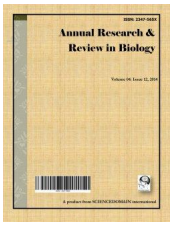

Annual Research & Review in Biology

23(4): 1-11, 2018; Article no.ARRB.38907
ISSN: 2347-565X, NLM ID: 101632869

Genetic, Protein and FTIR Spectroscopic Comparison of Anterior and Posterior Deciduous Dental Pulp for Subsequent Obtention of SHED

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VSM, GJVZ and MMMM participated in the conception and design of the study, carried out the experiments, did the statistical analysis, interpreted the data and drafted the manuscript. Authors RJDM and MRL acquired and interpreted some data and made a critical revision of the manuscript. Authors RRR and OGA carried out the experiments, acquired and interpreted some data. All the authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/38907
Editor(s):
(1) Jean-Marie Exbrayat, Professor, Universite Catholique de Lyon, France.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Priya Thomas, Annoor Dental College & Hospital, Kerala University of Health Sciences, India.
(2) Franco Ferrante, University of Genoa, Italy.
(3) Khushbu Yadav, Nepal Dental Hospital & Technical Research Institute, Nepal.
Complete Peer review History: http://www.sciencedomain.org/review-history/23006

Original Research Article

Received 19th November 2017
Accepted 31st January 2018
Published 3rd February 2018

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ABSTRACT

Background: Stem cells from Human Exfoliated Deciduous teeth (SHED) were identified by Miura in 2003. SHED have been described as a suitable, accessible and potential source for regenerative medicine and therapeutic applications. However, the best group of deciduous teeth for the obtention of stem cells (SCs) has not been established. Therefore, this research aimed to determine the dental organs group from which SHED can be obtained with higher potentiality, considering their biomolecular features.
Methodology: Deciduous teeth from 64 healthy children were collected and divided into two groups: anterior and posteriors. Dental pulp tissue was removed to determine their genetic, phenotypic, and spectroscopic profiles by RT-qPCR, immunofluorescence, and Fourier Transform Infrared (FTIR) spectroscopy respectively.
Results: The results showed a higher gene (*CD73* and *NANOG*) and protein (NANOG and SOX2) expression of mesenchymal and pluripotent markers in anterior SHED. *CD146* gene expression between the two groups shows no statistical significant difference. Furthermore, the analysis of deciduous dental pulps by FTIR spectroscopy showed spectral bands related to biological samples, indicating the higher state of potentiality in anterior deciduous dental pulps.
Conclusion: The deciduous dental pulp harbor a heterogenous population of SCs with different potentiality; however, the expression of multipotent and pluripotent markers was higher in the pulps from anterior deciduous teeth respect to posterior deciduous teeth. The storage and obtention of SHED from anterior teeth is more recommended respect to posterior teeth. However, it is necessary to analyze more stem cell markers and to study the differentiation capability of SHED.

Keywords: Adult stem cells; deciduous teeth; dental pulp; SHED; regenerative medicine.

1. INTRODUCTION

The regeneration process is essential for many organisms including humans, and regenerative medicine has become the leading field of biomedical research due to its possible implications for the treatment of chronic degenerative diseases [1]. Cell therapy is one of the disciplines of regenerative medicine and depends on cell culture techniques to generate cells that will replace the morphological structures, tissues, and functions [2].

Currently, Stem Cells (SCs) seem to be the basis of cell therapy and they are characterized by self-renewal properties that allow them to generate more SCs that differentiate into various cell lineages when cultured under appropriate conditions. According to their origin, these cells are classified as Embryonic Stem Cells (ESCs) or Adult Stem Cells (ASCs) and based on their differentiation potential, SCs are classified as totipotent, pluripotent, multipotent and unipotent [3].

Although ASCs or postnatal SCs have less potentiality that ESCs, they are considered to be multipotent as they retain two fundamental characteristics of SCs: self-renewal for limited periods when they are stimulated to proliferative cell cycle, and capability of multilineage differentiation. Moreover, due to ethical concerns associated with the use of ESCs, the interest in ASCs research has increased over the years [4].

ASCs have been isolated from many tissues and organs including dental tissue. Several mesenchymal SCs types have been identified and isolated from oral tissues, but Stem Cells from Human Exfoliated Deciduous teeth (SHED) seems to generate the best possible results regarding the potentiality and proliferative capacity [4]. These cells were isolated by Miura in 2003 and were described as a highly proliferative population of clonogenic cells capable of differentiating into at least three different cell lines including osteo/odontogenic, adipogenic and neurogenic cells [4,5].

SHED have been described as a suitable, accessible and potential alternative source for regenerative medicine and therapeutic applications [6]. The obtainment of deciduous teeth is simple and convenient, with little or no trauma, because dental exfoliation in humans is a genetically regulated event that occurs between 5 to 12 years [7]. Furthermore, the deciduous teeth can be obtained easily without increasing the patient morbidity, and few ethical considerations are requested.

SHED are a heterogeneous multicellular population of SCs and express mesenchymal (*CD73*, *CD105*, *CD146*, *CD166*) and pluripotent (*OCT-3/4*, *NANOG*, *REX-1*, *SOX2*, and *CD117*) SCs markers [4,8,9,10]. *CD73* expression has been linked to the regulatory phenotypes of T and NK cells, and it has been shown that CD73/adenosine pathway involves the immunomodulatory function of MSCs in autoimmune responses [11]. *CD146* is a key cell adhesion protein in vascular endothelial cell activity and angiogenesis, and play a pivotal role as a cell marker, useful in identification of genuine MSCs. Its expression defines MSCs with higher multipotency and has been linked to SCs derived from bone marrow, placenta, adipose tissue and umbilical cord. In fact, *CD146*-expressing MSCs clones from multiple organs were found to exhibit trilineage potency [12].

NANOG, a pluripotent marker, is a transcription factor whose expression is observed in the morula and inner cell *mass,* but is absent in unfertilized oocytes, 2 to 16 cell embryos, early morula, and trophectoderm. *NANOG* is downregulated when organogenesis is initiated at the time of embryo implantation. Its silencing leads to the differentiation of ESCs into trophectoderm and extraembryonic endodermal lineages, along with a downregulation of *OCT-4*. *SOX-2* is included in the SOX B1 group of transcription factors having single high-mobility group DNA-binding domain. Together with *OCT-4* and *NANOG*, *SOX-2* plays a role in the maintenance of ESCs pluripotency. Its expression is first observed during the morula stage, followed by the inner cell mass, epiblast, and cells from the extraembryonic ectoderm [13].

Several studies have isolated SCs from different dental regions; however, these cells differ in the expression pattern of SCs markers and consecutively on their potential of differentiation [6,10,14,15].

SHED represent a population of postnatal multipotent SCs that could be more immature than the postnatal stromal SCs populations previously analyzed [16]. This could be attributed to the fact that dental pulp of deciduous teeth is present before birth, maintaining an active niche rich in SCs, which has not been affected by the cumulative effect of genetic and environmental factors [9].

Therefore, these cells seem to be the ideal source to repair damaged dental structures, induce bone regeneration and possibly could be used to treat neural tissue injuries or degenerative diseases [17-19].

Since there are insufficient literature studies to establish the best group of deciduous teeth for the obtention of SCs, this study is set to analyze the dental pulp tissue from anterior and posterior deciduous teeth, comparing their genetic, phenotypic, and spectroscopic profiles by RT-qPCR, immunofluorescence, and Fourier Transform Infrared (FTIR) spectroscopy respectively. This study is therefore designed to determine the dental organ group from which SHED can be obtained with high rate of potentiality.

2. MATERIALS AND METHODS

2.1 Study Population and Sample Collection

The current study was conducted in the “Unidad de Especialidades Odontologicas” of the National Defense Ministry-Mexico in the period from December 2016 to June 2017. 64 dental pulp tissues from anterior and posterior deciduous teeth of healthy children aged between 5 and 10 years were collected. They were further sub-grouped: 32 pulp tissues (16 anteriors and 16 posteriors) were used to analyze gene expression, 16 pulps (8 anteriors and 8 posteriors) to study protein expression and 16 pulps (8 anteriors and 8 posteriors) to examine the spectroscopic profile. It is important to mention that the biomolecular assays were performed *in-situ* within the dental pulp tissue without the isolation of cells.

Healthy deciduous teeth from children aged between 5 and 10 years with retained deciduous teeth or teeth close to natural exfoliation, with tooth mobility class I and II or with root resorption that not exceeded 2/3 of root length were included in the study group. Teeth with carious lesions, dental restorations or alterations on its development were excluded from the study. Dental organs characteristics considered for the current study are shown in Table 1.

Written informed consent for the dental extraction and participation in the study were obtained from their legal guardians. The Institutional Human Research Ethical Committee approved the protocol and the informed consent forms. All experiments have been examined and approved by the appropriate ethics

Table 1. Dental organs characteristics used in the study

Anterior deciduous teeth				Posterior deciduous teeth			
Donator	**Age**	**Gender**	**Dental organ number (in FDI)**	**Donator**	**Age**	**Gender**	**Dental organ number (in FDI)**
1	6	F	81	1	9	F	74
2	6	F	71	2	9	F	74
3	8	F	52	3	9	F	75
4	8	M	62	4	10	F	54
5	6	F	51	5	10	F	55
6	7	M	51	6	10	M	64
7	8	F	83	7	10	F	84
8	9	M	82	8	10	F	55
9	9	M	53	9	10	F	54
10	9	M	73	10	10	F	84
11	9	F	73	11	11	M	55
12	10	M	63	12	11	M	75
13	10	M	73	13	11	M	65
14	10	M	83	14	11	F	55
15	10	M	63	15	11	M	75
16	5	F	71	16	8	F	74
17	8	M	62	17	9	M	64
18	6	F	81	18	8	F	84
19	9	M	63	19	9	M	64
20	6	F	51	20	10	F	65
21	10	M	53	21	9	M	74
22	9	F	83	22	9	F	84
23	5	F	81	23	9	F	64
24	7	M	62	24	11	M	75
25	7	F	82	25	9	M	54
26	5	M	81	26	9	F	74
27	6	F	51	27	10	F	85
28	7	M	71	28	9	F	54
29	7	F	61	29	10	F	64
30	9	F	73	30	9	F	84
31	7	F	51	31	11	F	55
32	9	M	53	32	9	M	74

committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.2 RT-qPCR Assays

Dental pulp tissue was removed from each tooth, and total RNA was isolated with Trizol reagent according to the manufacturer's protocol (Invitrogen, CA USA). The integrity of RNAs samples was checked by electrophoresis in non-denaturing agarose gels. RNA concentrations were determined spectrophotometrically at 260 nm. Isolated RNA was treated with DNase RQ1 RNase-Free (Promega WI USA) to avoid genomic DNA contamination. Total RNA DNase treated was reverse transcribed using GeneAmp Kit (Applied Biosystems CA, USA), according to the manufacturer's protocol. RT-qPCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA), monitoring in real time the increasing of fluorescence using the SYBR Green PCR Master Mix (Applied Biosystems CA, USA). Immediately after the amplification, melt curve protocols were performed to ensure that self-complementary primer-dimmers formations and other non-specific products were minimized or eliminated. RT-qPCR assays were analyzed in triplicate. Primer expresses software for Real-Time PCR

ver 3.0 Applied Biosystems was used to design the primers for RT-qPCR (Table 2).

The relative quantification was calculated using CT method, which uses the arithmetic formula $2^{-\Delta\Delta CT}$ [20]. To validate the $2^{-\Delta\Delta CT}$ method we verified that the efficiency of the amplification of target genes and the internal control *GAPDH*, were equal.

Table 2. Oligonucleotides used in real-time quantitative PCR (RT-qPCR)

Gene	Sequence
CD146	F: ACCCTGAATGTCCTCGTGACC
	R: TGAGGCCAGTGGTTGTGTTG
CD73	F: GAGGTGGCGCACTTCATGA
	R: GTGGCCCCTTTGCTTTAATG
NANOG	F: CTGTGATTTGTGGGCCTGAA
	R: TGTTTGCCTTTGGGACTGGT
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: GCCTTCTCCATGGTGGTGAA

F: forward 5' – 3' and R: reverse 5' – 3'

2.3 Immunofluorescence Staining

Expressions of proteins in anterior and posterior dental pulp tissues were analyzed by immunofluorescence. All tissues were fixed for 30 minutes using 4% paraformaldehyde (Sigma-Aldrich) and then washed with phosphate buffer solution (PBS) twice. Subsequently, cells membranes were permeabilized with 0.1% Triton (Sigma-Aldrich). Tissues were then washed with PBS and incubated with blocking protein (Dako, Glostrup, Denmark) for 20 minutes to inhibit nonspecific staining. Immunocytochemical staining was done using the following rabbit primary antibodies: anti-NANOG (1:200; Abcam, Cambridge, UK) and anti-SOX2 (1:250; Abcam). Primary antibodies were incubated overnight at 4°C. Samples were then washed with PBS twice and the conjugated secondary antibody Dylight 488 goat anti-rabbit (1:200, Abcam) was incubated for 45 minutes. Finally, samples were washed with PBS and coverslipped with 10% glycerol. Microscopic observations were carried out with a fluorescence microscope (Ti-U Eclipse, Nikon, Japan).

The software Image-Pro Premier (MediaCybernetics, MD, USA) was used to measure cell fluorescence of NANOG and SOX2. All fluorescent cells in a field employing a 20X objective within the defined regions of interest were selected. The color intensity was measured in a single channel, obtaining the mean intensity of each cell, which was analyzed and averaged according to the tissue group that they belonged (anterior or posterior dental pulp). For each of the three biological replicates one field was analyzed.

2.4 Fourier Transform Infrared Spectroscopy Analysis

FTIR spectral analysis of anterior and posterior deciduous dental pulp tissues were conducted in the spectral range between 400-4000 cm^{-1} using an FTIR spectrometer Bruker Vertex 70 in the Attenuated Total Reflection (ATR) sampling mode. Each dental pulp was obtained and deposited onto the surface of the ATR crystal and dried at room temperature for about 10 minutes to eliminate water excess. The infrared radiation propagated along the crystal to obtain the corresponding spectra, which were the average of 120 data acquisitions.

2.5 Statistical Analysis

Statistically significant differences in gene and protein expression between groups for the different stem cells markers were analyzed using t-student test. All statistical analysis were developed using Sigma Stat statistical software ver 2.0 SPSS, Inc., and the significance level was $p<0.05$.

3. RESULTS

3.1 Gene Expression

Relative expression levels of the studied genes from anterior and posterior dental pulp groups are shown in Fig. 1. No statistically significant difference between the two groups for the expression of *CD146* gene was observed; however, higher expression levels of *CD73* and *NANOG* genes were found in anterior-pulps respect to posterior-pulps, 8.9 and 2.5 fold respectively.

3.2 Protein Expression

Protein expression was analyzed by immunofluorescence in anterior and posterior dental pulp tissues. In agreement with the gene expression results, fluorescence intensity confirmed higher expression of pluripotent markers (NANOG and SOX2) in anterior-pulp tissues (Fig. 2). Quantitative analysis of the mean fluorescence intensity in deciduous dental

pulp is summarized in Fig. 3. It is important to mention that 5 to 8 SOX2-positive cells in an entire histological section of a deciduous dental pulp were detected.

3.3 FTIR Analysis

FTIR spectra of anterior and posterior deciduous dental pulps showed absorption bands related to different types of biomolecules including lipids, proteins, carbohydrates, and nucleic acids, which are common findings in biological samples.

Fig. 4A shows the raw FTIR spectra of anterior and posterior deciduous dental pulp tissues depicted in the amide I proteins region (1600-1700 cm^{-1}) related to C=O stretching vibration. Broadening and increase in the intensity of the amide I band of anterior dental pulps compared with the corresponding amide I band of posterior dental pulps was observed, which is related to a higher number of components of the secondary structure of proteins such as β-sheets (1634 cm^{-1}) and α-helices (1650 cm^{-1}).

Likewise, Fig. 4B shows the raw FTIR spectra of anterior and posterior deciduous dental pulps depicted in the glycogen and phosphate groups regions (1030-1080 cm^{-1}). The band at 1030 cm^{-1} is related to glycogen, and the band at 1080 cm^{-1} is associated with symmetrical stretching vibrations of PO2 phosphodiester groups. In the figure, it is possible to observe an increase in the intensity of posterior dental pulps spectrum compared with the corresponding spectrum of anterior dental pulps.

4. DISCUSSION

Since the first report of the derivation of human pluripotent ESCs in 1998, a significant number of studies have focused in the obtention of these cells and have explored adult tissues containing SCs with ESCs characteristics [21]. About this, recently, a small group of ASCs with pluripotent SCs surface markers such as NANOG and OCT-4 has been reported, called very small embryonic-like stem cells [8,22].

In addition, it has also been reported that SHED are in a more undifferentiated state than SCs from adult dental pulp, because they express CD117 (receptor for stem cell factor I, typical for pluripotent cells), retaining their undifferentiated state even after extended culture periods and cryopreservation, without affecting their biological and immunological properties [4]. Based on the above concept, we deliberated the current study to analyze the ideal dental organs for SHED obtention, through different techniques according to their genetic and biochemical potentiality profiles.

In this study, a higher gene (*CD73* and *NANOG*) and protein (NANOG and SOX2) expression of mesenchymal and pluripotent markers in anterior deciduous dental pulps was observed, suggesting that the anterior exfoliated deciduous teeth harbor SCs with higher potentiality; attributing the reason to less time span of anterior teeth in the oral cavity and to the fact that SCs decline with age [23-26]. Moreover, anterior teeth are less affected by changes in the external environment such as temperature and mechanical forces of mastication. Besides, anterior teeth maintain a constant blood supply during development due to its anatomy of a single large root. In contrast, posterior teeth have multiple sources, and the root canals are so narrow that could be affecting the cell irrigation required to maintain the viability of SCs.

Furthermore, we also compared the gene expression levels in anterior and posterior dental pulp tissues, and similar to other reports a higher gene expression of *NANOG* followed by *CD73* and *CD146* was detected [27-29].

Pluripotent and multipotent markers on anterior and posterior dental pulps were detected, agreeing with the results reported by Aghajani *et al.* who immunodetected mesenchymal and pluripotent SCs markers on SHED [27], the results of the present study are also in accordance with Kashyap (2015) who stated that SHED may not be a single-cell type, and maybe a heterogeneous population of cells from the pulp. On the other hand, Kashyap has mentioned that the number of cells isolated from the deciduous canines is more compared to other types of teeth [30]. In this study, from 5 to 8 SOX2-positive cells in anterior deciduous pulp tissue were detected; this could be related to the long unresorbed roots associated with canines, which harbor more pulp tissue in comparison to other teeth. In addition, Miura et al. [5] have reported the obtainment of 12-20 SCs by cell sorting in cell suspension consisting of dental pulp components, results that are quite similar to the obtained in this work, once we observed from 5 to 8 cells in an entire histological section of dental pulp.

Respect to infrared vibrational spectroscopy, in this study the amide I proteins, glycogen, and phosphate groups regions were analyzed in the deciduous dental pulps based on the fact that spectral bands related to cellular potentiality can be determined in these regions [3,31,32].

In addition to that, amide I proteins region from 1600 to 1700 cm^{-1} in the anterior deciduous pulps spectrum showed an increase in the relative intensity of components associated with the secondary structure of proteins as β-sheets and α-helices compared to the posterior pulp spectrum. This suggested a higher potentiality in anterior pulps, agreeing with other works in which we found a higher number of β-sheets and α-helices in pluripotent stem cells than in differentiated cells [3,31].

Furthermore, according to our previous works and Walsh et al., the spectral interval from 1030 to 1080 cm^{-1}, glycogen and symmetric phosphate stretching vibrations, showed an increase in the intensity of the posterior deciduous dental pulps

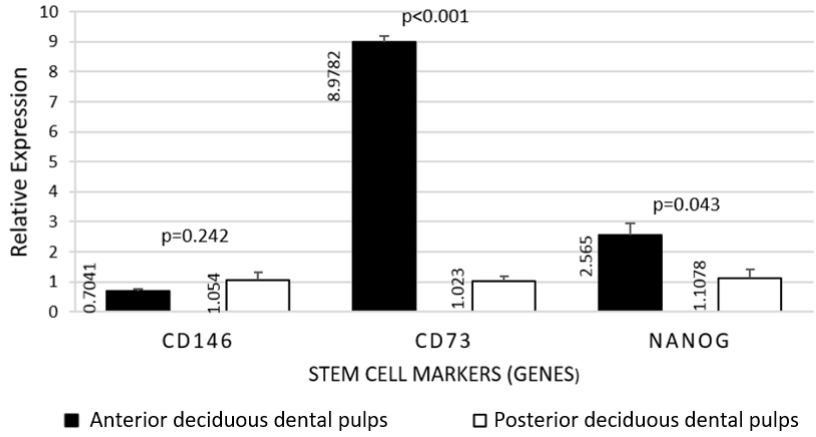


Fig. 1. Relative gene expression of *CD146*, *CD73,* and *NANOG* in anterior and posterior deciduous dental pulps. Triplicate Real-Time qPCR was performed. Expression levels were normalized against *GAPDH*

Bars represent means ± SD. All samples were analyzed three times.
(p) Represents the value of statistical significance in relative gene expression between anterior and posterior deciduous pulps for each gene

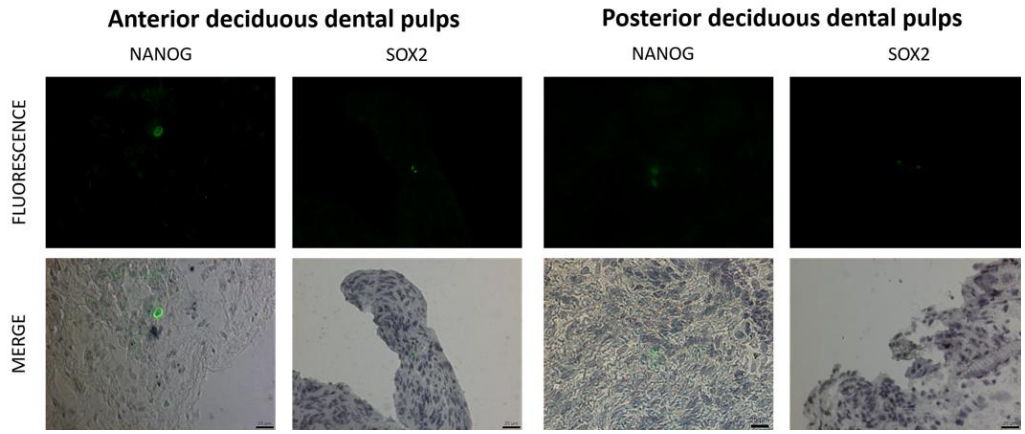


Fig. 2. Representative images of immunofluorescence analysis on deciduous dental pulps. Immunodetection of pluripotency markers (NANOG and SOX2), evidencing a higher protein expression on anterior stem cells from human exfoliated deciduous teeth (SHED)

(N = 8, with three biological replicates, 200X)

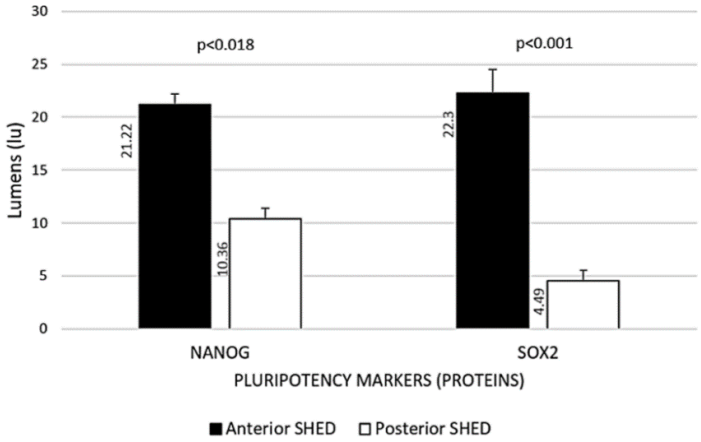
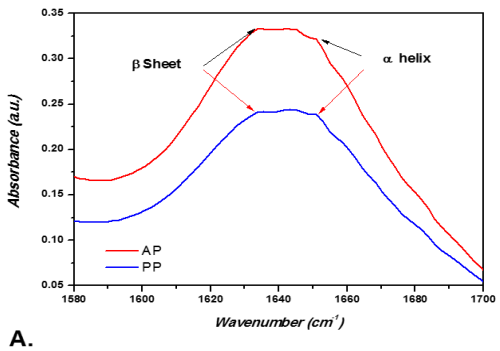
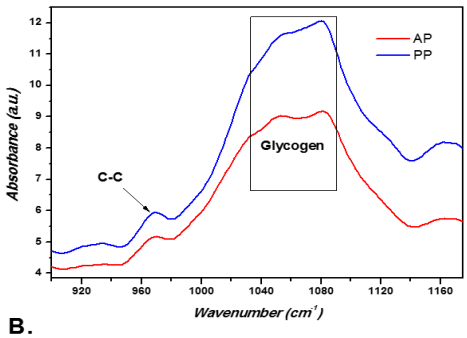


Fig. 3. Quantitative analysis of the mean fluorescence intensity on stem cells from human exfoliated deciduous teeth (SHED). The graph displays the quantification of fluorescence of the pluripotent proteins (NANOG and SOX2) on anterior and posterior SHED

Bars represent means ± SD. (p) Represents the value of statistical significance in protein expression between anterior and posterior SHED. (N = 8, with three biological replicates)



A.



B.

Fig. 4. Fourier transform infrared (FTIR) spectra of anterior and posterior deciduous dental pulps. A. Amide I groups from proteins (1600-1700 cm^{-1}). B. Glycogen and phosphate groups (1030-1080 cm^{-1}). AP: anterior pulps, PP: posterior pulps

(N = 8, with three biological replicates)

compared to anterior dental pulps. This could be assigned to the absorbance of glycogen which starts to increase during the potentiality decrease or cell differentiation, due to glycogen synthase kinase-3 (Gsk3) being activated and thereby an increase glycogen levels in these process [33,34]. Besides, this region also constitutes a good indicator of alterations in the secondary structure of DNA, indicating this region as pivotal in the differentiation process [35,36], confirming the greater state of potentiality in SHED from anterior pulps.

With the above-mentioned and similar to the results established by Martinez-Saez *et al.* and Arora et al. the deciduous dental pulps showed pluripotent markers (NANOG and SOX2), despite the fact that SHED are considered multipotent. Anterior deciduous dental pulp tissues presented better pluripotent molecular and spectroscopic profiles that make them exceptionally valuable for cell replacement therapies and regenerative medicine. Nevertheless, some other features need to be studied, such as morphology, expression of stem cell markers like SOX2, STRO-1, CD105, CD117 and OCT-3, trilineage differentiation *in vitro* and derivation of embryoid bodies [37,38].

5. CONCLUSION

The current study highlights that the dental pulp tissues from deciduous teeth harbor SCs with different potentiality. However, the expression of

multipotent and pluripotent markers was higher in the pulp from anterior deciduous dentition respect to posterior deciduous dentition. The storage and obtention of SHED from anterior teeth is more recommended respect to posterior teeth. However, it is necessary to analyze more stem cell markers and to study the differentiation capability of SHED.

ACKNOWLEDGEMENTS

The authors thank the "Unidad de Especialidades Odontológicas", the legal guardian of each participant, as well as Dr. Liliana Cortes Rangel and Bs.C. Jose Antonio Contreras Rozo who contributed to the recollection of the biological samples. The A022-2017 program supported this work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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