Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 1, January - March 2013, p. 313 - 321

BEHAVIOURAL CHANGES AND PLASTIC POTENTIAL ALTERATION OF DENTAL PULP STEM CELLS EXPOSED TO HIGH GLUCOSE CONCENTRATIONS

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Considering the major interest in stem cells tissue engineering, our aim was to investigate the osteogenic differentiation of dental pulp stem cells when exposed to glucose enriched medium. Dental pulp derived stem cells were isolated and differentiated into osteoblasts showing how, under the influence of cellular stress factors, their behaviour changes. Molecular analysis was performed to assess the expression of characteristic osteogenic markers. After isolation, and culture of mesenchymal stem cells, cell viability testing and immunophenotyping was performed. Half of the cultured cells were placed on simple osteogenic differentiation media, the other half was placed on 4,5 g/l glucose enriched differentiation media. Cell viability of DPSC was over 90%. Cells showed a fibroblastoid phenotype, characteristic of mesenchymal stem cells. Starting day 15 of culture on simple osteogenic medium, approximately 20% of the cells changed their shape, showing morphological aspects of osteoblasts. An intense positive response can be seen for CBFA1 protein, alkaline phosphatase and a decreased but present signal for OSTERIX and OSTEOCALCIN. Shifts in cell dimension, cell density, unequal spaces and various cell shapes were found in the glucose enriched media. Taken together, we consider that high glucose disturbs the osteogenic differentiation of DPSC cells, in a supplemented medium.

(Received December 14, 2012; Accepted January 31, 2013)

Keywords: Teeth, Pluripotent cells, Cell viability, Cell metabolism changes, Osteogenic differentiation.

1. Introduction

During the odontogenesis the same cells forming the odontoblasts, coming from the Hertwig lamina, are involved in the development of several but different hard-tissues, including crown and root dentin, cementum and alveolar bone [1] ; their physiologic activity does not end with the eruption because for DPSCs it has been hypothesized a role in resorption of deciduous teeth too[2] . After Gronthos studies [3] other researchers confirmed how these stem cells, under specific stimuli [4], differentiated into multiple cell types, including neurons, adipocytes and chondrocytes [5,6] although the main commitment remains to form bone [7-9]. Dental pulp stem cells showed differentiation profiles similar to those showed during bone differentiation [10] and this event make them very interesting as a model to study the osteogenesis [11]. Due to their high proliferation rate and efficiency in producing bone chips, DPSCs seem to be the best candidates to study bone formation.

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Thus it is very important to see how DPSC proliferate and differentiate into different cell types but it is also important to in vitro assess how the proliferation and differentiation potentials are affected by different stress factors such as high glucose level, that are possible to occur in vivo.

During the in vitro ossification process, the DPSCs cells give rise to both osteoblasts and endotheliocytes, and to bone containing vessels, leading to the formation of an adult bone tissue after in vivo transplantation.

It has been also reported that the tissue culture of DPSCs in high glucose media results in a reduction of collagen synthesis [12], though no cell culture studies of DPSCs in high glucose media have been published. High glucose also reduces the proliferation and osteogenic differentiation of periodontal ligament cells [13].

Considering the major interest in developing tissue regeneration techniques using progenitor stem cells our aim was to investigate the osteogenic differentiation of dental pulp stem cells using, as cellular stress factor, glucose enriched medium. We show here that we can isolate pulp derived mesenchymal progenitor stem cells and differentiate them into osteogenic cells showing how, under the influence of cellular stress factors like high glucose their plastic potential is affected.

2. Material and methods

Chemicals and Reagents: Mesencult (Stem Cell Technologies), Dulbecco's modified eagle's medium (DMEM, Sigma Aldrich), Dulbecco's Phosphate buffered saline, trypsin-EDTA 0.25%, penicillin/ streptomycin, trypan blue (Sigma Aldrich, Redox Trading-Romania), plates Petrii (Nunc - Denmark), FCS (PromoCell, Heidelberg, Germany), anti-CD14, CD29, CD44, CD45, CD90, CD146 mAbs (BD Biosciences, Erembodegem, Belgium). The necessary apparatus consist in: Holten-type biosafety cabinet with laminar flow, reverse phase microscope Olimpus IX 70-type, Jouan Incubator, Jouan centrifuge, Artic freezer, Facs Sort flow-cytometer (BD Bioscience Heidelberg Germany) with Lysis II analysis software. Consumables used: sterile graded and transfer pipettes, sterile pipette heads, various capacities micropipettes, pipetting device, Neubauer chamber, 50 ml and 15 ml sterile centrifuge conical tubes, 0.5 ml microcentrifuge tubes for cell counting, gloves (latex, vinyl, nitrile), gown or protective equipment.

2.1 Harvesting and isolation of dental pulp stem cells

Dental pulp was harvested from teeth extracted for orthodontic purposes from subjects aged 12-17 years. This study was approved by the Internal Ethical Committee of the University of Medicine and Pharmacy Timisoara, Romania and respected all European understandings signed by our country (EU Directive 93/35/EEC, Amendment 76/768/EEC, directive 86/609 CEE), as well as the Romanian law (Law 471/2002, Ordonance 37/2002). All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki and written informed consent has been obtained from all the patients involved in the study.

Before extraction, each subject was checked for systemic and oral infection or diseases. Only disease-free subjects were selected. Each subject was pretreated for a week with professional dental hygiene. The extraction has been performed as less traumatically as possible in order to avoid dental damage, as well as to protect the alveolar bone.

After extraction, the pulp was extracted with a dentinal excavator or a Gracey curette the periodontal tissue and other soft tissues have been removed with a curette. The incisal and the apical parts were removed to facilitate the elimination of pulp chamber and root canal content.

The harvested tissue was transported in a tube containing PBS + 10% FCS + 1% penicillin / streptomycin and sectioned to obtain small pieces of approximately 0.5 mm^3. Isolation of mesenchymal stem cells has been done through simple plastic adhesion.

2.2 Culture of dental pulp stem cells

Mesenchymal stem cells were further cultured on basic culture media (Mesencult, Stem Cell Technology). The isolated cells were digested using 3 mg/ml collagenase IA solution for 2h at 37°C. After washing by centrifugation at 1600 RPM for 5 minutes, the cells were suspended in 10 ml PBS +10%FCS, followed by another centrifugation at 1600 RPM for 5 minutes. DPSCs were cultivated until they reached confluence, which can be seen after approximately 14-21 days. Afterwards cells have been immersed into Trypsin-EDTA washed by centrifugation and placed on other culture plates at a concentration of $1x10^5$ cells/ml, for further studies. The obtained cells were analyzed at the isolation moment and at different time points during their evolution.

The resulting pellet was once more suspended in 1ml PBS, counted and cell viability was assessed by using Trypan Blue solution. Dead cells with destroyed cell membrane were colored blue and normal cells stay uncoloured.

2.3 Immunophenotyping

Preparation of specimens for flow-cytometric analysis has been performed to define the phenotypical and functional properties of the mesenchymal stem cells. A FACS Sort flow-cytometer with Lysis II analysis software has been used. Data were examined by dedicated software (WinMDI). Statistical analysis of the resulting data has been performed with SPSS software for Windows 8.0; Log-Rank was the statistical test used. The value $p<0.01$ has been considered statistically significant. For the phenotypic analysis we have applied the following protocol. During the first phase, we have washed the mesenchymal stem cells 2x for 4min/1600 rpm with standard RPMI medium, adjusting the concentration at $1x10^5$ cells/ml and then we resuspended it in PBS 0.1% BSA 200µl/specimen. After that, we have performed the marking with monoclonal antibodies in 5 µl /specimen and put the specimens to incubate in darkness for 15 minutes. The next step consisted in the washing of specimens by centrifugation (resuspension in 2 x incubation volume [400 µl]), and at the end we resuspended them in 500 µl PBS 0.1% BSA. The resulting mesenchymal cells have been marked with monoclonal antibodies for further flow-cytometric study.

Flowcytometrical analysis and a combination of monoclonal antibodies (CD45FITC/CD14PE/CD34FITC/CD31PE/CD90FITC/CD29PE/CD44FITC/CD146PE/HLA-DRFITC/CD13PE/CD105PE/CD106PE*/isotype control FITC/isotypecontrolPE)* have been used to identify and quantify the mesenchymal stem cells.

Osteogenic differentiation of DPSC in normal and glucose enriched differentiation media

We studied the differentiation potential towards the osteogenic cell line. After day 14 of culture the first passage has been performed and cells have been further cultured on basic medium until day 21 (second passage) afterwards being immersed in Trypsin and then placed on proliferation medium for 24 hours. Afterwards osteogenic differentiation medium was added and cultures were observed for another 21 days.

Half of the cultured cells were placed on osteogenic differentiation media consisting of DMEM, 0,05Mm 2–phosphate–ascorbic acid, 10 Mm β–glicerolphosphate 10^{-7} M dexamethazone at a cellular density of 10^5 cells/cm^2. The other half was placed on the same differentiation media enriched with 4,5 g/lglucose. Cell morphology and mineralization using the Jakob von Kossa staining was evaluated. Molecular analysis was performed to assess the expression of characteristic osteogenic markers.

3. Results

3.1 Characterization of DPSCs

Following the isolation procedures, cell viability of DPSC has been over 90%. The cultured DPSC have been observed every second day at the inversed microscope. After the first week small conglomerates of adherent cells, oriented towards the edges of the plate could be seen

showing the fibroblast aspect. We have ascertained that the cells have a fibroblastic phenotype, with fine prolongations and tendency to dispose in networks, characteristic of mesenchymal stem cells (Fig.1).

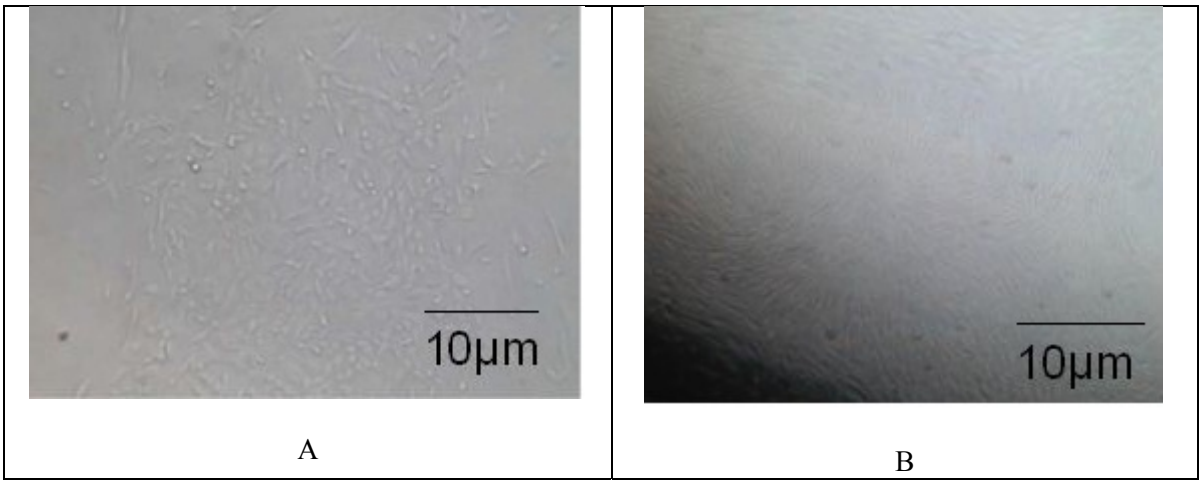

Fig. 1 A. Characterization of DPSCs on basic culture medium showing their fusiform aspects with a tendency to gather in concentric circles, day 12, magnification x10; B. Characterization of DPSCs on basic culture medium. Colonies having a vortex orientation facing the edges of the culture plate, day 21, magnification x 10.

3.2 Flow-cytometric analysis

Based on the analyzed marker combination we have established the following antigenic profile: CD45-CD14-CD34-CD31-CD13-CD90+CD29+CD44+CD146+ HLA-DR low+CD105+CD106+ (Table 1).

Table 1. Osteogenic markers expression. Expression of different mesenchymal stem cells characteristic markers after each passage.

Marker	P1	P2	P3
CD45	0,2%	0,1%	0,01%
CD14	0,2%	0%	0%
CD34	0,2%	0,03%	0%
CD31	0,3%	0,01%	3,8%
CD29	95,2%	95,4%	98,7%
CD90	99%	98,6%	95%
CD44	98,9%	99,7%	97%
CD146	91%	83,6%	87%
CD105	78%	77,4%	51,3%

The phenotype has been studied using CD14, CD29, CD44, CD45, CD90 and CD146 surface markers (Fig. 2A-D).

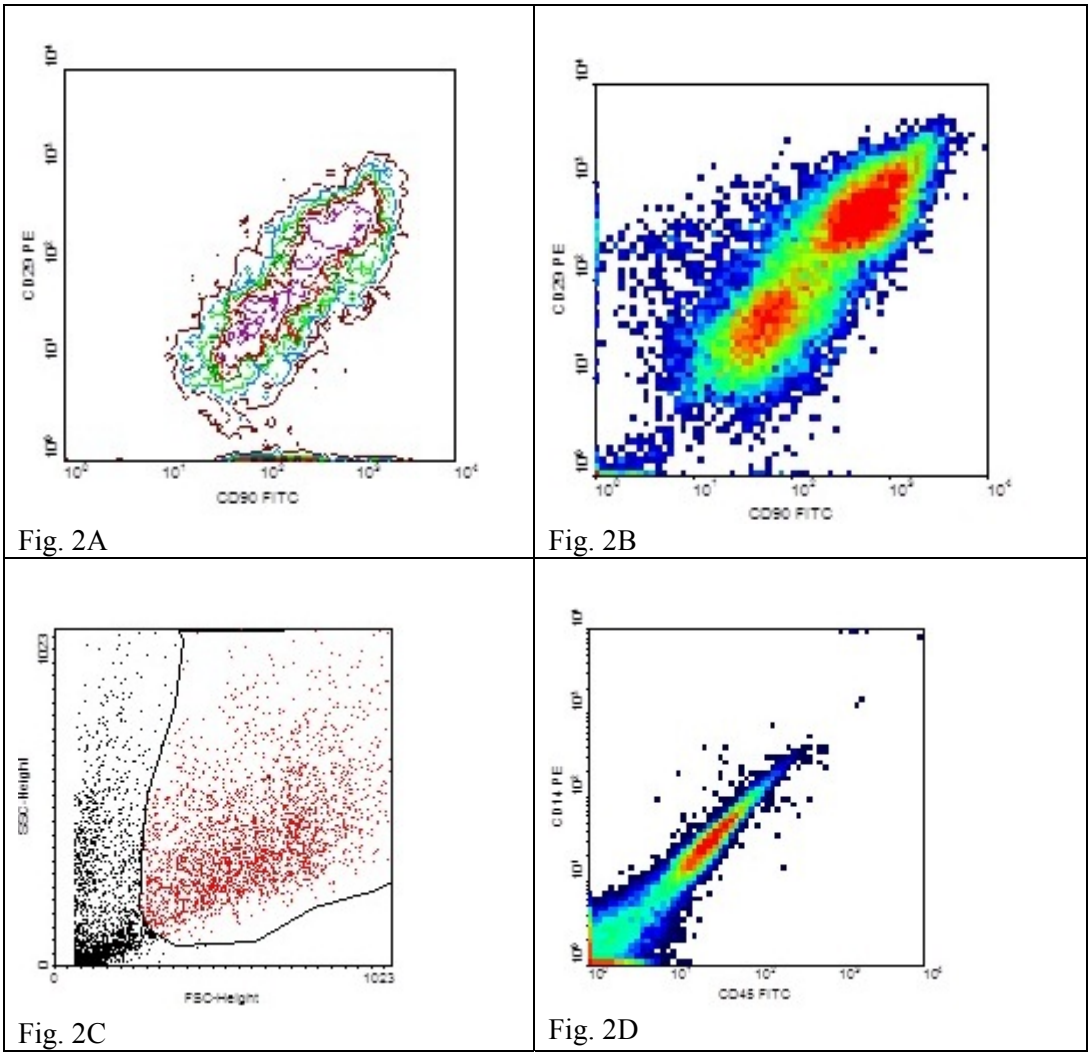

Fig. 2 A. B Most of the human mesenchymal cells are double-positive for CD90 and CD29; C. Flow-cytometric appearance of human mesenchymal cells; D. Human mesenchymal cells do not express CD14 and CD45

3.3 Morphological analysis of the osteogenic differentiated cells

After exposure in high and normal glucose medium, cell morphology has been observed with a phase contrast optical microscope. Starting day 15 of culture, approximately 20% of the cells cultured on simple differentiation medium changed their shape, becoming polygonal, showing morphological aspects of osteoblasts. Periodic evaluation of the plates has been performed until day 21 of culture observing an evident change of cell morphology. Cells got a rounded shape, polygonal having a tendency to gather in concentric circles (Fig. 3A, B).

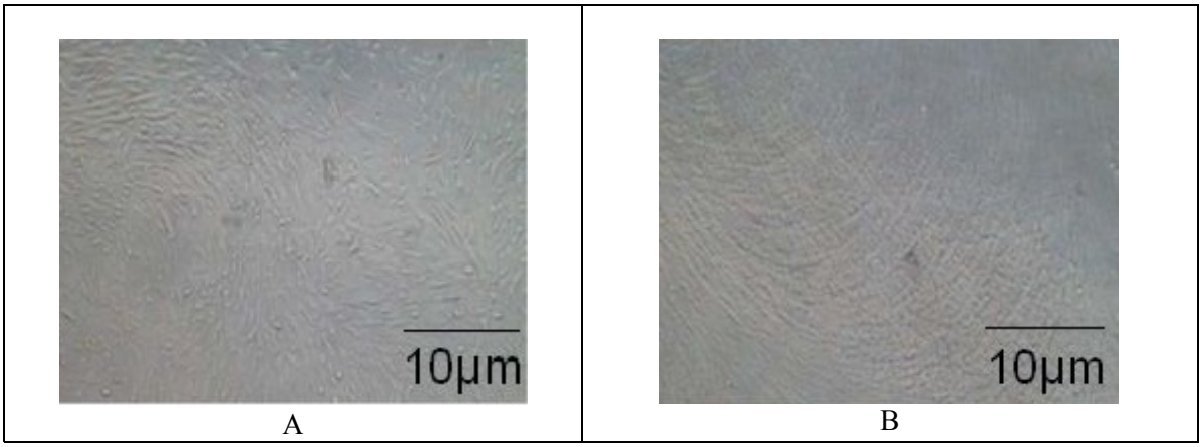

Fig. 3 A. Cell culture on normal differentiation medium, day 10, polygonal aspect, original magnification x 10; B. Cell colonies on normal differentiation media with concentrical disposal
showing characteristic of the osteogenic cell line, day 21 magnification x 10.

After the second passage the cell viability in high glucose medium was 40%. Cell dimensions vary upon the osmolar medium enriched with glucose, suggesting adjustments of cell metabolism. Shifts in cell density, increase of cellular volume, fader cellular outlines, consolidation of a dense cell network with unequal spaces and various cell shapes were found in the glucose enriched media (Fig. 4 A, B).

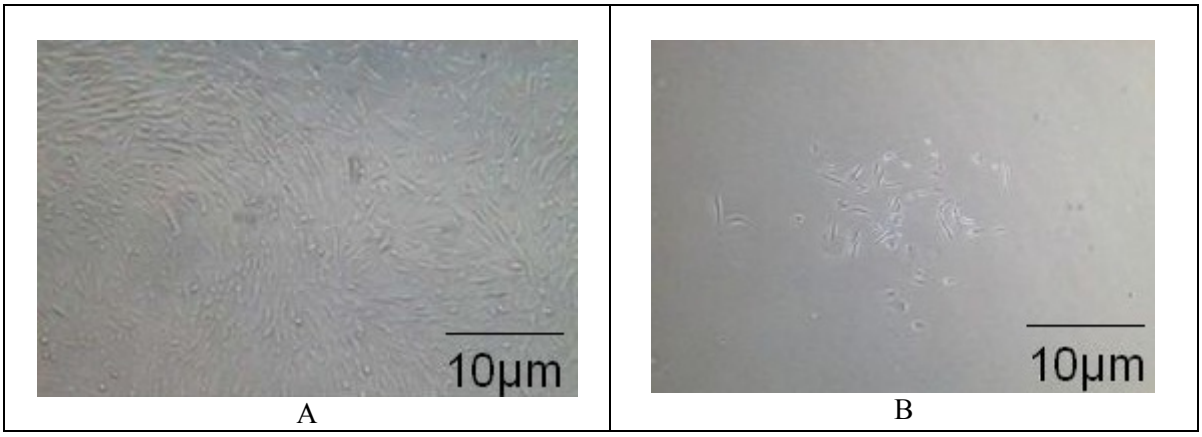

Fig. 4 A. Early signs of osteogenic differentiation in high glucose medium cells changing their shape in becoming rounded and polygonal, day 10, magnification x 10; B. Cell culture in high glucose media, third passage day 21, shift in cell density, unequal spaces in the cell network and
various cell shapes, magnification x 10.

3.4 Mineralized matrix deposits assessment

After 3 weeks in culture the mineral extracellular matrix deposits has been assessed through the Jakob von Kossa mineralization reaction indicating clear presence of extracellular mineralization areas on the normal glucose concentration cultures, whereas smaller amounts mineralized matrix has been observed on the cell cultures enriched with glucose.

3.5 Osteogenic markers expression

Molecular biology data, performed 3 weeks after induction confirm the expression of characteristic osteoblast markers on only normal glucose concentration cultures because in high concentration media cell density decreased after the third passage.

An intense positive response can be seen for CBFA1 protein, alkaline phosphatase and a decreased but present signal for OSTERIX and OSTEOCALCIN (Fig. 5).

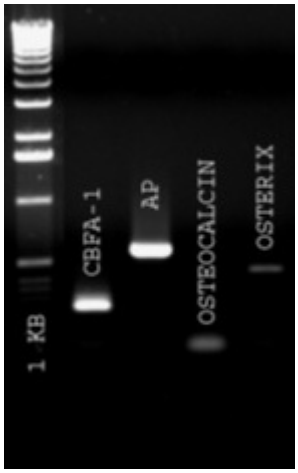

Fig. 5. Molecular analysis of osteogenic markers: CBFA1 protein, alkaline phosphatase, osterix, osteocalcin.

4. Discussion

In our study we isolated and cultured DPSC's, assessed their plastic potential by differentiating them into the osteogenic cell line and investigating their behavioural changes during the exposure to high glucose levels. If in normal medium cells showed adaptability and differentiation, in high glucose medium osteogenic differentiation was much lower, cells showing a constant decrease of viability after the third passage. Previous studies indicated that dental pulp has stem cells that contain not only odontogenic potential but also osteogenic capacity[14]. While pulp stem cells can be differentiated into multiple cell types, it has been reported that after a number of passages, pulp stem cells display only osteogenic capacity [15]. Intentional tooth replantation has shown that pulp tissue possesses tartrate resistant acid phosphatase-positive activity, and bone-like tissue is formed in the pulp chamber from DPSCs[14]. Mechanical stimuli, like pulsating fluid flow, causes pulp stem cells to differentiate into osteoblasts [16].

Our study showed that osteogenic markers are expressed after exposing DPSCs to osteogenic differentiation media. Alkalin phosphatase (ALP) serves as a useful marker for the transition from the proliferative period to matrix maturation in osteogenesis. In some studies, a time-dependent effect of osteogenic medium on ALP activity was observed. ALP activity increased in the control cells and in the cells induced for osteogenic differentiation after 3 days of culturing. ALP activity increased approximately fivefold after 1 week of culturing and continued to increase. After 14 days of culturing, the activity stopped rising and started to decrease. These results are supported by the findings of previous studies [17, 18].

Several previous studies showed that DPSC had high alkaline phosphatase activity and exhibited osteoblast markers such as osteocalcin release and mineralized matrix formations, when supplemented media containing ascorbic acid, β-glycerophosphate and/or dexamethasone were given.

In glucose enriched medium changes of proliferation and differentiation behaviour has been seen in our study, shown by a decrease of the cellular density, the increase of the cells

volume induced by the overrated nucleocytoplasmic volume, cytoplasmic vacuolisation, wiped cellular outline, with unequal intercellular spaces and variations in the shapes of the cells. These findings suggest that sustained high glucose concentration amounts alone inhibit the osteogenic differentiation potential of DPSCs. Decreased optical densities were seen on cell cultures in high glucose medium, and that means reduced cell viability. Our results suggest that dental pulp derived osteogenic cells respond in the same way other cells do regarding growth rate in high glucose concentration. Turner and Bierman [19], as well as Hehenberger and Hansson [20] stated that glucose was important for cell proliferation. They have shown that increasing glucose levels to 18 and 15.5 mM, respectively, increases fibroblast proliferation, whereas further increase lead to an inhibition of proliferation.The reason for the reduced proliferation rate in high glucose medium has been explained in detail. Hehenberger and Hansson [20] showed that a glucose amount of 15.5 mM and above inhibited fibroblast proliferation and induced resistance of growth factor such as IGF-I and EGF. This proliferation was more inhibited as the ratio of the glucose enriched media to normal media became larger. They stated that due to the amount of D-glucose in the media, the L-lactate levels increased in all cell types and that L-lactate production may play a role in the proliferation of fibroblast *in vitro*.

There is evidence that high glucose concentration impairs the responsiveness of odontoblasts to insulin-like growth factor stimulation *in vitro.* As mannitol does not have the same effect, this inhibition is specific to glucose [21]. This effect was not due to cytotoxicity of the glucose, because after seven days of incubation in high glucose concentration, cells that were transferred to normal glucose concentration resumed normal proliferation [21].

Based on previous studies, a supplemented medium containing 50 mg/ml of ascorbic acid, 10 mM of b-glycerophospate and 100 nM of dexamethasone was used to determine the effect of glucose on the mineralization of other cell cultures, like PDL [22-24]. These studies showed that the control PDL cells initiated mineralized nodules around day 14 [25]. Larger and much more mineralized nodules were formed as time passed. On day 21, mineralized nodules could be identified in the control group by the naked eye. Thus, PDL cells in the control group responded to supplemented media in the same way as other studies showed. The PDL cells in low glucose supplemented medium had a larger calcified area, five to six times bigger compared to the PDL cells in high glucose supplemented medium. The PDL cells in high glucose medium had decreased nodule formation, macroscopically and microscopically, on day 21, suggesting that differentiation into osteoblast-like cells was inhibited [26].

In order to obtain more information about the progenitors of hard tissue forming cells of the dental pulp, some studies compared the proliferation and differentiation of DPSC cultured in high glucose media with those in normal media [27]. The cultured cells were immunohistologically stained for the endothelial cell marker eNOS and mural cell markers such as NG2, calponin and α-SMA; those immunostainings were quantitatively examined. The time-dependent response behaviour of these markers was compared with that of the others, as well as with the behaviour of ALP activity, osteonectin, osteocalcin and DSPP.

The time-dependent expression manner of osteonectin in high-glucose media was no different from that in normal media in that there was no phase lag of expression peaks between high and normal glucose concentrations. On the other hand, DSPP indicated a clear phase lag due to the fact that odontogenesis is different from osteogenesis.

In conclusion, it is considered that high glucose disturbs the differentiation of DPSC cells, as well as osteoblasts, in a supplemented medium. The decreased cellular differentiation activity due to high glucose concentration amounts may compromise healing and regeneration.

Acknowledgements

The authors express their thanks to the staff of the University of Medicine and Pharmacy “Victor Babes” Timisoara, Romania. The Pathology Department of the University of Medicine and Pharmacy “Victor Babes” Timisoara, Romania, especially Liliana Vasile, deserve thanks for their continued excellence in tissue processing and for her assistance in the histomorphometric analysis.

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