UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

MAYARA BRINGEL DOS SANTOS

Isolation, characterization, and perspectives of use of stem cells from human exfoliated deciduous teeth (SHED) from children with cleft lip and palate

Isolamento, caracterização e perspectivas de uso de células-tronco de dentes decíduos humanos esfoliados (SHED) de crianças com fissuras labiopalatinas

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Orientadora: Profª. Drª. Maria Aparecida de Andrade Moreira Machado

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"Em algum lugar, algo incrível está esperando para ser descoberto".

Carl Sagan

RESUMO

Esta tese teve como objetivo apresentar dois artigos científicos. O Artigo 1, aborda uma revisão de estudos utilizando células-tronco de dentes decíduos humanos esfoliados (SHEDs) na regeneração óssea, salientando o uso em pacientes com fissuras labiopalatinas (FLP) e o Artigo 2, objetivou comparar a caracterização de SHED entre crianças com e sem fissuras labiopalatinas, a fim de analisar se as SHEDs autólogas podem ser consideradas uma opção para que, em breve, possam ser utilizadas para fins terapêuticos, como uma alternativa na reconstrução de fissuras alveolares em pacientes com FLP. No Artigo 1, foi possível observar que estudos *in vitro* e *in vivo* estão sendo realizados para que a regeneração óssea seja feita utilizando materiais não invasivos, com o uso de *scaffolds* e células-tronco como as SHEDs. Estudos *in vivo* mostraram que a SHED pode induzir a formação óssea, produzir dentina e expressar marcadores neuronais. No entanto, apesar de muitos esforços já feitos, por ser uma alternativa promissora que pode trazer benefícios para o tratamento e para a qualidade de vida de diversos pacientes, ainda há poucos relatos na literatura de aplicabilidade, principalmente em pacientes com FLP. No Artigo 2, tecidos pulpares foram obtidos de 6 dentes decíduos hígidos em estágio de esfoliação, de 6 doadores com idade entre 4 e 12 anos e divididos em dois grupos, sem fissura labiopalatina (Grupo Controle) e com fissura labiopalatina (Grupo Fissura). As células obtidas por cultura primária foram analisadas imunofenotipicamente, onde as que apresentaram expressões positivas dos marcadores de superfície celular CD105, CD73, CD90 e negativas de CD45, CD34, CD11b, CD19, HLA-DR (PE) foram coletadas após separação em Citometria de Fluxo. As células foram submetidas à diferenciação multilinhagem e foram analisadas morfologicamente. A diferença imunofenotípica entre os grupos foi analisada pelo teste t de Student e o nível de significância foi estabelecido em 5% ($p < 0,05$). Foi demonstrada adesão plástica, e altos níveis de marcadores CTM foram identificados em ambos os grupos, com expressão positiva de CD105, CD73 e CD90 (≥95% positivo) e ausência de marcadores de linhagem hematopoiética CD45, CD34, CD11b, CD19 e HLA-DR (≤2% positivo). As células pulpares isoladas em todas as amostras demonstraram capacidade de diferenciação adipogênica, osteogênica e condrogênica. A análise morfológica identificou núcleo ligeiramente menor e capacidade proliferativa menor no tempo avaliado no grupo Fissura. Houve diferença estatisticamente significante ($p = 0,042$) na comparação das SHEDs obtidas após *sorting* nos dois grupos, sendo maior no grupo Fissura. Após a confirmação dos critérios padrão para definição de CTM multipotentes em ambos os grupos, foi possível

confirmar o isolamento e a caracterização de SHED em todas as amostras. Esse achado sugere que SHEDs de crianças com FLP podem ser consideradas uma opção para investigações futuras sobre o uso autólogo de células em intervenção terapêutica. Não obstante, estudos futuros ainda são necessários para avaliar possíveis diferenças internas entre as células, analisar a curva de crescimento entre os grupos e quantificar a diferenciação osteogênica, visando identificar se a fissura poderia favorecer a regeneração óssea por meio da engenharia de tecidos.

Palavras-chave: Fissura Palatina. Fissua Labial. Células-Tronco Mesenquimais. Engenharia Tecidual.

ABSTRACT

Isolation, characterization, and perspectives of use of stem cells from human exfoliated deciduous teeth (SHED) of children with cleft lip and palate

This thesis aimed to present two scientific papers. Article 1 approached a review of studies using stem cells from human exfoliated deciduous teeth (SHEDs) in bone regeneration, highlighting the use in patients with cleft lip and palate (CLP). In Article 2, the objective was to compare the characterization of SHED between children with and without cleft lip and palate, to a view to analyzing whether autologous SHEDs may be considered as an option so that, soon, they can be used as an alternative in the reconstruction of alveolar clefts in patients with CLP. In Article 1, it was noted that in vitro and in vivo studies have been carried out so that bone regeneration can be performed with non-invasive materials, such as scaffolds and stem cells, including SHEDs. In vivo studies have shown that SHED can induce bone formation, produce dentin, and express neuronal markers. Although many efforts are being made because it is a promising alternative therapy that could bring benefits to the treatment and quality of life of many subjects, there are still few reports in the literature about its applicability, especially in subjects with CLP. In Article 2, dental pulp tissues were obtained from six healthy deciduous teeth in the exfoliation stage, from six donors aged between 4 and 12 years, and divided into two groups, without cleft lip and palate (Control Group) and with cleft lip and palate (Cleft Group). The cells obtained in the primary culture were analyzed immunophenotypically and collected those with positive expression of cell surface markers CD105, CD73, CD90 and negative for CD45, CD34, CD11b, CD19, HLA-DR (PE) after sorting in Flow Cytometry. The cells were subjected to multilineage differentiation and were morphologically analyzed. The difference immunophenotypic between groups was analyzed by Student's t-test, and the level of significance was set at 5% ($p < 0.05$). Plastic adhesion was demonstrated and high levels of MSC markers were identified in both groups, with positive expression of CD105, CD73, and CD90 (≥95% positive) and absence of hematopoietic lineage markers CD45, CD34, CD11b, CD19, and HLA- DR (≤2% positive). Pulp cells demonstrated adipogenic, osteogenic, and chondrogenic differentiation capacity. The morphological analysis identified a slightly smaller nucleus and proliferative capacity lower in the time evaluated in the Cleft group. There was a statistically significant difference ($p=0.042$) when comparing the SHEDs obtained after sorting from the two groups, being greater in the Cleft group. After confirming the standard criteria for defining multipotent MSCs in both groups, it was possible to confirm the isolation and characterization of SHED in all samples. These results suggest that SHEDs from children with

CLP can be considered an option for future investigations into the autologous use of cells in therapeutic intervention. Notwithstanding, future studies are still needed to evaluate possible internal differences between cells, analyze the growth curve between groups and quantify osteogenic differentiation, aiming to identify whether the cleft could favor bone regeneration through tissue engineering.

Key words: Cleft Palate. Cleft Lip. Mesenchymal Stem Cells. Tissue Engineering.

TABLE OF CONTENTS

1 INTRODUCTION

1 INTRODUCTION

Cleft lip and palate (CLP) are the most common and prevalent congenital anomalies affecting orofacial structures, with a worldwide incidence of approximately 1 in every 700 live births (SEIFELDIN, 2016; HIRAKI et al., 2020; VYAS et al., 2020). Despite the complex etiology, these anomalies may be caused by a combination of genetics and environmental factors (SEIFELDIN, 2016; CENTERS FOR DISEASE CONTROL AND PREVENTION-NCBDDD, 2020; VYAS et al., 2020). For the reconstruction of cleft area defects, the most accepted therapeutic intervention consists of secondary alveolar bone graft in the mixed dentition stage using autogenous iliac bone (POUREBRAHIM et al., 2013; BERGER et al., 2015; SEIFELDIN, 2016; MARTÍN-DEL-CAMPO; ROSALES-IBAÑEZ; ROJO, 2019; HIRAKI et al., 2020). However, this method has disadvantages as regards pediatric patients, where availability for bone collection may be limited, as it is an invasive process with potential risks for the donor area, such as infection, paresthesia, postoperative pain, and problems of healing (BERGER et al., 2015; AL-AHMADY et al., 2018; MARTÍN-DEL-CAMPO; ROSALES-IBAÑEZ; ROJO, 2019).

Tissue engineering strategies have become an alternative since they offer the possibility of using biomaterials directed to tissues and cells to be applied in the affected area to promote the regeneration of missing or damaged tissues (MARTÍN-DEL-CAMPO; ROSALES-IBAÑEZ; ROJO, 2019). Biomedical research and tissue engineering studies have shown great interest in using stem cells due to their excellent properties (MOREAU et al., 2007; KOSINSKI et al., 2020). Stem cells are undifferentiated cells that have the capacity for proliferation and self-renewal and can be classified as embryonic or somatic (BYDLOWSKI et al., 2009; CRISTANTE; NARAZAKI, 2011). Among somatic stem cells are mesenchymal stem cells (MSCs), which can be isolated from different locations, including adipose tissue, bone marrow, and dental pulp (COLLART-DUTILLEUL et al., 2015). MSCs can respond to external stimuli and give rise to countless different specialized cell lines, being responsible for tissue maintenance and repair (PINHEIRO et al., 2019).

Stem cells from exfoliated human deciduous teeth (SHED) in particular have been considered attractive due to their easy obtaining, their non-invasive collection, and availability of deciduous teeth, in addition to their multipotential proliferation and differentiation capacity

(MIURA et al., 2003). SHEDs present minimal risk of oncogenesis and high proliferative capacity and can even differentiate into several cell types such as neuronal cells, adipocytes, and odontoblasts (MIURA et al., 2003; TAGUCHI et al., 2019). As a non-invasive resource of cells, SHEDs are considered a promising tool for bone regeneration, with encouraging results proving their osteogenic potential in vivo studies, paving the way for new research possibilities (MIURA et al., 2003; NAKAJIMA et al., 2018; LEE et al., 2019; PINHEIRO et al., 2019; PRAHASANTI et al., 2019; HIRAKI et al., 2020).

Therefore, considering the importance of the topic and the lack of studies that analyze the SHEDs obtained from patients with CLP and that objectifies to develop a non-invasive method for the treatment of these patients, this work aims to expand the knowledge about autologous SHEDs and their possible use in bone regeneration of cleft lip and palate.

2 ARTICLES

2 ARTICLES

The articles of this thesis were written according to the submission guidelines of the corresponding journals.

- ✓ ARTICLE 1 - An overview on the use of SHED for bone regeneration of cleft lip and palate. (Submitted in Stem Cells Reviews and Reports)

- ✓ ARTICLE 2 - Characterization of stem cells from human exfoliated deciduous teeth (SHED) from children with cleft lip and palate.

2.1 ARTICLE 1 – An overview on the use of SHED for bone regeneration of cleft lip and palate.

Abstract

Over the years, stem cells have stood out in the scientific community for their outstanding properties. They are non-differentiated cells with the ability to proliferation and self-renewal and can be classified as embryonic or somatic. Among these, we highlight the stem cells of human exfoliated deciduous teeth (SHED) that represent a population of postnatal stem cells with extensive proliferation and multipotential differentiation. In vivo studies have shown that SHED can induce bone formation, produce dentin, and express neuronal markers. As well as being derived from extremely accessible tissue, they are also able to provide enough cells that may be used in clinical applications, cell transplants, and tissue engineering. Their properties indicate that SHED can become an alternative for the treatment of cleft lip and palate. By the fact that current techniques have disadvantages such as bleeding, nerve damage, aesthetic problems, pain, infection, and loss of tissue functions. Thereby, the purpose of this work is to present a review of studies using SHEDs in bone regeneration, emphasizing the treatment in patients with cleft lip and palate.

Key words: Cleft Palate. Stem Cells. Mesenchymal Stem Cells. Bone Regeneration. Tissue Engineering.

Introduction

Cleft lip, with or without cleft palate (CL/P), is the most prevalent congenital craniofacial defect originated by imperfect embryonic development of soft and hard tissues of the face area and oral cavity [1, 2]. The standard treatment performed usually at school age consists of a secondary graft with autogenous iliac bone, to establish a bone bridge forming the alveolar crest and thus closing the oronasal fistula [3, 4]. However, this method has limitations, such as bleeding, nerve damage, aesthetic problems, pain, infection, and loss of tissue functions [5, 6], which difficult the use the approach. Moreover, bone grafts using autologous cancellous bone may not fully integrate with the host bone and undergo resorption [7]. For this reason, a minimally invasive method to replace bone grafting and promote tissue regeneration has been broadly investigated.

Stem cells have stood out in the scientific community due to their outstanding properties. They are non-differentiated cells that have the capacity for proliferation and self-renewal [8, 9]. Mesenchymal stem cells (MSC) can be isolated from different locations, including adipose tissue, bone marrow, and dental pulp [10]. Stem cells of human exfoliated deciduous teeth (SHED) represent a population of stem cells with postnatal conditions able of extensive proliferation and multipotential differentiation [11]. In addition, they are stem cells with minimal risk of oncogenesis and high proliferative capacity, being able to differentiate into various cell types such as neuronal cells, adipocytes, and odontoblasts [11, 12]. In vivo studies have shown that SHED is apt to induce bone formation, produce dentin and express neuronal markers [11, 13]. As well as being derived from extremely accessible tissue, they are also able to provide enough cells that may be used in clinical applications, cell transplants, and tissue engineering [11]. Therefore, deciduous teeth began an ideal resource of stem cells to repair damaged dental structures, induce bone regeneration and possibly treat neural tissue injuries or degenerative diseases due to their higher accessibility [11, 13].

Thus, one of the conditions that could be benefited from the use of SHED in tissue engineering is the cleft lip and palate. Thereby, the purpose of this work is to present a review of studies using SHED in bone regeneration, emphasizing the treatment in patients with cleft lip and palate.

Cleft lip and palate prevalence

The global prevalence of orofacial cleft is estimated in 1 out of every 500–1000 births, with wide variations in patients treated between regions and countries [2, 14, 15]. Craniofacial defects affect the quality of life and self-esteem of individuals compromised with this disorder

[2]. These defects result not only in aesthetic problems but also in difficulties in speech, feeding, and breathing [7].

CL/P are more often unilateral, generally on the left side, and more common in males [16]. Already the cleft palate occurs more in females, and the majority are associated with other developmental anomalies [16]. Isolated CL/P is a complex trait that usually results from a combination of environmental factors, maternal metabolic imbalances, and infections during embryogenesis [2, 16]. Additionally, maternal folic acid deficiency during the periconceptional period, and exposure to alcohol or teratogenic drugs may also contribute to the occurrence of this disorder [2, 16].

Tissue engineering

Regenerative medicine has progressively advanced in recent years, emerging as hope for patients who can improve their quality of life by benefiting from new tissue engineering therapies, including bone regeneration [2]. The main goal of tissue engineering is to overcome the limitations of conventional treatments that are based on traditional reconstructive surgery or organ transplantation, by combining the triad of (1) cells with great growth potentials, such as stem cells, (2) biocompatible delivery vehicles, and (3) growth factors [17].

Tissue engineering demands biomaterials to promote the morphological and functional restoration of the tissue; therefore, advances in bone regeneration using 3D biometric scaffolds produced in different methods and materials have enabled the investigation of new tissue repair options in the CLP treatment [2]. It has been related that a scaffold with specific use for bone tissue regeneration must simulate the structure and biological function of a healthy tissue, which means have similar chemical compositions, hierarchical structure, as well as enabling cell propagation, transport of nutrients, and thus, facilitating adherence with osteoblasts [18]. Some biomaterials compounds, such as bioceramics, demineralized bone matrices, bioactive glasses, composite materials in combination with bioactive inorganic materials, among others, are considered suitable for new CLP treatments due to their biological properties such as osteoconduction, biocompatibility, chemical similarity with natural bone, improvement of proliferation and differentiation of osteoblasts [2, 18, 19].

Then, to occur bone reconstruction, osteoblasts need a scaffold and an induction mediator, so they can migrate to the defective area, initiating remodeling [20]. These scaffolds carry out a fundamental role in fixing cells and stabilizing blood clots, intending to prevent tissue damage during the initial regeneration phase [20].

Despite advances in the area, the complexity of the tissues and structures involved in

cleft palates, and the functional reconstruction of highly vascularized bones, such as the craniofacial region, is a major challenge in tissue engineering, as it requires a well-organized hierarchical vascular network [1, 17]. For this reason, many studies still need to be developed to overcome these challenges, promoting a more complete, organized, and successful regeneration.

Use of SHED in Tissue Engineering

Recent advances have been observed in tissue engineering studies to replace missing or damaged tissues with biomaterials with a high level of biocompatibility. This has encouraged researchers to use human stem cells in conjunction with supports derived from biomaterials [20, 21]. Among the cells that have been used for bone regeneration, there are SHEDs, which demonstrate differentiation capacity in osteoblasts [20].

Studies described in the literature have shown satisfactory results after using SHED for bone regeneration. A previous study investigated the effect of SHED transplantation after a bone defect was performed in the jaw of dogs [5]. After 12 weeks of the treatment, they observed bone neoformation both on the lingual part and on the defect floor, where it was compact bone [5].

Moreover, Nakajima and collaborators (2018) investigated bone regeneration after application of SHED compared to human dental pulp stem cells (hDPSCs) and bone marrow mesenchymal stem cells (hBMSCs) in an in vivo test performed after an artificially created bone defect in the calvaria of immunodeficient mice [13]. In this study, they concluded that the transplantation of SHED and hDPSCs induced bone formation in an approximate amount observed after the transplantation of hBMSCs [13].

In 2019, other two studies have also been reported. One of them produced and evaluated cell sheets (CSs) for cleft palate bone repair derived from human mesenchymal stem cells (hMSC) and SHED, which are two sources of osteogenic cells [7]. According to the authors, these CSs gave rise to in vitro calcification, indicating the osteogenic potential of these cells, in addition to expressing bone-specific osteogenic markers, Osterix (OSX), Osteocalcin (OCN), and Osteopontin (OPN), after insertion into cultured embryonic palatal shelves ex vivo and in vivo culture [7]. The other study by Prahasanti and collaborators analyzed the expression of biomarkers Osteoprotegerin (OPG) and the NF-Kb ligand-receptor activator (RANKL) after the use of a hydroxyapatite scaffold with a hydroxyapatite scaffold combined with SHED to correct an alveolar bone defect [20]. After in vivo analysis, it has been concluded that the

hydroxyapatite scaffold combined with SHED promoted an increase in OPG and decreased RANKL expression, proving their great potential as an effective biomaterial in the regeneration of the alveolar bone defect [20]. Since OPG protects the skeleton from excessive bone resorption by binding to RANKL and, thus preventing it from binding to your receiver RANK [20].

In addition, in 2020 another study proposed to evaluate the bone regenerative effects of SHEDs and conditioned medium (CM), which are paracrine factors secreted by MSCs during cell culture, generating potential for wound healing [4]. For this, bone defects were performed in calvaria of immunodeficient mice and then implanted stem cells or SHED conditioned medium (SHED-CM) [4]. Bone regeneration observed in this study was increased in defects treated with stem cells and CM compared to controls. Thus, SHED-CM proved to be more effective for the reconstruction of alveolar fissures in patients, as it regenerates bone in a non-invasive way [4].

All these results indicate the potential of using SHEDs, which may represent a new alternative to autologous bone transplantation in cleft palate reconstruction [8,10].

Use of dental stem cells obtained from patients with cleft lip and palate in bone differentiation

Dental pulp stem cells (DPSCs) has already been defined as all populations of stem cells in the dental pulp tissue, which includes populations such as SHEDs [22].

Thus, despite naming dental pulp stem cells (DPSCs), a recent study reports having obtained dental pulp cells from deciduous teeth from patients with CLP to compare the osteogenic potential of three different non-invasive sources of MSCs: stem cells from dental pulp (DPSCs), orbicularis oris muscle (OOMDSCs) and umbilical cord MSCs (UC-MSCs), to evaluate the applicability of a bioengineering alternative to traditional alveolar bone graft surgery in patients with cleft lip and palate (CLP) [17]. After comparison, they observed that of the three sources of obtaining MSC analyzed for bone tissue engineering for CLP patients, the best ones were DPSCs and OOMDSCs [17]. According to the study, these cells also presented better osteogenic potential than umbilical cord cells and the results indicate that the superior osteogenic potential showed in these MSCs is due to their neural crest cell origins [17].

Use of dental stem cells obtained from patients with cleft lip and palate in a clinical study

Recently, the use of stem cells from the deciduous dental pulp associated with a

hydroxyapatite collagen sponge (Bio-Oss Collagen® 250 mg, Geistlich) to close alveolar defects during the secondary dental eruption have been described [23]. Autologous DDPSCs were associated with the biomaterial and used to fill the alveolar defect, showing complete mineralization of the new bone and its integration with the intact bone limits after 12 months [23].

Notwithstanding, for being the first clinical report of alveolar bone tissue engineering using DDPSC in children, the study reports the small sample size and suggested that further randomized clinical trials are needed to substantiate these findings [23].

Cryopreservation

In the same way that SHEDs are easily obtained, they also have an advantage in their storage, since cells collected in childhood can be used in the future, through cryopreservation achieved by the creation and regulation of biobanks [11, 24–27]. Studies have shown that cryopreserved SHED maintained its characteristics of clonogenicity, self-renewal, multipotency, tissue regenerative capacity in vivo, in vitro immunomodulatory functions, and differentiation, equivalent to SHED that was not cryopreserved [24, 25].

Behnia et al. (2014) carried out a study using isolated and characterized SHEDs that were cryopreserved for 5 years. They proved that even after that time, the cells combined with scaffold were able to promote bone regeneration in the dog's jaws without generating any type of immune response, ratifying the osteogenic capacity of SHEDs even after a long period of cryopreservation [5]

Thus, knowing that cryopreservation does not interfere with the characteristics of these cells, adult patients with CLP without deciduous teeth could have SHEDs for use in bone regeneration. However, additional clinical studies are of paramount importance to achieve this technology [13].

Conclusion and Future Perspectives

It is noteworthy that many efforts have been conducted so that bone regeneration is performed using non-invasive materials, such as the use of scaffolds and stem cells such as SHEDs, which can bring benefits to the treatment and quality of life of several patients, including those with cleft lip and palate. However, despite being a promising alternative, there are still few reports in the applicability literature, especially in patients with CL/P, and hence, more studies are needed. Among such, the use of autologous SHEDs stands out since, due to the multifactorial origin of the cleft lip and palate, cells may present specific characteristics,

that are not yet identified, in comparison with the SHED of patients without CLP. Nevertheless, it reinforces that the properties of SHED, even in patients without cleft, indicate that the use of these cells to treat cleft lip and palate may be a successful alternative to existing methods.

Declarations

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Conflicts of Interest: The authors have no conflicts of interest to declare.

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2.2 ARTICLE 2 – Characterization of stem cells from human exfoliated deciduous teeth (SHED) from children with cleft lip and palate.

Abstract

This study compared the characterization of stem cells from human exfoliated deciduous teeth (SHEDs) between children with and without cleft lip and palate (CLP), aiming to analyze whether autologous SHEDs may be considered as an option so that, soon, they can be used as an alternative in the reconstruction of alveolar clefts in patients with CLP. Dental pulp tissues were obtained from six healthy deciduous teeth in exfoliation stage, from six donors aged between 4 and 12 years, and divided into Control and Cleft Group. The dental pulp cells obtained in the primary culture were analyzed immunophenotypically and collected those with positive expression of cell surface markers CD105, CD73, CD90 and negative for CD45, CD34, CD11b, CD19, HLA-DR (PE). The cells were subjected to multilineage differentiation and were morphologically analyzed. The difference immunophenotypic between groups was analyzed by Student's t-test, and the level of significance was set at 5% ($p < 0.05$). Plastic adhesion was demonstrated, and high levels of MSC markers were identified in both groups, with positive expression of CD105, CD73, and CD90 (≥95% positive) and absence of hematopoietic lineage markers CD45, CD34, CD11b, CD19, and HLA- DR (≤2% positive). Pulp cells demonstrated adipogenic, osteogenic, and chondrogenic differentiation capacity. The morphological analysis identified a slightly smaller nucleus and proliferative capacity lower in the time evaluated in the Cleft group. There was a statistically significant difference ($p=0.042$) when comparing the amount of cells obtained after immunophenotypic analysis, being greater in the Cleft group. After confirming the standard criteria for defining multipotent MSCs in both groups, it was possible to confirm the isolation and characterization of SHED in all samples, and the characterization between the SHEDs of both groups had no differences. These results suggest that SHEDs from children with CLP can be considered an option for future investigations into the autologous use of cells in therapeutic intervention.

Key words: Cleft Palate. Cleft Lip. Tooth, Deciduous. Mesenchymal Stem Cells. Stem Cell Research.

INTRODUCTION

Cleft lip and/or palate (CLP) are the most prevalent congenital anomalies affecting orofacial structures [1–3]. A cleft can be defined as a congenital abnormal gap in the upper lip, alveolus, or palate [1]. CLP can occur in different combinations or individually, being classified according to the extent of palatal involvement and has a worldwide incidence of approximately 1 in every 700 live births [1, 3]. The etiology of orofacial clefts is complex and multifactorial. It is believed that these anomalies may be caused by a combination of genetics and environmental factors [1, 3, 4]. Environmental or teratogenic risk factors include smoking, and the use of alcohol or medications such as retinoids, among others [1, 4]. Among the genetic factors, the origin can be syndromic, when associated with another malformation, or non-syndromic, in which cleft is an isolated characteristic and occurs in most individuals with cleft lip or palate, without a defined cause [1, 5].

For the reconstruction of alveolar bone defects, the most accepted therapeutic intervention consists of secondary alveolar bone graft in the mixed dentition stage using autogenous iliac bone [2, 3, 6–8]. However, this method has disadvantages for pediatric patients, where availability for bone collection may be limited, as it is an invasive process with potential risks for the donor area, such as infection, paresthesia, postoperative pain, and problems of healing [6, 8, 9]. Tissue engineering strategies have become an alternative since it can offer the possibility of using biomaterials directed to tissues and cells, to be applied in the affected area to provide tissue regeneration [6].

Studies have shown great interest in the use of mesenchymal stem cells as a stimulating factor for endogenous tissue regeneration for possible use in clinical applications [10, 11]. Stem cells from exfoliated human deciduous teeth (SHED) in particular have been considered attractive due to their easy obtaining, their non-invasive collection, and availability of deciduous teeth, in addition to their multipotential proliferation and differentiation capacity [12]. As a non-invasive source of cells, SHEDs are considered a promising tool for bone regeneration, with encouraging results proving their osteogenic potential in vivo studies, paving the way for new research possibilities [2, 12–16].

Thereby, this study compared the characterization of SHED between children with and without cleft lip and palate, aiming to analyze whether the autologous SHEDs from children with CLP do not have differences concerning cells obtained from children without CLP. And thus can be considered an alternative for use therapeutic in the reconstruction of alveolar clefts,

although genetic factors may be involved in orofacial malformation, and abnormal dentition development has already been reported [1].

MATERIAL AND METHODS

This study was approved by the Ethics Committee of the Bauru School of Dentistry (CAAE 30951220.8.0000.5417), and the Hospital for Rehabilitation of Craniofacial Anomalies of the University of São Paulo (HRAC/USP) (CAAE 30951220.8.3001.5441). All those responsible for the pediatric participants signed the Free and Informed Consent Term, and the minors signed the Free and Informed Assent Term prepared in an accessible language.

Subjects

For the isolation of SHEDs, dental pulp tissues were obtained from six healthy deciduous teeth in the exfoliation stage, presenting at least one-third of physiological root reabsorption, or with an indication of extraction [17]. The six samples collected were divided according to the participants between the Control and Cleft Groups (Table 1). Study participants in the Control Group were 6, 11, and 12 years old. While those in the Cleft Group were 4, 8, and 9 years old and presented different types of cleft lip and palate. Cleft Group teeth were extracted by a Pediatric dentistry upon request during plastic surgery for repair.

Table 1. Description of the samples, group, age, and teeth collected.

SAMPLE	GROUP	AGE	TOOTH
A	Control	11	53
B	Control	12	53
C	Control	6	72
D	Cleft	8	51
E	Cleft	4	52
F	Cleft	9	52

Cell Culture

After extraction each tooth was immediately stored in a falcon tube (Corning-Costar Corp., Corning, NY, United States) containing a solution with MEMα medium (Minimum Essential Medium α – Gibco, by Life Technologies®) supplemented with 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL amphotericin B (Antibiotic-Antimycotic (100X), Gibco, Life Technologies®) and 10 mg/mL gentamicin (Gibco, Grand Island, NY,

USA) on ice. In the biological safety cabinet, each pulp tissue was carefully removed by curettage [17]. To perform the explant technique to obtain primary culture, the pulp tissues were chopped into the smallest possible sizes with the aid of a scalpel [18]. Then, the tissues were left in a Petri dish with the same solution of MEMα medium supplemented with antibiotics, and antifungal for 40 minutes in a CO_2 incubator at 37ºC to eliminate possible contamination. After this period, the samples were centrifuged at 1200 rpm for 5 minutes. The supernatants were discarded, and each pellet was resuspended in 3ml of MEMα medium supplemented with 20% FBS (Fetal Bovine Serum - Gibco, Life Technologies®, South America), which were cultured in 25cm^2 culture flask (Corning-Costar Corp.) at 37ºC in an atmosphere of 5% CO_2 until reaching 70–90% confluence [19]. The culture medium was replaced every 3 days. After the first expansion, the cells were subcultured in 75cm^2 culture flask (Corning-Costar Corp.) to reach enough for use in the characterization.

SHED characterization

The cells between the first and third passage (P1 and P3) were dissociated with TrypLE™ (Gibco) at 37°C for 5 minutes and were analyzed by immunophenotyping. Each of the pulp cells obtained from the explant was evaluated by flow cytometry (BD FACSAria™ Fusion) using the Human MSC Analysis kit (BD StemflowTM, BD Biosciences, San Jose, CA, USA) to confirm that they were mesenchymal stem cells (MSCs). The kit enables the identification of MSCs by analyzing positive expressions of cell surface markers CD105 PerCP-Cy5.5, CD73 APC, CD90 FITC, and negative expressions of CD45, CD34, CD11b, CD19, HLA-DR PE [17, 20, 21]. All pulp cells were prepared according to the kit manufacturer's protocol, then the cells at 1×10^6 concentration marked with monoclonal antibodies passed through the flow sorting process, where the separation of pure and homogeneous cell populations occurs, according to predefined criteria. After that, the likely isolated and purified SHEDs were placed in a collection tube with culture medium to be expanded again and used in differentiation experiments to complete the criteria for defining human MSCs, according to the Committee on Mesenchymal and Stem Cells Tissues of the International Society for Cell Therapy (ISCT) [21].

Adipogenic Differentiation

In a 12-well plate (Kasvi, São José dos Pinhais, PR, Brazil), the cells from each sample were seeded in triplicate at a concentration of 1×10^4 cells, according to the manufacturer. After 24 hours of culture in MEMα 10% FBS medium, the cells were washed with DPBS (Dulbecco's

Phosphate-Buffered Saline, Gibco), and the culture medium was changed to specific adipogenic culture medium supplemented with growth factors, prepared according to the kit protocol (StemPro® Adipogenic Differentiation Kit; Gibco). The medium was replaced every 3-4 days for 14 days.

For evaluation, the cells were stained with oil red O (Oil Red O, Sigma Aldrich, St. Louis, MO). Briefly, all medium was removed, wells were washed twice with PBS (Phosphate-Buffered Saline, Gibco) and fixed with 60% isopropanol (Sigma Aldrich) for 5 minutes at room temperature. Then, the cells were stained with 0.5% oil red O for 15 minutes at room temperature. The washing was done once with 60% isopropanol, and distilled water twice. The observation of cellular structures and lipid vesicles was performed under inverted microscopy (Olympus CKX41) [16].

Osteogenic Differentiation

In a 12-well plate, the cells obtained from each sample were seeded in triplicate at a concentration of 1×10^4 cells. After 24 hours of culture in MEMα 10% FBS medium, the culture medium was changed to specific osteogenic induction medium supplemented with growth factors prepared according to the manufacturer (StemPro® Osteogenesis Differentiation Kit; Gibco), with replacement of the medium every 3-4 days for 21 days.

After 21 days in culture, the wells were washed with DPBS and the cells were fixed with 4% formaldehyde (Sigma Aldrich) for 30 minutes at room temperature. After fixation, the wells were washed twice with distilled water, then stained with a 2% alizarin red S solution (pH 4.2; Sigma Aldrich) for 3 minutes. For the final wash, each well was washed with DPBS three times. The formation of the mineralized extracellular bone matrix was observed by inverted microscopy.

Chondrogenic Differentiation

According to the manufacturer's protocol, in a 12-well plate, cells were seeded in triplicate at a concentration of 1×10^4 cells. After 24 hours of culture in MEMα 10% FBS medium, the culture medium was changed to specific chondrogenic differentiation medium supplemented with growth factors prepared according to the kit protocol (StemPro® Chondrogenic Differentiation Kit; Gibco), which was replaced every 3-4 days for 21 days.

After this period, to evaluate chondrogenic differentiation, alcian blue staining was performed to identify the proteoglycan or extracellular matrix released by chondrocytes [16]. The induction medium was removed from the cell cultures, which were washed with DPBS and

fixed with 4% formaldehyde for 30 minutes at room temperature. Then, the cells were washed with DPBS and stained with 1% alcian blue (Sigma Aldrich) for 30 minutes in the dark at room temperature. After being stained, the wells were washed three times with 0.1N hydrochloric acid (HCl), and distilled water was added to neutralize the acidity. The blue color, which indicates the synthesis of proteoglycans by chondrocytes, was observed with the aid of inverted microscopy.

Cell Morphology

For morphological evaluation of SHEDs, in a 6-well plate (Kasvi), the cells obtained from each patient were seeded at a concentration of 1×10^4 cells per well. After 24h in culture for adhesion in MEMα 10% FBS medium, the cells were washed with PBS, and the medium was replaced. After 72h of culture, cells were fixed in 4% formaldehyde for 30 minutes. Then, cells were washed three times with PBS, and then 1.5% crystal violet solution was added to each well for 5 minutes. The solution was removed, and the wells were gently washed under running water. Cell morphology was observed by an experienced examiner using an inverted microscopy.

Statistical analysis

Statistical analysis was obtained with Jamovi® (Jamovi® Statistical Software, Version 1.6). The normality test was applied and the difference between groups was analyzed by Student's t-test. The level of significance was set at 5% ($p < 0.05$).

RESULTS

Cell Culture

Samples collected from deciduous dental pulp were subjected to the explant technique, and the pulp cells were observed between 14 days after cell culture (Figure 1).

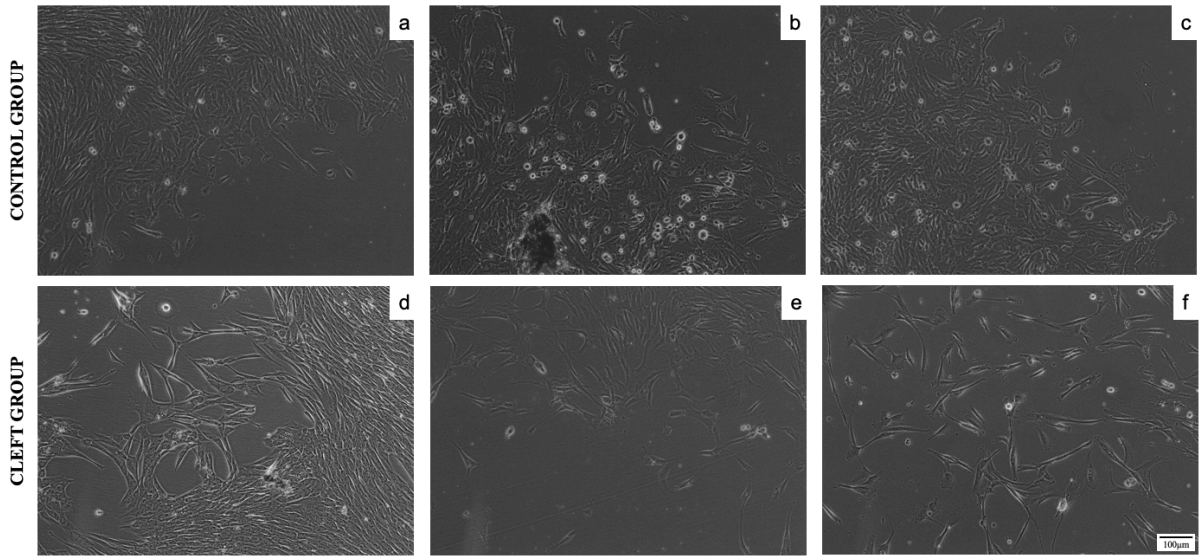

Figure 1. Adherent cell morphology after 14 days of isolation of deciduous tooth pulp tissues in Control (a, b, and c) and Cleft (d, e, and f) groups. There is a similarity in the fibroblastoid morphology in the samples. Scale bar= 100 µm.

SHED characterization

The immunophenotyping carried out by flow cytometry was analyzed using the FlowJo™ v10 software (BD Life Sciences).

Figure 2 shows the initial gates (size (FSC-A) by granularity (SSC-A)) performed for each sample of the two groups. After selecting the cells of interest negative for CD45, CD34, CD11b, CD19, HLA-DR (PE), and positive for CD90 (Figures 3A and 3C), they were separated and analyzed again for positivity for CD73 and CD105 (Figures 3B and 3D). Thus, only CD45-/CD34-/CD11b-/CD19-/HLA-DR- and CD105+/CD73+/CD90+ cells were collected and considered SHEDs.

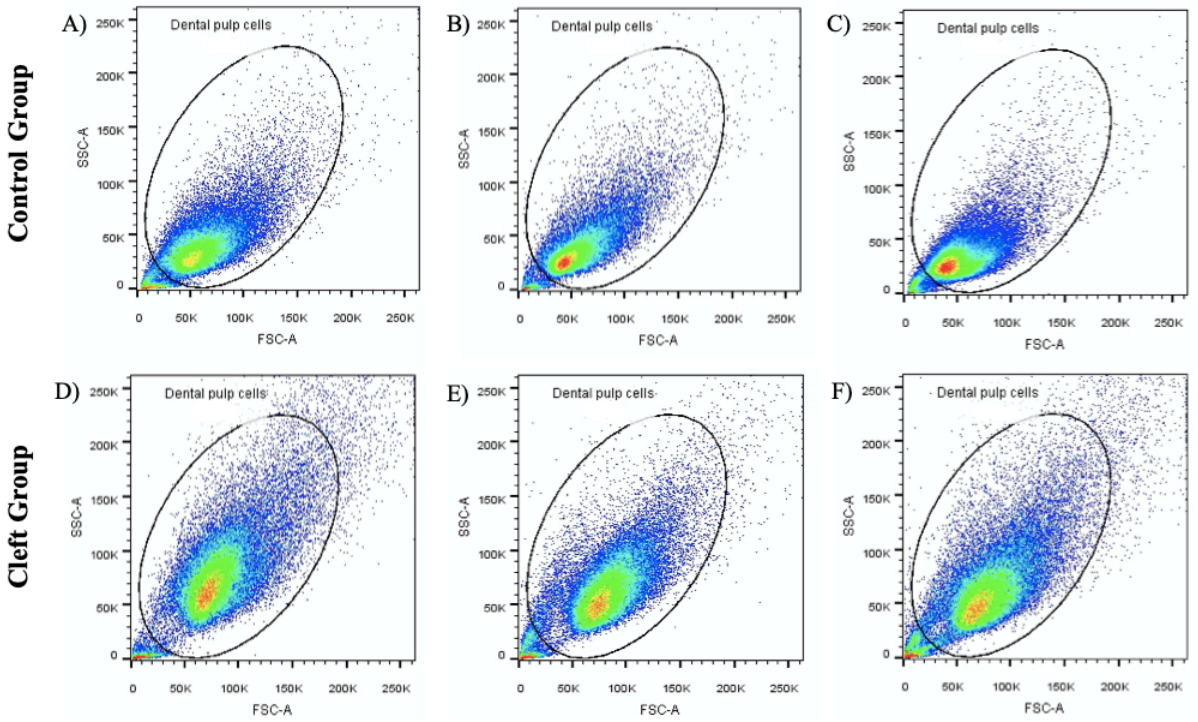

Figure 2. Dot plot obtained by flow cytometric analysis demonstrates the gates for cell analysis, as well as the difference between the size (FSC-A) and the granularity (SSC-A) of the cells from participants of the Control group (A, B, and C) and Cleft group (D, E, and F).

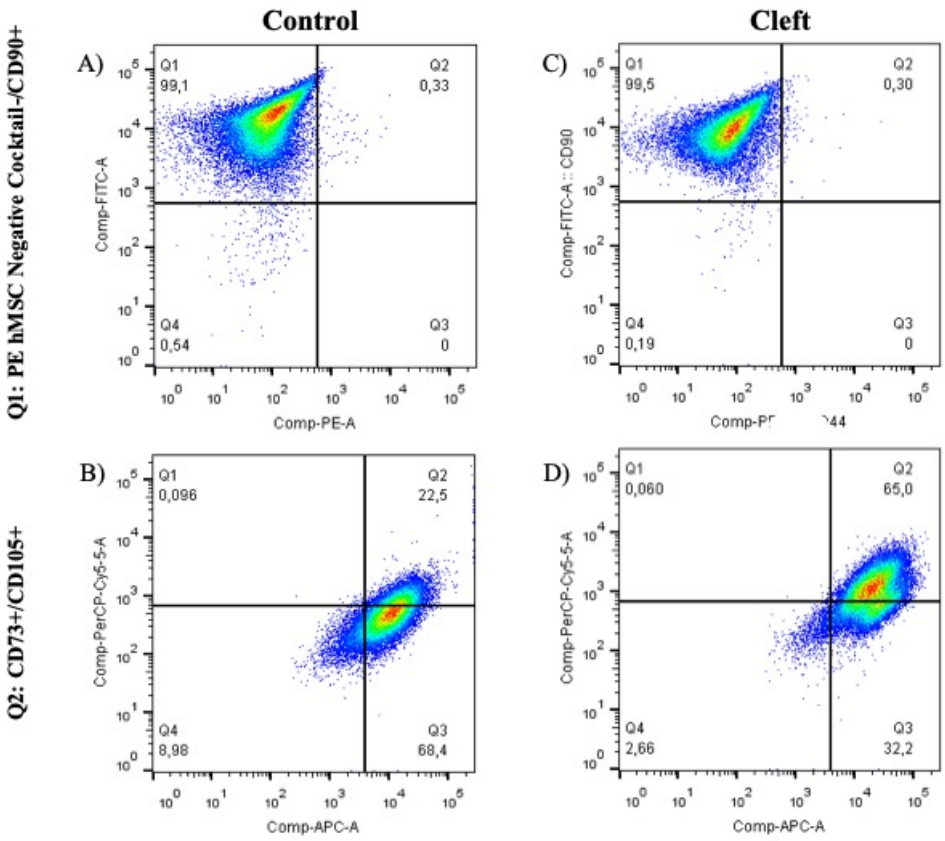

Figure 3. Dot plot obtained by flow cytometry analysis showing the gates for CD45-/CD34-/CD11b-/CD19-/HLA-DR- (PE hMSC Negative Cocktail)/CD90+ (A and C) and CD73+/CD105+ (B and D) cells analysis demonstrated by cells from participants of the Control group (A and B) and participants of the Cleft group (C and D).

The expression of positive and negative MSC markers observed in the SHEDs of each group was also demonstrated through histograms (Figure 4). And the average percentages of positive pulp cells for each surface marker were described by groups in Table 2.

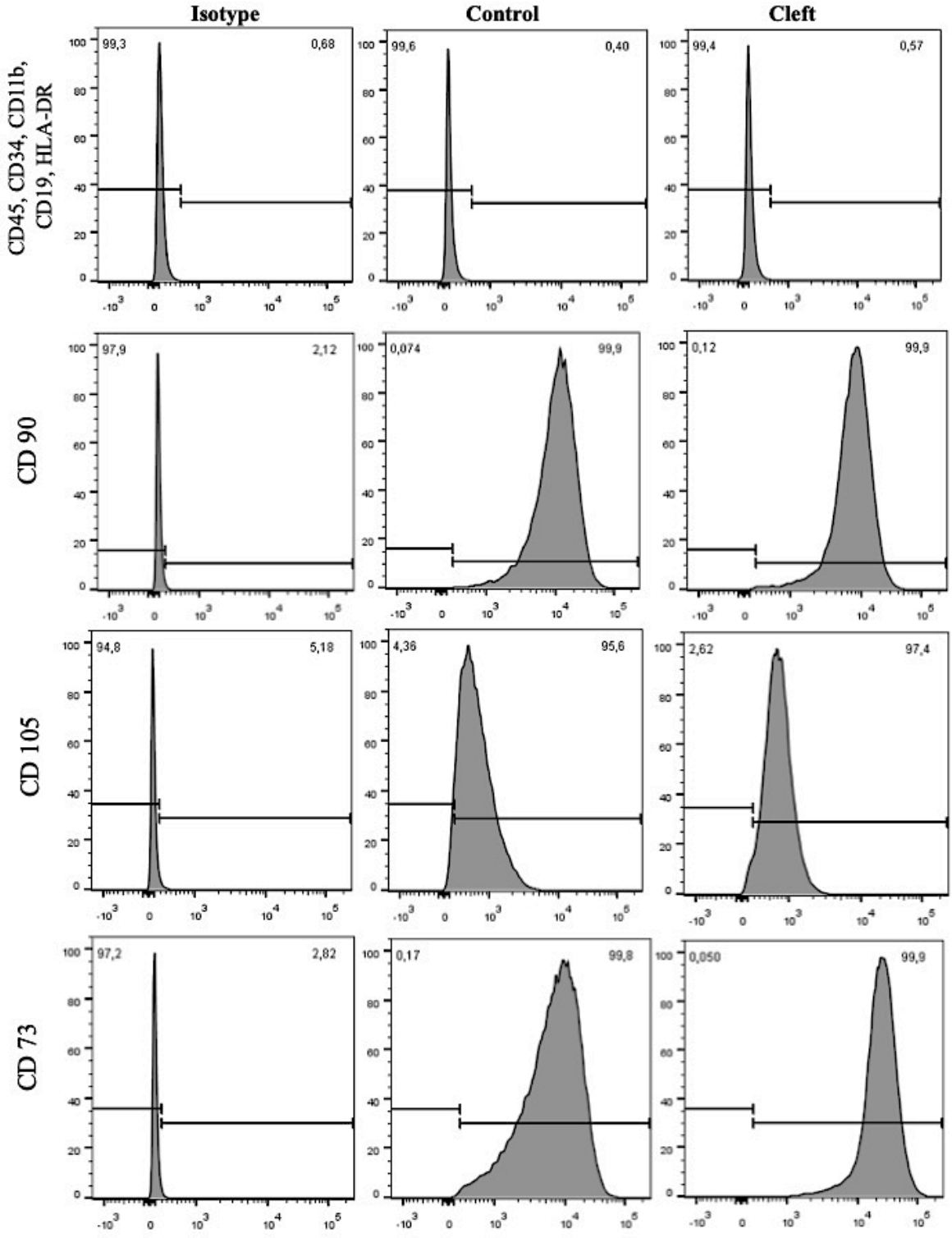

Figure 4. Flow cytometry histograms of negative (CD45, CD34, CD11b, CD19, HLA-DR (PE)) and positive (CD90 FITC, CD105 PerCP-Cy5.5, CD73 APC) expression of cell surface antigens in an expansion of MSCs derived from exfoliated human deciduous tooth pulp.

Table 2. Means of pulp cell populations positive for each MSC surface marker identified in the group samples after sorting flow process.

ANTIGEN	CONTROL GROUP	CLEFT GROUP
	Positive population mean (%)	**Positive population mean (%)**
CD 105+ PerCP-Cy™5.5	95.4	95.0
CD90+ FITC	99.8	99.7
CD73+ APC	99.8	99.8
CD45/CD34/CD11B/CD19/HLA-DR+ PE hMSC Negative Cocktail	0.5	0.5

Adipogenic Differentiation

Primary cultures of MSCs obtained from the pulp of human deciduous teeth exfoliated between the fourth and sixth passage (P4 and P6) were cultivated in adipogenic induction medium for 14 days. After this period, morphological alterations and the formation of intracellular lipid vesicles in cultured cells were observed, in both groups. Microscopy revealed the presence of lipid vesicles stained with Oil Red O, indicating differentiation into adipose cells, as shown in figure 5.

Osteogenic Differentiation

Primary cultures of MSCs between the fourth and sixth passage (P4 and P6) were induced for osteogenic differentiation for 21 days in osteogenic culture medium. In the end, after staining with 2% alizarin S red, the formation of the bone matrix was observed, in both groups, through inverted microscopy, as seen in figure 5.

Chondrogenic Differentiation

To perform chondrogenic differentiation, MSCs were cultured for 21 days in chondrogenic induction medium supplemented with growth factors. Then, they were stained with 1% alcian blue. Figure 5 identify the synthesis of proteoglycans by chondrocytes and the formation of the cartilaginous matrix, in both groups, through the blue color visualized by inverted microscopy.

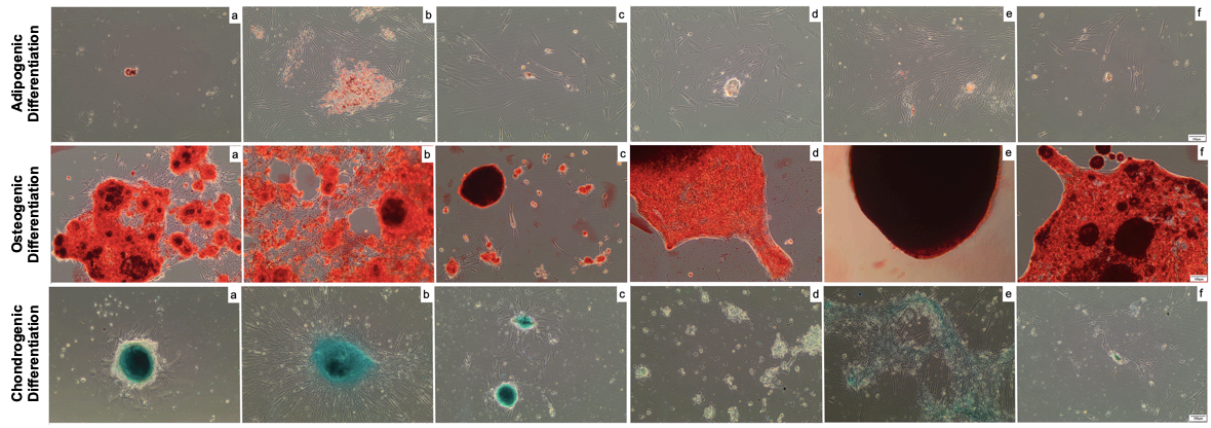

Figure 5. Formation of intracellular lipid vesicles observed in SHEDs after adipogenic induction for 14 days in Control (a, b, and c) and Cleft (d, e, and f) groups. Osteogenic differentiation after 21 days of induction, stained with alizarin red S, showing bone matrix formation in Control (a, b, and c) and Cleft (d, e, and f) groups. Chondrogenic differentiation after 21 days of induction, stained with 1% alcian blue indicating the synthesis of the cartilage matrix in Control (a, b, and c) and Cleft (d, e, and f) groups. Scale bar= 100 µm.

Cell Morphology

After 72 hours of cultivation of isolated and purified SHEDs between the fourth and sixth passage (P4 and P6), the morphology of the cells was observed by inverted microscopy. Figure 6 exhibits the morphology and the proliferation of each sample cultivated under the same conditions. The samples from the Control group showed a higher proliferation compared to the Cleft group.

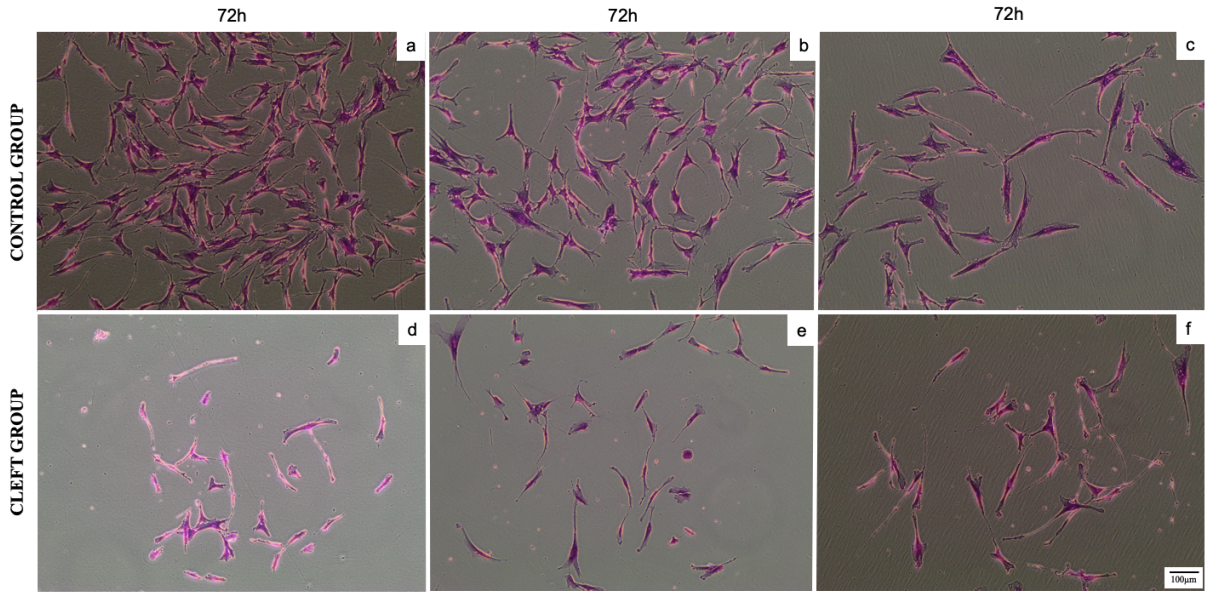

Figura 6. SHEDs culture after 72h in MEMα medium supplemented with 10% FBS, characterizing the morphology and showing the difference in cell proliferation between Control (a, b, and c) and Cleft (d, e, and f) groups. Scale bar= 100 µm.

Statistical analysis

Analysis of CD105 +, CD73 +, CD90 +, CD45-, CD34-, CD11b-, CD19, HLA-DR-(PE) surface markers revealed that of the total extracted cells in each group, 28.8% corresponds to the SHEDs in the Control Group, and 42.5% in the Cleft Group. The Shapiro-Wilk normality test showed that the data are normally distributed (p=0.133), and the Student t-test applied exhibited a statistically significant difference (p=0.042) between the groups (Table 3).

Table 3. Descriptive analysis of data obtained from both groups after Flow Cytometry and Student's t-test.

	Group	N	Mean	Median	SD	p
SHEDs	Control	6	28.8	26.3	9.56	0.042*
	Cleft	5	42.5	46.5	9.71	

*Indicate a statistically significant difference ($p < 0.05$)

DISCUSSION

In recent years, various studies have been conducted to evaluate the effectiveness of SHEDs for therapeutic purposes, emphasizing their use in bone regeneration through tissue engineering [2, 12–16, 22]. It stands out that because they originate from neural crest cells, SHEDs may have a tissue origin similar to mandibular bone cells, being an appropriate resource for regeneration of alveolar and orofacial bone defects [16, 22]. Furthermore, SHEDs have potential advantages for use in tissue engineering regenerative applications due to their multilineage differentiation capability and immunomodulatory properties [22, 23]. This work highlights the importance of comparing the characterization of SHEDs derived from the pulp of deciduous teeth of children with and without cleft lip and palate for possible autologous use, for all its potential already described and because they are an accessible source of cells during childhood, a period in which it is recommended surgical intervention for bone repair. The SHEDs obtained in both groups reached similar results in the characterization, being considered candidates for use in tissue engineering.

According to the ISCT, the minimal criteria to defining human MSC are plastic adherence, specific surface antigen expression, and potential for multipotent differentiation [21]. Our study proved the plastic adherence and revealed high levels of MSC markers in both groups, with positive expression of CD105, CD73, and CD90 (≥95% positive) and absence of hematopoietic lineage markers CD45, CD34, CD11b, CD19, and HLA-DR (≤2% positive). It has been reported that besides the quantification of fluorescence emission or biomarker

detection, other information can be drawn from the analysis of flow cytometry [24]. The selection of the population of interest in the dot plot, where forward scatter (FSC) is mainly related to cell size and side scatter (SSC) to cell granularity or complexity, revealed that there was a difference between the analyzed groups. As shown in Figure 2, different characteristics were observed in the dispersion of cell populations, with the Cleft Group having higher SSC values compared to the Control Group. There is a report in the literature that the SSC signal measured by flow cytometry is a variable correlated with membrane irregularity and internal cell granularity, such as cytoplasmic membrane wrinkling, number and shape of vesicles and mitochondria, development of endoplasmic reticulum, and structure from the cell nucleus [24]. This study also describes that there may be a permanent shuffling between the compartments of the pluripotent stem cells (PSCs) analyzed, with a tendency to apoptosis for cells with the highest SSC values and to differentiation for cells with the lowest SSC values, since that the SSC of PSCs drastically decreases after differentiation [24]. The authors suggest that SSC could be used as a simple and rapid tool to monitor loss of pluripotency during cell culture or differentiation of PSCs [24]. Despite not being pluripotent cells, the SHEDs of participants with CLP analyzed here showed greater granularity and this finding may be related to membrane irregularity or internal cell granularity since they were observed before differentiation. However, further studies are needed to evaluate and confirm these hypotheses.

Regarding the statistical analysis, a statistically significant difference was noted when comparing the pulp cells obtained after flow sorting process of the two groups. In the Cleft Group, the identification of cells with MSC antigen expressions was higher compared to pulp cells in the Control Group.

It has already been described some abnormal dental conditions during dentition development related to cleft lip and palate [1]. Studies propose that orofacial clefts and tooth development appear to have a similar genetic basis and thus, genetic mutations that lead to orofacial clefts can also result in a delay in tooth development [25, 26]. For this reason, differences between the characterization of SHEDs obtained from both groups could be expected. A study in Singapore reported that children with unilateral cleft lip and palate demonstrated delayed tooth maturation and a higher occurrence of asymmetric tooth pair formation of permanent teeth than children without CLP [27]. In contrast, Cesur et al. (2020) did not observe differences in dental ages between participants with unilateral CLP and healthy controls, nor asymmetrical tooth development in the lower teeth on the cleft or non-cleft side in individuals with CLP unilateral [26]. However, after evaluations performed using the Demirjian method, their study identified that the dental and chronological ages of individuals

were incompatible [26]. This last study corroborates another one conducted by Topolski et al. (2014), where after evaluating the dental development of Brazilian children and adolescents with and without cleft lip and palate, no significant delay in dental age was observed between the groups [28]. Given this, it is not possible to affirm that the dental and/or chronological age of the collected samples would alter the characterization of SHEDs between the groups in this study, even knowing that during embryological development, the formation of tooth germ is directly related to CLP concerning time and anatomical position [26, 29, 30].

Furthermore, among the standard criteria for characterization of MSCs, one of the relevant biological properties is their multipotent differentiation potential, in other words, the ability to differentiate into at least three types of tissues of the mesenchymal lineage [21]. Thus, in the present study, the pulp cells isolated in all samples demonstrated the capacity for adipogenic, osteogenic, and chondrogenic differentiation when exposed to the specific differentiation culture medium.

Adipogenic differentiation was confirmed by the change in cell morphology observed after positive staining for oil red O, being similar to adipocytes with the formation of lipid vesicles, as seen in a previous study [31]. In osteogenic differentiation, positive alizarin S red staining was detected, confirming that all samples differentiated. As calcium forms complexes with alizarin red S in the chelation process, calcium deposition from osteogenic lineage can be observed with this stain [31]. Thus, all samples showed calcium in the analyzed mineralized tissues. And, in chondrogenic differentiation, proteoglycans were found after staining with Alcian Blue. It has been reported that Alcian Blue stains acidic polysaccharides such as glycosaminoglycans in cartilage and proves the ability of these cells to secrete proteoglycan [31]. Our findings corroborate the results obtained by Yamashita et al. (2010) that related in their study that during the differentiation process, round-shaped cells eliminated from the aggregates and identified as chondrocytes formed cartilaginous aggregates [32].

Although it has already been reported that SHEDs do not directly differentiate into osteoblasts, these cells induce bone formation by recruiting osteogenic host cells in vivo [12]. And for that reason, deciduous teeth may not only guide the eruption of permanent teeth but may also be involved in inducing bone formation during this phase [12, 22]. Thereby, a limitation of this study that could be evaluated is the osteogenic potential between SHEDs of children with and without CLP. Additional studies that quantify and compare the extracellular bone matrices formed or the levels of gene expression associated with differentiation, including osteoblast marker genes, could verify whether there are differences between the groups. As a

result, it would be possible to investigate whether the genetic alterations involved in orofacial anomalies can interfere with or favor differentiation.

Other characteristics, such as morphology and cell proliferation, also had the tendency observed in this study. Despite the morphology of adherent cells being demonstrated after primary culture, cell morphology and proliferation were also observed after crystal violet staining, used for better visualization. The staining revealed that cell proliferation of SHED in the Cleft group was visibly lower compared to the Control group, maintained in cell culture for 72 hours under the same conditions. Nevertheless, although the proliferative capacity having been lower in the time evaluated and the morphological alterations, with a slightly smaller nucleus observed in the Cleft group, both had similar expressions of MSC antigens and multilineage differentiation potential.

Dental pulp and the orbicularis oculi muscles were recently considered the best sources for obtaining MSCs for bone tissue engineering for patients with CLP due to their better osteogenic potential compared to the umbilical cord [16]. SHEDs offer an easily accessible and non-invasive stem cell resource, with potential for use in regenerative medicine and therapeutic application [23, 33, 34]. Regardless of this, studies report that all primary human cells exhibit a finite proliferative capacity in culture and, after a limited number of cell divisions, enter cell senescence [23, 35]. This justifies the study proposed here so that patient-derived SHEDs can be considered for use in future therapies, emphasizing autologous application in patients with CLP.

CONCLUSION

Concerning the analyses of pulp cells isolated from deciduous teeth and confirmation of the standard criteria for defining multipotent MSCs in the two different groups, it was possible to confirm the SHED isolation and characterization in all samples. Our study showed that, although higher SHED values were found in the Cleft group during immunophenotyping, and a slower proliferation rate was observed in the morphological analysis, the characterization between the SHEDs of both groups had no differences. These results suggest that SHEDs from children with CLP can be considered an option for future investigations into the autologous use of cells in therapeutic intervention. Notwithstanding, future studies are still needed to evaluate possible internal differences between cells, analyze the growth curve between groups and quantify osteogenic differentiation, aiming to identify whether the cleft could favor bone regeneration through tissue engineering.

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Conflicts of Interest: The authors have no conflicts of interest to declare.

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3 DISCUSSION

3 DISCUSSION

In recent years, various studies have been conducted to demonstrate the effectiveness of SHEDs for therapeutic purposes, with emphasis on their use in bone regeneration through tissue engineering (MIURA et al., 2003; LIU et al., 2015; NAKAJIMA et al., 2018; LEE et al., 2019; PINHEIRO et al., 2019; PRAHASANTI et al., 2019; HIRAKI et al., 2020). Considered immature MSCs obtained from naturally exfoliated deciduous teeth, these cells offer an easily accessible and non-invasive stem cell source with potential for use in regenerative medicine, as they have multilineage differentiation capacity and immunomodulatory properties (BRAR; TOOR, 2012; KASHYAP, 2015; LIU et al., 2015; WANG et al., 2018). It is thought that because they originate from neural crest cells, SHEDs may have a tissue origin similar to mandibular bone cells, being an appropriate resource for the regeneration of alveolar and orofacial bone defects (LIU et al., 2015; PINHEIRO et al., 2019).

The comparison between the characterization of SHEDs derived from the pulp of deciduous teeth of participants with and without cleft lip and palate, for possible autologous use, had not yet been investigated and, considering the importance of the topic, this work aimed to expand knowledge about the use of SHEDs obtained from participants with CLP for bone regeneration

It has already been reported that primary human cells lose their proliferative capacity after a limited number of cell divisions, entering cellular senescence (HAYFLICK, 1965; WANG et al., 2018). This justifies the study proposed here so that the cells obtained from the patient can be used in future therapies, considering that regenerative medicine has progressively advanced, appearing as a hope for those who can benefit from bone regeneration, improving the living condition of these individuals (MARTÍN-DEL-CAMPO; ROSALES-IBAÑEZ; ROJO, 2019). The SHEDs of participants with and without cleft analyzed here showed no differences in characterization, and although additional studies are still needed, these cells become an alternative for further research that discusses the use of autologous SHEDs.

The principal purpose of tissue engineering is to overcome the limitations of conventional treatments of traditional reconstructive surgery or organ transplantation (PINHEIRO et al., 2019). Despite advances in the area, the functional reconstruction of highly vascularized bones, such as those in the craniofacial region, is a major challenge since it

depends on a well-organized hierarchical vascular network (MARTÍN-DEL-CAMPO; ROSALES-IBAÑEZ; ROJO, 2019). For this reason, many studies still require to be developed to overcome these challenges, promoting a more complete, organized, and successful regeneration.

4 CONCLUSIONS

4 CONCLUSIONS

Considering the analyses carried out, it is possible to conclude that:

Conclusion Article 1 - It is noteworthy that many efforts have been conducted so that bone regeneration is performed using non-invasive materials, such as the use of scaffolds and stem cells such as SHEDs, which can bring benefits to the treatment and quality of life of several patients, including those with cleft lip and palate. However, despite being a promising alternative, there are still few reports in the applicability literature, especially in patients with CLP and, hence, more studies are needed. Among such, the use of autologous SHEDs stands out, since, due to the multifactorial origin of the cleft lip and palate, cells may present specific characteristics, that are not yet identified, in comparison with the SHED of subjects without CLP. Nevertheless, it reinforces that the properties of SHED, even in subjects without cleft, indicate that the use of these cells to treat cleft lip and palate may be a successful alternative to existing methods.

Conclusion Article 2 - Concerning the analyses of pulp cells isolated from deciduous teeth and confirmation of the standard criteria for defining multipotent MSCs in the two different groups, it was possible to confirm the SHED isolation and characterization in all samples. Our study showed that, although higher SHED values were found in the Cleft group during immunophenotyping, and a slower proliferation rate was observed in the morphological analysis, the characterization between the SHEDs of both groups had no differences. These results suggest that SHEDs from children with CLP can be considered an option for future investigations into the autologous use of cells in therapeutic intervention. Notwithstanding, future studies are still needed to evaluate possible internal differences between cells, analyze the growth curve between groups and quantify osteogenic differentiation, aiming to identify whether the cleft could favor bone regeneration through tissue engineering.

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ANNEXES

ANNEX A – Approval by the Ethics Committee of the Bauru School of Dentistry

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da expressão gênica e síntese proteica de células-tronco derivadas da polpa de dentes decíduos de crianças com fissuras labiopalatinas após fotobiomodulação e uso de bandagem dentino/pulpar.

Pesquisador: Maria Aparecida de Andrade Moreira Machado

Área Temática:

Versão: 2

CAAE: 30951220.8.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.078.287

Apresentação do Projeto:

Trata-se de um projeto de Pesquisa de Doutorado apresentado ao Comitê de Ética em Pesquisa em Seres Humanos da Faculdade de Odontologia de Bauru e do Hospital de Reabilitação de Anomalias Craniofaciais, Universidade de São Paulo, intitulado "Análise da expressão gênica e síntese proteica de células-tronco derivadas da polpa de dentes decíduos de crianças com fissuras labiopalatinas após fotobiomodulação e uso de bandagem dentino/pulpar", da pesquisadora Profa. Dra. Maria Aparecida de Andrade Moreira Machado e equipe de pesquisa composta por Mayara Bringel dos Santos, Bianca Rapini Zalaf, Bárbara de Oliveira, Mariel Tavares de Oliveira Prado, Luciana Lourenço Ribeiro Vitor, Natalino Lourenço Neto, Cleide Felicio de Carvalho Carrara, Thais Marchini de Oliveira Valarelli, Cristiano Tonello e Nivaldo Alonso. O objetivo deste estudo será caracterizar e analisar a viabilidade e a proliferação celular, expressão gênica de RNAm e a cinética de expressão proteica de células-tronco de dentes decíduos esfoliados humanos provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar (BBio). Para tal serão selecionados até 3 crianças com fissura labiopalatina (regularmente matriculadas no HRAC/USP) e até 3 sem fissura labiopalatina, (atendidas na FOB/USP), com idade entre 5 e 7 anos, para extração de até 3 dentes decíduos por grupo. O estudo será dividido em duas etapas, onde: ETAPA 1 - Serão coletados o tecido pulpar provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas; será

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feita a cultura primária que permite identificar as células-tronco pela detecção da expressão dos marcadores de superfície celular; e caracterização das SHEDs através de Diferenciação Osteogênica; Diferenciação Adipogênica; e Diferenciação condrogênica; ETAPA 2 - Serão desenvolvidas as Bandagens de Bioestimulação dentino/pulpar (BBio); será realizada a irradiação com laser de baixa intensidade (LBI) e serão analisadas a viabilidade e a proliferação celular da SHED através do ensaio de MTT e Cristal Violeta; a avaliação da expressão de RNAm de DMP-1, DSPP, ALP e Col I por RT-PCR e expressão da síntese proteica de DMP-1, DSSP, ALP e Col I pelo método de ELISA após fotobiomodulação e uso de bandagem dentino/pulpar.

Objetivo da Pesquisa:

Objetivo Primário:

O objetivo deste estudo será caracterizar e analisar a viabilidade e a proliferação celular, expressão gênica de RNAm e a cinética de expressão proteica de células-tronco de dentes decíduos esfoliados humanos provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar (BBio).

Objetivo Secundário:

Caracterizar e comparar SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas; Analisar a viabilidade e a proliferação celular de SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar; Analisar a expressão gênica de RNAm para DMP-1, DSPP, ALP e Col I de SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar; Analisar a cinética de expressão das proteínas para DMP-1, DSPP, ALP e Col I de SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos da pesquisa ao participante relacionados ao procedimento de exodontia, pode incluir mordedura de lábio, sangramento e desconforto pós-operatório, que caso ocorram podem ser sanados no controle pós-operatório oferecido pelo profissional especialista que executará o procedimento.

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Continuação do Parecer: 4.078.287

Por fim, relacionado a utilização de suas células, o risco neste momento seria a exposição de dados do participante sem a autorização prévia do mesmo. Entretanto estará a salvaguardo pela Instituição e seus pesquisadores.

Benefícios:
Os benefícios ao participante da pesquisa, além da exodontia de um dente com indicação clínica ou ortodôntica por um profissional especialista qualificado, também residem na aceleração do tratamento ortodôntico que indicou a exodontia, além de exame clínico completo prévio a exodontia e encaminhamento do paciente para os devidos tratamentos dentro das clínicas pertinentes da FOB/USP e do HRAC/USP, caso haja outras necessidades.

Comentários e Considerações sobre a Pesquisa:
Trata-se de uma pesquisa bem interessante. Não existe nada que torne a pesquisa inviável do ponto de vista ético, uma vez apresentados todos os documentos necessários para a realização da mesma.

Considerações sobre os Termos de apresentação obrigatória:
Foram apresentados todos os documentos necessários para que seja avaliada a presente pesquisa. Ou seja: O projeto, carta de encaminhamento, orçamento, cronograma, folha de rosto, o TCLE que será encaminhado aos responsáveis pelos menores, o Termo de assentimento, o documento de cessão dos dentes dos menores e os respectivos termos de aquiescência.

Recomendações:
Veja a lista de inadequações. E também recomendamos a pesquisadora que, caso seja do seu interesse, retorne o protocolo de pesquisa para nova análise a este comitê no prazo de 30 dias, conforme dispõe na norma operacional nº 001/2013 do CNS (item 2.2. alínea "E") de 30 de setembro de 2013. Bem como no Regimento deste CEP no artigo 6º alínea III.2 de 31 de março de 2015.

Conclusões ou Pendências e Lista de Inadequações:
A presente pesquisa foi analisada por este CEP em reunião realizada no dia 06 de maio de 2020. Naquela reunião a pesquisa foi considerada com pendencias para sua realização. A pesquisadora reenvia o projeto para uma nova análise e observa-se que todas as pendencias foram esclarecidas. Entretanto, o TCLE apresentado para análise necessita de nova correção. A pesquisadora esclarece nesse novo documento apresentado para análise que "as células obtidas do dente extraído serão

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Continuação do Parecer: 4.078.287

utilizadas na pesquisa e as restantes serão descartadas. " Mediante essa nova informação, informe nesse documento o que a pesquisadora fará com as células tronco obtidas do dente extraído depois que a pesquisa estiver concluída. Na sequência corrija outras informações no TCLE que, mediante essa nova informação, não são mais cabidas neste documento. Por exemplo: Veja essa informação: "Ao ter seu material biológico armazenado em um laboratório para o uso em pesquisas, será mantido sigilo e privacidade do participante durante todas as fases da pesquisa, bem como nas células armazenadas, havendo..." ou esta informação "O armazenamento seguirá normas específicas e regulamentadas e nenhum..." Se não há armazenamento de células, retire essas informações. Considerando a necessidade de apresentar um novo documento, sou de parecer pelo não início da pesquisa até que esse documento seja apresentado.

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 03/06/2020, via Google Meet, devido à pandemia da COVID-19 e por orientações da CONEP, com base nas normas éticas da Resolução CNS 466/12. No entanto solicita correções no TCLE, conforme descrito no item "Conclusões ou Pendências e Lista de Inadequações" e o envie na forma de Notificação, acompanhado de um ofício resposta. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1536305.pdf	14/05/2020 15:28:26		Aceito
Outros	Term_Cessao_Dentes_Hum_novo.doc	14/05/2020 15:24:31	Maria Aparecida de Andrade Moreira Machado	Aceito
Outros	Carta_Resposta.doc	14/05/2020 15:23:40	Maria Aparecida de Andrade Moreira Machado	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_de_Pesquisa_cronograma.docx	14/05/2020 15:20:44	Maria Aparecida de Andrade Moreira Machado	Aceito

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Continuação do Parecer: 4.078.287

TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Menor_novo.doc	14/05/2020 15:19:21	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Pesquisadores	HRAC_Term_Comp_Tornar_Publico_Dest_Mat.pdf	16/04/2020 11:43:22	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Pesquisadores	HRAC_Term_Comp_Pesq_Resp.pdf	16/04/2020 11:41:34	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Instituição e Infraestrutura	HRAC_Term_Aquiesc_Coparticipante.pdf	16/04/2020 11:33:46	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Pesquisadores	Declaracao_de_Compromisso_do_Pesq.pdf	15/04/2020 17:56:13	Maria Aparecida de Andrade Moreira Machado	Aceito
Folha de Rosto	FolhaDeRostoASSINADA.pdf	15/04/2020 17:54:09	Maria Aparecida de Andrade Moreira Machado	Aceito
Outros	Check_listCEP_2019.pdf	15/04/2020 17:32:11	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Instituição e Infraestrutura	Termo_de_aquiescencia_EXTERNO_HRAC.pdf	15/04/2020 17:18:41	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Instituição e Infraestrutura	FOB_Termo_de_aquiescencia_Ciencias_Biologicas.pdf	15/04/2020 17:17:10	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Instituição e Infraestrutura	Termo_de_aquiescencia_CIP.pdf	15/04/2020 17:15:22	Maria Aparecida de Andrade Moreira Machado	Aceito
Declaração de Instituição e Infraestrutura	Termo_de_aquiescencia_Depto.pdf	15/04/2020 17:11:03	Maria Aparecida de Andrade Moreira Machado	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termo_Assentimento.docx	15/04/2020 17:08:20	Maria Aparecida de Andrade Moreira Machado	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

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USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

BAURU, 09 de Junho de 2020

Assinado por:
Juliana Fraga Soares Bombonatti
(Coordenador(a))

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ANNEX B – Approval by the Ethics Committee of the Hospital for Rehabilitation of Craniofacial Anomalies of the University of São Paulo (HRAC/USP)

USP - HOSPITAL DE REABILITAÇÃO DE ANOMALIAS CRANIOFACIAIS DA UNIVERSIDADE DE SÃO PAULO -HRAC/USP

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da expressão gênica e síntese proteica de células-tronco derivadas da polpa de dentes decíduos de crianças com fissuras labiopalatinas após fotobiomodulação e uso de bandagem dentino/pulpar.

Pesquisador: Maria Aparecida de Andrade Moreira Machado

Área Temática:

Versão: 1

CAAE: 30951220.8.3001.5441

Instituição Proponente: Hospital de Reabilitação de Anomalias Craniofaciais da USP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.133.187

Apresentação do Projeto:

O projeto de pesquisa de doutorado com o título: "Análise da expressão gênica e síntese proteica de células -tronco derivadas da polpa de dentes decíduos de crianças com fissuras labiopalatinas após fotobiomodulação e uso de bandagem dentino/pulpar" da pesquisadora responsável Profa. Dra. Maria Aparecida de Andrade Moreira Machado é apresentado a esse Comitê para ser avaliado em seis aspectos éticos.

A pesquisadora anexou o seguinte resumo:

"O objetivo deste estudo será caracterizar e analisar a viabilidade e, proliferação celular, expressão gênica de RNAm e a cinética de expressão proteica para DMP-1, DSSP,ALP e Col I de células-tronco de dentes decíduos esfoliados humanos (SHED) provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar. As amostras serão obtidas por meio de cultura primária de SHED. O tecido pulpar será removido e armazenado em MEM suplementado com 20% de Soro Fetal Bovino (SFB) até atingirem confluência. Após caracterização, as SHED serão avaliadas de acordo com os seguintes grupos experimentais:

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Grupo 1 - Laser de Baixa Intensidade na dosimetria de 2,5J/cm2;
Grupo 2 – Laser de Baixa Intensidade na dosimetria de 3,7J/cm2;
Grupo 3 – Bandagem de bioestimulação dentino/pulpar;
Grupo 4 - Laser de Baixa Intensidade na dosimetria de 2,5J/cm2 + Bandagem de bioestimulação dentino/pulpar;
Grupo 5 - Laser de Baixa Intensidade na dosimetria de 3,7J/cm2 + Bandagem de bioestimulação dentino/pulpar;
Grupo controle (positivo) - MEM+10% SFB;
Grupo controle (negativo) - MEM+1% SFB.
Serão realizados os experimentos de proliferação e viabilidade celular pelo método do MTT e Cristal de Violeta. A expressão gênica de DMP-1, DSPP, ALP e Col I será avaliada pelo método RT-PCR. Será avaliada a síntese de proteínas e quantificada pela produção de DMP-1, DSPP, ALP e Col I. Os experimentos serão realizados em triplicata biológica e repetido três vezes. Após as análises, os dados serão coletados e devidamente descritos por meio de gráficos e tabelas. As médias e desvio padrão serão testados quanto a normalidade dos dados. Se os dados apresentarem distribuição normal, a análise será realizada pela ANOVA a dois critérios, seguido pelo teste de Tukey. Se os dados não apresentarem distribuição normal, será utilizada a análise Kruskal-Wallis, complementada pelo teste de Dunn. Será adotado nível de significância de 5% para que as diferenças sejam consideradas estatisticamente significativas."

Objetivo da Pesquisa:

A pesquisadora descreve como objetivo geral:
"O objetivo deste estudo será caracterizar e analisar a viabilidade e a proliferação celular, expressão gênica de RNAm e a cinética de expressão proteica de células-tronco de dentes decíduos esfoliados humanos provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar (BBio)."

Como objetivos específicos a pesquisadora acrescenta:
"1- Caracterizar e comparar SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas;
2- Analisar a viabilidade e a proliferação celular de SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da

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Continuação do Parecer: 4.133.187

bandagem dentino/pulpar;
3- Analisar a expressão gênica de RNAm para DMP-1, DSPP, ALP e Col I de SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar;
4- Analisar a cinética de expressão das proteínas para DMP-1, DSPP, ALP e Col I de SHED provenientes de polpas de dentes decíduos de crianças com e sem fissuras labiopalatinas após fotobiomodulação e uso da bandagem dentino/pulpar."

Avaliação dos Riscos e Benefícios:
A pesquisadora relata:
"Os riscos da pesquisa ao participante relacionados ao procedimento de exodontia, pode incluir mordedura de lábio, sangramento e desconforto pós-operatório, que caso ocorram podem ser sanados no controle pós-operatório oferecido pelo profissional especialista que executará o procedimento. Por fim, relacionado a utilização de suas células, o risco neste momento seria a exposição de dados do participante sem a autorização prévia do mesmo. Entretanto estará a salvaguardo pela Instituição e seus pesquisadores. Os benefícios ao participante da pesquisa, além da exodontia de um dente com indicação clínica ou ortodôntica por um profissional especialista qualificado, também residem na aceleração do tratamento ortodôntico que indicou a exodontia, além de exame clínico completo prévio a exodontia e encaminhamento do paciente para os devidos tratamentos dentro das clínicas pertinentes da FOB/USP e do HRAC/USP, caso haja outras necessidades."

Comentários e Considerações sobre a Pesquisa:
É um projeto de doutorado e de coparticipação(FOB-HRAC). A amostra será composta por 06 pacientes sendo 03 da FOB e 03 do HRAC com idade entre 5 à 7 anos onde serão extraídos até 03 dentes em fase de exfoliação ou por indicação ortodôntica. Os dentes serão encaminhados para o laboratório onde serão realizados os procedimentos para a obtenção das células.

Considerações sobre os Termos de apresentação obrigatória:
Foram anexados os seguintes termos:
- TERMO DE CESSÃO DENTES HUMANOS,
- TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO,

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Continuação do Parecer: 4.133.187

- TERMO DE ASSENTIMENTO LIVRE E ESCLARECIDO

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

Uma vez que o projeto em análise não apresentou comprometimento ético recomendo sua aprovação por esse colegiado.

Considerações Finais a critério do CEP:

O pesquisador deve atentar que o projeto de pesquisa aprovado por este CEP refere-se ao protocolo submetido para avaliação. Portanto, conforme a Resolução CNS 466/12, o pesquisador é responsável por "desenvolver o projeto conforme delineado", se caso houver alterações nesse projeto, este CEP deverá ser comunicado em emenda via Plataforma Brasil, para nova avaliação.

Cabe ao pesquisador notificar via Plataforma Brasil o relatório final para avaliação. Os Termos de Consentimento Livre e Esclarecidos e/ou outros Termos obrigatórios assinados pelos participantes da pesquisa deverão ser entregues ao CEP. Os relatórios semestrais devem ser notificados quando solicitados no parecer.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Outros	Term_Cessao_Dentes_Hum_novo.doc	14/05/2020 15:24:31	Maria Aparecida de Andrade Moreira Machado	Aceito
Outros	Carta_Resposta.doc	14/05/2020 15:23:40	Maria Aparecida de Andrade Moreira Machado	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_de_Pesquisa_cronograma.docx	14/05/2020 15:20:44	Maria Aparecida de Andrade Moreira Machado	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Menor_novo.doc	14/05/2020 15:19:21	Maria Aparecida de Andrade Moreira Machado	Aceito
Outros	Check_listCEP_2019.pdf	15/04/2020 17:32:11	Maria Aparecida de Andrade Moreira Machado	Aceito
TCLE / Termos de	Termo_Assentimento.docx	15/04/2020	Maria Aparecida de	Aceito

Endereço: Rua Silvio Marchione, 3-20
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Continuação do Parecer: 4.133.187

Assentimento / Justificativa de Ausência	Termo_Assentimento.docx	17:08:20	Andrade Moreira Machado	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BAURU, 03 de Julho de 2020

Assinado por:
Renata Paciello Yamashita
(Coordenador(a))

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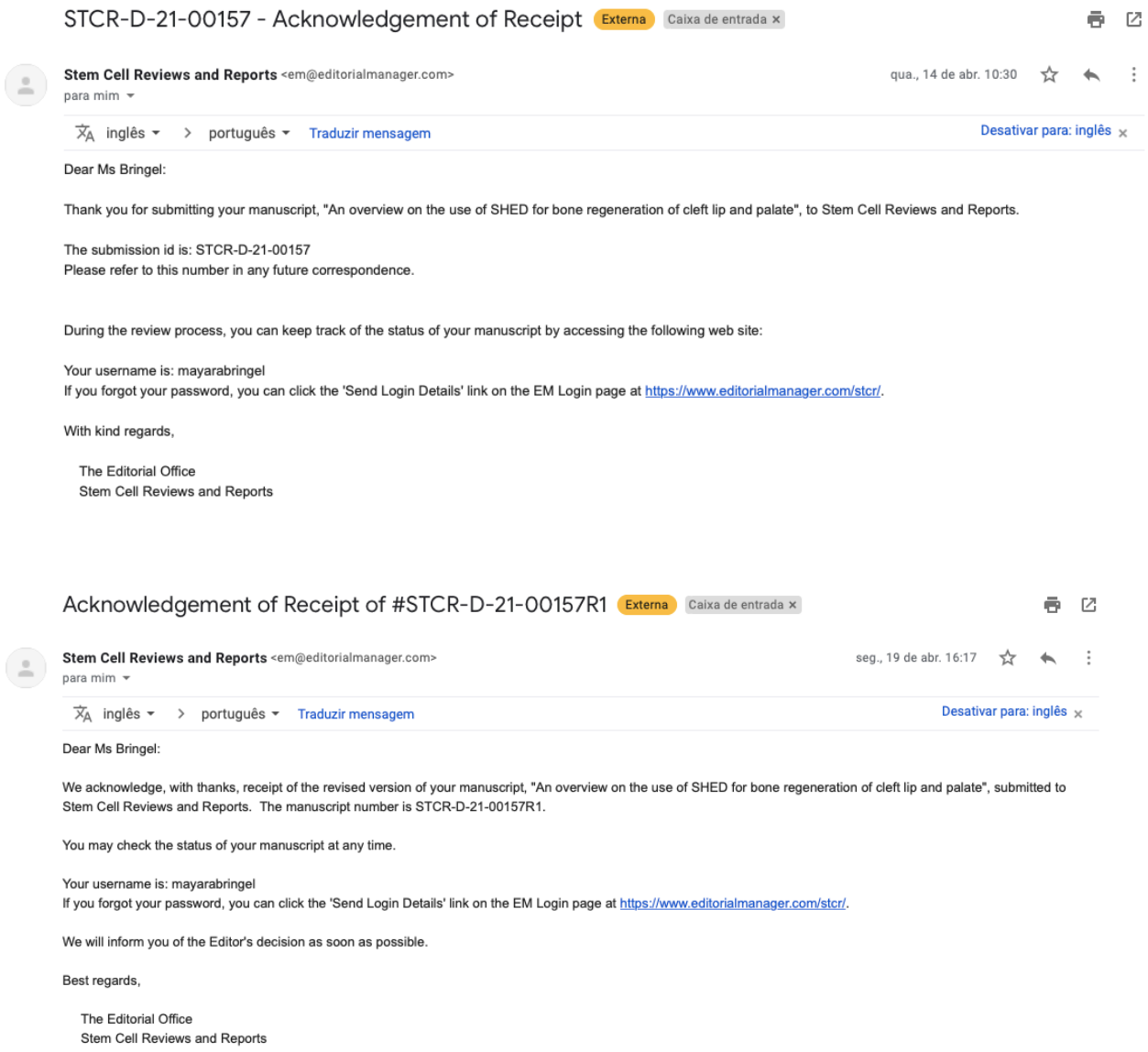
ANNEX C – Submission to Stem Cell Reviews and Reports



STCR-D-21-00157 - Acknowledgement of Receipt Externa Caixa de entrada ×

Stem Cell Reviews and Reports <em@editorialmanager.com> qua., 14 de abr. 10:30
para mim

inglês › português Traduzir mensagem Desativar para: inglês ×

Dear Ms Bringel:

Thank you for submitting your manuscript, "An overview on the use of SHED for bone regeneration of cleft lip and palate", to Stem Cell Reviews and Reports.

The submission id is: STCR-D-21-00157
Please refer to this number in any future correspondence.

During the review process, you can keep track of the status of your manuscript by accessing the following web site:

Your username is: mayarabringel
If you forgot your password, you can click the 'Send Login Details' link on the EM Login page at https://www.editorialmanager.com/stcr/.

With kind regards,

The Editorial Office
Stem Cell Reviews and Reports

Acknowledgement of Receipt of #STCR-D-21-00157R1 Externa Caixa de entrada ×

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Dear Ms Bringel:

We acknowledge, with thanks, receipt of the revised version of your manuscript, "An overview on the use of SHED for bone regeneration of cleft lip and palate", submitted to Stem Cell Reviews and Reports. The manuscript number is STCR-D-21-00157R1.

You may check the status of your manuscript at any time.

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We will inform you of the Editor's decision as soon as possible.

Best regards,

The Editorial Office
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