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Cryopreservation of dental and periodontal stem cells and their potential for tissue regeneration

Criopreservação de células-tronco dentais e periodontais e seu potencial para a regeneração tecidual

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ABSTRACT

The aim of this systematic literature review was to discuss the influence of cryopreservation on stem cells obtained from dental and periodontal tissues. According to the literature, cryopreservation permits the maintenance of living tissues for long periods of time, guaranteeing cell viability and function. With the advances of stem cell research using cells isolated from dental and periodontal tissues – such as dental pulp, periodontal ligment, apical papilla, and exfoliated deciduous teeth – a paradigm shift in dental practice is possible, as new treatment possibilities involving cell-based therapy and tissue engineering could provide feasible clinical alternatives. The studies reviewed demonstrate that cryopreservation of dental and periodontal cells under controlled conditions permits their complete recovery after thawing and the maintenance of morphological characteristics and cell proliferation potential, representing a possibility for future application in regenerative medicine.

Keywords: Tooth; Stem cells; Cryopreservation; Cell culture techniques; Regeneration.

RESUMO

O objetivo desta revisão sistemática da literatura foi discutir a influência da criopreservação sobre células-tronco obtidas a partir de tecidos dentais e periodontais. De acordo com a literatura, a criopreservação permite a manutenção de órgãos vivos por longos períodos de tempo, garantindo a sua viabilidade e função. Com o avanço das pesquisas em células-tronco, utilizando células isoladas de tecidos dentais e periodontais – tais como a polpa dental, o ligamento periodontal, a papila apical e o dente decíduo esfoliado – a quebra de paradigmas na prática odontológica é possível, uma vez que novos métodos de tratamento envolvendo terapia celular e engenharia tecidual poderão proporcionar alternativas clínicas viáveis. Os estudos revisados demonstraram que a criopreservação de células dentais e periodontais sob condições controladas permite a sua completa recuperação após o descongelamento, garantindo a manutenção das suas características morfológicas e do potencial de proliferação celular, o que representa uma possibilidade para aplicação futura na medicina regenerativa.

Palavras-chaves: Dente; Células-tronco; Criopreservação; Técnicas de cultivo celular; Regeneraçã

INTRODUCTION

Undifferentiated mesenchymal cells, also known as stem cells, have the ability to self-renew and to produce at least one highly specialized cell type. There are two types of stem cells: pluripotent embryonic stem cells that are derived from the embryoblast during the blastula phase and that are able to give origin to all cell lines of the body, and multipotent or unipotent cells, called adult stem cells, which give origin to specific cell types[1]. The main advantage of embryonic stem cells is their ability to proliferate and differentiate into different cell types (plasticity). However, there are limitations such as the genetic instability of these cells, the need for transplantation of these cells to immunocompromised hosts, and the risk of formation of teratomas, in addition to ethical issues. In contrast, adult stem cells have the advantage of being

autogenic, there are no associated moral limits, and these cells respond to host growth factors. However, adult stem cells also present limitations such as the fact that they are not pluripotent and the difficulty in isolating, purifying and culturing these cells *in vitro*, in addition to their smaller quantity in tissues[2-3].

Several studies have isolated stem cells derived from oral tissues such as dental pulp[4,5-6-7-8-9], exfoliated deciduous teeth[6], apical papilla[10], and periodontal ligament[11,12-13-14-15]. The identification of this cell population in dental tissues has raised interest in the regenerative potential of these cells[16] and their application to tissue engineering, or bioengineering[17].

Recent studies have focused on tissue engineering, a therapeutic approach that comprises an interdisciplinary research field where principles of engineering and biological sciences are used to develop biological substitutes that are able to restore, maintain or improve the function of tissues or organs[18]. Bioengineering is used for the construction or regeneration of damaged or lost tissues – secondary to degenerative diseases, trauma, cancer, periodontal disease – such as bone tissue, and is based on cell therapy involving the use of mesenchymal stem cells in combination or not with biomaterials[19].

The need to maintain organs alive for long periods of time without the loss of cell function has led to the investigation of the process of cryopreservation[20]. So far, it remains unknown to what extent different cell types are able to maintain their differentiation capacity and morphofunctional properties after a long period of cryopreservation. This knowledge is important to evaluate the long-term storage potential of these cells and their subsequent use in tissue regeneration therapies[21].

Within this context and in view of the important role of stem cells in tissue reconstruction processes[16], the objective of this systematic literature review was to discuss the influence of cryopreservation on dental stem cells considering their possible future application in protocols designed to restore, maintain or improve the function of tissues and organs.

METHODS

In order to gather as many studies as possible on the issue, the strategy for data collection aimed to identify papers which discussed the influence of cryopreservation on stem cells obtained mainly from dental tissues. The following sources were searched looking for scientific studies: (i) electronic databases (MEDLINE, Science Direct, Scopus, and Scielo); and (ii) cited reference searches. The search strategy used the following terms: cryopreservation, dental pulp, exfoliated deciduous teeth, apical papilla, and periodontal ligament.

All relevant papers published between 1990 and 2010, with no language restrictions, were considered for this review. Articles were eligible if they were conducted in cell culture or animal models. Studies were excluded for the following reasons: absence of methodological details; lack of relevant information such as number of cases in the sample, time of cryopreservation not reported; and technical impairment to access full text article.

RESULTS AND DISCUSSION

The process of cryopreservation has been known for decades and its objective is to stop all biological functions of living tissues in a reversible and controlled manner at an ultra-low temperature (usually about -196°C)[25]. The cooling of cells to temperatures slightly above 0ºC reduces their metabolism without eliminating it, with these cells continuing to undergo progressive deterioration at a lower rate. In addition, various metabolic pathways are differently affected by low temperatures, a factor also contributing to the loss of viability of these cells when stored at these temperatures for a very long period of time[22].

The term "vitrification" is frequently used to describe cooling protocols that cause an extreme increase in the viscosity of an extracellular solution, i.e. turning into a glass, without the formation of ice crystals. Normally, this requires rapid cooling in the presence of high concentrations of cryoprotective agents. However, the use of controlled-rate cooling whose aim is to turn a cell into a more vitreous state by controlled dehydration, an idea proposed by Pegg and Diaper in 1990, has only recently gained consistent support[23].

The cell damage that occurs during cryopreservation using controlled-rate cooling can be explained by three main factors: osmotic damage due to the inflow

and outflow of water during the addition and removal of cryoprotective agents, mechanical damage due to the formation of intracellular ice crystals[24], and effects of the solutes generally described as chemical damage caused by an increase in the intracellular concentration of ions as a consequence of freezing. The extent of damage caused by these factors varies according to the concentration of the cryoprotectant, type of cooling, and heating sequence[23].

Two procedures are necessary to prevent this damage: use of a freezing rate that is ideal for the type of cell studied, and application of cryoprotective agents that minimize the formation of ice crystals and reduce the intensity of cellular dehydration[22]. In order to avoid rapid intracellular freezing, the living tissue should be cooled slowly enough as to permit that its water content approaches steady-state before it reaches the temperature of solidification[20]. Cryoprotective agents such as glycerol and dimethylsulfoxide (DMSO) effectively protect against cellular damage caused by freezing, minimizing or neutralizing the formation of intracellular ice crystals and reducing the osmolarity of cell membranes[25].

Glycerol is an atoxic cryoprotective agent. Even at high concentrations, this agent reduces the denaturation of proteins when cells are exposed to low temperatures. Glycerol penetrates the cell slowly, a fact limiting its use for the cryopreservation of different cell types. Another widely used cryoprotective agent is DMSO, a hydroscopic compound of low molecular weight. DMSO is the cryoprotectant most frequently used for cell cryopreservation. Concentrations of this agent of 5 to 10% are generally employed. The function of this agent is essentially colligative, i.e., the capture of water-free molecules which reduces the amount of ice formed, lowering the freezing temperature and increasing the vitrification point. At room temperature, this substance penetrates tissues and cells more rapidly than glycerol, an appreciable advantage. However, DMSO is more toxic than glycerol at this temperature[22].

According to Temmerman et al. (2006)[26], Bartlett and Read were the first to perform experiments on the cryopreservation of dental tissue, in 1972. The authors extracted tooth germs from rats and stored the tissues at -196°C. After thawing, the tissues were transplanted into the eyes of rats. The authors observed that the dental structures developed well, indicating that the cells of these tissues survived the freezing process. Several other animal experiments on cryopreservation and autotransplantation of dental tissues have been conducted, but numerous questions remain regarding the possible reactions of dental tissue to these procedures.

The cryopreservation of cells and tissues, especially those of the reproductive system, has significantly improved over the past few years, but so far only hematopoietic stem cells are routinely cryopreserved and then successfully used for transplantation[21]. According to Oh et al. (2005)[20], cryopreservation does not seem to exert a negative influence on the viability and differentiation capacity of periodontal ligament fibroblasts when controlled-rate freezing is used. In addition, DMSO guaranteed the survival of most of these periodontal cells. In that study, alkaline phosphatase activity, a biomarker of periodontal ligament fibroblasts, was analyzed by immunohistochemistry to evaluate the differentiation capacity of these cells after freezing and thawing. The results showed no significant differences in the intensity of alkaline phosphatase staining between the control and experimental (frozen teeth) groups.

Seo et al. (2005)[27] used human periodontal ligament to test the hypothesis that the cryopreserved periodontal ligament contains recoverable postnatal stem cells. These cryopreserved undifferentiated mesenchymal cells maintained the characteristics of non-cryopreserved periodontal ligament cells, including expression of the cell surface marker STRO-1, cell colony formation, differentiation potential, cementum-/periodontal ligament-like tissue regeneration, and a normal diploid karyotype. The study demonstrated that postnatal stem cells can be recovered from cryopreserved periodontal ligament, providing an accessible clinical approach for the subsequent utilization of these cryopreserved tissues.

In another study, Papaccio et al. (2006)[21] transplanted stem cells obtained from the dental pulp of rats and their osteoblasts differentiated *in vitro* after 2 years of cryopreservation. The cells were frozen in cryogenic vials with 10% DMSO in liquid nitrogen for 2 years. After this period, the cells were rapidly thawed, transferred to cell culture flasks, and incubated at 37°C in

an atmosphere containing 5% CO_2. The *in vitro* tissue samples of recovered osteoblasts were transplanted subcutaneously into the back of rats. The transplants were removed after 4 weeks, fixed in formalin, decalcified in 10% EDTA, and embedded in paraffin. The results of that study provided evidence that dental pulp stem cells and their osteoblasts differentiated *in vitro* can be easily cryopreserved and recovered, with no significant difference in the proliferation of cells maintained at conventional and cryopreservation temperature. The time necessary for recovery and reinitiation of proliferation ranged from 2 to 3 days, indicating that, like fresh cultures, these osteoblasts are able to rapidly produce bone matrix. Analysis of the cryopreserved cells showed the absence of cell death due to apoptosis, although a small number of cells were lost by necrosis due to the formation of lethal intracellular ice. These characteristics render dental pulp stem cells a potentially useful and reliable source of cells for tissue repair.

Zang et al. (2006)[28] evaluated the multilineage differentiation potential of stem cells isolated from dental pulp of human third molars after cryopreservation. The cells were isolated using enzymatic procedures and frozen in liquid nitrogen for 1 month. After cryopreservation, all cultures of dental pulp cells were recovered and only those maintained for 25 passages were selected. At the 26th passage, cells expressing the stem cell marker STRO-1 were cultured in induction media for neurogenic, osteogenic/odontogenic, adipogenic, myogenic and chondrogenic differentiation, and analyzed morphologically and by immunohistochemistry and RT-PCR for specific marker genes. The results showed a proliferation and differentiation potential of these cells under the influence of five different induction media. The authors concluded that dental pulp cells of the third molars may serve as a source of multipotent stem cells for autologous tissue regeneration and cell-based therapies, even after cryopreservation.

Perry et al. (2008)[29] showed that stem cells extracted from the dental pulp of human third molars remain viable after 24 hours and for at least 5 days after tooth extraction when the teeth are stored at 4°C in sterile flasks containing 20 mL HypoThermosol, Mesencult basal medium or phosphate-buffered saline (PBS). These findings indicate that immediate processing of the material is not required for successful dental stem cell banking, a fact facilitating the development and improvement of future protocols. Cryopreservation of isolated dental pulp resulted in the recovery of all (100%) stem cells, whereas 70% were recovered when whole teeth were cryopreserved. A standard cryopreservation protocol was used: each 2-mL cryogenic vial contained 10% DMSO, 1.3 mL Mesencult medium, and a dental pulp stem cell suspension containing 0.5 to 1.5 x 10^6 cells. The suspension was cooled at 1°C/min to -85°C and stored in liquid nitrogen. The same general freezing procedures were used for whole teeth, with two exceptions: whole teeth were frozen in 15-mL cryogenic vials and kept in cryoprotectant solution for 1 h at 4°C before freezing to facilitate penetration of the cryoprotectant into the tissue. The cells and teeth were frozen for at least one month. The results showed the feasibility of banking cryopreserved dental pulp stem cell and whole teeth for future applications in regenerative medicine.

The immediate cryopreservation of tissues for subsequent cell isolation and culture is more practical than the immediate isolation and culture of these cells. According to Woods et al. (2009)[30], the isolation of stem cells from dental pulp is laborious, time consuming and expensive, and cryopreservation of whole teeth or isolated dental tissue may therefore be more advantageous for banking. In that study, the authors concluded that cell viability after cryopreservation is not limited by the concentration of frozen cells, at least up to 2 x 10^6 cells/mL. In addition, dental pulp stem cells expanded in culture can be stored without a significant difference at -85ºC or -196ºC for a period of 6 months. These cells maintain their capacity to differentiate into osteogenic, chondrogenic and adipogenic lines, indicating the preservation of functionality after thawing. Satisfactory results regarding post-thaw cell culture were also obtained with the isolation and cryopreservation of enzymatically digested dental pulp extract. Cell recovery was slightly higher than 85%. The cells isolated after thawing maintained their morphological characteristics and developmental capacity and demonstrated a potential of trilineage differentiation. The isolation and cryopreservation of dental pulp tissue for subsequent enzymatic digestion

yielded the most satisfactory results in terms of post-thaw cell culture.

The survival and maintenance of the function of periodontal cells, including mesenchymal stem cells, are essential for good prognosis of a grafted tooth. In this respect, cryopreservation does not seem to exert any negative influence on the regeneration capacity of the periodontal ligament. According to Temmerman et al. (2006)[26], the use of DMSO in combination with controlled-rate freezing guarantees the survival of numerous periodontal cells and, although the repair process is slower, periodontal regeneration of cryopreserved teeth was similar to that seen for immediately transplanted cells, with no signs of progressive root resorption. These results indicate that cryopreserved teeth are adequate for clinical use since periodontal cells remain viable after cryopreservation. In another study, Temmerman et al. (2008)[25] examined the effect of controlled cryopreservation on periodontal ligament fibroblast cultures obtained from human third molars and demonstrated that the three parameters important for cell activity, i.e., membrane integrity, growth capacity, and expression of alkaline phosphatase, were not influenced by cryopreservation. According to the authors, the presence of a healthy and functional periodontal ligament increases the success rate of this procedure.

In a more recent study, Temmerman et al. (2010)[31] evaluated the *in vitro* viability of isolated human pulp of third molars after cryopreservation. The study was divided into three experiments. In the first experiment, pulp tissues were isolated and divided into horizontal segments and each segment was then cultured separately to evaluate the existence of differences in the growth capacity of fibroblasts derived from the coronal, medial and apical portion of pulp tissue. In the second experiment, the isolated pulp tissues were divided into two portions (mesial and distal): one part was cryopreserved for 30 days before culture and the other part was cultured immediately to compare the growth capacity of these tissues. In the third experiment, whole third molars were cryopreserved for a period of 6 to 11 months. After thawing, the dimension of the apical foramen was measured and pulp was isolated, segmented horizontally and cultured for comparison of growth capacity. The results of the first two experiments showed no significant difference in growth capacity between fibroblasts derived from different pulp segments of the same tooth (without cryopreservation) or between cryopreserved and non-cryopreserved fibroblasts. Thus, the viability of isolated pulp tissue can be maintained during cryopreservation if a cryoprotectant and standard procedures are used. The third experiment revealed a positive correlation between the dimension of the apical foramen and pulp viability after cryopreservation. A minimum dimension of 9.42 mm^2 permits the cryoprotectant to penetrate sufficiently and to protect the pulp tissues from apex to crown, with the observation of a viability of 90.9%.

In a study designed to optimize cryopreservation protocols for dental pulp stem cells, Woods et al. (2009)[30] found that DMSO at concentration of 0.5, 1.0 and 1.5 M yielded better results in terms of cell viability than propylene glycol and ethylene glycol at the same concentrations. In addition, the study showed that cryopreservation (with 10% DMSO) of tissue extract rather than enzymatically digested cells yields better results. The authors believe that digesting tissue first before cryopreservation damages the cell membrane structure to some degree. In that study, cryopreservation of whole teeth did not produce satisfactory results.

Ding et al. (2010)[32] studied undifferentiated mesenchymal cells from apical papilla of human third molars cryopreserved for 6 months with three cryoprotectants: 10% DMSO plus 90% FBS, 10% glycerol plus 90% FBS, and 10% ethylene glycol plus 90% FBS. The following parameters were analyzed: cell proliferation rate, colony-forming efficiency, multilineage differentiation potential, expression of mesenchymal stem cell markers, karyotype, and immunological properties. The study demonstrated that the biological and immunological properties of cryopreserved undifferentiated mesenchymal cells from apical papilla were not affected by the protocols used, supporting the feasibility of cryopreservation of these cells in liquid nitrogen.

CONCLUSION

The studies reviewed demonstrate the feasibility of cryopreservation of stem cells derived from dental and periodontal tissues for future application in regenerative medicine. Cryopreservation of these cells

under controlled conditions permits their complete recovery after thawing and the maintenance of morphological characteristics and cell proliferation potential.

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