Original Article

The Differentiation Potential of Human Natal Dental Pulp Stem Cells into Insulin-Producing Cells

(dental / diabetes / human / insulin / stem cells)

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Abstract. Mesenchymal stem cells have the ability to differentiate into insulin-producing cells, raising the hope for diabetes mellitus treatment. The aim of this research was to study the ability of stem cells from discarded natal teeth to differentiate into insulin-producing cells. Two vital human natal teeth were obtained from a healthy 2-day-old female. Stem cells from the dental pulp were isolated, cultured under xenogenic-free conditions, propagated and characterized. Proliferative activity, population doubling time and viability were measured, and the multipotent differentiation ability was investigated. A two-step protocol was used to induce the human natal dental pulp stem cells to differentiate into insulin-producing cells. Phenotypic analysis was done using flow cytometry. Immunohistochemistry was performed to detect insulin and C-peptide. *PDX1, HES1* and *Glut2* gene expression analysis was performed by quantitative reverse transcription-polymerase chain reaction. Human natal dental pulp stem cells were able to undergo osteogenic, chondrogenic and adipogenic differentiation upon exposure to the specific differentiation media for each lineage. Their differentiation into insulin-producing cells was confirmed by expression of C-peptide and insulin, as well as by 975.4 % higher expression of PDX-1 and 469.5 % higher expression of HES1 in comparison to the cells cultivated in standard cultivation media. Glut2 transporter mRNA was absent in the non-differentiated cells, and differentiation of the stem cells into insulin-producing cells induced appearance of the mRNA of this transporter. We were the first to demonstrate that stem cells obtained from the pulp of natal teeth could be differentiated into insulin-producing cells, which might prove useful in the stem cell therapy for type 1 diabetes.

Introduction

Despite advances in the treatment of diabetic patients, diabetes mellitus remains one of the most serious health care problems in our civilization. Using transplants of Langerhans islets in cell-based therapy of type 1 diabetes mellitus has shown major limitations because of the side effects associated with immunosuppressive therapy and insufficient supply of islets (Moshtagh et al., 2013).

The discovery of stem cells (SCs) and their successful differentiation into insulin-producing cells (IPCs) have raised a new hope for all diabetic patients. Recent research has shown that embryonic cells can differentiate into IPCs. However, this approach has raised many concerns regarding tumour formation, immune rejection and ethical approval (Limbert and Seufert, 2009). Therefore, much effort has been made to find new sources for generating IPCs, and various types of SCs have shown the differentiation potential into IPCs including hepatic SCs, umbilical cord blood SCs, and bone marrow-derived mesenchymal stem cells (MSCs). The scarcity of the donor pool and the invasive procedures required to isolate and culture these cells have limited their use (Govindasamy et al., 2011).

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Abbreviations: α-MEM – α modification of minimal essential medium, CD – cluster of differentiation, CXCR4 – chemokine receptor type 4, DAPI – 4,6-diamidino-2-phenylindole, DMEM – Dulbecco's modified Eagle's medium, FCS – foetal calf serum, HBSS – Hank's balanced salt solution, hNDPSCs – human natal dental pulp stem cells, IPCs – insulin-producing cells, Isl-1– insulin gene enhancer protein, ITS – insulin-transferrin-sodium-selenite supplement, MSCs – mesenchymal stem cells, Ngn-3 – neurogenin 3, OCT – octamer-binding transcription factor, Pax – paired box, PBS – phosphate-buffered saline, PD – population doublings, PDL – periodontal ligament, Pdx-1 – pancreatic and duodenal homeobox 1, PRP – platelet-rich plasma, qRT-PCR – quantitative reverse transcription-polymerase chain reaction, SCs – stem cells, TGF-β1 – transforming growth factor β1.

MSCs derived from dental tissues are good candidates for cell therapy because they are accessible, multipotent, with significant plasticity and various immunomodulatory effects (Moshtagh et al., 2013; Sawangmake et al., 2014). Advances in cell therapy have shown a great interest in identifying methods to direct dental pulp SC differentiation *in vitro* toward therapeutically functional IPCs because of their immunomodulatory functions via secretion of active molecules and/or direct interaction with immune cells, implying the advantage for cell therapeutic purposes (Li et al., 2014). Many studies have examined the proliferation and differentiation capacity of MSCs derived from dental pulp (Wang et al., 2017). Deriving stem cells from the pulp of discarded teeth such as natal and/or neonatal teeth have been proved to be feasible (Huang et al., 2008).

The presence of teeth at birth (natal teeth) or within one month after birth (neonatal teeth) is a rare incidence and occurs in 1 : 2000 to 1 : 3500 live births, because the normal eruption of primary teeth starts six months after birth (Uzamis et al., 1999; Seminario and Ivancakova, 2004). Although the aetiology of early eruption of these teeth is still unknown, a number of factors have been related to their occurrence (Basavanthappa et al., 2011). Natal and/or neonatal teeth represent a source of SCs that can differentiate and self-renew to produce new SCs, which could have a great potential in cell-based therapy. These teeth are usually discarded as they may have a negative functional and physiological impact on the child and his parents (Kadam et al., 2013).

Human natal/neonatal dental pulp stem cells have been shown to express various MSC, markers and differentiate into adipogenic, osteogenic chondrogenic, myogenic and neurogenic lineages (Karaöz et al., 2010).

The feasibility and ease of isolation of human natal/neonatal dental pulp SCs and their demonstrated ability to differentiate toward specific cell lines raises the possibility that these cells could form a basis for future cell-based therapies.

The aim of this study was to isolate and characterize human natal dental pulp stem cells (hNDPSCs) and explore, for the first time, their ability to differentiate into IPCs using xenogenic-free culture medium in an attempt to define a new approach in cell-based therapy for type 1 diabetes.

Material and Methods

Isolation and cultivation of hNDPSCs

The study was approved by the Ethical Committee of the University Hospital Hradec Králové ref. 200712 S01P. The donation and the use of blood products in the study were also approved by the same Committee ref. 201011 S14P.

Two vital human natal teeth were obtained from a healthy 2-day-old female under local anaesthesia and complete aseptic conditions. Prior to donation, the patient's legal representative (mother) was informed of the nature of the study and signed the informed consent. Extracted teeth were disinfected and transported to the culture lab at 4 °C, fully immersed in a transportation media composed of 1 ml of Hank's balanced salt solution (HBSS) (Invitrogen, Waltham, MA), 9 ml water for injection (Bieffe Medital, Grosotto, Italy) and antibiotics to guard against any contamination. Antibiotics added included: 20 μg/ml gentamicin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2.5 μg/ml amphotericin (Sigma-Aldrich Co., St. Louis, MO), and 100 U/ml penicillin (Invitrogen).

Pulpal tissues were retrieved through the wide apical foramina, cut into small pieces and enzymatically dissociated in a solution of enzymes collagenase (0.2 mg/ml; Sevapharma, Prague, Czech Republic) and dispase (2 mg/ml; Gibco, Thermo Fisher Scientific, Foster City, CA), PBS (Invitrogen) and HBSS in the 1 : 1 : 1 : 1 ratio for 50 min at 37 °C. Following centrifugation (600 *g*), the obtained cell pellet was resuspended, seeded and cultured in tissue culture flasks (Sarstedt, Newton, NC) in a media whose composition is based on α-MEM (Invitrogen) with 2% platelet rich plasma (PRP). For enhancing the proliferation rate, the culture medium was supplemented with 10 ng/ml epidermal growth factor (PeproTech, London, UK), 10 ng/ml platelet-derived growth factor (PeproTech) and 50 mM dexamethasone (Bieffe Medital). Ten μl of ITS/ml (Bieffe Medital) was added to increase the nutrients' utilization and 0.2 mM L-ascorbic acid (Bieffe Medital) was added to protect against oxidative stress and free oxygen radicals. The culture medium was supplemented with essential amino acid glutamine (Invitrogen in final concentration 2 % and antibiotics – 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 20 μg/ml gentamicin (Invitrogen) – to guard against bacterial infection. Culture plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every three days until cells reached 70% confluence, and then the cells were passaged.

Ten samples of PRP were obtained from the Transfusion Department, University Hospital Hradec Králové, Czech Republic, and pooled to eradicate the inter-individual differences. All donors were examined and fulfilled conditions to be considered as blood sample donors for medical usage.

HNDPSC characterization

For measuring the basic biological characteristics (population doubling and population doubling time), the cells were regularly counted using Z2 Coulter (Beckman Coulter, Miami, FL). The number of viable cells was counted using a Vi-Cell analyzer (Beckman Coulter) in the 2nd and 10th passages.

Flow cytometry

The phenotype was analysed in the hNDPSCs harvested in the 5th passage. The cells were first detached and then stained sequentially using different primary immunofluorescent antibodies. The percentage of posi-

tive cells was determined as a percentage of cells with higher fluorescence intensity than the upper 0.5 % of isotype immunoglobulin control. The following classification criteria to detect the various markers were: < 10 % no expression, 11–40 % low expression, 41–70 % moderate expression and > 71 % high expression. The phenotype analysis was performed in Cell Lab Quanta (Beckman Coulter), using the following antibodies: anti-CD29 (Chemicon, clone: TDM29), anti-CD44 (Caltag, clone: MEM-85), anti-CD73 (BD Pharmingen, clone: AD2), anti-CD90 (Chemicon, clone: F15-42-1), anti-CD105 (Caltag, clone: SN6), anti-OCT3-4 (eBiosciences, clone: EM92) and anti-CXCR4 (Caltag, clone: 12G5).

HNDPSCs obtained from the 3rd passage were frozen and stored. After the cells bypassed the Hayflick limit, the hNDPSCs' ability to differentiate into osteoblast-like cells, chondroblast-like cells and adipocytes was verified. All differentiation experiments were compared with a negative control.

The osteogenic induction medium was composed of the standard cultivation medium described above supplemented with 10 mM glycerophosphate (Bieffe Medital). For verification of osteogenic differentiation of hNDPSCs, hNDPSCs that were cultivated in the differentiation media for 21 days were fixed with 4% paraformaldehyde, embedded in paraffin and stained using the alizarin red technique to demonstrate the presence of osteogenic matrix and calcium deposits.

The adipogenic induction of the hNDPSCs was induced by using Adipogenic BulletKit (Lonza, Basel, Switzerland) according to the manufacturer's recommendation. The Oil Red O staining was performed to visualize the intracellular lipid droplets, which indicates successful adipogenic differentiation. Adipogenic differentiated cells were fixed using 10% formalin for 1 h, washed with distilled water and finally immersed for 2 h in a solution of 0.18% Oil Red O.

For chondrogenic differentiation, the cells were cultivated in micro mass cultures in non-adherent culture flasks. To induce the differentiation, cells were cultivated in the standard cultivation media enriched with 50 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) for 21 days. The chondrogenic pellets were fixed with 4% paraformaldehyde, embedded in paraffin and stained with Alcian blue to assess chondrogenic differentiation by visualization of acid mucopolysaccharides, which are highly presented within the chondrogenic matrix.

Protocol for MSCs differentiation into insulin-producing cells

For differentiation, the hNDPSCs obtained from the 6th passage were used. The two-step differentiation protocol described by Thatava et al. (2006) was used. The first step took three days and the cells were cultivated in the medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 55 nM Trichostatin-A (Sigma-Aldrich). The second step took additional seven days. The cells were cultivated in a medium composed of DMEM (Sigma-Aldrich) and DMEM/F12 (Sigma-Aldrich) in ratio 1 : 1, supplemented with glucose in a final concentration of 25 mM, 10% foetal calf serum (FCS) and 10 nM glucagon-like peptide (Sigma-Aldrich).

Immunocytochemistry staining

Fixation of cells that were cultured for 10 days in the pancreatic β-cell differentiation media in 2-well Permanox Chamber Slides Nunc (Sigma-Aldrich) was done in cold methanol for 15 min. After fixation, cell cultures were permeabilized with 0.1% Triton-X100 (Sigma-Aldrich) in PBS. The samples were incubated with primary antibodies. Insulin rabbit monoclonal antibody (C27C9, Cell Signaling Technology, Danvers, Massachusetts, ♯3014) at 1 : 400 dilutions for 24 h and C-peptide rabbit polyclonal antibody (Cell Signaling Technology, ♯4593) at 1 : 100 dilutions for 24 h, both with human, mouse and rat species reactivity, were applied. After washing with Dulbecco's phosphate buffered saline containing Tween 20, cells were incubated with secondary antibodies for 45 min in the dark at room temperature. As secondary antibodies, donkey anti-rabbit conjugated with cyanine 3 (Cy3) (Jackson ImmunoResearch, Europe Ltd, Newmarket, UK) were used. DAPI (0.1 mg/ml) was used for nuclei counterstaining. Samples were examined using a BX51 Olympus microscope equipped with an Olympus DP71 digital camera (Tokyo, Japan).

Gene expression

PDX1, HES1 and *Glut2* gene expression analysis was performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in the 7500 HT Fast Real-Time PCR System (Thermo Fisher Scientific). RNA was isolated using the TRI reagent (Sigma-Aldrich). The isolated RNA was converted into cDNA via a High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Ten ng of cDNA was loaded into each reaction consisting of TaqMan Fast Universal PCR Master Mix and pre-designed Taq-Man Gene Expression Assay (Thermo Fisher Scientific). The following gene expression assays were used: Hs00236830_m1 for *PDX1* (NCBI Reference Sequence: NM_000209.3), Hs00172878_m1 for *HES1* (NCBI Reference Sequence: NM_005524.3), and Hs01096908_m1 for *GLUT2* (NCBI Reference Sequence: NM_000340.1) (Thermo Fisher Scientific). The reactions were performed in duplicate. Data were normalized to the *18S* (Thermo Fisher Scientific) housekeeping gene. The relative expression ratio was then calculated from Ct as previously described (Hirsova et al., 2013).

Results

Characterization of hNDPSCs

During the long-term cultivation, we reached a total of 60.59/60.06 PD and the average DT was 55.96 +/–

15.38 / 57.69 +/– 17.91 h (lineage 1/2). Viability was measured in the 2nd and 10th passages and the results showed high viability rate: 97.2 %, 92.0 % / 93.4 %, 91.8% (lineage 1 – 2nd, 10th passages / lineage 2 – 2nd, 10th passages). During the study, we did not observe any signs of spontaneous differentiation, and cultivation was terminated within the 15th passage. The cultivated cells kept their morphology during the entire study. The cells were spindle shaped, mostly with two processes (Fig. 1A,C).

Phenotypic analysis was performed in the 5th passage and showed high positivity for all tested stem cell surface markers. The percentage of positive cells in lineage 1 / 2 order were CD29 – 99.76 / 99.98, CD44 – 99.72 / 99.98, CD73 – 99.72 / 99.95, CD90 – 99.87 / 99.99, CD105 – 99.95 / 99.98, OCT3-4 – 99.74 / 99.80, CXCR4 – 99.02 / 98.57.

Regarding the multipotent differentiation properties, cells were cultured in osteogenic medium for 21 days. hNDPSCs produced the extracellular matrix (Fig. 2A).

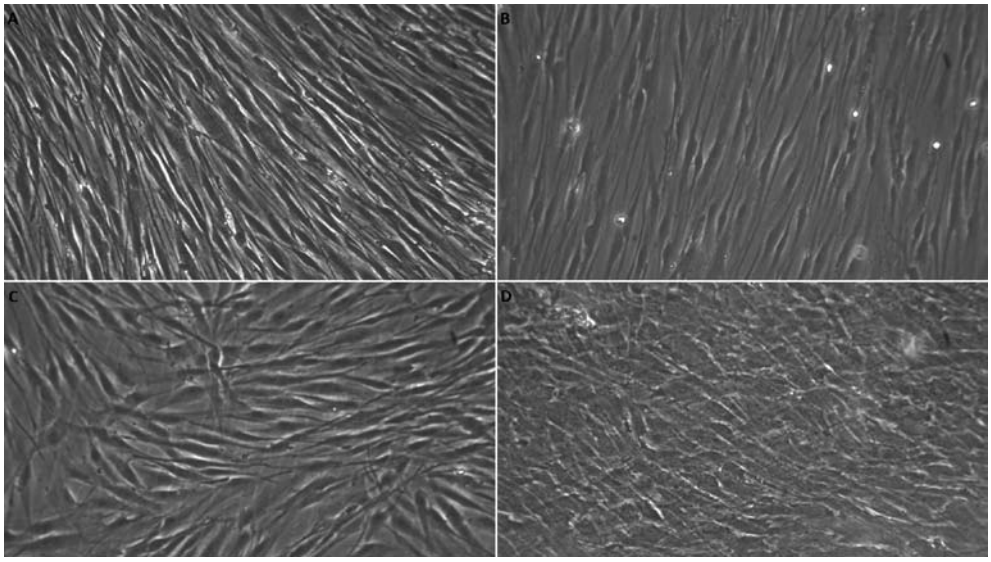

Fig. 1. **A**) hNDPSCs cultivated in standard cultivation media for 2 days. **B**) hNDPSCs cultivated in pancreatic β-cell differentiation media for 2 days. **C**) hNDPSCs cultivated in standard cultivation media for 11 days. All cells in figures A+B+C kept similar fibroblast-like morphology. **D**) hNDPSCs cultivated in pancreatic β-cell differentiation media for 11 days. Cells became flattened and more square-shaped. Phase contrast microscopy, magnification 200×

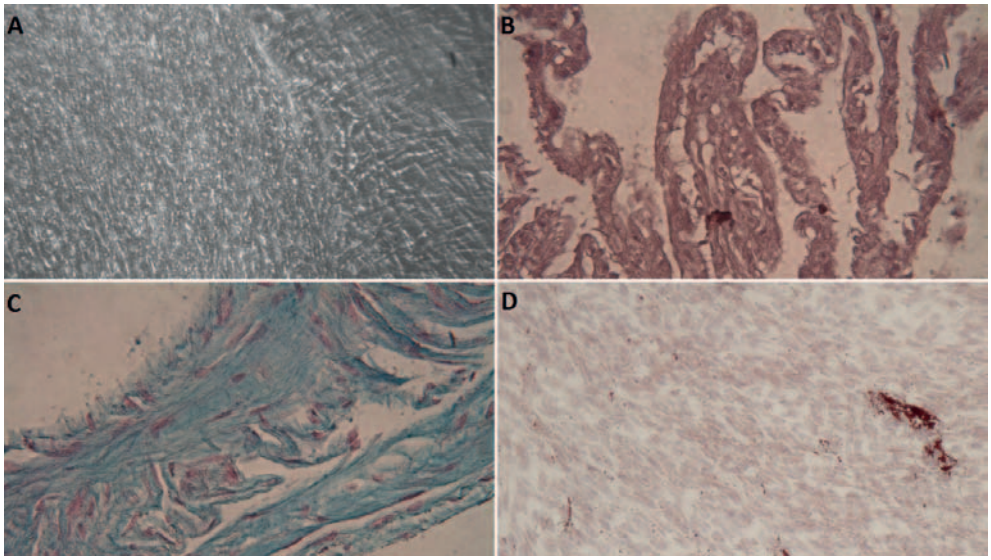

Fig. 2. **A**) hNDPSCs cultivated in osteogenic medium after 21 days produced high amounts of extracellular matrix. **B**) Micro mass of hNDPSCs cultivated in osteogenic media after 21 days and stained by alizarin red, which stained the osteoid tissue. **C**) Micro mass of hNDPSCs exposed to chondrogenic media after Alcian blue staining to visualize acid mucopolysaccharides and glycosaminoglycans. **D**) hNDPSCs cultivated for 4 weeks in adipogenic media after Oil Red O staining. Oil droplets can be seen as brown dots within the cells. Phase contrast microscopy, magnification 100×

Using the alizarin red staining technique we proved the presence of osteoid matrix with calcium deposits (Fig. 2B). For chondrogenic differentiation, cell pellets that were induced to differentiate into chondrocytes stained positively with Alcian blue (Fig. 2C), and the presence of glycosaminoglycans and acid mucopolysaccharides was demonstrated. For adipogenic differentiation, the presence of lipid droplets was confirmed by Oil Red O staining (Fig. 2D).

Differentiation into IPCs

After exposure of hNDPSCs to pancreatic β-cell differentiation media, the cells kept their standard morphology until the 2nd day (Fig. 1A,B). After the 3rd day, the cells started to change their morphology to become more flattened and lose their fibroblast-like character (Fig. 1D). Moreover, soon after inducing the differentiation, the cells stopped proliferating. hNDPSCs cultivated in the standard cultivation media (control) kept their fibroblast morphology during the entire cultivation period (Fig. 1C).

To confirm the insulin expression of IPCs at the protein level, the undifferentiated and differentiated cells were subjected to immunocytochemistry. Using the primary antibody against C-peptide (Fig. 3A,B) and insulin (Fig. 3D,E), we visualized the presence of these substances within the cells. Insulin and C-peptide were homogeneously spread within the cell cytoplasm, but insulin showed a slightly higher density around the cell nuclei. In contrast, all of the undifferentiated cells with unchanged spindle-like shape were negative for insulin and C-peptide (Fig 3C,F).

Cells treated with differentiation media demonstrated 975.4 % higher expression of PDX-1 mRNA and 469.5 % higher expression of HES1 mRNA in comparison to the cells cultivated in standard cultivation media. Glut2 transporter mRNA was absent in the non-differentiated cells and IPC differentiation induced appearance of this transporter.

Discussion

The discovery of stem cells and their successful differentiation into insulin-producing cells (IPCs) gave a new hope for the treatment of diabetes mellitus as they overcome the limitations of donor availability and rejection of β-cell transplantation (Moshtagh et al., 2013). Several studies have demonstrated the differentiation capability of various types of stem cells into IPCs. However, many of them require immunosuppression and others have yielded controversial results with their ability to secrete insulin (Lee et al., 2006).

Despite their rare incidence, neonatal teeth resemble a usually discarded source of SCs that have similar characteristics with other mesenchymal stem cells and can be easily isolated and propagated to be used in SC therapy. To our knowledge, this is the first research to investigate the potential of dental pulp derived from neonatal cells to differentiate into insulin-secreting cells.

Several protocols were used for directed differentiation of MSCs into IPCs. The protocol used in the present study was chosen as per recommendation of Gabr et al. (2014), who compared the relative efficiency of three differentiation protocols and recommended the use of this two-step one because of its simplicity and the short duration needed for differentiation.

In this research, SCs obtained from the dental pulp of a 2-day-old female infant have demonstrated the charac-

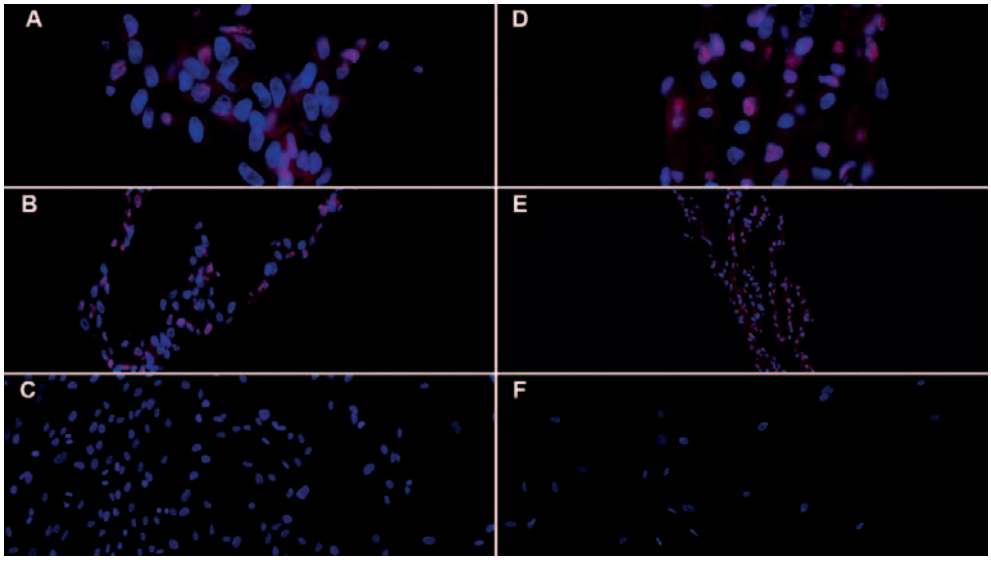

Fig. 3. hNDPSCs cultivated for 10 days in two-step IPC differentiation media. hNDPSCs stained using antibodies against C-peptide (**A**+**B**), C-peptide negative control (**C**), insulin (**D**+**E**) and insulin negative control (**F**). Blue colour marked the cell nuclei stained by DAPI, red colour indicates the presence of tested markers stained by secondary antibody conjugated with cyanine 3. We can see high positivity for both tested markers. Images were acquired using a BX51 Olympus microscope equipped with an Olympus DP71 digital camera, magnification for B+D+E+F 100× and for A+D 400×.

teristics of mesenchymal stem cells as they adhered to plastic dishes, formed colonies, self-renewed, and expressed mesenchymal specific markers CD29, CD44, CD73, CD 105, OCT 3-4, CXCR4. In addition, hNDPSCs were shown to exhibit multipotent differentiation properties into adipocytes, chondrocytes and osteocytes *in vitro*.

Several studies have demonstrated the potential of pulp cells from deciduous teeth to form SCs. SCs obtained from the pulp of deciduous teeth have demonstrated increased pluripotency and greater plasticity than adult derived SCs, and have therefore become increasingly popular to study (Govindasamy et al., 2011). Our work is in line with previous research that attempted to isolate and culture hNDPSCs for the first time, where the ease of differentiation of these cells into adipose, bone, cartilage, muscle and nerve cells was demonstrated and related to the greater plasticity and diverse immune-phenotypic characteristics of these cells (Karaöz et al., 2010).

The present study demonstrated successful *in vitro* differentiation of hNDPSCs into IPCs that were morphologically similar to pancreatic islet cells and were shown to express insulin and C-peptide, as confirmed by immunofluorescence staining. Moreover, IPCs demonstrated higher expression of PDX-1 and HES1 in comparison to the cells cultivated in standard cultivation media, and newly occurring mRNA for Glut2, which was not detectable in the negative control, confirming the differentiation of hNDPSCs into IPCs.

This is in line with previous research that has shown that SCs from other dental origin were capable of secreting insulin. In previous work investigating the pluripotency of periodontal ligament (PDL) cells, evidence suggested that SCs obtained from PDL cells can differentiate into pancreatic cells capable of insulin secretion (Huang et al., 2009). Our results are supported by a pioneer study of 2011, where researchers succeeded for the first time in differentiating dental pulp stem cells into islet-like aggregates, confirming their results by dithiozone-positive staining as well as by the expression of C-peptide, Pdx-1, Pax4, Pax6, Ngn3 and Isl-1 (Govindasamy et al., 2011). Our experiment, however, did not include the glucose challenge experiment performed in that study, and would therefore need additional trials to confirm their value for clinical applications. Similarly, a feasibility study of the differentiation potential of dental tissue-derived MSCs toward IPCs demonstrated that stem cells derived from human pulp cells exhibited a significant increase in PDX-1, NGN-3, and insulin mRNA expression and were considered to have better differentiation potential toward IPCs than stem cells derived from human periodontal ligament (Sawangmake et al., 2014)

It is worth mentioning that human PRP was used instead of FCS as culture supplement in the medium used for the cultivation and differentiation processes in this study. Although FCS is the most widely used component during long-term cultivation, scientists have started to work on animal component-free medium for SC differentiation. MSCs cultured in media containing high concentrations of FCS have demonstrated karyotypic instability, metabolic and morphological changes, and pose a risk of disease transmission and immunological reaction in human recipients, making them improper for use in human medicine (Bruinink et al., 2004; Halme and Kessler, 2006; Dictus et al., 2007; Suchánek et al., 2013). Higher concentrations of FCS have been shown to display critical side effects on cell chromosomal stability, while media with low concentrations of FCS have led to low cell proliferation rate (Suchánek et al., 2013). In a recent study, the use of 10% PRP test sera as cultivation medium has enhanced the proliferative ability of SCs from human exfoliated deciduous teeth more than the use of FCS and human plasma, suggesting that these cells can be cultivated in media supplemented with allogenic PRP, as they retain their proliferative and multidifferentiation capacity (Suchánek et al., 2016).

Conclusion

The present study documents the potential of dental pulp stem cells obtained from natal teeth to differentiate into insulin-producing cells that could prospectively be used for the treatment of diabetes mellitus.

Acknowledgement

The authors declare no conflict of interest.

References

Basavanthappa, N.N., Kagathur, U., Basavanthappa, R. N., Suryaprakash, S. T. (2011) Natal and neonatal teeth: a retrospective study of 15 cases. *Eur. J. Dent.* **5**, 168-172.

Bruinink, A., Tobler, U., Halg, M., Grűnert, J. (2004) Effects of serum and serum heat-inactivation on human bone derived osteoblast progenitor cells. *J. Mater. Sci. Mater. Med.* **15**, 497-501.

Dictus, C., Tronnier, V., Unterberg, A., Herold-Mende, C. (2007) Comparative analysis of in vitro conditions for rat adult neural progenitor cells. *J. Neurosci*. *Methods* **161**, 250-258.

Gabr, M. M., Zakaria, M. M., Refaie, A. F., Khater, S. M., Ashamallah, S. A., Ismail, A. M., El-Badri, N., Ghoneim, M. A. (2014) Generation of insulin-producing cells from human bone marrow-derived mesenchymal stem cells: comparison of three differentiation protocols. *Biomed. Res. Int*. 2014:832736.

Govindasamy, V., Ronald, V. S., Abdullah, A. N., Nathan, K. R., Ab Aziz, Z. A., Abdullah, M., Musa, S., Kasim, N. H., Bhonde, R. R. (2011) Differentiation of dental pulp stem cells into islet-like aggregates. *J. Dent. Res.* **90**, 646-652.

Halme, D. G., Kessler, D. A. (2006) FDA regulation of stem cell-based therapies. *New Engl. J. Med.* **355**, 1730-1735.

Hirsova, P., Karlasova, G., Dolezelova, E., Cermanova, J., Zagorova, M., Kadova, Z., Hroch, M., Sispera, L., Tomsik, P., Lenicek, M., Vitek, L., Pavek, P., Kucera, O., Cervinkova, Z., Micuda, S. (2013) Cholestatic effect of epigallocatechin gallate in rats is mediated via decreased expression of Mrp2. *Toxicology* **303**, 9-15.

Huang, A. H., Chen, Y. K., Lin, L. M., Shieh, T. Y., Chan, A. W. (2008) Isolation and characterization of dental pulp stem cells from a supernumerary tooth. *J. Oral Pathol. Med.* **37**, 571–574.

Huang, C. Y., Pelaez, D., Dominguez-Bendala, J., Garcia-Godoy, F., Cheung, H. S. (2009) Plasticity of stem cells derived from adult periodontal ligament. *Regen. Med.* **4**, 809-821.

Kadam, M., Kadam, D., Bhandary, S., Hukkeri, R. Y. (2013) Natal and neonatal teeth among cleft lip and palate infants. *J. Maxillofac. Surg.* 4, 73-76.

Karaöz, E., Doğan, B. N., Aksoy, A., Gacar, G., Akyüz, S., Ayhan, S., Genç, Z. S., Yürüker, S., Duruksu, G., Demircan, P. C., Sariboyaci, A. E. (2010) Isolation and in vitro characterization of dental pulp stem cells from natal teeth. *Histochem. Cell Biol.* **133**, 95-111.

Lee, T. C., Barshes, N. R., Agee, E. E., O'Mahoney, C. A., Brunicardi. F. C., Goss, J. A. (2006) The effect of whole organ pancreas transplantation and PIT on diabetic complications. *Curr. Diab. Rep.* **6**, 323-327.

Li, Z., Jiang, C. M., An, S., Cheng, Q., Huang, Y. F., Wang, Y. T., Gou, Y. C., Xiao, L., Yu, W. J., Wang, J. (2014) Immunomodulatory properties of dental tissue-derived mesenchymal stem cells. *Oral Dis.* **20**, 25-34.

Limbert, C., Seufert, J. (2009) In-vitro (re)programming of human bone marrow stromal cells toward insulin-producing phenotypes. *Pediatr. Diabetes* **10**, 413-419.

Moshtagh, P. R., Emami, S. H., Sharifi, A. M. (2013) Differentiation of human adipose-derived mesenchymal stem cell into insulin-producing cells: an in vitro study. *J. Physiol. Biochem.* **69**, 451-458.

Sawangmake, C., Nowwarote, N., Pavasant, P., Chansiripornchai, P., Osathanon, T. (2014) A feasibility study of an in vitro differentiation potential toward insulin-producing cells by dental tissue-derived mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* **452**, 581-587.

Seminario, A. L., Ivancakova, R. (2004) Natal and neonatal teeth. *Acta Medica (Hradec Kralove)* **247**, 229-233.

Suchánek, J., Kleplová, T. S., Kapitán, M., Soukup, T. (2013) The effect of fetal calf serum on human dental pulp stem cells. *Acta Medica (Hradec Kralove)* **56**, 142-149.

Suchánek, J., Suchánková Kleplová, T., Řeháček, V., Browne, K. Z, Soukup, T. (2016) Proliferative capacity and phenotypical alteration of multipotent ecto-mesenchymal stem cells from human exfoliated deciduous teeth cultured in xenogeneic and allogeneic media. *Folia Biol. (Praha)* **62**, 1-14.

Thatava, T., Ma, B., Rohde, M., Mayer, H. (2006) Chromatin-remodeling factors allow differentiation of bone marrow cells into insulin-producing cells. *Stem Cell* **24**, 2858-2867.

Uzamis, M., Olmez, S., Ozturk, H., Celik, H. (1999) Clinical and ultrastructural study of natal and neonatal teeth. *J. Clin. Pediatr. Dent.* **23**, 173-177.

Wang, C. H., Lin, Y. T., Lin, Y. J. (2017) A survey of natal and neonatal teeth in newborn infants. *J. Formos. Med. Assoc.* **116,** 193-196.