Proliferative Capacity and Differentiation Potential of Isolated Postnatal Human Dental Pulp Stem Cells in Diabetic Patients

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ABSTRACT

Stem cells are characterized by the ability to form clones, self-renew and differentiate into different types of cells. Stem cells derived from human dental pulp (DPSCs) have been shown to differentiate into odontoblasts in vitro after using Bone Morphogenetic Protein -2 (BMP-2). The aim of the present study was to explore the variations in the differentiation potential of the DPSC into odontoblasts in diabetic adults; it also aimed at investigating the possible influence of the origin of stem cells (coronal and radicular pulp) on their differentiation potential. Pulp tissue was extirpated from healthy individuals as well as from controlled and uncontrolled diabetic patients. Pulp tissue was divided into coronal and radicular parts and each were cultured for 30 days and then BMP-2 was added. Alizarin red staining was performed to confirm mineralization. RT-PCR was used to analyse expression of mineralization markers Dentin sialophosphoprotein (DSPP) & Enamelysin. The results of this study showed that DPSCs of both healthy and diabetic groups had stem cell properties. DPSCs of the coronal pulp have a more proliferative capacity than that of the radicular pulp. Controlled diabetes mellitus provides relatively unfavourable conditions for the DPSCs to proliferate and differentiate while uncontrolled diabetes produces more deleterious effect on the DPSCs capacity for proliferation and differentiation. Expression of DSPP and enamelysin was positive in healthy and controlled diabetics groups, while in the uncontrolled diabetic group it was negative. It is concluded that DPSCs of the coronal pulp have a more proliferative capacity than that of the radicular pulp, controlled diabetes mellitus provides relatively unfavourable conditions for the DPSCs to proliferate and differentiate and uncontrolled diabetes produces more deleterious effect on the DPSCs capacity for proliferation and differentiation.

Keywords

Bone Morphogenetic Protein - 2, Dental pulp, Diabetes Mellitus, Stem cells, Odontoblasts.

Introduction

Current progress in stem cell and tissue engineering technologies is now providing significant supporting evidence for its future clinical applications. Craniofacial tissue engineering promises the regeneration or de novo formation of dental, oral, and craniofacial structures lost due to congenital anomalies, trauma, or diseases [1]. For successful tissue engineering attempts, three main elements must be fulfilled, progenitor/stem cells, inductive morphogenetic signals (growth factors) and the extracellular matrix scaffold [2]. Stem cells are defined as clonogenic, self-renewing cells which are capable of generating one or more specialized cell types [3].

The first type of dental stem cell was isolated from the human pulp tissue and termed postnatal dental pulp stem cells (DPSCs) [4]. Subsequently, 4 more types of dental stem cells were isolated and characterized including stem cells from exfoliated deciduous teeth (SHED) [5], periodontal ligament stem cells (PDLSCs) [6], and dental follicle precursor cells [7] and stem cells from apical papilla (SCAP) [8].

Dental pulp stem cells (DPSCs) were detected within the “cell rich zone” of dental pulp [9]. Researchers managed to isolate dental pulp stem cells (DPSCs) from extracted human third molars. Pulp tissue was gently separated from the crown and root of healthy human impacted third molars and DPSCs were isolated [4].

The DPSCs were capable of forming ectopic dentin and...
associated pulp tissue in vivo. Stem cells were re-established in culture from primary DPSC transplants and re-transplanted into immunocompromised mice to generate a dentin-pulp-like tissue, demonstrating their self-renewal capability. DPSCs were also found to be capable of differentiating into adipocytes and neural-like cells [3]. It was found that DPSCs could be induced to undergo uniform differentiation into smooth and skeletal muscle cells, neurons, cartilage and bone cells under different culture conditions [10]. Furthermore, it was demonstrated that DPSCs have a general gene expression pattern similar to that of mature native odontoblasts [11]. Moreover, DPSCs were considered a good candidate for organ transplantation [12] as they were proved to have a high proliferative activity and could be cryopreserved with retaining their multi-potential differentiation ability [13].

As DPSCs cells have property to differentiate into odontoblasts, these cells can be used directly for dental therapy as well as very well used as in vitro model system to evaluate or optimize newly developed bioactive materials for future dental therapy. Regenerative property of the pulp-dentin complex is mainly depending on formation of tertiary dentin, reactionary dentin and reparative dentin. There are two different approaches implemented in regeneration of dentin by the use of tissue engineering techniques. The first approach includes a device which can use as a filling material into a deep cavity of tooth with partial layer of dentin on top of the pulp. In this process, they used some growth factors or molecules that can form reparative dentin. The second approach is to put scaffold on open pulp along with odontoblast-like cells to grow on it. These cells will synthesis reparative dentin. This is somewhat a difficult and challenging approach and is being studied extensively for curing dental disorders. It is mandatory that the scaffold used for clinical application should have capacity to adhere the cells to the surface of this scaffold and should proliferate and differentiate these dental pulp cells into dentin forming odontoblasts. It is also essential to have a good mobility of these cells on this scaffold. Similarly after implantation, the scaffold should be replaced by regenerated tissue without alteration of volume and size of this scaffold material [14].

Characterization of a dentinogenic response in DPSCs after odontogenic differentiation requires demonstration of mineralization markers. It is widely assumed that Osteocalcin (OCN), osteonectin, alkaline phosphatase (ALP), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP) and Enamelysin have been used as mineralization markers for odontoblast/osteoblast-like differentiation of DPSCs [4,13]. Dentin sialophosphoprotein (DSPP) and Enamelysin are a major marker of odontoblast differentiation. DSPP is a major non-collagenous dentin specific protein expressed and secreted by odontoblasts. It is an essential protein for normal tooth development [15]. MMP-20 (enamelysin), The matrix metalloproteinase-20 (MMP-20) also known as Enamelysin was discovered in the enamel organ, and has also been detected in odontoblasts during dentin formation [16].

The effect of cryopreservation on DPSCs characteristics was evaluated by isolating dental pulp cells from, seven days old, non-cryopreserved and cryopreserved human deciduous teeth and culturing them simultaneously [17]. It was found that there was no change in differentiation and immunophenotype properties of both cells. There was a change in the morphology, proliferative capacity of cryopreserved cells than non-cryopreserved cells [17].

The lineage commitment and differentiation of dental stem cells during dentin/pulp tissue repair and regeneration markedly differ depending on multiple factors. Apart from their intrinsic gene expression and epigenetic profiles, the cellular fate of dental pulp-derived stem cells seems more likely to be determined by the bioactive signalling molecules within the local microenvironment [18]. Currently, a diverse array of inductive growth factors have been identified implicated in important cellular events during dentinogenesis and pulp tissue regeneration and they are either released from the degraded dentin matrix, derived from the etched platelets within induced blood clot during revitalization procedures or artificially synthesized in vitro and locally delivered into engineered tissue [19].

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor beta superfamily. The human genome encodes 20 different types of BMPs. The roles of BMPs in embryonic development and cellular functions in adult animals have been extensively studied in recent years [20]. BMPs also play a critical role in tooth morphogenesis [21]. In craniofacial development, they have been implicated in the inductive interactions between dental epithelium and mesenchyme in a stage-dependent, reiterative manner. BMPs 2, 4 and 7 were expressed in dental epithelium, and it was proved that recombinant BMPs 2 and 4 could be used as a substitute for dental epithelium in inducing mesenchyme differentiation. BMPs were also expressed in the enamel knot and were associated with differentiating odontoblasts and ameloblasts. BMP-3 and BMP-7 have been immunolocalized in developing PDL, cementum and alveolar bone [22]. Recombinant human BMP-2 and BMP-4 could induce new dentin formation. Recombinant human BMP-2 and BMP-4 were capped with dentin matrix on amputated pulp. After two months, the amputated pulp was filled with tubular dentin. The amount of dentin formed was markedly diminished when dentin matrix alone was implanted. These findings imply that recombinant human BMP-2 and BMP-4 induced the differentiation of adult pulp cells into odontoblasts [23].

**Aim of the study**

The aim of the present study was to explore the variations in the differentiation potential of the DPSC into odontoblasts in diabetic adults (whether well or poorly controlled). It also aimed at investigating the possible influence of the origin of stem cells (coronal and radicular pulp) on their differentiation potential. This would pave the way for studies on tissue engineering or vital pulp therapy.

**Materials and Methods**

Impacted Human third molars from healthy males as well as teeth indicated for extraction from diabetic male patients were collected.
Teeth collection was from the outpatient clinics of faculty of oral & dental medicine & the dental clinics of the National Institution for Diabetes, Cairo - Egypt. The age of the selected subjects was ranging between 30 and 55 years (average 42.5 years). A brief medical history was taken from all patients and then they were subjected to chair side random glucose analyses using identification strips. Then the extracted teeth were assigned in one of the three groups (Ten teeth each):

- **Group I (control group):** This included the pulps removed from impacted third molars extracted from individuals with normal blood glucose level.
- **Group II (controlled diabetic group):** Which included pulps of molars extracted from diabetic patients following control measures.
- **Group III (uncontrolled diabetic group):** Included pulps removed from molars extracted from diabetic patients not following diabetes control measures.

Each group was further subdivided into two sub-groups: Group A (coronal pulp) and Group B (radicular pulp). Radicular and coronal parts of the extracted pulp tissue of all teeth were separated using a sterile scalpel and then each part separately was minced into approximately 2x2x1mm fragments. Pulp tissue was then digested in IV 0.1% collagenase (Sigma C-5138, St. Louis, MO, USA). Then the medium was incubated for 15 min and centrifuged (10 min at 1000 rpm). Supernatant was removed and the medium was cultured in T-25 flasks containing DMEM supplemented with 15% fetal calf serum and antibiotic combination (100u/ml penicillin & 100 µg/ml streptomycin) and then incubated in 5% CO2 incubator at 37ºC. Culture medium was changed once every four days and cultured cells in the flasks were monitored everyday using inverted phase contrast light microscope. Passage was done when cells reached 80-90% confluence. For cell counting a hematocytometer (ERMA 3485, Tokyo, Japan) was used. For this, 10µL of specimens of each flask, were pipette into each chamber of the two chambers of the hematocytometer. For the colony-forming efficiency assay, day 8 cultures were monitored using inverted phase light microscope. Passage was done when cells reached 80-90% confluence. For cell counting a hematocytometer (ERMA 3485, Tokyo, Japan) was used. For this, 10µL of specimens of each flask, were pipette into each chamber of the two chambers of the hematocytometer. For the colony-forming efficiency assay, day 8 cultures were monitored using inverted phase light microscope. Passage was done when cells reached 80-90% confluence.

**Results**

**Isolation and culturing of DPSCs**

Cell proliferation of cells in all groups was evident, however difference in cell number and proliferation rates were detected (Figure 1).

![Figure 1: Cultured DPSCs on day 8 of the coronal & radicular pulp of the three experimental groups revealing the increase in cell number in the coronal pulp versus the radicular pulp of the three groups. Notice the decrease in cell number & colonies in groups II & III than that in control group I.](image)

The comparison of the coronal segments among all groups (coronal healthy, coronal controlled and coronal uncontrolled). One Way Analysis of Variance (ANOVA) showed the presence of a statistically significant difference among the three groups (P ≤ 0.001) Table 1.

<table>
<thead>
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<th>Comparison</th>
<th>Mean Difference</th>
<th>t</th>
<th>P Value</th>
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Table 1: Pairwise multiple comparison procedures of the coronal segments among all groups.

As for the comparison of the radicular segments among all groups (radicular healthy, radicular controlled and radicular uncontrolled). One Way Analysis of Variance (ANOVA) showed the presence of a statistically significant difference among the three groups (P ≤ 0.001) Table 2.

<table>
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<th>P Value</th>
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</table>

Table 2: Pairwise multiple comparison procedures of the radicular segments among all groups.

**Number of cells**

One Way Analysis of Variance (ANOVA) showed the presence of a statistically significant difference between mean number of cells among all experimental groups (P ≤ 0.001) Graph (1).

**Graph 1:** Bar chart showing the mean values for Number of cells after 30 days in all groups.
Odontogenic Differentiation

Staining with Alizarin red

All studied and control groups were stained with alizarin red staining, results revealed negative staining in control groups while positive staining was evident in studied groups. Variations in amount of staining was detected among studied groups, being more in coronal than radicular groups. Moreover, healthy groups showed the highest amount of calcification followed by controlled diabetic and then uncontrolled diabetic group which showed the least amount of calcification (Figure 2).

Figure 2: Alizarin red staining in all experimental groups, revealing positive staining in all groups. Alizarin calcified nodules were more prominent in coronal than radicular pulp of all groups. The calcified deposits were discrete & sparse in groups II & III.

RT-PCR

RT-PCR revealed negative expression in all control groups. Positive expression of enamelysin and DSPP was detected in healthy and controlled diabetic groups. While in uncontrolled diabetic group, negative expression of both markers was evident (Figure 3).

Discussion

In previous studies, human dental pulp stem cells were isolated from adults [9,13]. However; detailed characterization between coronal and radicular parts of the pulp, as well as between healthy and diabetic patients has not been documented. The present study is the first to investigate and compare the proliferative and differentiating capability between the coronal and radicular pulps in both healthy and diabetic patients.

It has been reported by several investigators that dental pulp tissue has shown the presence of a population of highly proliferative stem cells. [3,23,24]. This is in agreement with the current work, as shown by the high number of cells produced and their clonogenic nature following cell- doubling as well as estimating colony- forming efficiency. In the present study DPSCs were successfully isolated from the coronal and radicular pulp of healthy adults, diabetic controlled and diabetic uncontrolled adults. Cells proliferation and confluence occurred in all dishes of the three groups included in this study. However, the number of cells and colonies in coronal pulp was higher than that of radicular pulp in the three studied groups. These results were statistically confirmed, where a statistically significant difference was present between the coronal and radicular DPSCs of the three groups, with a p value ≤ 0.001.

Diabetes could directly affect the pulp tissue [25]. In fact, metabolic alterations in pulp tissue such as altered levels of nitrite and kallikrein have been shown in diabetic rats. Decreased collagen concentration has also been shown [26]. Our results are in agreement with another study performed where histological changes in pulp tissue such as reduction in collagen fibers and fibroblasts densities after 3 months of diabetes were detected in their experiments [27]. Also in agreement, a decreased concentration of collagen in dental pulp after 1 and 3 months of diabetes onset in rats was found in previous experiments [26].

It was reported that cells having a higher proliferative activity would be more susceptible to apoptosis [28]. Furthermore, the apoptotic cells in the human pulp were investigated and were found to be more intense in the coronal than the radicular region [29]. Therefore, it was assumed that coronal pulp has more proliferative cells than the radicular part. Pulps of diabetics tend to age more readily because of obliteratorative endarteritis and because the dental pulp has limited or no collateral circulation in the fully developed teeth [29]. Data of the present research is in agreement with the previous studies, since the number of cells and colonies in the coronal pulp was always ahead than that in the radicular pulp. Moreover, the values were always higher in the healthy group compared to the diabetic groups.
Previous researches utilized different stains for determination of minerals. Alizarin red was among the most used stains to confirm successful formation of calcified nodules in monolayer cultures [30]. Alizarin red was used as a histochemical marker in the present study for determination of calcium mineral and confirms mineralized nodule formation. After 30 days of addition of BMP-2, both experimental and control dishes were stained by Alizarin red stain to evaluate the calcification potential of DPSCs cells.

Results of the current study revealed that the long term culture (30 days) of DPSC in the presence of BMP-2 demonstrated the capacity to form Alizarin red positive nodules. Staining was positive in all the experimental dishes, while it was negative in the control ones. This result is in agreement with previous work done by other investigators who used Alizarin red stain to confirm the effect of BMP-2 in odontogenic differentiation of dental pulp stem cells [31]. The expression of Alizarin was variable among the three groups of the present research. The revealed expression was in the form of condensed nodules in both groups I and II; however it was in the form of scattered deposits in group III.

The RT-PCR revealed the expression of both DSPP & Enamelysin markers in the BMP-2 supplemented groups (I and II) only, compared with the control groups which were not supplemented with the BMP-2. This indicated that BMP-2 enhanced differentiation of pulp stem cells into odontoblasts. On the other hand, expression of DSPP and Enamelysin was negative in the uncontrolled diabetic group III. The mineralization capacity of DPSCs and identifying the potential markers of odontoblasts differentiation have been previously investigated and it was found that DSPP expression was upregulated over time throughout the culture time with significant upregulation on day 21 in odontogenic induction [30]. The latter finding is in accordance with the finding of the current research, though we investigated the marker expression only at the day 30 after the BMP-2 supplement, since monitoring the DSPP expression at different intervals throughout the culture time was not the aim of the present study. The negative expression of both markers in group III could be referred to the deleterious effect of diabetes on the dental pulp as previously mentioned.

Interestingly, in the current research though Alizarin red staining confirmed mineralized nodules formation in the three experimental groups, DSPP and Enamelysin immunomarkers were positive in both groups I and II only, and negative in the uncontrolled diabetic (group III). The negative expression of DSPP and Enamelysin in (group III) could be attributed to the unfavourable microenvironment created by the uncontrolled diabetes in the dental pulp, which in turn lead to the formation of calcified tissue other than dentin.

Our study showed that DPSCs obtained from the healthy and diabetic dental pulp have a proliferative capacity and could form hard tissue in vitro. Many teeth are extracted and discarded in the dental clinics; therefore, the DPSCs may be available for hard tissue regeneration, such as the repair of damaged teeth or bone tissue. Moreover, techniques to isolate human DPSCs and manipulate their growth under defined in vitro conditions have to be established and optimized before therapy can become a clinical reality for caries and endodontic therapy.

References
17. Lindemann D, Werle SB, Steffens D, et al. Effects of


