# Behaviour of human dental pulp stem cell in high glucose condition: impact on proliferation and osteogenic differentiation

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**Behaviour of human dental pulp stem cell in high glucose condition: impact on proliferation and osteogenic differentiation.**

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**<u>Running title:</u> Dental stem cells and glucose condition**

Abbreviations: AR (Alizarin red), BM (bone marrow), DMEM (Dulbecco's modified Eagle's medium), DMSO (Dimethysulfoxide), DPSCs (dental pulp stem cells), FBS (fetal bovine serum), HG (high glucose), LG (low glucose), MSCs (mesenchymal stromal/stem cells), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), PBS (Phosphate Buffered Saline), PDLSCs (periodontal ligament stem cells), SCAPS (stem cells from the apical papilla), SHEDs (stem cells from human exfoliated deciduous teeth),

**Abstract**

*Objective:* The aim of this study is to investigate the changes of human dental pulp stem cell (hDPSC) viability, proliferation and osteogenic differentiation in high glucose condition.

*Design:* After 21 days of culture in low (5.5 mM) and high (20 mM) glucose medium, hDPSC viability and proliferation were assessed with respectively the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst assays.

To investigate the influence of glucose on osteogenic differentiation hDPSCs were cultured for 28 days in low or high glucose medium with osteoinductive cocktail. Mineralization was examined by alizarin red staining/quantification and the expression of osteogenic-related genes [*Runt-related transcription factor 2* (*RUNX2), Osteocalcin (OCN), Collagen 1A1 (COL1A1)*] analyzed by RT-qPCR.

*Results:* We observed no significant difference ($p > 0.05$) on hDPSC proliferation or cell viability between low or high glucose groups. We did not highlight a significant difference after alizarin red staining and quantification between hDPSCs cultured with high or low glucose concentration in the culture medium. In the same manner, high glucose concentration did not appear to modify osteogenic gene expression: there was no significant difference in osteogenic-related gene expression between high or low glucose groups.

*Conclusion:* Proliferation, viability, and osteogenic differentiation of hDPSCs were not changed by high glucose environment.

**Key words:** dental pulp stem cells; cell proliferation; osteogenic differentiation; glucose; bone tissue engineering

## 1. Introduction

The main purpose in tissue engineering is to reconstruct native tissue by associating progenitor or mesenchymal stromal/stem cells (MSCs) and a suitable scaffold. Some studies have shown that MSCs represent attractive cells for bone tissue engineering (Le Blanc & Pittenger, 2005; Neuss et al, 2008; Zhang et al, 2009). MSCs can be obtained from different sources, but bone marrow MSCs (BM-MSCs) are widely considered as a gold standard (Polymeri, Giannobile & Kaigler, 2016). BM-MSCs have been successfully used to repair critical-size bone defects *in vivo*, and their use is well-documented for years (Quarto et al, 2001; Wexler et al, 2003). However, BM-MSCs present some disadvantages (harvested by invasive and painful surgical procedure), and other sources of MSCs have been considered.

Among the wide family of MSCs of human origin such as adipose tissue-derived MSCs or umbilical cord-derived MSCs, dental pulp stem cells (DPSCs) are MSCs that originate from the cranial neural crest and reside within the perivascular niche of the dental pulp. DPSCs were first isolated and described in the early 2000s (Gronthos, Mankani, Brahim, Robey & Shi, 2000) and represent a valuable non-invasive source of MSCs for tissue engineering or cell therapies (Kichenbrand, Velot, Menu & Moby, 2019). Interestingly, they are easily accessible with limited morbidity in the operation site and are often obtained from tooth extraction for orthodontic reasons. DPSCs have immune privilege and anti-inflammatory properties (Kichenbrand, Velot, Menu & Moby, 2019), display high growth rate (Alge et al, 2010), multilineage differentiation ability (Zhang, Walboomers, Shi, Fan, & Jansen., 2006) and so can regenerate various tissues. The osteogenic potential of DPSCs was described previously (Cha et al, 2015; Chamieh et al, 2016; Giuliani et al, 2013; Laino et al, 2006[a]; Ling et al, 2015; Wongsupa, Nuntanaranont, Kamolmattayakul, & Thuaksuban, 2017). DPSCs are capable of differentiating into osteoblasts *in vitro* after an induction by dexamethasone, ascorbic acid and

β-glycerophosphate supplementation (Cha et al, 2015; Laino et al, 2006[b]). They are able to form woven bone *in vitro* and *in vivo* by secreting abundant extracellular matrix (Laino et al, 2006[b]). This osteogenic potential of DPSCs has been demonstrated in rat models (Chamieh et al, 2016; Ling et al, 2015), rabbit models (Wongsupa, Nuntanaranont, Kamolmattayakul & Thuaksuban, 2017) and in human studies to repair jaw defects (Giuliani et al, 2013). This osteogenic potential is therefore of major interest, notably in the field of oral and maxillofacial defect therapeutics.

Despite the considerable promise offered by MSCs to repair damaged bone tissue, bone constructs in human trials often present modest or short-lived benefits. The key to overcoming the limits after transplantation may lie in better understanding the influence of biochemical microenvironment and local factors such as hypoxia or glucose concentration. Glucose plays a major role in modifying cells proliferation and differentiation *in vitro* (Stolzing, Coleman & Scutt, 2006; Weil, Abarbanell, Herrmann, Wang & Meldrum, 2009). Pathological conditions such as diabetes mellitus are associated with hyperglycemic environment that impacts osteoblasts and bone formation (Kalaitzoglou, Popescu, Bunn, Fowlkes & Thrailkill, 2016). Thus, diabetic patients are known to present an increased risk of delayed bone healing, that lead to major issues especially in the field of oral and maxillo-facial surgery (Jiao, Xiao & Graves, 2015). Bone tissue engineering in those patients require using MSCs that can overcome high glucose conditions in order to improve the benefits of bone constructs. Elucidating the impact of glucose concentration on cells culture is essential to enhance cell survival and differentiation potential. *In vitro,* MSCs can be amplified and differentiated toward different cell lineages for regenerative therapies by applying culture media of different composition, notably various glucose concentrations (Saki, Jalalifar, Soleimani, Hajizamani, & Rahim, 2013). The effects of glucose concentration have been investigated on several types of MSCs, including BM-MSCs (Elseberg et al, 2012; Li et al, 2007; Stolzing, Bauer & Scutt, 2012; Wang et al, 2013; Zhang

et al, 2017), adipose-derived mesenchymal stem cells (Liang et al, 2012), periodontal ligament stem cells (PDLSCs) (Kato et al, 2016; Seubbuk, Sritanaudomchai, Kasetsuwan & Surarit, 2017), tendon-derived stem cells (Lin et al, 2017), embryonic stem cells (Yang, Shen, Reece, Chen & Yang, 2016) or stem cells from the apical papilla (SCAPs) (Wang, Wang, Lu & Yu, 2019). To date, existing studies on the influence of glucose on MSC proliferation and differentiation exhibited quite puzzling results and did not provide a clear answer to the exact influence of glucose.

Furthermore, only one work focused on the impact of glucose on DPSC functioning (Kanafi, Ramesh, Gupta & Bhonde, 2013), and its repercussion on DPSC osteogenic differentiation has not yet been clearly studied.

Therefore, in this work, we aimed to investigate the changes of DPSC proliferation, viability and osteogenic differentiation in high glucose environment. We chose two glucose concentrations that corresponds to the routinely used culture medium (5.5 mM and 20 mM).

## 2. Materials and methods

### *2.1. Isolation and culture of human DPSCs (hDPSCs)*

#### *2.1.1. Isolation of hDPSCs*

Impacted third molars were collected from healthy donors (aged 13–17 years) after receiving written informed consent from the patients and their parents, according to the amended Declaration of Helsinki and following a protocol approved by the French Ministry of Higher Education and Research (CELSORDINO: ID-RCB: 2017-A00860-53). Only teeth between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) were collected. The explant method was used to isolate hDPSCs as described previously (Ducret et al, 2015; Hilkens et al, 2013). Briefly, dental pulps were gently extirpated from teeth and cut

into small fragments. Explants were placed into 6-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

*2.1.2. Culture of hDPSCs*

DPSCs migrated from the pulp explants were amplified ($5x10^3$ cells/$cm^2$) in the same medium and maintained in a humidified atmosphere of 5% $CO_2$ at 37°C with a medium changes two times a week. They were either frozen after two passages or used to confirm the MSC phenotype by flow cytometry. Cells were negative for the hematopoietic surface markers CD34, CD45 and positive for CD73, CD 90 and CD105 (data not shown). Cells isolated from three different donors were used.

*2.2. Assessment of cell proliferation*

Post-thaw P3-DPSCs were plated in 6-well plates at conventional seeding density ($5x10^3$ cells/$cm^2$) and cultured for 21 days, in a humidified atmosphere of 5% CO2 at 37°C, shared in two groups: 1) low glucose DMEM (LG-DMEM) with a glucose concentration of 5.5 mM; 2) high glucose DMEM (HG-DMEM) with a glucose concentration of 20 mM. Media were purchased from Gibco and supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B (Sigma-Aldrich). The medium was changed every 3–4 days.

Cell proliferation was evaluated by measuring DNA concentration. This last was assessed using Hoechst 33342 fluorescent stain at days 1, 3, 7, 14 and 21. Cell amounts were determined by a fluorometric quantification of DNA using Hoechst assay. Cells were trypsinized (TypLE®, Gibco), centrifuged at 300*g* during 10 min and diluted in Hoechst buffer, followed by three cycles of freezing (−80 °C) and thawing (37 °C). After cell lysis, Hoechst 33342 fluorescent dye (0.1 µg/ml, Sigma-Aldrich, USA) was added. Sample supernatant (200 µl) was taken off, and the fluorescence was measured at a wavelength of 350–461 nm with a Varioskan™ LUX

multimode microplate reader. A DNA standard curve was realized to determinate the DNA concentration in the samples. The DNA absorbances were used to normalize the cell viability.

*2.3. Evaluation of cell viability*

Cell viability was evaluated by analyzing the mitochondrial activities of the hDPSCs. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to quantitatively assess the number of viable hDPSCs grown in the two culture conditions (LG-DMEM and HG-DMEM). Post-thaw P3-hDPSCs were seeded in 96-well plates at conventional seeding density ($5\times10^3$ cells/$cm^2$) in a humidified atmosphere of 5% $CO_2$ at 37°C and were cultured for 1, 3, 7, 14 and 21 days. The medium was changed every 3–4 days. The MTT solution was prepared by dissolving MTT in phosphate buffered saline (PBS), after which it was filtered and sterilized. After the predetermined time intervals (1, 3, 7, 14 or 21 days), cells were incubated with 5 mg/ml MTT (Sigma) for 4 hours at 37 °C. The MTT solution was discarded and the blue formazan crystals formed, representative of cell viability, were solubilized from the cells by incubation with dimethylsulfoxide (DMSO) for 5 minutes at 37°C. Aliquots of the resulting solutions were transferred to new plates. The absorbance was measured at 540 nm using a Varioskan™ LUX multimode microplate reader. The MTT absorbance normalized by Hoechst absorbance was calculated for each sample.

*2.4. Osteogenic differentiation of hDPSCs*

To evaluate the effect of glucose concentration on osteoblast differentiation, hDPSCs were incubated in HG or LG osteogenic culture medium (DMEM supplemented with 10% FBS, pyruvate, penicillin/streptomycin, amphotericin B and osteogenic cocktail dexamethasone (100 nM), ascorbic acid (50 µM), β-glycerophosphate (10 mM)). hDPSCs at passage 3 were cultured for 28 days in 6-well plates at conventional seeding density ($5\times10^3$ cells/$cm^2$) and in a

humidified atmosphere of 5% $CO_2$ at 37°C. The medium was changed every 3–4 days. We used as negative control DMEM without osteogenic differentiation cocktail.

### *2.4.1. Alizarin red staining*

The formation of calcium nodules in osteogenic differentiated hDPSCs was assessed using alizarin red (AR) staining, according to Stanford (Stanford, Jacobson, Eanes, Lembke & Midura, 1995). After culture under osteogenic-inducing conditions for 28 days in 6-well plates, hDPSCs were washed three times with PBS (pH 7.4), fixed with 4% paraformaldehyde for 15 min, incubated with 2% AR solution (Sigma-Aldrich) and finally rinsed three times with PBS. The calcium nodules were visualized under conventional microscopy (10× magnification). To quantify the mineralization, we extracted calcified nodules at low pH and neutralized with ammonium hydroxide, as previously described (Gregory, Gunn, Peister & Prockop, 2004). Briefly, cultures were destained with acetic acid (10%v/v) for 30 minutes at room temperature. The monolayer was scraped off, heated at 85°C for 10 minutes, cooled with ice for 5 minutes and centrifuged at 20,000g for 15 minutes. Resulted supernatant was removed, and pH adjusted (4.1-4.5) with ammonium hydroxide.

The absorbance values at 405 nm were detected using a microplate reader, and AR staining concentrations were calculated according to its standard curve. Three independent experiments were performed in triplicates. We used as negative control DMEM without osteogenic differentiation cocktail.

### *2.4.2. RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)*

To analyze osteogenic-related gene expression, total RNA was isolated at day 28 by adding Quiazol reagent (QIAzol®Lysis Reagent, Qiagen) and extracted from cultured hDPSCs using Direct-zol ™ RNA MiniPrep (Zymo Research). About 50 ng of total RNA were reverse

transcribed into complementary deoxyribonucleic acid (cDNA) using the cDNA synthesis kit (iScript™ Reverse Transcription Supermix for RT-qPCR, Biorad). The RT-qPCR was performed by using iTaq™ Universal SYBER® Green Supermix (Biorad) and system StepOne Plus Apparatus (Real Time PCR System, Applied Biosystem, USA) to compare the expression of Runt-related transcription factor 2 (*RUNX2*), osteocalcin (*OCN*) and collagen 1A1 (*COL1A1*) genes (Table 1) between cells cultured with different glucose concentrations.

Gene expression levels were obtained from cells of three different patients, cultured in triplicate. Gene expression levels were calculated by the comparative delta CT method ($2^{-\Delta ct}$ formula) after being normalized to the CT value of the ribosomal protein housekeeping gene (*RP29*).

### *2.5. Statistical analysis*

Data are expressed as mean ± standard error or medians (alizarin red assay). Each experimentation has been made in triplicate and was repeated independently three times ($n$ = 3). Differences between groups were analyzed on ranks using Mann-Whitney and Kruskal-Wallis (alizarin red assay) nonparametric tests (GraphPad Prism software (GraphPad Inc, CA, USA)). $P$ values lower than 0.05 were considered to be statistically significant.

## 3. Results

### *3.1. Effects of high glucose concentration on cell proliferation and viability*

In Fig. 1A, results of Hoechst assay exhibited the influence of glucose concentration on hDPSC proliferation. DPSCs presented an increase in DNA quantity until 7 days and a decrease after. At 7 days, hDPSCs cultured with LG medium seemed to show higher DNA quantities (3.49 UI *vs* 2.31 UI) compared to those cultured in HG medium, but this difference was not significant

($p$ = 0.70). Then, there was no significant difference ($p$ > 0.05) after Hoechst assay between hDPSCs cultured in HG medium or LG medium.

MTT assay highlighted a progressive increase of cell metabolic activity until 21 days of culture (Fig. 1B). Whatever the glucose concentration, no significant difference ($p$ > 0.05) was observed in cell metabolic activity, whatever the kinetics point.

### *3.2. Osteogenic differentiation under different glucose concentrations*

The osteogenic ability of hDPSCs was evaluated by the detection of mineralized bone nodules and expression of genes involved in bone development (*COL1A1*, *RUNX2*) and mineralization (*OCN*).

#### *3.2.1. Alizarin red staining*

After 28 days of culture, AR staining depicted the presence of precipitated calcium in hDPSCs cultured with osteogenic differentiation medium (Fig. 2A). Staining and then mineralization seemed visually more intense with HG medium than LG medium.

Mineralization quantitative assay highlighted that the increase of glucose concentration did not induce greater formation of precipitated calcium ($p$ = 0.40) (Fig. 2B).

#### *3.2.2. Expression of osteogenic-related genes*

To investigate the influence of glucose concentration on hDPSC differentiation, we evaluated the modifications of gene expression of *RUNX2* (early osteogenic marker), *OCN* (late osteogenic marker) and *COL1A1* at day 28 in HG group and LG group. No significative differences (*RUNX2*, $p$ = 0.10; *OCN*, $p$ = 0.33; *COL1A1*, $p$ = 0.99) were observed in gene expression of these three genes between the two groups (Fig. 3).

## 4. Discussion

Human mesenchymal stem cells play a major role in regenerative medicine and offer great promise in the field of bone tissue engineering. Among MSCs, DPSCs represent an interesting therapeutic option for mineralized tissue regeneration, and there has been a growing interest in their use over the last years (Batouli et al, 2003; Gronthos et al, 2002). Indeed, DPSCs are easily accessible and can differentiate into multiple lineages including osteoblasts (Cristaldi et al, 2018). It also has been demonstrated that DPSC potential in terms of proliferation and ability to induce mineralization is greater than BM-MSC potential (Alge et al, 2010). There is an imperative need for further research on DPSCs to better understand their cell function in order to improve their *in vivo* therapeutic use. A better understanding and modulation of some factors of DPSC local microenvironment such as glucose concentration could ameliorate cell proliferation and differentiation (Salazar-Noratto et al, 2020). Glucose concentration is a parameter that can easily be adjusted *in vitro* via the culture medium. Improving cell function and survival depending on glucose concentration seems particularly relevant, given the involvement of hyperglycemia in many pathologies, in which stem cell therapies could be helpful. In our study, we aimed to compare two glucose concentrations (5.5 mM and 20 mM) in terms of influence on DPSC proliferation, viability, and differentiation. We chose to focus on these glucose concentrations because they correspond to the routinely used culture medium. We chose one glucose concentration that corresponds to normal condition (5.5 mM) and one considered as high (20 mM), both in reference to *in vivo* serum glucose concentration and *in vitro* glucose concentration of cell culture medium. In fact, 5.5 mM glucose concentration is usually considered as normal glucose serum condition and corresponds to LG culture medium *in vitro*. Glucose concentration of 20 mM is considered *in vitro* as HG culture medium and is compared to hyperglycemic environment that can be found *in vivo* notably in diabetic patients (Kato et al, 2016). In the literature, several studies have investigated the influence of glucose

on MSCs, but their results are divergent or inconsistent (Kanafi, Ramesh, Gupta & Bhonde, 2013; Kato et al, 2016; Li et al, 2007; Lin et al, 2017; Seubbuk, Sritanaudomchai, Kasetsuwan & Surarit, 2017; Stolzing, Coleman & Scutt, 2006; Stolzing, Bauer & Scutt, 2012; Wang et al, 2013; Wang, Wang, Lu & Yu, 2019; Weil, Abarbanell, Herrmann, Wang & Meldrum, 2009; Yamawaki, Taguchi, Komasa, Tanaka & Umeda, 2017; Yang, Shen, Reece, Chen & Yang, 2016; Zhang et al, 2017). To our knowledge, among those studies, only one work is available on DPSCs (Kanafi, Ramesh, Gupta & Bhonde, 2013).

The present work first studied the influence of glucose in hDPSC proliferation and viability. We found no significant differences in terms of viability between hDPSCs cultured with LG medium and hDPSCs cultured with HG medium for 21 days. The proliferation rate was also similar between the two groups ($p > 0.05$). It seems to demonstrate that HG concentration in cell culture medium did not increase or had deleterious effect on the hDPSC proliferation and viability. Those results are consistent with a previous study on DPSCs that showed no significant difference at day 8 in terms of proliferation rate and cell viability depending on glucose concentration (Kanafi, Ramesh, Gupta & Bhonde, 2013). In our work, proliferation rate has been measured until day 21, but glucose effects seemed to be the same both on short- and long-term exposure. Similar results were obtained with BM-MSCs (Weil, Abarbanell, Herrmann, Wang & Meldrum, 2009) in a work on glucose short-term exposure (2 days) with no significant difference depending on glucose concentration. Other studies on HG effects performed on other dental sources of MSCs displayed various results. Depending on studies, HG inhibited (Kato et al, 2016; Wang, Wang, Lu & Yu, 2019) or promoted (Salazar-Noratto et al, 2019; Seubbuk, Sritanaudomchai, Kasetsuwan & Surarit, 2017) proliferation of SCAPs (Wang, Wang, Lu, & Yu, 2019), PDLSCs (Kato et al, 2016; Seubbuk, Sritanaudomchai, Kasetsuwan & Surarit, 2017) or stem cells from human exfoliated deciduous teeth (SHEDs) (Kanafi, Ramesh, Gupta & Bhonde, 2013). The same contradictory results were observed

concerning BM-MSCs with either boosting (Deschepper et al, 2013; Elseberg et al, 2012; Kim, Heo & Han, 2006; Li et al, 2007, Wang et al, 2013) or suppressing (Keats & Khan, 2012; Stolzing, Bauer & Scutt, 2012; Zhang et al, 2017) effect of HG on cell proliferation. These different outcomes could be explained by several factors such as the variation in delay between the cell seeding and the achievement of the proliferation assay (from 4 days to 4 weeks depending on studies (Deschepper et al, 2013; Elseberg et al, 2012; Kanafi, Ramesh, Gupta & Bhonde, 2013; Kato et al, 2016; Kim, Heo & Han, 2006; Keats & Khan, 2012; Li et al, 2007; Stolzing, Bauer & Scutt, 2012; Seubbuk, Sritanaudomchai, Kasetsuwan & Surarit, 2017; Wang et al, 2013; Wang, Wang, Lu & Yu, 2019; Zhang et al, 2017), variation of cell seeding densities, the individual donors' variable trends (Li et al, 2007) or the differences between types of stem cells.

The second part of our work focused on the effects of glucose concentration on osteogenic differentiation and mineralization of human DPSCs. Studies demonstrated that hDPSCs can differentiate into osteoblast lineage in the presence of osteoinductive agents (Papaccio et al, 2006). The osteogenic differentiation of stem cells is regulated by specific molecules and factors of the microenvironment. Among these, the role of glucose on osteogenesis was mainly studied on BM-MSCs, and the results in the literature are confusing. Therefore, some studies have shown that HG culture medium enhanced the osteogenic differentiation of BM-MSCs (Li et al, 2007; Yamawaki, Taguchi, Komasa, Tanaka & Umeda, 2017), PDLSCs (Seubbuk, Sritanaudomchai, Kasetsuwan & Surarit, 2017) or SCAPs (Wang, Wang, Lu & Yu, 2019). In contrast, some other studies demonstrated that HG concentration suppressed or reduced BM-MSC (Stolzing, Bauer & Scutt, 2012; Wang et al, 2013) or PDLSC (Kato et al, 2016) osteoblastic differentiation. To our knowledge, no study compared the effects of LG and HG medium on osteogenic differentiation of DPSCs. One study obtained similar osteogenic differentiation of SHEDs or DPSCs in presence of hypoxia or HG (qualitative Von Kossa

staining) (Kanafi, Ramesh, Gupta & Bhonde, 2013). However, the osteogenic differentiation potential of DPSCs was not compared between HG and LG.

In our work, the mineralization assay by alizarin red staining on DPSCs after 28 days of culture showed no significant difference ($p > 0.05$) between cells cultured with HG osteoinductive medium (20 mM) or LG osteoinductive medium (5.5 mM). We obtained for both groups mineralized nodules, and even if the staining visually seemed stronger with the HG medium, the quantitative assay did not indicate significant difference ($p > 0.05$) between the two groups. Then, we reviewed how glucose concentration could influence the expression of several osteoblastic-related genes: *RUNX2, OCN* and *COL1A1*. *RUNX2* is a transcription factor which is essential in the early stages of DPSC osteogenic differentiation (Yu et al, 2007). *OCN* is a later-stage marker of mineralized bones (Bai et al, 2010). *COL1A1* is a gene whose expression is closely correlated with the osteoblast proliferation stage (Nakashima & de Crombrugghe, 2003). Our results showed no significative difference ($p > 0.05$) between osteoblastic-related gene expression for hDPSCs induced with LG osteogenic medium and induced with HG osteogenic medium after 28 days of differentiation. These results confirmed previous results obtained with mineralization assay. Then, HG concentration appears to have no boosting or suppressive effect on hDPSC osteogenic-related gene expression.

To conclude, our work has not established evidence of neither boosting nor deleterious impact of high glucose on hDPSC behavior. hDPSCs cultured in HG medium did not exhibit a higher proliferation rate, viability or osteogenic differentiation compared with those cultured with LG medium. Despite the lack of numerous studies in the existing literature about the effects of glucose concentration on DPSCs, especially about DPSC osteogenic differentiation, we can formulate the following assumptions. First, the different type of stem cells used in the experiments could explain the different results. Secondly, cells were exposed to osteoinductive

medium for different culture times. Thus, in our work, stem cells were cultured for 28 days in presence of osteoinductive media *vs*. 7 (Kanafi, Ramesh, Gupta & Bhonde,, 2013), 14 (Stolzing, Bauer & Scutt, 2012) or 21 days in other studies (Kato et al, 2016; Wang et al, 2013). Finally, Li et al. (2007) have performed only qualitative alizarin red staining after 28 days of BM-MSC culture with HG or LG osteoinductive medium.

Further investigations are required for a better understanding of the molecular mechanisms of glucose on DPSCs. Nevertheless, this finding seems to highlight an interesting resistance of hDPSCs against high glucose concentration and could be promising in bone tissue engineering in pathological conditions such as diabetes mellitus.

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**Declaration of competing interest**

The authors declare that they have no conflict of interest.

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**Figure captions**

**Fig. 1. Effect of different concentrations of glucose (low (5.5mM) or high (20mM) glucose concentration) on hDPSC proliferation and metabolic activity**. DPSCs were cultured for 21 days. A. hDPSC DNA quantification (µg/µL), B. Measurement of MTT absorbance at 540 nm and normalized by DNA absorbance. Results are mean ± SE (n=3).

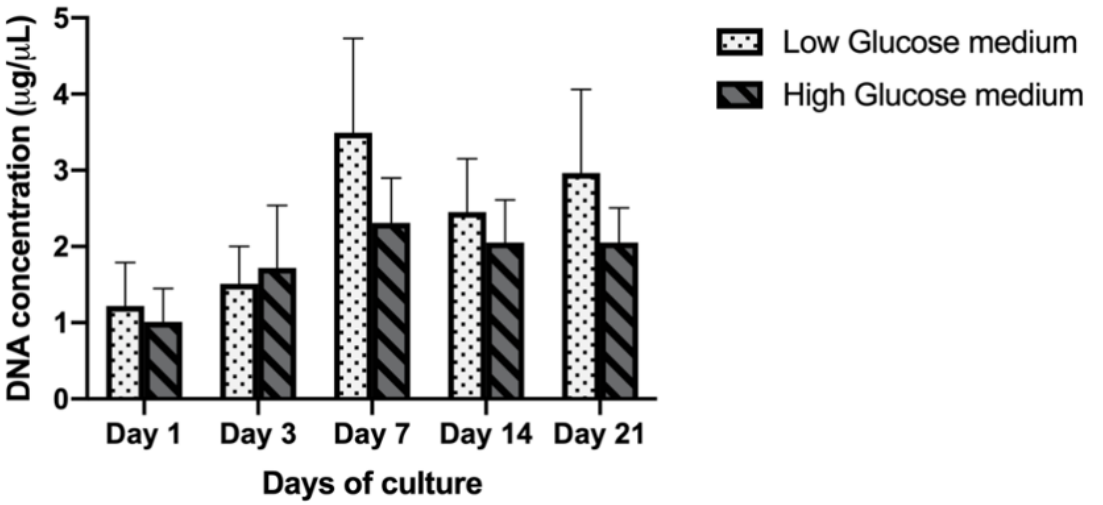
**Fig. 2. Effect of glucose concentration on the formation of mineralized bone nodules.** A. Alizarin red staining of hDPSC cultured for 28 days in basal medium (A.1.), low glucose (LG, 5.5 mM) osteogenic induction medium (A.2.) and high glucose (HG, 20 mM) osteogenic induction medium (A.3.). B. Quantification of alizarin red staining for hDPSC cultured for 28 days in LG- or HG-medium supplemented (LGD and HGD) or not (control, basal medium) with osteogenic factors, *a:* $p<0.05$ versus basal medium. Results are medians (n=3).

**Fig. 3. Effect of glucose concentration on osteogenic gene expression in hDPSC**. The expression of *RUNX2*, *OCN* and *COL1A1* genes in hDPSC was measured using real-time PCR. There was no significative difference ($p>0.05$) at day 28 between low glucose (5.5 mM) *vs* high glucose (20 mM) groups in *RUNX2*, *OCN*, and *COL1A1* expression in DPSCs. Results are mean ± SE (n=3)

**Table 1. Primers used for reverse transcription-quantitative polymerase chain reaction analysis**

| Genes | Primers | Sequences (5'-3') |
|---|---|---|
| *RUNX2* | Forward | CCCGTGGCCTTCAAGGT |
| | Reverse | CGTTACCCGCCATGACAGTA |
| *OCN* | Forward | GTGCAGAGTCCAGCAAAGGT |
| | Reverse | TCAGCCAACTCGTCACAGTC |
| *COL1A1* | Forward | AGGTGCTGATGGCTCTCCT |
| | Reverse | GGACCACTTTCACCCTTGT |
| *RP29* | Forward | AGATGGGTCACCAGCAGCTGTACTG |
| | Reverse | AGACACGACAAGAGCGAGAA |

A

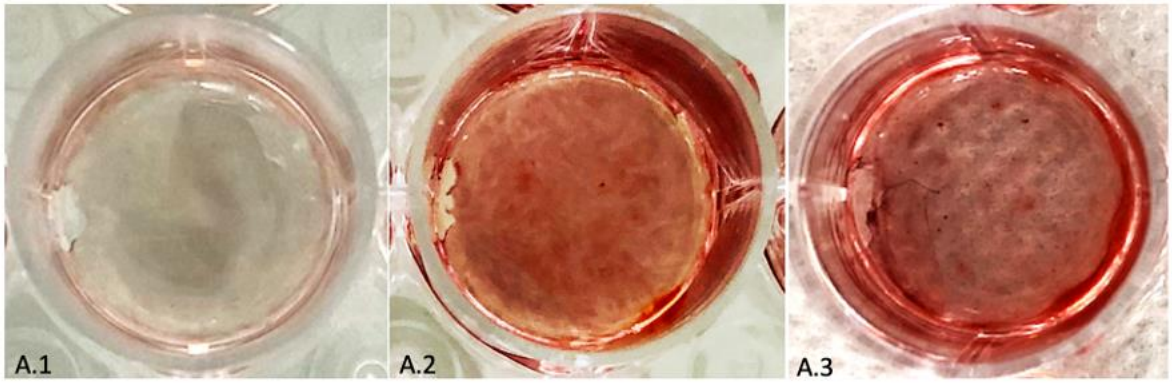

DNA concentration (μg/μL)
5
4
3
2
1
0
Day 1
Day 3
Day 7
Day 14
Day 21
Days of culture
Low Glucose medium
High Glucose medium

B


Normalized MTT absorbance
0.6
0.4
0.2
0.0
Day 1
Day 3
Day 7
Day 14
Day 21
Days of culture
Low Glucose medium
High Glucose medium

**Figure 1**

A.


A.1
A.2
A.3

B.

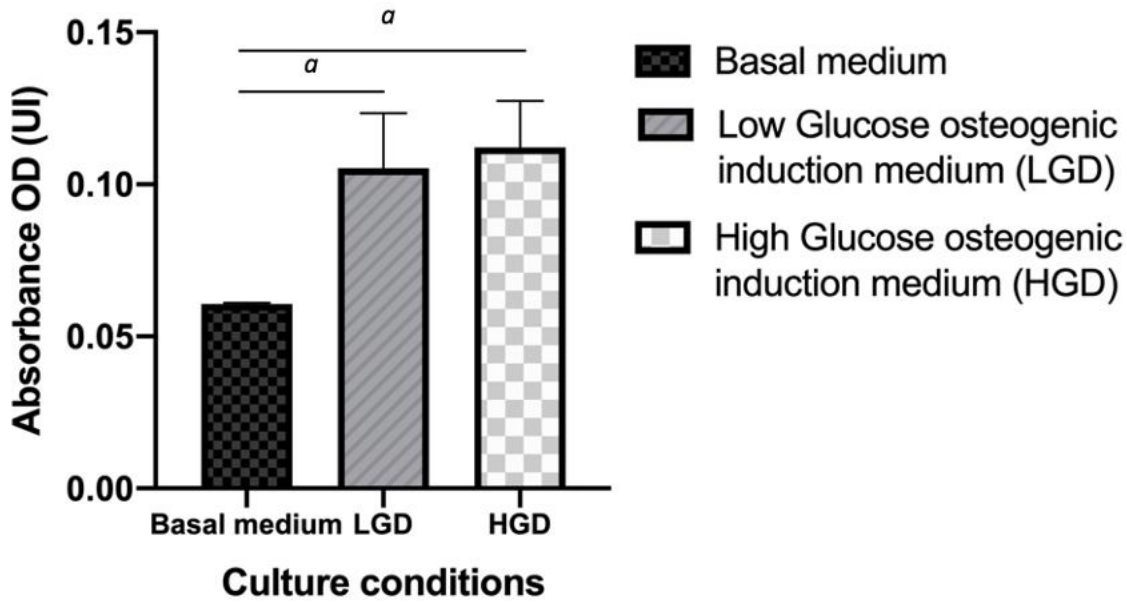

a
a
0.15
0.10
0.05
0.00
Absorbance OD (UI)
Basal medium
LGD
HGD
Culture conditions
Basal medium
Low Glucose osteogenic induction medium (LGD)
High Glucose osteogenic induction medium (HGD)

**Figure 2**

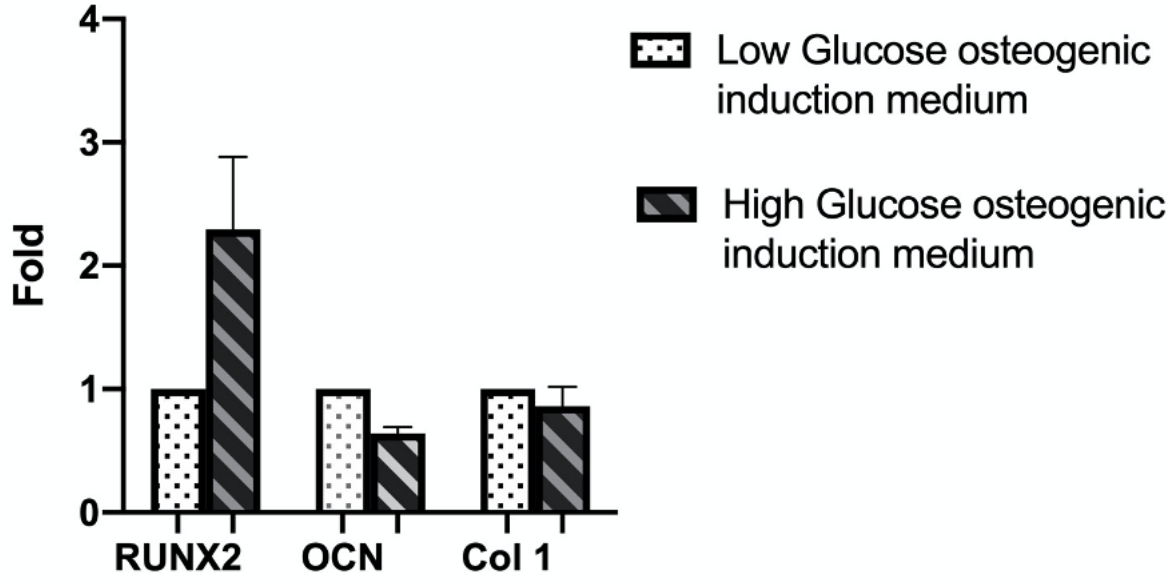

Fold
4
3
2
1
0
RUNX2
OCN
Col 1
Low Glucose osteogenic induction medium
High Glucose osteogenic induction medium

**Figure 3**