Review Article

Effect of Cryopreservation on the Structural and Functional Integrity of Human Periodontal Ligament Stem Cells: A Systematic Review

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Abstract

Aim: The aim of the present systematic review was to assess the effect of cryopreservation on the human periodontal ligament (PDL) stem cells and their ability for periodontal regeneration. **Materials and Methods:** An electronic search without time restrictions was conducted up to August 2017 in indexed databases using the combination of different keywords including cryopreservation, cryofixation, vitrification and human periodontal ligament stem cells. The exclusion criteria included reviews, commentaries, letters to the editor, interviews and updates. The relevant articles were included and data extraction was processed. **Results:** Dimethyl sulphoxide was used as a cryoprotectant in all the studies which yielded good results. The magnetic freezing proves to be better than the normal freezer. The cryopreserved cells showed no significant difference for viability, proliferation, and regenerative capacities as compared to the fresh human PDL stem cells (PDLSCs), *in vitro* as well as *in vivo*. **Conclusion:** Cryopreservation of the human PDLSCs would serve as an opportunity for future regenerative therapy for the periodontium.

Keywords: Cryopreservation, human periodontal ligament stem cells, periodontal regeneration

Introduction

Periodontal diseases lead to destruction of the periodontium including hard and soft tissues. Any surgical or non-surgical periodontal therapy aims to stop the progression of the disease and regenerate the structural and functional loss. This is quite challenging because of the complex apparatus composed of different tissues including bone, cementum and periodontal ligament (PDL). All the treatment modalities for periodontal regeneration, which includes guided tissue regeneration, grafts, growth factors and host-modulating agents, have shown limited success. Hence, alternative regenerative treatment modalities are highly desirable. Recent reports have focussed on cell-based regenerative approaches using stem cells.[1]

Stem cells are undifferentiated cells capable of self-renewal and differentiation into multiple functional cell types.[2] A stem cell possesses three main characteristics. They are self-renewing and can produce many generations of cells identical to itself through mitosis without becoming aneuploid, even after long periods of inactivity. Stem cells are undifferentiated and carry the cell markers of unspecialised cells. Finally, they are able to differentiate into specialised cells. These qualities allow stem cells to proliferate and regenerate missing or compromised tissues.[1,3] Based on their differentiation potential, stem cells can be categorised as totipotent, pluripotent, multipotent and unipotent.[1] Adult stem cells have been isolated from a variety of tissues, including bone marrow, brain, liver, lungs, breast, skin, skeletal muscles, hair follicles and teeth.[2]

National Institute of Dental and Craniofacial Research has shown the presence of adult stem cells in PDL of the permanent teeth that maintains a high differentiation capacity and also developed a protocol for their isolation and culture. The protocol includes segregation of the stem cells from extracellular matrix, seeding

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onto culture plates with specific medium and incubation at 37°C in 5% CO_2 atmosphere. PDL proves to be an easily available and effective autogenous source for heterogenous undifferentiated mesenchymal cells that can differentiate into fibroblasts, osteoblasts and cementoblasts, which help mediate the periodontal regeneration.[4] A group of cells from PDL express mesenchymal stem cell surface markers such as STRO-1 and CD146. These cells have an ability of self-renewal and multipotency, and hence, they can differentiate into cementoblasts/osteoblasts, adipocytes and collagen-forming cells.

To standardise the isolation and preparation methods, the International Society for Cellular Therapy proposed the following criteria to identify human stem cells: (1) adherence to plastic when maintained in standard culture conditions; (2) expression of markers including CD105, CD73 and CD90, and lack of expression of haematopoietic cell markers such as CD45, CD34, CD14 or CDI1b, CD79 alpha or CD19 and human leucocyte antigen-DR and (3) capacity to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*.[1]

Cryopreservation is the process of preserving cells or whole tissues by cooling them to sub-zero temperatures. At these freezing temperatures, biological activity is stopped as are any cellular processes that lead to cell death. The cells can be successfully stored long term with cryopreservation and still remain viable for use. These cells can be cryopreserved for an extended period, and when needed, carefully thawed to maintain their viability. Two approaches, vitrification and slow freezing, have been tried to achieve cryopreservation without cell damage. Vitrification is a process by which the cells freeze quickly before ice crystals can form.[5]

Historical background

An experiment on large mammal cloning published in 1997 was first of its kind and provided new impetus towards regenerative medicine using stem cells. An entire adult ewe with exact phenotype and genotype of its founder organism was successfully cloned. This reminded that DNA carries the genetic information and an adult could be recapitulated from a postnatal somatic cell. This technique of nuclear transfer could be used to create raw materials to replace defective or senescent tissue as a natural extension of the biologic stem cells.[6]

The current research indicates that dental stem cells may have the potential to regenerate bone, the PDL and possibly teeth. Thus, appropriate preservation of these dental cells is imperative for medical and dental application.[2]

Techniques for preservation of periodontal ligament stem cells

Cryopreservation has been extensively studied as a viable solution to the long-term storage of various biomaterials, one of which is the stem cells. Cryopreservation of the teeth could constitute a clinically relevant method for storage of teeth for extended periods of time, provided the healing *in vivo* is equal to that of unfrozen teeth following replantation or transplantation.[7]

Various other methods for preservation of stem cells have been tried. The two most used are as follows:

1. Vitrification
2. Using electric fields
3. Using magnetic freezing.

Materials and Methods

Focussed question

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines were used to conduct this systematic review. A specific question was developed according to the Population, Intervention, Control and Outcomes format. The addressed focussed question was 'effect of cryopreservation on human PDL stem cells (PDLSCs) and their efficacy for regeneration of the periodontium'.

Eligibility criteria

A study was considered eligible for inclusion if the study was conducted using human PDL and were cryopreserved. The exclusion criteria included (a) qualitative and/or quantitative reviews, commentaries, letters to the editor, interviews or updates, (b) studies conducted using any other dental tissues than PDL or whole teeth transplantations and (c) studies which used PDL from animals.

Literature search protocol

To identify relevant studies, a structured and logical electronic search without time limitation up to August 2017 in PubMed (National Library of Medicine), Scopus, Cochrane and MEDLINE was performed. The following Medical Subject Headings (MeSH) were used: (1) cryopreservation, (2) cryofixation, (3) vitrification, (4) human periodontal ligament and (5) periodontal ligament stem cells. Boolean operators (OR, AND) were used to combine the above-mentioned keywords. To minimise the potential for reviewer bias, titles and abstracts of studies identified using the above-described protocol were independently screened by two reviewers (RP and MS) and checked for agreement. Full-texts of studies judged by title and abstract to be relevant were read and independently evaluated for the stated eligibility criteria. Reference lists of original studies were searched to identify any articles that could have been missed.

Results

Study selection

Initially, 56 articles were identified. Titles and abstracts were screened from which the duplicate articles and those not fulfilling the inclusion criteria and 49 such articles were excluded. The rest seven articles were read and four articles were identified from their references. After complete reading, 11 articles were selected for the analysis [Figure 1].

General characteristics

All the participants of all the included studies had indications for extraction of the healthy teeth either due to impaction or

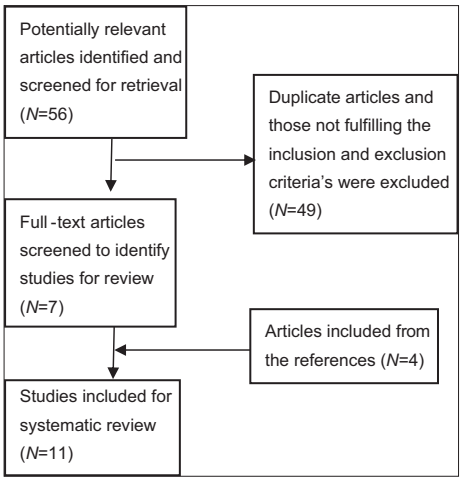

Figure 1: Study selection.

for orthodontic treatment. Prior informed consent was taken from the participants in all the included studies.

All the studies incubated the cells at 37°C with 5% CO_2 except for one study where the incubation was done with 10% CO_2. The preservation method and materials of all the included studies have been mentioned in Table 1.

Biological properties

All the outcomes measured in all the included study such as viability, proliferative capacity, histologic structure, surface markers, osteogenic and adipogenic potential, collagen forming potential, gene expression, alkaline phosphate potential, migration, phenotype and the clinical applications were considered and reviewed. Only one study evaluated the alkaline phosphate potential which showed no significant difference between the cryopreserved and freshly extracted stem cells. Similarly, only one study evaluated the phenotype which showed no alteration. One study evaluated the migration which showed that the cryopreserved cells took twice the time. One study evaluated the surface markers which showed the presence of STRO-1, TCFBR-1 and BSP [Table 2].

Discussion

A number of studies have already shown the differentiating and regenerative capabilities of fresh human PDLSCs. Preserving these stem cells would be a more practical approach for clinical use.[8] It is very important to take care that the extracted tooth or PDL tissue obtained from the tooth does not dry off and should be treated immediately.[9] The present systematic review was done to evaluate the effect of cryopreservation on the viability, vitality and regenerative capacity of PDLSCs. A lot of factors can influence the viability of such cryopreserved PDLSCs such as pre- and post-freeze processing, temperature variations and storage duration.[8] Bartlett and Reade in 1972 concluded that cryopreservation, if carried out under controlled conditions, would leave the intricate intracytoplasmatic functions either undamaged or amenable for recovery. Kristerson *et al*. in 1976, Oh *et al*. in 2005 and Stevenson *et al*. in 2004 said that cryopreservation has no negative effect on the viability or even remain transiently active in damaged cells.[10] The studies included in the present systematic review also showed that cryopreservation does not affect the viability.[11,12,13]

Studies show that adhesion and proliferation of the cells are not affected by the cryopreservation[8,14-17] However, the rates differ at different time intervals and indicate their expressive capacity for growth and multiplication under specific conditions.[2,4,15,18,19]

Permeation by the cryoprotectant and condensation are very essential. Slow permeation would expose the cells to the chemical toxicity of cryoprotectant. Slow freezing is he equilibrium freezing as it helps in exchange of fluids between the extra- and intra-cellular spaces which results in safe freezing without serious osmotic and deformation effects to cells. Low concentration of cryoprotectants may not cause serious osmotic or toxic damage, but, would be insufficient to avoid the ice crystal formation in the cells. On the other hand, vitrification is based on conversion from a fluid to solid by increase in viscosity without phase change and crystallisation of water.[5] The extremely high cooling rate and high concentration of cryoprotectant would cause high toxic damage. Studies show that magnetic exposure on cells prevents intracellular ice crystal formation by causing vibrations.[20] Typically, the vitrification medium comprises complex mixture of solutes and none of the components exceeds its putative toxic concentration. The medium consists of an essential permeating cryoprotectant such as dimethyl sulphoxide (DMSO), ethylene glycol (EG), acetamide or propylene glycerol, supplemented with a macromolecule like Ficoll 70 and a small molecule like sucrose which reduce the toxicity as they are non-permeating. Sucrose (saccharides) also reduce the toxicity and contribute to osmolarity.[5] This requires a high concentration of cryoprotectants that are usually toxic to most cells. On the other hand, conventional slow freezing requires a low, relatively non-toxic, concentration of cryoprotectants always associated with cell injury from ice formation and prolonged exposure to cryoprotectants.

Jackson *et al*. showed that 2.45 GHz microwave radiation could reduce the amount of ice formed during attempted vitrification of EG solutions. Sun *et al*. studied freezing in the presence of electric fields oscillating at frequencies between 1 and 200 kHz and found ice crystal domain size to be minimised at a frequency of 50 kHz.[21] The water molecules have an intrinsic electric dipole moment which makes the water a dielectric substance. When an electric field is applied, the molecules rotate. By this mechanism, the oscillating electric fields heat pure water. To achieve a rapid and uniform warming, radio or microwave frequency is used. Further, the effect that static electric fields can nucleate ice formation has been used to prevent intracellular ice formation during freezing. This is achieved by applying kilovoltage to electrodes in direct contact with supercooled water. An electric field strength on the order of 5 109 V/m is necessary to nucleate bulk supercooled water into cubic ice.

Table 1: General characteristics

Author	Storage before PDL separation	Preservation Medium	Preservation method and Time	Culture	Trypsinization
Seo *et al*. 200[58]		FCS + 10% DMSO	Liquid nitrogen 4°C 3-6 months	α-MEM+15% FCS+100uML ascorbic acid 2-phosphate+2mML glutamine+100U/mL penicillin+100 µg/mL streptomycin	
Temmerman *et al*. 2007[10]	450ml DMEM+19ml FCS + Rapid solution [10,000 U/mL penicillin/streptomycin + 5 mg/ml fungizone + 40 mg/ml gentamycin] 4°C, 24 h	FCS + 10% DMSO	Liquid nitrogen -196°C 1 day	1.5 ml culture media [optimem I supplemented with 2% Ultroser G, 1% glutamine, 1% penicillin/ streptomycin, 5% FCS]	0.25% trypsin + 0.08% EDTA
Kaku *et al*. 2010[17]		1ml 10% DMSO	CAS magnetic freezing -150°C 7 days	α-MEM [10% FCS, 32U/ ml penicillin G, 250 µg/ml amphotericin B, 60 µg/ml kanamycin]	0.1% trypsin/EDTA
Min *et al*. 2010[9]	PBS	DMEM, 10% FBS, 100U/ml penicillin G, 100ug/ml streptomycin, 0.25 µg/ml fungizone, 10% DMSO 1 hr	Liquid nitrogen -196°C 1 week	DMEM, 10% FBS, 100U/ ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml fungizone	
Kawata *et al*. 2010[21]		BAMBANKER 2, Lymphocyte	CAS magnetic freezing -40°C 3 days	α -MEM [10% FCS, 32 U/ ml penicillin G, 250 µg/ml amphotericin B and 60 µg/ml kanamycin]	
Dissanayake *et al*. 2010[5]	F-medium [DMEM + Ham's nutrient mixture] F-12, 10% FBS, 100 units/µl penicillin, 0.3 µg/ml fungizone and 100 µl/ml streptomycin	1) Conventional [80% F-medium, 10% FBS, 10% DMSO] 2) Vitrification [40% ethylene glycol, 18% Ficoll 70, 0.3 M sucrose]	Liquid nitrogen -196°C 2 weeks	20 ml F-media [DMEM+Ham's nutrient mixture]	1ml 0.25% trypsin + 0.08% EDTA
Kamada *et al*. 2011[22]		10% DMSO	CAS magnetic freezing -150°C 3 days	α-MEM [10% FCS, 32 U/ ml penicillin G, 250 ug/ml amphotericin B, 60 ug/ml kanamycin]	0.1% trypsin/EDTA
Vasconcelos *et al*. 2011[4]	5ml α-MEM 4°C Maintenance [α-MEM, 10,000 IU/ml penicillin, 10,000 µg/ml streptomycin, 100 mg/ml gentamycin, 250 µg/ml amphotericin B	FBS+10% DMSO	-85°C 30 days	α-MEM, 15% FBS	
Abedini *et al*. 2011[23]		5ml 10% DMSO	CAS magnetic freezing -150°C 5 years	α-MEM, 10% FBS, 32 U/ ml penicillin G, 250 µg/ml amphotericine B, 60 µg/ml kanamycin	
Kim *et al*. 2015[24]	F-medium [DMEM, Ham's nutrient mixture F-12, 10% FBS, 100 units/µl penicillin, 100 µl/ml streptomycin, 0.3 µg/ml fungizone	10% DMSO	Liquid nitrogen -196°C 7 days	20 ml F-medium	1 ml 0.25% trypsin, 0.08% EDTA
Li *et al*. 2017[25]		90% FBS, 10% DMSO 4°C	Liquid nitrogen -80°C 24 h	α-MEM, 15% FBS, 2 mmol/L glutamine, 100 U/ ml penicillin, 100 µg/ml streptomycin	

Table 2: Biological properties

Author	Viability	Proliferative capacity	Histologic structure	Osteogenic , Adipogenic and Collagen forming potential	Gene expression	In vivo application
Seo *et al*. 2005[8]	40% cells recovered	High for 12 h	Normal structure However, anisokaryosis, variable sizes of nuclei and clumping of cells observed	Alizarin-red +ve nodules: calcium accumulation Oil-red O +ve: lipid laden fat cells Well organized collagen fibers	Normal G-Banded Karyotype	Typical cementum/ PDL structure Thin layer of cementum on [HA/ TCP] Human specific mitochondria Positive cementoblasts/ cementocytes Positive for anti-type I collagen and BSP antibody staining
Temmerman *et al*. 2007[10]	No statistical significance*	No statistical significance*	NE	NE	NE	NA
Kaku *et al*. 2010[17]	Higher in magnetic cryopreserved	No cell appearance in normal freezed group	Nuclei, mitochondrion and endoplasmic reticulum well retained in magnetic cryopreserved. Destroyed structure in normal freezed	NE	NE	NA
Min *et al*. 2010[9]	NE	NE	Elongated spindle shaped.	NE	FGFR2 mRNA was two-folds downregulate	NA
Kawata *et al*. 2010[21]	96% in -30°C group (highest)	Observed at 48 h Confluent by 40 days	NE	NE	NE	NE
Dissanayake *et al*. 2010[5]	More viability of conventional medium as compared to vitrification medium.	Maximum viability observed with conventional method	Spindle shaped and elongated appearance.	NE	NE	NE
Kamada *et al*. 2011[22]	NE	NE	NE	NE	No significant difference in expression of collagen type I mRNA Significant level of alkaline phosphatase mRNA (P<0.05)	Replanted in rat. No significant difference.
Vasconcelos *et al*. 2011[4]	NE	No statistical difference Proliferative capacity maintained for 30 days	NE	NE	NE	NE
Abedini *et al*. 2011[23]	NE	No statistical difference	NE	NE	No statistically significant difference in expression of collagen type I, ALPase and VEGF, mRNAs	NE
Kim *et al*. 2015[24]	No statistical difference	No statistical difference	Fibroblast like appearance. No statistical difference	NE	NE	NE

Contd...

Table 2: Contd...

Li *et al*. 2017[25]	No statistical difference	No statistical difference	No statistical difference 1) ECM intact 2) Uniform 2-dimensional structure 3) Fibronectin, Type I collagen and Integrin Present abundantly	Kossa staining: nodules representing calcium accumulation Oil Red o staining: lipid laden adipocytes No statistical difference Sirius Red staining: network of collagen type I and type III No statistical difference	No significant difference 1) RUNX2, OSX, OCN [osteogenic markers] PPARY2, LPL [adipogenic markers] confirmed 2) chromosomal arrangement, shifts in kinetochove position, G Band position and chromosome length	Odontoblast like cells Bone like matrix

NE - Not evaluated. *No significant difference between Cryopreserved and Non-cryopreserved stem cells

It is difficult to electrically align water molecules to cause freezing, but it is easier to electrically disturb them to alter or prevent freezing.[21]

At temperature below 4°C, ice crystal formation starts and this leads to a weak electric current which may disrupt cell membranes. The water molecules instantly freeze when a magnetic field is exerted. Some studies have concluded that lower DMSO concentration and shorter pre-equilibration time are required for magnetic freezing.[22]

The hold-time plays an important role in allowing the cryoprotectant to osmose into the cell without exposing them to the cryoprotectant for too long. Similarly, a suitable plunging temperature protects the cells from intracellular ice crystal formation or excessive dehydration.[20]

Of all the cryoprotectants, the least toxic is EG and glycerol followed by DMSO and propylene glycol and the most toxic is acetamide. All the studies included in this systematic review used 10% DMSO in varying quantities. Four studies used DMSO alone and in others studies it was used in combination with other mediums: two studies used foetal calf serum, four studies used foetal bovine serum (FBS), one study used F-medium, vitrification medium and one study used Dulbecco's modified Eagle's medium, penicillin, streptomycin and fungizone.[22]

In 1985, Schwartz *et al.* showed that DMSO at a concentration of 10% was the best cryoprotectant.[10] A combination of serum and DMSO maintains the accessibility between the frozen medium and cellular structures.[8] DMSO is known to have a drawback of its inherent cytotoxicity that has detrimental effect on cell viability, and hence, it is advisable to reduce its concentration to ensure maximum post-thaw cell yield. FBS comprises a mixture of growth factors, proteins, carbohydrates, cytokines, and indispensable nutrients that plays an important role to maintain the biological properties and reduce the risk of cell damage during the freezing procedure. Concentration as high as 90% have been seen to cause cell damage. The protein content may still remain intact in the cells after washing and may modify the surface markers. Following these reasons, the use of FBS is not recommended.[22]

Five studies cryopreserved the PDL using liquid nitrogen alone at different temperatures and magnetic cryopreservation was used in three studies for different time periods.[22]

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Seo *et al*. in 2005 found the STRO-1 characteristics of cryopreserved cells similar to that of fresh cells and hence concluded that cryopreserved PDLSCs may be derived from a population of perivascular cells. In 2004, they also observed that cryopreserved PDLSCs also showed a heterogeneous nature that may indicate the different developmental stages or different PDL cells lineages analogs with non-frozen cells. There have been studies which show that these cells have a variable capacity to generate cementum.[8]

A study done by Basdra and Komposch in 1997 concluded that PDL fibroblasts showed intense alkaline phosphatase staining which indicated the capacity to the cells to differentiate into osteoblasts and cementoblasts.[4]

Gene expression analysis of the preserved cells is carried to measure any changes that may affect the regenerative process, as especially those involving biological processes such as cell communication, cell growth, maintenance, cell death, differentiation and proliferation. Geiss *et al*. in 2000 showed twofold up- and down-regulation of the genes following 1 week of cryopreservation. Such changes could be a cause of RNA degradation.[9] No difference has been observed in the collagen type I mRNA between the cryopreserved and fresh cells.[23,24]

A report by Draper *et al*. in 2004 and correspondence from Buzzard *et al.* suggest that hES cell lines propagated *in vitro* for even a few months can develop an abnormal karyotype.[25]

Limitations

The studies included in this systematic review have used different time intervals for preservation with vide ranges and variations in the techniques. Furthermore, the clinical use and results should be studied.

Conclusion

The cryopreservation of the human PDLSCs does not affect the structural and functional properties. Further studies with *in vivo* and clinical application should be done. This may provide us with new therapeutic techniques for future regenerative use.

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Conflicts of interest

There are no conflicts of interest.

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