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Preserving human cells for regenerative, reproductive, and transfusion medicine

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Abstract

Cell cryopreservation enables maintaining cellular life at sub-zero temperatures by slowing down biochemical processes. Various cell types are routinely cryopreserved in modern reproductive, regenerative, and transfusion medicine. Current cell cryopreservation methods involve freezing (slow/rapid) or vitrifying cells in the presence of a cryoprotective agent (CPA). Although these methods are clinically utilized, cryo-injury due to ice crystals, osmotic shock, and CPA toxicity cause loss of cell viability and function. Recent approaches using minimum volume vitrification provide alternatives to the conventional cryopreservation methods. Minimum volume vitrification provides ultra-high cooling and rewarming rates that enable preserving cells without ice crystal formation. Herein, we review recent advances in cell cryopreservation technology and provide examples of techniques that are utilized in oocyte, stem cell, and red blood cell cryopreservation.

Keywords

Cryopreservation; Vitrification; Regenerative medicine; Transfusion medicine; Reproductive medicine

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Conflict-of-interest statement

Dr. Utkan Demirci is a founder of, and has an equity interest in: (i) DxNow Inc., a company that is developing microfluidic and imaging technologies for point-of-care diagnostic solutions, and (ii) Koek Biotech, a company that is developing microfluidic IVF technologies for clinical solutions. Dr. Utkan Demirci's interests were viewed and managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

1 Introduction

The need to cryopreserve cells is an escalating clinical problem due to high demands of various cell types in clinical medicine including human oocytes, stem cells (SCs), and red blood cells (RBCs) [1]. In reproductive medicine, oocyte cryopreservation has emerged as a viable option to maintain female fertility [2, 3]. The ability to preserve oocytes for a long time would maintain fertility options for female patients who suffer from pathological conditions (e.g., premature ovarian failure, cysts, and tumors) or receiving anticancer therapy such as chemo/radio-therapy or other gonadotoxic therapy [2, 3]. In regenerative medicine, human SC therapy is one of the promising therapeutic approaches [4]. Particularly, hematopoietic SCs, human mesenchymal stem cells (hMSCs), human embryonic stem cells (hESCs), and umbilical cord SCs are being utilized in treating various diseases such as cardiovascular diseases, diabetes, immune-modulatory disease, and cancer [5–9]. According to a recent report from clinical trials database by National Institute of Health (http://clinicaltrials.gov/), 123 clinical trial sites have been registered for evaluating hMSC therapy throughout the world. The global SC market is forecasted to reach $63.8 billion by 2015 [10]. With an increasing use of stem cells in therapeutics and drug screening, it has become important to cryopreserve human SCs for a continuous quality-controlled supply and transportation between different sites [11]. In transfusion medicine, the demand of blood products has constantly increased during the last decade and the current blood biopreservation is approximately a $11–12 billion market in the United States alone as recently reported by US Blood & Organ Banks Market Research Report. According to the latest National Blood Collection and Utilization Survey Report compiled by U.S. Department of Health and Human Services, among 15.5 million units of blood that were collected in 2011, 13.8 million units were transfused, leaving ~1.7 million units to be discarded mainly due to short shelf-life (*i.e.*, 42 days) [12]. This limited shelf-life storage resulted in significant blood waste (~382 million USD annually) [12]. Furthermore, millions of health complications resulted from the local blood shortages in the clinical settings [13]. According to this survey report, up to 3.3% of US hospitals have reported delays in the elective surgeries due to blood inventory shortage [12]. New technologies in RBC cryopreservation would have a significant impact on blood supply system, thus reducing frequent blood shortage, outdating of blood units, as well as decreasing the incidence of post-transfusion complications.

Cell cryopreservation is a process to maintain cellular life at extremely low temperatures. During cryopreservation, a chemical substance (cryoprotective agent, CPA) is utilized to protect the cellular structures from damage during cooling and rewarming processes. Two main groups of CPAs are used: (i) intracellular CPAs that penetrate the cell membrane such as dimethyl sulfoxide (DMSO or Me_2SO), glycerol, and 1, 2-propanediol; and (ii) extracellular CPAs that do not penetrate the cell membrane such as large molecular weight polymers and sugars (e.g., hydroxyethyl starch (HES), polyvinyl pyrrolidone (PVP), and poly (ethylene glycol) (PEG))[14–17]. Although both groups have shown to be useful in protecting the cellular components during cryopreservation, controlled addition and removal of such CPAs is necessary to prevent cell lysis, differentiation or toxicity [11, 15]. Recently, synthetic anti-freeze (glyco)protein and bio-inspired cryo-agents that are isolated from

extremophylic bacteria, such as ectoine and trehalose are being investigated for preserving mammalian cells [1, 18–21].

Slow and rapid freezing are the two conventional approaches utilized in clinical practice for cell cryopreservation [15]. In more commonly used slow freezing method, cells or tissues are cooled down at a rate of ~1°C/min and eventually stored at −80 °C [22]. Intracellular ice formation is reduced in slow freezing as water gets enough time to diffuse into extracellular solution and new equilibrium point state is achieved. On the other hand, in slow freezing, cells are exposed for a longer time to high CPA concentrations resulting in potentially damaging effects [23]. Further, during slow freezing, cells squeeze into channels between ice crystals. As temperature decreases, the ice crystals grow to close the channels. The growing ice crystals exert mechanical forces on squeezed cells resulting in cryo-injury [24, 25]. In contrast during rapid freezing, cells or tissues are cooled down at high freezing rates (60–120°C/min) [26]. During cooling process, ice is formed in extracellular region that removes water from solution in the form of ice. This removal of water increases the CPA concentration in the remaining solution. To achieve new equilibrium state, intracellular water diffuses to extracellular solution. At high cooling rates, water does not get enough time to diffuse to extracellular region leading to the formation of intracellular ice crystals. The intracellular ice formation results in various adverse changes, which are collectively referred as a “cryo-injury” or “cryo-damage” [27]. Cryo-injury leads to loss of cell viability or compromises cell function by damaging cell membrane, morphology, and cytoskeletal components [28–31].

Vitrification has emerged as an alternative approach to conventional freezing methods to minimize cryo-injury. Vitrification method involves ultra-fast cooling rates by submerging cells in liquid nitrogen (LN_2) (−196 °C) or LN_2 vapor (−165 °C) [15]. During vitrification the cell transforms rapidly into a glass-like solidification status (i.e. vitreous) where ice crystallization is avoided [15, 32]. However, the high CPA concentration that is required to achieve vitrification results in osmotic dehydration to cells. New vitrification approaches have emerged as alternative techniques, which have shown the ability to significantly reduce cryo-injury (Table 1) [33]. Utilizing these minimum volume vitrification approaches have enhanced the cooling/rewarming rates up to 700,000 °C/min [34, 35] and reduced the CPA concentration that is required to achieve vitrification [26, 33]. In this review, we describe the current methodologies in cell cryopreservation focusing on vitrification of oocytes, SCs, and RBCs as these cell types have broad and significant applications in medicine.

2 Oocyte Cryopreservation and Reproductive Medicine

Currently oocyte cryopreservation has been achieved by slow freezing and vitrification methods. [36]. When oocytes are cryopreserved using the slow freezing method for *in vitro* fertilization, low pregnancy rates are reported (13%) [31, 37–41]. This outcome has been attributed to the permanent damage to cryopreserved oocytes such as misalignment of chromosomes and hardening of the zona during slow freezing [28–30]. To minimize oocyte damage during cyropreservation, minimum volume vitrification techniques such as closed-pulled straw, cryoloop, and cryotop have been developed (Figure 1). These methods have efficiently improved the pregnancy rates (36–61%) [41–46].

Closed-pulled straw is one of the oocyte vitrification techniques that utilize minimum volume approach to enhance the cooling and rewarming rates. About 4 to 6 oocytes are pulled into a straw using a syringe and placed into LN_2. Air segments before and after oocyte solution inside a straw protect oocytes form direct contact LN_2 [47]. According to a recent clinical study, oocytes vitrified using closed-pulled straw method showed 81% survival after rewarming [41]. These oocytes were further used in intracytoplasmic sperm injection and showed successful embryo development and clinical pregnancy rates up to 38% [41] (Figure 1A–F). The cryoloop technique is another method that has provided a uniform and rapid exchange of heat during the cooling process due to the reduced sample volume (<1 μl) (Figure 1G). It has demonstrated the ability to relatively increase oocyte survivability compared to the straw-based approach [48]. However, the cleavage rate and blastocyst development after *in vitro* fertilization was significantly reduced. On the other hand, cryotop is currently used as one of the most common carrier-based vitrification methods. This approach is based on coating a polyethylene strip with small sample volume (<1 μl) that is sufficient to cover the intended cell to cryopreserve (Figure 1H). Following submerging in LN_2 the strip is enclosed with plastic cover to protect the cryopreserved oocyte during cryo-storage. Due to the minimum sample volume and the thin strip, this approach has provided high cooling and rewarming rates up to 40,000 C°/min [34]. This is a major advantage particularly during the rewarming process as this could prevent crystallization reducing the chance of cryo-injury. Recent reports have highlighted that during the cryopreservation, rewarming rate is more critical than cooling rate, as cryo-injury is more likely to occur during the former process [49–52]. Recently, four clinical randomized trails demonstrated high cell survival rate after rewarming (90–97%) when this approach was used [43–46]. This approach has demonstrated a significant increase in pregnancy rates (36–61%) compared to slow freezing methods [43–46].

In addition to these carrier-based systems that require manual oocyte handling and skilled operators, carrier-free droplet generation methods are being developed that increase cooling and rewarming rates due to the absence of a bulky carrier [33, 53]. In a recent report, the ejector-based droplet vitrification platform has been developed to cryopreserve mouse oocytes in nanoliter droplets [54]. High oocyte survival (89.9%) and cleavage rates (97%) were reported after rewarming compared to the fresh oocytes [54]. The reported results are based on mouse oocyte vitrification; further evaluation with human oocytes is required.

Overall, minimum volume vitrification technologies have shown high oocyte survival rates and pregnancy outputs in clinical settings. These results are promising for establishing universal oocyte banking in the future, which would lower the cost and eliminate the waiting period for matching donors. As minimum volume vitrification methods are recently developed, a thorough evaluation will be needed to analyze the long term potential effects of vitrification on children born using cryopreserved oocytes.

3 Stem Cell Preservation and Regenerative Medicine

The ability of SCs to differentiate into various cell types have found applications in many areas including drug screening, regenerative medicine, and tissue engineering [5, 7, 55–58]. These applications require a continuous supply of SCs that can be achieved by using

cryopreservation technology. Current SC cryopreservation protocols involve slow-freezing using DMSO alone or combined with other CPAs such as glycerol and proline, along with animal/human serum [59–63]. In the case of hMSC cryopreservation, DMSO based slow freezing protocols provide cell recovery up to 90% after cryopreservation [64]. The current research focus in hMSC cryopreservation is on clinical safety by avoiding or reducing the DMSO concentration and eliminating the use of animal/human serum in cell cryopreservation protocols. There is a wide range of DMSO concentrations used without any clear guidelines and scientific rationale as revealed by a study of 444 European Group for Blood and Marrow Transplantation centers [65]. The potential side effects related to indiscriminate transfusion of cryopreserved cells along with DMSO intended for transplant therapy include cardiovascular failure and respiratory distress such as bradycardia, hypotension, respiratory arrest and fatal arrhythmias [65]. In addition, many reports have shown neural lineage differentiation when DMSO is used to cryopreserve SCs [66–68]. Only 1% DMSO has been reported to induce differentiation in ESCs to mesendoderm [69, 70]. Clinically employed protocols such as the New York Blood Bank Protocol washed out the DMSO and serum before infusion [71, 72]. Washing out DMSO and serum from the cells significantly decreases infusion-related toxicity, however, some adverse reactions have also been reported after using these washing protocols [65, 73, 74]. Utilizing alternative CPAs in combination with minimal amount of DMSO, or alone, are being investigated [75, 76]. To reduce the DMSO concentration, PVP and HES have been used in combination with DMSO for cryopreservation [75, 76]. Recently, the ability to protect the hMSCs from cryo-damage has been reported using a bio-inspired ectoin solution [18]. This study reported the use of ectoin with serum-free cryo-medium to preserve hMSCs by following a slow freezing approach. Although the viability of cryopreserved cells was 72% post-thawing, developing a DMSO-free and serum-free cryopreservation approache for SCs would make it more suitable for clinical therapy. In another effort to eliminate animal serum, a serum-free CPA (7.5% DMSO, 2.5% PEG), 2% serum albumin) has been developed where fetal bovine serum is replaced with serum albumin [64]. The hMSC cells showed 82.9% post-thaw viability which was comparable to when animal serum was used with 10% DMSO.

Besides the use of non-toxic CPAs in SC cryopreservation, vitrification has become an attractive approach due to its higher cell survival rate compared to slow freezing especially for adherent SCs such as hESC [77, 78]. Vitrification of mouse ESCs has been demonstrated using Cryotip and Quartz microcapillary approaches [32, 66, 79]. However, these two methods suffer from low throughput. The efficiency of these techniques has to be further investigated in hESCs. Alternatively, a surface-based vitrification technique using Thermanox© coverslip has been developed (Figure 2A) [80]. In this method a bulk quantity of adherent hESC can be preserved efficiently, exhibiting a higher survival rate after rewarming (89%) compared to slow frozen colonies (51%). This approach allows a precise handling and storage of SCs. The cryopreserved hESC cells were also tested for their ability to differentiate using staining antibodies against pluripotent markers such as Oct-4 and Tra-1-81. Approximately 80% (±12%) of the cryopreserved cells were positive for pluripotency compared with 81% (±13%) in unfrozen control colonies [80]. In another study, a bulk vitrification method based on cell strainer was utilized to cryopreserve large quantities of hESC clumps (Figure 2B) [81]. In these two methods, the cells have to be in

contact with LN_2. Although this is potentially considered to increase the possibility of contamination, LN_2 can be sterilized by utilizing ultra-violet radiation or sterile filters, which would minimize such contamination risks [82–84]. In summary, various CPA formulations and vitrification methods have been investigated for SC cryopreservation that showed good cell recovery after rewarming. As new batch of serum-free and DMSO-free CPA formulations are being developed, there is a need to standardize the SC cryopreservation methods in terms of CPA choice and used concentrations.

4 Red Blood Cell Preservation and Transfusion Medicine

Recent studies have underscored questions about the clinical effectiveness of blood units biopreserved using refrigeration [85, 86]. For instance, increased mortality and morbidity rates were reported in compromised patients after cardiac surgery [87]. These complications were correlated with longer storage of the blood units (> 14 days), which suggests the responsibility of progressive adverse changes seen in stored RBCs [87]. Cryopreservation of RBCs would offer an alternative approach to address the current challenges in the field of transfusion medicine by providing the extended means of RBC preservation and reducing storage damage. Currently, there are two methods used clinically for RBC cryopreservation: high glycerol-slow freezing and low glycerol-rapid freezing methods [88, 89]. The high glycerol/slow freezing technique (common in the USA and Canada) utilizes 40% (w/v) glycerol in conjunction with a freezing rate of ~1°C/min and storing at −80 °C. The low glycerol/rapid freezing method (routinely used in Europe) involves the application of 10–20% glycerol and rapid freezing rates (60–120°C/min) by submersing the CPA containers in LN_2 or LN_2 vapor (−196 °C or −165 °C, respectively). Glycerol is a penetrating CPA that efficiently prevents the formation of ice crystals during RBC cryopreservation [90]. For glycerol based freezing methods, it is important to remove the intracellular glycerol after thawing to minimize the RBC hemolysis following transfusion [15, 91, 92]. This deglycerolization process requires multiple washing steps where about 15% cells are hemolysed [93, 94]. Recently published results affirm the adverse changes in RBC morphology after cryopreservation and deglycerolization [94]. Such changes negatively affect RBC function by reducing its deformability, which allows cells to flow through ultra-thin capillaries to oxygenate target tissues [95]. Despite these limitations, glycerol based cryopreservation is being used effectively in clinics. To overcome the challenges associated with glycerol-based cryopreservation, alternative materials including anti-freeze proteins and their synthetic mimics such as poly(vinyl alcohol) (PVA) are being investigated for their potential use in transfusion medicine. PVA is appealing because of its properties to inhibit ice recrystallization [96]. PVA is known for its minimal toxicity and is already approved by Food and Drug Administration for dietary use [97]. Recently, PVA is used at significantly low concentrations (0.1% wt) compared to glycerol (rapid freezing (20% wt) or slow freezing (40% wt)) for blood cryopreservation and enabled cell recovery of >40% after rewarming [1]. Although >40% is a low cell recovery, cryopreservation of RBCs at extremely low PVA concentrations is achieved due to its ability to inhibit ice crystallization without dynamic ice shaping [1]. Alternative CPAs such as Ectoine have shown good results with other cell types and are being explored in transfusion medicine to limit washing steps. Ectoine is expensive as compared to glycerol but it is a biocompatible [98, 99] low

molecular weight solute which is currently used in low concentrations (0.1%–1%) for protein and cell freezing [68, 100], hence, multiple washing steps may not be required.

Vitrification has not been well investigated for transfusion medicine because of throughput challenges. Recently, a scalable method for blood cryopreservation has been reported where cells are cryopreserved in microdroplets generated by an ejector at low glycerol concentrations (~ 23%) [26]. Each ejector processes 0.2 mL of blood in a minute, but making arrays of ejectors would enable high throughput cryopreservation of RBCs.

During RBC cryopreservation, blood needs to be stored at −80 °C or even lower temperatures, which makes the whole process economically less feasible. There is also a high cost associated with the complex processing requiring trained personnel. For these reasons, currently, blood is cryopreserved in limited amount only for natural calamities, rare blood groups, and military settings [91, 101].

5 Future prospects and conclusions

The integration of nano- and micro-scale technologies with bio-inspired and synthetic materials has brought innovative ideas targeting the existing challenges in cryobiology [102]. For instance, a recent finding that PVA at extremely low concentrations works as an effective inhibitor for ice recrystallization, can be adapted to cryopreservation field to address the toxicity issues of conventional CPAs. Utilizing biocompatible materials at low CPA concentrations simplifies cryopreservation protocols by reducing multiple CPA unloading steps.

Manipulating cells in minimal volume droplets of CPA increase cooling and warming rates, which would potentially reduce cell damage. This minimal volume approach would potentially eliminate the requirements for high CPA concentrations to achieve cell vitrification and minimize osmotic shock and toxicity. Minimal volume vitrification techniques are successfully being applied for oocyte and SC cryopreservation. Although some of the reported vitrification techniques have automation potential, alternative methods should be investigated to meet the high throughput requirements for commercial applications. To reduce the cost associated with storing the vitrified cells at extremely low temperatures, there is a need to investigate cell lyophilization or freeze-drying technologies for cell preservation at room temperature. Technologies to store cells in dry form are especially needed in transfusion medicine where blood is stored in large volumes. Overall, these emerging technologies open new avenues to address today’s challenges in cell cryopreservation enabling new applications in tissue engineering, regenerative medicine, personalized medicine, drug screening, and bio-banking.

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List of abbreviations

CPA	Cryoprotective agent
SCs	Stem cells
RBCs	Red blood cells
hMSCs	Human mesenchymal stem cells
hESCs	Human embryonic stem cells
DMSO or Me_2SO	dimethyl sulfoxide
HES	Hydroxyethyl starch
PVP	Polyvinyl pyrrolidone
LN_2	Liquid nitrogen
PEG	Poly (ethylene glycol)
PVA	Poly (vinyl alcohol)

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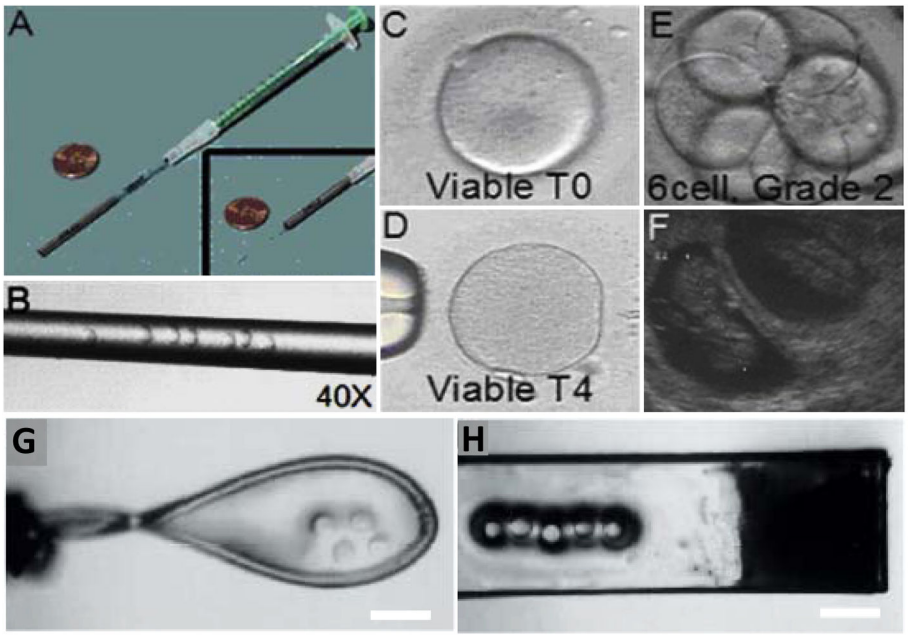

Figure 1.

Minimum volume vitrification methods. (A) An image of closed-pull straw system. (B) Magnified image of straw with oocytes loaded inside. The straw diameter is 200 μm. (C) Image of a viable oocyte just after warming (T1 stage). (D) Image of a viable oocyte 4 hours after warming (T4 stage), just before intracytoplasmic sperm injection. (E) Image of a Day 3 human embryo at 6 cell grade 2 stage. (F) Sonogram showing a ten week clinical pregnancy following a transfer of 3 embryos. A–F are reprinted by copyright permissions from [41]. (G) Cryoloop carrier loaded 4 human oocytes. (H) Cryotop carrier loaded with 5 oocytes. Scale bars indicate 400 μm. G and H are reprinted by copyright permissions from [108].

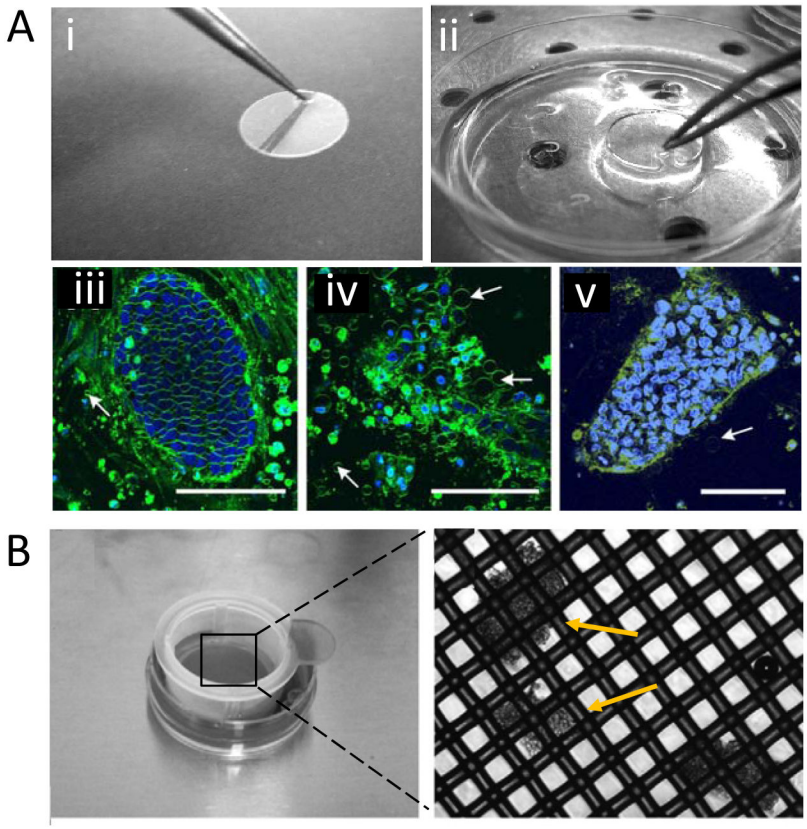

Figure 2.

(A) Surface-based vitrification method. (i) A modification of Thermanox cultivation disc with a small tip to handle with tweezer. (ii) Disc incubation in CPA. Multi photon laser scanning micrographs of hESC-colonies (iii) Control colony, (iv) Cryopreserved colony using slow rate freezing, and (v) Cryopreserved colony using surface based vitrification. Fewer membrane vesicles (arrows) were observed in vitrified sample. Scale bars indicate 100 μm. Reprinted by copyright permissions from [80]. (B) Bulk vitrification method. hESC cell clumps were loaded on the nylon mesh of a cell strainer and incubated in CPA. The inset (right) shows the magnified view of nylon mesh with cell clumps indicated by arrows. Reprinted by copyright permissions from [81].

Table 1

Summary of vitrification systems used in RBCs, SCs and Oocytes.

Cell Type	Vitrification techniques	CPA	CPA concentration (%)/CPA level (M)	Freezing rate °C/min	Carrier-free method	Clinically in Use	Viability	Sample Volume/Size	Ref.
RBCs	Minimum Volume Vitrification	Glycerol	23% (2.5M)	NA	NA	No	NA	200μL/min per ejector	[26, 103]
Stem Cells	Open pull Straw	DMSO/Ethylene glycol	10–20%/10–20%	16,700	No	No	> 70%	1–20μL	[104]
	Bulk Method	DMSO/Ethylene glycol	10–20%/10–20%	NA	Yes	No	> 94%	NA	[81]
	Quartz Microcapillary	1,2-Propanediol/Trehalose	2M/0.5M	250,000	No	No	> 70%	~3 mm (inner diameter)	[32, 33]
	Surface-based method	DMSO/Ethylene glycol	10–20%/10–20%	NA	Yes	No	> 89%	NA	[80]
Oocyte	Conventional Straw	DMSO/Acetamide/Propylene glycol/Polyethylene glycol	20.5%/15.5%/10%/6%	2500	No	Yes	> 80%	0.25 mL	[105]
	Cryoloop	DMSO/Ethylene glycol	10–20%/10–20%	700,000	No	Yes	> 90%	0.5–0.7 mm (inner diameter)	[106]
	Cryotop	DMSO/Ethylene glycol	15%/15%	40,000	No	Yes	< 90%	<1 μL	[34, 107]
	Nanoliter Vitrification	DMSO/Ethylene glycol	4–8%/4–8%	N/A	Yes	No	> 89%	~ 1 nL	[54]