# Cryopreservation of Dental Stem Cells

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ABSTRACT
Nowadays, regenerative and reparative medicine has grown in popularity. Dental stem cells are easily accessible source of adult stem cells. They can be harvested by a tooth extraction or spontaneous deciduous tooth exfoliation. They have to be isolated, expanded and stored until time they would be needed for individual stem cell therapy. Cryopreservation is both a short-term and long-term storage of tissues or cells at sub-zero temperatures. There are several methods of cryopreservation requiring different technologies. The objective of this review is to compare them and highlight their advantages and disadvantages.

KEYWORDS
cryopreservation; dental stem cells; freezing protocol

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## INTRODUCTION

Stem cells (SCs) have opened promising future in regenerative medicine, because of their two remarkable features known as self-renewal and multilineage differentiation. Based on the SCs origin, they are classified into Embryonic stem cells (ESCs) and Adult (postnatal) stem cells (ASCs). The advantages of ASCs are less ethical concerns, low immunogenicity and less tumorigenic potency than their embryonic counterparts (1). ASCs have been isolated from many human tissues so far, such as dermis (2), peripheral blood (3), adipose tissues (4), intestine (5), cartilage (6) and bone marrow (7). Several types of ASCs have been identified in dental related tissues. ASCs reside in specialized micro-environment called "niche", which regulates stem cell behavior and maintains a balance between cell death and self-renewal (8).

Dental related tissues represent an easily accessible source of ASCs. Embryologically, human teeth develop from reciprocal interaction between the dental ectoderm (oral epithelium) and the neural crest-derived mesenchyme (dental mesenchyme) (9). Most of the dental tissues have dental mesenchyme origin – dentin, dental pulp, periodontal ligaments, alveolar bone. Enamel is a dental ectoderm derivative. Due to their ectomesenchymal origins, dental stem cells (DSCs) may display characteristics of both mesoderm and ectoderm (10).

Up until now, seven different types of DSCs have been isolated. They are classified in two major groups, dental pulp-related stem cells and periodontium-related stem cells. Dental pulp-related stem cells are: Dental Pulp Stem Cells (DPSCs) (11); Stem cells from Human Exfoliated Deciduous teeth (SHED) (12); and Stem Cells from Apical Papilla (SCAP) (13), and Human Natal Dental Pulp Stem Cells (NDP-SCs) (14). Periodontium-related are: Periodontal Ligament Stem Cells (PDLSCs) (15), Dental Follicle Progenitor Cells (DFPCs) (16), Gingival Mesenchymal Stem Cells (GMSCs) (17). DPSCs were first identified by Gronthos et al. in 2000. And 3 years later, in 2003, Miura et al. first isolated SHED.

As it has been already mentioned, DSCs are easily accessible by a tooth extraction among younger individuals or when a primary tooth is exfoliated. However, these tooth extractions or spontaneous exfoliations usually occur in a period of life when there is normally no need for a stem cell therapy. Therefore, it is necessary to isolate, expand and store stem cells until the time when they would be required.

## PRINCIPLES OF CRYOPRESERVATION

Cryopreservation is a process of sustaining the viability of cells and tissues by freezing and storing them at sub-zero temperatures, when biochemical reactions do not occur (18). DSCs can be subject to an irreversible damage during the freezing or thawing process, known as a freezing injury (19). An exact mechanism is poorly understood, but in general, the irreversible changes to the DSCs are explained by extracellular and intracellular forming of ice crystals. There are two key mechanisms. The first one occurs, when DSCs are cooled slowly, and the extracellular ice crystal formation causes an osmotic efflux of water from cells. This mechanism increases the concentration of intracellular solutes, which can lead to an osmotic damage because of the solute toxicity. The other one happens, when DSCs are cooled rapidly. Because of that, there is not enough time for water to leave the cells and the intracellular formation of ice crystals causes mechanical and structural damages to the cells (20).

Other freezing-associated stress is a creation of reactive oxygen species (ROS), which are possible triggers for apoptosis (21).

To avoid that, there is a cryoprotective agent (CPA) incorporated into the freezing medium to protect DSCs during both the freezing and thawing process. The major effects of CPAs are to optimize the cooling rate and to block the formation of ice crystals by binding to nuclei and slowing down ice crystals growing (22).

## CRYOPRESERVATION OF WHOLE TEETH

Although the main area of interest of short or long-term cryopreservation studies of DSCs is the cryostorage of dental stem cell lineages after their successful isolation and expansion from the dental related tissues, there have been several attempts to cryopreserve whole, both deciduous and permanent, intact teeth. Dental stem cell isolation and expansion in a lab environment is expensive, time consuming and risky for contamination and spontaneous differentiation (23). The hypothesis, why to bank intact whole teeth, was to postpone these procedures till later time, when DSCs would be actually required. However, many limitations remain in this method as well, which make its clinical use practically impossible. One of the problems is the low percentage of viable DSCs obtained from cryopreserved deciduous or permanent teeth after the thawing process. Liedemann at al. showed in their study that DPSCs were obtained from cryopreserved deciduous teeth only with a 30% culture rate, whereas from non-cryopreserved teeth, this rate increases to 61% (23). In another research, Woods et al. used permanent immature teeth with not fully developed roots and they observed only a 20% isolation rate after thawing. For them, 3 out of 10 cryopreserved teeth did not contain any stem cells showing morphological characteristics of DPSCs or they exhibited no cell growth whatsoever. Plus, in post-thawing observations the cryopreserved DPSCs had a low proliferation rate and round-shaped cytoplasm, compared with spindle-shaped cytoplasm of non-cryopreserved group (23). The low isolation rate might be associated with low penetration and diffusion of the CPA into the center of the dental pulp and therefore the insufficient protection from the ice crystal formation. Because of that, deciduous teeth with no visible root resorption or permanent teeth with fully developed roots cannot be used. On the other hand, root resorption or open root apices provide a way of penetration for the CPA. Yet the proliferation rate remains very low, in particular to meet the clinical demands. There was also an attempt to modify the freezing protocol by using Nd:YAG laser piercing, which makes micro-holes into the tooth surface and facilitate penetration of CPA (25). The

isolation rate of DPSCs during this novel method was similar to the rate of non-cryopreserved DPSCs. However, the costs and difficulty were significantly higher. The other causes of DPSC damage may be due to the tooth fractures that might occur after low temperature exposure during freezing process. For now, this method does not show repeatable and satisfiable results in DPSC cryopreservation and it does not allow its application in therapeutic purposes.

## INTERNATIONAL LICENSED DENTAL STEM CELL BANKS

According to recently published studies, the cryopreservation of isolated lineages of DSCs remains the method with the most successful cryorecovery results. This conclusion is very important in answering the question if cell banks should bank whole intact teeth or the isolated stem cell populations. Another hypothesis can also be that a minimal manipulation with the pulpal tissues before the freezing might yield better results of post-thawed viable DPSCs. Wood et al. disproved this idea; DPSCs digested from post-thaw pulp tissue did not proliferate at the same rate observed in their fresh counterparts. It took at least twice as long (24).

Current number of licensed dental stem cell banks is very low. Historically the first one was built in Japan at Hiroshima University in 2005 (26). The National tooth bank was opened soon after in 2008. Another was open in Norway thanks to the collaboration between Norwegian Institute of Public Health and the University of Bergen (27). There are also commercially licensed tooth banks in the UK and the USA.

## CRYOPRESERVATION PROCEDURE OF ISOLATED POPULATION OF DSCS

When DSCs are intended to use for therapeutic purposes the entire cryopreservation thawing protocol must follow standards of good manufacturing practice (cGMP).

## TOOTH COLLECTION

In general, each vital tooth both deciduous and permanent might be a potential source of dental related stem cells. However, one of the major problems associated with cryopreservation is that the degrading process of dental related tissues starts immediately after the tooth is extracted or exfoliated. Several previous studies established the maximum time frame for stem cell isolation to be 120 hours post extraction (24, 28). The longer time frame from the tooth extraction, the lower post thawing cell recovery efficiency is observed (28). A submersion of the tooth into a sterile hypotonic phosphate-buffered saline solution while the tooth is transported to a laboratory for DSC harvesting and isolation prevents dental tissue necrosis to begin. This solution precludes tooth dehydration. Transported teeth have to be kept in hypothermic conditions (at 4 °C), which is being referred to as a sustention.

A tooth extraction, as a future source of DPSCs, is a common surgical procedure, which can be performed at any age. Dental pulp tissues from wisdom teeth are the most common source of DPSCs among adults. The second most common are premolars. In particular, first premolars are often extracted during an orthodontic treatment of frontal teeth crowding. In general, the efficiency of DPSC harvesting and isolation is higher when the permanent teeth are immature and they do not have fully developed roots. In deciduous teeth, as sources of SHED, vital pulp tissues are mainly found in clinical crowns. Their roots must not be resorbed more than 1/3 of the original length. The grater root resorption might have an negative impact on the viability of SHED (29).

Harvested teeth should be washed out in commercially available bactericidal solutions, e. g. chlorhexidine, to minimize the bacterial contamination. It helps to further reduce the risk of cross-contamination (24).

## DENTAL PULP STEM CELL ISOLATION AND EXPANSION

As soon as a tooth is collected and transported to the laboratory, the dental crown is gently separated from the root to open the pulp chamber and harvest the pulp tissue. There are several methods how to perform that allowing for a minimal damage to the cellular components. First, undeveloped roots in permanent teeth or resorbed roots in deciduous teeth have largely open apexes (usually more than 2 mm). These open roots create a way to obtain pulp tissues through them without a necessity of splitting the crown from the root. The other approach, if the apical foramina are too narrow, is to use special forceps or diamond burs and gently open the pulp chamber in the cervical third of the tooth, at the enamel – cementum junction. Indeed, the possibility of an irreversible damage to pulp tissues is higher with this method. Afterwards, the dental pulp is gently separated from the pulp chamber and root canal walls and minced into small pieces.

In order to isolate DPSCs there are also two approaches. DPSCs can be isolated either by an enzymatic digestion (ED) method (30, 31) or a spontaneous outgrowth (OG) method (32, 33).

To perform the first method, pulp tissue fragments are digested by submerging them into a solution of enzymes collagenase type I and dispase, or trypsin enzyme to obtain single cell suspension. The second approach, on the other hand, is based on a spontaneous stem cell overgrowth from minced pulp tissue pieces. Several researchers have tried to answer a question which method has better efficiency of cell proliferation rate or unchanged morphological and phenotypic properties of isolated stem cells. Unfortunately, while some researchers concluded that stem cells isolated by OG have lower proliferation rate and weaker stem cells marker expression (34), some others concluded no differences in those aspects regardless of the isolation method used (35). Thus more detailed studies are further required to answer this question.

## CRYOPRESERVATION SOLUTION

The high-water content of tissues and cells is one of the leading determinants of physical changes during the freezing or thawing phase of cryopreservation. During sub-zero temperatures formation of ice crystals occurs, which can lead to a fatal damage to tissues and cells.

A cryoprotective agent (CPA) is incorporated into the cryopreservation medium in order to protect stem cells from the ice crystal formation. Major attribute of CPAs is determined by their ability to reduce the freezing and thawing point and therefore optimize the freezing rate (22). Currently used CPAs are divided into two major groups. First one are low molecular weight substances, for example glycerol, ethylene (propylene) glycol, dimethyl sulfoxide (DMSO), which can penetrate a cell cytoplasmic membrane, prevent the formation of ice crystal nuclei and slow down the ice crystal growth inside cells (36). Contrary, the second group includes substances with high molecular weight, for example, dextran, hydroxyethyl starch (HES), polyvinylpyrrolidone and polyvinyl alcohol. They remain in the extracellular space and allow for cell dehydration and minimization of the intracellular ice crystal formation, and stabilize the cellular membrane (22).

A freezing medium containing DMSO as the CPA has been widely used in both in vitro and in vivo, since its high efficiency in cell protection was documented. The review of recent literature has shown 10% concentration of the DMSO as the most effective. However, there are some documented concerns about its cytotoxicity and related side effects such as sickness if it is introduced to patients as part of the stem cell therapy (36).

Interestingly, even the knowledge of these side effects has not stopped the usage of DMSO as the CPA, because there is not such as effective alternative. The theory, how to decrease DMSO concentration and the cytotoxic effect as well, is its combination with substances with the high molecular weight. In some recent studies the combination of DMSO and HES has been documented as an effectual alternative in stem cell cryopreservation (22, 37).

Another question is the optimal cell concentration for cryopreservation to maximize the post-thaw cell viability. Woods et al. at indicated that 1.0 – 1.5 × $10^6$ cell (with 10% DMSO as the CPA) yielded optimal results (24).

Furthermore, a xenogeneic serum is the most commonly incorporated into the standard used freezing medium. However, the presence of the fetal calf serum/bovine serum is against standards of cGMP, in particular in the human stem cell therapy. Therefore, one of the major interests of further researchers should be to comprehensively examine the efficiency of the serum-free freezing medium that contains the non-toxic CPA in DSC cryopreservation.

## FREEZING PROTOCOL

There are several techniques of the freezing protocol. However, a choice among those methods depends on resources available and experience of scientists and technicians.

## CONTROLLED-RATE FREEZING

The goal of minimizing the potential for stem cell damage by dehydration and ice crystal formation during cryopreservation led to the introduction of controlled-rate freezing methods. The slow freezing rate (SRF), 1–2 °C per minute, is generally considered as optimal for maintaining stem cell viability during cryopreservation (38, 39). Other method, ultra-slow freezing rate (USFR), is the highly controlled rate of 0.3 to 0.6 °C per minute, which is more costly (40). Current studies have shown significantly higher post-thaw cell growth and viability using those methods, in contrast with the rapid freezing method described below. For instance, Huynh et al. concluded that controlled-rate freezing methods resulted in a significantly higher number of viable cells, both in 5% and 10% DMSO (79.7% and 79.0% respectively) compared with the rapid freezing method (41). At the same time, Naaldijk et al. observed the effect of different freezing rates during cryopreservation of rat mesenchymal stem cells and they determined, on the other hand, that the rapid freezing protocol is no less effective in maintaining post-thaw viability of MSC compared to the controlled rate freezing method. They also showed, that different effect of freezing protocols are only observable 3 days after thawing and beyond (37).

In addition, another problematic issue is that these methods cannot be scaled up to provide uniformity of temperature to all vials during large scale banking. Thus, further development in this area is necessary (42).

## UNCONTROLLED-RATE FREEZING

The alternative approach of cryopreservation is an uncontrolled rate freezing protocol. The CPA and tissue or stem cell samples are first precooled to 4 °C and then they are directly deposited into the freezer at −80 °C or into liquid nitrogen (43). This process is considered to be a simpler, cheaper alternative that requires no individual training or specific technology. Current research has determined uncontrolled rate freezing to be an efficient method in long-term cryopreservation of DPSCs (44). It also concluded no post-thaw effect on cell viability, multipotency, proliferation and differentiation. However, the efficiency of uncontrolled rate freezing protocol has so far only been studied in the presence of 10% DMSO as the CPA.

## RAPID FREEZING (VITRIFICATION)

The principle of the rapid freezing protocol is based on the formation of an arrested liquid state and a glasslike solidification in presence of a high concentration of the CPA (45). This method has been well described in the cryopreservation of human embryonic stem cells, oocytes, sperms, which are particularly sensitive to cryoinjuries. For instance, Jadoon et al. concluded that the greater amount of vitrified oocytes survived cryopreservation through rapid freezing than slowly frozen (SRF) oocytes (70.3% vs. 12.5%) in their study in 2015 (46). On the other site, there are very

few published papers concerning the usage of the rapid freezing method in cryopreservation of DSCs. Huynh et al. compared the post-thaw cell viability and the growth rate of DPSCs cryopreserved by controlled-rate freezing and rapid freezing method in 5% and 10% DMSO. Regardless the percentage of DMSO used, DPSCs cryopreserved for 6 months by the latter method did not regrow after thawing. In comparison to it, the former method resulted in significant higher number of viable cells and the more rapid growth rate (47). Unfortunately, this approach also seems to be unsuccessful in cryopreservation of whole intact teeth, due to progressive root resorption and the damage of DSCs embedded in the hard dental tissues (48). The concept of this technique is to isolate small volume of the tissue specimen, which is going to be cryopreserved, and to place them on vitrification carriers, such as cryoleaf, cryoloop, or ministraw, directly into a liquid nitrogen (49, 50). It is a very simply technique. Interestingly the possible toxic effect of the DMSO as the CPA can be minimized by rapid freezing rate (51). Unfortunately, just the small volume of samples and relatively high volume of liquid nitrogen for each vitrification, caries limit this technology to scale up for wider clinical use.

## MAGNETIC FREEZING

Magnetic cryopreservation is considered to be a controlled slow-rate freezing technique. Programed freezers are supplied with a magnetic field of 0.01 mT and they cool at the rate of 0.5 °C/min until the temperature reaches −32 °C (52). Afterwards, the cryopreserved samples are stored in a −150 °C freezer. The magnetic field has been proven to lower water aggregation during freezing. This is advantageous as formation of ice crystals as well as a generation of unwanted weak electric current are both reduced. Therefore, the electric current cannot disrupt cell membranes, which allows for a better protection of cells during freezing (53). It has been published so far that this technique can be effective in the cryopreservation of both, whole intact teeth as well as isolated DPSCs. Lee SY, Sun CH, Kuo TF et al. observed the effect of the magnetic cryopreservation on intact rat teeth and pulpal tissues in the presence of different concentration of the DMSO as the CPA. Still, they observed a problematic penetration of the CPA into the coronal part of the pulp tissue while cryopreserving intact teeth, however, a better penetration to the root portion of the teeth has been seen. At the end, they concluded that the magnetic cryopreservation is still an effective method for intact tooth and pulp tissue banking compared to the others. It requires lower concentration of DMSO and shorter pre-equilibration time. Finally, it allows higher biological activity of the recovered DPSCs from post-thawed tooth and pulp tissue (53). DPSCs isolated from the magnetic cryopreservation contained 73% of viable cells (54). Additionally, this method also revealed successful results for cryopreservation of DPSCs in presence of 3% DMSO and a serum-free cryopreservation medium (SFM). This seems very promising for good manufacturing practice (cGMP) and therefore for further application in human regenerative medicine (52).

## OPTIMAL STORAGE TEMPERATURE

The viability of post-thawed DPSCs, stored for 1 week, 1 month or 6 months at either −85 °C or −196 °C, was ≥ 90% for all storage lengths and both temperatures. No variations in the doubling time or in the differential potential were significant statistically (24).

## CELL THAWING

There have been very few research papers concerning the optimal thawing method or the thawing temperature. The most widespread standard procedure, that has been utilized so far, is a 37 °C water bath. It has been proven that this technique removes ice crystals efficiently and the potential cell damage is minimal (28, 55). On the other hand, water environment brings a high risk for bacterial or fungicidal contamination. Other method, that has been studied up to now, is the application of dry heat. These studies identified that samples thawed using dry heat had similar viabilities and clonogenic potential to those thawed using the standard water procedure (56, 57). The different thawing temperature (0, 20 and 37 °C for 20 min) did not uncover any statistically significant changes on post-thawed cells (58). However, this is only a one study and this area ought to be examined more comprehensively before a generally valid conclusion can be drawn.

In any case, The CPA should be washed out from cryovials after thawing due to its cytotoxic effect, DMSO in particular. This approach has been standardized in thawing of cells of bone marrow and peripheral blood. The problematic step of the CPA removal is that cells first shrink and then swell, which may cause their damage as well. Because of it a stepwise removal of the CPA, using graded isotonic solutions, is recommended. However, recently published studies concerning cryopreservation and thawing of DPSCs incorporated either the stepwise removal of the CPA or no rinsing steps at all.

## CONCLUSION

Over the recent years, reparative and regenerative medicine has been grown in popularity. DSCs represent a relatively simple to obtain sources of adult stem cells with minimal harm to the donor individuals. Both, cryopreserved intact tooth or DSCs, can be further use in individual regenerative cell and tissue therapy. As such, the most optimized cryopreservation protocol for both has to be established. However, the question what optimal is remains to be addressed. What is the optimal concentration of the CPA? Is there any non-toxic CPA or serum-free freezing medium, which has no cytotoxic effect and yields efficient results for the post-thaw cell viability? What is the optimal cryopreservation method with successful results, but is not overly costly and does not require specific technology? Among other questions there is an area of the long-term cryopreservation (>10 years) and its negative effects on cryopreserved tissues and cells. It is clear, that an optimal cryopreservation protocol remains to be a relatively un-

explored field with lots of questions and less answers and further comprehensive research is required before valid conclusions can be drawn.

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