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REVIEW ARTICLE

Comparison of Isolation, Expansion and Cryopreservation Techniques to Produce Stem Cells from Human Exfoliated Deciduous Teeth (SHED) with Better Regenerative Potential

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Abstract: Mesenchymal stem cells (MSCs) are adult stem cells that are gaining worldwide attention for their multi-potential use in tissue engineering-based regenerative medicine. They can be obtained from numerous sources and one of the excellent sources is the dental tissue, such as stem cells that are extracted from the human exfoliated deciduous teeth (SHED). SHED are considered ideal due to their inherent characteristics, including the capability to proliferate quickly with minimal oncogenesis risk, multipotency capacity and their ability to suppress the immune system. On top of these positive cell traits, SHED are easily accessible with the patient's safety assured, posing less ethical issues and could also provide a sufficient number of cells for prospective clinical uses. This is primarily attributed to their ability to differentiate into multiple cell linages, including osteoblasts, odontoblasts, neuronal cells, adipocytes, as well as endothelial cells. Albeit SHED having a bright future, there still remains an obstacle to develop reliable experimental techniques to retain the long-term regeneration potential of the stem cells for prospective research and clinical applications. Therefore, this review aims to describe the various isolation, expansion and cryopreservation techniques used by researchers in this stem cell field. Optimization of these techniques is crucial to obtain distinct SHED culture with preserved stem cell properties, which enable more reproducible results that will be the key for further stem cell therapy development.

Keywords: Human exfoliated deciduous teeth (SHED), isolation technique, expansion technique, cryopreservation technique, Mesenchymal Stem Cells (MSCs), regenerative potential.

1. INTRODUCTION

By definition, stem cells are clonogenic cells with the self-renewal capacity and multi-lineage differentiation [1]. Two decades ago, stem cells derived from human exfoliated deciduous ('baby') teeth (SHED) were acknowledged as a novel source of adult tissue stem cells that present distinctive features, including multiple cell linages differentiation capability, high population doubling, as well as proliferation rate. SHED represent a unique population of multipotent stem cells first identified by Gronthos *et al.*, (2000) from the remaining pulp of exfoliated deciduous incisor teeth obtained from children aged 7 to 8 years old.

Unlike other postnatal stem cells such as bone marrow stromal cells, which are extracted from the alveolar bone, SHED offer attractive advantages as they are readily accessible, naturally exfoliated and do not involve an invasive extraction method, hence assuring patient's safety with limited legal and ethical considerations [2]. Regardless of their source of origin, SHED possess the same colony-forming efficiency and multilineage differentiation ability as other niches such as umbilical cord mesenchymal stem cells (UCMSCs), gingival mesenchymal stem cells (GMSCs) and bone marrow stromal stem cells (BMSCs). Intriguingly, the SHED exhibits higher proliferation capacity in comparison with BMSCs, UCMSCs, GMSCs and dental pulp stem cells (DPSCs), hence making it an ideal candidate for tissue regeneration-related cellular therapies [3-5]. This is probably due to the active state and high telomerase activity of SHED during the replacement of deciduous teeth by permanent teeth [6]. Moreover, SHED, when compared to their DPSCs predecessors, have advantages for tissue engineering, which allows fast *in-vitro* expansion before re-implantation of the tissue. Nevertheless, both SHED and DPSCs have similar properties in terms of their cell morphology, cell surface markers (CD13, CD29, CD44, CD73, CD90, CD146, CD166 and HLA-ABC) expression, differentiation ability and cell cycle distribution, indicating that SHED should be provided similar opportunities like DPSCs for future human tissue regeneration and transplantation applications [2, 7].

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Thus far, SHED have been widely studied by numerous researchers, indicating their infinite potential SHEDin the field of tissue engineering for regenerative medicine purposes (Fig. **1**). This could be because SHED were shown to be correlated with the neural crest cells, through the exhibition of a wide range of neural cell-associated markers, such as beta III tubulin, nestin, GAD, NFM, GFAP, NeuN and CNPase [4]. Physiologically, neural crest cells have an important function in the development of several cell types such as smooth muscle, nerve cells, pigment cells, bone and craniofacial cartilage during embryonic growth [8]. Hence, SHED are assumed to be a practical candidate for tooth and facial bone regeneration, as well as for treatment of nerve tissue related injuries and degenerative diseases. In a study by Seo *et al.*,, SHED that could differentiate into osteoblast-like cells were transplanted into immunocompromised mice and had successfully repaired parietal tissue defects through the generation of bony tissue [9]. On the other hand, SHED were also capable of restoring critical-size mandibular defects in a large animal model, especially swine [10]. Remarkably, a recent clinical study showed the successful regeneration of a three-dimensional whole dental pulp by implanted SHED that further developed sensory nerves and blood vessels in both animal and human study models, thus demonstrating the potential of SHED as an alternative source of stem cells in human orofacial bone regeneration [11].

In recent years, stem cell transplantation in association with a standard autografting technique has been a trending approach in the restoration of extensive nerve damage and brain injuries. Numerous studies on the use of SHED in repairing nerve have been carried out. For example, an investigation involving autografting of nerve cells in combination with SHED and polyglycolic acid fibre-based tube (PGAt) showed improved recovery of the *Wistar* rats' facial nerves [12]. Furthermore, the transplantation of SHED showed the capability of improving locomotor recovery of rats through the preservation of myelin sheaths and neuronal filaments, promoting transected axon's regeneration and replacement of lost cells *via* the differentiation into mature oligodendrocytes [13]. Besides restoring nerve damage, transplantation of SHED and SHED-conditioned medium (SHED-CM) has contributed to significant neurological and pathophysiological recoveries in mice with hypoxia-ischemia brain injury, whereas another independent study demonstrated its potential in promoting migration of endogenous neural progenitor cells (NPC), which thus facilitated reversal of focal cerebral ischemic damages in affected rats [14, 15]. Notably, SHED also demonstrated potential in the treatment of neurodegenerative diseases, including Parkinson's and Alzheimer's diseases. Transplanted SHED were successfully differentiated into specific dopaminergic neuronal cells, therefore improving the apomorphine-evoked rotation of behavioural disorders in Parkinson's disease rat models [16]. On the other hand, SHED could improve cognitive function in an Alzheimer's disease mouse model through repressing β-amyloid plaques-induced pro-inflammatory responses and triggered anti-inflammatory responses [17].

The convenient accessibility and extensive differentiation potential of SHED have portrayed their unlimited possibility for their contribution toorgan restoration and transplantation in order to overcome the issue of organ donor shortage. Thus, research works were performed to differentiate SHED into other cell types such as islet-like cell clusters (ICCs) and SHED-converted hepatocyte-like cells (SHED-HLCs). Indeed, these studies led to improved kidney and liver functions in mice with streptozotocin (STZ)-induced diabetes and CCl_4-induced chronic liver fibrosis with factor VIII (F8)-knock out [18, 19]. Furthermore, SHED also helped in organ injuries recovery by promoting wound healing in ischemic kidney injury through suppression of inflammatory cytokines and MCP-1 levels while elevating HGF expression, attenuating inflammation and apoptosis to protect the heart from acute ischemic injury, as well as regulating anti-inflammatory M2-like macrophage and BLM-induced pro-inflammatory response that promotes lung regeneration in bleomycin-induced mice [20-22].

Intriguingly, while SHED are able to differentiate into adipogenic and osteogenic cells, they are also capable of exhibiting significant immunomodulatory activities. This finding spurred further investigations where SHED were transplanted to effectively treat the SLE-associated disorder induced in an MRL/*lpr* mice model [6]. Besides stem cell transplantation, SHED-CM transplantation has also been studied frequently, especially for the treatment of autoimmune diseases. Previous studies have shown the potential of SHED-CM and its main component, secreted domain of ectodomain sialic acid-binding IG-like lectin-9 in treating complications present in rheumatoid arthritis (RA) and multiple sclerosis mouse models [23, 24].

Development of reliable techniques for stem cell isolation, culturing and cryopreservation is crucial not only for further research, but also for potential clinical applications, especially to retain stem cells differentiation potential and proliferation rate. However, several common problems were faced by different scientists while handling SHED. Tissue degradation activity during the tooth extraction step until the SHED isolation step had inadvertently led to a drastic attenuation of SHED viability after tooth storage. This indicates that the tooth needs to be processed immediately to yield higher SHED viability [25]. Besides that, media composition used for the culture of SHED is one of the most discussed issues due to the conspicuous impacts on maintaining the high proliferation rate, differentiation potential and senescence stability. Hence, efforts on optimizing and modifying conditions or supplements of culture media in order to improve the long-term expansion of SHED and their self-renewal properties are deemed as highly challenging [26]. The use of traditional cryopreservation methods with DMSO as a cryoprotectant agent also led to risks of morphological alterations and reduced proliferation rate [27]. Moreover, Wood *et al.*, (2009) reported that isolated SHED from a preserved third molar tooth and digested dental pulp tissues did not produce consistent results among samples [28].

The potential of SHED as an autologous and non-controversial source for stem cell regenerative therapy is indeed an encouraging alternative strategy for future clinical applications. Nonetheless, optimization of current research techniques to isolate, expand and preserve SHED is necessary to obtain distinct SHED cultures with preserved stemness properties. This would, in turn, lead to more consistent results, which is a key consideration for further development of stem cells therapy.

2. SHED ISOLATION TECHNIQUES

SHED are considered a promising source of stem cell due to their isolation process that is relatively simple, non-invasive and does not cause any traumatic experience to the patient. Unlike isolation of human dental pulp stem cells (hDPSCs) that involves extraction from either permanent adult teeth or non-exfoliated deciduous teeth, SHED found in exfoliated primary teeth seem to offer a better option for stem cells reserve [29, 30]. Regardless of the source, however, establishing an isolation method that retains enhanced *in vitro* expansion potential of dental pulp stem cells is crucial in order to maintain their high proliferation rate [31]. Among the isolation techniques currently used by different researchers to obtain stem cells from exfoliated deciduous teeth are through (i) enzyme digestion and (ii) outgrowth or direct culture method using primary culture. In both of these methods, the collected teeth need to be cleansed and sterilized first, followed by cutting of the cementum-enamel junction with a clean dental fissure bur to expose the pulp chamber.

2.1. Cleansing and Sterilization of Deciduous Teeth

Teeth sterilization before and during teeth extraction processes is very important to avoid contamination of the subsequent cell culture. In different studies, the patients were asked to rinse their mouth with 0.12% or 0.2% chlorhexidine for 60 secs prior to tooth extraction [32, 33] because chlorhexidine is an antiseptic agent that has been commonly used as a mouthwash solution since it is effective against a broad spectrum of bacteria, some yeasts, as well as viruses [34]. Alternatively, iodine solution was used as a disinfection agent when surgical procedures were performed to extract the tooth [32, 35] due to its antimicrobial activities and lower chances for development of resistance [36]. After this initial step, the extracted tooth was soaked in cold, sterile phosphate-buffered saline (PBS) that was occasionally supplemented with 1% antibiotic (penicillin/streptomycin) and immediately transported back to the laboratory for processing [32, 33]. In one study which had used carious teeth, the plaques and calculus were first removed before washing the teeth under running tap water. After that, 1% povidone-iodine solution was used to soak the teeth for 60 secs before finally rinsing it with PBS containing 1% antibiotic and antimycotic [37].

2.2. Isolation of SHED from Deciduous Teeth

2.2.1. Enzyme Digestion Method

A popular method used for SHED isolation is *via* the enzyme digestion method to separate stem cells from the surrounding dental tissues. Many of the recent studies reportedly employed this enzyme digestion method to successfully isolate SHED. After sterilizing the teeth, the dental pulp tissue was commonly extracted using appropriate tools such as endodontic files. Subsequently, the minced remnants of pulp tissues which are rich in collagen fibers from the deciduous teeth, were enzymatically digested using a mix of collagenase type I and dispase enzymes [38-40]. This was according to the protocol first used by Gronthos *et al.*, (2000) who separated the pulp tissue from the root and crown, followed by digesting the pulp tissue in an enzymatic solution containing 4 mg/ml dispase and 3 mg/ml collagenase type I for 1 hr at 37°C [2]. In a more recent study, the researcher used 4 mg/ml collagenase type I containing 5% fetal calf serum (FCS) for tissue digestion [41]. Most of the time, the choice of enzymes for digestion heavily relies on the major extracellular matrix composition of the tissue being investigated. In the case of dental pulp tissue that is rich in collagen type I [42], collagenase type I and dispase enzymes were frequently chosen since both the enzymes have better effectiveness against collagen type I fibers. Both the enzymes dissolve the collagens at a specific pH value and temperature [43]. Under optimum digestion conditions, the enzymes will cause the least damage to the cells and hence, are ideal enzymes to be used for tissue digestion [44].

With slight variations, Raoof *et al.*, (2014) had also compared SHED' isolation efficiency between two enzymatic digestion methods, (i) regular tissue digestion with collagenase type I/dispase enzymes alone and (ii) enzymatic tissue digestion followed by cells fixation under a coverslip in the medium [32]. Using the enzyme digestion only method, they observed that a few cell colonies with homogenous morphology were detectable after 4 days. However, with the second method involving enzyme digestion and fixation, the SHED were isolated more successfully with about 60% efficiency after 2 days [32].

An advantage of this method is that the isolated SHED are more heterogeneous [45], and hence they may display greater potential for multiple cell lineages differentiation. This speculation was proven by the study of Jeon *et al.*, (2014) who discovered enhanced cell proliferative capacity, colony-forming ability, as well as higher MSC markers expression in SHED derived from the enzymatic digestion method. Thus, they concluded that SHED derived from this method possess a higher degree of stemness [46].

2.2.2. Outgrowth or Direct Culture Method

In the outgrowth method, dental pulp tissues were cut into smaller fragments with an approximate dimension of 2×2×1 mm. These tissue explants were initially grown in smaller culture dishes (4-wells or 6-wells plate) supplemented with Dulbecco's modified Eagle's medium (DMEM) con-

taining 10% fetal bovine serum (FBS), 1% antibiotic–antimycotic, and 1% non-essential amino acids. The culture medium was replaced on the following day and subsequently every 2 days. Once those outgrown cells reached confluence, they were transferred to larger culture flasks (5x10 cm) as passage 1 cells. Subsequent cultures were passaged continuously at a 1:3 ratio [37, 47]. In a similar outgrowth method described by Raoof *et al.*, (2014), a longer period of about 12 days was needed to obtain sufficient numbers of cells and this method only has an isolation efficiency of about 10%. Moreover, cell migration through the periphery was noticeable in this method and colonies with heterogeneous morphology with a barrel-shaped spindle and round configuration were observed. Cells isolated by this method also showed a relatively lower proliferation rate as compared to those isolated *via* the enzymatic digestion method [32].

In a more recent study, the outgrowth method was modified slightly, whereby sterile coverslips were placed over the tissue explants (size $\leq$ 0.5 mm) to immobilize them. Within 3 weeks of culturing, SHED growth from the dental pulp tissue explants could be seen, with most cells demonstrating regular fibroblast-like morphology [48]. Another report had documented the use of the outgrowth method from deciduous dental pulp, dental pulp and periodontal ligament tissues whereby a mixed population of primary cells was obtained. In this study, immunomagnetic separation was required to further isolate stem cells from this mixed population by using the MSC marker, STRO-1 [49].

Although SHED' isolation using outgrowth method is more time-consuming, it is relatively easy to perform, cost-effective and convenient, without the need to use digestive enzymes and straining the cells with a filter. Furthermore, this method does not require much starting material and thus could be suitable for SHED isolation as deciduous teeth usually contain a limited amount of pulp tissue.

2.3. Comparison of the Different SHED Isolation Method

In terms of the efficiency of method, Raoof *et al.*, (2014) concluded that digestion followed by fixation of pulp tissue pieces with a coverslip resulted in successful stem cells isolation after 2 days, in comparison with the digestion only method (4 days) and the outgrowth method (10-12 days) [32]. Meanwhile, several studies have demonstrated a variation in results in terms of stem cells morphology and phenotypes maintenance. Huang *et al.*, (2006) discovered that stem cells isolated using the enzyme digestion method retained a higher proliferative capacity than those isolated through the outgrowth method [47]. This finding was also supported by Jeon *et al.*, (2014) who showed increased SHED proliferation ability when they were isolated using the enzyme digestion method. On top of that, the cells also had enhanced colony-forming unit capability and adipogenic differentiation ability. Contrarily, however, SHED derived from the outgrowth method seemed to demonstrate higher osteogenic differentiation potential, in both *in vivo* and *in vitro* setting [46]. On the other hand, another study had shown no significant differences in proliferation rate and cell morphology when comparing the stem cells isolated either *via* enzyme digestion or outgrowth methods [45]. Based on the above observations, it is highly possible that the different isolation techniques led to the existence of different populations of SHED. Nonetheless, it is noteworthy that apart from the isolation methods, such a difference could also be attributed to the effects of different culture medium supplements being used during the SHED expansion phase.

2.4. Factors Affecting the Success Rate of SHED Isolation

Other than the isolation technique used, other factors could have affected the efficiency and success of isolating SHED [50]. SHED' isolation is usually considered as successful when the stem cells were able to adhere to the culture vessels with the formation of cell colonies. On top of that, the stem cells culture should be able to propagate to the third passage (P3) with no contamination [50]. Studies have shown a greater success rate for isolation of SHED from extracted deciduous teeth (82%) as compared to hDPSCs from the permanent teeth (70%), indicated by earlier confirmation of isolated SHED than hDPSCs [50]. In terms of tooth type, deciduous anterior incisors resulted in a higher SHED isolation success rate at 72.4%, in contrast to the deciduous molars that only had 61.4% success rate, which could possibly be attributed to the increased contamination risk [50]. In addition, the authors had found that patients' age when teeth were extracted, remaining dental root's length, as well as mechanical stress may also play an important role in determining the success rate of SHED isolation [50].

3. SHED EXPANSION TECHNIQUES

Although researchers have successfully isolated stem cells from exfoliated deciduous teeth, the challenge remains in the expansion techniques, as SHED may require a longer time to form an initial colony [51]. Besides, there is apparently a slow but ongoing loss of SHED stemness and spontaneous SHED differentiation, with concomitant genetic instability and cell apoptosis that occur when the cells are cultured for a prolonged period of time. This, therefore, warrants a deeper understanding of how culturing techniques may affect the growth of SHED. Currently, more studies are ongoing to optimize and establish new procedures to increase reproducibility and to ensure the maintenance of dental stem cell cultures with well-defined characteristics for clinical use. In this section, we will be discussing the various factors in the current techniques that could affect the expansion of human dental stem cells.

3.1. Basal Media

Micro-environmental niche in which SHED grow plays a huge role in determining the success rate of cell culture. Among the numerous basal media being studied, knock out--Dulbecco's modified Eagle's medium (DMEM-KO) and alpha-minimum essential medium (α-MEM) were reported to be more superior than DMEM-F12 and DMEM-low glucose (DMEM-LG) for maintaining the phenotypes and stemness

properties in a long-term SHED culture [52]. Although both DMEM-F12 and α-MEM basal media consist of similar nutrient compositions, including ascorbic acid, proline and vitamin B12, α-MEM that has a lower glucose level appeared to be better suited for SHED growth in comparison to DMEM-F12. Meanwhile, although DMEM-LG has a low glucose concentration, it lacks an important vitamin known as biotin that helps in cell growth *via* stimulating glucose consumption and succinic acid production [53]. This might therefore explain why stem cells aged and reached senescence state much quicker when cultured in DMEM-LG in comparison with other basal media types albeit having similar glucose concentration with those from α-MEM.

3.2. Serum Supplementation

Besides basal media, the serum also has an essential function in the culture and expansion of SHED. It is currently still a debatable subject in this research sphere whether to use an animal- or a human-derived serum. In the current setting, the most commonly used culture medium to isolate stem cells and for subsequent stem cell expansion, involves supplementation with animal-based serum or animal-derived matrix constituents. An example of the culture medium routinely used by most researchers is Matrigel, which contains essential nutrients to support cell growth [54]. However, animal-derived reagents may give rise to ensuing health issues such as severe immune response, risk of infections and the potential of pathogen contamination. On the other hand, another group of researchers showed that the use of a growth culture medium containing 1.25% human serum constituents could replace FBS for the isolation and expansion of a stem cell population from dental pulp tissues that are morphologically and phenotypically consistent [55]. Other similar studies also demonstrated the successful expansion of dental pulp-derived stem cells using StemPro® MSC and DMEM/F12 media added with 15-20% of human serum, respectively. Interestingly, Saeed *et al.*, (2017) reported from his research that 10% human platelet-rich fibrin (hPRF) exudate could be a good replacement for 10% FBS in the culture medium for human dental stem cell expansion. They found that 10% hPRF exudate was excellent for cell proliferation, whereas 1% hPRF was recommended for osteo-differentiation activity in order to prevent zoonosis risk which is associated with the use of FBS [56].

Moreover, the presence or absence of FBS had also been found to impact the capability of SHED to differentiate into multiple cell types, such as the mesenchymal lineages (including osteoblasts, chondroblasts, and adipocytes) or the neural lineages [57, 58]. Studies showed that human dental stem cells are able to co-differentiate synergistically into osteoblast and endothelial cell phenotypes when cultured in FBS-containing media [57, 59]. Besides, Huang *et al.*, (2009) and several other researchers have reported successful cases of SHED isolation with subsequent differentiation into MSCs using embryonic stem cell medium supplemented with 20% FBS [6, 60, 61].

3.3. Basic Fibroblast Growth Factor (bFGF) Supplementation

Basic fibroblast growth factor (bFGF) is a signaling protein encoded by the FGF2 gene and is a crucial entity in the heparin-binding protein family, which regulates growth, migration and differentiation of many neuroectodermal- and mesodermal-based cell types [62]. In the context of cell differentiation, osteogenic medium supplemented with bFGF had been shown to inhibit alkaline phosphatase enzymatic activity as well as human dental stem cell mineralization. On the other hand, bFGF seemed to be a crucial factor that could stimulate neurogenic differentiation of dental stem cells [63]. This study reported an increased size of neurospheres with concurrent enhanced expression of neurogenic markers when cultured in the presence of bFGF. Meanwhile, the use of FGFR inhibitors or PLCgamma was able to reverse the bFGF-induced differentiation into neuronal cells [63]. Based on these findings, it could be suggested that bFGF present in the culture medium may play a role in determining the fate of SHED.

A separate study had demonstrated that prolonged *in vitro* culture of SHED will lead to a reduction of stemness characteristics as marked by the decreased expression of pluripotent stem cell markers, including NANOG, OCT4 and REX1 [64]. Moreover, a long term culture of cells *in vitro* (> passage 10) will also lower the colony-forming unit ability of SHED [64]. Both reductions in stemness properties and colony-forming ability are the two major hurdles of *in vitro* SHED culture. Nevertheless, this group of researchers had demonstrated the use of bFGF in culture media to solve these cell growth problems as bFGF could effectively increase stem cell markers expression and colony-forming unit capability in both the short and long term cultures of SHED [64]. This observation is further supported by other researchers who reported promotion of colony-forming unit ability in SHED isolated from inflamed dental pulp tissue from human deciduous teeth after treatment with FGF-2 [65]. On the other hand, blocking of endogenous bFGF expression or the receptor activity had been shown to decrease colony-forming capacity in SHED [66].

3.4. Plating Density

Another important expansion factor that is often being overlooked by researchers involves the plating density of SHED, which would eventually determine the ultimate yield. Plating density of 800–1,000 cells/cm^2 was reported to enhance the proliferation activity of stem cells, thus producing a higher cell yield [52]. Similarly, Bartmann *et al.*, (2007) had also found that reducing seeding density could boost stem cell propagation [67]. The lower plating density of the cells that had resulted in an improved cell expansion ability could possibly be due to the phenomenon of decreased contact inhibition [68].

3.5. Extracellular Matrix (ECM) Proteins

Optimum expansion of stem cells frequently depends on their adhesion ability in the culture dishes, which can be en-

hanced *via* pre-coating extracellular matrix (ECM) proteins onto plastic surfaces or by using culture dishes with peptide modified surfaces and synthetic polymeric cations [69]. However, there are also a number of studies that have reported an impact of these adhesive proteins or culture systems on the SHED' growth and phenotypes. A study demonstrated that the use of poly-D-lysine was not able to sustain attachment and proliferation of dental stem cells as the majority of the cells remained in the suspension form [70]. On the other hand, they found that collagen-coated dishes have a better capacity to sustain stem cell growth, but these coated dishes had resulted in an alteration of cell morphology [70]. Meanwhile, ECM derived from dental pulp stem cells was found to perform better than Matrigel in enhancing growth and maintaining the stemness of induced pluripotent stem cells (iPSCs) generated from dental stem cells, thus indicating an important function of ECM components [71].

4. SHED CRYOPRESERVATION TECHNIQUES

Cryopreservation is a technique for long-term storage of cells by cooling to sub-zero temperatures, particularly -196 °C at which the biological activities of the cells are halted. To protect the cells from cryo-injury, cryoprotective agents such as DMSO and glycerol are widely employed. The exact mechanism by which cryoprotective agents work remains unclear, but it has been suggested that these agents partially replace intracellular water and prevent dehydration during the freezing process [72]. In addition, the rate of freezing is a critical issue during cryopreservation as it commonly affects cell viability. The formation of ice crystals could be observed inside the cells if the freezing rate is too rapid, resulting in necrotic cell death during thawing. In contrast, a slow freezing rate may cause cellular shrinkage due to the loss of intracellular fluid. Therefore, rate-controlled freezing is crucial to reduce cell damage [73].

Table 1. Effect of cryopreservation conditions on SHED.

Source	Medium + Cryoprotectant Agent	Cooling Protocol	Storage Temperature	Storage Duration	Findings	References
Cryopreserved intact teeth	Foetal bovine serum + 10% DMSO	1hr at 4°C followed by controlled freezing (1°C/min from 4°C to -80 °C)	-196°C (Liquid nitrogen vapour phase)	1-8 months	No significant changes between the fresh SHED in terms of proliferation rate, morphology, MSC markers, osteogenic and adipogenic differentiation ability, and quality of bone formation during *in vivo* transplantation	[86]
Cryopreserved intact teeth	Foetal bovine serum + 10% DMSO	1hr at 4°C followed by controlled freezing (1°C/min from 4°C to -80 °C)	-196°C (Liquid nitrogen vapour phase)	1-9 months	Cells cryopreserved < 3 months have similar outgrowth ability and cell viability as the fresh SHED. Low outgrowth ability (46%) and cell viability (70%) for cells cryopreserved > 3 months.	[83]
Cryopreserved intact teeth	Foetal bovine serum + 10% DMSO	1hr at 4°C followed by controlled freezing (1°C/min from 4°C to -80 °C) for 24hr	-196°C (Liquid nitrogen vapour phase)	7 days	Lower SHED isolation success rate (30%) compared to non-cryopreserved group (61%). The cells demonstrated reduced proliferation rate with altered morphology	[27]
Cryopreserved SHED	Foetal bovine serum + 10% DMSO	2hr at 4°C followed by 18hr at -20°C	-80°C	1 and 6 months	Similar properties in terms of cell viability, proliferation rate and cell cycle profile to fresh SHED	[82]
Cryopreserved pulp tissues	Foetal bovine serum + 10% DMSO	4°C followed by overnight incubation at -80°C	-196°C (Liquid nitrogen vapour phase)	25-30 months	No changes to stem cell properties including self-renewal ability, multipotency, clonogenicity, expression of stem cell markers, *in vitro* immunomodulatory function as well as *in vivo* tissue regenerative capacity between cryopreserved and SHED isolated from fresh tissues	[85]
Cryopreserved SHED	Foetal bovine serum + 10% DMSO	Not specified	-196°C (Liquid nitrogen vapour phase)	unspecified	Similar proliferation rate to fresh SHED, maintain stem cells property and multipotency	[84]

Undeniably, the cryopreservation conditions for stem cells are critical in order to preserve their viability, proliferation rate and plasticity for tissue regeneration. Since the discovery of SHED in 2003, the potential applications of SHED in tissue engineering have been aggressively investigated. Nevertheless, most studies did not indicate whether the cells used for the experiments were derived from freshly harvested or cryopreserved SHED [74-79]. To the best of our knowledge, we have summarised studies that investigated various cryopreservation methods and their effects on SHED (Table **1**). DMSO (10%) appears to be the only cryoprotective agent investigated by the studies. Other permeating (*e.g*. glycerol) and non-permeating cryoprotective agents (*e.g*. polyvinylpyrrolidone, sugars) were not included to compare their cryoprotective efficacy with DMSO. Although these cryoprotective agents are intended to provide cellular protection as implicated by their name, cryoprotectants, in fact, can also cause harm to the cells. In terms of freezing rate, most studies performed a controlled freezing rate to –80 °C and subsequently storing the cells at the liquid nitrogen vapour phase (-196 °C).

MSCs intended for clinical use are usually immersed in 5% or 10% DMSO containing saline solution with the addition of a protein (such as human serum albumin), followed by controlled freezing at a rate of 1°C/min to 5°C/min until approximately -100 °C before finally stored at the liquid nitrogen vapour phase [80]. However, this protocol was optimised to store hematopoietic stem cells and lymphoid cells rather than MSCs [81]. Despite the absence of optimised cryopreservation protocol for MSCs, particularly for SHED, it appears that the abovementioned protocol did not severely affect the viability, proliferation rate and multipotency of cryopreserved SHED in comparison to the freshly harvested SHED, be it from cryopreserved intact teeth or isolated SHED [82-85]. Ma *et al.*, (2012) reported that SHED cryopreserved for more than 2 years (25-30 months) at -196 °C still shared similar stem cell characteristics, including self-renewal capacity, clonogenicity, expression of stem cell markers, multipotency, *in vitro* immunomodulatory function, as well as *in vivo* tissue regenerative capacity when compared to SHED isolated from the fresh tissues [85]. In contrast to other studies, Ji *et al.*, (2014) demonstrated that SHED cryopreserved for more than 3 months had resulted in a significant drop in outgrowth ability (46%) and cell viability (70%) [83]. Likewise, Lindemann *et al.*, (2014) found that the cryopreserved cells displayed lower proliferation rate and altered cell morphologies but retained the defined MSC markers identified by flow cytometry analysis and differentiation into adipocytes, chondrocytes and osteocytes [27]. This was a little surprising to us because the reported storage period at -196 °C was only for 7 days, which was much shorter than in other studies (ranging from 1-30 months).

Unfortunately, for long-term cryopreservation, SHED stored at -196 °C might incur a high maintenance cost. So, Ginani *et al.*, (2016) conducted an experiment to evaluate the effect of SHED storage at -80 °C for a period of 1 and 6 months, respectively [82]. The results demonstrated that cryopreserved SHED retained a high viability rate (98%), preserved defined MSC markers and were able to be differentiated into adipocytes and osteocytes. These findings were consistent with the study by Wood *et al.*, (2009) on dental pulp stem cells isolated from the permanent teeth [28] and hence, suggested that stem cells that were grown in culture could be stored at -80°C for a minimum of 6 months without compromising SHED's viability and functionality. The authors claimed that the protocol is simpler, more convenient, and incurs lower cost, thus making it more accessible to most research laboratories. It should be taken into consideration that long-term cryopreservation at -80°C is still not a standard practice for cell storage.

Practically, deciduous teeth are cryopreserved prior to SHED isolation. Nevertheless, this might result in a lower cell yield [27, 83]. Lindemann *et al.*, (2014) reported that only a 30% SHED isolation rate was achieved in comparison to non-cryopreserved deciduous teeth (61%) after cryopreserving the intact teeth for 7 days [27]. Correspondingly, Ji *et al.*, (2014) also observed that cryopreservation for 3–9 months led to reduced cell activity and cell viability [86]. Taken together, for the conventional cryopreservation method, cryopreservation of isolated SHED is recommended instead of cryopreserving intact teeth to achieve a higher isolation success rate. The cells can be immersed in FBS + 10% DMSO with a controlled freezing temperature to -80°C before being transferred into a liquid nitrogen tank for no more than 2 years.

Efforts have been attempted to reduce the toxicity of DMSO cryoprotectant. A magnetic field, particularly Cell Alive System (CAS) and Static Magnetic Field (SMF) have been reported to be able to overcome the issues encountered in conventional cryopreservation. CAS consists of a programmed freezer equipped with an oscillating magnetic field to stimulate the vibration of intracellular and extracellular water molecules, thus preventing the formation of ice crystals during the freezing process. This system has been used for cryopreservation of human adult whole teeth [87], human periodontal ligament cells (PDLs) [88-91] and hDPSCs [92]. To date, there is no report on the effect of CAS on SHED's viability and plasticity. Lee *et al.*, (2012) [92] indicated that the optimum storage medium for magnetic cryopreservation of DPSCs is 3% DMSO containing serum-free cryopreservation medium, in comparison to serum with 3% DMSO. These post-thawed magnetically cryopreserved DPSCs have a higher survival rate (> 70%), express MSC markers with no DNA damage, and are able to differentiate into osteocytes and adipocytes. Nevertheless, the research group did not optimize other CAS parameters (such as intensity, hold-time and plunging temperature) for the storage of hDPSCs. Kaku *et al.*, (2010) found that a 15 min hold-time, 0.01mT of magnetic field and a plunging temperature of -30°C resulted in the highest survival rate (> 75%) of cryopreserved PDLs (in complete growth media + 10% DMSO) compared to other conditions tested. Based on these findings, whole teeth were then cryopreserved for 1 year using the same storage conditions as above. The viability of PDLs isolated from the whole teeth is high (> 90%) and could

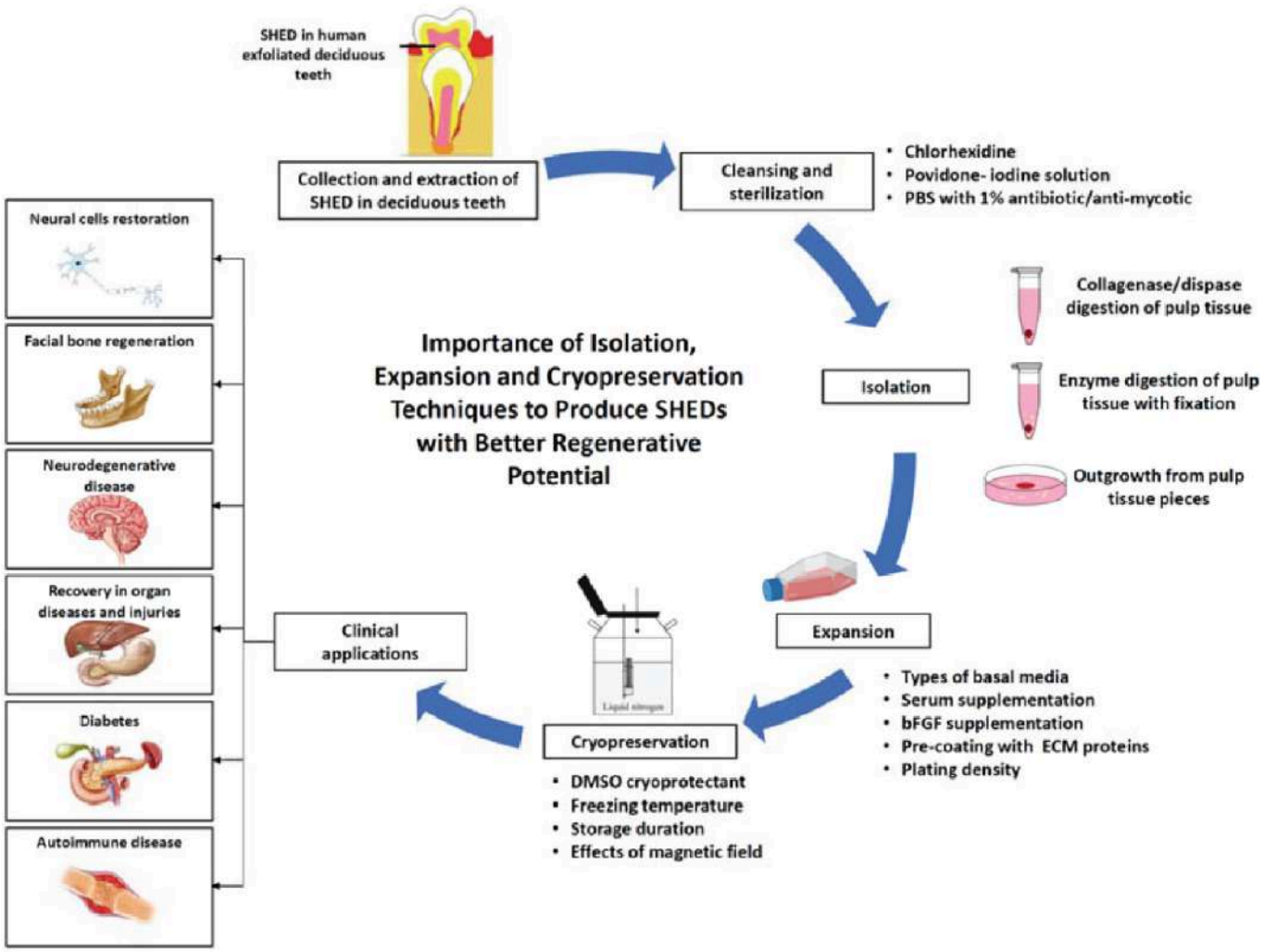

Fig. (1). Preparation of stem cells derived from human exfoliated deciduous teeth (SHED) for its therapeutic potentials. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

grow as quickly as a new tooth [89]. Another study assessed the cryoprotective effects of SMF on hDPSCs during cryopreservation, comparing the absence and presence of DMSO [93]. Generally, hDPSCs were frozen using 0.4-T or 0.8-T SMF before they were stored at -196 °C for 24 hr. Their findings showed that the control group, cryopreserved cells without the use of DMSO and SMF, only led to about 5% survival rate of hDPSCs. Meanwhile, the use of SMF had improved the hDPSCs survival rate to 15% when the cells were similarly stored using DMSO-free cryoprotectant agent. However, approximately 30%-50% survival rate of hDPSCs was observed when the cells were cryopreserved with 3% and 10% DMSO in combination with MSF. This observation could be due to the improved membrane stability attributed to MSF which had helped to resist damages that are potentially caused by ice crystals formed during the freezing process. In view of the lower cell viability, it is therefore not recommended to use DMSO-free medium to store the cells. Overall, magnetic field cryopreservation can be recommended as an effective and reliable method to store SHED. Nonetheless, further study is necessary to optimise the magnetic system for SHED as this smaller amount of cryoprotectant could be beneficial for the clinical application of post-thawed cells in the field of regenerative medicine.

FUTURE PERSPECTIVE AND CONCLUSION

Since SHED can be found in naturally disposed tissues without causing notable morbidity to the patients as well as with minimal ethical concerns, they are considered as the hidden treasure in the tissue engineering field for regenerative medicine. In addition, numerous evidences point to the huge therapeutic potential of SHED due to their multiple cell lineages differentiation and immune modulation capabilities, hence creating an overwhelming opportunity for the utilization of SHED in tissue regeneration. However, SHED need to be well isolated, expanded and cryopreserved with

their regenerative potential retained in order to be clinically applicable. For future improvement of the isolation techniques, the final clinical use of the stem cells should be taken into consideration during the selection of the method since different isolation conditions may lead to a varying differentiation aptitude as a result of the heterogeneous population existence in the dental pulp tissue. For instance, if the intended use is for dental regeneration, then the outgrowth method can be used. On the contrary, if the multipotent property of SHED is desired, then the stem cells should be isolated using the enzyme digestion method, with subsequent addition of specific growth supplements and growth influencers in the culture medium, such as bFGF, vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP) to maintain the stemness of the SHED or to specifically promote differentiation into desired specialized cells. In this context, the culture expansion system plays a critical role as it is well known that primary cells have a limited growth capacity after which, they will enter the senescence phase. Besides, stem cells frequently change their morphology and phenotype during *in vitro* expansion as a form of adaptation in response to the micro-environmental factors. Thus, more detailed investigations are needed to study and evaluate each of the underlying factors, or a combination of these factors that can produce optimum cell expansion *in vitro* with stemness characteristics maintained before SHED-based therapies can become a clinical reality. This is because an adequate number of stem cells need to be obtained for SHED to be applicable for tissue engineering and regenerative medicine in the clinical setting. Although it is also usually overlooked, the potential risks for clinical use of SHED also require further investigations to ensure the safety of the patients who will be benefiting from the regenerative capability of these stem cells

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CONFLICT OF INTEREST

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