goat anti-mouse (cat. no A21050, ThermoFisher Scientific) respectively, both of which were diluted to 2 µg/mL in PBS with 1.5% normal blocking serum for 1 h at room temperature in the dark. The nuclei were then stained using 4'-6-diamidino-2-phenylindole (DAPI) (cat. no D9542, Sigma-Aldrich). Following triple PBS washes for 5 min each time in the dark, coverslips were mounted on glass slides with a permanent mounting solution (cat. no S3023, Dako). Finally, stained cells were examined under a fluorescence microscope.

ELISA analysis of conditioned media (passages 2-10)

The DPSCs were seeded into T25 culture flasks at a cell density of 1×10^6 and were cultured in DMEM with 10% FBS until 70–80% confluency is reached. The culture medium was then discarded, and the cells were washed with PBS 2 times, followed by the addition of 1.6 mL fresh media to the cells for a further 24-hour incubation period. Conditioned media and cell lysates were then collected. The levels of VEGF, BDNF, GDNF, and NT-3 were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the protocols of the manufacturer (BDNF and NGF from Promega; VEGF and NT-3 from R&D Systems).

To test BDNF and NT-3 levels: 50 µL of standards and samples were added to the respective coated reaction cups and incubated at room temperature for 2 h. For BDNF, the binding solution was added without washing the plate, whereas, with NT-3, binding solution was only added after the plates were washed. Plates were then incubated at room temperature for 1 h and 1.75 h respectively. After washing the plates, respective substrates were added at room temperature for 30 min, followed by termination solution and absorbance measurement at 450 nm within 30 min.

To test VEGF and NGF levels: 200 µL of standards and samples were added to the coated reaction cup and incubated at room temperature for 2 h. After washing the plates, VEGF and NGF binding solutions were added and incubated at room temperature for 2 and 1.75 h respectively. After washing the plates, respective substrates were added and incubated at room temperature for 30 min, then the termination solution was added and absorbance was measured at 450 nm within 30 min.

Statistical analysis

Statistical analysis was performed by a statistical package and expressed as mean±standard deviation (SD). The distinction of the proportion of cytokines was processed via the student's t-test for statistical calculation. $P<0.05$ was considered statistically significant. Graphs were prepared with Prism version 8.0 (Graph Pad Software).

RESULTS

Morphological characterization of DPSCs

After 5 days of primary culture, a small number of elongated spindle-shaped cells surrounding and growing out of the tissue fragments were observed. Eight days later, cells fused together and exhibited characteristic polygonal and spindle-shaped morphology. They were seen to have clear nuclei with a huge chromatin core, together with a granular aggregation in the cytoplasm.

After cells were selected via limited dilution in 96-well plates, monoclonal cells were seen to significantly grow and appeared as elongated spindle-shaped cells with a swirling growth pattern.

Proliferation of hDPSCs

From P2-P10, cells maintained an optimal growth curve (see Fig. 1). The proliferative capacity of hDPSCs peaked at P4 ($P<0.05$).

Identification of hDPSCs

Cell surface markers were detected using flow cytometry to determine the mesenchymal origin of the hDPSCs. The following markers were positively expressed at respective percentages; CD29 at 91.5%, CD73 at 94.8%, CD90 at 96.7%, whereas the hemopoetic stem cell marker,

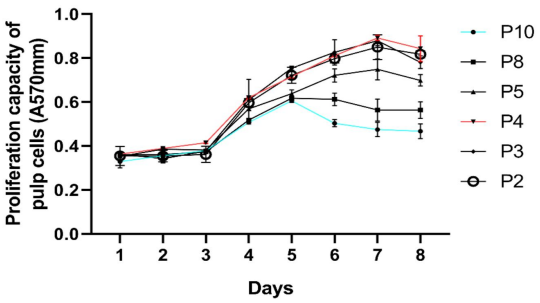

Fig. 1. Proliferation curves of human dental pulp stem cells at different passages.

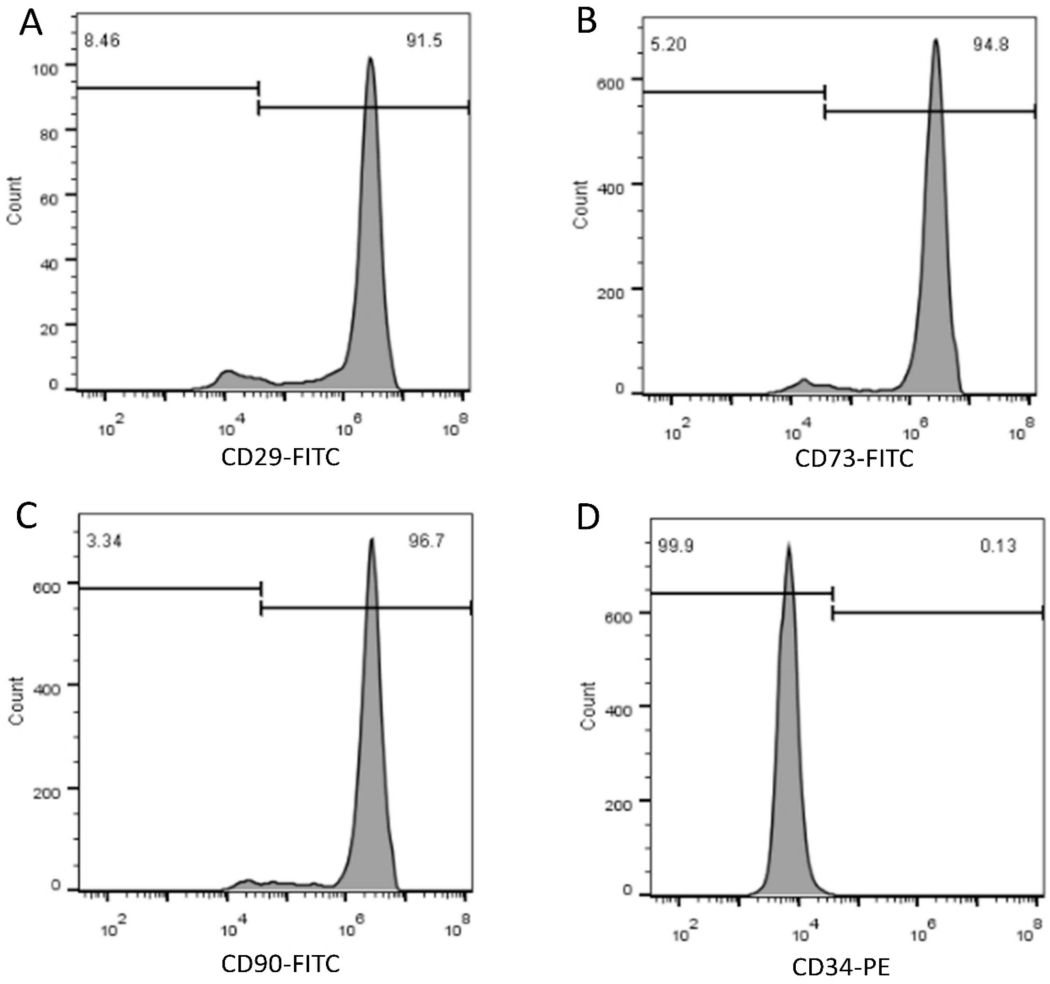

Fig. 2. Flow cytometry detection of passage 3 dental pulp stem cells.
Cells expressed mesenchymal surface markers CD29 (91.5%, A), CD73 (94.8%, B), and CD90 (96.7%, C), while hardly expressing the hematopoietic stem cell surface marker CD34 (0.13%, D).

CD34 was minimally expressed at a percentage of 0.13% (see Fig. 2).

Most hDPSCs were positive for the cell surface marker STRO-1 (in Fig. 3).

Cellular identification based on differentiation potential

Before neural differentiation induction, hDPSCs displayed a fibroblast-like appearance with an elongated and flattened body. Along the induction process, differentiating hDPSCs started to change their morphology towards neuron-like cells with multiple extensions. At the end of the differentiation period of 21 days, the expression of β-tubulin (neuronal-specific tubulin) and MAP-2 (mature neurocyte) was detected (in Fig. 3). Lack of neuronal staining in some cells with nuclei stained with DAPI denoted the specificity of the immunofluorescence staining.

Changes of hDPSCs secretion of cytokines through passages 2-10

ELISA showed that the peak secretion of VEGF, BDNF, NGF at P3 of hDPSCs. Significant differences between the VEGF, NGF, and BDNF groups (P<0.05) (see Fig. 4B,D,F). hDPSCs secretion of NT-3 peaked at P2 (P<0.05) (see Fig. 4H).

DISCUSSION

The central and peripheral nervous systems govern all activities of the body and perform important physiological functions. While it is established that damage to central nerves is irreversible, peripheral nerves do possess repair functions, their role is however limited, and therefore, severe nerve damage ultimately leads to irreversible

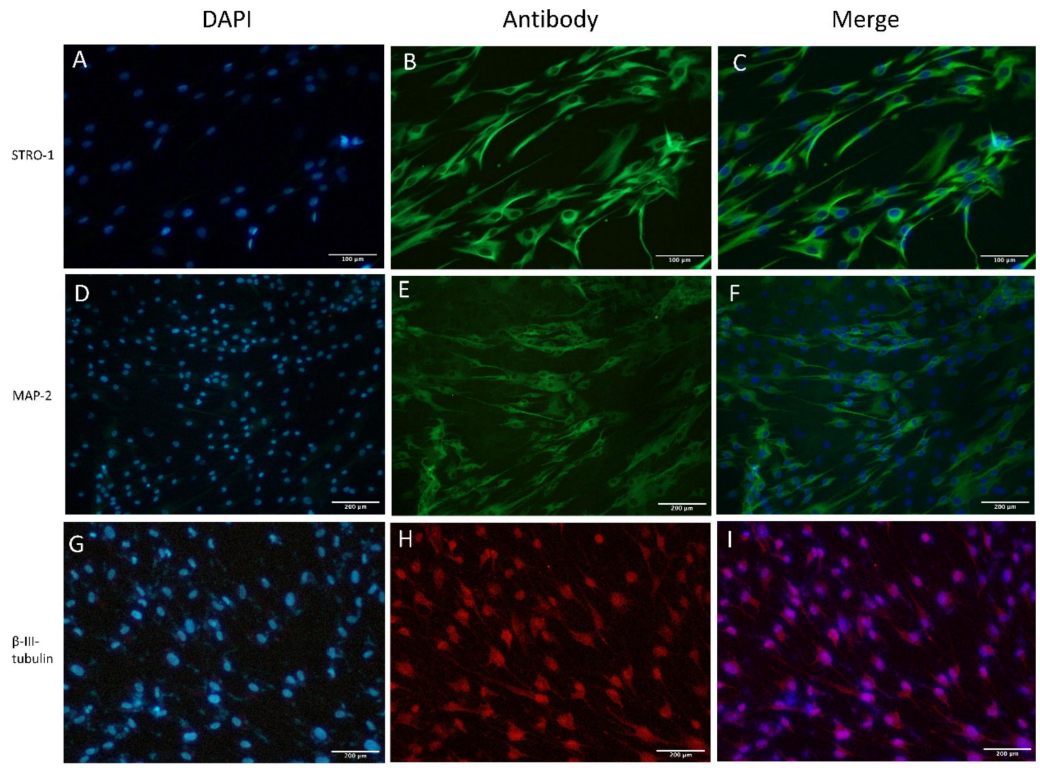

Fig. 3. Induced neural differentiation of passage 3 dental pulp stem cells *in vitro.*
Nuclei of the cells were stained with DAPI (blue) shown in Fig. (A, D, G). STRO-1 was marked by immunofluorescence (green) shown in Fig. B. hDPSCs were stained with both DAPI and STRO-1. in Fig. C. Expression of MAP-2 stained in green shown in Fig. E. Expression of β-III-tubulin stained in red shown in Fig. H.

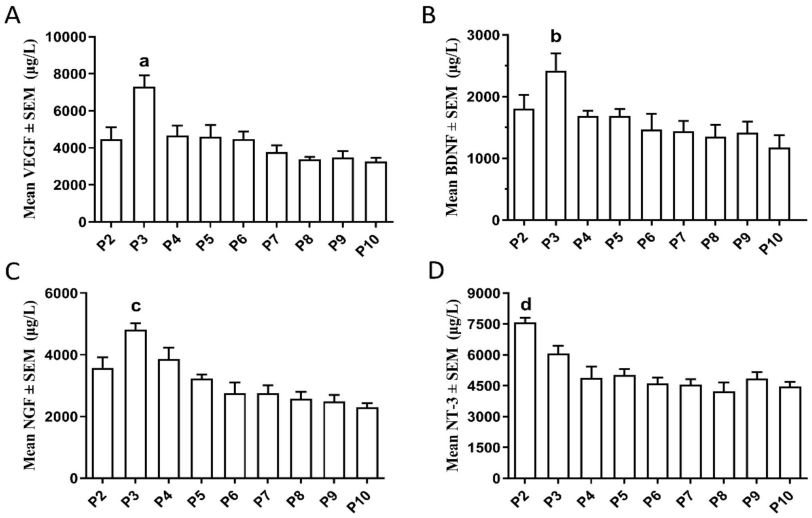

Fig. 4. Secretion of vascular endothelial growth factor, brain-derived nerve growth factor, nerve growth factor, and neurotrophin-3 from human dental pulp stem cells at different passages.
A, B, C, and D are the secretion levels of VEGF, BDNF, NGF, and NT-3 from P2-P10 DPSCs Compared with other passages [a]$P<0.05$; [b]$P<0.05$; [c]$P<0.05$.

dysfunction. Stem cells are characterized by self-renewal and multi-directional differentiation ability, and in recent years a variety of stem cells have been confirmed to have the potential to differentiate into neuronal cells, amongst which are the hDPSCs. hDPSCs possess a potent differentiation ability, as well as favorable paracrine effects, rendering them a therapeutic means for the treatment of nerve injuries.

Furthermore, hDPSCs not only express conventional neuronal markers such as nestin, glial fibrillary acidic protein, and neuron-specific nuclear protein, but they also express further markers such as nucleolin, glial fibrillary acidic protein, neuron-specific nuclear protein (NeuN), and S-100. Moreover, they secrete a variety of neurotrophic factors, such as BDNF, GDNF, and other NGFs which play important roles in neuroprosthetics[15]. BDNF and GDNF act directly on dopaminergic neurons and have strong nutritional repair effects that promote recovery of dopaminergic neurons after injury. These neurotrophic factors affect themselves and the surrounding microenvironment through autocrine and paracrine effects, creating a microenvironment conducive to cellular growth and neuronal differentiation.

It has been shown that the biological properties of MSCs up to 10 passages are stable. Yu Jinhua et al. showed that Stro1+-labeled hDPSCs obtained by immune magnetic bead sorting were highly expressed at P9 compared with P1. Nevertheless, hDPSCs at the P1 were observed to highly express the following dentin-specific genes; dentin sialophosphoprotein (DSPP), dentin sialoprotein (DSP) in addition to osteogenic-specific genes such as alkaline phosphatase (ALP) and osteopontin (OPN), suggesting that P1 hDPSCs are potent in terms of multipotential differentiation that is directed toward dentin, bone tissue and cartilage formation, whereas hDPSCs at P9, i.e. at a more advanced passage are particularly more inclined towards osteogenic differentiation. The aforementioned data denotes that the advancement of in vitro passages has a directional effect on the differentiation potential of hDPSCs.

Moreover, the results of ELISA showed that hDPSCs secreted the highest level of VEGF at P3, peaking up to 7300 μg/L. VEGF is an important regulator of physiological and/or pathological vasculogenesis and angiogenesis, and plays an important regulatory role in the proliferation, survival, migration and infiltration of vascular endothelial cells. Since hDPSCs can also promote vascular regeneration by expressing VEGF, thereby further promoting and contributing to axonal growth and Schwann cell proliferation. More specifically, VEGF-A is one of the key factors in angiogenesis, and in a study where differentiated hDPSCs were implanted into a rat model with a 15 mm sciatic nerve defect, VEGF-A was found to be responsible for enhancing angiogenesis and promoting the inward growth of the initial nerve projections[16].

Moving on to the neurogenic paracrine effect, ELISA results showed that the level BDNF secreted by the 3rd passage of hDPSCs was 2421 μg/L. BDNF is physiologically widely distributed in the central nervous system (CNS) and plays an important role in the survival, differentiation, growth and development of neurons during the development of the CNS, while also preventing neuronal death and contributing to the reversal of the pathological state of neurons. BDNF is a neurotrophic factor that promotes biological effects such as regeneration and differentiation of injured neurons, and is also necessary for the survival and normal physiological functions of mature neurons in the central and peripheral nervous system. Consequently, changes in the expression level of BDNFs are closely related to various neurological diseases, such as Depression, Epilepsy, Alzheimer's, Parkinson's and Huntington's disease[17].

The ELISA results showed that the 3rd passage of hDPSCs secreted the highest level of NT-3, which reached 7571 μg/L. NT-3 were observed to significantly increase the number of neuritis βIII-tubulin+ retinal cells and the length of neural dendrites in co-cultures in vitro. It was also observed, that retinal cell activity decreased when treated with NT-3 receptor inhibitors, demonstrating the protective effect of NT-3 on RGCs (ref.[10]). Moreover, retinal ganglion cells (RGC) are known to express a large number of neurotrophic factors, including NGF, BDNF, NT-3 and GDNF, which can bind to the RGCs receptors and thereby enhance not only the survival of RGCs, but also axonal regeneration[18]. hDPSCs may also be used as a resource in treating retinal injury because the paracrine effect of hDPSCs entails the relevant expression of the aforementioned neurotrophic factors.

ELISA results also showed that the 3rd passage of DPCSs secreted the highest level of NGF which reached 4814 μg/L. Moreover, NGF is one of the earliest discovered factors of the neurotrophin family and are known to maintain the survival of sensory neurons, promote the growth of nerve fibers, as well as increase the expression of sensory neuropeptide genes. In the retina, NGF is produced and utilized by the retinal ganglion cells, bipolar neurons, and glial cells in a local paracrine or autocrine manner. Furthermore, Colafrancesco et al.[19] found that NGF protects the retina by reducing ganglion cell apoptosis. During the development of the visual system, NGF and its receptor are both highly expressed in visual centers and can influence neuronal proliferation, survival, and selective apoptosis. Moreover, NGF activates signaling pathways by binding to tropomyosine receptor kinase (TrkA) receptors to maintain neuronal survival and differentiation. In a guinea pig model of Form-Deprivation Myopia (FDM), Davis et al.[20] used immunohistochemistry and fluorescence quantitative PCR to detect the protein and gene levels of NGF and TrkA, respectively. Results showed that the expression of NGF and TrkA proteins and nucleic acids decreased in the retina with the prolongation of shape perception deprivation time. Information regulation in retinal cells promoted differentiation to a functional retinal developmental stage. Moreover, Spalding et al[21] found that NGF and TrkA were expressed in both neuronal and non-neuronal cells in the retina of a guinea pig model of FDM. The experiments only discussed the secretion level of cytokines from the 2nd through the 10th passage of hDPSCs. Therefore, the secretion level of cytokines of subsequent passages still

needs to be clarified; and the clinical applications of hDPSCs also need further investigation in terms of indications and contraindications. In addition, further research on the exact mechanism of paracrine secretion of hDPSCs and the factors influencing paracrine function, as well as how to use this potential function of stem cells in a reasonable and effective manner, are issues that need to be resolved.

CONCLUSION

In summary, our study discussed the biological characteristics of hDPSCs and the cell passages optimal for cytokines secretion, all of which provided new ideas for the clinical translation of cellular therapy.

ABBREVIATIONS

hDPSCs: Human dental pulp stem cells; VEGF: Vascular endothelial growth factor; BDNF: Brain derived neurotrophic factor; NGF: Nerve growth factor; NT-3: Neurotrophin-3.

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Ethics approval and consent to participate

Administrative permissions were acquired by our team to access the data used in our research. The study protocol was approved by the Guiyang Stomatological Hospital that approved the study. Accordingly, all teeth were coded with number and all personal identification of the patients were removed. All parent or guardian of participants provided written informed consent for using their teeth which otherwise would have been discarded as waste.

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6 Chapter 6: Publication in *In Vivo*, April 2023

Characterization of Dental Pulp Stem Cell Populations in the Teeth of Patients With Neurofibromatosis Type 1 - Therapeutic Potential for Bone Tissue Engineering

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Personal contribution:

The following research article has been published in ***In Vivo***. I am the first author of the paper. I conducted the experiments and data collection and analyzed the results (raw data analysis and statistical analysis). I contributed to writing the manuscript draft and editing during the revisions.

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Characterization of Dental Pulp Stem Cell Populations in the Teeth of Patients With Neurofibromatosis Type 1 - Therapeutic Potential for Bone Tissue Engineering

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Abstract. *Background/Aim: Neurofibromas (NF) are the most common benign nerve sheath tumors in the tongue, gingiva, major salivary glands, and jaw bones. Nowadays, tissue engineering is a revolutionary technique for reconstructing tissues. To explore the feasibility of using stem cells derived from NF teeth to treat orofacial bone defects, the differences in cell biological properties between an NF teeth group and Normal teeth group. Patients and Methods: The intra-dental pulp tissues from each tooth were extracted. The cell survival rates, morphology, proliferation rates, cell activity, and differentiation abilities were contrastively analyzed between the NF teeth group and Normal teeth group. Results: Between the two groups, there were no differences in the primary generation (P0) cells (p>0.05), the cell yield, and the time required for the cells to grow out of the pulp tissue and attach to the culture plate. Furthermore, no differences were found at the first generation (passage) between the two groups in colony formation rate and cell survival rate. The proliferation capacity, cell growth curve, and surface marker expression of dental pulp cells was not altered in the third generation (p>0.05). Conclusion: Dental pulp stem cells from NF teeth were successfully obtained and were not different from normal dental pulp stem cells. Although, clinical research using tissue-engineered bone to repair bone defects is still in its infancy, it will eventually enter the clinic and become a routine means of bone defect reconstruction treatment as related disciplines and technologies develop.*

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Key Words: Neurofibromatosis type 1, dental pulp stem cells, plexiform neurofibroma, adipogenicity, osteogenicity.

Peripheral nerve sheath tumors (PNST) can develop sporadically or be an indicator of a syndrome. In syndromic PNST, neurofibromas are the hallmark of neurofibromatosis type 1 (NF1), an autosomal dominant tumor suppressor gene disease (1). Numerous neurofibromas may occur in the skin of NF1 patients (cutaneous neurofibromas), which can be very noticeable and aesthetically detrimental. Another PNST observed almost exclusively in NF1 is the plexiform neurofibroma (PNF), a neoplasm that probably develops during the embryologic phase of life. PNF frequently arises from larger nerves and can infiltrate into adjacent organs. PNF is considered as a precancerous condition, *i.e.*, a precursor to malignant peripheral nerve sheath tumors (MPNST). An estimated 50% of all patients with MPNST have NF1 mutations, and the diagnosis of MPNST is a major factor in the reduced life expectancy of these patients compared to the general population (1, 2).

Schwann cells or their precursors are the tumor cells of PNST in NF1 (2). Schwann cells derive from the neural crest (NC) (3). Both neurogenic tumors and many other findings in

the NF1 patient can be interpreted as NC cell (NCC) differentiation disorders (4). Indeed, differentiation disorders of the NCCs explain numerous diseases and the relatively common syndrome NF1 is classified as a group of disorders of the NC (5). Therefore, NF1 is addressed as an important representative of the so-called neurocristopathies (6). The assignment implies that the *NF1* gene product neurofibromin has many more functions in addition to the known tumor suppressor gene function. It was previously pointed out that *NF1* gene is a histogenesis control gene (7, 8). Considering this characteristic of the *NF1* gene, targeting of histogenesis and cell repair of NF1 patients in certain regions can be a suitable treatment for tumors or specific dysplastic conditions of the syndrome, for example pseudarthrosis of long bones (9, 10).

Due to the multipotent capacities of NCCs, some areas of NF1 research focus on the differentiation ability of adult stem cells (SC), which originate from NC. SC isolated from different tissues are used to analyze the differentiation potential of *NF1*-mutated cells (11-15). A well-known source of SC is the dental pulp (DPSC) (16). DP is source of SC, for which intact teeth (especially wisdom teeth) are extracted and used for research purposes after medical evaluation of the indications to extract the teeth. DPSC can differentiate into several cell types in cell culture and provide a valuable tool for understanding cellular differentiation (17-21) and the consequences of differentiation disruption (22, 23). The first studies on DPSC from NF1 patients showed their differentiation capacity (24-26). The aim of this study was to examine the differentiation capacity of DPSC from NF1 patients depending on a syndrome-specific oral environment.

Materials and Methods

Cultivation of human dental pulp cells.

Extraction of teeth. Eight teeth were extracted from two male NF1 patients. One of the teeth was extracted from the site of a facial plexiform neurofibroma. In order to assess the differentiation potential of the DPSC from NF1 patients, ten wisdom teeth of healthy teenagers (19-21 years old) were collected from November of 2020 to June of 2021 in our hospital. The teeth were removed and immersed in Dulbecco's Modified Eagle medium (DMEM, Cat. No. 41965-049, Gibco, Leicestershire, UK) with 10% fetal bovine serum (FBS, Cat. No. 10500-064, Gibco, Carlsbad, CA, USA) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Cat. No. 15140-148, Gibco) at 4˚C to keep the pulp alive because of the time and travel required to transfer it from the clinic to the laboratory. Experiments were conducted within a range of 4 h. Teeth were immersed in 4˚C Dulbecco's phosphate buffered saline (DPBS, Cat. No. 14190-094, Gibco) solution (including 2×10^5 U/l penicillin and streptomycin) for 30-60 min to disinfect the teeth.

Teeth derived from NF1 patients served as the experimental group (NF teeth group) whereas teeth from healthy individuals served as the normal control (Normal teeth group). In the following step, the intra-dental pulp tissues were divided into 0.5 mm^3 pieces and emerged in 10% FBS/DMEM medium.

Cultivation of human dental pulp cells. Tissues from each group were distributed in 24-well plates and incubated at 37˚C in a 5% CO_2 humidified incubator. When the cells reached confluency, 0.05% trypsin- ethylenediaminetetraacetic acid (EDTA, Cat. No. 25300-054, Thermo Fisher, Waltham, MA, USA) was added and the cells were passaged at a density of $5\times10^3/cm^2$. For each group, the time required for cellular outgrowth and attachment after the insertion of tissue blocks was recorded.

Calculation of cell yield/harvest. The primary dental pulp cells were digested with 0.05% trypsin at 37˚C for 3 min after reaching 80-90% confluency. The dental pulp cells were then put into 50 ml tubes and centrifuged (Eppendorf 5810R, Hamburg, Germany) for 10 min at room temperature at 241×g. The cells were resuspended in DMEM after the supernatant was discarded. Cell counting and the following equation were used to determine the number of cells: Primary cell yield=Total cell counted/4×dilution factor$\times10^4\times$volume of cell suspension.

Colony-forming efficiency. A culture dish with a diameter of 10 cm was seeded with 1×10^3 first generation (passage) dental pulp cells from each group. Every three days, the culture media was replaced with fresh media. Two weeks later, the medium was discarded, and the dental pulp colonies were fixed with 95% methanol for 15 min, rinsed with DPBS, stained with Giemsa solution (Cat. No. 48900, Sigma-Aldrich, Buchs, Switzerland) for 10 min, and then washed three times with DPBS to remove any remaining Giemsa staining solution. More than 50 dental pulp cell colonies were noted. Three aliquots of cells were analyzed in parallel. The following equation was used to calculate the colony-forming efficiency: Colony-forming efficiency=number of successfully formed colonies/number of seeded cells×100%.

Cell survival rate after trypan blue staining. After being stained for two min with 0.4% trypan blue (Cat. No. 15250-061, Gibco) first generation dental pulp cells of each group were examined under an inverted microscope. From a total of 500 dental pulp cells, the number of living cells that remained unstained after five min was counted. The cell survival rate was then determined using the equation shown below:

Cell survival rate=number of unstained surviving cells/500×100%.

Cell survival rate after live-dead staining. First generation dental pulp cells were taken from each group and seeded on tissue culture coverslips (TCC, Cat. No. 83.1840.002, Sarstedt, Nümbrecht, Germany) at a density of 8×10^4/ml in 12-well plates that equals to 8×10^4 cells per well and maintained at 37˚C in a 5% CO_2 incubator for 3 h. Five hundred μl of fluorescein diacetate working solution (FDA) and 60 μl of propidium iodide (PI) (50 g/ml in PBS) were added to each well. Samples were examined under a fluorescent microscope (Nikon ECLIPSE Ti-S/L100, Düsseldorf, Germany) following 3 min of incubation at room temperature and a DPBS rinse. The cell survival rate was determined using the following equation: Cell survival rate=number of green-stained cells/number of total cells×100%

Evaluation of proliferation with the MTS assay. Third generation dental pulp cells were seeded into 96-well plates at a density of 2×10^4/ml, or 2×10^3 cells per well, and then cultured at 37˚C in a

Table I. *Primer sequences of adipogenic and osteogenic induced gene expression.*

Primer	Direction	Sequence	Length of products (bp)
LPL	Forward	ACAAGAGAGAACCAGACTCCAA	76
	Reverse	GCGGACACTGGGTAATGCT	
PPAR-γ	Forward	GGGATCAGCTCCGTGGATCT	186
	Reverse	TGCACTTTGGTACTCTTGAAGTT	
Type I collagen	Forward	GGACACAATGGATTGCAAGG	441
	Reverse	AACCACTGCTCCACTCTGG	
Osteocalcin	Forward	GGCGCTACCTGTATCAATGG	110
	Reverse	GTGGTCAGCCAACTCGTCA	
GAPDH	Forward	GAGTCAACGGATTTGGTCGT	185
	Reverse	GACAAGCTTCCCGTTCTCAG	

5% CO_2 incubator. The proliferation of cells was assessed every day for eight days using the MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (Cat. No. G1111, Promega, Madison, WI, USA). In each group, cells from three wells were submitted to MTS colorimetric examination. After a 3-h incubation, the absorbance was measured using a microplate reader (Thermo Fisher Scientific) at a wavelength of 490 nm. Each well received 20 μl of MTS reagent.

Multi-differentiation potential. Adipogenic differentiation. Dental pulp cells at the third generation (P3) were seeded at a density of 2×10^4/ml, corresponding to 4×10^4 cells per 35 mm dish. Adipogenesis induction medium (DMEM containing 10% FCS, 5 g/ml insulin, 0.5 mmol/l 3 isobutyl-1 methylxanthine, and 10 mol/l dexamethasone) was used to cultivate the cells for 21 days until they attained 60-70% confluency. The medium was changed every 3 days. On the 22nd day, the cells were fixed with paraformaldehyde (PFA; Electron Microscopy Sciences, Fort Washington, PA, USA) for 15 min and washed twice with DPBS. Then, 0.5% Oil Red O working solution (Cat. No. O1391-250ML, Sigma-Aldrich, St. Louis, MO, USA) was used to stain the induced cells at room temperature for 10 min. After staining, lipid droplets in the cytoplasm were observed and photographed under an inverted microscope after the cells had been washed twice with DPBS.

Quantitative analysis of adipogenic differentiation. To remove the remaining staining solution, stained cells on dishes were washed three times with DPBS. The lipid droplets were then dissolved in each dish using 2 ml of isopropanol (Cat. No. 34965-1L, Honeywell, Hamburg, Germany), and the dishes were gently shaken until the solution was evenly colored. A microplate reader (Thermo Fisher Scientific) was used to measure the absorbance of the staining solution at a wavelength of 540 nm after being placed into 96-well plates at a rate of 100 μl per well. Each sample was analyzed in triplicates.

Osteogenic differentiation. Third generation dental pulp cells were seeded at a density of 2×10^4/ml in 3.5 cm diameter culture dishes. After the cells reached 60-70% confluency, they were grown in osteogenic induction medium (DMEM containing 10% FCS, 10 mmol/l glycerophosphate, 5 mmol/ml ascorbic acid, and 1 mol/l dexamethasone) for 21 days. The medium was changed every three days. On the 22nd day, the cells were fixed with paraformaldehyde for 15 min before being thrice rinsed with DPBS. The induced cells were then stained at room temperature for 15 min using a 0.1% Alizarin red S solution (Cat. No. GT6383, Glentham, Carsham, UK). After staining, the cells were washed twice with DPBS before the stained calcium nodules were viewed and captured on camera using an inverted microscope (Olympus IX71, Olympus Corp., Tokyo, Japan).

Quantitative analysis of osteogenic differentiation. To get rid of the remaining staining solution, stained cells on dishes were washed three times with DPBS. Once the colored calcium nodules were completely dissolved, 1 ml of 10% acetic acid (Cat. No. 2289.1000, Geyer GmbH, Hamburg, Germany) was added to each dish. The dishes were then gently shaken. To neutralize the acetic acid, an equal volume (1 ml) of 10% ammonium hydroxide was added. The solution was transferred into 96-well plates, and a microplate reader (ELx800 Absorbance Microplate Reader, BioTek, Bad Friedrichshall, Germany) was used to measure absorbance at a 405 nm wavelength.

Adipogenic and osteogenic-induced gene expression. Total RNA from differentiated dental pulp cells was extracted using TRIzol reagent (Cat. No. 15596026, Ambion, Austin, TX, USA), and the quantity was determined using a spectrophotometer (model V 530, UV/Vis Spectrophotometer, Jasco, Japan) and 1% agarose gel electrophoresis. Using the GoScriptTM RT reagent Kit (Cat. No. A5001, Promega) following the manufacturer's instructions. The extracted total RNA was reverse transcribed into cDNA and analyzed by reverse transcription PCR. We chose type I collagen, osteocalcin, and lipoprotein lipase (*LPL*) as adipogenic genes. Type I collagen and *PPAR*- were chosen as osteogenic genes. *GAPDH* was used as an internal control. Primer sequences and product lengths are shown in Table I.

Ethics approval and consent to participate. The investigations of anonymized data were performed in accordance with Hamburgisches Gesundheitsdienstgesetz (Hamburg Healthcare Act).

Statistical analysis. To examine differences in mean values between the two groups, a student *t*-test was employed. Values were considered significant at $p<0.05$. For statistical analysis, SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) was utilized.

Results

Morphological evaluation of human dental pulp stem cells (hDPSCs). Morphological evaluation of hDPSCs: hDPSCs

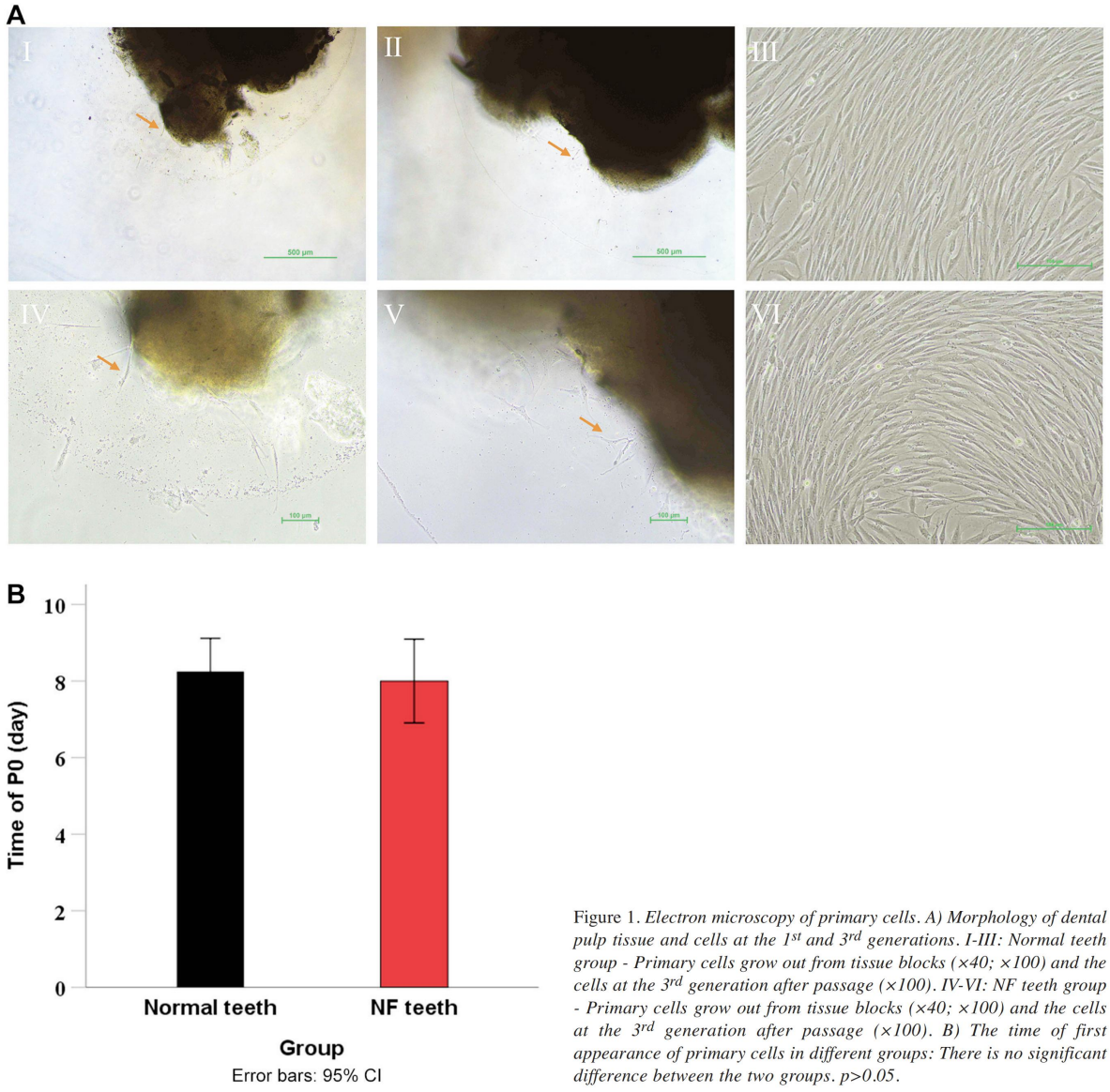

Figure 1. *Electron microscopy of primary cells. A) Morphology of dental pulp tissue and cells at the 1st and 3rd generations. I-III: Normal teeth group - Primary cells grow out from tissue blocks (×40; ×100) and the cells at the 3rd generation after passage (×100). IV-VI: NF teeth group - Primary cells grow out from tissue blocks (×40; ×100) and the cells at the 3rd generation after passage (×100). B) The time of first appearance of primary cells in different groups: There is no significant difference between the two groups. p>0.05.*

have a fibroblast-like morphology and seem as triangular or spindle-shaped cells under the microscope. In terms of cell morphology, there was no discernible difference between the two groups (the NF teeth group and Normal teeth group) (Figure 1A). In approximately 7 days of cultivation, the primary dental pulp cells were radically migrated out, forming a "growth halo" around the tissue block. In addition, no differences were observed between two groups in the time required to migrate out of the tissue block (Figure 1B) (p>0.05).

Cell yield. There was no difference in the number of harvested cells (p>0.05). The number of primary dental pulp cells harvested in the normal teeth group was $(11.725\pm1.601)\times10^5$, whereas that of the NF1 teeth group was $(11.333\pm1.341)\times10^5$ (Figure 2).

Comparison of proliferative rates. The MTS method was used to examine the proliferation rates of hDPSCs produced from normal teeth or NF teeth from day 1 to day 8 of culture of the pulp tissue. Between the two groups, there was no discernible

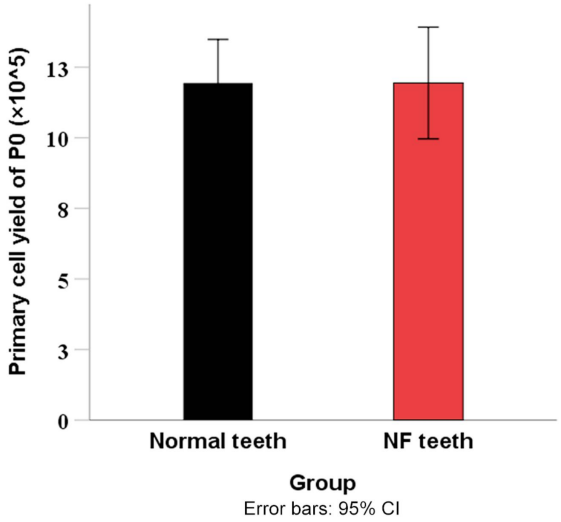

Figure 2. *Number of harvested cells from (black) normal teeth and (red) NF teeth. Number of harvested viable cells (gain) did not significantly differ (p>0.05).*

difference in the ability of cells to proliferate, no evidence of obvious differences in Giemsa staining was identified by visual observation (Figure 3A), including cell cloning formation and growth curve (p>0.05) (Figure 4B and C).

Survival rate. There was no discernible difference in the cell survival rate between the two groups after trypan blue and live-dead staining (p>0.05) (Figure 3A, B and C).

Evaluation of the multipotent differentiation capacity. After osteogenic and adipogenic induction for 3 weeks, third generation cells were stained (Figure 5A), and the corresponding absorbance was determined at various wavelengths (osteogenic—405 nm, adipogenic—540 nm)using a spectrophotometer. The results showed that there was no difference in the capacities for osteogenic and adipogenic differentiation between the two groups (Figure 5B), corresponding to the results of PCR gel electrophoresis (Figure 5C).

Discussion

This study reveals the adipogenic and osteogenic differentiation potential of DPSC of NF1 patients is similar to those healthy controls. Obtaining and further processing of DPSCs was not influenced by topographical peculiarities. Analogous to the apparently lack of influence of PNF in NF1 on the shape (27) and number of teeth (28, 29), and the capacity of oral diffuse-plexiform neurofibroma to differentiate extraosseous displaced odontogenic tissue to a tooth crown (30), DPSCs derived from teeth embedded in an oral PNF can be differentiated in the same way as teeth without any apparent contact to a PNST. In both situations, NF1 derived DPSCs differentiate identical to controls.

The area of oral and maxillofacial medicine urgently demands innovative treatment techniques to restore damaged tissues functionally and aesthetically. In recent years, the disciplines of SC research and regenerative medicine have seen tremendous development and groundbreaking discoveries. It has recently been reported that dental tissue-derived stem cells stand out as a significant stem cell source for bone regeneration in oral and maxillofacial surgery, craniofacial abnormalities, and orthopedics (31, 32). Furthermore, SCs can be collected intraorally from tooth pulp and periodontal ligaments. Our previous research has shown that dental SCs have the advantages in myogenic differentiation, odontogenic/osteogenic differentiation, and chondrogenic differentiation (33, 34).

PNF of the oral cavity often affects the trigeminal and upper cervical nerves (35). PNF has been documented to develop in the oral cavity on the tongue, lip, palate, gingiva, major salivary glands, and maxillary bones. PNF of the trigeminal nerve often leads to characteristic deformations of the jaw. The enlarged mandibular foramen is a radiologically conspicuous sign of a dorsal differentiation disorder through an adjacent PNF (36), as is the relatively frequently reported enlargement of the mandibular canal (37). However, the development of teeth in the orofacial PNF region is often disturbed in that the mesial migration of certain permanent teeth is incomplete or completely absent and the roots of the teeth are deformed due to the lack of bony space, but the visible structure of the tooth is not impaired (27).

NF-1 is significant because of the differences in clinical presentation, therapy, and prognosis. The current therapy for neurofibroma is total excision. These tumors are not radio-sensitive and have limited benefit from surgical treatment (38).

However, is it possible that stem cells existing at these sites have somewhat different properties from those in normal teeth. In the presented study, we compared multilineage potentials of dental pulp-derived mesenchymal stem cells isolated from dental pulps of two types of teeth (normal and PNF-associated).

We succeeded in culturing and separating original DPSCs from the tissue block using the limiting dilution method. It has been shown that, as is the case normal pulp tissues, stem cells are also present in NF teeth.

The ability to maintain self-renewal and differentiation is critical for the growth and effective use of stem cells in therapeutic settings (39). There was a concordance between the two kind of stem cells in their morphological and biological characteristics. MTS assay and colony-forming efficiency assay was used to assess the proliferation ability

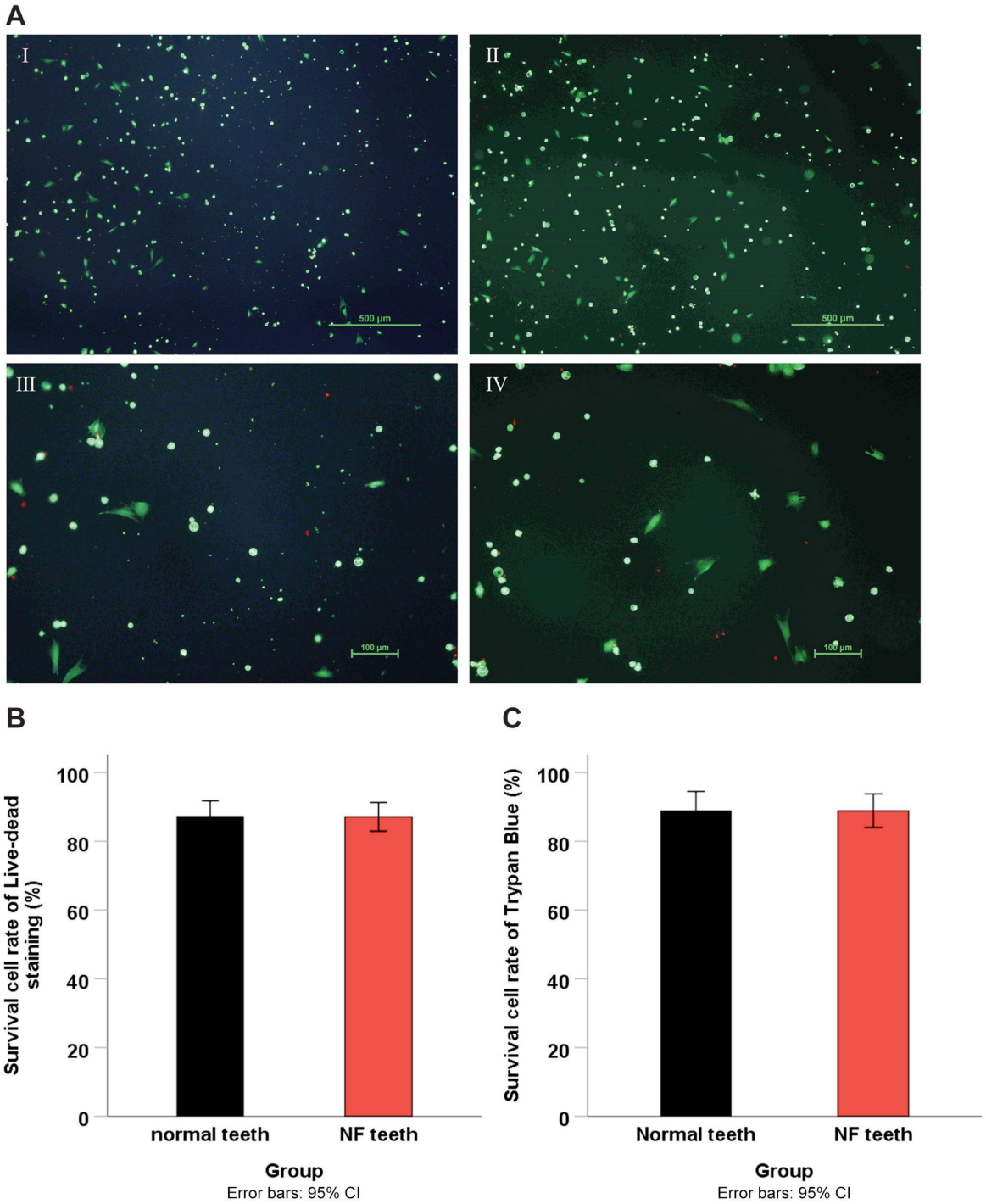

Figure 3. *Comparison of cell survival rate: A) Live-dead staining results of dental pulp cells; I: normal teeth group (×40); II: NF teeth group (×40); III: normal teeth group (×100); IV: NF teeth group (×100). B) The survival rate assayed using live-dead staining in each group. C) The survival rate assayed using trypan blue staining in each group.*

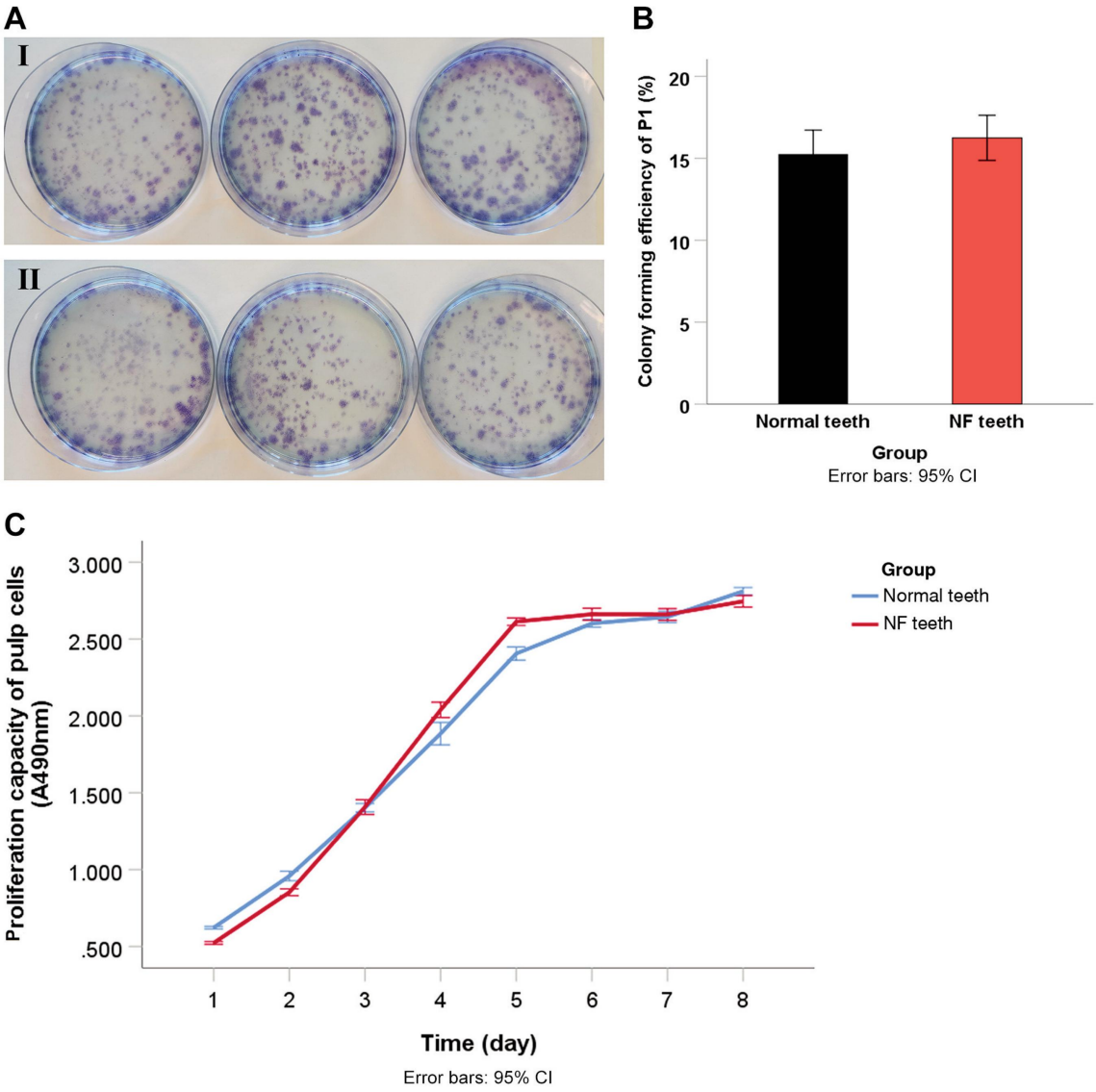

Figure 4. *Proliferation ability of primary dental pulp cells in each group. A) Giemsa staining results of dental pulp cells; I: normal teeth group; II: NF teeth group. B) Colony-forming efficiency. C) Cell growth curve (MTS assay).*

and colony forming potential; the intrinsic ability to display self-organizing morphogenetic properties in *ex vivo* culture may represent a general property of pulp tissue stem cells.

Traditionally, the potential of mesenchymal stem cells to develop into three lineages (osteogenic, chondrogenic, and adipogenic) has been used to assess their suitability for orthopedic and aesthetic regeneration applications (40). Collagen type I (COL I) is an early matrix mineralization marker, whereas Osteocalcin (OSC) is an osteogenic maturation marker (41); they indicate the differentiation and maturation of the bone, respectively. We have previously shown that dental pulp cells express bone-related genes (ALP, osteocalcin, collagen I) and can form mineralized nodules *in vitro* (42). The current cells derived from NF teeth were shown to possess a series of characteristics such as the ability to develop mineralized nodules *in vitro* and express

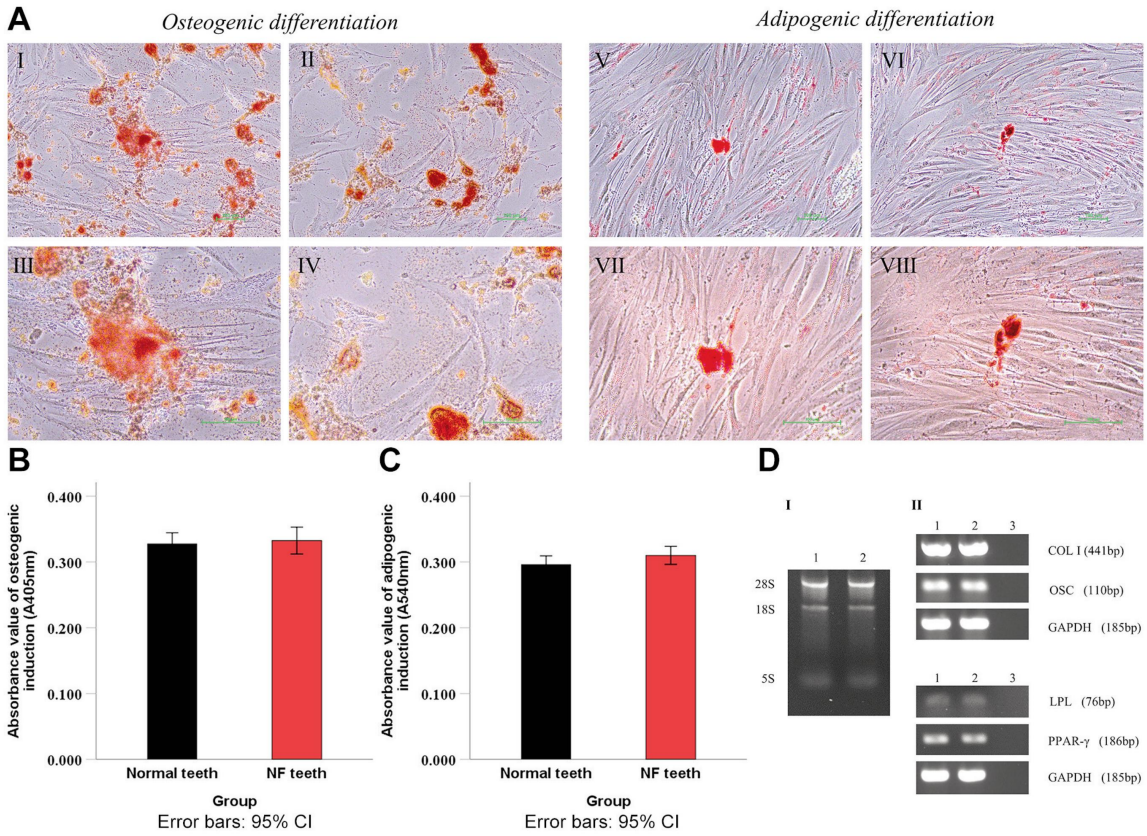

Figure 5. *Differentiation potential of dental pulp cells. A) Representative microscopic images from adipogenic and osteogenic differentiation assays. I-IV: Osteogenic differentiation. I: Normal teeth group (×40); II: Normal teeth group (×100); III: NF teeth group (×40); IV: NF teeth group (×100); V-VIII: Adipogenic differentiation. V: Normal teeth group (×40); VI: Normal teeth group (×100); VII: NF teeth group (×40); VIII: NF teeth group (×100). B) The absorbance value at 450 nm of the dissolved solution after Alizarin Red S staining in the two groups. C: The absorbance value at 540 nm of the dissolved solution after Oil Red O staining in the two groups. D: Gel electrophoresis results. I: RNA electrophoresis in the two groups: 1. Normal teeth group; 2: NF teeth group. II: Electrophoresis results regarding the expression of osteogenic and reference genes in each group. 1: Normal teeth group; 2: NF teeth group; 3: Negative control group; III: Electrophoresis results regarding the expression of adipogenic and reference genes in each group. 1: Normal teeth group; 2: NF teeth group; 3: Negative control group.*

the bone-associated markers OSC and COL I. The osteogenic differentiation capacity of these cells was commensurable with the bone-forming activity of normal pulp stem cells. Both mesenchymal stem cells were examined for their ability to undergo adipogenic differentiation. The most assessed genes αρε peroxisome proliferator activated receptor γ (PPAR-γ) and lipoprotein lipase (LPL). Like osteogenic differentiation ability, the adipogenic one showed same lipid vacuoles (Oil Red staining) in mesenchymal stem cells from NF and normal teeth.

DPSCs have been successfully studied in NF1 patients (24, 25). DPSCs obtained from NF1 patients can be differentiated into several specifications, for example chondrogenic, osteogenic or adipogenic (24, 25). In principle, this means that autogenous sources of cell and tissue regeneration are available for NF1 patients (11). This and previous studies show that the constitutive loss of the NF1 gene does not affect the ability of DPSCs to differentiate according to the chosen conditioning of the environment (43-45). Until now, it was unknown whether DPSCs from teeth in this tumor area have the same differentiation capacity as DPSCs from NF1 patients who have not developed this facial tumor.

NF1 is a disease with a plethora of signs and symptoms. For example, bony changes (36) are just as much a part of the spectrum of the syndrome as the often-plentiful accumulation of fat cells in body regions affected by diffuse

neurofibromas (46). Basic research is of great importance for understanding the cellular and molecular basis of the disease and develop therapeutic strategies, for example in skeletal regeneration.

The presented initial results indicate that the differentiation capacity of DPSCs is the same between the two groups. Beyond the experimental evidence, this finding is interesting for the qualification of the NF1 gene as a histogenesis control gene (7). Even under the artificial conditions of cell culture, alteration of the genetic status by *NF1* mutation does not prevent the differentiation of the DPSCs. This result may provide an opportunity for further basic research on the re-habilitation of these often severely disfigured patients by regenerative cell culture techniques. This can be achieved, for example, by the selective generation of SCs from DPSCs or other sources of SCs, which are used to stabilize the connective tissue. Especially diffuse PNF are often characterized by a significant lack of elasticity of the affected body region. Surgical treatment options do not guarantee stable results in terms of body shape and volume of the reduced tumors (38). Supportive measures are highly desirable. With the potentially unlimited differentiation of SCs derived from NF1 patients, there is a wide field of SC application in the reconstructive surgery of NF1 patients.

Conclusion

In this study, dental pulp stem cells derived from NF teeth were successfully obtained and found not to be different from normal dental pulp stem cells for comparison. However, the research in constructing tissue-engineered bone to reconstruct bone defects in the clinical setting is still in the exploratory stage, and there are still many challenges to realize its clinical application, such as how to obtain sufficient amount of high quality and high purity stem cells, how to improve the vascularization and physical and mechanical properties of tissue-engineered bone, and how to promote the effective integration of tissue-engineered bone tissue with host bone tissue. Along with the continuous development of related disciplines and technologies, the existing challenges will be gradually overcome, and tissue-engineered bone will eventually enter the clinic and become a routine means of bone defect reconstruction treatment.

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Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

MY: conceived the study, supervised the experiments, and drafted the manuscript. WW: conceived the study, supervised the experiments, and drafted the manuscript. SF: data evaluation and manuscript preparation. US: data evaluation and manuscript preparation. MG: analyzed the data and revised the manuscript. REF: conceived the study, supervised the experiments, and revised the manuscript. RS: performed the data collection. HF: conceived the study, designed the data evaluation, and revised the manuscript. All Authors read and approved the final manuscript.

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7 Chapter 7: Publication in *American Journal of Translational Research*, September 2020

Inducing differentiation of human dental pulp cells toward acinar-lineage

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Personal contribution:

The following research article has been published in ***American Journal of Translational Research***. I am the first author of the paper. I conducted the experiments and data collection and analyzed the results (raw data analysis and statistical analysis). I contributed to writing the manuscript draft and editing during the revisions.

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Original Article

Inducing differentiation of human dental pulp cells toward acinar-lineage

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Abstract: Objective: To induce acinar-differentiation from human dental pulp cells for potential application in aiding treatment of dry-eye syndromes. Method: Human dental pulp cells were co-cultured with human submandibular gland acinar cells using a transwell construction for 2 weeks. The two populations of cells were physically separated while chemical and biochemical components can be exchanged. Fibroblasts were included as a negative control. Expression of amylase, cytokeratin 8 and vimentin were examined by immune-staining. Amylase activity was measured using an AMS Assay Kit. Result: Cobblestone-like islands, a feature of acinar cells, appeared in the dental pulp cells which were co-cultured with salivary gland cells for one week and increased in number and size after two weeks. Antibody detected amylase in 30 and 50% of the pulp cells 1 and 2 weeks in the co-culture, respectively. Cytokeratin 8 increased while vimentin decreased. All these changes indicate an acinar-like differentiation of the dental pulp cells. None of these changes were observed in fibroblasts which were also co-cultured with salivary gland cells, indicating that the acinar-like differentiation is specific for the dental pulp cells. Neither of the changes were observed in dental pulp cells when not co-cultured with the salivary gland cells, indicating that induction is specific and essential. Conclusions: Human dental pulp cells have the potential to differentiate into acinar-like cells which may provide an autologous source for cellular therapy for dry-eye syndromes.

Keywords: Dental pulp cells, differentiation, salivary glands, regenerative, fibroblasts, dry-eye syndromes

Introduction

Sjogren syndrome is a chronic autoimmune disease of the exocrine glands associated with dryness of the eyes and mouth that severely impairs the quality of life [1, 2]. The prevalence is estimated as 0.1~4.8% in Europa and 0.77% in China [3, 4].

The lacrimal gland is the most common site involved. Impairment of secretion from the acinar cells can lead to symptoms including burning sensation, serious ocular conjunctiva, susceptibility to corneal infection and ulcers, and in server cares, even blindness [5].

The saliva gland is the second most common site involved. Patients with salivary gland hypofunction often suffer from conditions including dry mouth, dysphagia, rampant caries, susceptibility to fungus infection and malnutrition, all leading to poor quality of life [6].

So far, there is no established curative treatment. The current measures mainly aim to ameliorate the symptoms which does not solve the problems [7]. For some severe cases of dysfunctioning acinar gland, transplantation of the mandibular gland is an option to prevent loss of vision and to enable the patients a normal life to certain extent [8]. However, this major surgical procedure demands extensive experience and skill from the surgeon. More importantly, such transplantation leads to damage of the donor site, and is therefore controversial. In addition, since the saliva gland is also affected in the Sjogren syndrome, the transplanted gland is expected to function only for limited time period [9].

Advance in regenerative medicine raises the hope of using autologous cells and tissues for compensating and supporting dysfunctional organs and tissues. In the case of the Sjogren syndrome, injecting autologous acinar-cell-like cells may provide a strategy for treating dry eyes or at least for ameliorating the symptoms. Recently, bioengineered lacrimal glands from embryo-stem/progenitor cells have been successfully transplanted into mice which produced acini and ducts [10]. Other studies injected stem/progenitor cells from the lacrimal gland to the defect ones with improvement in structure and function of the latter in mice. However, using such embryonic or lacrimal gland stem/progenitor cells is not realistic in humans. An alternative practicable strategy would be inducing other adult human pluripotent cells into acinar-cell-like cells.

Human dental pulp cells are cells from roots of teeth which exhibit features of pluripotent stem/progenitor cells and therefore provide a promising cell source for tissue engineering and regeneration medicine [11]. Many people will likely have some of their teeth, for example and especially, their wisdom teeth, extracted for clinical reasons or prevention purpose sometime in their life. Cells cultured from the teeth of a patient are autologous to this patient which salvages the problem of host rejection. Dental pulp cells can be induced into osteogenic, adipogenic and chondrogenic cells. We therefore hypothesized that dental pulp cells can also be induced into the lineage of acinar cells [12]. However, there is no established cultural condition for such induction *in vitro*.

The present study was designed to test the feasibility of inducing acinar-differentiation from dental pulp cells by co-culturing them with cells derived from mandibular gland. We used a transwell construction which prevents mix of the two types of cells while enables chemical exchange and interaction. As acomparison, we included a human fibroblast culture.

Materials and methods

Culturing human salivary gland cells

At submandibular sialadenectomy surgeries carried out in our Hospital for patients with blocked saliva glands, the removed glands were saved from biowaste for the study. The study protocol was approved by the local authority and all patients gave written consent for using their removed gland specimen for the study. All specimens were anonymized for privacy protection. The salivary glands were dissected into small fragments in the laboratory and used to culture the gland cells by the explant method. The gland cells were identified by labeling with an antibody for the enzyme amylase (Amylase-G10 1:150, cat NO. SC466-57, SANTA Inc, USA) and were used for inducing the pulp cells.

Culture and purification of human dental pulp cells

Human dental pulp tissues were obtained from teeth extracted at our hospital for medical indications. The primary culturing was carried out using the explant methods. Expression of CD29 and CD44 was examined using flow-cytometry. Pluripotent of the pulp cells were checked by their differentiation into adipogenic and osteogenic cells which were stained with Oil Red-O and Alzerin red [12].

Inducing acinar differentiation using a transwell construction

The dental pulp cells and the gland cells were co-cultured in a transwell plate (4-μm pore size, Corning, USA) where 4×10^4 acinar cells were seeded in the upper inserts and 1×10^4 pulp cells in the lower wells (**Figure 1A**). Transwell membrane inserts have 0.4 μm pores which prevent migration of cells while enable exchange of the medium with component secreted by the cells. Media in both the upper and the lower chambers were changed every two days. The co-culture was continued for 2 weeks. Morphology of the cells were observed daily. After one and two weeks, expression of amylase, cytokeratin 8 (CK8; cat. no. ab9023; dilution, 1:800; Abcam, USA) and vimentin (anti-vimentin 1:1000, cat# ab8069, Abcam, USA) were examined using antibodies and counter-staining with DAPI or the hematoxylin.

Amylase activity

To measure amylase activity, the upper inserts with inducing cells were removed and the medium in the lower differentiating cells changed to medium without color pH indicator. After 3 days, the supernatants were used for measuring amylase activity using the AMS assay kit (Nanjing jiancheng Corp., Nanjing, China). The

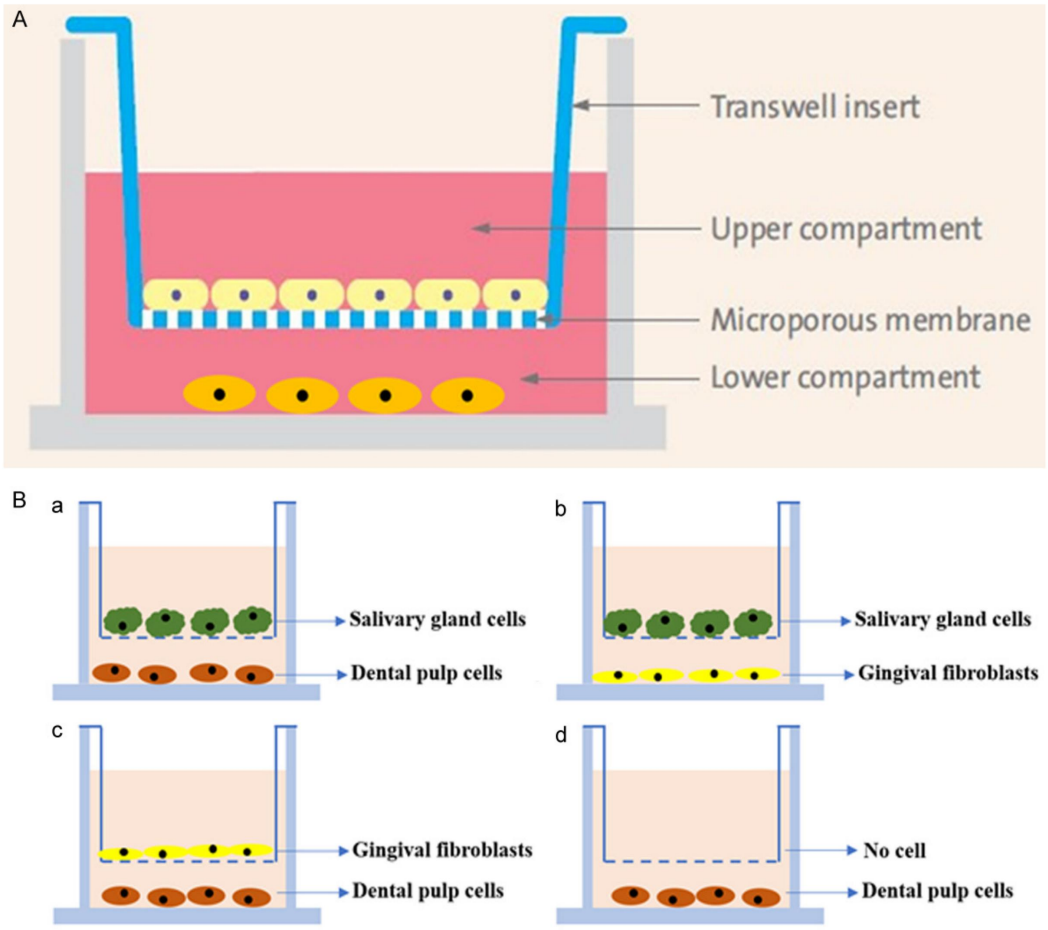

Figure 1. Study design. A: The transwell-construction. Two populations of cells were cultured in the upper and lower compartment, separated by a microporous membrane which allows passage of chemical and biochemical molecules. B: 4 combinations of the inducing and differentiating cells.

activity was calculated as: Amylase activity (U/dl) = (blank OD value-test OD value)/blank OD×80. For each sample, the measurement was carried out in 4 replicates. The values were given in means and standard deviation. Comparing the amylase activities in two samples was carried out using the t-test with two-tailed hypothesis. The significant level was set at 0.05.

Results

Human salivary gland cells and dental pulp cells

Salivary gland cells migrating from the explants in primary culture were observed in phase contrast microscope. After two days of plating the dissected fragments of the gland tissue, cells migrating from the explants were observed. These cells appeared in polygonal shape which is typical for epithelial gland cells. After 6 to 8 days, these cells reached 80-90% confluence.

Production of amylase is one of the most characteristic function of salivary gland acinar cells. Immuno-staining revealed that cells in culture were positive for amylase in their cytoplasm.

5 to 7 days after plating the explants of dental pulp tissue, cells started migrating from the explants. In the primary dental pulp cells, the morphology of the cells was from ellipse to a spindle, strip or irregular shape.

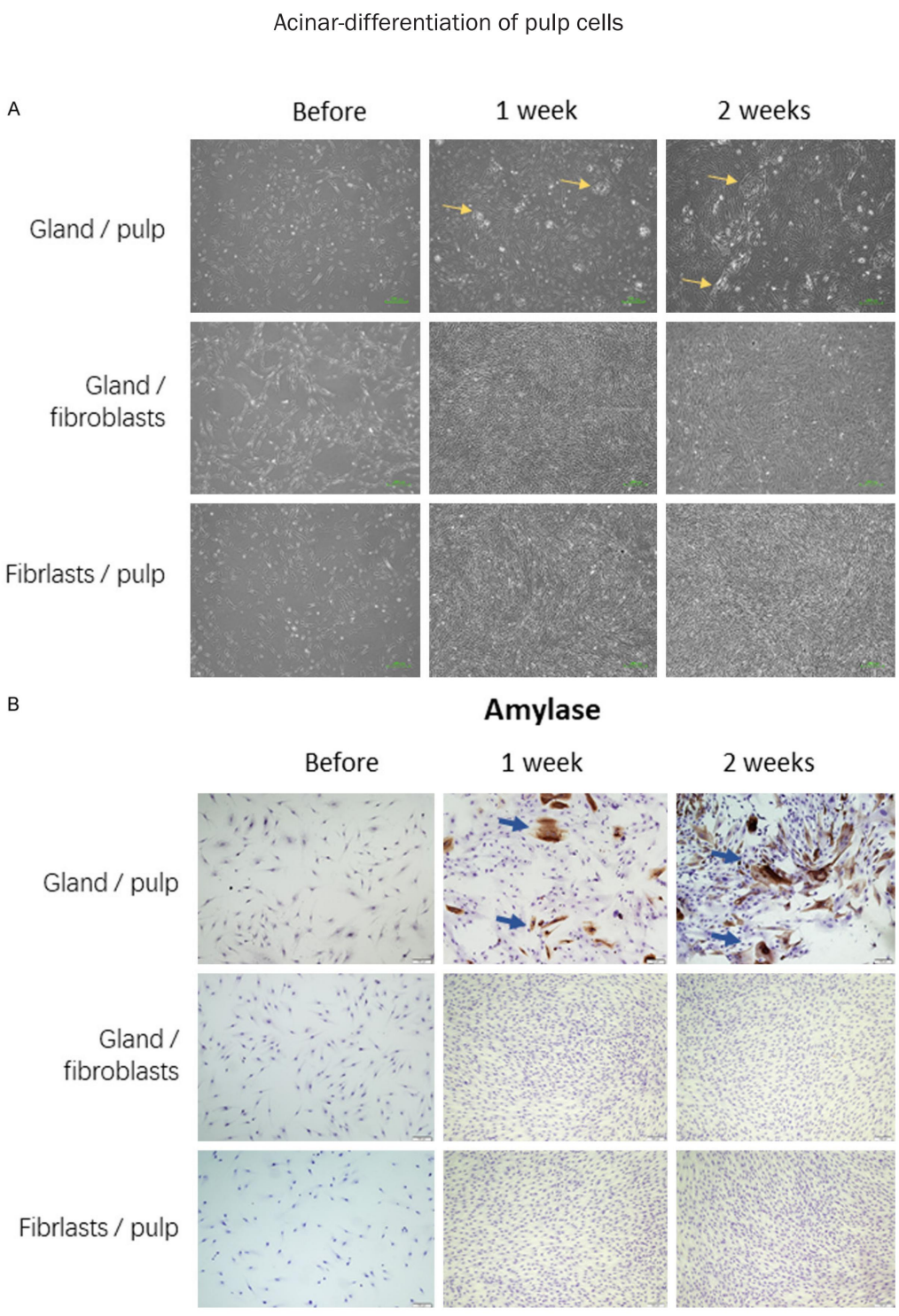

Figure 2. Acinar-like differentiation of the dental pulp cells co-cultured with salivary gland cells (gland/pulp): island-like structures were visible (A) and amylase-positive cells were evident (B). These chances were not visible in the human gingival fibroblasts co-cultured with salivary gland cells (gland/fibroblasts) and not in the dental pulp cells without salivary gland cells (fibroblasts/pulp).

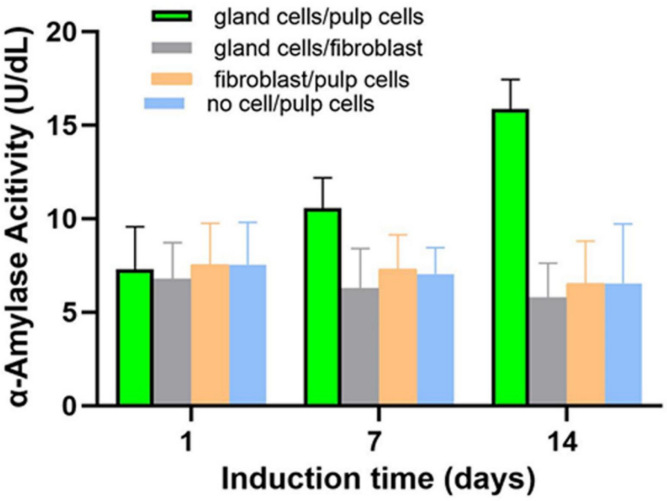

Figure 3. Amylase activity increased in the supernatant of the dental pulp cells which have been co-cultured with salivary gland cells (green bars) for 1 (P=0.06) and 2 weeks (P<0.001). To exclude the amylase activity from the salivary gland cells, the inserts with inducing cells were they were removed and the cells in the lower compartments were cultured for another 3 days before the measure. No such increase was measured in the supernatant of the human gingival fibroblast which has been co-cultured with salivary gland cells (gray bars). Consequently, the amylase activity of the gland-co-cultured pulp cells was significantly higher (P=0.02 on day 7 and P<0.001 on day 14). Without co-culturing with the salivary gland cells, the amylase activity did not increase in the dental pulp cells neither (yellow bars).

Acinar-like differentiation of pulp cells

The primary dental pulp cells and the gland cells obtained as above were cultured together in a transwell construction which enables exchange of chemical and biochemical components of the two physically separated cell populations. The gland cells were in the upper insert and the pulp cells in the lower wells (**Figure 1A**). After one week, morphology change began in the pulp cells (**Figure 2A**). Acinar cell-like polygonal shaped cells appeared among the elongated ovate pulp cells. These acinar cell-like cells packed together compactly, forming an island-structure (**Figure 2A**, yellow arrows). On top of the islands, bubbles were visible which were possibly secretion from the cells. Proportion of these acinar cell-like cells, size and number of the cell-islands, and bubbles over them further increased with time. However, 3 weeks after the co-culture, the cell-islands started to detach the surface.

Amylase is a characteristic feature of acinar cells. Immunostaining indeed revealed expression of amylase in approximately 30% of the pulp cells with induction of gland-cells in the upper insert for one week (**Figure 2B**, blue arrows). After two-weeks, the amylase positive cell population increased to approximately 50%. In contrast, none of the fibroblasts was positive for amylase. Similarly, no pulp cells without acinar-induction by gland-cells in the upper chambers were positive for amylase expression (**Figure 2B**).

In concordance, increased amylase activity was measured in the supernatant of pulp cells co-cultured with the gland cells (**Figure 3**, green bar). This increase did not reach the significant level (P=0.06) for one week co-cultured, but became highly significant (P<0.001) after two weeks co-culture. In contrast, no such increase in amylase activity was measured in the two negative controls (**Figure 3**). For example, the amylase activity of the gland-induced fibroblasts was significantly lower than that of the gland-induced pulp cells (P=0.02 and <0.001 for one and two weeks co-culture).

As further supporting evidence for the acinar-like differentiation, cytokeratin, an epithelial marker, was also increased in the dental pulp cells which were co-cultured with the gland cells. In concordance, vimentin, a mesenchymal marker, control, decreased indicating that the dental pulp cells were losing the stem/progenitor features in the acinar-differentiating process.

This acinar-like differentiation potential seems to be a specific feature of dental pulp cells, since no morphological and expression changes were observed in the fibroblasts induced under the identical condition (**Figures 2-4**). Instead, the fibroblasts continued to grow. In another negative control, the pulp cells were co-cultured with fibroblasts in the upper inserts,

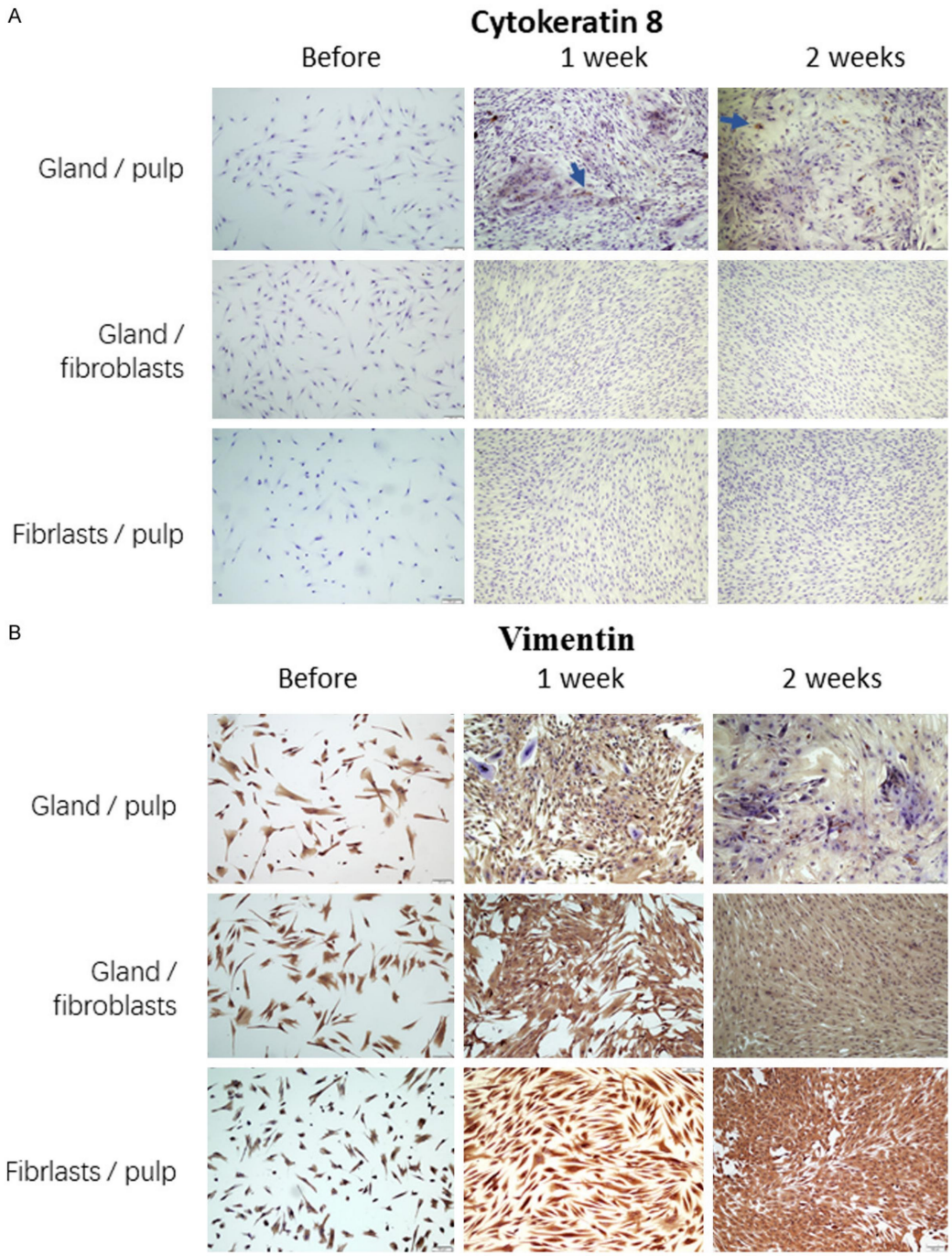

Figure 4. A: Increasing expression of cytokeratin 8, a marker for epithelial cells, in the acinar-like differentiating pulp cells acinar-like differentiation of the dental pulp cells co-cultured with salivary gland cells (gland/pulp). B: Decreasing expression of vimentin, a mesenchymal marker, in the acinar-like differentiating pulp cells acinar-like differentiation of the dental pulp cells co-cultured with salivary gland cells (gland/pulp). These chances were not visible in the human gingival fibroblasts co-cultured with salivary gland cells (gland/fibroblasts) and not in the dental pulp cells without salivary gland cells (fibroblasts/pulp).

no acinar-like differentiation was observed (**Figures 2-4**).

Discussion

We succeeded in inducing acinar-like differentiation from human dental pulp cells. The induction is realized by co-culturing salivary gland cells in a transwell construction. Since the two populations of cells share the same medium, the acinar-differentiation of the pulp cells in the lower wells was likely induced by factors and components secreted from the salivary gland cells in the upper inserts.

For induction of differentiation in various lineages such as the adipogenic and osteogenic ones, conditions have been established which are based on a special combination of small molecules. However, for acinar-differentiation, no such simple conditions are known. A recent study succeeded in enriching lacrimal gland cells from human embryonic stem cells by transfecting synthetic messenger RNA for several transcription factors [13]. However, transfection is not a practicable approach for medicine application.

Co-culturing is an alternative strategy for inducing differentiation. Indeed, differentiation into the acinar-lineage has been achieved from adipose stromal cells when co-cultured with salivary gland cells [14]. However, no detail of the co-culturing construction was given in that study and no transwell is mentioned. It is possible that the two kinds of cells were cultured in mixture.

In the present study, we used a transwell construction to carry out the “co-culturing”. This construction enables exchange of chemicals and biochemical molecules. However, the membrane of the trans-inserts does not allow passage of the cells. Therefore, the two types of cells remain physically separated. An important advantage of this semi-co-culture is that the two types of cell can have different origins. While the pluripotent cells in the lower wells can be autologous for application without host rejection, the inducing cells in the upper inserts can have a different origin. In the present study, human salivary gland cells were used as the inducing cells. However, other sources of inducing cells and tissues are also theoretically possible, for example, cell lines derived from human or non-human gland tissues. Alternatively, conditioned medium of gland cells may also have effect in inducing acinar-differentiation of pluripotent cells.

We used a ratio of 4 to 1 of the inducing cells to the differentiating cells. However, it is possible, that other ratios may also work. Furthermore, since the acinar-like differentiation is reached in approximately 30% of the pulp cells, it is possible that these cells will secrete factors and therefore enable the condition for further differentiation of other pulp cells. In addition, future studies also need to explore the potential of other types of pluripotent cells including bone marrow cells and adipose stromal cells in this acinar-differentiation.

Human stem/progenitor cells are postulated to have some immune-suppressing activity [15, 16]. Therefore, in addition to their potential acinar-function after the induction, dental pulp cells may also contribute to suppress some dysregulated immunity which damages the original lacrimal cells in the dry-eye syndrome.

In summary, results of the present study demonstrated the feasibility of inducing acinar-differentiation from human dental pulp cells by co-culturing them with salivary gland cells using a transwell construction without mixing the two cell populations. The acinar-like cells may have application potential in cellular therapy for dry-eye syndromes as autologous cell source.

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Disclosure of conflict of interest

None.

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8 Chapter 8: Publication in *Cells*, July 2021

Evaluation of the Effects of Human Dental Pulp Stem Cells on the Biological Phenotype of Hypertrophic Keloid Fibroblasts

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Personal contribution:

The following research article has been published in ***Cells***. I am the first author of the paper. I conducted the experiments and data collection and analyzed the results (raw data analysis and statistical analysis). I contributed to writing the manuscript draft and editing during the revisions.

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Article

Evaluation of the Effects of Human Dental Pulp Stem Cells on the Biological Phenotype of Hypertrophic Keloid Fibroblasts

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Abstract: Objective: Despite numerous existing treatments for keloids, the responses in the clinic have been disappointing, due to either low efficacy or side effects. Numerous studies dealing with preclinical and clinical trials have been published about effective therapies for fibrotic diseases using mesenchymal stem cells; however, no research has yet been reported to scientifically investigate the effect of human dental pulp stem cells (HDPSCs) on the treatment of keloids. The objective is to provide an experimental basis for the application of stem cells in the treatment of keloids. Methods: Human normal fibroblasts (HNFs) and human keloid fibroblasts (HKFs) were cultured alone and in combination with HDPSCs using a transwell cell-contact-independent cell culture system. The effects of HDPSCs on HKFs were tested using a CCK-8 assay, live/dead staining assay, quantitative polymerase chain reaction, Western blot and immunofluorescence microscopy. Results: HDPSCs did not inhibit the proliferation nor the apoptosis of HKFs and HNFs. HDPSCs did, however, inhibit their migration. Furthermore, HDPSCs significantly decreased the expression of profibrotic genes (CTGF, TGF-β1 and TGF-β2) in HKFs and KNFs ($p < 0.05$), except for CTGF in HNFs. Moreover, HDPSCs suppressed the extracellular matrix (ECM) synthesis in HKFs, as indicated by the decreased expression of collagen I as well as the low levels of hydroxyproline in the cell culture supernatant ($p < 0.05$). Conclusions: The co-culture of HDPSCs inhibits the migration of HKFs and the expression of pro-fibrotic genes, while promoting the expression of anti-fibrotic genes. HDPSCs' co-culture also inhibits the synthesis of the extracellular matrix by HKFs, whereas it does not affect the proliferation and apoptosis of HKFs. Therefore, it can be concluded that HDPSCs can themselves be used as a tool for restraining/hindering the initiation or progression of fibrotic tissue.

Keywords: human dental pulp stem cells; fibroblast; co-culture; keloid

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1. Introduction

During wound healing, a dynamic balance of synthesis and the degradation of collagen usually results in either a physiological or a pathological scar. A pathological scar can be described as a fibroproliferative disease caused by the hyperproliferation of fibroblasts and excessive synthesis of the extracellular matrix (ECM) in the process of dermis wound healing following skin trauma or serious burns [1–3]. Moreover, pathological scars can be classified into the following two types: hypertrophic scar and keloid. The former is predominantly localized above the original wound region, with a reddish or

pinkish appearance, which can sometimes be pruritic. Furthermore, a hypertrophic scar usually regresses after years to form a matured scar [4,5], whereas the situation is strikingly different when the latter is involved. Although a keloid is a non-malignant disease, keloids almost always overgrow onto the surrounding skin, where they can often lead to malignant manifestations such as pain, ulceration, secondary infection, active angiogenesis and even carcinogenesis [6]. Such symptoms not only impact the quality of life, but they can also be highly unsightly, contributing further to psychological disturbances and corresponding social distress.

Despite numerous existing treatments for keloids, such as surgical removal, hormonal therapy, laser treatment or radiotherapy, as well as interventional sclerotherapy, the responses in the clinic have been disappointing due to either low efficacy or side effects, or a combination of both [7]. In order to address the aforementioned existing and urgent clinical need, new and more efficient therapeutic strategies are in order.

Numerous studies dealing with preclinical and clinical trials have been published about effective therapies for fibrotic diseases using mesenchymal stem cells, including the following: bone-marrow-derived mesenchymal stem cells (BMSCs) [8–10] and adipose derived mesenchymal stem cells (ADSCs) [11]. Human dental pulp stem cells (HDPSCs) are a type of adult stem cell that possess self-renewal, self-replication and multi-differentiation properties, where they can differentiate into a variety of mesodermal tissue cells, such as chondrocytes, osteoblasts, cardiomyocytes as well as adipocytes [12]. However, no research has yet been reported to scientifically investigate the effect of HDPSCs on the treatment of keloids.

The objective is to provide an experimental basis for the application of stem cells in the treatment of keloids. A co-culture method was set up to investigate the influence and mechanism of dental pulp stem cells on keloid fibroblast properties, such as cell proliferation, migration, collagen synthesis, invasion and apoptosis.

2. Materials and Methods

2.1. Cell Isolation and Cultures

Isolation of human dental pulp stem cells was performed using the explant growth method. Extracted teeth were obtained from patients (aged 18–25 years) with informed consent at the Guiyang Hospital of Stomatology (Guiyang, China). The retrieved dental pulp tissue was cut with sterilized scissors into small pieces and the tissue pieces were then plated onto 100-mm cell culture dishes in DMEM with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)/streptomycin (100 µg/mL) at 37 °C and 5% CO_2. Cells were further grown until confluence was reached. Human dental pulp stem cells were obtained using a limiting dilution method, a method previously established by our group [13]. Only passages 3–5 of dental pulp stem cells were used in this study.

Seven tissue samples (0.5 cm^3) of keloids and seven corresponding normal skin tissue samples were used as a reference (Table 1). All tissue samples were taken from the maxillofacial area of patients in the same hospital with informed consent. The samples were classified into 2 groups (keloid and normal skin) based on clinical and pathological diagnosis. Furthermore, pathological characteristics were diagnosed upon staining with Hematoxylin and Eosin (H&E) with the following findings: (1) the dermal layer of the skin is markedly thickened; (2) disappearance of skin appendages; (3) collagen fibers begin to coarse into thick bundles. Finally, fibroblasts from keloid and normal skin isolation were also performed using the explant growth method. Samples were cut into 0.5 mm^3 tissue pieces. Each tissue piece was then cultivated onto 100-mm cell culture dishes in DMEM with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)/streptomycin (100 µg/mL) at 37 °C and 5% CO_2. Cells were harvested until confluence was reached. Cells from passages 3–5 were used for this study.

Table 1. Patient epidemiological data.

Subject	Age	Sex
1	29	F
2	38	M
3	21	M
4	32	M
5	27	F
6	25	F
7	33	M

2.2. Flow Cytometry Was Used to Detect the Surface Markers of HDPSCs

HDPSCs at passage 3 were trypsinized by 0.25% trypsin, washed twice with PBS and resuspended at a concentration of 1×10^9/L in culture medium, according to the minimum criteria set by The International Society for Cellular Therapy in 2006 for defining multipotent MSCs [14]. The antibodies used in the experiments were CD19 (561295; BD Biosciences, San Jose, CA, USA), CD44 (560531; BD Biosciences, San Jose, CA, USA), CD45 (560368; BD Biosciences, San Jose, CA, USA), CD90 (555595; BD Pharmingen, San Jose, CA, USA), HLA-DR (560652; BD Biosciences, San Jose, CA, USA), CD29 (17-0299; eBiosciences, San Jose, CA, USA), CD73 (561014; BD Biosciences, San Jose, CA, USA), CD105 (560819; BD Biosciences, San Jose, CA, USA), CD73 (561014; BD Biosciences, San Jose, CA, USA), CD34 (560940; BD Biosciences), CD105 (560819; BD Biosciences) and CD11b (11-0113; eBiosciences, San Jose, CA, USA). In flow cytometry tubes, 1 mL of the cell suspension was collected with 5 μL of each of the anti-fluorescein isothiocyanate-conjugated antibodies. Cells were incubated at 4 °C for 1 h in the dark, and the cell surface markers were then analyzed on a BD Fortessa (BD Biosciences) instrument. All data analysis was conducted using FlowJo (v.10) software (FlowJo, Ashland, OR, USA).

2.3. Transwell Co-Culture Systems

In our project, 6-well Transwell systems, where cells shared medium without making any contact with transwell inserts (0.4-micrometer pore size), were employed (Figure 1A). Culture media was composed of DMEM with the following constituents: 10% FBS, 1% glutamine and 1% penicillin/streptomycin. There were the following four culture conditions in this project: (Figure 1B) HNFs monoculture, (Figure 1C) HDPSCs/HNFs co-culture, (Figure 1D) HKFs monoculture and (Figure 1E) HDPSCs/HKFs co-culture. In the monoculture groups, HNFs or HKFs were seeded in the bottom chambers. In the co-culture groups, HNFs or HKFs were seeded into the bottom chambers, and HDPSCs into the top chambers. The ratio of cells in the upper chamber to cells in the lower chamber was 1:1.

2.4. Cell Morphology

Cells were photographed under an inverted microscope (ECLIPSE Ts2-FL, Nikon, Japan) at days 1, 3, 5, and 7.

2.5. Cell Proliferation

Both normal and keloid fibroblasts were each individually seeded into 6-well plates at a concentration of 1×10^4 cells per well. In this study, cellular proliferation was analyzed using the CCK8 assay (Dojindo, Kumamoto, Japan) according to manufacturer's instructions. After removal of the supernatant, 660 μL of CCK8 solution (60 μL CCK8:600 μL medium) was added to each well and incubated in the incubator for 2 h on days 1, 3, 5 and 7. Then, a 100-microliter volume of the supernatant was separated by centrifugation and transferred into a fresh 96-well plate. Readings at wavelengths of 450 nm were recorded using a Fluostar Omega plate reader, and a standard curve was then plotted against the readings of the standards.

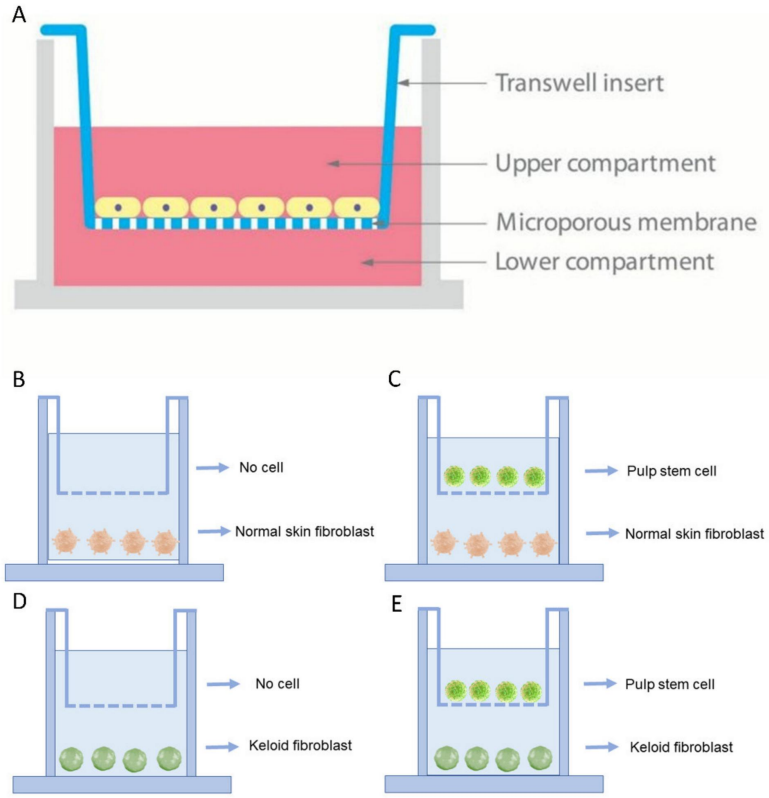

Figure 1. Study design. (**A**): A schematic design of the Transwell co-culture model was established using Transwell chambers with a 0.4-micrometer pore size that allows for the passage of chemical and biochemical molecules. (**B**–**E**): 4 combinations of the inducing and differentiating cells.

2.6. Collagen Synthesis Detection

Both normal and keloid fibroblasts were each individually seeded into 6-well plates at a concentration of 5×10^5 cells per well. Supernatants were retrieved from 6-well plates after 48 h when cells reached confluence and were handled according to the instructions of Hydroxyproline Assay Kit (MAK008; Sigma Aldrich, St. Louis, MI, USA). A total of 1.5 mL of the supernatant was harvested and resuspended in 0.05 mL of digestion solution at 37 °C for 3 h. Then, 0.5 mL of solution A was transferred into the mixture for 10 min and 0.5 mL of solution B was added and allowed to react for 5 min, followed by the addition of 1 mL of solution C at 60 °C for 15 min. Absorbance was then read at a wavelength of 550 nm. Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline (0–100 mg/mL).

2.7. Wound Scratch Assay to Detect the Cell Migration Ability

Normal and keloid fibroblasts were seeded individually into 6-well plates at a concentration of 5×10^5 cells per well. When the cells reached 90% confluency, 200 µL sterile pipette tips were used to scratch the bottoms of the 6-well plate culture wells. After rinsing out the floating cells using PBS, cells were starved for 24 h in serum-free medium. In the test group, transwell chambers were seeded with a concentration of 5×10^5 of dental pulp stem cells. The wound area was recorded using a light microscope camera after scratching at 0 and 24 h. Finally, the rate of cellular migration was quantified using image J software V1.51 (NIH software, Bethesda, MD, USA). The migration rate of cells was measured using the following formula: $(W_{0h} - W_{24h})/W_{0h} \times 100\%$.

2.8. Live and Dead Staining

Live and dead cells were assessed using LIVE/DEAD® staining kits (Cat# L3224; Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions, where 500 µL of staining solution was added to each well after the chamber was removed at days 1, 3, 5 and 7, the cells were then incubated in the dark at room temperature for 10 min, and then photographed with the employment of immunofluorescence microscopy. Five images, one from the center and four from the periphery, were obtained from each well using an Olympus inverted fluorescence microscope. Live and dead cells were counted using Image J software, and the rates of cell survival were calculated as Live cells/(Live + Dead cells)%.

2.9. Western Blot

Normal and keloid fibroblasts were seeded in six-well plates at a density of 5×10^5 cells/well and transwell chambers were seeded with a concentration of 5×10^5 of dental pulp stem cells cultured in complete medium for 24 h. The medium was discarded and the fibroblast in the lower chamber washed 3 times with phosphate buffered saline (PBS). Total cell lysates from different groups were obtained by lysing the cells in a RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease inhibitors (100 µM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 10 µM pepstatin and 2 mM EDTA). The protein content was quantitated using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (50 µg/lane) were separated via 4–20% SDS-PAGE and transferred to nitrocellulose membranes (Pall Life Sciences, Pensacola, FL, USA). Membranes were blocked with 2% Bovine Serum Albumin (BSA) for 1 h at room temperature and probed overnight at 4 °C with primary antibodies against CTGF (ab6992), α-SMA (ab7817), TGF-β1 (ab64715), TGF-β2 (ab66045) and collagen I (ab292), which were products of Abcam (Cambridge, UK), in a humidified chamber. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) (cat. nos. AS014 and AS003; ABclonal Biotech Co., Ltd., Woburn, MA, USA) at room temperature for 2 h, and were visualized using an enhanced chemiluminescence system (EMD Millipore, Billerica, MA, USA). Fluorescent secondary antibodies were then added and immunoblots were thereafter imaged with a two-channel (at 700 plus 800 nm) IR fluorescent Odyssey CLx imaging system (LI-COR®, Lincoln, NE, USA). Results were quantified using image J software.

2.10. Real-Time Quantitative PCR (RT-qPCR)

Normal and keloid fibroblasts were seeded in six-well plates at a density of 5×10^5 cells/well and transwell chambers were seeded with dental pulp stem cells at a concentration of 5×10^5 and cultured in complete medium for 24 h. The medium was discarded and the fibroblast in the lower chamber was washed 3 times with phosphate buffered saline (PBS). Thereafter, cells were harvested using a cell scraper after the addition of a lysis buffer. RNA was consequently extracted through the following steps: lysing cells in Trizol reagent (Cat No. 15596-026, Life Technologies, Carlsbad, CA, USA), followed by extracting RNA in trichloromethane, and then precipitating it in isopropanol, and finally resuspending it in RNase-free water. The RNA concentration and purity levels were determined using a Nanodrop2000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Total RNA (2.5 µg) was subjected to cDNA synthesis using a qScript cDNA SuperMix (Quanta BioSciences, Beverly, MA, USA) through the following consequent cycles: firstly at 25 °C for 5 min, followed by 42 °C for 30 min and finally at 85 °C for 5 min. A real-time PCR was performed to determine the mRNA levels of TGF-β1, fibrinogen, α-SMA and GAPDH using SYBR Green Master MIX (ABI, Vernon, CA, USA). For a relative mRNA expression, the 2ΔΔcq method, in which ΔCq = each corresponding Cq value − minimum Cq value, was calculated.

2.11. Statistical Analyses

The data are shown as the mean ± standard deviation (SD) from at least three independent repeated experiments. Student *t*-test was used to analyze the differences in mean values between the two groups. Significant differences were defined as $p < 0.05$. All statistical analyses were performed using Graph Pad Prism software (Graph Pad Prism, San Diego, CA, USA; RRID:SCR_002798) version 7.0a.

3. Results

3.1. Clinical and Pathological Characteristics

The samples were classified into two groups (keloid and normal skin). Compared with normal skin tissue, the keloid is harder in texture, possessing either a round or oval structure, and involving excessive growth beyond the boundary of the originally wounded skin, thereby invading neighboring normal tissues. See Figure 2.

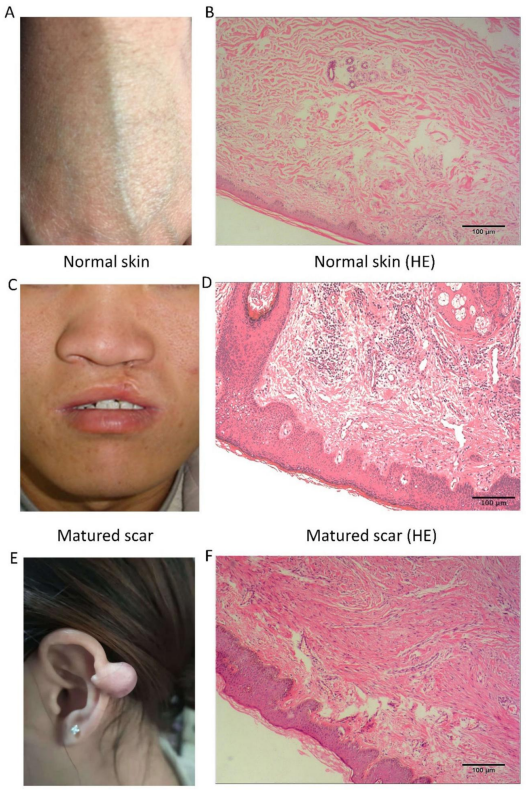

Figure 2. Gross and hematoxylin and eosin (H&E) staining image of normal skin, matured scar and keloid. (**A**) Normal skin. (**B**) H&E staining of normal skin. (**C**) Matured scar. (**D**) H&E staining of matured scar. (**E**) Keloid. (**F**) H&E staining of keloid.

3.2. Identification of Stem Cells

To confirm that these HDPSCs were of mesenchymal origin, cell surface markers were detected using flow cytometry to determine the mesenchymal origin of the HDPSCs. The following markers were positively expressed at the respective percentages: CD29 at 99.30%, CD73 at 99.60%, CD105 at 99.50% and CD90 at 99.60%, whereas the hemopoietic stem cell marker, CD19 at 0.43%, CD44 at 0.17%, CD45 at 0.74%, HLA-DR at 0.14%, CD34 at 0.11% and CD11b at 0.19%. See Figure 3.

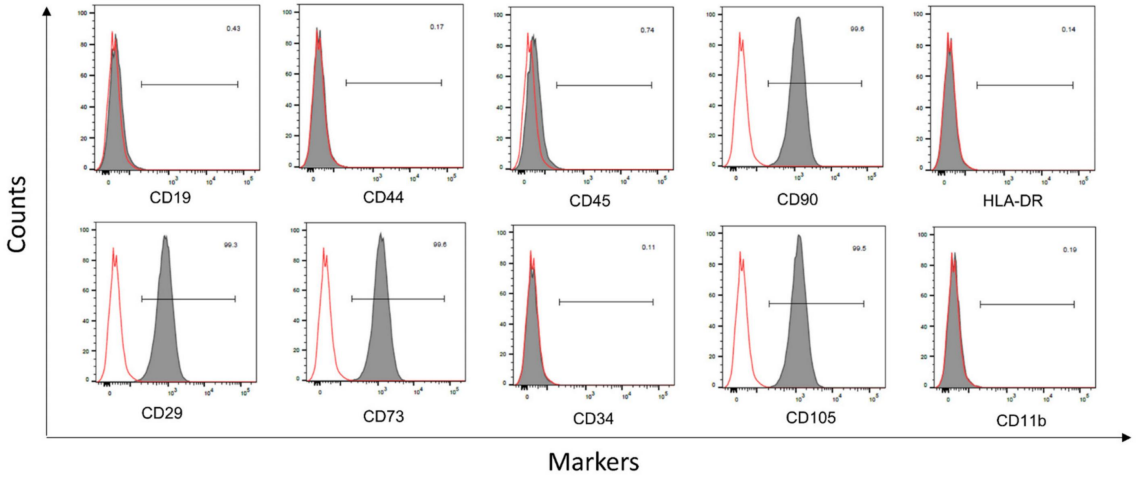

Figure 3. Flow cytometric results of passage 3 HDPSCs. Representative histograms showing antigen expression in bone-marrow-derived MSC. From left to right CD19, CD44, CD45, CD90, HLA-DR, CD29, CD73 CD105, CD73, CD34, CD105 and CD11b. Black-filled histogram: antigen expression; solid red line: auto-fluorescence control.

3.3. HDPSCs Did Not Inhibit the Proliferation of HNFs and HKFs

Compared to the HKFs cultured alone, the CCK-8 assay showed that no difference in the proliferation of HKFs was observed in the HDPSCs/HKFs co-culture group at days 1, 3, 5, and 7 ($p > 0.05$, *t*-test; $n = 7$, mean ± SD). Moreover, as a positive control, there was also no detected difference in the proliferation of HNFs in the DPSCs/HNFs co-culture group at days 1, 3, 5, and 7 ($p > 0.05$, *t*-test; $n = 7$, mean ± SD). However, the proliferation rate of HKFs was much higher than that of HNFs on the third day (Figure 4A). Compared with the control group, the morphology of the fibroblast was still elongated, and there was no variation between the two groups.

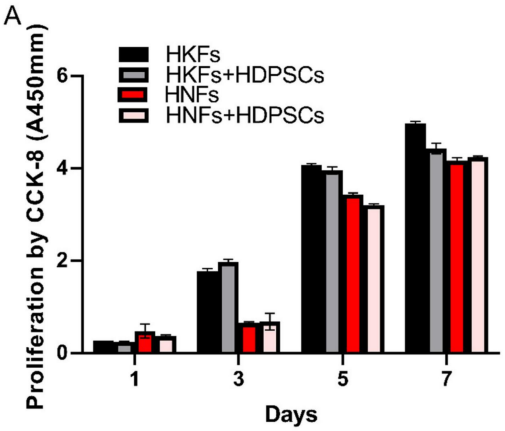

Figure 4. *Cont.*

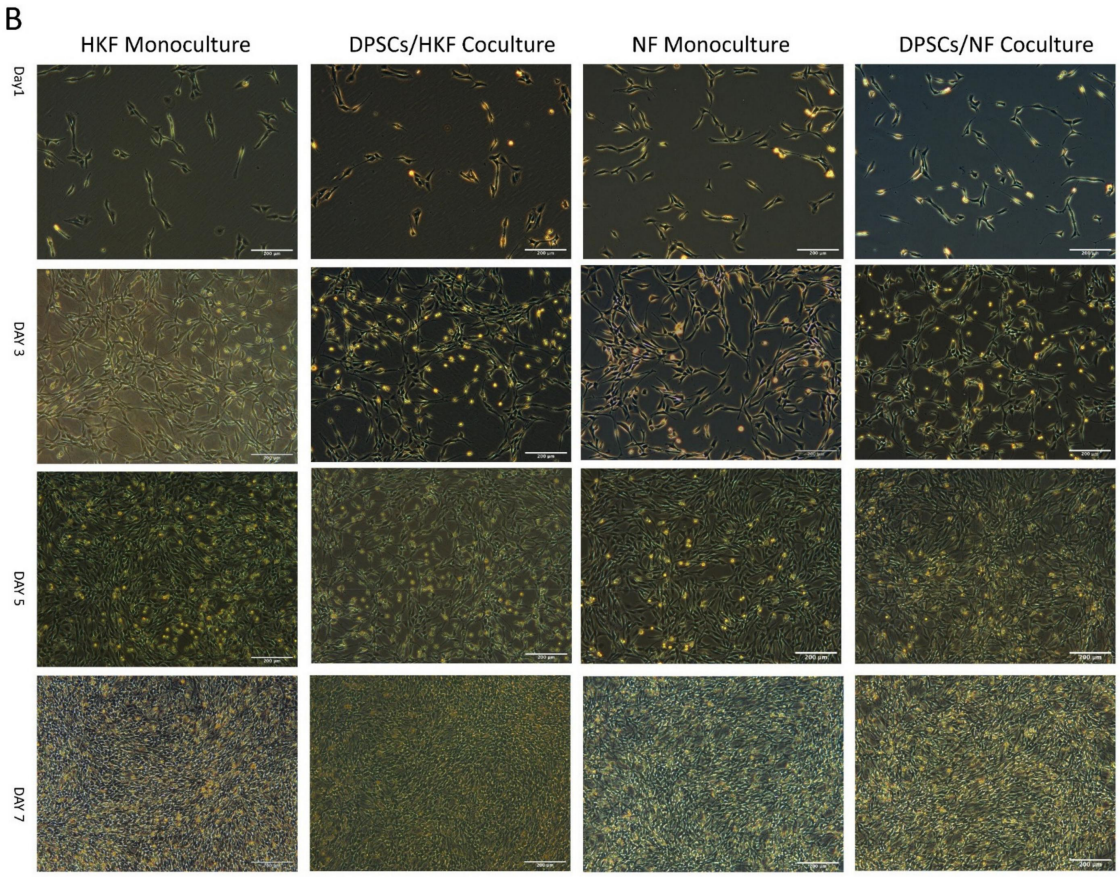

Figure 4. (**A**): The cell counts (proliferation) were analyzed using CCK-8 kits (Dojindo) on days 1, 3, 5, and 7. (**B**): The morphology of the fibroblasts was determined on days 1, 3, 5, and 7 by direct observation with a light microscope. Scale bar: 200 μM. HNFs: human normal fibroblasts, HKFs: human keloid fibroblasts, HDPSCs: dental pulp stem cells.

3.4. HDPSCs Did Not Influence the Apoptosis of HNFs and HKFs

Our results indicate that the cellular apoptosis of HNFs and HKFs was not affected by HDPSCs. As shown in Figure 5, with respect to cell apoptosis, both HKFs and HNFs were not affected through their coculture with HDPSCs ($p > 0.05$). This result further validates the cell proliferation results.

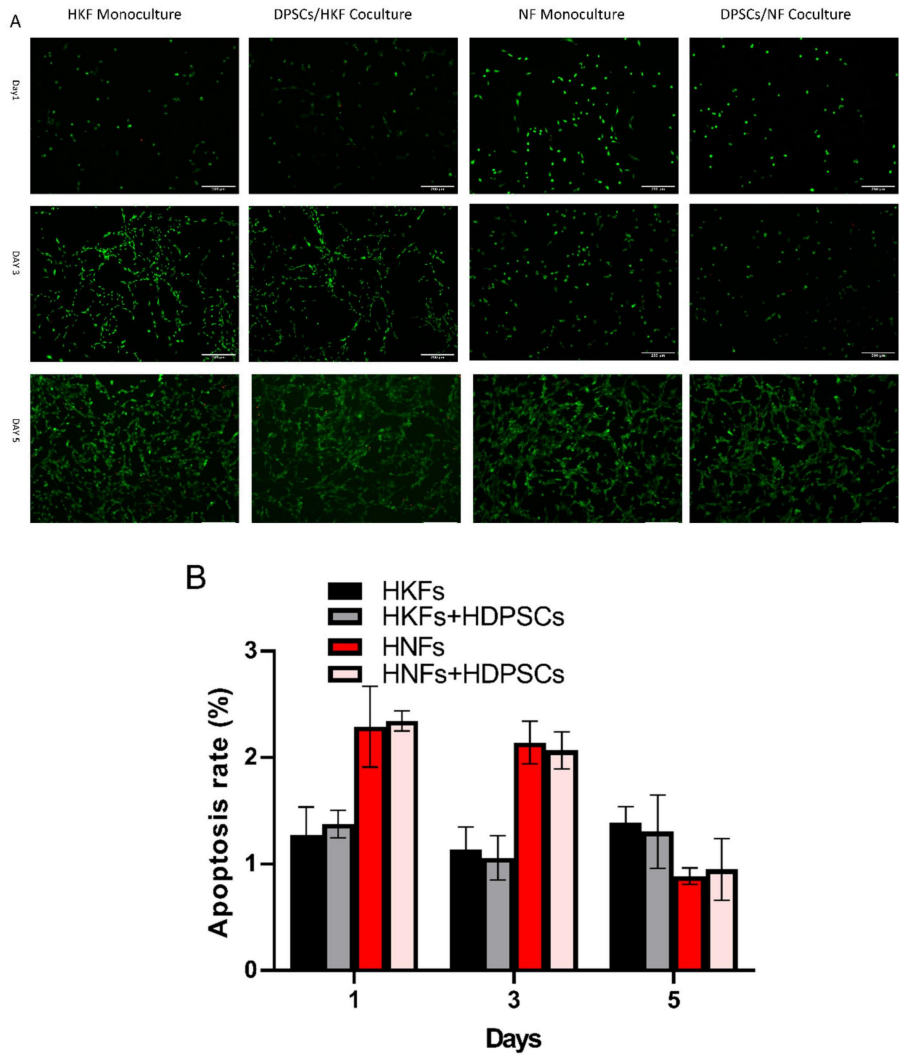

Figure 5. Apoptosis rate of HNFs and HKFs in mono and co-cultures. HDPSCs do not induce apoptosis in HNFs or HKFs. (**A**): Vital cells stain green with Calcein-AM, while dead cells stain red with propidium iodide on days 1, 3, and 5. Scale bar: 200 µm. (**B**): The quantification of cell apoptosis/necrosis using the percentage of PI-positive cells/AM-positive cells. (Mean q-SD, $n = 5$, Student t-test, $p < 0.05$).

3.5. The Effects of HDPSCs on the Migration of HKFs and HNFs

The wound scratch assays showed that there was only a difference in the cell migration ability in the co-culture group of the human keloid fibroblast after 24 h of culture ($p < 0.05$). As a positive control, cellular migration of the HNFs remained uninfluenced ($p > 0.05$). See Figure 6.

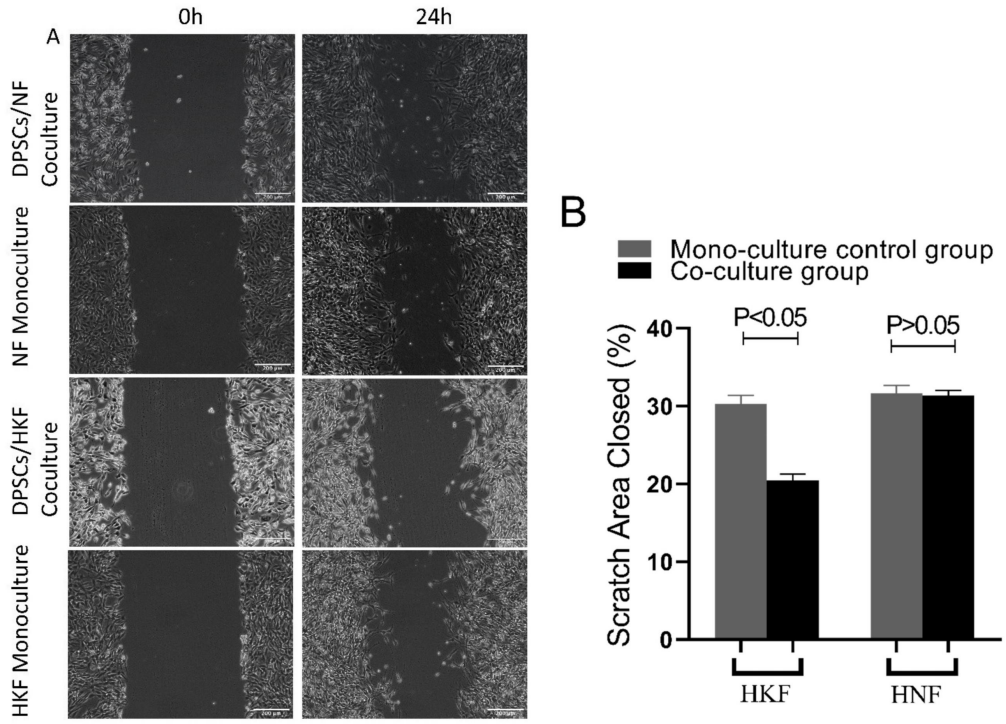

Figure 6. Cellular migration in scratch area. (**A**): Pictures were taken at 0 and 24 h, a magnification of 100× was used. (**B**): Percentage of wound area recovery by migrated cells was quantified by Image J. Significant differences were only detected in the co-culture group of the human keloid fibroblast after 24 h ($p < 0.05$, *t*-test; $n = 7$, mean ± SD).

3.6. Inhibited Expression of Fibrosis-Associated Gene Phenotype and Protein Expression in HKFs and KNFs

Compared with solely cultured cells, only the expression levels of CTGF in HNFs were observed to be unaffected ($p > 0.05$, *t*-test; $n = 7$, mean ± SD), whereas the expression levels of CTGF, TGF-β1 and TGF-β2 in HKFs and KNFs at the mRNA and protein levels were significantly inhibited when co-cultured with the HDPSCs ($p < 0.05$, *t*-test; $n = 7$, mean ± SD). These findings indicate that both the transcriptional and posttranslational levels were inhibited. See Figure 7.

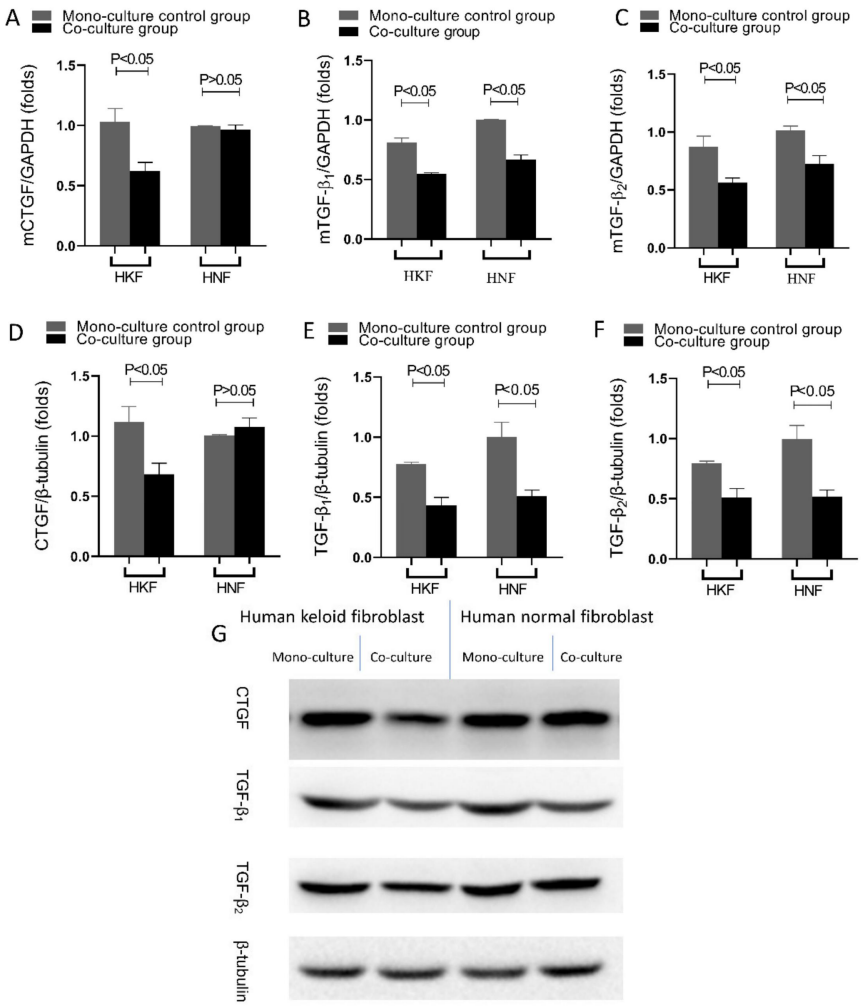

Figure 7. HDPSCs attenuated the pro-fibrotic phenotype of HKFs and HNFs. After 24 h of culture, cells were subjected to RT-qPCR and Western blot. Quantification of (**A**) CTGF, (**B**) TGF-β1 and (**C**) TGF-β2 gene expression, normalized to GAPDH expression (**D**–**F**). Quantification of (**D**) CTGF, (**E**) TGF-β1 and (**F**) TGF-β2 protein levels, normalized to β-tubulin expression. (**G**) Gels were analyzed by immunoblotting using the indicated antiserum.

3.7. HDPSCs Inhibits Extracellular Matrix Synthesis of HKFs and HNFs

Compared with solely cultured cells, the expression levels of collagen I in HNFs were observed to be unaffected ($p > 0.05$, *t*-test; $n = 7$, mean ± SD), whereas the expression levels of collagen I and α-SMA in HKFs and KNFs at the mRNA and protein levels were significantly inhibited when co-cultured with the HDPSCs ($p < 0.05$, *t*-test; $n = 7$, mean ± SD). These findings indicate that both the transcriptional and posttranslational levels were inhibited. See in Figure 8A–D,F.

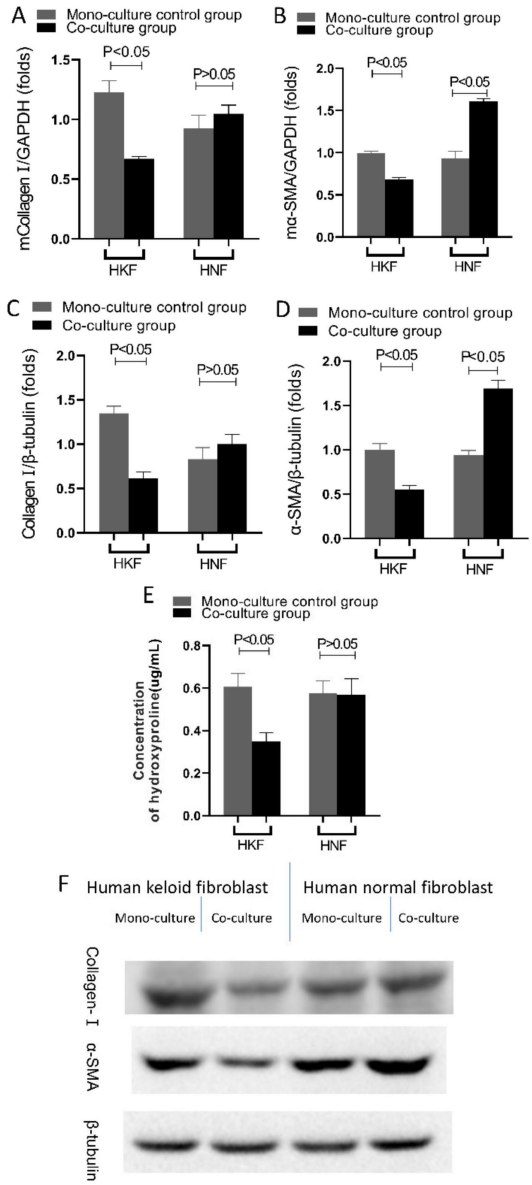

Figure 8. HDPSCs inhibits extracellular matrix synthesis of HKFs and HNFs. (**A**) After 24 h of culture and then subjected to RT-qPCR and Western blot. Quantification of (**A**) Collagen I, and (**B**) α-SMA gene expression, normalized to GAPDH expression. Quantification of (**C**) Collagen I, and (**D**) α-SMA protein levels, normalized to β-tubulin expression levels (**E**) Cell culture supernatants were collected and tested for hydroxyproline content. (**F**) Gels were analyzed by immunoblotting using the indicated antiserum.

There was a significant decrease in the resultant hydroxyproline concentrations of the HKFs co-culture group ($p < 0.05$, *t*-test; $n = 7$, mean ± SD), whereas no significant changes were detected in the other three groups ($p > 0.05$, *t*-test; $n = 7$, mean ± SD). See in Figure 8E.

4. Discussion

Wound repair is a complex process that often leads to the formation of scars following traumatic skin injuries. This process is associated with the functions of various cells, such as fibroblasts, endothelial cells, macrophages and lymphocytes [15–17], among which the biological behavior of fibroblasts is considered to be a key factor in the scar formation process. Numerous studies have shown that fibroblasts, which happen to be the main constituents of keloid tissue, have the ability to over proliferate and are accompanied by incomplete apoptosis, together with the abnormal synthesis of collagen, which overall results in the continuous proliferation of keloid tissue [18,19]. Therefore, the inhibition of fibroblast proliferation and induction of apoptosis in keloid tissues can majorly reduce keloid tissue proliferation and thereby delay disease progression, which is important for the improvement and treatment of keloid scars. Therefore, understanding the biology of keloid fibroblasts is important for the treatment of keloids.

Mesenchymal stem cells (MSCs) are an important member of the stem cell family, which have been used to treat scar formation-related diseases such as pulmonary fibrosis. Moreover, great progress has been made where they have also been used to inhibit cardiac scar formation through the secretion of various cytokines [20]. Studies have shown that stem cells have been used in animal models and in a few clinical trials for the regeneration of diseased organs. Furthermore, stem cells have been shown to improve tissue repair by secreting interleukins such as interleukin 6, interleukin 8, interleukin 10 and other proteins that are suitable for inducing tissue regeneration [21,22]. Recent studies have shown that adipose-derived stem cells are able to inhibit mRNA expression levels of COL1A1, transforming growth factor β1, connective tissue growth factor and alpha actin 2 (ACTA2) in renal fibrosis tissues, thereby playing a therapeutic role in renal fibrosis [23]. Whether or not dental pulp stem cells can play a role in the repair of skin scars by inhibiting the proliferation and migration of keloid fibroblasts through the secretion of cytokines has not been clearly reported thus far.

Dental pulp stem cells, human keloid fibroblasts and human normal skin fibroblasts were isolated using the tissue explant method. The molecular mechanisms of action of dental pulp stem cells on the proliferation, migration and apoptosis of keloid fibroblasts were investigated using a transwell co-culture. The histology of keloid scars is highlighted by the secretion and deposition of large amounts of extracellular collagen [24]. Furthermore, both skin keloids and fibrous tumors are pathologically fibrous connective tissue lesions with large extracellular collagen deposition [25]. In this study, we also found that the co-culture of HDPSCs inhibited the migration of HKFs and HNFs but did not inhibit the proliferation of either HKFs and HNFs, nor did it induce the apoptosis of HKFs and HNFs. Our experimental data are similar to the results of a recent study reporting that the conditioned medium and cell lysates of human-derived WJ-MSCs inhibit the migration of human HKFs [26]. In comparison with this report, we used HDPSCs, which have the advantages of being easily accessible with minimal patient harm, and the fact that they can be used autologously. However, studies reporting opposing results also exist [27], in which the conditioned medium of human WJ-MSCs could promote HKFs proliferation in a paracrine manner through an indirect transwell co-culture treatment system. We hypothesize that the reasons for these different results are attributed to the different types of MSCs used, the different cell treatment cultures and different assays, all of which impact the following biological behaviors of keloid fibroblasts: migration, proliferation and collagen secretion, in addition to the apoptosis involved in wound repair after skin injury. Therefore, by inhibiting the migration and proliferation of keloid fibroblasts, keloid formation is also consequently inhibited [28]. Moreover, it has been shown that the inhibition of matrix metalloproteinase expression in keloid fibroblasts can inhibit the migration ability of keloid fibroblasts and, therefore, function as a keloid treatment aid [29]. Furthermore, similar findings have been reported where inhibition of mTOR protein expression in keloid fibroblasts can also inhibit the migratory ability of keloid fibroblasts and thereby further inhibiting the development of keloids [30]. Similarly,

and consistent with the aforementioned studies, the results of the present study revealed that dental pulp stem cells are also able to inhibit the migration of keloid fibroblasts in vitro, thereby suggesting that dental pulp-derived stem cells can promote wound healing by inhibiting scar formation.

The expression of anti-fibrotic and pro-fibrotic genes is closely related to the pathogenesis of fibrotic diseases, which in another way confirms that hyperplastic scars and keloids are classified as fibrotic diseases. TGF-β1 and TGF-β2 overexpression is an important cause for excessive scar proliferation and fibrosis, and studies have shown that the targeted reduction in TGF-β1 and TGF-β2 expression in hyperplastic scars and keloids can inhibit scar proliferation and achieve clinical therapeutic effects [29,30]. Therefore, TGF-β1 and TGF-β2 have also become the targets of numerous studies that tackle the treatment of hyperplastic scars and keloids. Moreover, CTGF is a marker protein of fibrotic diseases in which it promotes both the proliferation of fibroblasts as well as the secretion and deposition of extracellular matrix proteins, such as collagen I and fibronectin [31,32]. In the current study, it was found that after 24 h of HDPSC co-culture with HKFs, both the gene and protein expression of TGF-β1, TGF-β2 and CTGF were significantly reduced in HKFs. This is in accordance with studies that have shown MSCs as being capable of secreting cytokines in order to alter some biological phenotypes of fibroblasts, such as fibrotic and proliferative phenotypes, through paracrine functions [33,34]. Increasing evidence thereby suggests that the paracrine function of MSCs is an important potential mechanism for their cellular therapeutic function. Thereupon, after being injected into the body, MSCs can, on the one hand, inhabit the area of tissue damage through the processes of chemotaxis, proliferation and, eventually, differentiation by evolving into the cell type required for the secretion of extracellular matrix that would be needed to repair the damage in the recipient area; on the other hand, MSCs, upon entering the body, can exhibit a paracrine function, which entails the secretion of cytokines and nutrient-active substances required for repairing the damage, and inducing the body's self-generated cells to repair the tissue damage. The SMAD signaling pathway is a downstream mediator of TGF-β. After phosphorylation, the phosphorylation of R-SMAD 3 is upregulated in the keloid, whereas the downregulation of R-SMAD 3 significantly reduces procollagen gene expression in keloid fibroblasts. I-SMAD 6 and I-SMAD 7 inhibit the action of TGF-β. SMAD 6 also inhibits the binding of SMAD 4 and R-SMAD. The expression of I-SMAD 6 and 7 is reduced in keloid fibroblasts. By inhibiting the TGF-β1-SMAD signaling pathway and activating TLR7 or SMAD 7, keloid formation can be significantly reduced. The toll-like receptor signaling pathway plays a protective as well as a destructive role. After skin injury, toll-like receptors (TLR) are combined as damage-related molecular patterns (DAMP) to enable the innate immune system to respond to sterile tissue damage. As the concentrations of several pro-inflammatory and pro-fibrotic cytokines in macrophages increase in response to TLR stimulation in macrophages, fibroblast gene expression and TGF-β responses change, leading to an increased collagen production.

A very distinctive feature of proliferative scars and keloid tissues is the excessive deposition of the extracellular matrix [35]. There are two main mechanisms that can lead to an excessive extracellular matrix deposition, one being an increase in the extracellular matrix synthesis, and the other being a decrease in the extracellular matrix degradation. Compared to normal skin, the amount of collagen synthesis in hyperplastic keloid scars is three times higher, while keloid scars can reach up to 20 times higher [4]. In our experiments, it was further observed that due to the co-culture with DPSCs, both HSFs and HKFs displayed a reduction in extracellular matrix synthesis, as shown by the reduced expression of collagen type I, α-SMA and hydroxyproline. Moreover, in the extracellular matrix of scar tissue, collagen type I is normally the predominantly present collagen type. Furthermore, the detection of hydroxyproline content in cell culture media is recognized as a reliable indicator of the ability of fibroblasts to synthesize collagen. Relevantly, it was previously reported that both bone marrow MSCs and dermal MSCs were able to inhibit collagen synthesis and the expression of α-SMA in keloid fibroblasts [27].

Finally, our findings are consistent with previous studies by showing that dental pulp stem cells were able to inhibit the expression of pro-fibrotic genes and keloid fibroblast collagen synthesis in vitro. Furthermore, this study revealed that dental pulp stem cells inhibit the migration of keloid fibroblasts. Nevertheless, the specifically secreted cytokines that influence the biological behavior of keloid fibroblasts need to be further investigated by subsequent experiments.

5. Conclusions

The co-culture of HDPSCs inhibits the migration of HKFs and the expression of pro-fibrotic genes, while promoting the expression of anti-fibrotic genes. HDPSCs' co-culture also inhibits the synthesis of the extracellular matrix by HKFs, whereas it does not affect the proliferation and apoptosis of HKFs. Therefore, it can be concluded that HDPSCs can be used as a tool for restraining/hindering the initiation or progression of fibrotic tissue.

Author Contributions: M.Y.: conceived the study, supervised the experiments and drafted the manuscript; O.A.N.: conceived the study, supervised the experiments and drafted the manuscript; L.-L.F.: data evaluation and manuscript preparation; L.-M.C.: data evaluation and manuscript preparation; M.G.: analyzed the data and revised the manuscript; R.E.F.: analyzed the data and revised the manuscript; R.S.: performed the data collection; H.-C.F.: conceived the study, designed the data evaluation and prepared the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Administrative permissions were acquired by our team to access the data used in our research. The Ethics approval Research Ethics Committee number: GYSKLL-KY-20161223-01. Accordingly, all teeth were coded with number and all personal identification of the patients were removed.

Informed Consent Statement: All parent or guardian of participants provided written informed consent for using their teeth that otherwise would have been discarded as waste.

Data Availability Statement: The data are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare that they have no conflicting interests.

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9. Discussion

9.1 5% DMSO offers better protection and lower toxicity for hDPSCs cryopreservation

(This part is about the publication doi: 10.21873/invivo.12049. See page 19 of this dissertation).

Stem cells are undifferentiated cells with significant self-renewal abilities and multi-differentiation potential. Stem cells exist in embryonic as well as adult tissues. They can be categorized into embryonic and adult stem cells. Human dental pulp stem cells (hDPSCs) are adult stem cells originating from dental pulp tissue. As a result, they demonstrate the potential for self-renewal and multi-differentiation. Their differentiation into osteoblasts, odontoblasts, adipocytes, muscle cells, and neurons is influenced by microenvironment modulation. hDPSCs play a vital role in tooth regeneration, nerve repair, and bone tissue engineering. The dental pulp is an accessible and plentiful source of stem cells for tissue regeneration, suitable for translational medicine.

Stem cells isolated from exfoliated deciduous or routinely extracted permanent teeth can be produced in large quantities for diverse applications[113]. Consequently, the critical challenge is to establish an effective and trustworthy tissue inventory system to preserve this effortlessly accessible and extremely precious dental pulp tissue. Research has demonstrated that cryopreservation is an efficacious technique for biological tissue samples to be preserved for an extended period of time[114]. Isolating stem cells with biological activities rather than genes from frozen tissues requires more than simple preservation in liquid nitrogen. It is essential to develop and refine methods for tissue cryopreservation to guarantee the successful cultivation and utilization of stem cells in the future[115].

Dimethyl sulfoxide (DMSO), as the principal agent in cell cryopreservation, serves as a crucial osmotic cell protectant. Cryopreservation at extremely low temperatures (-198°C) can avert damage from ice crystal formation in intracellular fluid, fluctuations in osmotic pressure, and disruption of cellular structure. However, although DMSO is the most effective cryopreservation protectant for cells, it is also a harmful chemical agent, particularly evident during cell recovery and culture[116]. Research indicates that a residual content of 10% DMSO in the culture media results in nearly 100% inhibition of cellular proliferation. At a concentration of 0.1%, the inhibition rate is 35%.DMSO negatively impacts cell growth even at a concentration of 0.004%[117]. Furthermore, DMSO's considerable skin penetration and volatility, which are indicative of its status as a "universal solvent," may pose health risks to the operator. Therefore, reducing the dosage and frequency of DMSO during cell freezing and resuscitation may mitigate the risks associated with transplantation procedures and enhance

operational safety. The traditional concentration of DMSO was decreased from 10% to 5% aiming to mitigate direct cytotoxicity resulting from the repetitive freezing and thawing of cells and the DMSO itself. The dosage of DMSO could be meticulously regulated from the sample collecting phase, enhancing the frozen storage method.

To objectively assess the efficacy of the cryopreservation system utilizing 5% DMSO on hDPSCs, the experiment established a positive control group (lacking cryopreservation solution) and a negative control group (cryopreservation system containing 10% DMSO). Assessments were made regarding cell morphology, stem cell-specific markers, capacity of cell self-renewal, and the potential for multi-directional differentiation. It was demonstrated that in the experiment assessing the duration required for primary hDPSCs to migrate from dental pulp explants, the migration time for cells in both cryopreserved groups was significantly prolonged compared to the positive control group. However, the data distinctly illustrated that the migration time in the group comprised of 5% DMSO was much lower compared to the group comprised of 10% DMSO ($P<0.05$). This result was likewise observed in the primary cell yield assay. The experimental group's statistics remained in a median position when compared to the other two groups. The reason for such a result is attributable to the use of primary hDPSCs as the research subject and aimed at examining the effect of thawing on the initial stages of in vitro cell isolation. It has been proven that freezing influenced the initial stage of cell isolation and culture [49], resulting in an extended adaptation period and reduced cell yield. Furthermore, the proportion of DMSO added significantly influenced the previously mentioned two features. In a later series of experiments, the third passage of cells was utilized, as these cells may be naturally purified during passage, making it the most appropriate for clinical application. Therefore, in vitro cell culture can not only guarantee cell quantity but also serve as a reliable representation, provided that the biological activity remains unaffected by the cryopreservation of tissue from donors. It can establish an academic basis for future transplantation in clinical therapies and enhance treatment safety. The study found no significant differences ($P>0.05$) in various biological characteristics between hDPSCs preserved by two cryopreservation methods and freshly extracted hDPSCs. This aligns with prior study findings, indicating that cryopreservation does not alter the fundamental biological properties and functionality of stem cells[118]. In conclusion, cryopreservation influenced the isolation and cell yield of primary hDPSCs. However, suitably decreasing the DMSO addition ratio can mitigate this impact. The biological activity of hDPSCs is not compromised by cryopreservation post-passage, and they can be safely utilized in subsequent transplantation procedures. In terms of

overall cell yield and the duration needed for in vitro culture, the 5% DMSO cryopreservation system surpassed the 10% DMSO system and represented one of the more optimal options for hDPSCs preservation. Finally, it is advisable to select a 5% DMSO rather than 10% to mitigate its direct cytotoxic effects on cells during the passage process, hence minimizing cell loss.

9.2 Direct cryopreservation of pulp tissue is a viable method for hDPSCs preservation

(This part is about the publication doi: 10.5507/bp.2020.061. See page 28 of this dissertation).
In tissue engineering, the selection of the most suitable seed cells is essential. hDPSCs are extensively studied stem cells that meet the strict requirements for an ideal source of seed cells in tissue engineering and cell therapy[119, 120]. In hDPSCs culture, cryopreservation in liquid nitrogen is a conventional storage technique[121, 122]. Furthermore, advancements in biotechnology are continually enhancing this method[123, 124]. hDPSCs retain viability and differentiation potential after two years of cryopreservation [125]. Perry et al.demonstrated that intact dental pulp tissue cryopreserved at 4 °C was successfully cultured for a duration of up to five days[126]. Our study established the viability of cryopreserving fragments of human dental pulp tissue and the long-term preservation of hDPSCs isolated from these fragments using liquid nitrogen (-196°C) cryopreservation. The proliferation and differentiation potential of cells cultured from cryopreserved tissue fragments were comparable to those derived from fresh tissue fragments. The cryopreserved cells from explants exhibited a two-day growth delay compared to the other group. The result is consistent with previous studies, indicating that following extended cryopreservation, native hDPSCs necessitate an adaptation period for growth[127].
Currently, there is no data on the total cell yield, as the cultures were still in the 3-5 passage stage. It is anticipated that certain cells will sustain damage during cryopreservation, resulting in a total yield that is inferior to that of fresh tissue. A plausible rationale for our achievement in acquiring viable cells from cryopreserved dental pulp fragments is the loose packaging structure of hDPSCs, which facilitates the complete penetration of the cryoprotectant DMSO, similar to that observed in a single-cell suspension. A further potential explanation pertained to the limited sample volume (<0.5cm^3). As mentioned above, it is recommended to minimize the volume of dental pulp fragments to the greatest extent possible. This strategy may not be applicable to other tissues. Our study aims to isolate single cells for culture purposes. Consequently, maintaining the initial structure and cellular arrangement of the tissue is not essential. The delayed proliferation of cells produced from cryopreserved explants could be

attributed to changes in the structure and organization of cells throughout the cryopreservation process. Despite a minor delay in cell growth, viable cells derived from cryopreserved tissue fragments exhibited characteristics comparable to those found in fresh tissue fragments.

We did identify congruent characteristics regarding proliferation, adipogenic, and osteogenic differentiation in matched fresh and frozen tissue fragments. Importantly, fibroblasts as negative controls exhibited a complete absence of differentiation under identical conditions. This provides compelling evidence that the observed differentiation was exclusive to human hDPSCs. Our findings aligned with existing literature indicating that cryopreservation does not alter biological features, structure, or mechanical and biological properties[118].

The main aim of this work was to evaluate the viability of cryopreserving dental pulp fragments for the subsequent hDPSCs culture and the dental pulp tissue was, therefore, sectioned into pieces on a sterile culturing bench in the laboratory to avert contamination. We are currently investigating the viability of preparing dental pulp tissue in a conventional clinical environment devoid of a laboratory sterile bench. Dental pulp tissue affected by severe infections can be preserved during tooth extractions at dental or oral clinics. The sole requisite apparatus is a household -20°C refrigerator, which is used to temporarily store tissue fragments, followed by their transfer to a -80°C refrigerator at an opportune moment, and ultimately their placement in liquid nitrogen for long-term preservation.

9.3 Direct cryopreservation of treated teeth is feasible for preserving hDPSCs

(This part is about the publication doi: 10.3390/ijms231911485. See page 36 of this dissertation).

This study introduces a novel cryopreservation technique using varying DMSO concentrations to preserve third molars, which could provide hDPSCs for donors. To assess the efficacy of the newly developed cryopreservation method, we extracted 18 third molars and isolated cell lineages, thereafter analyzing the disparities between frozen and unfrozen teeth. The study found that cell growth duration was significantly longer in the T1 and T2 DMSO frozen groups than in fresh dental pulp tissue, indicating that higher DMSO concentrations extend the growth period of primary cells. The T1 and T2 frozen groups produced significantly fewer primary cells than group C. Nonetheless, cryopreserving hDPSCs at −80 °C for one month post-first passage did not affect cell morphology, including size, cytoplasmic density, and nuclear features. The results from colony formation efficiency, cell viability, and MTS assays indicated that the novel cryopreservation method preserved hDPSC activity. We evaluated the surface antigens of hDPSCs from teeth preserved using a

novel cryopreservation technique, following ISCT protocols. It was found that CD45 and CD34 expressions were negative across all three groups, whereas CD73, CD90, and CD105 expressions were positive, consistent with prior research[128]. The surface antigens of these cells biologically correspond to those of mesenchymal stem cells (MSCs), indicating that the cryopreservation procedure does not adversely affect the biological characteristics of hDPSCs. After 21 days of induced differentiation, the frozen group of hDPSCs retained their ability to differentiate into adipose and osteogenic tissues, as indicated by calcium and lipid accumulation. Moreover, the mRNAs linked to osteogenesis, specifically ALP, RUNX2, COL I, and OSC, were not downregulated at the observed time points (7, 14, and 21 days). Three groups did not exhibit any discernible variation in their ALP detection results. The differentiation potential of hDPSCs preserved in DMSO was retained post-passage, as indicated by changes in osteogenic gene expression levels over the course of osteogenic induction.

This study demonstrates that the novel cryopreservation method diminished the yield of primary hDPSCs and prolonged their initial emergence period. This may be because the cryopreservation method may diminish the interaction between dental pulp tissue and cryoprotectants that preserve tooth integrity, leading to an inadequate quantity of safeguarded cells. DMSO, a commonly utilized cryoprotectant, may present slight hazards at concentrations below 10%, despite its capacity to inhibit the production of ice crystals in cells and preserve their structural and functional integrity post-thawing[129, 130]. Conversely, the freezing process did not notably affect the proliferation and differentiation abilities of passaged hDPSCs. This may be because the one-month freezing period may not have been sufficiently prolonged, resulting in minimal cell damage. Furthermore, the dental tissue of the donor chosen for the present study exhibited considerable cellular resilience, enabling the cells to endure physical and chemical stressors.

Alveolar bone loss due to dental implants, tumor surgery, trauma, and periodontitis has significantly increased the demand for bone regeneration treatments in individuals over 40 years old[131]. Consequently, hDPSCs are regarded as a valuable biological resource for regenerative medicine and tissue engineering[132]. Age-related declines in MSC number and capacity for self-renewal have been documented in studies[133]. As MSCs age, their proliferation and differentiation capabilities diminish as a result of telomere shortening[134], DNA damage, and epigenetic alterations in transcriptional regulation[134, 135]. The increased formation of secondary dentin and root canal mineralization in older adults leads to significant dental pulp tissue atrophy, making the extraction of hDPSCs challenging[136]. Therefore, the

preservation of a patient's younger hDPSCs in advance is crucial for maintaining their stem cell functionalities, including proliferation and differentiation potential. Our preservation technique allows for the freezing and storage of a large number of hDPSCs, maintaining their optimal stem cell properties and maximum proliferation capacity.

Our laboratory previously demonstrated that dental pulp tissues can be cryopreserved for a month in 5% and 10% dimethyl sulfoxide (DMSO) media at −196 °C. Additionally, human dental pulp stem cells (hDPSCs) exhibited similar proliferation, growth, and differentiation capacities in both frozen and unfrozen groups[137]. This discovery provides important insights into the extensive and enduring preservation of dental pulp tissues. Nevertheless, the absence of sterile conditions in surgical areas of clinics and hospitals continues to pose a risk of tissue contamination due to environmental constraints. Furthermore, the majority of dental clinics do not have the capability to cryopreserve tissues and cells at −196 °C using liquid nitrogen. Our method corroborates earlier findings that hDPSCs preserved in 10% DMSO at −80 °C for 1-5 years maintain significant capability[138, 139]. This aligns partially with research indicating that hDPSCs maintain functionality after being stored at −85 °C for six months [140]. Ginani F et al. also discovered that human deciduous teeth cells could maintain similar cell viability and proliferation rates for up to six months after six months of storage at −80 °C in 10% DMSO[141], suggesting that the whole-tooth cryopreservation method might still be applicable for the preservation of deciduous teeth.

The multipotent differentiation capabilities of stem cells are essential for their therapeutic use. Dental mesenchymal stem cells (DMSCs) are ideal for cell engineering and tissue regeneration due to their ability to differentiate into endodermal, mesodermal, and ectodermal lineages. Yanasse et al. The combination of human hDPSCs and platelet-rich plasma (PRP) formed a stem cell scaffold that notably enhanced articular cartilage repair in a rabbit model [142]. Wang et al. In a mouse model of amyotrophic lateral sclerosis (ALS), injecting either the secretion proteome or the carrier (DMEM) from cultured human dental pulp stem cells (hDPSCs) significantly extended survival and overall lifespan following disease onset[143]. Li et al.and Carter et al.HGF-modified hDPSCs were cultured and intravenously transplanted into a rat model of ulcerative colitis, demonstrating their ability to inhibit inflammation and promote proliferation by differentiating into ISC-like cells, thus reducing oxidative stress damage[144]. Hata et al. Injected cultured hDPSCs into nude mice with diabetic polyneuropathy led to notable enhancements in nerve conduction velocity, blood flow, and sensory perception thresholds[145]. In addition, hDPSCs offer significant advantages in terms of accessibility compared to other stem cell sources. Third molars are the primary source for

stem cell cryopreservation among various teeth. Carter et al. report that third molar impaction occurs in 18–68% of cases, with 41% identified as mesioangular impactions, leading dentists to frequently advise prophylactic extraction[146]. This study focuses on impacted third molars that are not fully erupted, and whose extraction does not affect oral masticatory function. Impacted third molars can cause gum swelling, ulcers, damage, and decay of the second molars, as well as periodontal and osseous diseases around them. They are linked to pathological changes, including pericoronitis, root resorption, periodontitis, caries, and cyst or tumor development[147]. Retaining impacted wisdom teeth over time can elevate the risk of pathologies in adjacent structures, and their removal at an older age may result in more frequent and severe complications[148]. Consequently, the preventive extraction of asymptomatic, healthy wisdom teeth, regardless of their impaction status, has traditionally been deemed a suitable treatment strategy[149].

Premolars, particularly those extracted during orthodontic procedures for severe crowding and Class II malocclusions, are the second most common source of human dental pulp stem cells (hDPSCs) after impacted third molars. The typical age of these patients ranges from 10 to 16 years[150]. Multiple teeth are the third most common source of hDPSCs[151]. Biologically, any healthy tooth, including the specified categories, can provide hDPSCs. Premolars extracted during orthodontic treatment are a frequent source of dental pulp cells[151]. When teeth are well-preserved after extraction, dental pulp cells can serve as a valuable and versatile source of stem cells for organ reconstruction and tissue regeneration, rather than being considered biological waste. In conventional cryopreservation, extracting dental pulp is crucial, as improper methods can contaminate dental tissues. Furthermore, as preservation time increases, the number of dental pulp stem cells isolated from extracted teeth decreases[152]. Research shows that dental pulp stem cells can stay viable in vitro for up to 12 hours or overnight[124], representing the minimum exposure duration in a sterile surgical environment. Our study demonstrates that cryopreserving entire teeth at −80°C is a viable method that improves the retrieval of viable dental pulp stem cells, reduces contamination risk during post-extraction processes, and maintains the cells' original phenotype. Moreover, this approach allows us to reduce the requirements and costs associated with hDPSCs preservation, creating better conditions for establishing a dental stem cell bank and future clinical applications.

However, due to time constraints and limited samples, further research into the maximum duration of complete tooth cryopreservation remains challenging. Resolving this issue would enhance the flexibility of whole-tooth cryopreservation programs, allowing their

implementation at the optimal time for cellular function in therapeutic applications. Future research will aim to enhance the understanding of hDPSC preservation.

9.4 Comparison of the neurogenic differentiation performance of hDPSCs at different passages

(This part is about the publication doi: 10.5507/bp.2021.058. See page 55 of this dissertation).

The central and peripheral nervous systems regulate all bodily activities and perform essential physiological functions. Although central nervous system damage is permanent, peripheral nerves have limited regenerative abilities. Consequently, severe nerve damage can lead to irreversible functional impairments. Stem cells possess the ability for self-renewal and differentiation into multiple lineages. In recent years, various stem cells, including hDPSCs, have been proven to possess potent differentiation capabilities and beneficial paracrine effects, making them promising therapeutic agents for treating neuronal diseases and nerve injuries.

Moreover, hDPSCs express both traditional neuronal markers, including nestin, GFAP, and NSE, and additional markers such as NeuN and S-100. They also release neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and other nerve growth factors (NGFs), which are essential for nerve repair processes[25].

BDNF and GDNF directly act on dopaminergic neurons, providing robust trophic support that facilitates the recovery of these neurons following injury. Neurotrophic factors promote cell growth and neuronal differentiation by acting autocrine and paracrine on their own cells and the surrounding microenvironment.

Research indicates that the biological properties of mesenchymal stem cells remain stable up to 10 passages. According to studies by Jin Hua et al., hDPSCs marked with Stro1+ obtained through immunomagnetic bead sorting show higher expression levels at passage 9 (P9) compared to passage 1 (P1). This suggests that P1 hDPSCs possess the potential for multipotent differentiation toward dentin, bone, and cartilage formation, while P9 hDPSCs, representing a later stage, show a greater propensity for osteogenic differentiation. These data imply that extended in vitro culturing can specifically influence the differentiation potential of hDPSCs.

ELISA results indicate that hDPSCs secrete the highest level of VEGF at passage 3 (P3), measuring 7300 micrograms per liter. VEGF is a key regulator of both physiological and pathological angiogenesis and vasculogenesis, significantly influencing endothelial cell proliferation, survival, migration, and infiltration. hDPSCs enhance vascular regeneration via

VEGF expression, which in turn supports axonal growth and Schwann cell proliferation. VEGF-A plays a crucial role in angiogenesis. In a study involving the transplantation of differentiated hDPSCs into a rat model with a 15 mm sciatic nerve defect, VEGF-A was observed to enhance angiogenesis and facilitate the initial inward growth of nerve fibers[153].

Regarding the neurogenic paracrine effects, ELISA results indicated that the BDNF levels secreted by third-passage hDPSC cells were 2421 μg/L.BDNF is extensively present in the central nervous system (CNS) and is vital for CNS development, impacting neuron survival, differentiation, growth, and development. It also prevents neuronal death and aids in reversing pathological neuronal states. BDNF, a neurotrophic factor, is crucial for neuron regeneration, differentiation, survival, and normal physiological function.

Variations in BDNF expression are strongly linked to neurological disorders such as depression, epilepsy, Alzheimer's, Parkinson's, and Huntington's diseases[154].

ELISA analysis revealed that third-passage hDPSCs secreted NT-3 at a peak concentration of 7571 μg/L.NT-3 significantly increases the number and dendritic length of βIII-tubulin+ retinal cells. It was also observed that the activity of retinal cells decreases when an NT-3 receptor inhibitor is used, demonstrating NT-3's protective effects on retinal ganglion cells (RGCs) [Reference 10]. RGCs express various neurotrophic factors such as NGF, BDNF, NT-3, and GDNF. These factors can bind to receptors on RGCs, enhancing not only their survival but also promoting axonal regeneration[155].

hDPSCs can also be used for treating retinal damage due to their paracrine effects, which include the expression of the aforementioned neurotrophic factors. ELISA results further indicated that the secretion of NGF by third-passage hDPSCs was the highest, reaching 4814 micrograms per liter. NGF, an early-identified neurotrophin, supports sensory neuron survival, nerve fiber growth, and sensory neuron peptide gene expression. In the retina, retinal ganglion cells, bipolar neurons, and glial cells locally produce and utilize NGF via paracrine or autocrine mechanisms. Moreover, Colafrancesco et al. [156]found that NGF can protect the retina by reducing the apoptosis of ganglion cells.

NGF and its receptors are prominently expressed in the visual centers during visual system development, affecting neuronal proliferation, survival, and selective apoptosis. Furthermore, NGF maintains neuronal survival and differentiation by activating signaling pathways through its binding with the tropomyosin receptor kinase A (TrkA) receptor. In the form-deprivation myopia (FDM) model in guinea pigs, Davis et al.[157]employed immunohistochemistry and quantitative PCR to assess NGF and TrkA protein and gene expression levels. The study found that extended form deprivation led to a reduction in NGF and TrkA protein and nucleic

acid expression in the retina.

Information regulation within retinal cells facilitates differentiation during the developmental stages of retinal function. Additionally, Spalding et al.[158]demonstrated that NGF and TrkA are present in both neuronal and non-neuronal cells within the guinea pig retina in the FDM model. The experiments analyzed cytokine secretion levels from the second to the tenth passage of hDPSCs. Further investigation is needed to clarify cytokine secretion levels in subsequent passages and to explore the clinical applications, indications, and contraindications of hDPSCs. Moreover, the exact mechanisms of hDPSCs' paracrine functions, factors influencing these functions, and how to effectively utilize this potential capability of stem cells require further investigation.

9.5 hDPSCs from neurofibroma-affected and normal teeth have similar biological properties

(This part is about the publication doi: 10.21873/invivo.13113. See page 63 of this dissertation).

Innovative treatment techniques are urgently needed in oral and maxillofacial medicine to restore the function and structure of damaged tissues. Stem cell research and regenerative medicine have recently experienced significant advancements and groundbreaking discoveries[159]. Recent reports highlight dental tissue-derived stem cells as a promising source for bone regeneration in oral and maxillofacial surgery, craniofacial abnormalities, and orthopedics. Furthermore, stem cells can be collected intraorally, including from tooth pulp and periodontal ligaments. Prior studies have demonstrated that dental stem cells exhibit advantages in myogenic, odontogenic/osteogenic, and chondrogenic differentiation [160, 161]. Furthermore, stem cells can be collected intraorally, including from tooth pulp and periodontal ligaments. Our previous studies have demonstrated that dental stem cells exhibit advantages in myogenic, odontogenic/osteogenic, and chondrogenic differentiation[162, 163].

Oral cavity neurofibromas frequently involve the trigeminal and upper cervical nerves[164]. Neurofibroma can occur in the oral cavity, affecting areas such as the tongue, lip, palate, gingiva, major salivary glands, and maxillary bones. Due to the substantial size of the inferior alveolar nerve bundles, most intraosseous forms are reported in the posterior mandible, with fewer occurrences in the maxilla[165]. NF-1 is important due to its distinct clinical presentation, treatment, and prognosis. The standard treatment for neurofibroma involves complete surgical removal. These tumors are not radiosensitive and have little benefit from treatment [166]. The tumor will be removed in bloc with the surrounding tissues including

bone and teeth. There is a need for autologues tissue repair in patients suffering from tumor predisposition syndrome. Therefore, cell culture studies on the differentiation capability of stem cells are a first step towards improved reconstructive measures in a severely affected body region (haploinsufficiency and second hit of NF1 gene in the tumor region).

Is it possible that stem cells present at these sites possess distinct properties compared to those in normal teeth? This study compared the multilineage potentials and proteomic profiles of mesenchymal stem cells (MSCs) derived from the dental pulp of normal and NF teeth.

We successfully cultured and isolated original dental pulp stem cells using the tissue block method with limiting dilution. Research has demonstrated that similar to normal pulp tissues, stem cells are also found in NF teeth.

Maintaining self-renewal and differentiation is essential for stem cell growth and therapeutic application[167]. The two types of stem cells exhibited similar morphological and biological characteristics. The MTS and CFU assays were employed to evaluate proliferation and colony-forming potential, suggesting that the inherent self-organizing morphogenetic properties in ex vivo culture may be a characteristic of pulp tissue stem cells.

The potential of MSC stem cells to differentiate into osteogenic, chondrogenic, and adipogenic lineages traditionally serves as a measure of their suitability for orthopedic and aesthetic regeneration applications[168]. Collagen type I (COL I) serves as an early marker for matrix mineralization, while Osteocalcin (OSC) indicates osteogenic differentiation and maturation of bone[169]Previous research demonstrated that dental pulp cells express bone-related genes, such as ALP, osteocalcin, and collagen I, and are capable of forming mineralized nodules in vitro[137]. Cells from NF teeth exhibit characteristics including in vitro mineralized nodule formation and expression of bone-associated markers such as Osteocalcin (OSC) and Collagen type I (COL I). The osteogenic differentiation potential of these cells was comparable to the bone-forming activity of normal pulp stem cells. The adipogenic potential of both MSCs was evaluated. The most commonly assessed genes were peroxisome proliferator-activated receptor γ (PPAR-γ) and lipoprotein lipase (LPL). Similar to osteogenic differentiation, adipogenic differentiation in MSCs from NF teeth also exhibited lipid vacuoles, as evidenced by Oil Red staining.

This study successfully obtained dental pulp stem cells from NF teeth, which were comparable to normal dental pulp stem cells. The development of tissue-engineered bone for clinical bone defect reconstruction remains exploratory, facing challenges such as acquiring ample high-quality stem cells, enhancing vascularization and mechanical properties, and ensuring effective integration with host bone tissue. As related disciplines and technologies

advance, existing challenges will be gradually overcome, allowing tissue-engineered bone to eventually become a standard clinical method for bone defect reconstruction.

9.6 Inducing differentiation of human dental pulp cells toward acinar-lineage

(This part is about the publication PMID: 33042457. See page 75 of this dissertation).

We successfully induced acinar-like differentiation in human dental pulp cells. This induction was achieved by co-culturing with salivary gland cells in a transwell setup. Since both cell populations shared the same medium, the acinar differentiation of the dental pulp cells in the lower chamber likely resulted from factors and components secreted by the salivary gland cells in the upper insert.

To induce differentiation into various cell lines such as adipocytes and osteocytes, conditions based on specific combinations of small molecules have been established. However, no such straightforward conditions exist yet for acinar differentiation. A recent study successfully enriched lacrimal gland cells from human embryonic stem cells by transfecting synthetic messenger RNA encoding several transcription factors[170]. Nonetheless, transfection is not a feasible method for medical applications due to its complexities and potential for unintended effects.

Co-culturing represents another strategy for inducing differentiation. Adipose stromal cells differentiated into the acinar lineage when co-cultured with salivary gland cells[171]. However, that study did not provide details on the co-culture setup, nor did it mention the use of a transwell. It is possible that the two cell types were mixed together in the culture.

In this study, we utilized a transwell structure for co-culture, which allows the exchange of chemical substances and biochemical molecules while the semi-permeable membranes prevent cell passage. Thus, Cells of the two types are physically separated from one another. A significant advantage of this semi-co-culture approach is that the two types of cells can originate from different sources. While the pluripotent cells in the lower chamber can be autologous, and suitable for applications without causing host rejection, the inducer cells in the upper insert can be from different sources. In our study, human salivary gland cells were used as inducer cells. However, other inducer cells and tissue sources are theoretically possible, such as cell lines from human or non-human glandular tissues. Alternatively, the conditioned medium from gland cells might also influence the acinar differentiation of pluripotent cells.

We adopted a ratio of 4:1 between inducer cells and differentiating cells. However, other ratios may also be effective. Additionally, as approximately 30% of dental pulp cells achieved

acinar-like differentiation, these cells could secrete factors that facilitate further differentiation of other dental pulp cells. It would be interesting to explore the possibilities of other types of pluripotent cells in the future, including bone marrow cells and adipose stromal cells, in this type of acinar differentiation.

There are currently two main hypotheses regarding the mechanisms of mesenchymal stem cell multilineage differentiation: the microenvironment induction theory and the cell fusion theory. The study utilized co-culturing to differentiate human dental pulp stem cells (hDPSCs) into salivary gland-like cells. Literature indicates that co-culturing acinar cells with bone marrow mesenchymal stem cells (BM-MSCs) in a Transwell system can prompt BM-MSCs to differentiate into salivary acinar-like cells that secrete alpha-amylase. Cardiomyocyte lysate enhances BM-MSC proliferation and differentiation into cardiomyocyte-like cells.

In the experiments on culturing acinar cells, we incorporated 10% fetal bovine serum (FBS) into DMEM/F12 culture medium. This effectively preserved the biological functions of the acinar cells, creating necessary conditions for the subsequent proliferation of salivary acinar cells and successful induction of BM-MSCs into salivary acinar-like cells.

In the acinar cell culture experiments, 10% fetal bovine serum (FBS) was added to the DMEM/F12 medium. This effectively preserved the biological functions of the acinar cells, creating necessary conditions for the subsequent proliferation of salivary acinar cells and successful induction of BM-MSCs into salivary acinar-like cells.

In this study, the experimental group used lysate prepared from submandibular gland cells (SMGCs) to induce BM-MSCs. Immunocytochemistry revealed amylase expression in the cytoplasm of these cells, whereas the control group, which continued to be cultured in the original medium, showed no amylase expression. This indicates that in the simulated microenvironment of the submandibular gland acinar cells lysate, the interaction of BM-MSCs with the extracellular matrix of SMGCs, various cytokines, and enzymes, leads to the regulation of key genes that determine their differentiation path. Although we simulated the submandibular gland environment and induced the expression of the submandibular gland-specific secretory protein, alpha-amylase, in dental pulp mesenchymal stem cells, further investigation is required to identify the specific cytokines and gene action sites involved in this induction. Moreover, the difference between the amylase protein secreted by the induced submandibular gland-like cells and that secreted by normal submandibular gland cells warrants further study.

Human stem/progenitor cells are considered to possess certain immunosuppressive activities[172, 173]. Therefore, aside from their potential to perform acinar functions post-

induction, dental pulp cells may also help suppress some dysregulated immune responses that damage the original lacrimal gland cells in conditions such as dry eye disease.

This study shows that acinar differentiation is achievable by co-culturing human dental pulp cells with salivary gland cells in a transwell setup, maintaining separate cell populations. Acinar-like cells may serve as an autologous cell source with potential applications in cell therapy for dry eye disease.

9.7 Effects of hDPSCs on the Biological Phenotype of Hypertrophic Keloid Fibroblasts

(This part is about the publication doi: 10.3390/cells10071803. See page 75 of this dissertation).

The intricate process of wound healing frequently results in scar formation following skin injuries. Scar formation involves various cell types such as fibroblasts, endothelial cells, macrophages, and lymphocytes[174-176]. The biological behavior of fibroblasts is regarded as a crucial variable. Research indicates that fibroblasts, as primary components of scar tissue, can excessively proliferate, exhibit incomplete apoptosis, and synthesize collagen abnormally, contributing to persistent scar tissue growth.[177, 178]. Inhibiting fibroblast proliferation and inducing apoptosis in scar tissue can significantly reduce scar tissue growth, thereby slowing disease progression and playing a crucial role in scar improvement and treatment. Hence, the treatment of scar keloids requires an understanding of the biological characteristics of fibroblasts in scar tissue.

Mesenchymal stem cells (MSCs), a crucial component of the stem cell family, are extensively used in the treatment of scar-related conditions such as pulmonary fibrosis. Furthermore, they have shown remarkable potential in reducing cardiac scar formation by secreting a variety of cytokines.[179]. Research involving animal models and clinical trials indicates that stem cells have the potential to regenerate damaged organs. Stem cells enhance tissue repair by secreting cytokines like interleukin-6, interleukin-8, and interleukin-10, along with other proteins crucial for tissue regeneration[180, 181]. Recent studies suggest that adipose stem cells can suppress mRNA expression of COL1A1, TGF-β1, CTGF, and ACTA2 in renal fibrosis tissue, offering a therapeutic approach to kidney fibrosis. The ability of dental pulp stem cells to secrete cytokines that inhibit the proliferation and migration of keloid fibroblasts, contributing to skin scar repair, has not been previously documented.[182].

Human dental pulp stem cells (hDPSCs), scar keloid fibroblasts (HKFs), and normal skin fibroblasts (HNFs) were isolated via the tissue explant method. We investigated the molecular mechanisms by which hDPSCs influence HKF proliferation, migration, and apoptosis using a

Transwell co-culture system. Scar keloids are histologically defined by significant extracellular collagen secretion and deposition[183]. Both skin scar keloids and fibromas are fibroconnective tissue disorders characterized by excessive extracellular collagen deposition[184]. Additionally, we found that co-culturing with hDPSCs inhibited HKF and HNF migration, but they did not suppress proliferation or induce apoptosis. Our experimental data align with recent studies indicating that conditioned medium and cell lysates from human Wharton's Jelly-derived MSCs (WJ-MSCs) can inhibit the migration of human HKFs[185]. In contrast to the previous report, we employed hDPSCs due to their easy accessibility, minimal invasiveness, and suitability for autologous applications. Contrary findings have been reported, indicating that the conditioned medium from human WJ-MSCs can enhance HKF proliferation via a paracrine mechanism using an indirect Transwell co-culture system[186], We propose that the varying results may stem from the use of diverse MSC types, cell processing and culturing methods, and assay techniques, which could influence the migration, proliferation, collagen secretion, and apoptosis of scar keloid fibroblasts involved in wound healing after skin injury. Therefore, by inhibiting the migration and proliferation of scar keloid fibroblasts, it is also possible to suppress the formation of scar keloids[187]. Furthermore, studies have shown that inhibiting the expression of matrix metalloproteinases in scar keloid fibroblasts can reduce their migratory capacity, thus serving as an adjunct treatment for scar keloids [183]. Research indicates that suppressing mTOR protein expression in scar keloid fibroblasts reduces their migration, thereby inhibiting scar keloid development[188]. Our study supports the notion that dental pulp stem cells can inhibit the migration of keloid fibroblasts in vitro, implying their potential role in promoting wound healing by preventing scar formation.

The expression of genes associated with anti-fibrotic and pro-fibrotic activities is integral to the development of fibrotic diseases, reinforcing the classification of hypertrophic scars and keloids as such. Excessive scarring and fibrosis are primarily caused by the overexpression of TGF-β1 and TGF-β2. Research indicates that targeting the expression of TGF-β1 and TGF-β2 in hypertrophic scars and keloids can effectively inhibit scar proliferation, leading to beneficial clinical outcomes [188, 189]. Consequently, TGF-β1 and TGF-β2 are pivotal targets in hypertrophic scar and keloid treatment research. CTGF is a marker protein for fibrotic diseases, enhancing fibroblast proliferation and the secretion and deposition of extracellular matrix proteins such as type I collagen and fibronectin.[190, 191]. The study demonstrated that co-culturing human dental pulp stem cells (hDPSCs) with human keloid fibroblasts (HKF) for 24 hours significantly decreased the gene and protein expression of

TGF-β1, TGF-β2, and CTGF in HKFs. This supports evidence that MSCs, through paracrine functions, can secrete cytokines and modify fibroblast phenotypes, including fibrosis and proliferation [192, 193]. Growing evidence indicates that the paracrine function of MSCs is a key mechanism underlying their therapeutic effects. After injection, MSCs migrate to injury sites via chemotaxis, proliferate, and differentiate into cells that produce the extracellular matrix essential for tissue repair. Additionally, upon entering the body, MSCs can exhibit paracrine functions, secreting cytokines and bioactive substances needed for tissue repair, thereby inducing the body's own cells to repair tissue damage. The SMAD signaling pathway is a downstream mediator of TGF-β, with R-SMAD3 being upregulated in scar keloids following phosphorylation, while downregulation of R-SMAD3 significantly reduces the expression of procollagen genes in keloid fibroblasts. I-SMAD6 and I-SMAD7 suppress TGF-β activity. SMAD6 prevents the interaction between SMAD4 and R-SMAD. Keloid fibroblasts exhibit decreased expression of I-SMAD6 and I-SMAD7. By inhibiting the TGF-β1-SMAD signaling pathway and activating TLR7 or SMAD7, the formation of scar keloids can be significantly reduced. The Toll-like receptor (TLR) signaling pathway plays both protective and destructive roles. Following skin injury, Toll-like receptors (TLRs) interact with damage-associated molecular patterns (DAMPs), facilitating the innate immune system's response to sterile tissue damage. TLR stimulation in macrophages elevates pro-inflammatory and pro-fibrotic cytokine levels, altering fibroblast gene expression and TGF-β responses, thereby enhancing collagen production.

Hypertrophic scars and keloid tissues are characterized by excessive extracellular matrix deposition. Excessive extracellular matrix deposition can result from either increased synthesis or reduced degradation.[194]. Collagen synthesis is three times higher in hypertrophic scars and can be up to twenty times higher in keloids compared to normal skin [195]. Our experiments demonstrated that co-culturing human scar fibroblasts (HSF) and human keloid fibroblasts (HKF) with hDPSCs led to reduced extracellular matrix synthesis, evidenced by lower expression levels of Type I collagen, α-SMA, and hydroxyproline. Moreover, in the extracellular matrix of scar tissues, Type I collagen is usually the predominant type of collagen present. Measuring hydroxyproline content in cell culture medium reliably indicates fibroblasts' collagen synthesis capability. Previous reports indicate that both bone marrow and dermal MSCs can suppress collagen synthesis and α-SMA expression in keloid fibroblasts[186].

In conclusion, our findings align with prior research, demonstrating that dental pulp stem cells can inhibit pro-fibrotic gene expression and collagen synthesis in keloid fibroblasts in vitro. The study demonstrated that dental pulp stem cells inhibit keloid fibroblast migration. In future experiments, keloid fibroblasts' specific secreted cytokines must be investigated for their influence on their biological behavior.

From an ethical standpoint, embryonic stem cell research is highly controversial due to its destructive impact on embryos, leading to opposition from some groups. Human dental pulp stem cells, as adult stem cells, are not subject to the same ethical constraints. Regarding material collection, obtaining samples from naturally exfoliated deciduous teeth or extracted wisdom teeth is relatively simple and feasible, causing minimal harm to donors (children), and is more readily accepted by their parents. Personalized collection and storage of SHED (stem cells from human exfoliated deciduous teeth) for autologous transplantation and regenerative therapy can significantly minimize the risks of immune rejection and cross-infection. Moreover, storing stem cells before the onset of disease provides lifelong protection for individuals, which is one of the guiding principles for establishing stem cell banks. SHED banks are available for use by the donor's close relatives, including grandparents, parents, and siblings. Research by Arora et al. indicates that the storage cost of SHED banks is only one-third that of cord blood cell banks. Adult stem cells, such as SHED and cord blood-derived stem cells, complement each other in their multipotent differentiation capabilities and potential for clinical applications. Cord blood stem cells can generate essential blood components, including red and white blood cells and platelets, for treating various hematological disorders. Unlike hematopoietic stem cells, SHED uniquely contributes to both soft and hard tissue regeneration.

9.8 Concluding remarks

In conclusion, our research has successfully demonstrated the immense potential of human dental pulp stem cells (hDPSCs) in regenerative medicine. Through comprehensive studies, we optimized cryopreservation techniques, ensuring that hDPSCs maintain their viability and differentiation potential even after long-term storage. This advancement, particularly with the whole teeth, opens up new possibilities for the efficient storage and future therapeutic use of hDPSCs.

Moreover, our investigation into the differentiation capabilities of hDPSCs has revealed their significant versatility. From bone tissue engineering to neurological therapies, hDPSCs have shown robust osteogenic, adipogenic, and neural differentiation potential. Additionally, their

ability to differentiate into acinar-like cells offers promising therapeutic avenues for treating glandular disorders, such as dry-eye syndrome. Furthermore, our findings on hDPSCs' role in inhibiting fibrosis and keloid formation suggest their potential as a novel approach to treating fibrotic conditions.

Overall, these studies emphasize the diverse applications of hDPSCs, from bone regeneration and neurological repair to glandular restoration and fibrosis treatment. The combination of their multipotent nature and the feasibility of long-term cryopreservation positions hDPSCs as a valuable tool for future clinical applications in personalized and regenerative medicine.

10. Abbreviations

ADSCs	Adipose-Derived Stem Cells
ALP	Alkaline Phosphatase
ALS	Amyotrophic Lateral Sclerosis
AMS	Amylase Assay Kit
AP-1	Activator Protein-1
ATP	Adenosine Triphosphate
BMSCs	Bone Marrow Mesenchymal Stem Cells
BMP2	Bone Morphogenetic Protein-2
BDNF	Brain-Derived Neurotrophic Factor
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
BSA	Bovine Serum Albumin
C/N	Carbon-to-Nitrogen Ratio
CBF	Core-Binding Factor
CD	Cluster of Differentiation
CD44	Cluster of Differentiation 44
CD73	Cluster of Differentiation 73
CD90	Cluster of Differentiation 90
CD105	Cluster of Differentiation 105
COL I	Type I Collagen
CNTF	Ciliary Neurotrophic Factor
cNF	Cutaneous Neurofibromas
CTGF	Connective Tissue Growth Factor
CXCL1	C-X-C Motif Chemokine Ligand 1
CXCR6	C-X-C Chemokine Receptor Type 6
DAPI	4',6-Diamidino-2-Phenylindole
DMD	Duchenne Muscular Dystrophy
DMSCs	Dental Mesenchymal Stem Cells

DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DPSC	Dental Pulp Stem Cell
DSPP	Dentin Sialophosphoprotein
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate
GCs	Glucocorticoids
G-CSF	Granulocyte-Colony Stimulating Factor
GDNF	Glial Cell-Derived Neurotrophic Factor
hDPSCs	Human Dental Pulp Stem Cells
hMSCs	Human Mesenchymal Stem Cells
iPSC	Induced Pluripotent Stem Cell
H&E	Hematoxylin and Eosin
HA	Hydroxyapatite
HGF	Hepatocyte Growth Factor
HLA	Human Leukocyte Antigen
HKFs	Human Keloid Fibroblasts
HNFs	Human Normal Fibroblasts
HUVECs	Human Umbilical Vein Endothelial Cells
IGF	Insulin-Like Growth Factor
IL-1β	Interleukin-1β
IL-6	Interleukin-6

ISC	Intestinal Stem Cell
JAK2	Janus Kinase 2
LFA-1	Lymphocyte Function-Associated Antigen 1
LPL	Lipoprotein Lipase
MAP	Mitogen-Activated Protein
MAPKs	Mitogen-Activated Protein Kinases
MCP-1	Monocyte Chemoattractant Protein-1
MEF	Mouse Embryonic Fibroblasts
MI	Myocardial Infarction
MMPs	Matrix Metalloproteinases
MPNST	Malignant Peripheral Nerve Sheath Tumors
mRNA	Messenger RNA
miRNAs	MicroRNAs
MSCs	Mesenchymal Stem Cells
NF1	Neurofibromatosis Type 1
NF2	Neurofibromatosis Type 2
NF-kB	Nuclear Factor-kB
NGF	Nerve Growth Factor
NOD	Non-Obese Diabetic
NT-3	Neurotrophin-3
OSC	Osteocalcin
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PNF	Plexiform Neurofibroma
PNST	Peripheral Nerve Sheath Tumors
PI3K	Phosphatidylinositol-3-Kinase
PLGA	Poly Lactic-co-Glycolic Acid
PRP	Platelet-Rich Plasma

PSS	Primary Sjögren's Syndrome
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RUNX2	Runt-Related Transcription Factor 2
SCF	Stem Cell Factor
SDF-1	Stromal Cell-Derived Factor-1
SEM	Scanning Electron Microscopy
SIC	Substituted Isochroman
SOC	Organic Carbon
SOD	Superoxide Dismutase
SS	Sjögren's Syndrome
STAT3	Signal Transducer and Activator of Transcription 3
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
TRAP	Tartrate-Resistant Acid Phosphatase
UC	Ulcerative Colitis
VEGF	Vascular Endothelial Growth Factor
WNT	Wingless/Integrated Signaling Pathway

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12. Zusammenfassung auf Deutsch und auf Englisch

Zusammenfassung

Unsere Forschungsgruppe hat eine Reihe von Studien zu menschlichen Zahnmark-Stammzellen (hDPSCs) durchgeführt, wobei der Schwerpunkt auf der Optimierung ihrer Kryokonservierung, dem Verständnis ihres Differenzierungspotenzials und der Untersuchung ihrer therapeutischen Anwendungen in der regenerativen Medizin lag.

Zunächst haben wir verschiedene Kryokonservierungsmethoden untersucht, um optimale Bedingungen für die Erhaltung der Lebensfähigkeit und Funktionalität von hDPSCs zu ermitteln. Durch den Vergleich frischer hDPSCs mit solchen, die in 5% und 10% DMSO konserviert wurden, stellten wir fest, dass 5% DMSO bessere Bedingungen für die Erhaltung der Zellviabilität und ein schnelleres Zellwachstum im Vergleich zu 10% DMSO bot. Dennoch zeigten sowohl kryokonservierte als auch frische Zellen ähnliche Koloniebildungsraten, Überlebensraten und Differenzierungspotenziale. Diese Ergebnisse zeigten, dass die Kryokonservierung, insbesondere mit 5% DMSO, eine praktikable Langzeitlagerlösung für Stammzellen ist. Aufbauend auf diesen Erkenntnissen haben wir die Machbarkeit der Kryokonservierung ganzer Zahnmarkgewebe über längere Zeiträume untersucht. Nach der Lagerung von Gewebefragmenten in 10% DMSO für über einen Monat stellten wir fest, dass die aus diesen konservierten Geweben gewonnenen Zellen ihre biologischen Eigenschaften, einschließlich Proliferation und Differenzierungspotenzial, beibehielten. Wichtig ist, dass es keine signifikanten Unterschiede in den biologischen Eigenschaften zwischen Zellen aus frischen und kryokonservierten Geweben gab, was die Praktikabilität der Kryokonservierung von Gewebefragmenten für die zukünftige klinische Anwendung bestätigt.

Basierend auf diesen Erkenntnissen entwickelten und testeten wir einen neuartigen Ansatz zur Kryokonservierung ganzer Zähne. Diese Methode ermöglicht die Erhaltung von hDPSCs in ihrer natürlichen Umgebung und bewahrt ihre Lebensfähigkeit und multipotenten Eigenschaften, einschließlich ihrer Fähigkeit zur Differenzierung in osteogene und adipogene Linien. Dieser Ansatz bietet eine kostengünstige und effiziente Lösung für die Langzeitlagerung von hDPSCs und macht sie für therapeutische Anwendungen ohne sofortige Extraktion aus dem zahn leicht verfügbar.

Zusammen bilden diese Studien eine kohärente Entwicklung von der Optimierung der Kryokonservierungsbedingungen für einzelne Zellen über die Erhaltung größerer Gewebesegmente bis hin zur Kryokonservierung ganzer Zähne. Jede Stufe baut auf den vorhergehenden Erkenntnissen auf und erhöht die Praktikabilität der Verwendung von

hDPSCs in regenerativen Therapien. Unsere Forschung zeigt, dass hDPSCs ihre Lebensfähigkeit und ihr Differenzierungspotenzial über verschiedene Kryokonservierungsmethoden hinweg beibehalten, wodurch neue Möglichkeiten für ihre Anwendung in langfristigen klinischen Anwendungen eröffnet werden.

In einer weiteren Studie untersuchten wir die Zytokinsekretion von hDPSCs in verschiedenen Passagen. Passage 3 wurde als optimaler Zeitpunkt identifiziert, da sie die höchsten Sekretionsniveaus von VEGF, BDNF und NGF aufwies, was sie besonders für neurologische Therapien geeignet macht. Darüber hinaus behielten hDPSCs, die von Patienten mit Neurofibromatose Typ 1 (NF1) stammen, ähnliche osteogene und adipogene Differenzierungskapazitäten wie jene gesunder Individuen, was auf ihr Potenzial für den Einsatz in der Knochengewebsrekonstruktion hinweist.

Wir haben auch die Fähigkeit von hDPSCs zur Differenzierung in azinusartige Zellen nachgewiesen, wenn sie mit Speicheldrüsenzellen co-kultiviert werden. Diese azinäre Differenzierung, gekennzeichnet durch eine erhöhte Amylaseaktivität und die Expression epithelialer Marker, deutet auf potenzielle therapeutische Anwendungen bei Drüsenerkrankungen wie dem trockenen Auge hin.

Schließlich ergab unsere Forschung zur Rolle von hDPSCs bei der Behandlung von Keloiden, dass die Co-Kultivierung von hDPSCs mit Keloidfaserblasten (HKFs) die Expression pro-fibrotischer Gene und die Synthese der extrazellulären Matrix hemmt, während gleichzeitig die Expression antifibrotischer Gene gefördert wird. Diese Erkenntnisse deuten darauf hin, dass hDPSCs einen neuartigen Ansatz zur Verhinderung und Behandlung von Fibrose und Keloidbildung bieten könnten.

Insgesamt unterstreichen diese Studien unserer Gruppe die Vielseitigkeit von hDPSCs in der regenerativen Medizin und demonstrieren ihr Potenzial in einer Vielzahl therapeutischer Anwendungen, einschließlich der neurologischen, knochen- und drüsenbezogenen Regeneration sowie der Behandlung von Fibrose. Die Fähigkeit, hDPSCs erfolgreich zu kryokonservieren, erhöht zusätzlich ihren Nutzen für die langfristige klinische Anwendung.

Summary

Our research group has conducted a series of studies on human dental pulp stem cells (hDPSCs), focusing on optimizing their cryopreservation, understanding their differentiation potential, and investigating their therapeutic applications in regenerative medicine.

Initially, we investigated various cryopreservation methods to determine optimal conditions for preserving hDPSCs while maintaining their viability and functionality. By comparing fresh hDPSCs with those preserved in 5% and 10% DMSO, we found that while 5% DMSO provided better conditions for maintaining cell viability and faster cell outgrowth compared to 10% DMSO, both cryopreserved and fresh cells demonstrated similar colony formation, survival rates, and differentiation potential. These results indicated that cryopreservation, particularly with 5% DMSO, is a viable long-term storage solution for stem cells. Building on these findings, we further explored the feasibility of cryopreserving entire dental pulp tissues for extended periods. After storing tissue fragments in 10% DMSO for over a month, we found that cells derived from these preserved tissues retained their biological characteristics, including proliferation and differentiation potential. Importantly, there was no significant difference in the biological properties between cells cultured from fresh and cryopreserved tissues, confirming the practicality of cryopreserving tissue fragments for future clinical use.Taking these insights a step further, we developed and tested a novel approach to cryopreserving whole teeth. This method allows for the preservation of hDPSCs in their natural environment, maintaining their viability and multipotent characteristics, including their ability to differentiate into osteogenic and adipogenic lineages. This approach offers a cost-effective and efficient solution for long-term storage of hDPSCs, making them readily available for therapeutic applications without the need for immediate extraction.

Collectively, these studies form a cohesive progression from optimizing cryopreservation conditions for individual cells to preserving larger tissue segments, ultimately leading to whole-tooth cryopreservation. Each stage builds upon the previous findings, enhancing the practicality of using hDPSCs in regenerative therapies. Our research demonstrates that hDPSCs maintain their viability and differentiation potential across various cryopreservation methods, opening new possibilities for their use in long-term clinical applications.

In another study, we examined cytokine secretion by hDPSCs across different passages. Passage 3 was identified as the optimal stage, with the highest levels of VEGF, BDNF, and NGF secretion, making it particularly suitable for neurological therapies. Furthermore, hDPSCs derived from patients with neurofibromatosis type 1 (NF1) retained similar

osteogenic and adipogenic differentiation capabilities as those from healthy individuals, indicating their potential for use in bone tissue engineering.

We also demonstrated the ability of hDPSCs to differentiate into acinar-like cells when co-cultured with salivary gland cells. This acinar differentiation, characterized by increased amylase activity and epithelial marker expression, suggests potential therapeutic applications for glandular disorders such as dry-eye syndrome.

Finally, our research into hDPSCs' role in keloid treatment revealed that co-culturing hDPSCs with keloid fibroblasts (HKFs) inhibits the expression of pro-fibrotic genes and extracellular matrix synthesis while promoting anti-fibrotic gene expression. These findings indicate that hDPSCs may offer a novel approach to preventing and treating fibrosis and keloid formation.

Overall, these studies from our group underscore the versatility of hDPSCs in regenerative medicine, demonstrating their potential across a range of therapeutic applications, including neurological, bone, and glandular regeneration, as well as fibrosis treatment. The ability to successfully cryopreserve hDPSCs further enhances their utility in long-term clinical use.

13. Acknowledgements

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Time flies like a horse, days and months are like falling flowers and flowing water, in a flash, the bits and pieces on the academic road converge into this moment of farewell. At this moment, I would like to express my deepest gratitude to all those who have given me knowledge, courage, and inspiration. Every experience is my precious memory. Every challenge is a ladder for my growth. Although the road ahead is unknown, I will take your teachings and blessings with me as I bravely move on to a new journey.

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05/2013-08/2015 Effects of anticoagulation intervention on the success rate of skin flap grafting and thrombosis complications in patients with oral cancer.

07/2011-05/2013 Long-term Evaluation of Airway Changes by Cone-beam Computed Tomography in Patients with Skeletal Class III Malocclusion Treated by Orthodontic-bimaxillary Surgery.

Clinical study of Maxillary Reconstruction with Fibula flap.

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Papers & Publications

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Activities:

06/2024 Attend the International Conference On DENTISTRY AND ORAL HEALTH 2024. Gave one presentation entitled <Harnessing the power of dental pulp stem cells: exosomes as a promising therapy for keloid inhibition>

05/2024 Attend the Conference der 19, Life Science Studierendentagung Hamburg. Gave one presentation entitled <Harnessing the power of dental pulp stem cells: exosomes as a promising therapy for keloid inhibition>

07/2022 Attend the Conference HEALTHCARE & STEM CELL 2022. Gave one presentation entitled <Evaluation of the effects of human dental pulp stem cells on the biological phenotype of hypertrophic keloid fibroblasts>

05/2019 Attend the 69. Jahrestagung der Arbeitsgemeinschaft für Kieferchirurgie & 40. Jahrestagung des Arbeitskreises für Oralpathologie und Oralmedizin. Gave five presentations entitled <Effects of anticoagulation intervention on success rate of skin flap grafting and thrombosis complications in patients with oral cancer>

<Study on correlation between oral squamous cell carcinoma coagulation function level and late recurrence and transfer>

<Preliminary study on the effect of triptolide on human mononuclear macrophage function in acidic microenvironment>

<Application of three-dimensional reconstruction of the enhanced CT with iPlan CMF software in head and neck neoplasms>

<Application of Computer-assisted Navigation in Oral and Maxillofacial Tumor Adjacent to Skull Base>

05/2019 Attend the 24th International Conference on Oral & Maxillofacial Surgery. Gave two presentations entitled

<A preliminary study on vasculogenic mimicry in oral squamous cell carcinoma>

<Application of three-dimensional marking technique in diagnosis and treatment of mandibular malignant>

01/2019 Attend the International Meeting on Precision Oncology and Personalized Medicine for Head and Neck Cancer. Gave presentations entitled

<Inhibition of long non-coding RNA prostate cancer-associated ncRNA transcript 1 suppresses proliferation and metastasis of tongue squamous cell carcinoma cells via the activation of p21 >

Awards and Personal Honor:

1. 2020 Merit scholarships for international students enrolled at Universität Hamburg
2. 2021 Merit scholarships for international students enrolled at Universität Hamburg

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Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

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