STEFANIE BRESSAN WERLE

EFEITO DA HIPÓXIA NA PROLIFERAÇÃO, APOPTOSE E EXPRESSÃO DE GENES DE PLURIPOTÊNCIA DE CÉLULAS-TRONCO MESENQUIMAIS DA POLPA DE DENTES DECÍDUOS HUMANOS

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Sonhe com o que você quiser.
Vá para onde você queira ir.
Seja o que você quer ser, porque você possui apenas uma vida e nela só temos uma chance de fazer aquilo que queremos.
Tenha felicidade bastante para fazê-la doce. Dificuldades para fazê-la forte.
Tristeza para fazê-la humana.
E esperança suficiente para fazê-la feliz.

(Clarice Lispector)

RESUMO

A capacidade de diferenciação em diversos tipos celulares e a facilidade na obtenção com o mínimo de dano invasivo ao doador tornam as células-tronco provenientes da polpa de dentes decíduos esfoliados uma promessa para a engenharia de tecidos. No entanto, a pequena quantidade de células isoladas da polpa de dentes decíduos humanos é um dos principais obstáculos para a terapia celular, em que um grande número de células é crucial. A tentativa de aprimorar o microambiente da cultura celular, para promover uma expansão que comporte a aplicabilidade clínica, é um passo importante para a pesquisa e para o futuro da engenharia tecidual. O presente estudo teve por objetivo avaliar o efeito da hipóxia (3% de oxigênio) na proliferação, apoptose e pluripotência de células-tronco mesenquimais provenientes da polpa de dentes decíduos exfoliados (SHEDs). Primeiramente, foi realizada uma revisão de literatura abordando os conceitos de hipóxia, bem como o seu efeito sobre o cultivo das células-tronco com origem em tecidos dentais. O artigo original apresentou a caracterização de SHEDs (n=5), de acordo com a Sociedade Internacional de Terapia Celular. Para avaliação da atividade metabólica, o ensaio de WST-8 foi realizado após 1, 3, 5, 7 e 14 dias de cultivo em hipóxia ou normóxia. O uso do anticorpo Ki67 foi utilizado para analisar a proliferação em citômetro de fluxo. A análise da apoptose foi mensurada com Annexin V/ iodeto de propídeo após 1, 4 e 7 dias, nos grupos hipóxia e normóxia. A expressão de genes relacionados à pluripotência celular (Oct4, Sox2 e Nanog) foi mensurada através de PCR quantitativo após 24 horas e 7 dias. Para a comparação estatística entre os grupos, o teste t de Student foi aplicado. Não foram observadas diferenças entre a atividade metabólica, apoptose e proliferação das SHEDs cultivadas em normóxia ou hipóxia ($p>0,05$). O ensaio de WST-8 demonstrou o crescimento exponencial para todas as culturas, no período analisado. A maior fração de células em proliferação foi observado no quarto dia de análise em ambos os grupos, sendo que foi observada uma reduzida fração de células mitóticas nos dias 1 e 7. A expressão de Oct4, Sox2 e Nanog foi significantemente maior no grupo hipóxia ($p<0,01$). Esses achados apontam o aumento nos marcadores de pluripotência, possibilitando a maior capacidade de diferenciação celular, como a principal vantagem do cultivo das SHEDs em situação de hipóxia.

Palavras-chave: células-tronco, dente decíduo, hipóxia, polpa dental.

ABSTRACT

Stem cells from exfoliated deciduous teeth are a promising source of cells for tissue engineering due to their possibility of differentiation into many cell types and their isolation abilities with minimal damage to the donor. However, the small quantity of isolated cells from tooth pulp is a major obstacle for cellular therapy. To improve the microenvironment of the cell culture for promoting expansion is an important step for tissue engineering research. This study has aimed to evaluate the effect of hypoxia (3% oxygen) in the proliferation, apoptosis and pluripotency of mesenchymal stem cells from human exfoliated deciduous teeth (SHEDs). Firstly, a review of the literature addressing concepts of hypoxia and its effect on stem cells derived from dental tissue is presented. The original paper presents the characterization of SHEDs according to the International Society of Cellular Therapy (n =5). To evaluate the metabolic activity, the WST-8 assay was performed after 1, 3, 5, 7 and 14 days of cultivation under a hypoxic or normoxic environment. The Ki67 antibody was used to examine the proliferation by flow cytometry. Analysis of apoptosis was measured using AnnexinV/ Propidium iodide after 1, 4 and 7 days in the hypoxia or normoxia groups. The expression of pluripotency related genes (Oct4, Sox2 and Nanog) was measured by quantitative PCR after 24 hours and 7 days. The statistical comparison between the groups was performed with Student t test. No differences were observed in metabolic activity, apoptosis and proliferation of SHEDs cultivated in normoxic or hypoxic ($p > 0.05$). The WST-8 showed exponential growth for all the samples throughout the duration of the experiment. The major fraction of proliferative cells was observed on the fourth day in both groups, while a small fraction of mitotic cells was found on days 1 and 7. The expression of Oct4, Sox2 and Nanog was significantly higher in the hypoxia group ($p < 0.01$) in 24 hours and in 7 days. These findings point to the increase in pluripotency markers, related to the differentiation capacity, as the main advantage of SHED cultivation under a hypoxic environment.

Keywords: deciduous tooth, dental pulp, hypoxia, stem cells.

SUMÁRIO

LISTA DE ABREVIATURAS

ADSC – (*Adipose Derived Stem Cell)* Célula-tronco derivada de tecido adipose

BMSC – (*Bone Marrow Stem Cell*) Célula-tronco da medulla óssea

cDNA – *(Complementar DNA*) DNA complementar

DPSC – (*Dental Pulp Stem Cell)* Células-tronco da polpa dental

DMEM/Hepes – Dulbecco's Modified Eagle Medium

FBS – (*Fetal bovine serum*) Soro fetal bovino

HIF – *(Hypoxia-inducible Factor)* Fator induzível de hipóxia

ISCT – (International Society for Cellular Therapy) Sociedade Internacional de Terapia Celular

MSC – (*Mesenchymal Stem Cell*) Célula-tronco mesenquimal

PBS 1X – (*Phosphate Buffer Saline)* Tampão fosfato-salino

PCR – *(Polymerase Chain Reaction)* Reação em cadeia da polimerase

PDLSC – (*Periodontal derived Stem Cell*) Célula-tronco do ligamento periodontal

RNA – *(Ribonucleic Acid)* Acido ribonucleico

SCAP – (*Stem Cells from Apical Papilla*) Célula-tronco da papila apical

SHED – (*Stem Cells from Human Exfoliated Deciduous Teeth)* Células-tronco da polpa de dentes decíduos humanos esfoliados

TGFβ1 – *(Transforming Growth Factor-beta 1)* Fator de crescimento transformador beta 1

VEGF – *(Vascular endothelial Growth Factor)* Fator de crescimento endotelial vascular

WST-8 – *(Water-soluble tetrazolium salt)* – sal tetrazólio solúvel em água 8

1. INTRODUÇÃO

Desde a caracterização das células-tronco da polpa de dentes decíduos esfoliados (SHED – *Stem Cells from Human Exfoliated Deciduous teeth*) em 2003, a pesquisa com a utilização dessas células em diferentes estratégias de engenharia tecidual vem sendo crescente. A capacidade de diferenciação em diversos tipos celulares e a facilidade na obtenção com mínimo dano invasivo ao doador tornam as SHEDs uma fonte atrativa para utilização em pesquisas na área de medicina regenerativa (Kanafi et al., 2013; Kawashima 2012). No entanto, a pequena quantidade de células isoladas a partir da polpa (Miura et al.,2003) constitui uma limitação para sua utilização em terapias celulares, em que um grande número de células é fundamental (Cicione et al, 2013; Haque et al., 2013).

A expansão das culturas celulares *in vitro* é usualmente realizada em normóxia (21% de oxigênio - O_2), enquanto que a condição *in vivo* varia entre 2 e 9% de O_2 (Haque et al., 2013). Tem sido reportado que a alteração desse parâmetro pode levar à senescência celular precoce, instabilidade genética e diminuição da capacidade de proliferação (Haque et al., 2013; Muscari et al., 2013), uma vez que o ambiente oxigenado à 21% de O_2 pode ser tóxico às células pelo estresse oxidativo e pela produção de espécies reativas de oxigênio (ROS - *reactive oxygen species*) (Agata et al., 2009; Roy et al., 2012; Cicione et al., 2013).

Câmaras de hipóxia têm sido utilizadas para o cultivo celular, mostrando altas taxas de proliferação em comparação à normóxia em culturas de células-tronco provenientes de diversos tecidos humanos (Volkmer et al., 2010; Adesida et al. 2012; Roy et al., 2012). No entanto, o efeito da hipóxia depende principalmente da tensão de oxigênio, tempo de exposição à hipóxia e fonte de células-tronco, o que produz uma grande influência no comportamento celular (Werle et al., 2016).

Até o presente momento, existe pouca informação sobre o efeito da hipóxia no cultivo das SHEDs. Esse estudo revisa os conceitos de hipóxia, a resposta celular à baixa tensão de oxigênio e o efeito da hipóxia sobre o cultivo de células-tronco isoladas de tecidos dentais. Além disso, investiga o efeito da hipóxia sobre a capacidade de proliferação, apoptose e pluripotência das SHEDs.

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Review

The effects of hypoxia on *in vitro* culture of dental-derived stem cells

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ABSTRACT

The culture of cells under hypoxia is considered one of the hot topics of tissue engineering, especially when exploring the proliferation capacity, a critical step for cellular-based therapies. The use of *in vitro* hypoxic environment aims to simulate the oxygen concentrations found in stem cell niches. Dental tissues are attractive sources of stem cells, as they are obtained from discarded tissue, after third molar extraction and exfoliation deciduous teeth, respectively. However, small amounts of cells are obtained from these sources. Thus, optimizing the *in vitro* conditions for proliferation and differentiation of these cells is essential for future regenerative strategies. This review presents a summary of the results regarding the effect of hypoxia on dental-derived stem cells after an electronic search on PubMed databases. The studies show increased differentiation potential and paracrine action of dental-derived stem cells under hypoxic environment. There are controversies related to proliferation of dental-derived stem cells under induced hypoxia. The lack of standardization in cell culture techniques contributes to these biases and future studies should describe in more detail the protocols used. The knowledge regarding the effect of hypoxia on dental-derived stem cells needs further clarification for assisting the clinical application of these cells.

Contents

1. Introduction

The advances in cell-based therapies and regenerative medicine have aroused interest in the factors that control stem cell characteristics (Muscari et al., 2013; Maul, Chew, Nieponice, & Vorp, 2011; Stolzing, Bauer, & Scutt, 2012; Bakopoulou et al., 2015; Li, Peng, & Ding, 2015; Fafián-Labora et al., 2015; Naaldijk et al., 2015). *In vivo*, the stem cell niche is regulated by growth factors, cytokines and low oxygen tension (Nombela-Arrieta et al., 2013; Simon & Keith, 2008). To mimic this environment *in vitro*,

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simulating hypoxia is one of the hot topics of tissue engineering research (Bornes, Jomha, Mulet-Sierra, & Adesida, 2015; Fotia, Massa, Boriani, Baldini, & Granchi, 2015; Hutton & Grayson, 2016; Wakai et al., 2015), which aims at improving proliferation rates (Bornes et al., 2015; Fotia et al., 2015; Grayson, Zhao, Izadpanah, Bunnell, & Ma, 2006; Grayson, Zhao, Bunnell, & Ma, 2007; Forristal, Wright, Hanley, Oreffo, & Houghton, 2010; Choi et al., 2014; Wu et al., 2013a; Zhang et al., 2014; Peng, Shu, Lang, & Yu, 2015) and differentiation potential of stem cells (Bornes et al., 2015; Fotia et al., 2015; Grayson et al., 2006; Adesida, Mulet-Sierra, & Jomha, 2012) as well as enhancing their viability after transplantation (Yan et al., 2012; Zhu, Sun, Zou, & Ge, 2014; Bader et al., 2015).

Hypoxia is considered to be one of the key factors in maintaining cell plasticity and self-renewal (Basciano et al., 2011; Forristal et al., 2010; Grayson et al., 2006, 2007). However, cellular response to low oxygen depends on stem cell source (Dionigi, Ahmed, Pennington, Zurakowski, & Fauza, 2014; Bigot et al., 2015) and environment conditions (Yuan, Guan, Ma, & Du, 2015a).

Dental tissues are a promising source of stem cells for regenerative medicine, because they present advantages such a high proliferative and multidifferentiation potentials (Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Miura et al., 2003; Bakopoulou et al., 2011; Huang et al., 2008). Moreover, they are attractive because these tissues are usually discarded after tooth extraction and physiologic resorption, as occurs in cases of impacted third molars or primary teeth exfoliation, respectively.

Different portions of dental tissues are sources of populations of adherent mesenchymal stem/stromal cells with shared similar characteristics. These populations are named distinctly if they are isolated from the dental pulp of permanent (DPSC) (Gronthos et al., 2000) and deciduous teeth (SHED) (Miura et al., 2003), periodontal ligament (PDLSC) (Shi et al., 2005), dental follicle (DFPC) (Morsczeck et al., 2005) or apical papilla (SCAP) (Huang et al., 2008). The exact location of the putative stem cell population in teeth is still unknown. Studies in mice have shown that a very primitive population of quiescent stem cells is located at the periarterial niche in the neurovascular bundle of incisors (Zhao et al., 2014). Under homeostasis or after acute injury, periarterial cells are activated and give rise to a subpopulation of actively dividing cells that will differentiate into odontoblast or pulp mesenchyme and contribute to the healing process (Shi & Gronthos, 2003; Ishikawa, Ida-Yonemochi, Nakakura-Ohshima, & Ohshima, 2012).

Recent *in vivo* studies have tested the therapeutic potential of stem cells from dental tissues (Iohara et al., 2008; Nishino et al., 2011; Yamaza et al., 2010). Several researchers focus on the regeneration of dental pulp (Iohara et al., 2011; Rosa, Della Bona, Cavalcanti, & Nör, 2012; Rosa, Zhang, Grande, & Nör, 2013; Wang, Zhao, Jia, Yang, & Ge, 2013) or bone defects due to the intrinsic osteogenic potential presented by these cells (Abuarqoub, Awidi, & Abuharfeil, 2015; Daltoé et al., 2014; Kim et al., 2012; Riccio et al., 2012), which is probably related to their niche (Fuchs, Tumbar, & Guasch, 2004). Despite dental stem cells having been tested using animal models for the treatment of non-dental diseases (De Berdt et al., 2015; Gomes et al., 2010; Mita et al., 2015; Moshaverinia et al., 2014; Riccio et al., 2012), their differentiation into cells of a non-mesenchymal origin is still questionable and the beneficial effects are attributed to the paracrine factors secreted by these cells (Ankrum, Ong, & Karp, 2014; Ishikawa et al., 2015; Wakayama et al., 2015). Regardless of the clinical application of stem cells from dental tissues, hypoxia can represent a perspective for optimizing the characteristics of stem cells prior to their clinical use. The aim of this review has been to gather the available knowledge regarding the effects of hypoxia on the *in vitro* characteristics of dental-derived stem cells and discuss the benefits of low oxygen expansion for the clinical application of these cells.

2. Hypoxia and cell metabolism

Cellular metabolism under normoxia is conducted by mitochondria. In this condition, adenosinetriphosphates (ATPs) are generated through oxidative phosphorylation (Malda, Klein, &

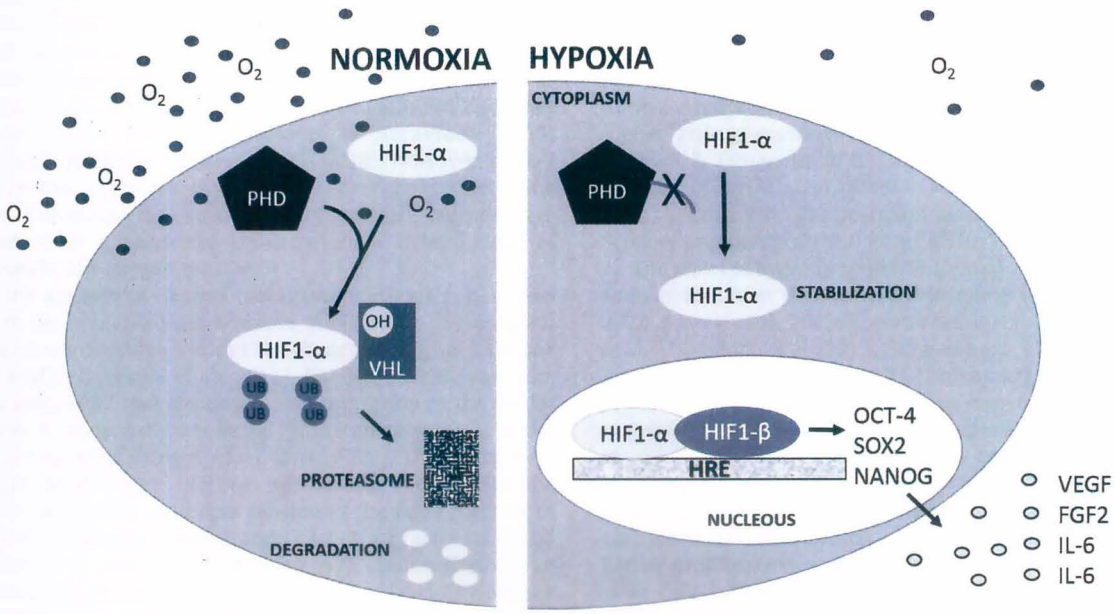

Fig. 1. Biochemical pathways of Hypoxia-inducible factor 1 (HIF1) in normoxia and hypoxia. When oxygen is abundant (O_2) the HIF-1a subunit is immediately hydroxylated by prolyl hydroxylase enzymes (PHD) which increases HIF-1a recognition by the von Hippel-Lindau (VHL) protein. After ubiquitination by a complex of proteins the HIF-1a is degraded by proteasomes in the cytoplasm of the cells. Alternatively, when low oxygen is established (hypoxia), the HIF-1a is stabilized and accumulates in the nucleus of the cells, where it heterodimerizes with HIF-1b. The HIF-1 dimer along with other proteins bind to specific regions of the genome called hypoxia-responsive elements (HRE) and promote the transcription of the target genes. UB: ubiquitin.

Upton, 2007), and reactive oxygen species (ROS) are produced (Zhou et al., 2013). Although necessary in many cell processes, the excessive production of ROS has deleterious effects on cell structures, causing damage to DNA proteins and lipids (Busettil, Rubio, Dollé, Campisi, & Vijg, 2003). An imbalance on ROS production has been associated with an increase in cellular senescence (Cicione et al., 2013; Chaudhari, Ye, & Jang, 2014) and a decrease in pluripotency (Haque, Rahman, Abu Kasim, & Alabsi, 2013). Under hypoxia (<5% O2), the Krebs cycle is suppressed. Thus, glycolysis becomes responsible for the production of ATP, even in a smaller amount than is produced by the mitochondria (Malda et al., 2007) without the production of ROS (Chaudhari et al., 2014). The cellular response to hypoxia is guided by hypoxia-inducible factors (HIFs), which are heterodimeric transcription factors composed of the alpha and beta subunits. The HIF-1α protein is continuously expressed under normoxia; however, the exposure to oxygen tension (>5%) leads to degradation of the protein by oxygen-activated prolyl hydroxilase domain containing proteins (PHDs). Under hypoxia, PHDs are blocked and the HIF-1α is stabilized and enters into the nucleus, where it heterodimerizes with the HIF-1β subunit. The HIF-1 complex binds to hypoxia response elements (HRE) in the genome and initiates the transcription of target genes (Fig. 1) (Fandrey et al., 2006; Muscari et al., 2013). HIF-1α accumulation occurs a few minutes after exposure to low levels of oxygen, decreasing its expression after 48 h (Forristal et al., 2010). Thus, the primary response to hypoxia is guided by HIF-1α and long-term response by HIF-2α (Forristal et al., 2010). The expression of HIF-1α modulates several signaling pathways, increasing the expression of genes involved in self-renewal and pluripotency, such as SOX2, OCT4 and NANOG (Forristal et al., 2010; Petruzzelli, Christensen, Parry, Sanchez-Elsner, & Houghton, 2014). It also regulates angiogenesis-related genes, stimulating tissue neovascularization (Aranha et al., 2010; Chen et al., 2014; Vanacker et al., 2014) and proinflammatory/anti-inflammatory related factors (IL-6 and IL-8) (Chen et al., 2014).

Since the beginnings of *in vitro* cell culture there has been concern about mimicking the environment experienced by cells *in vivo*. The microenvironment that surrounds and governs stem cell fate is known as the stem cell niche, in which stem cells are present in an undifferentiated and self-renewable state (Schofield, 1983). In the last few decades there has been a great improvement in the development of culture medium and different matrix substrates as well as soluble factors that optimize cell proliferation and their maintenance (Villa-Diaz, Ross, Lahann, & Krebsbach, 2013; Laitinen et al., 2016; Rungsiwiwut et al., 2016). However, not all the factors that compose the niche have received the same attention. Currently, the vast majority of cell cultures are performed under a normoxia ambient which is composed of approximately 21% oxygen tension.

With the advance of oxygen measuring methods it has been possible to determine oxygen tensions *in vivo*. The physiological oxygen concentration varies from 1 to 14%, depending on the tissue or organ analyzed (Haque et al., 2013). Although highly vascularized, it is speculated that the oxygen concentration in the dental pulp is low. A study with rats found approximately 3% O_2 in the pulp tissue (Yu, Boyd, Cringle, Alder, & Yu, 2002). The use of two-photon phosphorescence lifetime microscopy allowed for the absolute measurement of oxygen tension in the bone marrow of living mice. An average concentration of 1.8% oxygen was found outside the blood vessels, corroborating with studies that use an oxygen electrode for measuring (Spencer et al., 2014). The oxygen concentration *in vitro* culture under normoxia is 4–10 times higher than in the physiological environment (Haque et al., 2013). Thus, there has been an increasing interest in the growth of cells in hypoxia over the last few years.

The in vitro behavior of different populations of stem cells has been tested using hypoxic conditions. Currently, different strategies can be used, from varying the oxygen tension using a mixture of gases to the use of pharmacologic agents that simulate hypoxia. The first approach requires hypoxic chambers, commonly found in research laboratories around the world (Aranha et al., 2010; Gong, Quan, Jiang, & Ling, 2010; Kanafi, Ramesh, Gupta, & Bhonde, 2013; Brennan, Rexius-Hall, Elgass, & Eddington, 2014; Zhou, Fan, & Xiao, 2014; Sakdee, White, Pagonis, & Hauschka, 2009). More accurate systems to control oxygen tension can be achieved by the use of hypoxia workstations, in which stable oxygen levels are maintained even during media exchange, but this is generally restricted to laboratories specialized in hypoxia research (Brennan et al., 2014). Moreover, other agents that mimic hypoxia have been used in the literature, such as colbalt chloride ($CoCl_2$)(Osathanon et al., 2015; Yuan et al., 2015b) and deferoxamine (DFO) (Jiang et al., 2014; Weng, Li, Li, Yang, & Sheng, 2010) that act by inducing HIF expression and nitric oxide (NO) production. NO is a regulator of cell metabolism and inhibits cytochrome C oxidase (CcO), competing with oxygen in the final step of the mitochondrial transport chain (Beltran-Povea et al., 2015). The stabilization of HIF-1 also can be achieved through the use of novel propyl hydroxylase inhibitors that block the enzymatic activity (PHD) or interfere with its substrate binding site (Forristal & Levesque, 2014).

3. Hypoxia and stem cell culture

Since embryos experience a low oxygen tension of 3–5% *in vivo*, the benefits of cultivating human embryonic stem cells (ESC) have already been tested and established (Forristal et al., 2010; Ezashi, Das, & Roberts, 2005; Forristal et al., 2013). Hypoxia promotes an increase in ESC proliferative capacity, maintaining cells in a pluripotent state more efficiently and reducing spontaneous differentiation in long-term cultures (Forristal et al., 2010; Petruzzelli et al., 2014). Hypoxia also induces re-entry of committed cells into pluripotency state (Mathieu et al., 2013).

Hypoxia has also been tested for improving reprogramming protocols of iPS cells. Endothelial cells reprogrammed by the transduction of transcription factors in hypoxic conditions increased the number of colonies by 2.5-fold compared to normoxia (Panopoulos et al., 2012). Similar results were obtained for the reprogramming of mice embryonic fibroblasts and human dermal fibroblasts, showing that low oxygen tension promotes higher reprogramming efficiencies (Yoshida, Takahashi, Okita, Ichisaka, & Yamanaka, 2009) and accelerate the generation of iPS colonies (Shimada, Hashimoto, Nakada, Shigeno, & Nakamura, 2012) because HIFs are upstream regulators of NANOG, OCT4 and SOX2 expression (Forristal et al., 2010; Petruzzelli et al., 2014).

The effect of hypoxia on mesenchymal stem cells from different sources has been demonstrated in many studies (Adesida et al., 2012; Aranha et al., 2010; Bornes et al., 2015; Choi et al., 2014; Drela et al., 2014; Fotia et al., 2015; Grayson et al., 2006, 2007; Peng et al., 2015; Zhang et al., 2014). The advantages of hypoxia in cell proliferation was reported for bone-marrow-derived mesenchymal stem cells (Adesida et al., 2012; Bornes et al., 2015; Grayson et al., 2006, 2007). When exposed to 2–3% oxygen, the cells increased their proliferation (Adesida et al., 2012; Volkmer et al., 2010) and achieved the cumulative population up to ten times compared to cells cultured in normoxia (Fehrer et al., 2007). A higher proliferation potential was also observed in mesenchymal stem cells isolated from the umbilical cord under 5% of oxygen (Drela et al., 2014) and in the placental mesenchymal stem cells (Mathew, Rajendran, Gupta, & Bhonde, 2013). Adipose-derived mesenchymal stem cells submitted to hypoxia (2–5% O_2) increased the proliferation potential (Choi et al., 2014; Kang, Kim, & Sung,

2014) and osteogenic and chondrogenic differentiation capacities (Choi et al., 2014).

However, hypoxia is a variable that could have unequal effects on cells from different sources (Bigot et al., 2015; Dionigi et al., 2014) and donor age (Iida k Takeda-Kawaguchi, Tezuka, Kunisada, Shibata, & Tezuka, 2010). Moreover, the hypoxia duration (short or prolonged) (Potier et al., 2007; Yuan et al., 2015a) and the oxygen level (severe or mild hypoxia) (Cicione et al., 2013; Yuan et al., 2015a) are factors that influence the results.

4. Hypoxia and the dental stem cell proliferation potential

Regarding stem/stromal cells derived from dental tissue, the effect of hypoxia (1–3%) has been evaluated in stem cells from apical papilla (SCAP) (Bakopoulou et al., 2015; Vanacker et al., 2014; Yuan et al., 2015b) stem cells from periodontal ligament (PDLSC) (Wu et al., 2013a, 2013b), dental pulp stem cells (DPSC) and stem cells from exfoliated deciduous teeth (SHED) (Kanafi et al., 2013).

One of the limitations in the use of dental stem cells for cell therapy is the small amount of available tissue. The number of colony-forming units is estimated to be approximately 12–20 cells per dental pulp from incisors (Miura et al., 2003). Thus, the cells need to be extensively expanded *in vitro* before reaching an appropriate number for therapeutic purposes. Different amounts of cells (at least half million) have been used for pulp engineering strategies in animal models (Kawashima, 2012), and for filled full premolar human root canal, 6×10^5 cells/ml seems to be the required quantity for seeding onto scaffolds (Rosa et al., 2013). However, consecutive passages *in vitro* can have detrimental effects on cell characteristics (Duailibi et al., 2012). Phenotypic changes, genetic instability and senescence have previously been associated with long-term cultures of mesenchymal stem cells, especially in 20% of oxygen (Estrada et al., 2012). Therefore, early passage cells are preferred for clinical applications (Sensebé et al., 2013).

Stem cell proliferation is commonly determined by a variety of methods. Cell counting using image programs is a direct method for proliferation assay; however, the randomization of images needs to be well assessed in order to be representative. Accurate multiparametric flow cytometry assays with BrdU or Ki67 content are used to determine the fraction of cells in each cell cycle (Vignon et al., 2013) but also present bias, since the cell trypsinization might affect the results (Massey, 2015). Finally, indirect methodologies, such as WST (water-soluble tetrazolium salts) or MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) evaluate the cell metabolic activity through the absorbance values, correlated to living cell numbers (Kanemura et al., 2002; Braghirolli, Zamboni, Acasigua, & Pranke, 2015; Werle et al., 2016). This last methologies are used in hypoxic experiments but needs caution as the number of mitochondria is reduced in hypoxic conditions (Basciano et al., 2011) and could potentially affect the results, leading to erroneous conclusions (Sakdee et al., 2009).

The beneficial effect of hypoxia (2% O_2) on PDLSC proliferation was determined by cell count after 7 days in cultivation (Zhang et al., 2014), corroborating with stem cells from other sources in the same (Grayson et al., 2007) and different experimental courses (Bornes et al., 2015; Chen et al., 2014; Fehrer et al., 2007; Forristal et al., 2010).

The culture of DPSC in 72 h under 1% or 21% oxygen did not show differences in proliferation rates measured by WST-1 assay (Aranha et al., 2010). The hypoxic effect (1% O_2) on the proliferation rate of SCAP has also been shown recently (Vanacker et al., 2014). The use of life imaging for PDT calculation (populations doubling time) did not result in differences when compared to the normoxic group after 72 h. However, the oxygen tension used in these studies, near 1% (severe hypoxia), may have affected the results in comparison with 2–2.3%. In this context, it is not possible to affirm the effect of hypoxia on SCAP or DPSC with a single study using severe hypoxia in a short-term assay.

When the proliferation potential of SHED and DPSC is compared under hypoxia (2.3% O_2), the SHED showed a higher proliferation capacity when evaluated using the PDT method for 8 days (Kanafi et al., 2013). This outcome found that hypoxia reproduces the differences in proliferation between SHED and DPSC at normoxia (Miura et al., 2003).

Regarding dental pulp cells, not characterized as stem cells, Iida and colleagues (Iida k Takeda-Kawaguchi et al., 2010) demonstrated that the proliferation rate of DPSC is not dose-dependent in relation to oxygen concentration. Different oxygen concentrations were tested using a cell counting method. The highest proliferation rate was found with 3% oxygen. Cells cultivated with 1% and 10% oxygen had the same growth rate of cells in normoxia. Interestingly, the differences on population doubling time between the normoxia (21% O_2) and hypoxia (3% O_2) groups were noticed only at a low density of cells. This result, corroborating with a study that evaluated the proliferation in 14 days using a cell counting method, showed an increase in proliferation rates in cultures growing under 3% oxygen compared to cultures under normoxia (Sakdee et al., 2009). However, a recent study found no difference in proliferation rate of cells cultured in hypoxia or normoxia, but the oxygen tension (2%) and the methodology (MTT) used were different (Zhou et al., 2014).

Therefore, the available literature regarding the effect of hypoxia on the proliferation capacity of dental stem cells is limited. Moreover, the conflicting results may be attributed to the different methodologies and oxygen concentration employed (Table 1). Further studies are required to elucidate the effect of hypoxia on proliferation as the optimization of this characteristic improves the prospects for using these cells in tissue engineering.

Table 1
Studies evaluating viability/proliferation and plasticity capacities of stem cells from dental tissues under hypoxic conditions.

Study	[O_2]	Cell Type	Duration of hypoxia (days)	Method of analysis/Differentiation	Effect on cells
(Zhang et al., 2014)	2%	PDLSC	7	Cell count/	Increased
				Osteogenic	Increased
(Vanacker et al., 2014)	1%	SCAP	3	PDT/	Unaltered
			28	Osteogenic and neurogenic	Increased
(Kanafi et al., 2013)	2,3%	DPSC and SHED	8	PDT	Unaltered
(Wu et al., 2013a)	2%	PDLSC	1	MTT	Increased
			9	Osteogenic	Increased
(Wu et al., 2013b)	2%	PDLSC	9	Osteogenic	Increased
(Aranha et al., 2010)	1%	DPSC	3	Wst-1	Unaltered

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PDT: Population doubling time; Wst-1: Water soluble tetrazolium salts 1.

5. Hypoxia and dental stem cell differentiation

Although high cell numbers are required for clinical therapy, it is essential that cells submitted to hypoxia are still capable of differentiation. Generally, stem cells increase mitochondrial activity during the differentiation process (Zhang, Marsboom, Toth, & Rehman, 2013; Mandal, Lindgren, Srivastava, Clark, & Banerjee, 2011; Chen, Shih, Kuo, Lee, & Wei, 2008; Cho et al., 2006). Mitochondrial activity depends on the oxygen levels and oxygen reduction can inhibit the differentiation process (Choi et al., 2014; Fehrer et al., 2007; Malladi, Xu, Chiou, Giaccia, & Longaker, 2006). However, the studies that evaluated the hypoxic effect on differentiation potential of dental-derived stem cells showed an improvement of this capacity (Vanacker et al., 2014; Wu et al., 2013a, 2013b; Zhang et al., 2014) (Table 1).

The upregulation of osteogenic (RUNX2, ALP, TGF1β) and neurogenic-related genes (CNP,SNAIL, NSE) was observed for SCAP cultures under hypoxia (Vanacker et al., 2014). In the same way, PDLSC showed an increase of ALP activity and RUNX2, Sp7, osteocalcin and osteopontin expression (Wu et al., 2013a, 2013b).

The hypoxic effect on DPSC and SHED has not yet been investigated. However, the inhibition of osteogenic differentiation under 3% of oxygen on dental pulp cells, accompanied by a decrease in the expression of osteogenic-related genes, such as alkaline phosphatase (ALP), dentin matrix acidic phosphoprotein (DMP1) and dentin sialophosphoprotein (DSPP), was established by Iida and collegues (Iida k Takeda-Kawaguchi et al., 2010).

6. Hypoxia and mesenchymal stem cell surface markers

Among the criteria proposed by the International Society for Cellular Therapy to define human mesenchymal stem cells, is the surface markers profile. It has been established that mesenchymal stem cells must express the surface markers CD73, CD90 and CD105 and have a lack of expression of CD45, CD34, CD11b or CD14, CD79a or CD19 and HLA-DR (Dominici et al., 2006).

Until now, few studies have investigated the effect of hypoxia on the cell surface marker expression of stem cells from dental tissues. No changes on the expression of CD105 were reported under hypoxic conditions (Vanacker et al., 2014). Markers that had their expression modified by hypoxia are STRO-1 and CD133. The number of STRO-1 and CD133 positive cells increased and decreased under hypoxic condition, respectively (Sakdee et al., 2009). STRO-1 is mesenchymal stem cell marker, used for the identification of primitive dental pulp stem cells (Gronthos et al., 2000). The STRO-1 expression varies from 0.02% to 9.56% (Bernardi et al., 2011; Gronthos et al., 2000; Miura et al., 2003; Sakdee et al., 2009) in human pulp stem cell cultures and decreases at later passages (Bernardi et al., 2011). CD133 is considered a cancer stem cell marker and is also expressed in different types of stem cells (Irollo & Pirozzi, 2013).

7. Hypoxia and tissue engineering

One of the current strategies in tissue engineering research is the hypoxic preconditioning of stem cells prior to transplantation in order to improve viability (Bader et al., 2015; Yan et al., 2012) and allow for cell differentiation under specific stimulus (Fotia et al., 2015; Volkmer et al., 2010; Zhang et al., 2014). Soon after transplantation, the cells experience a hypoxic-schemic environment until full vascularization is established. What occurs for most of the protocols is that undifferentiated cells are cultivated under normoxia and are submitted to hypoxia *in vivo*, at the moment that they would start to differentiate. The effects of this approach are not completely understood but this environment change could lead to an inhibition of *in vivo* differentiation. Thus, preconditioning the cells under hypoxia before initiating differentiation protocols, or prior to transplantation, shows promising results in relation to their ability to differentiate under low oxygen conditions (Volkmer et al., 2010). The advantages of the preconditioning of dental stem cells in order to improve differentiation potential remains to be tested in further studies.

Moreover, hypoxia has an effect on the modulation of the paracrine activity of stem cells causing up-regulation of several secretory factors such as FGF2, TGF-β, VEGF, IL-8 and IL-6 in stem cells derived from cord blood (Bader et al., 2015), adipose tissue (Kang et al., 2014), apical papilla (Vanacker et al., 2014) and dental pulp (Aranha et al., 2010). Some authors consider the paracrine action as the major mechanism responsible for the improvement found in cell-based therapies (Ng, Kuncewicz, & Karp, 2015); since cells cultured in normoxia die within a few days after transplantation into ischemic tissue or 3D scaffolds, due to limited availability of oxygen and nutrients. (Potier et al., 2007) Culture of DPSC (Aranha et al., 2010), PDLSC (Wu et al., 2013a, 2013b) and SCAP (Bakopoulou et al., 2015; Vanacker et al., 2014; Yuan et al., 2015b) under hypoxia upregulated the expression of VEGF, even when exposed to severe hypoxia (1% of oxygen).

Thus, the hypoxic preconditioning of cell cultures is recommended for regenerative medicine (Sart, Ma, & Li, 2014), upregulating paracrine factors that stimulate angiogenesis and the secretion of factors associated with cell homing and migration (Muscari et al., 2013). However, the required time and oxygen tension for preconditioning is still unknown.

8. Final considerations

Stem cells from dental tissues have the ability to differentiate into various cell types and their culture under hypoxia may be an alternative to improving their differential potential.

The positive effect of hypoxia on the paracrine function of stem cells from dental pulp has been shown by different research groups (Vanacker et al., 2014; Wu et al., 2013a; Yuan et al., 2015b) which is of interest from the perspective of cell-based therapies (Ishikawa et al., 2015; Wakayama et al., 2015).

The DPCS, SHED and SCAP are stem/stromal cells presenting high proliferation potential (Gronthos et al., 2000; Huang et al., 2008; Miura et al., 2003) when compared to bone marrow stem cells (BMSCs). The expressive increased proliferation potential under hypoxia observed in BMSC and other stem cell sources was not observed in dental-derived stem cells, which suggests that hypoxia may not have a relevant effect on the proliferation of these cells. Furthermore, studies that used oxygen tensions near to 1%, which is considered as severe hypoxia or short course assays, deserves cautious interpretation as this parameters could affect the outcome.

The post-transplant survival of stem cells is a barrier to overcome for successful regenerative strategies. Understanding stem cell metabolism under hypoxia would help to improve *in vitro* expansion protocols and optimize the best approach for the preservation of the regenerative capacity of the cells after *in vivo* transplantation (Sart et al., 2014; Zhang et al., 2014).

The knowledge regarding the effect of hypoxia on stem cells needs to be expanded by future researches to establish the real benefits of this condition for the cultivation and differentiation of cells. In order to facilitate future comparisons, studies should describe in more detail the protocols used for hypoxia induction and cell maintenance. Moreover, the degree of differentiation would benefit from analysis by quantitative methods, allowing for statistical analysis. This will help to clarify the controversial results obtained to date on tissue engineering and in cell therapy fields.

Conflict of interest

None declared.

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Title: Upregulation of pluripotency of stem cells from human deciduous teeth by hypoxia

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Abstract

Cultivation under hypoxia promotes different responses in mesenchymal stem cells and has been producing promising results for clinical applications. Pulp tissue from deciduous teeth is a source of stem cells which has high proliferative potential and is feasible for isolation. This study has evaluated the effect of hypoxia on proliferation, apoptosis and expression of pluripotent related genes of stem cells from human exfoliated deciduous teeth (SHED). Cells were isolated from the dental pulp of exfoliated deciduous teeth and characterized as mesenchymal stem cells, in accordance with the International Society for Cell Therapy (n=5). Cells from the hypoxia group were cultivated under hypoxia (3% oxygen) and compared to the normoxia group (21% oxygen). The proliferation was evaluated by Ki67 antibody up to 7 days, while metabolic activity was measured by wst-8 assay up to 14 days. The apoptosis cells were analyzed by Annexin V and PI (propidium iodide) staining in 24h, 4 and 7 days. The expression of pluripotent genes (Oct4, Sox2 and Nanog) was quantified by qPCR after 24h or 7 days cultivated under hypoxia or normoxia. No differences in metabolic activity, proliferation rate and apoptosis of SHED cultivated under hypoxia or normoxia ($p>0.05$) were observed. The expression of pluripotent genes was significantly higher after 24h and 7 days of exposure to hypoxia ($p<0.01$). These findings indicates the increase of pluripotency within 7 days as being the main advantage of SHED culture in hypoxia.

Key-words: dental pulp, deciduous tooth, stem cells, hypoxia

Introduction

The stem cell niche is defined as a location with low oxygen tension (Shoefiled 1983) and hypoxia has been recognized as the key factor for maintaining the undifferentiated state and plasticity of stem cells (D´Ipollito et al. 2006; Grayson et al., 2006). The culture under a hypoxic environment (<%5 oxygen) has shown increased proliferation rate of mesenchymal (Grayson et al., 2006; Fehere et al., 2007; Grayson et al.,2007; Choi et al.,2014; Zhang et al., 2014; Peng et al., 2015) and embryonic (Ezashi et al., 2005; Forristal et al., 2010 and 2013) stem cells. Moreover, the hypoxia modulates the pluripotency of stem cells (Forristal et al., 2010; Petruzelli et al., 2014; Zhou et al. 2014). The effect on proliferative potential and pluripotency seems to be related to cellular adaptive response to hypoxia, which include the hypoxia-inducible factor 1α (HIF-1α), stabilization and the transcription of several genes that modulate cell characteristics, such as pluripotency, self-renewal and angiogenesis (Haque, 2013).

Stem cells from human exfoliated deciduous teeth (SHED) are mesenchymal stem cells with a high proliferative potential (Miura et al., 2003; Wang et al., 2012) and differentiation capacity in several lineages. The use of SHED has been successfully reported in dental (Rosa et al., 2012) and medicine regenerative strategies (Kawashima, 2012). Moreover, SHED are an attractive source because they are usually discarded after physiologic resorption of the teeth.

However, the low number of cells obtained from the pulp tissue (Miura et al., 2003) is one of the limitations of this promising source of stem cells for use in clinical therapies (Haque 2013). Furthermore, the progenitor population comprises less than 1% of the cells isolated from dental pulp (Gronthos et al., 2000). In this way, the clinical applications of SHEDs requires expansion through consecutives passages, but this process involves time and detrimental effects on cells, such as senescence and genetic instability (Duailibi et al., 2012).

The standard culture *in vitro* of SHED is carried out in atmospheric oxygen tension (21%) (Miura et al., 2003; Wang et al., 2012; Werle et al., 2015), which is considerably higher than physiological tension (Yu et al., 2002). The aim of this study has been to evaluate the hypoxic effect on proliferation, apoptosis and pluripotency of SHEDs.

Materials and Methods

Ethical concerns

Sound deciduous teeth (molars and canines) with at least 1/3 of physiologic root resorption and intended for extraction were selected from 5 donors, aged 8 to 12 years old. The tooth extraction was conducted at the Children and Youth Dental Clinic, School of Dentistry, Federal University of Rio Grande do Sul (UFRGS), Brazil. This research was conducted in accordance with the ethical standards of the Resolution of the National Council on Ethics in Research (n. 466/2012) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The Research Committee (n. 26237) approved the protocol of this study.

Isolation and cultivation of cells

One tooth was obtained from each donor. Mesenchymal stem cell isolation was performed as previously described (Werle et al., 2015). The cells were retrieved by enzymatic digestion using 0.2% type I collagenase solution (Gibco, Grand Island, NY) for 60 min at 37 °C. After centrifugation at 800×g for 10 min the pellet was resuspended and seeded onto a 12-well plate. The cells were cultivated in Low-glucose Dulbecco's modified Eagle medium (DMEM) supplemented by 10 % fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin and 100 □g/mL streptomycin (Gibco, Grand Island, NY. When confluence was reached, the cells were trypsinized using 0.25% trypsin-EDTA solution (Sigma, St. Louis, USA) and transferred to new tissue culture flasks. At the fifth passage (P5) under normoxic environment (21% O_2) the cells were characterized as MSCs. All experiments were performed at P5.

Characterization of MSCs

The expression of cell surface markers was evaluated by flow cytometry (FACSAria III - BD Bioscience, San Jose, USA). The cells from each sample (n=5) were trypsinized and ressuspended in PBS at 10^6 cells/ml. The cells were incubated with the following fluorochrome-conjugated antibodies: anti-CD14-FITC, -CD34-FITC, -CD45-APC, -CD73-PE, -CD90-FITC and -CD105-APC (BD Biosciences) for

30 min at 4°C. The differentiation potentials of osteogenic, chondrogenic and adipogenic lineages were performed by incubation with specific induction media for 4 weeks.

Hypoxia induction

To simulate hypoxia, a sealed chamber (Stem Cell Technologies) was flushed with a gas mixture of 3% O_2, 5% CO_2 and92% N_2 at 20 L/min for 4 minutes. The chamber was sealed and maintained at 37°C. The gas flush interval was defined by the pH alterations of culture medium of cells, in a pilot study. After 24 hours of gas flushing, the pH did not show variances, inferring the stability of CO_2 up to 24 hours (ΔpH 0.012) inside the chamber. The culture medium was changed every 4 days. During the media changes, the hypoxia-cultivated cells were exposed for a short period (<10 minutes) in a normoxic environment. The control group was maintained under normoxia (21% O_2 and 5% CO_2) in regular incubators at 37°C.

HIF-1alpha staining

The cells cultivated under normoxia or hypoxia (n=5) for 48 hours were fixed in 4% paraformaldehyde for 30 minutes. These cells were incubated with PBS containing 1% BSA and 0.1% triton X-100 solution for 30 minutes at room temperature. The cells were incubated with anti-HIF1alpha antibody (Sigma, St. Louis, USA) and diluted in the above described solution for 2 h. The cells were extensively washed and incubated with Alexa Fluor 488 anti-rabbit IgG (Life Technologies) for 30 minutes. The nuclei were stained with DAPI. The images were analyzed by fluorescence microscopy.

Metabolic activity assay

A water-soluble tetrazolium monosodium salt cell counting Kit-8 (wst-8, Sigma-Aldrich, St. Louis, MO) was used for evaluation of the cellular metabolic activity through the experimentation course (1, 3, 5, 7 and 14 days). A total of $1x10^3$ cells/wells were seeded on 24-well plates and incubated under normoxia or hypoxia. Analysis was performed in triplicate, according to the manufacturer's instructions. The absorbance was measured at 450 nm on a spectrophotometer (SpectraMax M2 - Molecular Devices Sunnyvale, USA). Appropriate controls (background fluorescence) were performed for each day of analysis. The absorbance of the test was subtracted from the absorbance

of the control, resulting in the final absorbance. Values were transformed in percentages for each culture (n=5).

Proliferation assay

A total of 1.25 x 10^5 cells were seeded and cultivated under hypoxia and normoxia (n=5). The analysis of proliferation was performed on days 1, 4 and 7 after seeding. The cell suspensions were fixed in 4% paraformaldehyde for 30 minutes at room temperature and then permeabilized with PBS 0.1% Triton X-100 and 1 % BSA for 30 minutes. The cells were stained with mouse anti-human Ki67 antibody (Dako, Glostrup, Denmark) for 1 hour followed by Alexa Fluor 488 anti-mouse IgG (Invitrogen, Oregon, USA) and maintained for 30 minutes in dark incubation. Samples were processed by flow cytometry (FACSAria III - BD Bioscience, San Jose, USA). Data was analyzed using FACSDiva v. 6.1.3 – BD Bioscience, San Jose, USA).

Apoptosis Detection

The frequencies of cells undergoing apoptosis and necrosis were determined using the ApoDETECT Annexin V-FITC Kit (Invitrogen, Frederick, USA). A total of 1.25 x 10^5 cells were seeded and cultivated under hypoxia or normoxia (n=5) for 1, 4 or 7 days. Cell processing was performed according to the manufacturer's instructions. The cells were analyzed by flow cytometry (FACSAria III - BD Bioscience, San Jose, USA).

Gene Expression (qPCR)

The expression of Oct4, Sox2 and Nanog was quantified in the cells under normoxia or hypoxia. Cells were plated in 25 cm^2 tissue culture flasks for analysis after 24 h and 7 days (n=3). The RNA was extracted using Trizol reagent and resuspended in diethyl pyrocarbonate-treated water. Quantification and purity of the RNA was measured using Nanodrop ND-2000 and the cDNA was synthesized using M-MLV Reverse Transcriptase kit (Invitrogen). qPCR reactions were prepared using Power SYBR® Green qPCR Master Mix kit (Invitrogen Co, Carlsbad, CA, EUA). Each 25 µL sample was composed of 12.5 µL of qPCRSupermix, 0.5 µM of each primer (Table 1) and 1 µL of cDNA, diluted 1:3 in water. qPCR cycle conditions were as follows: 95° C for 10 minutes for denaturation followed by 40 cycles at 95° C for 10 s, 60° C for 1 minute and the final step at 70° C for 10 minutes. All the samples

were run in triplicate. Gene expression was normalized by Actb expression and comparisons were performed using the normoxia groups as a reference.

Statistical analyses

Analyses were performed using SPSS 2.0 (IBM Corporation, USA). Data was presented as the mean± SD. The independent samples t test was used to verify differences in metabolic activity, proliferation, apoptosis and gene expression between the normoxia and hypoxia groups at courses evaluated. The significance level was 5%.

Results

Characterization of MSCs

Isolated cells presented the capacity of plastic attachment and the expected morphology. Alizarin red, Alcian blue and Oil Red O staining of the cells confirmed their differentiation capacity into cells of osteogenic, chondrogenic and adipogenic lineages, respectively (data not shown). The mean of mesenchymal surface markers was 98.8% (±2.1) for CD90, 97% (±3.2) for CD73 and 88.4% (±9.4) for CD105. The expression of the hematopoietic markers was 2.4% (±3.9)for CD14, 0.03% (±0.08) for CD34 and 0.06% (±0.1) for CD45.

HIF-1alpha staining

The cell cultures from both groups showed positive staining for HIF-1α. For the SHEDs cultivated under hypoxia, the fluorescence was concentrated in the cell nuclei, as for DAPI colocalization. The SHEDs cultivation under normoxia presented the HIF-α fluorescence staining mainly in the cytoplasm (Fig 1A).

Metabolic activity assay

Cells from both groups presented an exponential growth from day 1 to day 14 (Fig 1B). The absorbance values range were 0.09±0.05 on the first day to 2.75±0.03 on the fourteenth day of analysis for the normoxia group and 0.09±0.09 to 2.84±0.17 for the hypoxia group. Absorbance values were transformed into percentages for statistical analysis. There were no differences in metabolic activity between the hypoxia and normoxia groups throughout the course of the experimental ($p>0.05$).

Proliferation assay

The average frequencies of $Ki67^+$ cells on day 1 were 5.1±2.4% and 6.8±3.5% for the normoxia and hypoxia groups, respectively. On day 4, an increase in the frequency of mitotic cells was observed in both group (43.5% for normoxia – 42.1% for hypoxia), followed by a significant reduction on day 7 (21.4% for normoxia– 20.8% for hypoxia) (Fig 1C and D). There was no statistical differences between the normoxia and hypoxia groups ($p>0.05$) throughout the trial period.

Apoptosis Detection

The frequency of non-apoptotic, early apoptotic, late apoptotic and necrotic cells was accessed by Annexin V and propidium iodide staining (Fig 2A). There were no differences between the groups on the time course. The mean of living cells was higher on day 1 for the normoxia (95.9±0.8) and hypoxia (95.7±1.3) groups ($p>0.05$).

Gene Expression (qPCR)

The upregulation of pluripotency related genes was found in the hypoxia group at 24h and 7 days of analysis compared to normoxia group ($p<0.01$) (Fig 3). Relative quantification values of Sox2, Oct4 and Nanog at day 1 were upregulated compared to day 7 ($p<0.05$). The fold changes were 21.3±9.4, 7.5±1.1, 6.4±1.1 for Sox2, Oct4 and Nanog after 24h cultivated under hypoxia, respectively. After 7 days under hypoxia the fold changes were 9.1±3.2, 4.3±1.6, 3.9±1.6for Sox2, Oct4 and Nanog..

Discussion

The SHEDs from the hypoxia group showed a significant increased expression of pluripotency factors (Oct4, Sox2 and Nanog) compared to the normoxia group. Despite of reduction on expression on day 7 compared to 24 hours under hypoxia, these genes keeping upregulated in relation to normoxia. These results are in accordance with other studies that have tested the expression of pluripotency genes in stem cells from other sources (Petruzzeli et al., 2006; Grayson et al., 2007; Forristal et al., 2010; Zhou et al, 2014).

The increase in the expression of pluripotency markers can represent an advantage of the cultivation of SHEDs under hypoxia. Since the progenitor population comprises less than 1% of the cells isolated from dental pulp (Gronthos et al., 2000),

hypoxia could increase the fraction of the pluripotent cells (Huang et al., 2014) and prolong the culture life span (Fehrer et al., 2007).

The up-regulation of pluripotency markers seems related to HIFs actions. The HIF-1α is continuously expressed, but is degraded by oxygen-activated prolyl hydroxilase domain containing proteins (PHD) in normoxia (Muscari, 2013). We evaluated the HIF-1α gene expression under hypoxia and normoxia and was not observed differences between groups (data no shown). When uin hypoxia, the PHD is blocked and HIF-1α heterodimerizes with subunit HIF-1β into the nuclei (Petruzelli et al., 2006). The complex of HIFs 1, 2 and 3 subunits are responsible for the transcription of more than a thousand genes (Haque 2013), including genes related to pluripotency (Grayson et al., 2007, Forristal et al., 2010, Zhou et al., 2014).

A recent study with DPSCs (dental pulp stem cell) described that the co-overexpression of Oct4 and Nanog acts as an up-regulator of the proliferation rate (Huang et al., 2014). Despite the increase in expression levels of pluripotency genes on SHEDs under hypoxia, their proliferation rate and cell viability did not present statistical differences compared to the normoxic groups.

Several studies use the cell metabolic activity to infer proliferation as the absorbance correlates to the number of living cells (Kanemura et al., 2002). However, the metabolic activity in the hypoxia group may have been underestimated due the possible inhibition of up to 75% on mitochondria biosynthesis in oxygen tensions below 5% oxygen (Basciano et al., 2011). Aranha and colleagues (2010) found no differences in proliferation when DPSCs were cultivated under 21% or 1% oxygen, despite the oxygen tension is lower than the used in the present study.

The assessment of proliferative potential was performed using Ki67 antigen, which is a non-histone protein generally accepted as the most reliable cell proliferative marker because it is expressed in all active cell cycle phases (G1, S, G2 and M), and absent in the G0 phase (Kim et al., 2015). The frequency of mitotic cells was similar between the hypoxia and normoxia groups at all times of analysis, corroborating the result found for BMSC under 3% and 21% oxygen (D´Ippolito et al., 2006). The frequencies of Ki67+ cells in both groups at day 4 were similar to the values found by SHEDs after 3 days in cultivation under normoxic environment (Wang 2012). Lower frequencies of proliferating cells on day 1 and 7 seem to be related to cell adaptation

post trypsinization (Bernardi et al., 2011) and culture confluence (Balint et al., 2015), respectively.

Taking these analyses together, the 3% oxygen tension used in this study does not appear to stimulate the proliferation of SHEDs. No previous study has compared these cells under hypoxic and normoxia environments. Comparing the results with cells from other sources, this result does not corroborate with studies that show a positive effect of hypoxia on the proliferation of mesenchymal stem cells from adipose tissue (ADSC) (Choi et al.2014), periodontal ligament (PDLSC) (Zhang et al., 2014), and especially bone marrow (BMSC) (D´Ippolito et al., 2006; Fehere et al., 2006; Grayson et al., 2006 and 2007; Forristal et al., 2010; Bornes et al., 2011). However, the SHEDs presented a significantly higher proliferation rate when compared to BMSC (Miura et al., 2003; Wang et al., 2012) and it was not possible to observe changes in cellular metabolism during hypoxia in cells with a proliferative potential that is naturally high. Stem cells from apical papilla (SCAP), which have a high proliferation potential too, do not show differences when cultivated under hypoxia (1% oxygen) (Vanaker et al., 2014). Moreover, studies that have reported an increase in the proliferation rate have shown that this effect occurs just at low cell densities (Iida et al., 2010) or when the statistical analysis is performed to compare population doubling (PDT) per day, while the cumulative PDT shows no statistical differences (Bornes et al., 2011).

The flow cytometer is a rapid method for individual analysis of cell apoptosis and seems be appropriate for studying cell death. The Annexin V and PI (propidium iodide) assay has been used to discriminate early apoptotic cells from late apoptotic and necrotic ones, based on the determination of the translocation of phosphatidyl serine (PS) from the inner to the outer layer of the plasma membrane (Gorczyca et al., 1999; Vermes 2000).

The frequency of apoptotic cells cultivated in hypoxia was similar to the cells in normoxia after 7 days. This result is according to data shown by dental pulp cells in a hypoxic environment (5% oxygen) (Agata et al., 2008). It is generally accepted that cell death is more correlated with serum deprivation when an ischemic tissue is simulated (Potier et al., 2007, Zhu et al., 2006). Hypoxic preconditioning has recently been presented as an alternative for increasing post-transplant cell survival and for allowing cell differentiation at low oxygen tension (Wolkmer, 2010; Fotia et al., 2010,

Bornes et al.,2011; Zhang et al., 2014), as the site of transplantation presents ischemic characteristics until angiogenesis is established.

The hypoxia chamber was the choice to simulate the hypoxic environment in this study because it is small enough to be placed inside the incubator and does not require special equipment to use (Brennan, 2014). However, it is important to highlight that even the cells in the hypoxia group were exposed to intermittent normoxia during medium changes. The oxygen tension chosen in this study was based on the study of Iida et al. (2010), which tested oxygen tension varying from 1 to 21% for the cultivation of dental pulp cells. The effect of hypoxia was not dose-response dependent and 3% oxygen tensions showed an improvement the proliferation potential compared to normoxia. The gas flush interval (every 24 hours) was defined by the pH alterations of culture medium of cells under hypoxia. According Brodsky et al. (2013) the stability of CO_2 could be inferred by stability of pH.

These findings, thus, indicates the increase of pluripotency markers in 1 or 7 days as being the main advantage of SHED culture under 3% of oxygen. Therefore, the hypoxic effects on cell differentiation of SHEDs, as well as the effect of hypoxic preconditioning (Fotia et al., 2015; Wakai et al., 2015), needs further investigation. These results together could indicate the recommendation of SHEDs cultivated under hypoxia to improve their use for clinical application.

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Table 1: Real-time primers sequences, amplicon sizes, annealing temperatures and cycle number of PCR reactions.

Primer	Access number	Primer sequence 5′- 3′	Annealing temperature (°C)	Primer size (bp)	Amplicon size (bp)
OCT4	NM_001173531.1	GTGGAGGAAGCTGACAACAA	58	20	223
		GCCGGTTACAGAACCACACT	60	20	
SOX2	NM_003106	ACGGCAGCTACAGCATGA	59	18	104
		GACGTCGTAGCGGTGCAT	60	18	
NANOG	NM_024865.2	AATACCTCAGCCTCCAGCAG	59	20	175
		CTGGGGTAGGTAGGTGCTGA	60	20	
B-ACT	NM_001101	AGCACAGAGCCTCGCCTTT	60	20	77
		CGGCGATATCATCATCCAT	60	20	

Fig 1. HIF-1α staining, metabolic activity and proliferation rate of SHEDs under hipoxia and normoxia.

A) The fluorescence of HIF-1α (green) was concentrated in the cell nuclei for the SHEDs cultivated under 3% oxygen. The SHEDs cultivated under normoxia mainly presented the HIF-α fluorescence staining on the cytoplasm. B) The graph of metabolic activity shows the exponential growth for both groups during the experimental time, without statistical differences ($p>0.05$). C and D) Proliferation analyses by $Ki67^+$ cells showed no differences between the hypoxia and normoxia groups.

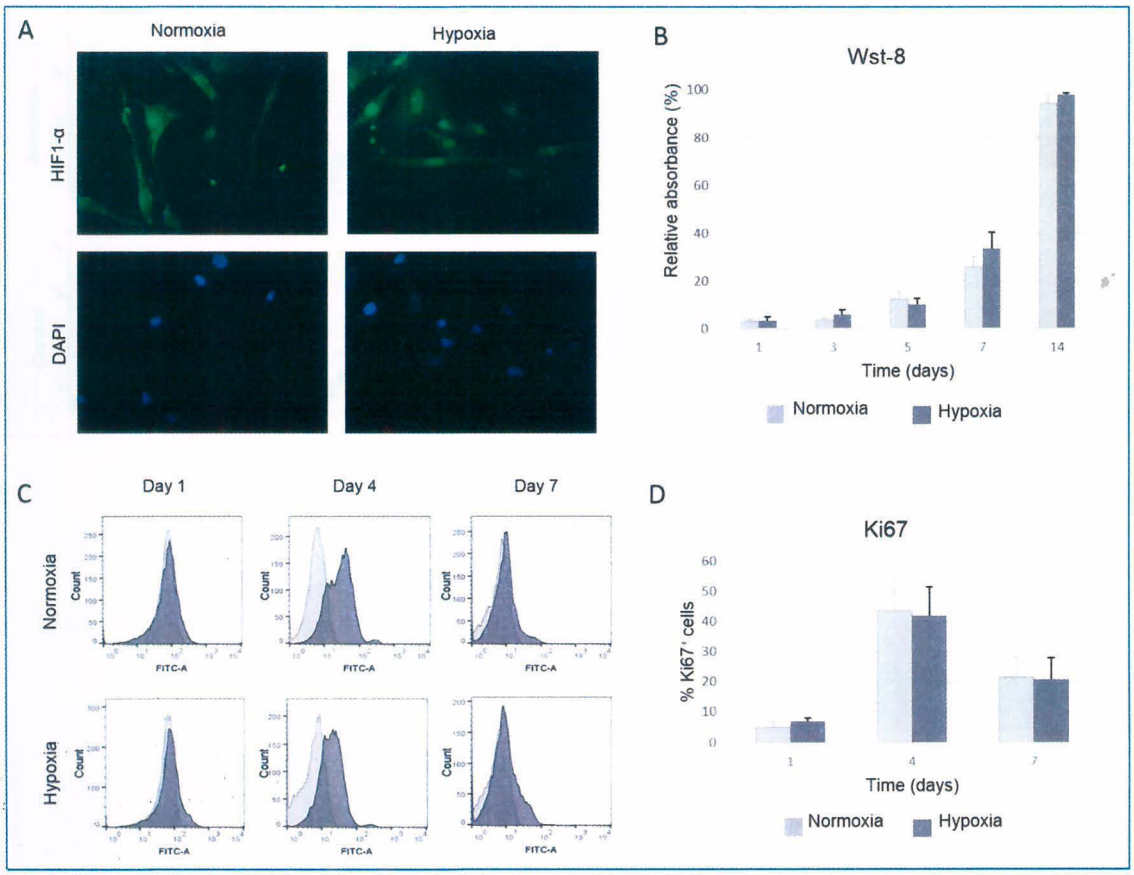


Fig 2. Apoptosis analyses.

A) Non-apoptotic cells were Annexin V-negative and PI-negative. Early apoptotic cells were Annexin V-positive but PI-negative and late apoptotic cells. Together with, necrotic cells were stained intensely with PI. B) There were no statistical differences in the frequency of cells in early or late apoptosis, as well in live and necrotic cells cultivated under hypoxia or normoxia ($p>0.05$).

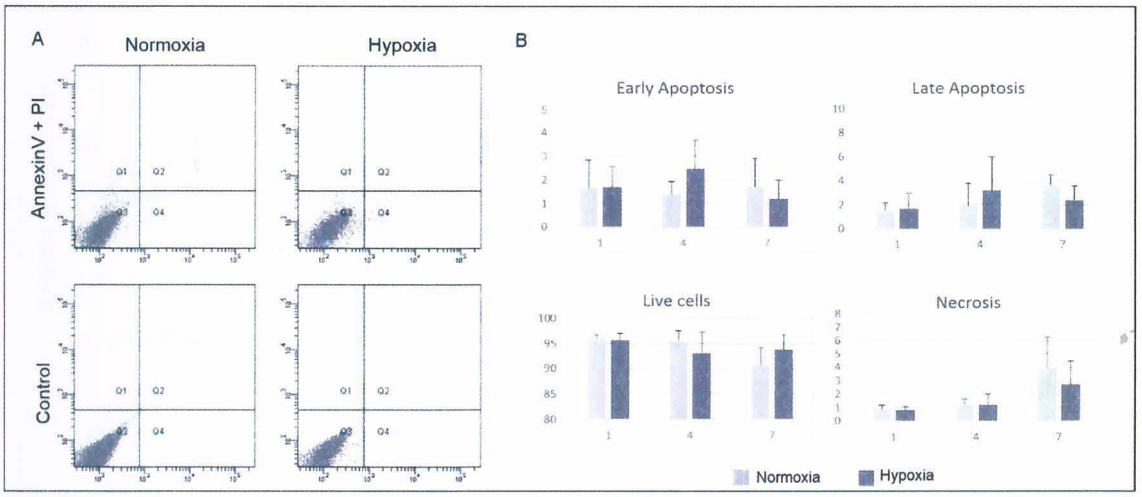
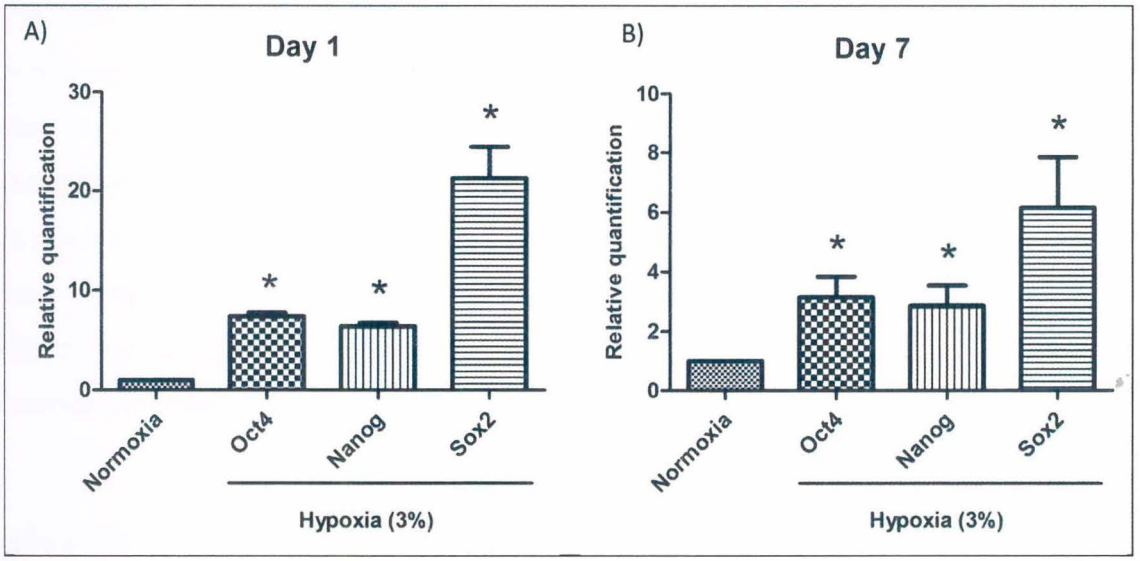


Fig 3. Pluripotency related genes.

The upregulation of Sox2, Oct4 and Nanog was observed in the hypoxia group on days 1 (A) and 7 (B). The reference value (normoxia group) was considered 1. Change fold above 2 is considered as a biological difference. The graphs show biological and statistical differences of gene expression by SHEDs cultured under hypoxia and normoxia.



*statistical difference between the hypoxia and normoxia groups ($p<0.01$).

4. CONSIDERAÇÕES FINAIS

Uma das limitações do uso das SHEDs em terapias celulares é o pequeno número de células obtido a partir da polpa remanescente em um dente em processo de esfoliação. O cultivo em hipóxia, tão promissor para algumas fontes de células-tronco mesenquimais, não afetou a capacidade de proliferação dessas células nos períodos avaliados. Portanto, novas estratégias podem ser buscadas para a otimização da proliferação celular. Uma outra hipótese a ser considerada é que as SHEDs possuem alta taxa de proliferação *in vitro* e que a limitação em se obter um número de células compatível com as terapias celulares está no pequeno volume de tecido pulpar, de onde as células são isoladas. Nesse sentido, é possível considerar que as SHEDs possam ser mais adequadas em aplicações para regeneração de pequenos defeitos ósseos e, especialmente, para a engenharia do tecido pulpar, em que um número consideravelmente menor de células é necessário.

A indicação do cultivo das SHEDs em hipóxia, com o objetivo de melhorar a aplicabilidade clínica dessas células, ainda é muito limitada. Estudos que avaliem outros benefícios do cultivo em hipóxia, tal como o pré-condicionamento previamente a um transplante, podem vir a apontar mais vantagens significativas dessa estratégia de cultivo para as SHED.

Nesse estudo, o cultivo das SHEDs em hipóxia aumentou a expressão de genes relacionados à pluripotência. Isso pode representar um benefício para a pesquisa em engenharia de tecidos, uma vez que pode gerar um aumento na fração de células pluripontetes o prolongamento do tempo de cultivo através das passagens celulares.

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ANEXO – Parecer de Aprovação do Comitê de Ética e Pesquisa

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito da hipóxia e supressão de soro animal na capacidade de proliferação e diferenciação de células-tronco pulpares de dentes decíduos humanos

Pesquisador: Luciano Casagrande

Área Temática:

Versão: 3

CAAE: 27111314.3.0000.5347

Instituição Proponente: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL/COMITÊ DE ÉTICA EM

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 619.411

Data da Relatoria: 17/04/2014

Apresentação do Projeto:

O projeto encontra-se apresentado de maneira completa. Em versões anteriores, a ausência de algumas informações dificultada o completo entendimento do projeto e, consequentemente, das suas implicações éticas.

Objetivo da Pesquisa:

O objetivo principal da pesquisa, bem como os secundários, encontram-se adequadamente descritos. Os ajustes realizados, a partir de pareceres anteriores, permitem a compreensão ainda mais ampla dos objetivos.

Avaliação dos Riscos e Benefícios:

Nesta versão, os riscos e benefícios estão adequadamente apresentados. Questões inerentes a procedimentos radiográficos (os autores explicam e justificam porque não farão parte do projeto), de anestesia, da condição de indicação e de isenção do profissional em relação à indicação de exodontia, bem como procedimentos de proteção ao sujeito estão adequadamente apresentadas. Nesta versão, os doadores (menores) têm um termo de assentimento que poderá (ou não) ser assinado.

Comentários e Considerações sobre a Pesquisa:

O projeto de pesquisa encontra-se adequadamente descrito. Ainda que haja, no termo de

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assentimento para o menor, terminologia um pouco complexa, partindo-se do pressuposto que haverá, também a concordância do responsável e uma explicação verbal, entende-se que possa ser aprovado.

Considerações sobre os Termos de apresentação obrigatória:

Os termos foram apresentados de maneira integral, adequando-se, portanto, às solicitações anteriores, que, majoritariamente tratavam das questões de TCLE e de assentimento do menor doador. Vale destacar que algumas informações solicitadas a respeito da fase (interpretada pelo parecerista como piloto) foi confirmada e trabalhada como tal nesta nova versão. Entende-se que, desta forma, esteja correta. Da mesma forma, os autores identificaram procedimentos laboratoriais de maneira mais detalhada, possibilitando uma melhor e mais fluente compreensão do desenvolvimento do projeto e suas consequências éticas. Houve, também, por parte dos autores, apresentação das possíveis fontes de fomento para o corrente projeto.

Recomendações:

Não existem recomendações adicionais à esta nova versão.

Conclusões ou Pendências e Lista de Inadequações:

Não existem pendências.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado.

PORTO ALEGRE, 17 de Abril de 2014

Assinador por:
MARIA DA GRAÇA CORSO DA MOTTA
(Coordenador)