DENTAL PULP STEM CELLS EXTRACTION, CULTURE AND CRYOPRESERVATION PROTOCOL

Exploratory Mode

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LIST OF ABBREVIATIONS

ATB Antibiotic mix - Penicillin/Streptomycin.

DMEM Dulbecco's Modified Eagle's Medium.

DMSO Dimethyl Sulfoxide.

DPSCs Dental Pulp Stem Cells.

FBS Fetal Bovine Serum.

PBS Phosphate Buffered Saline.

RPM Revolutions Per Minute.

RESUMEN

Durante las últimas décadas se ha evidenciado una creciente demanda de células madre, tanto para su investigación como para sus potenciales aplicaciones. Las células madre son altamente importantes en la ingeniería de tejidos y en la medicina regenerativa gracias a su capacidad de autorrenovarse y diferenciarse según su ubicación o su potencial. Estas células constituyen la base de la biología moderna y gracias a los estudios que se han venido haciendo con ellas se ha logrado cerrar la brecha existente en esta área del conocimiento.

La implementación de estas células es vital para mejorar la salud, calidad y esperanza de vida de la humanidad debido a que son pieza fundamental para el tratamiento de enfermedades isquémicas, degenerativas y diferentes traumas.

Gracias al impacto que ha causado el uso de estas células en la medicina regenerativa actual y el aumento en sus aplicaciones se ha visto necesario contar con biobancos, repositorios biológicos específicos para estas células y allí poder almacenarlas para su posterior uso con fines investigativos.

La función mencionada anteriormente de las células madre en la medicina regenerativa y la ingeniería de tejidos sirve como motivo para implementar un biobanco en la Universidad EIA en el cual se puedan almacenar las células que serán previamente extraídas de pulpa dental (considerado material de descarte), cultivadas y criopreservadas para poder llevar a cabo las diferentes investigaciones que se requieran para dichas áreas en esta institución.

El objetivo principal de este proyecto fue diseñar protocolos específicos de extracción, cultivo y criopreservación de las células madre de la pulpa dental para poder establecer una metodología estándar y así poder garantizar resultados óptimos, y constatar el adecuado funcionamiento del biobanco y la viabilidad de las células manipuladas.

Se eligieron las mejores condiciones de cultivo, incluyendo 1% ATB + 10% DMSO + DMEM y se evaluó la viabilidad de las células, y se obtuvo una viabilidad máxima de 99.1%. Luego, las DPSCs fueron congeladas bajo 2 condiciones diferentes, 10% DMSO y 10% Glicerol durante 1 y 2 meses, se evalúo la viabilidad después de la criopreservación y se observó que la muestra que mejor porcentaje de viabilidad mostró fue la que estaba sometida a 10%DMS y 1 mes de congelación.

Palabras clave: criopreservación; pulpa dental; células madre; DMSO; congelación.

ABSTRACT

During the last decades, demand for stem cells has increased noticeably due to various research and potential applications in regenerative medicine. Stem cells play a key role in tissue engineering and regenerative medicine due to their ability to self-renew and differentiate into various cell types depending on their location in the organism. These cells serve as one of the bases of modern biology and many studies are closing the knowledge gap in this field.

Stem Cells applications are fundamental in the ischemic and degenerative diseases and other traumas treatment; therefore, their implementation is inevitable as a mean to improve humanity's health quality and life expectancy. The impact of stem cells usage in regenerative medicine has brought up the necessity to establish biobanks, specific biological repositories for these cells, easing up their availability for later research purposes and translational medicine use.

Aforementioned stem cell's role in regenerative medicine serves as a reason to establish a cell deposit at EIA University. The stem cells used in this study were originated from extracted dental pulp (considered a discardable biomaterial), they were cultured and cryopreserved for further research projects in the field of regenerative medicine and tissue engineering at the same institution.

The main purpose of this project was to design specific protocols for extraction of teeth, culture and cryopreservation of stem cells, establishing a standardized methodology to guarantee appropriate results (cell viability maintenance) and well-established cellular deposit function.

The best culture conditions were chosen by evaluating different reagent concentrations, selecting 1% ATB + 10% DMSO + DMEM as it showed a maximum of 99,1% viability. Then, DPSCs were cryopreserved using two different conditions, 10% DMSO and 10% Glycerol during periods of one and two months, evaluating cell viability afterwards. The highest viability was reached by sample 2, using 10% DMSO and one month of cryopreservation. The study concluded that chosen conditions brought good results in terms of viability.

Keywords: cryopreservation; dental pulp; stem cells; DMSO; freezing.

INTRODUCTION

Stem cells have become very popular due to all the studies and discoveries that revolve around them, their multiple characteristics and benefits are reflected in various applications for which they are considered. It is common to find them in adipose tissue, spinal cord, umbilical cord, dental pulp, among others. In most cases, having access to them requires an invasive surgical procedure due to their location in the human body.

That is why dental pulp becomes somehow interesting as a source for stem cell extraction since teeth can be obtained in many ways: children's baby teeth when they fall out, waste from dental interventions, removal of wisdom teeth, among many other possibilities. In addition, it does not represent an extra risk for patients, dental pulp can be manipulated in a slightly simpler way than the rest of the tissues and, obviously, it represents a much less invasive process than the surgeries required to obtain this type of cells from other parts of the body.

The different protocols that are commonly used to extract, culture and cryopreserve dental pulp stem cells will be described below. In addition, the results obtained when performing this last procedure with two known cryoprotectants (dimethyl sulfoxide - DMSO and glycerol) will be presented to determine the best option to obtain a high cell viability at the end of the whole research. Further, a bibliographic review will be carried out to compare the protocols and results with the procedures used by different researchers in these areas of knowledge.

The aim of this study is to obtain protocols that ensure optimal results in terms of cell viability in order to be standardized and, somehow in the future use them for the implementation of a biobank with research purposes in Colombia, extracting biological material from donor patients from this geography to be used in areas such as regenerative medicine and tissue engineering. Mainly because in Colombia these biological material repositories are only found for therapeutic purposes.

It is worth mentioning that biological material used in this study was obtained through donor patients from a dental clinic in Medellín, after the donors signed an informed consent where the purposes of the project are explained to them.

1. PRELIMINARY

1.1 PROBLEM FORMULATION

During the last decades have increased exponentially the studies of stem cells and their application in the field of regenerative medicine and tissue engineering. Stem cells are undifferentiated heterogeneous populations of cells, which are unspecialized and they are characterized by their self-renewal potential and their plasticity to differentiate into various cell types in the organism. These main properties make stem cells a desired tool for forthcoming therapies of various diseases, moreover in regenerative medicine might support or totally replace the conventional therapies (Guhr et al., 2018).

These cells also play an important role in the progress and improvements in the field of tissue engineering. The use of stem cells helped to solve some obstacles/hurdles of the current treatments of some ischemic and degenerative diseases and their involvement in various therapies brought a new treatment modality, helping to build a fundamental part of a new biomedicine. Altogether the application of stem cells can greatly increase the health conditions and quality of life of many patients (Sykova & Forostyak, 2013).

The increasing demand of biobanks has arisen due to the immense use of these cells in regenerative medicine. These biological repositories provide the storage of biological tissue samples or cells. The cells have to be isolated, cultured and preserved with the ultimate purpose to be used for therapeutic purposes or research (Parodi, 2015). These biobanks can be maintained in both public or private institutions, such as university departments or companies (Bardelli, 2010).

Currently, the biobanks have to deal with several ethical dilemmas related to biological samples. There are specific international rules and recommendations that ensure preserving all the rights of donors or volunteers in research. These norms regulate the medical activity and biomedical studies in humans according to specific standards in each country. In Colombia, all the aforementioned regulations are followed, however, there are no specific rules for stem cell biobanks with research purposes. Those, which are placed in Colombia are focused simply on the storage of stem cells derived from umbilical cord blood for possible future treatments. These biobanks do not offer the cells for other purposes, therefore there is a high demand to create a stem cell bank only for research projects in this country, ensuring that the biological material of the donors will be used only for research purposes and implementing an informed consent that ensures voluntary participation (Veloza Cabrera, Wiesner Ceballos, Serrano López, Peñaranda Correa, & Huertas Salgado, 2010).

To implement a deposit for research purposes in Colombia it's pertinent to establish protocols considering all the international standards to validate the process itself and the results. In this project it is important to emphasize that the donors will not be affected by the removal of the tissue obtained from the dental pulp because this type of tissue is considered as a biological material designated for discard after the patients undergo specific dental

procedures. Moreover, significant amounts of stem cells can be obtained from this tissue and their isolation does not require extra medical interventions.

Due to a high demand of developing various scientific projects in the area of regenerative medicine and tissue engineering, EIA University requires a complete protocol for extraction, culture and cryopreservation to create a stem cell deposit only for research purposes.

1.2 PROJECT OBJECTIVES

1.2.1 GENERAL OBJETIVE

To establish a protocol for extraction, culture and cryopreservation of stem cells derived from dental pulp.

1.2.2 SPECIFIC OBJECTIVES

- To establish a methodology for isolation of dental pulp considering various incubation times handling the tooth and tissue.
- To determine the proper dental pulp stem cells culture conditions (different fetal bovine serum concentrations) that allow the establishment of their cryopreservation protocol.
- To examine, based on literature and the obtained results, the effect of different cryopreservants (DMSO and glycerol) and methods after storing them during one and two months, in order to maintain high viability.

1.3 STATE OF ART

1.3.1 Background

Nowadays in Colombia there exist different types of biobanks including the samples of blood and tissues, also biologic samples like viruses and bacteria and also there are stem cells biobanks. This data will be explained on the following lines marking a proper background for the use of biological material and regulations, however it's important to clarify that these entities are focused on storage and distribution of samples for therapeutic purposes.

The “Cruz Roja” National Blood Bank is one of the main blood banks in Colombia, which was founded in May of 1900 as a private non-profit institution in charge of obtaining, storing and distributing blood (Cruz Roja Colombiana, n.d.).

Tissue Bank, founded in 2003, is another non-profit oriented entity with standardized procedures for the manipulation of skeletal muscle and eye´s tissues. It is in charge of selecting, obtaining, processing, storing and distributing human tissue to use it in

orthopedics, maxillofacial surgery, neurology, ophthalmology and odontology transplants (Tissue Bank, n.d.).

“Cordón de Vida” is the first umbilical cord stem cell bank in Colombia. It has a complete specialized group to service the cryopreservation process of human tissues (Cordón de vida, n.d.).

The “Redcord” umbilical cord stem cells bank is a Colombian enterprise with 17 years of experience with advanced equipment and qualified staff for cryopreservation of these cells (Redcord, n.d.).

The “IDCBIS” is a research institute in regenerative medicine and cell therapy with a Blood, Tissue and Cell Bank, in favor of individual and collective health, linked to the District Secretary of Health (Instituto Distrital de Ciencia Biotecnología e Innovación en Salud, n.d.).

Some internationally significant stem cells biobanks are:

FOXG1, a biobank of The National Institute of General Medical Sciences focused on the global scientific acquisition of anonymous biological samples for investigation. They also study biopsies and possible mutations from the patients, especially children and provide information to patients about all the possible changes of their tissue simples (FOXG1 Research Foundation, n.d.).

Coriell Institute Stem Cell Biobank, founded in 2008, has as a main objective to study the pluripotent stem cells and their storage to finally distribute them for scientific studies. They insist that these cells can be only used for investigation and never for human treatments (Coriell Institute for Medical Research, n.d.).

Cambridge Blood and Stem Cell Biobank, founded in 2009, is a tissue bank that stores biological samples from both healthy and unhealthy donors. They also create a database that ease studies for patients with MPD and UK Myeloproliferative Disorders (Cambridge Blood and Stem Cell Biobank, n.d.)

These are examples of some of the world’s famous biobanks all under the same work model - with informed consent for donors to facilitate the storing and distribution of biological samples to improve research and future applications in regenerative medicine and tissue engineering.

1.3.2 Theoretical framework

○ 1.3.2.1 Stem Cells:

Stem cells are undifferentiated cells with self-renewal capability and differentiation potential that make them a pillar of the proper functioning of the organism since a whole human being can be created from these cells. Additionally, due to these characteristics they can play a significant role in the fight against many diseases including cancer. Nowadays, they are

extensively used in cellular therapy, regenerative medicine and tissue engineering as well as in biotechnology and pharmaceutics (McKee & Chaudhry, 2017).

- **1.3.2.2 Types of stem cells:**

They can be classified depending on their potency and tissue origin

- **1.3.2.3 By their potency:**

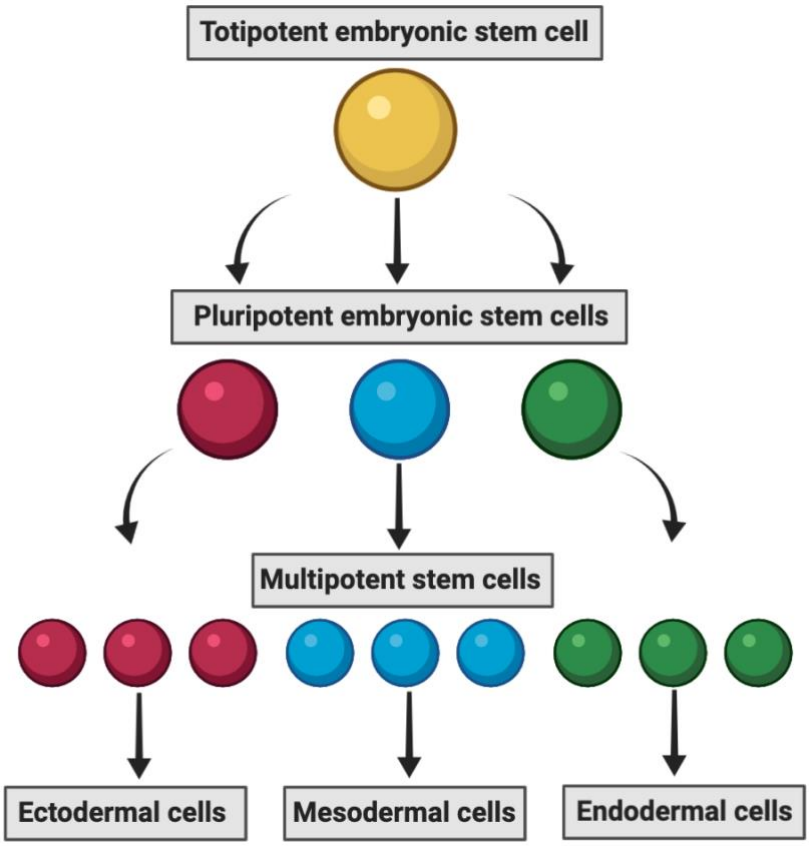

Figure 1. Graphic description of stem cell potency.

1.3.2.3.1 Totipotent: They can be obtained from the first stage of an embryo development and they can form a complete organism due to their differentiation capability (embryonic and extraembryonic tissue cells). They can be found in the zygote, morula and blastomeres (Lanza, 2004).

1.3.2.3.2 Pluripotent: These cells can differentiate in any lineage from embryo´s tissue, however they can't create an extraembryonic tissue. They are found in the blastocyst, which is formed between the fifth and sixth day after fertilization (Lanza, 2004).

1.3.2.3.3 Multipotent: They can differentiate into endoderm, ectoderm and mesoderm cells and can form a complete organism. They can be found in the bone marrow, umbilical cord blood, brain, skin, heart muscle, skeletal muscle, retina and pancreas and others (Lanza, 2004).

1.3.2.3.4 Unipotent: They can specialize into only one cell lineage, that is the reason why they are considered as low potency stem cells (Lanza, 2004).

- **1.3.2.4 By their tissue origin:**

1.3.2.4.1. Embryonic stem cells: They are able to form any cell, that means that they can specialize into any of the three cell lineages. They are found in the first stage of embryonic development and can be classified as pluripotent stem cells (Lanza, 2004).

1.3.2.4.2 Adult stem cells: They can be divided into 1) pluripotent and 2) unipotent stem cells. They cannot reverse their differentiation process, and therefore, when the cell lineage is formed, the specialized cells cannot be de-differentiated. They are in charge of maintaining and repairing the tissue they originate from. They are found in adult and umbilical cord tissues (Lanza, 2004).

1.3.2.4.3 Induced pluripotent stem cells: They are created from adult fully differentiated cells, which undergo specific procedures in vitro to get less differentiate state and they demonstrate the potency like an embryonic stem cell. They can be modified through cloning (transplantation of a nucleus to an enucleated somatic cell) or genetic alterations on an adult cell (introducing 4 stem cell genes to control the differentiation process) (Lanza, 2004).

- **1.3.2.5 Stem Cell Isolation:**

The isolation can vary depending on the tissue location and the further use of stem cells. Therefore, there are most abundant sources of these cells like: the embryo, bone marrow, adipose tissue, dental pulp or skin. Different techniques can be used to isolate these cells: 1) an explant formed from thin cuts of tissue, which allow the stem cells to leave the tissue in less invasive manner; 2) mechanical disaggregation, where is used the pressure to mechanically disaggregate the tissue using syringes, strainers or automatic dissociators; and 3) enzymatic disaggregation using various enzymes (trypsin, collagenase, accutase etc.) capable of dissolving a net of proteins in the extracellular matrix and release the stem cells from the tissue (Freshney, 2010).

- **1.3.2.6 Stem cells cell culture:**

After an isolation stem cells must be cultured in order to obtain well proliferating cells and to preserve viability as high as possible. Nowadays, there are a variety of options for cell culture but the most used one is the monolayer cell culture (2D). This type of cell culture is very well standardized and easy to use considering different variables like: medium, serum, growth factors, cryopreservation and of course the aseptic technique (without contamination).

Those conditions can change depending on the source of stem cells and the purpose of their application (Villa García-Torres, Flores-Hernández, & Santibáñez-Escobar, 2017).

- **1.3.2.7 Stem cells cryopreservation:**

This is the process to preserve cells and tissues in liquid nitrogen (-196C) using a low freezing rate at the beginning of the process. This technique provides the long-term storage of stem cells during guaranteeing the safe handling and biosafety requirements also for the distribution to clinic therapies and regenerative medicine (Pimentel-Parra & Murcia-Ordoñez, 2017).

- **1.3.2.8 Cryoprotectants**

Cryoprotectants are used to eliminate ice formation when freezing cells, there are two types: intracellular agents, which penetrate inside the cells preventing ice crystals formation and membrane rupture, among them are DMSO, glycerol, and ethylene glycol. The other agent is extracellular that acts by reducing the hyperosmotic effect present in freezing procedure (i.e sucrose, trehalose, dextrose, and polyvinylpyrrolidone) (De Lara Janz et al., 2012).

- **1.3.2.9 DMSO**

DMSO is the current gold standard for cell cryopreservation and is the most commonly used cryoprotectant for stem cells. The cryoprotective action of DMSO results from specific molecular interactions. Water and DMSO interact strongly and these interactions result in unique behavior during freezing, however several studies show that it can promote stem-cell differentiation in neuronal lineage and also presents cytotoxicity (Hornberger, Yu, McKenna, & Hubel, 2019).

- **1.3.2.10 Glycerol**

Glycerol is an oleochemical (chemical derived from natural oils and fats of both vegetable or animal origin) with some chemical and physical properties, which come up with a lot of important applications, cryoprotectant is one of them. Due to its low molecular weight, glycerol can penetrate a cell membrane, prevent the formation of ice crystals and slow down the ice crystal growth inside cells (Robergs & Griffin, 1998).

- **1.3.2.11 Biobank:**

A biobank is generally a biological repository where human, animal, bacteria and environmental samples can be found usually with a database filled with information about them. In other words, it is a place where biological samples (organs, blood, tissues, cells of different types) can be found stored and organized for research and therapeutic use but not for diagnosis (Parodi, 2015).

- **1.3.2.12 Regulations:**

Despite the fact that Colombia does not have specific regulations for the stem cells repositories, the constitution and functioning of biobanks must be ruled by Proyecto de Ley 237 of 2017. Those research-based biobanks are in charge of obtaining, processing, storing, transporting and assigning the biological human samples, and managing all the clinical and biological information (Congreso de la República de Colombia, 2017).

2. METHODOLOGY

Due to the fact that it is an exploratory project, the strategy chosen for the development of this work was experimental and investigative, which means that it was oriented to the consecutive fulfillment of stages, each one depending on the previous one.

The list of materials and reagents used for executing this project can be found at the end of this document as a **supplementary material 1**.

2.1 EXTRACTION OF SAMPLES

The patients (16-25 years old) were asked to voluntarily sign an informed consent before the procedure according to chosen inclusion and exclusion criteria attached in the annexes. Prior to surgery the patient's oral cavity was washed with alcohol and 2% chlorhexidine solution standardly used in dentist practice. The pre-molar tooth from the patient was extracted and immediately washed three times with phosphate buffered saline solution (PBS) in order to remove blood and solid debris. Consequently, the tooth was placed in 15 ml tube filled with Dulbecco's modified Eagle's Medium (DMEM) and 10% of antibiotic mix of penicillin/streptomycin (ATB) and put into refrigerated conditions at 4 °C during the transport to the laboratory, where the sample was processed in aseptic conditions.

2.2 STAGE 1

In order to fulfill the specific objective 1: "To establish a methodology for isolation of dental pulp considering various incubation times handling the tooth and tissue":

After the biological material was transferred to the laboratory, two samples were stored at 4ºC until specific timepoints from extraction (2 hours for first sample and 6 hours for the second one) were reached. The whole procedure was executed in the biosafety cabinet type II to ensure the aseptic conditions during the isolation process of the dental pulp stem cells (DPSCs). Cold distilled water was poured between the tooth and diamond disc to decrease the heat formed during the tooth's cutting. The tooth was cut longitudinally in a very careful manner not to damage the soft tissue. Dental pulp was removed by sharp tools like scissors or forceps, placing it on a Petri dish. The tissue was carefully cut into smaller pieces in order not to damage the cells and divided according to the objective 2 and cultured in Petri dishes. This procedure was executed on the first two patients´ samples. These underwent a procedure of isolation and culturing and the most suitable condition (depending on proliferation rate and viability) were applied to the rest of the patients' samples. **Figure 2** depicts the procedure in Stage 1.

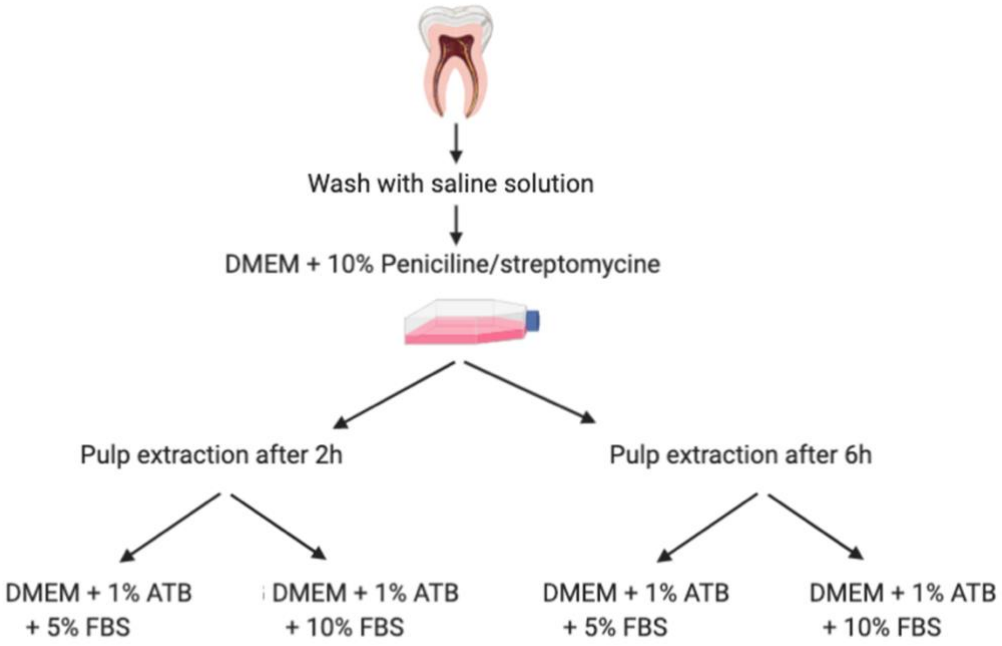

Figure 2. First stage of the proposed methodology for isolation, culturing and cryopreservation of dental pulp stem cells (DPSCs).

2.3 STAGE 2

In order to fulfill objective 2: "To determine the proper dental pulp stem cells culture conditions (different fetal bovine serum concentrations) that allow the establishment of their cryopreservation protocol":

One milliliter of complete medium consisting of DMEM (DMEM preparation protocol can be found as **supplementary material 2** at the end of this document), 1% of antibiotic solution (penicillin/streptomycin) and 5% or 10% of fetal bovine serum (FBS) were added to pieces of dissected tissue. It was essential to keep the minimal volume of medium in each Petri dish. It would not allow the tissue to float in order to ensure tissues´ and cells´ adherence to the plastic surface and on the other hand it still provided enough nutrients to the cells. After 24 hours the medium was changed for a fresh one to remove non-viable cells, residual erythrocytes and other cell/tissue debris. This process was carefully repeated every 2-3 days until the outgrowth of dental pulp stem cells from the tissue was seen. At day 14, cells were enzymatically disaggregated (subcultured or trypsinized) in order to evaluate the proportion of cells and their viability after isolation. Alongside this process, a comparison was made so the culture conditions for the other samples could be defined.

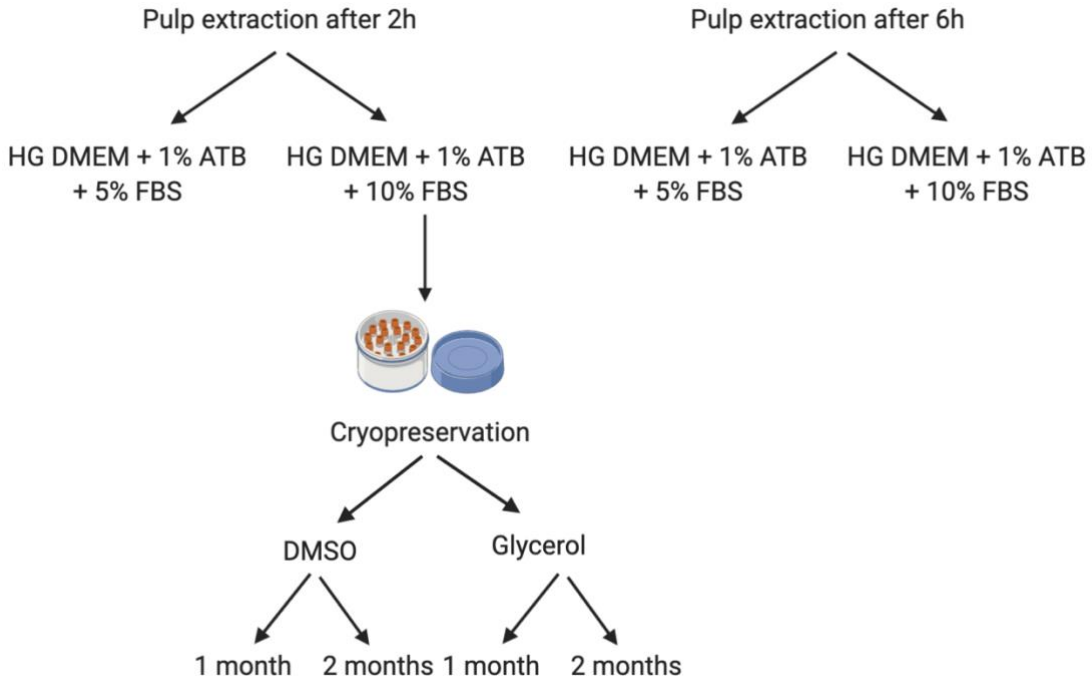

Figure 3. Second stage of the proposed methodology for isolation, culturing and cryopreservation of dental pulp stem cells (DPSCs).

2.3.1 Subculture

In order to establish the cell viability and concentration the subculture or trypsinization was performed on each sample.

The culture medium was carefully removed with a pipette in order to remove dead cells that were floating, not touching the living cells in order not to damage them. The medium was discarded in hypochlorite solution (10%), 1ml of PBS was added to cells to wash them because the serum blocks the trypsin used for enzymatic disaggregation of cells. The culture flask was gently shaken, and the liquid was removed. 700 µl of trypsin was added with a micropipette and the flask was taken to the incubator for 5 minutes at 37°C. The cell culture flask was observed every minute in order to observe the cells detaching from the plastic. Then, 2 ml of culture medium were taken with a micropipette and added to the flask as shown on **Figure 4A**, then the cells were resuspended and transferred into 15 ml tube with another 3 ml of media. The cells were centrifuged at 1200 rpm for 5 minutes. The medium was removed with a micropipette without touching the pellet and 1 ml of fresh DMEM was added to resuspend the DPSCs. At this point the cell viability and cell concentration were established. The residual cells were placed into a new culture flask and the cell culture medium was added to the final volume of 5 ml and placed in a humidified atmosphere

(**Figure 4B**) until the 90 % confluence and another subculture was needed in order to expand the cells.

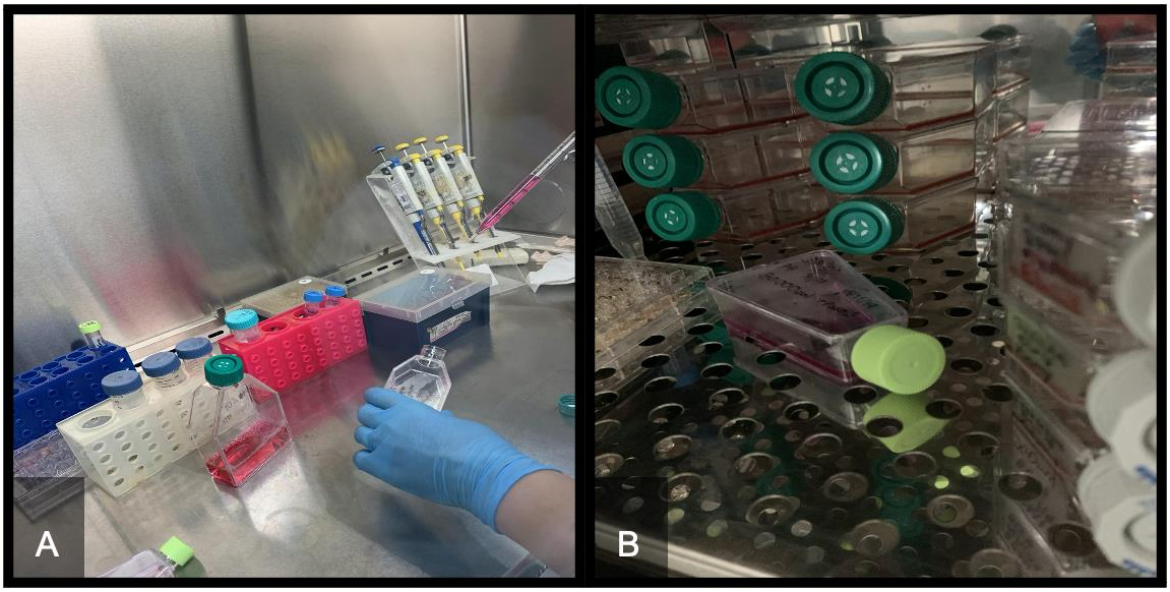

Figure 4. A. Addition of culture medium (DMEM) to DPSCs suspension for subculture. **B.** Incubation after adding DMEM to the cell suspension, the flask is placed horizontally in the incubator at 37°C until the next change of medium.

2.3.2 Cell viability analysis

We performed this simple assay after each passage, before and after the cryopreservation. Trypan blue exclusion assay determines the cell viability as described by Strober (2015). He described this test to be used to evaluate the number of viable cells based on a principle that live cells have intact cell surface, which excludes the trypan blue, these will appear as round and transparent cells in the microscope, whereas the dead cells with damaged cell membrane or apoptotic bodies will appear with stained cytoplasm in blue color. This staining provides the proportion of live and dead cells in a very simple, fast and cheap manner (Strober, 2015).

A Neubauer chamber was used consequently to perform the cell counting: briefly, the trypsinized cells were resuspended in 1 ml of culture medium and 10 µl of suspension was mixed with 10 µl of trypan blue in a test tube and mixed well to obtain a homogeneous solution. This mixture was carefully loaded onto the improved Neubauer chamber as shown in **Figure 5**, and then it was observed by a light microscope. Cell viability and cell concentration were counted.

Figure 5. Preparation of the assembly of the Neubauer chamber after each subculture in passage 3 to be taken to the microscope. (Superior Marienfeld, n.d.).

2.4 STAGE 3

In order to fulfill objective 3: “To examine, based on literature and the obtained results, the effect of different cryopreservants (DMSO and glycerol) and methods after storing them during one and two months, in order to maintain high viability”.

The most suitable cell culture conditions from the objective 2 were applied to the rest of the biological samples (Two samples of the same donor with DMSO (1M, 2M) and one sample with glycerol (1M), at the end there were three cryovials for one donor). The dental pulp stem cells which reach the 3rd or 4th passage, were trypsinized and the cell viability and concentration was established. Two million of cells were mixed with 1ml of cryopreservative solution containing 10% of DMSO and 10% of glycerol in a solution of DMEM/FBS. The cryovials were placed into a controlled freezing container (Nalgene® Mr. Frosty, Sigma-Aldrich, USA) filled with isopropyl alcohol, ensuring a gradual freezing rate of 1ºC per minute. Finally, the cells were stored for 1 and 2 months in the vapor phase of liquid nitrogen. Subsequently after reaching each time point the cells were thawed in a water bath at 37 ºC for 1 minute. The cells and cryoprotectants were diluted in a 4 ml of complete media, transferred into 15 ml tubes and centrifuged at 1200 rpm for 5 minutes. The pellet was resuspended in 1 ml of complete medium and the cell viability was analyzed using the staining with trypan blue. Subsequently, cells were placed in complete media and cultured for 3 days. The cells were trypsinized, and the cell concentration was evaluated.

2.4.1 Review of literature

Due to the small number of samples used in this study the results were compared with the current state of art of literature. Different scientific databases were used to obtain access to various journals, books and articles during the research in order to obtain a broader view of

the state of the art of dental pulp stem cells (DPSCs), different methods of extraction, culture and preservation that have been used and the materials used to cryopreserve them. The sources of information were mainly: Science Direct, SciELO, Scopus, PubMed.

In order to obtain the expected information, different search strategies were used, including certain phrases: “dental pulp stem cells + cryopreservants”; “dental pulp stem cells + freezing conditions”; “dental pulp stem cells + biobank”. The information obtained was filtered and revised.

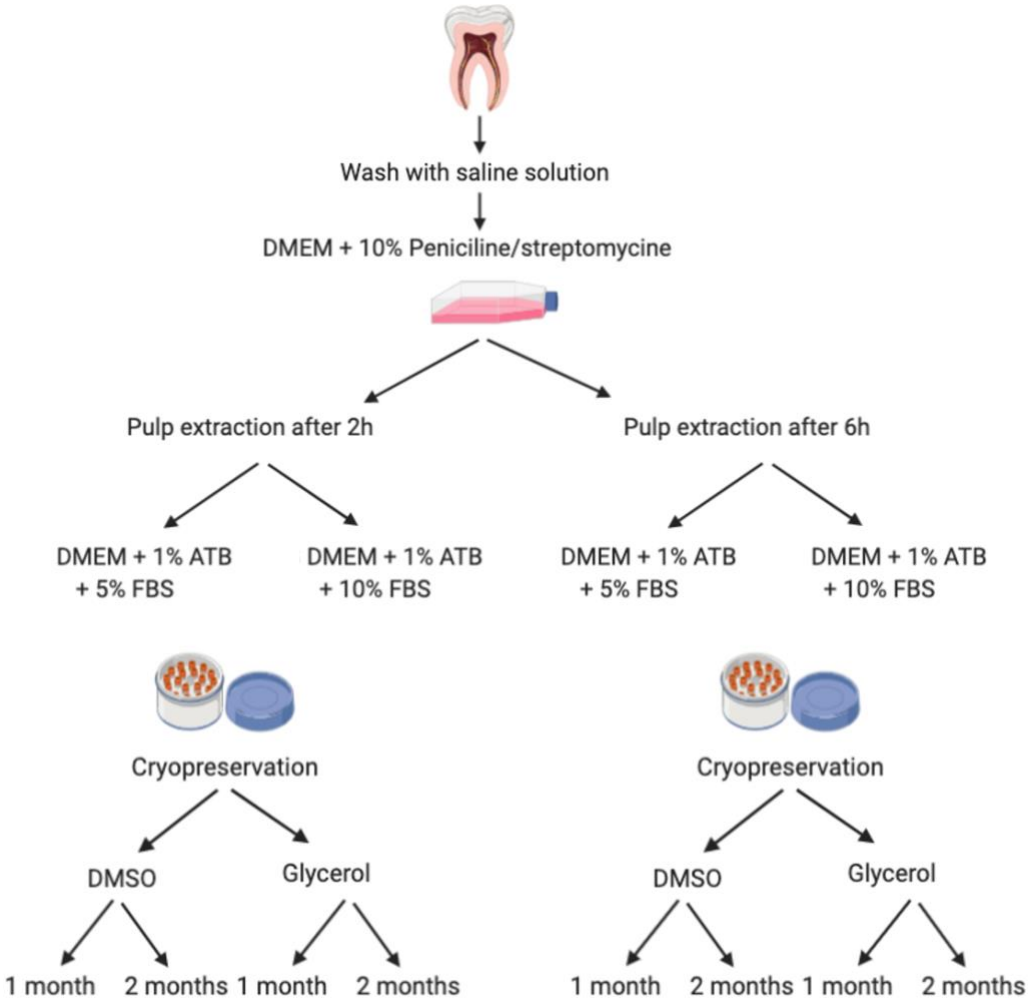

Figure 6. Complete scheme representing the proposed methodology for isolation, culturing and cryopreservation of dental pulp stem cells (DPSCs).

3. RESULTS AND DICUSSION

The presented work focuses on accomplishing three main goals: establishing the isolation conditions of dental pulp tissue, selecting the most suitable cell culture conditions for dental pulp stem cells and examining the effect of different cryoprotectants on cellular viability.

3.1 DENTAL PULP EXTRACTION

In collaboration with the dentist Andrea Osorio and the Sonri Dent clinic, 2 pre-molar teeth were extracted from patients within age 16-25 years. Briefly, removed teeth were placed into 15 ml tubes with DMEM and 10% of antibiotics solution (ATB), placed at 4 °C while transported to the laboratory to preserve them until the time points of 2 hours (sample 1A) or 6 hours (sample 1B) were reached and the pulp extraction was performed (2A).

In the aseptic conditions both teeth samples were cleaned from the residual soft tissue (**Figure 7A**) and underwent the same procedure for dental pulp extraction after selected timepoints. The teeth were carefully longitudinally cut with the diamond disc (**Figure 7B** and **Figure 7C**) in order not to damage the tissue. The dental pulp was removed from each part of the tooth with a sharp tool (**Figure 7D**) and placed on a Petri dish (**Figure 8**).

All necessary precautions were taken into account like correct lubrication for diamond disk and careful handling of the tool while cutting, preventing heating up of the dental pulp, ensuring the protection of the delicate tissue. The sterile conditions were maintained in order to prevent potential contamination.

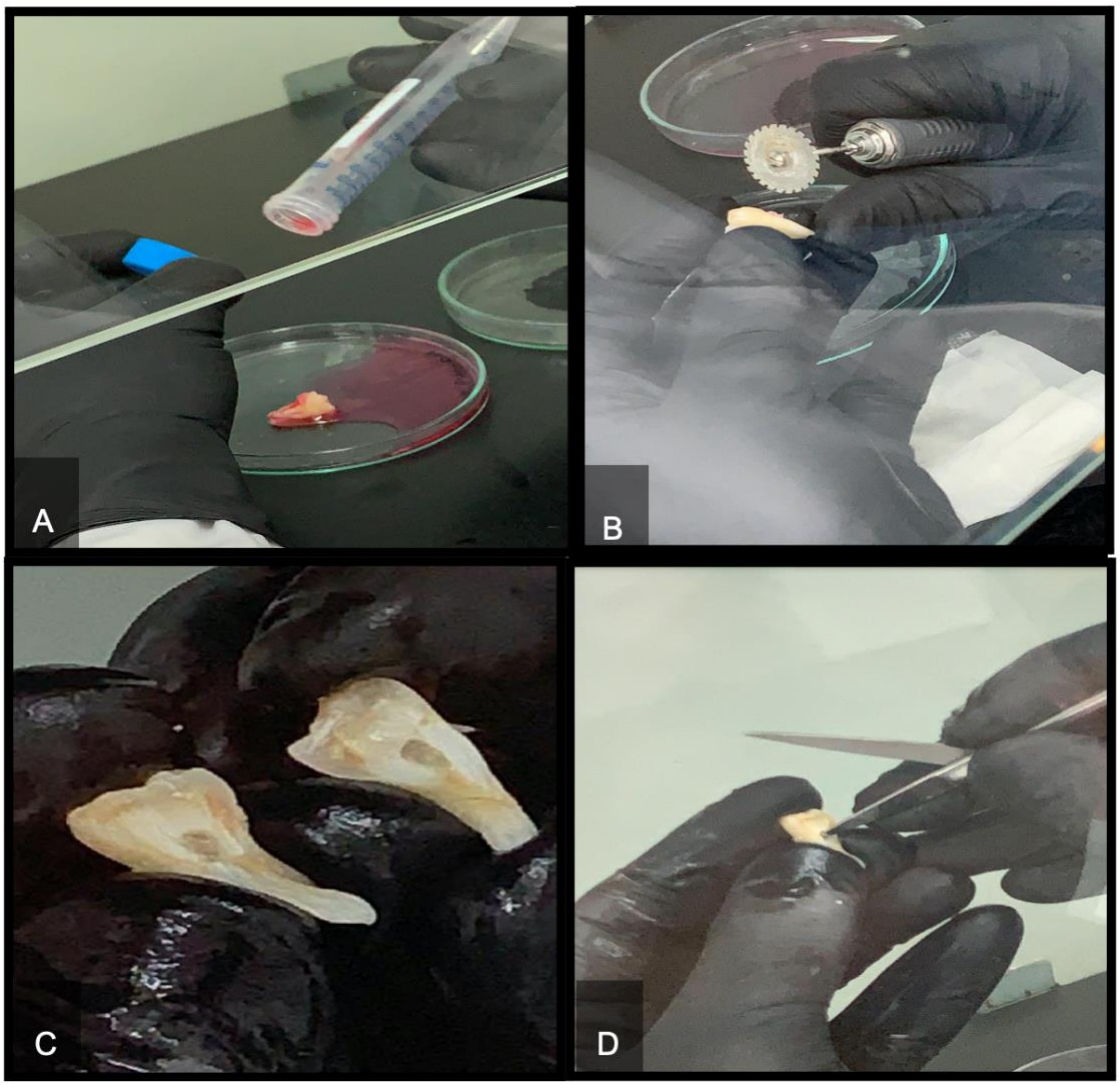

Figure 7. **A.** Extracted tooth after being transported from the clinic to the laboratory. It was stored in a plastic test tube and placed in a Petri dish to start the procedure**. B**. A diamond disc was used to cut the tooth, this tool is used to ensure a clean cut and that the tooth does not break. **C**. The tooth was cut lengthwise to avoid cutting or burning the dental pulp. The stem cells are located in that small hole that can be seen in the center of the tooth. **D**. The dental pulp was extracted with a sharp element.

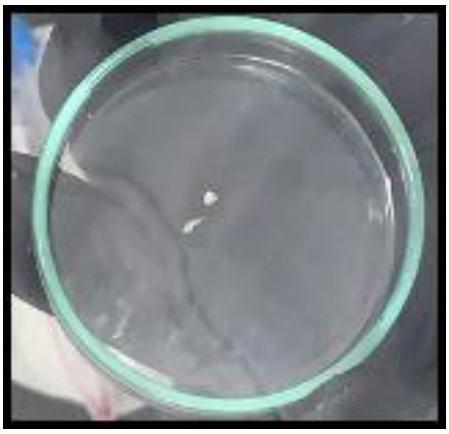

Figure 8. Dental pulp after extraction, placed in a Petri Dish.

Each dental pulp tissue isolated after specific time point (named: sample 1A (2 hours after isolation) and sample 1B (6 hours after isolation)) was placed into specific culture media containing different concentrations of fetal bovine serum of 5% and 10% respectively (as stated in Methodology, stage 2). The medium was added to slightly cover the tissue to help its adherence to the plastic surface and each Petri dish was placed into a humidified atmosphere at 37 °C. Every 2-3 days the medium was replaced until at day 14 the first subculture was performed in order to establish the cell concentration, viability and debris-free cell culture assurance. On **Figure 9** the outgrowth of DPSCs from the tissue is shown (sample 1A and 1B) in different cell culture media (containing 10% or 5% of FBS) after 3 days of culturing.

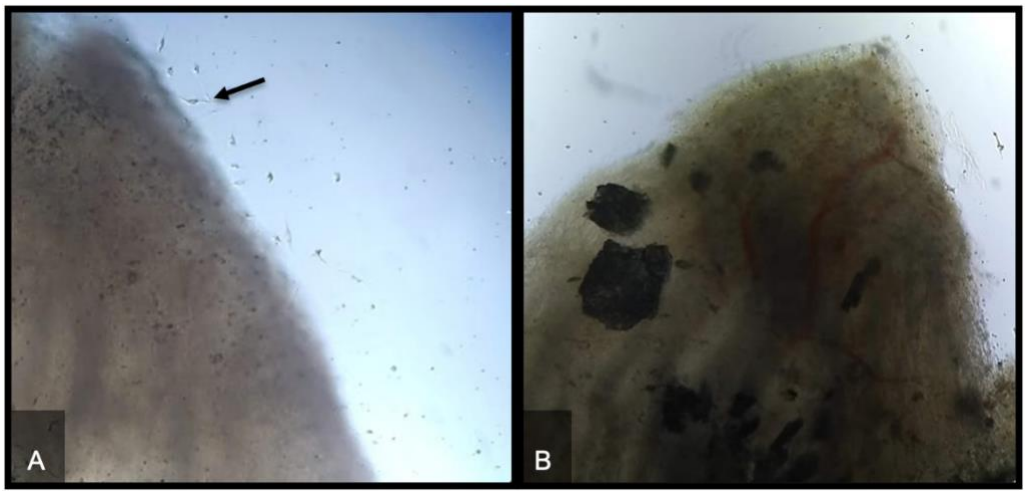

Figure 9. Dental pulp tissue with the DPSCs outgrowth 3 days after the isolation in different culture media. **A.** Sample 1A: growing in DMEM + 10% FBS + 1% ATB. **B.** Sample 1B: growing in DMEM + 5% FBS + 1% ATB. Arrow shows the viable cells. Magnification 5x.

Figure 10 shows the DPSCs (sample 1A) after 14 days of culturing at 90% confluence.

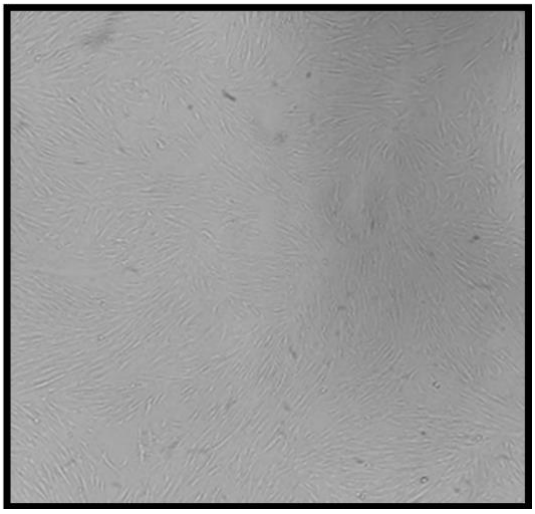

Figure 10. DPSCs before the subculture show the typical fibroblast-like morphology of dental pulp stem cells. Magnification 5x.

Sample 1A was used in a cell culture media with 10% of FBS for further experiments and in the rest of the document is stated simply as Sample 1. It was possible to isolate DPSCs from 5 more samples named Sample 2-4, unfortunately sample 5 and 6 got the fungal contamination shown on **Figure 11** so they were excluded from further experiments.

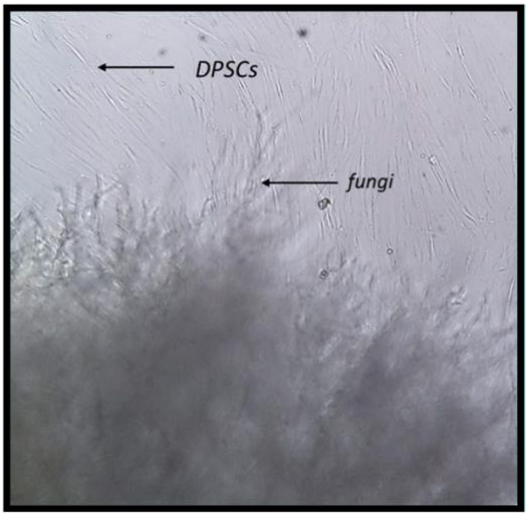

Figure 11. This is how the cell culture of the contaminated sample 5 looks like, the DPSCs are translucent and the fungal contamination is depicted by darker hyphae.

3.2 CELL VIABILITY ANALYSIS BEFORE CRYOPRESERVATION

After 14 days of culturing the subculture was performed and cell viability and concentration was counted.

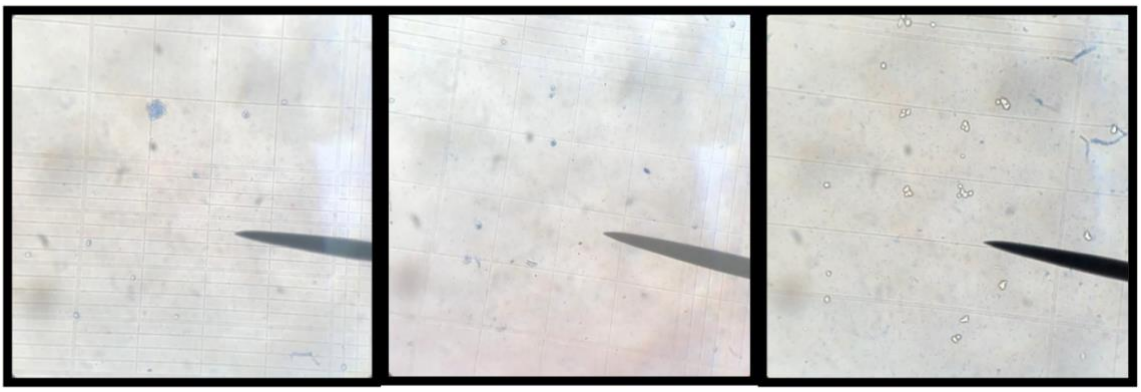

Figure 12. Counting DPSCs in the viable samples under the microscope with Neubauer chamber before cryopreservation. Magnification 10x.

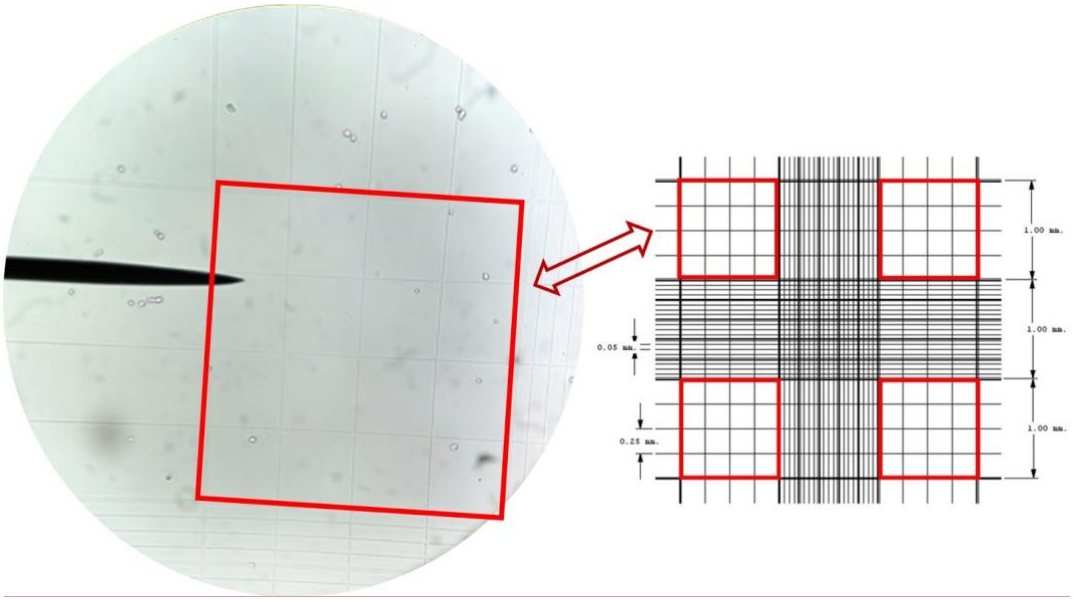

Figure 13. Illustration of cell counting using the improved Neubauer chamber. The cells were counted through the calculation of the average of the four squares marked in red and using this value later in the formulas.

3.2.1 Calculation of cell viability and cell concentration

Cell concentration was established like the number of living cells concentration per milliliter as shown on the first formula, and cell viability was calculated as shown on the third formula, taking into consideration the total number of cells as Viable Cell Count + Non-viable Cell Count.

$$Viable\ Cell\ Count\ = \frac{Number\ of\ Live\ Cells\ Counted}{Number\ of\ Large\ Corner\ Squares\ Counted} \times Dilution\ Factor$$

$$Non-viable\ Cell\ Count\ = \frac{Number\ of\ Dead\ Cells\ Counted}{Number\ of\ Large\ Corner\ Squares\ Counted} \times Dilution\ Factor$$

$$Percentage\ of\ Viability = \frac{Number\ of\ Viable\ Cells}{Total\ Number\ of\ Cells} \times 100$$

Figure 14 shows the percentage average of cell viability using the trypan blue exclusion assay and **Figure 15** depicts the cell number counted from the concentration of cells/ml counted from each sample's condition.

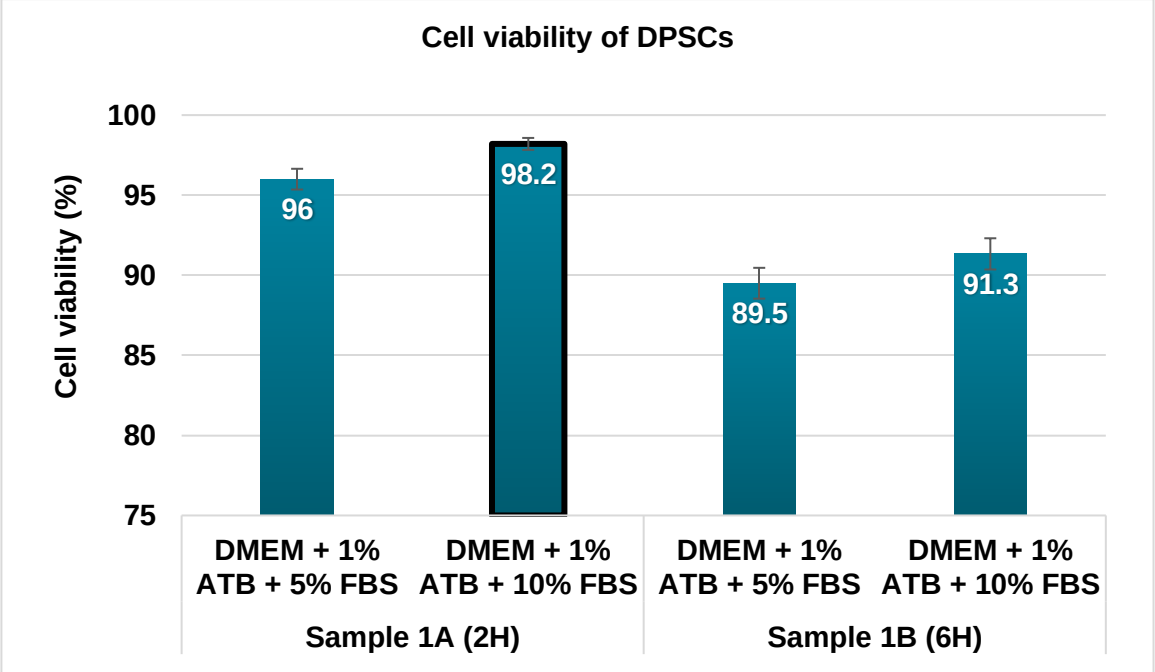

Figure 14. Cell viability of sample 1A and 1B in different culture conditions. The most suitable conditions are highlighted by a black line. *Values are expressed as averages of triplicates ±standard deviation.

The highest viability was reached by the sample 1A isolated after 2 hours from the tooth extraction and cultured in medium with 10% of fetal bovine serum - the viability was 98.2 %. These cells showed about 8.7% increased viability compared to the lowest value of 89.5%. This value was obtained from the cells of Sample 1B, which was isolated 6 hours after the extraction and cultured in medium only with 5% of FBS. Also, the cells concentration correlated with the cell viability as shown on **Figure 15.** The highest cell number of 464.194 cells was obtained from the same sample conditions. The lowest cell number was registered by Sample 1B (5% FBS) and it was only 295.033 cells after 14 days since the pulp isolation.

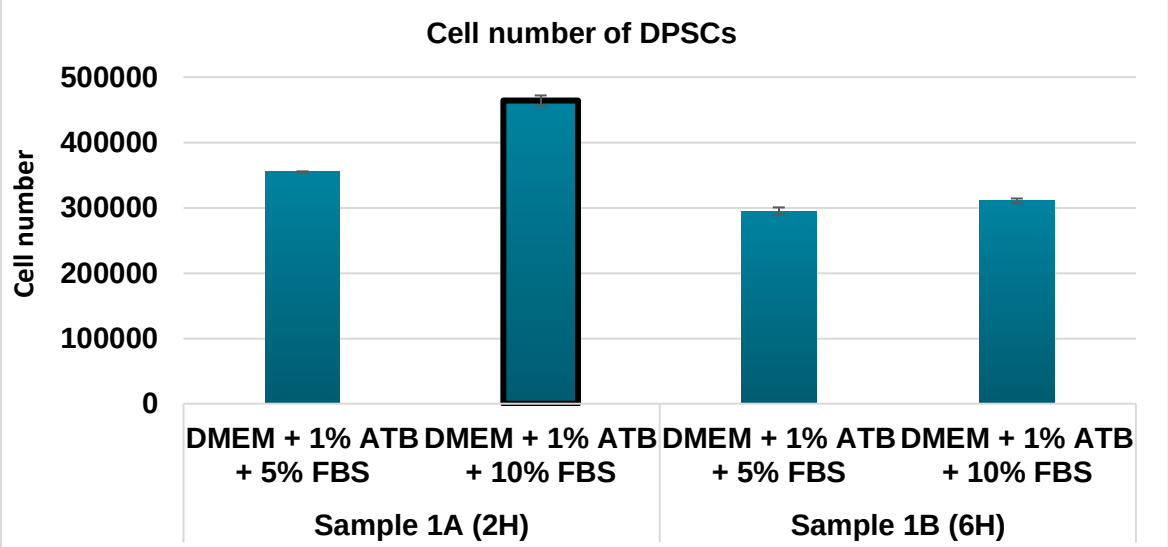

Figure 15. Sample 1A and 1B cell number in different culture conditions. The most suitable conditions are highlighted by a black line. *Values are expressed as averages of triplicates ± standard deviation.

From the obtained data it can clearly be seen that the most suitable cell culture conditions for DPSCs can be stated as following: the tissue has to be isolated after 2 hours of a tooth removal and cells cultured in a media consisting of DMEM,1% of ATB and 10% of FBS. Our findings were also reported by Khasawneh et al. (2019) who reported stem cells, when taken directly from the patient's biological material, have high requirements of FBS in order to guarantee their viability; this is caused by the cell's need for a variety of the biomolecules that the serum can provide, simulating the physiological fluid that serves to evoke the in vivo conditions of the human body. As described by other groups, a concentration of 5% FBS is not enough to stimulate the healthy stem cells for obvious reasons - they are not immortal and cancerous cells can grow in lower concentrations of serum as it was also showed in this study. The higher concentration of fetal bovine serum guarantees their high viability in vitro and the suitable conditions for their manipulation before and during experimenting (Khasawneh, Al Sharie, Abu-El Rub, Serhan, & Obeidat, 2019).

Moreover, it is strongly recommended to manipulate the tooth and its tissue in the shortest time possible because the longer the tooth is kept still, more dead cells will appear due to the removal

from the blood circulation and the lack of nutrients and oxygen. Some studies show that after 24 hours the cells might be completely dead and not suitable for the further use, as it was confirmed by Alsulaimani et al. (2016) in their study (Alsulaimani, Ajlan, Aldahmash, Alnabaheen, & Ashri, 2016).

According to the results, the rest of the samples were manipulated after 2 hours from the tooth isolation and kept in the most suitable cell conditions with 10% fetal bovine serum.

3.3 CRYOPRESERVATION OF DPSCs

The cryopreservation medium was prepared by mixing 90% of fetal bovine serum with 10% of DMSO or glycerol. Both solutions were filtered by using 0.22 µm nitrocellulose filters and stored at -20 C until used for cryopreservation and thawed prior to use.

The DPSCs in passage 3 were trypsinized, the number of cells and viability was established as depicted in **Figure 16** and **Figure 17**.

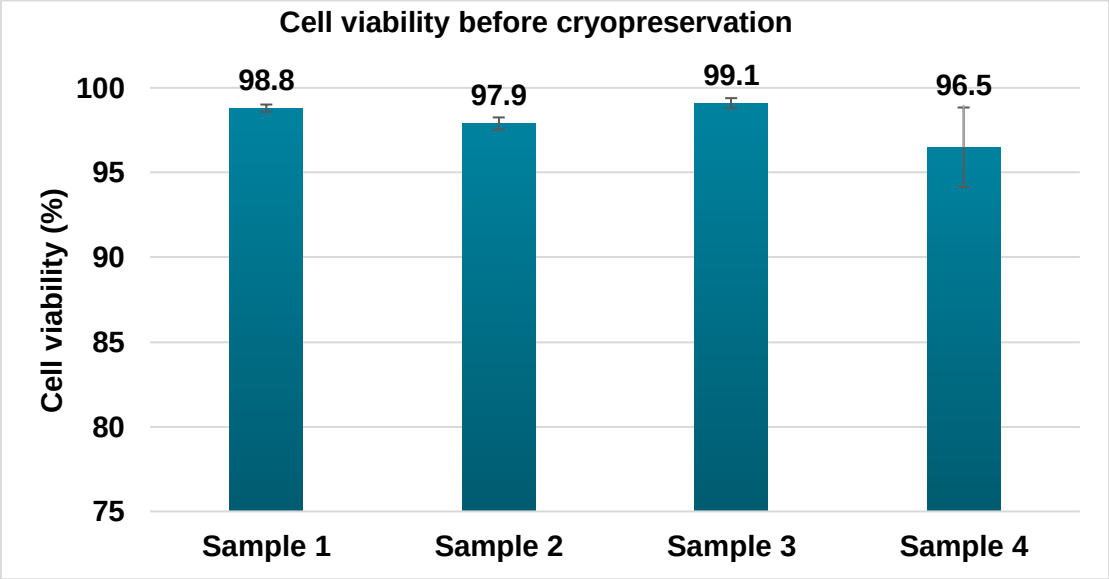

Figure 16. Cell viability in the four samples before cryopreservation. *Values are expressed as averages of triplicates ± standard deviation.

The lowest viability (96.5%) was showed in Sample 4 and generally it was observed that these DPSCs cells proliferated slower compared to the rest of the samples.

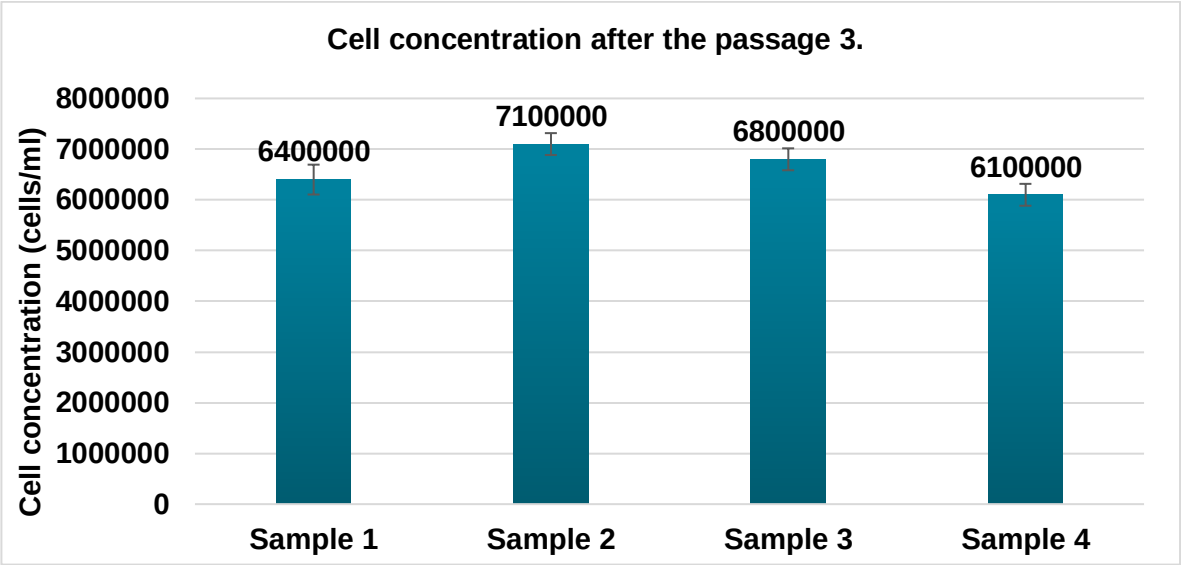

Figure 17. Cell number in the four samples after passage 3**.** *Values are expressed as averages of triplicates ± standard deviation.

The cells generally showed lower proliferation than expected. These cells usually show high proliferative potential, but in this work, the doubling time of cells was not considered, nor the investigation of possible contamination from Mycoplasma, one of the most serious bacteria found in cell culture, as mycoplasma is very difficult to detect while infections with other bacteria, yeast or fungi are quickly recognized. This could trigger the stop of cell proliferation due to a massive infection (Gignac et al., 1992). It is worth mentioning characterization techniques for future studies are highly recommended in order to confirm isolated cells are indeed stem cells, since the low proliferation can indicate the presence of other types of cells like fibroblasts.

After passage 3 cryopreservation of the cells started, therefore, the cell number had to be determined in order to divide the cells into categories according to the specific objective number 3. The cells were supposed to be divided into 4 groups according to the time and used cryopreservants in which they would be stored in liquid nitrogen: 1) DMSO, 1 month; 2) DMSO, 2 months; 3) Glycerol, 1 month and, 4) Glycerol, 2 months.

According to the cell number of DPSCs after the third passage and given time it could only be created the first three categories. It was decided to cryopreserve two-millions cells per vial as Ferro et al. (2014) proposed in their study (Ferro, Spelat, & Baheney, 2014). Briefly, this number of cells were centrifuged, and their pellet was resuspended with cryopreservants into groups 1), 2) and 3). Finally, all the cryovials were placed into a freezing container named Mr. Frosty, which helped to achieve a slow freezing process **(Figure 18)**. This container was put into a freezer at -80°C and after 48 hours the cryovials were transferred into a tank filled with liquid nitrogen at -196 °C until the specific time point was reached.

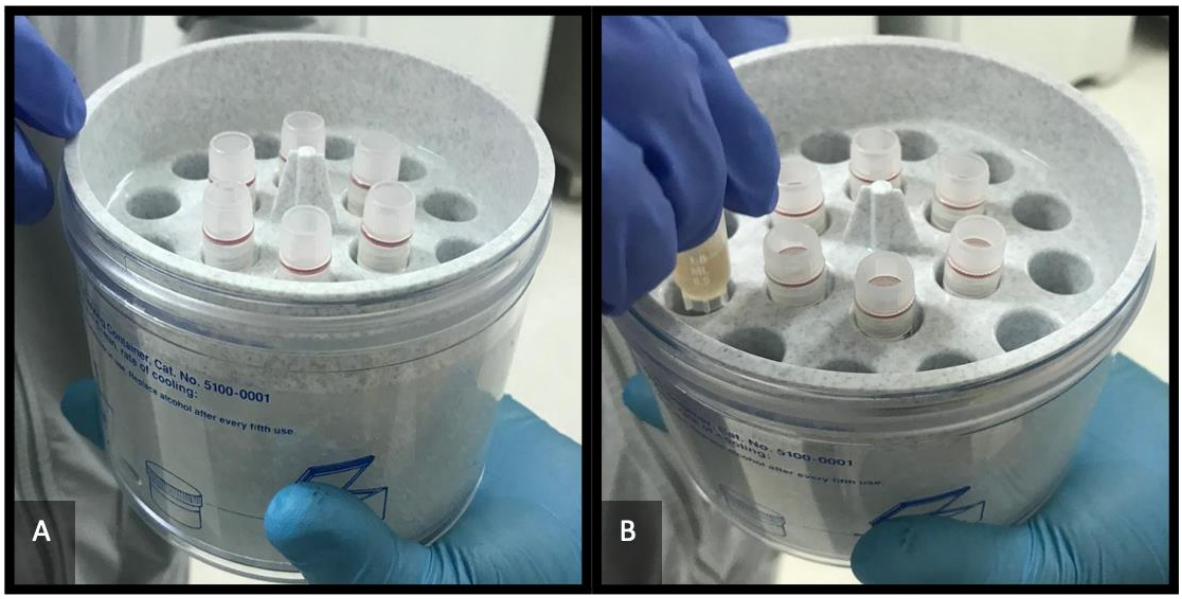

Figure 18. A. Nalgene Mr. Frosty™ Freezing Container. **B.** Inserting the cryovial with DPSCs+ DMSO + FBS in Mr. Frosty™.

3.4 CELL VIABILITY ANALYSIS AFTER CRYOPRESERVATION

After reaching 1 and 2 months since the freezing, the cells were carefully thawed and the cell viability was counted using trypan blue assay as stated previously.

The remaining cells were seeded on the culture flasks, placed in a humidified atmosphere for 3 consecutive days and again the cell viability and cell number were established in order to compare the conditions before and after the cryopreservation.

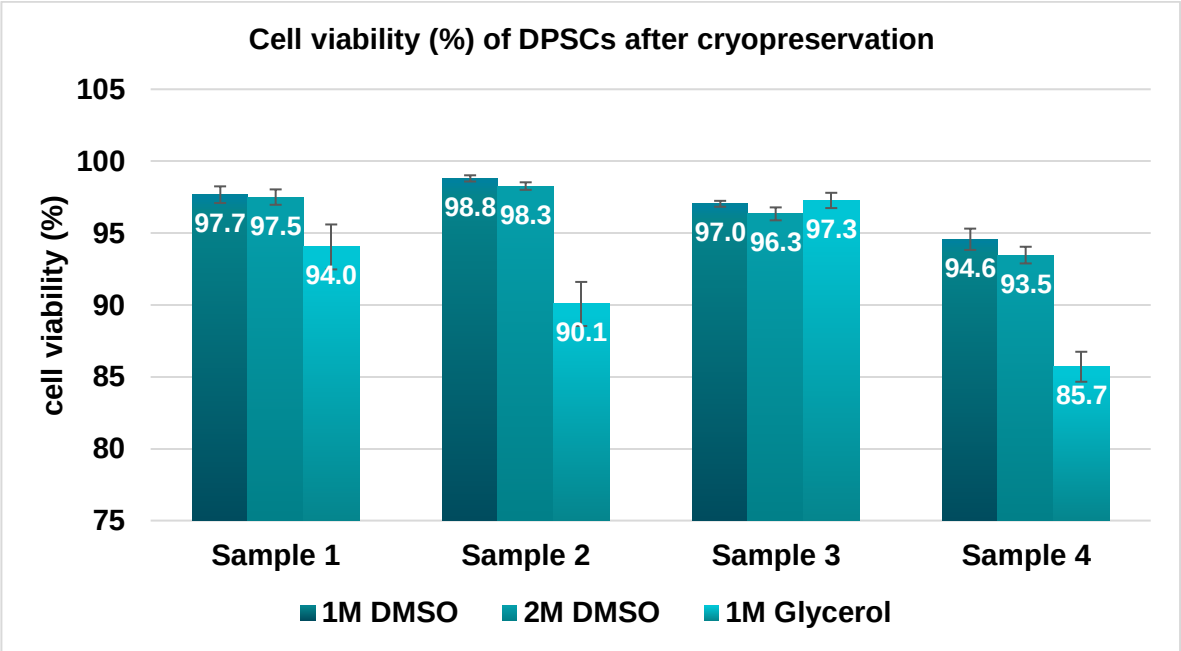

Figure 19. Cell viability of samples 1, 2, 3 and 4 after cryopreservation using different cryoprotectants for one and two months. *Values are expressed as averages of triplicates ± standard deviation.

Figure 19 shows the differences between cell viability right after thawing the cells based on used cryopreservants and time point. It was not possible to cryopreserve samples with glycerol during two months due to the small number of cells. The highest viability of DPSCs was reached by using DMSO as a cryopreservant with slight differences if used for 1 month or 2 months. Clearly, the other substance glycerol shows lower viability from 97,3% up to 85,7%, but the cells were still considered healthy. From these data it is also obvious that Sample 4 exhibits way lower viability than the rest of the samples. This can also be explained by a few factors. Some oral diseases such as Pulpitis and Periodontitis could trigger programmed cell death (Huang, Zhan, Yang, & Hou, 2020) and affect colony-forming efficiency and proliferation rate (Sun et al., 2014) respectively. Besides, senescence could decrease cell proliferation and donor's age could also show affections (Stanko, Altanerova, Jakubechova, Repiska, & Altaner, 2018). Furthermore, it is possible that contamination of Mycoplasma as it was stated previously, could influence the number of live cells, it would be necessary to examine all the samples for this possible pathogen in order not to contaminate any other cells in the deposit.

Three days after the recovery from the thawing process the cells were trypsinized again and cell viability and concentration were counted, in order to see if the cells were capable of reestablishing their vital functions. To emphasize, 2 million cells were cryopreserved, and the following **Figure 20** and **Table 1** show the number of cells after cell culturing.

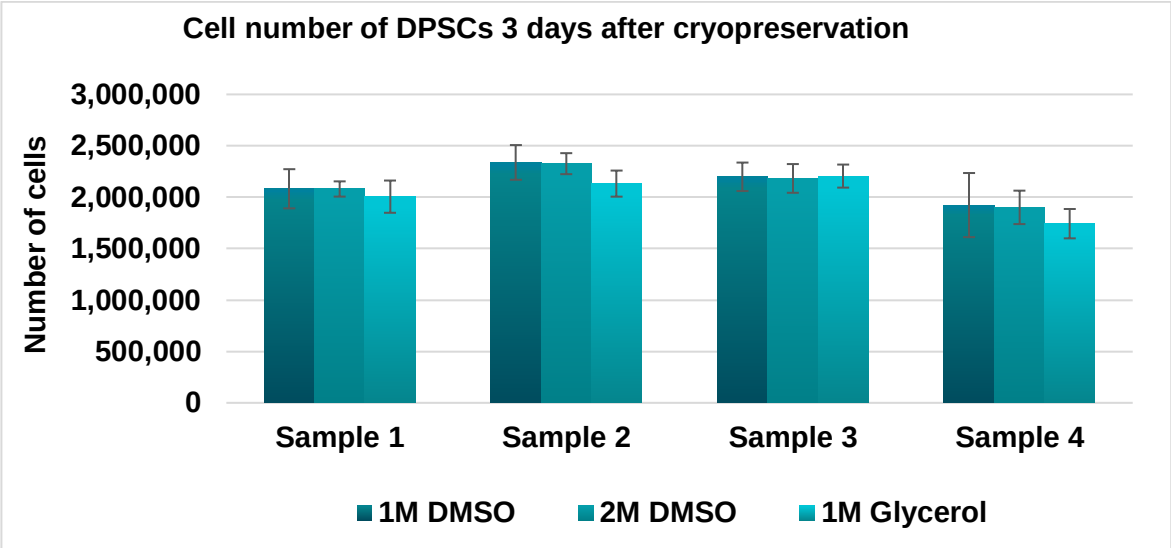

Figure 20. DPSCs concentration in samples 1, 2, 3 and 4 three days after cryopreservation using different cryoprotectants (DMSO and Glycerol) for one and two months. *Values are expressed as averages of triplicates ± standard deviation.

Time	*1M*	*2M*	*1M*
Cryopreservant	DMSO	DMSO	Glycerol
Sample 1	2 082 133	2 079 999	2 005 333
Sample 2	2 338 266	2 326 432	2 132 366
Sample 3	2 198 666	2 182 799	2 205 466
Sample 4	1 923 533	1 901 166	1 742 566

Table 1. Number of cells in samples 1, 2, 3 and 4 after one and two months of cryopreservation using different cryoprotectants (DMSO and Glycerol) after the thawing and 3 days of culturing.

Based on the results shown in Table 1, it is possible to conclude that the DPSCs are highly capable of rapidly recovering from such stressful conditions like thawing, increasing the cell number in as short of a period as three days. The only sample which showed decreased proliferation and did not even reach the number of cells registered before freezing was Sample 4, and this might be explained by the factors described previously.

Also, slight differences were presented in the effect of cryopreservation of DPSCs using two different cryoprotectants and from these preliminary results, it is concluded it would be better to work with the DMSO. This substance showed its capability of preserving cells without decreasing the cell viability and quick cell recovery after freezing capacity. (Conde et al., 2016).

3.5 REVIEW OF LITERATURE

Lately, some research groups have studied dental pulp as a potential source for stem cells, focusing their efforts in finding the best protocols for isolation, culture and cryopreservation of these cells (DPSCs), in order to storage them in biobanks and use it for research or therapies purposes. Results of recently published studies of DPSCs cryopreservation have been promising, showing good recovery post-thaw, however these results are still preliminary (Woods et al., 2009).

As mentioned at the methodology, different search strategies were used, including certain phrases: “dental pulp stem cells + cryopreservants”; “dental pulp stem cells + freezing conditions”; “dental pulp stem cells + biobank”. Different studies related to isolation, culture and cryopreservation of DPSCs are cited below aiming to compare their methods and results with the ones used in this work.

3.5.1 Isolation of stem cells

The objective of this procedure performed by Woods et al. (2009) is to optimize the cryopreservation of DPSCs cultures and the cryoprotective agent, the cryopreservative concentration, the concentration of frozen cells and the storage temperatures, in addition to evaluating the growth, surface markers and differentiation of the DPSCs obtained. In order to achieve what they are aiming for, they started by the collection and transport of the extracted samples. The collected teeth were put in PBS and taken to the laboratory to be handled within 24 hours. For processing and tissue recovery the teeth were washed with different solutions such as saline solution, polyvinylpyrrolidone-iodine (PVP-I), PBS with sodium thiosulfate, Listerine used as an antiseptic and finally in sterile PBS, and then the dental pulp was extracted. To continue with tissue digestion, it was necessary to place the dental tissue in a mixture of saline solution with Collagenase type I and II, thermolysin and neutral protease at 37°C for 15-30 minutes, after this time medium was added and the cells were seeded in Mesencult (Woods et al., 2009).

3.5.2 Culture of stem cells

Continuing with the methodology of Woods et al. (2009), for the culture, it was chosen to sow the corresponding to a tooth digested by flask and they were incubated at 37°C with 5% of CO2, the culture medium was changed 3 times a week for 14 days, the cells were dissociated with trypsin; counts were made and a subculture was carried out under the same

conditions, with flow cytometry it was confirmed if they could be called MSC. To give rise to cell differentiation, cells were seeded in 6cm plates to be cultured over a period of 3-4 weeks with a specific culture medium for differentiation (Mesencult) that serves for osteogenic and adipogenic differentiation. For chondrogenic differentiation, Mesencult+ dexamethasone+ ascorbic acid+ phosphate+ beta-glycerophosphate+ TGFBeta3 was used (Woods et al., 2009).

On the other hand, Huynh et al. (2017), to reach their goal, to simplify the collection, isolation and cryopreservation of DPSCs to establish stem cell banks, they implemented a protocol that consisted of placing the extracted teeth in saline solution with gentamicin or DMEM with antibiotics, extracting the pulp and placing it in a flask with culture medium and antibiotics to culture it until the desired confluence was reached. The cultures were kept at 37°C and 5% of CO_2. Flow cytometry was carried out and then alive cell count was made (Huynh, Le, Doan, Ngo, & Tran, 2017).

3.5.3 Cryopreservation of stem cells

Cryopreservation is a necessary process when there are cells or tissues that need to be stored while maintaining its viability, reaching sub-zero temperatures when biochemical reactions don't occur. However, cells can suffer during freezing or thawing process, it might cause crystals formation inside or outside cells, creating an osmotic efflux of water from cells, increasing the concentration of intracellular solutes, which can lead to an osmotic damage because of the solute toxicity, and also it might occur when cells are cooled rapidly and mechanical and structural damages can appear due to the crystals. To prevent this possible harm, usually there is a cryoprotective agent included into the freezing medium, which protects cells during the whole process, optimizes the cooling rate and blocks ice crystals formation (Pilbauerová & Suchánek, 2018).

There are different types of cryoprotectants according to their molecular weight, for example, glycerol or DMSO are substances with low molecular weight that can penetrate the membrane and prevent the ice crystals formation, differently, high molecular weight substances as dextran or polyvinyl alcohol, do not penetrate the membrane but remain in the extracellular area allowing cell dehydration and decreasing ice crystal formation (Pilbauerová & Suchánek, 2018).

According to the report made by the UNC Lineberger comprehensive cancer center (2007), in order to fulfil the objective of cryopreserving and recovering the cells, techniques that ensure rapid freezing, but slow thawing must be chosen. To achieve this, a programmable speed freezer and an isolated container were used, and then in a final concentration of no more than 20% and, as a first option for cryopreservation, dimethyl sulfoxide (DMSO) at 7-10% concentration; and the second option was adding 10% of glycerol to the medium. The cells were collected by trypsinization, placed into cryovials and stored in liquid nitrogen. The first method, programmable speed freezer, consisted in programming the system to start the freezing stage at a temperature of 4°C, introduce the cryovials in the cells and then the program starts to lower the temperature at a speed of 1°C/minute until it reaches -30°C. At this point, the program receives the command to cool at 0.5°C/minute until it reaches -50°C

and then to reset the ratio to 1°C/minute until it reaches -100°C, the cells were stored in liquid nitrogen. The second method, an isolated container, consisted of a polystyrene container where the vials and walls were totally isolated and taken to a freezer at -70°C, it is a slow process due to the isolation, it can take several days. Once the process is completed, the cells can be taken to liquid nitrogen. For thawing, the cryovials were placed in a container of distilled water preheated to 37°C for 1-2 minutes, the cells are resuspended in fresh culture medium and the culture medium is changed after 24-48 hours to remove any residual cryoprotectant (Lineberger, 2007).

Taking up the study of Huynh et al. (2017), for cryopreservation they prepared a freezing medium with DMEM/F12, 10% PBS, 1% L-Glutamine and 1% antibiotic) and DMSO between 5-10%, they were placed in cryovials, left at room temperature for 15-40 minutes and 4 freezing conditions were established: 1. (F5) DMSO at 5% and directly to liquid nitrogen (-196°C), 2. (F10) DMSO at 10% and directly to liquid nitrogen, 3. (S5) DMSO at 4%, a pre-cooling process and freezing chamber (Mr. Frosty™) filled with isopropyl alcohol and passed through a freezer at -80°C for 24 hours before being moved into liquid nitrogen, 4. (S10) DMSO at 10% with a freezing chamber with a -1°C/minute ratio. Then, the cells were thawed after 6 months by soft agitation in a 37°C water bath until the ice was completely melted, the cryoprotectant was removed by centrifugation and the supernatant was discarded, after that, the cells were resuspended and cultured. Placing the tooth in the gentamicin solution reduced the DPSCs contamination (2.3%) and the cells showed normal stem cell molecular markers. When measuring viability, conditions F5 and F10 did not grow after thawing (10.8% and 32.1% respectively), in F5 and F10 they did see conclusive results (79.7% and 79.0% respectively) , being F5 the condition with the highest viability in the following two weeks of culture after obtaining a confluence of 80% but in F10 the cells stopped growing in that period of time, which leads to the conclusion that slow and controlled freezing methods give better results when it comes to viability than rapid cooling. It can also be concluded that the use of gentamicin has benefits in preventing contamination of samples, in addition to establishing that the use of 5% DMSO is the best alternative for cryopreservation due to the results of the S5 condition (Huynh et al., 2017).

For the cryopreservation process of Woods et al. (2009), different cryoprotectants in different concentrations were used as ethylene glycol (EG), dimethyl sulfoxide (DMSO), propylene glycol (PG); the cells were stored in 2ml cryovials (1 million cells/cryovial), and cooled at a rate of -1°C/minute with the technique of immersing the cryovials in isopropanol inside a mechanical freezer of -85°C for 24 hours to be taken directly to liquid nitrogen and left frozen for one month. For thawing, the cryovials were taken to a water bath at 37°C, the cells were washed with a culture medium to remove the cryoprotectant and then centrifuged. A trypan blue exclusion assay was performed to identify dead cells. The result of this experiment was that a viability of approximately 90% was obtained with DMSO, which led to the conclusion that it was a much better option than PG and EG. It was also determined that DPSCs can be stored between -85°C and -196°C for approximately six months without losing functionality (Woods et al., 2009).

The purpose of this study made by Conde et al. (2016) is to review the literature on different cryopreservation protocols and whether they can affect the biological functions of cells. The

search was made under the PICO model (population, intervention, comparison, outcome), the information presented was obtained from PubMed and the ISI Web of Science until April 2015. The data found were: it is very common to see that the most used amount of DMSO for DPSCs is 10% and the lowest is 3%, besides, using 10% glycerol or 10% ethylene glycol has the same effects regarding cellular properties as using the same amount of DMSO, but they assure that this is the most recommended because it shows a cellular viability of 90% after thawing. The most applied technique has been rapid freezing (-80°C) for 1 year. If the magnetic field freezer technique is used it is recommended to use 3%-5% DMSO and it can be a positive influence because it prevents the formation of crystals because it immediately freezes all the water molecules. Another viable and reliable option to store DPSCs is mechanical freezing (-85°C) for 6 months. There is a question about the use of FBS, because it has a mixture of growth factors, proteins, carbohydrates, cytokines and others that are indispensable for the in vitro development of cells, and it also reduces the cellular risk in the freezing and thawing processes but, on the other hand, it is said that a medium free of FBS is also a good option to carry out cryopreservation because risks related to the transmission of animal pathogens that can affect the antigenic response of the patient after the process of implantation of these can be avoided. This alternative is widely used when magnetic fields are to be used because it has been seen that they present less apoptotic activities and, also, it is recommended to use a medium without this component if you want to cryopreserve porcine DSC for one year. In conclusion, cryopreservation with 10%-20% DMSO is the best option for two-year periods because it allows preserving cell viability, multipotency capacity, karyotype and surface stem cell markers, although it is not possible to accurately predict the behavior of the cells when they are subjected to very high storage periods, so it is recommended to interpret and follow these conditions very carefully (Conde et al., 2016).

According to recent literature, 10% DMSO is the most used cryoprotectant, however it has been proved that these cryoprotectant might induce some side effects as cytotoxicity and the addition of FBS is self-defeating for therapeutic purposes, that is why some researchers have focused their efforts in finding new methods for cryopreservation improving the process with other technologies described below.

The standard slow-freezing procedure with 10% DMSO could not be the best option for stem cell-based therapies because DMSO is cytotoxic, instead, Lee et al. (2012) evaluated a new technique called Magnetic cryopreservation, which uses a programmed freezer with a magnetic field, this procedure has been able to obtain 73% viable cells. Materials and methods are described below. “One million DPSCs were placed in the programmed freezer supplied with a magnetic field of 0.01 mT at –5°C and maintained for 15 min and then cooled at a rate of –0.5°C/min until the temperature reached –32 ° C. After the freezing procedure, the cells were stored in a –150°C freezer (MDF-11561; Sanyo) for 1 week. After 1-week storage, the NMF and MF cells were immediately thawed in a 37°C water bath with gentle agitation. The cells were then washed dropwise with the complete culture medium to remove the cryoprotectant. Followed by centrifugation at 500 g for 5 min”. They also needed to determine the required concentration of DMSO for magnetic cell cryopreservation and after some experiments they found out that cell viability after magnetic cryopreservation using 3% DMSO was comparable to the one obtained with 10% DMSO (Lee et al., 2012).

Title	Authors	Cryoprotectant	Freezing technique	Cryopreservation	Thawing technique	Conclusion
General protocol for the cryopreservation of mammalian cells	UNC Lineberger	7-10% DMSO	Programmable speed freezer	Liquid nitrogen	Container of distilled water preheated to 37°C	-
		10% Glycerol	Isolated polystyrene container			
Simplified conditions for storing and cryopreservation pf dental pulp stem cells	Nam Cong-Nhat Huynh, Son Hoang Le, Vu Nguyen Doan, Lan Thi Quynh Ngo, Ha Le Bao Tran	5-10% DMSO	5% DMSO - directly to liquid nitrogen	Liquid nitrogen	Water bath at 36°C until the ice was completely melted and soft agitation	Slow and controlled freezing methods give better results and 5% DMSO is the best alternative for cryopreservation
			10% DMSO - directly to liquid nitrogen			
			4% DMSO - Mr. Frosty			
			10% DMSO - freezing chamber			
Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use	Erik J. Woods, Brandon C. Perry, J. Jeffrey Hockema, Lindsay Larson, Dan Zhou, W. Scott Goebel	Ethylene glycol	Immersing the cryovials in isopropanol inside a mechanical freezer	Liquid nitrogen	Water bath at 37°C	DMSO reached the higher viability result so it's the best option to cryopreserve DPSCs
		DMSO				
		Propylene glycol				
Does cryopreservation affect the	Marcus Cristian Muniz Conde, Luiz	3-10% DMSO	Mechanical freezing	-	-	10-20% DMSO is the best option for two-

biological properties of stem cells from, dental issues?	Alexandre Chisini, Guillermo Grazioli, Alejandro Francia, Rodrigo Varella de Carvalho, Jose Carlos Bernedo Alcázar, Sandra Beatriz Chavez Tarquinio, Flávio Fernando Demarco	10% glycerol				year periods of cryopreservation
		10% ethylene glycol	Rapid freezing			
Magnetic cryopreservation for dental pulp stem cells	Sheng-Yang Lee, Guo-Wei Huang, Jau-Nan Shiung, Yen-Hua Huang, Jiiang-Huei Jeng, Tzong-Fu Kuo, Jen-Chang Yang, Wei-Chung Vivian Yang	3-10% DMSO	Programmed freezer supplied with magnetic field	Freezer	Water bath at 37°C with gentle agitation	Magnetic cryopreservation is thus a reliable and effective method for storage of DPSCs. The smaller amount of DMSO required in SFM for cryo-preservation is beneficial for the clinical applications of post-thaw cells in regenerative medicine.

Table 2. Different cryopreservation techniques found during the review of literature.

4. CONCLUSIONS AND FINAL CONSIDERATIONS

Human molars were processed, implementing conditions that were believed to provide the highest viability of DPSCs and the most suitable requirements for the subsequent storage of DPMSC. These cells were cultured under different conditions, 10% FBS remained through the whole experiment as it was observed to be an adequate amount, considering its importance in the human body in vivo condition simulation. In addition, cell viability using 10% DMSO was as high as expected according to the literature (however, glycerol also showed a similar percentage of viability) and it may represent a better option for cryopreservation with therapeutic purposes, as it is less cytotoxic than DMSO (Conde et al., 2016).

Although in general it was obtained a good percentage of cell viability after cryopreservation, sample 4 shows a lower viability in comparison with the other samples. The factors that could have had a great influence on these results are the donor's age, oral health's quality and some diseases the donor could have suffered from. One of the diseases that influence cell viability and proliferation is pulpitis, known as one of the most prevalent oral inflammatory diseases, responsible for programmed cell death (Huang et al., 2020). In a similar fashion, periodontitis, also an oral disease, causes periodontal inflammation, affecting colony-forming efficiency and proliferation rate (Sun et al., 2014). In addition, dental pulp may suffer from other pathologies such as mycoplasma, an extracellular infection in the membrane that may contribute to lower cell proliferation, however, the necessary techniques to identify this affection could not be performed in this preliminary stage of the study.

In order to obtain more reliable results it becomes necessary to perform characterization techniques such as Fluorescence-activated cell sorting (FACS), Alkaline phosphatase (ALP) and Alizarin red-S (ARS) as a mean to confirm the presence of stem cells after dental pulp isolation (Lee et al., 2012). As an example, low cell proliferation found in Sample 4 might have also been explained by the presence of a higher rate of Fibroblast to Stem cells. Given the case one of the techniques listed above had been used, the situation would have been easily spotted and as a result, the sample would have been rejected, providing far better-grounded results.

At the end, samples could only be cryopreserved for 1-2 months period due to the current COVID-19 pandemic and lack of access to the laboratory. To support our results and aiming to obtain more theoretical basis for future practices, additional information on other cryopreservation techniques was searched in scientific databases with the sole intention of encouraging further studies as a mean to establish the most appropriate cryopreservation conditions using DPSCs, a stem cell type that can easily be obtained through noninvasive

procedures, and which importance can’t be stressed enough as they have the ability to bring numerous benefits to both human health development and many research fields.

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6. SUPPLEMENTARY MATERIAL

SM 1. MATERIALS AND REAGENTS USED

All Chemicals were sterile and purchased from Sigma-Aldrich (USA) if not stated otherwise. All solutions, which required preparation were filtered to be suitable for the cell culture conditions.

Reagents

- Dulbecco's modified Eagle´s medium (DMEM): 4.5 g/l glucose, L-glutamine, Phenol Red (Gibco®, ThermoFisher, USA)
- Fetal bovine serum (FBS) (Microgen Ltda., Colombia)
- Penicillin/Streptomycin antibiotic mix (ATB): penicillin: 10.000 U; streptomycin: 10 mg/ml
- Trypsin/EDTA: 0.05% w/v trypsin; 0.02% w/v EDTA (Gibco®, ThermoFisher, USA)
- Phosphate buffered saline (PBS): 8 g/l NaCl, 1.52 g/l Na2PO4 x 12 H2O, 0.2 g/l KH2PO4, 0.2 g/l KCl, pH: 7.2 – 7.4
- Freezing medium: 90% FBS + 10% DMSO or glycerol
- Trypan blue: (InvitrogenTM, USA)
- DMSO
- Glycerol

SM 2. DMEM PREPARATION PROTOCOL

1. Take 13.48 g/liter of DMEM powder and 3.7 g/liter of sodium bicarbonate and mix them

2. Add 1 liter of sterile water is

3. It must be filtered because the mixture was not completely sterile in order to add the fetal bovine serum. A 0.22 um filter is used for this because that size does not allow fungi, cells or bacteria to pass through. It is important not to touch the part with squares because it damages the membrane.

    a. Sterilize the filter that you are going to use (you must be very careful with the color of the packaging because it changes color with temperature)

        I. First part of the filter: this is the non-sterile part, where the liquid is poured

        II. Lower part: sterile part, where the filtered liquid falls

    b. A vacuum pump forces the liquid down through the filter, observing the membrane not to dry out.

4. Pipette the liquid into a clean, sterile bottle.

7. ANNEXES

ANNEX 1. CONSENTIMIENTO INFORMADO

ANNEX 2. FICHA ÉTICA