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Review Article

Principles of Cryopreservation and Applicabilities in Intestinal Organoids

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1. Abstract

Cryobiology is known as a study of low temperatures effects in tissues and cells, which means understand influences on decreasing temperature and pressure in the freezing medium and how the cell membrane reacts to these variables. Cryopreservation emerged by the principles of Cryobiology as a technology capable of preserving the composition and viability of cells indefinitely, at low temperature below water fusion point. However, the low temperature can lysis a cell membrane and destroy it. At the same time, the low temperature can also preserve the cell, avoiding the formation of ice crystal in the cytoplasm. To maintain long term organoids is a great challenge because in the cryopreservation and thawing process there is an important loose of cells and, consequently, the culture does not work anymore. It means a waste of materials and time, loss of unique cell lineages, and even the collection of new samples. Thus, it has been an obstacle to maintaining long-term cultivation of some species and cell types, as each one has its membrane potentials. It is not a simple process and to solve this problem is necessary more knowing about Cryopreservation. The objective of this review is disserted about cryopreservation proprieties and how this can meddle in long live culture intestinal organoids and integrity of cell membrane, cytoplasm, and nucleus, as well as the current organoid applicability and diverse of results some experiments, had in different intestinal organoids cryopreservation protocols.

2. Introduction

It is possible to generate tissues with organization and function in plates, as it self-regenerates stem cells in vivo and ex vivo. It's like “mini-organs”, called organoids. Today, it's possible to isolate cells from the intestine, liver, heart, brain, kidney, and other organs to generate a culture. The organoids have great applications in many areas such as the study of cell regulation, growth, and differentiation; a better understanding of the physiology of normal processes and diseases; therapy of genetic diseases, evaluation in cancer therapy, analysis of drugs toxicity; models to replace the animals, and others [1, 2, 6, 8].

However, challenges have been faced with regard to cell culture in laboratories in the manipulation and keeping of different organoids. The cryopreservation is a critical point where cells can die since the cooling rates can preserve cells or kill them. The freezing of cells is contradictory, because in cryosurgery the low temperature is used to eliminate cells, or they can be maintained at low temperature for long periods (1-2 years).

Cryopreservation allows the long term storage of cells, which has a number of applications. Clini-

cally, cells may be stored by a patient for their own use at a later date. Cells may also be banked to facilitate a donation to other patients. Having stored the cells, they can be distributed, making it easier to coordinate patient care, and avoiding the need to synchronize donor and recipient. The ability to store cells reduces wastage of cells if they cannot be used fresh, and thereby increases the supply of transplantable material. Cell storage is also valuable in scientific research allowing archiving of material, repeated experiments from the same tissue source, and, by allowing distribution of stored samples, facilitates research collaboration [3, 11].

It is important to understand the principles of cryopreservation and thawing to better the application of protocols. There are several basic facts with regards to freezing, thawing, and cryoprotection that will be useful for an understanding of the various routes to the successful cryopreservation of cells. Therefore, as in most fields, the details of ice formation and growth can be complex and occasionally controversial [4, 10]. Tissue/organ preservation is a challenge for tissue engineering in clinical application, especially for complex tissues or organs which constitute different cell types in an organized structure [5, 6, 10-11].

One of the cryopreservation applications is in long term organoid culture regarding the difficulty in maintaining the cell membrane equilibrium due to low temperature, freezing time, and speed and medium components effects. In general, cryopreservation of multi cellular cultures displaying cell-to-cell and cell-to-matrix interactions differs significantly from that of dissociated single-cell suspensions. Those interactions influence both intracellular ice formation and intercellular ice nucleation upon freezing, making cryopreservation of complex multi cellular structures or tissues less feasible compared to single-cell suspensions [1-2, 6-8]. Furthermore, in-plate cryopreservation of 3D cultures faces challenges such as post-thaw matrix retrieval. Thereby, it represents a critical point in intestinal organoid culture and it is necessary to understand the cryopreservation mechanisms and how do they affect the cells to establish the best protocol for each organoid culture type.

2.1. Properties of Freezing

Cells can respond in different ways among these varieties, which will affect their survival after freezing and thawing [4, 10]. When cells are transferred from 37°C to -196°C of liquid nitrogen the freezing can produce intra and extracellular ice crystals and changes in cell chemical composition which can be fatal to them [9]. The formation of extra and intra crystals depends on the freezing rate, temperature, and solution concentration. Initially (Figure 1), cells are kept suspended in a cryopreservation solution at room temperature (or below). As the sample is cooled, the formation of ice is initiated (either spontaneously or by seeding). The formation of ice in an extracellular solution removes water in ice form from the solution. This removal of water produces an abrupt change in the concentration of the unfrozen portion of the extracellular solution [4, 10].

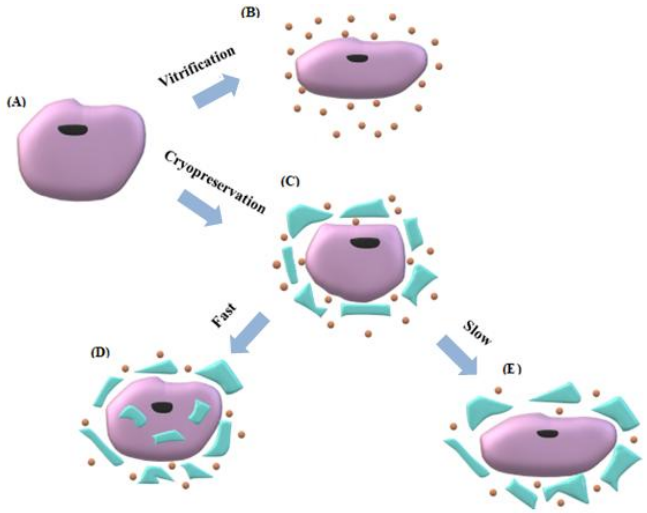

Figure 1: Methods of freezing cells. (A) The cell in the culture medium is in a saturated solution at room temperature and can be frozen using: (B) Vitrification where it is put in vitrification solution (ultra hypersaturated with cryoprotectants) and quickly subjected to ultra low freezing rates; (C) Cryopreservation where the cell is put in cryopreservation solution (hypersaturated with cryoprotectants) and submitted in (D) Fast freezing rate, where intra and extracellular ice crystals are formed, these crystals in the cytoplasm lysing the cellular membrane causing a drop in viability after thawing. (E) The slow freezing rate has the formation of ice crystal medium, which turns it more hypersaturated (added cryoprotectant and formation of ice crystal). The cells start a dehydration process, searching by homeostasis with the medium.

Table 1: Properties of methods to freeze cells.

	Cell in culture	Vitrification	Slow	Fast
			Cryopreservation	Cryopreservation
Solution	Homeostasis	Ultra Hypersaturated	Hypersaturated	Hypersaturated
Solute: Solvente	Solute ~ Solvente	Solute > Solvente	Solute > Solvente	Solute > Solvente
Freezing Rate	-	>10.000ºC/min	> 1-2 ºC/min	>3-5 ºC/min
Risk of crystal formation	-	none	Extra-cellular	Intra and extra-cellular

The difference in each freezing mechanism influences the viability of the cells, which have formed ice crystals in the cytoplasm that will be lysed by them. (Table 1) shows the differences between freezing cell methods and their particularities. The rate of freezing was widely debated in the literature. The controlled rate freezing technique is still considered standard, mainly due to the fact that heat liberation at the transition or eutactic point (about 4°C) is deemed detrimental to the stem cell population. At this point, water molecules within the frozen unit are in a precise molecular order, which eventuates in the thermodynamic liberation of fusion heat [4, 10-11, 42].

In the long term culture, the main mechanism of freezing organoids was cryopreservation at a slow freezing rate. In many studies, it was shown that organoids had better survival than at a slow freezing

rate [6-8, 35, 63]. The most common methods for cryopreservation involve a slow freezing process (>1ºC/min) and a penetrating cryoprotectant, such as DMSO to avoid intracellular ice formation, which is the major cause of cell death during vitrification. However, during the warming process, the ice crystal formation might still be induced by devitrification [6, 12-13]. The crystal formation from devitrification, which might disrupt the cell alignment within tissues or organs, cannot be avoided during the conventional cryopreservation procedure [6].

Also, problems as disruptive ice propagation between inter-connected cells and insufficient cell dehydration and cryoprotectants (CAPs) penetration during slow cooling and ice-recrystallization during the warming process have all been noted. Cooling multi cellular systems down to deep sub-zero temperatures has proven far more challenging, especially when large volumes of biomass require cryo-banking. These problems began to be understood during early attempts to cryopreserve whole organs or large complex tissues. They were mainly related to the formation of extracellular ice in liquid spaces within the tissue, such as within small capillary blood vessels inside an organ, which physically destroyed the internal structure [4, 14-15].

2.2. Cell Concentration

Studies have demonstrated that a high cell concentration followed by cryopreservation had a decrease of cell viability because it could result in toxicity to cells. This was partially attributed to the total volume and the cryopreservatives in the solution [11, 16-17]. Hence, the initially proposed concentration of cryopreserved cells was suggested to be not over $2 \times 10{-}7$/ml NC [10, 11, 18].

A variety of hypotheses were presented to explain the increased damage to frozen cells at high concentrations. It is believed to be due to the mechanical stresses present during the heating of other tan drowning in slow cooling rates. The exact mechanisms are still controversial, and more studies will be needed to distinguish between cell-specific effects and those which are solution dependent [10].

2.3. Membrane Permeability

The permeability of the membrane to water and penetrating CPAs will influence the osmotic response of cells during the use and removal of the cryopreservation solution [10-11, 40]. During freezing, the permeability of the membrane will be important in determining the water content of the cell, which in turn will influence the probability of formation of crystals in the cytoplasm or extent of cell dehydration [4, 10, 40].

To better understand the effects of the environment on the plasma membrane, prior knowledge about binding effects, especially omoscopy (Figure 2) is necessary. In a situation of equilibrium, the cell is in homeostasis with the culture medium which means it has an equilibrium hydroeletrolict and where there is no significant difference between the concentrations of medium intra and extracellular. When the cells are put in a freezing solution, added CAPs and reducing of temperature with the formation of crystal ice in the extracellular medium, all these factors cause changes in the concentration of the medium (hyper saturated). Thus, cell stress occurs followed by dehydration, as a way of maintaining osmotic balance. The addition of CAPs leads to an increase in osmotic pressure, at the same moment as the increasing of membrane plasmatic pores and finally, increasing of osmose flux [4, 10, 26, 40].

The osmotic pressure can be calculated by Van't Hoff equation (1), where it is possible to observe the relation of proportionality between temperature (T) and molarity (M) with osmotic pressure (π), directly proportional. Where "R" represents a universal constant of the perfect gases and "i" Van't Hoff factor [10, 19-20]. The Boyle-Van't Hoff relation is here derived via classical thermodynamics, assuming that the chemical potential of water is equal across a membrane permeable to water only [19].

$$\pi: M.RTi \qquad (1)$$

M: Molarity (mol/L)

R: Universal constant of the perfect gases (0,082 atm . L. mol^{-1}. K^{-1} or 62,3 mm Hg L. mol^{-1}. K^{-1})

T: Absolute temperature (Kelvin)

i: Van't Hoff factor

Due to the importance of membrane permeability in the response of a cell to freezing, a lot of work was done in order to measure the permeability of the membrane and to model the transport of water during freezing. With this assumption in mind, the connection between membrane hydraulic permeability (LP) and temperature can be determined by using the Arrhenius equation (2) [10, 20-21]:

$$L_p = L_{pg}[cpa] exp\left(-\frac{E_{Lp}[cpa]}{R}\left(\frac{1}{T}-\frac{1}{T_R}\right)\right) \qquad (2)$$

Where *L*pg [cpa] is the reference membrane permeability at a reference temperature, *E*Lp [cpa] is the apparent activation energy or the temperature dependence of the cell membrane permeability, *R* is the universal gas constant, TR (typically 0~ for freezing studies). This equation shows the relationship of the reversibility of Lp with T, they are not proportional. Other than ELp [cpa] with Lp`s where both increase and decrease together, but not in the same proportion [10]. Another factor relevant to the description of the water transport characteristics of cells is the osmotically inactive volume fraction, Vb. This quantity represents the fraction of solids and water within the cells that is not available for transport out of the cell during dehydration. Vb is a static property of the cell, which typically is determined independently of Lpg and ELp [10].

It's important to know each cell has a different potential membrane

permeability which can change when the cell in a culture medium suffers differentiation, with culture time the cells increased significantly in volume (twofold to threefold) and Vb decreased, on the other hand, Lpg and ELp increased. An example is the results of preliminary studies cited by Hubel (1997) in which they determined the permeability parameters for different blood cells, demonstrating how these cells respond differently to freezing (Table 2) [10, 64].

Table 2: Cryobiophysical Characteristics of Blood Cells.

Cell Type	L_{pg}	E_{Lp}	Vb
	(10 $^{13}m^3$/NS)	**(kj/mol)**	**(%)**
Erythrocytes	$1.27(10)^{-12}$	16.3	17
Granulocytes	$6.7(10)^{-14}$	218	35
Lymphocytes	$6.3(10)^{-14}$	14.3	35
Monocytes	$4.9(10)^{-14}$	61	26
Fonte: Adapted from Hubel (1997).			

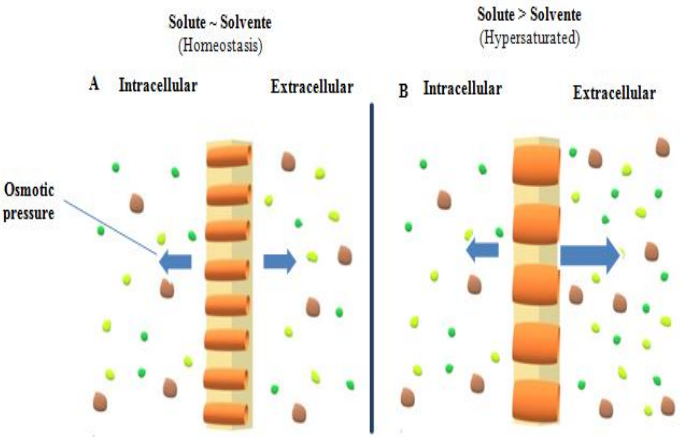

Figure 2: Represents the principles of omoscopy and its effects on the plasma membrane. (A) In the beginning, the cells are in culture medium with equilibrium solvent/solute concentrations (osmose). There is no difference in the flow of fluid in and out, the cell is not dehydrated or grows by the influence of the extracellular environment. (B) In a second moment, when the cells are transferred to the freezing medium and the CAPs are added, there is an increase in the osmotic pressure in the plasma membrane. As it means that the cells began to lose fluid to the extracellular environment (dehydration), it does not mean that the cell will only leak fluid but then the osmotic pressure is bigger. There is an increase of membrane plasmatic pores with effected of high osmotic pressure.

Comparing the values of Elp, Vb, and Lpg between cells greater membrane permeability in Erythrocytes than in other cells was observed. So, for the same rate of cooling the lymphocytes, monocytes, and granulocytes present little or no dehydration in contrast to the erythrocytes which presented extensive dehydration. The distinct difference between the permeability of the membrane indicates that it will be difficult to freeze these types of cells together and reach high rates of survival [10, 64].

In Added, other points should be considered as the rate of water efflux between intracellular to extracellular medium as the conventional membrane-limited transport model with two well-mixed compartments which the solute transport neglected, the state of the system can be described by a nondimensional volume V, representing the volume of osmotically active intracellular solution, normalized to its initial value, Vo [3, 4]. Under the assumption that cell is always spherical, the rate of change of the normalized osmotically active volumes is given by [24, 65-66]:

$$\frac{dv}{dt} = -\frac{[(1-b)V+b]^{2/3}}{1-b} SJ_w \qquad (3)$$

(or)

$$J_w = \frac{L_p}{V_w} . (\mu^{in} - \mu^{ex}) \qquad (4)$$

t: time

b: osmolitically inactive fraction of initial cell volume

S: initial surface-to-volume ratio of cell

Jw: volume flux of water from intra to extracelluarmediun

Lp: membrane water permeability

Vw: specific volume of water

μin:chemicalpotencial intracellular water

μex: chemical potencial extracellular water

2.4. Cryoprotectants (CAPs)

Cryopreservatives are necessary additives to stem cell concentrates since they inhibit the formation of intra and extracellular crystals and hence cell death. As the name implies, CPAs are substances that provide a protective benefit during the freezing process. The two most commonly used CPAs are glycerol (GLY) and dimethyl sulfoxide (DMSO). Other substances used include sugars, polymers, and alcohols [4, 26].

The standard CAPs are DMSO, which prevents freezing damage to living cells [27]. The first practical cryopreservation of cells dates to the seminal report by Polge and colleagues (1949) of successful preservation of fowl spermatozoa and, a year later, of red cells by Smith (1950). These investigators used glycerol, establishing it early on as effective CAPs. Glycerol was subsequently proven effective for platelets (PLTs) in a study made by Arnaud and Pegg (1989), which are a particular challenge because of their limited tolerance to osmotic excursions [4, 27-30].

CPAs can be divided into two different categories: substances that permeate the cell membrane and impermeable substances. For example, in the group of permeable is GLY, DMSO, the propanodiol (PROH) and etileno glycol (EG) and between not permeable is compost by sacarídeos, like trealose, a glycose, and macromolecules such as a polyvinylpyrrolidone and sodium hyaluronate. These groups have different mechanisms of action, permeable substances cause a dilution of cytoplasm since they are small molecules they can cross the plasmatic membrane and form hydrogen bonds with water molecules in the cytosol. Thereby, the water freezing point decrease and reduces the probability of the formation of ice crystal inside cells [10, 31].

On the other hand, CAPs impermeable provide a colligative effect in solution. This reaction can be explained by the Law of Raoult. François-Marie Raoult (1830-1901) was a physical chemist and his

experiments demonstrated that steam pressure is decreased by the presence of non-volatile substances, where the decrease is directly proportional to the quantity of solvent material [32]. When CAPs are added there was/is a change in molarity of the solution, the vapor pressure decreased/decreases then the boiling point increased/increases and, consequently, there is cryoscopic reduction. In addition, the osmotic pressure also increases proportionally to molarity.

The use of CAPs is not the solution for all cryopreservation problems because independent of the type of CAPs and your mechanism of action, the addition of solute changes the tonicity of the solution (hypersaturated solution) and a change in osmotic environment. If the cell is impermeable to the CPA, the cell will monotonically shrink to a new equilibrium volume. If the cell is more permeable to water than the penetrating CPA, the cells will shrink initially and then swell to a new equilibrium volume. For an isolated cell, these transient excursions in cell volume can cause damage and potentially lysis [34]. The maximum step change in CPA concentration that can be tolerated is a function of a variety of factors including the permeability and molar volume of a penetrating CPA and other properties of the cell membrane [10].

There are many protocols of organoids cryopreservation, using a diversity of CAPs being the principal: DMSO. Protocols of liver and pancreas organoids used DMSO [36]; Stomach and intestine with DMSO [37]; Jejum used 10% DMSO and 10% fetal bovine serum or recovery cell culture freezing medium [35]; mouse small intestinal organoids used 10% DMSO [38].

2.5. Vitrification

Vitrification is characterized by high concentrations of cryoprotectants CPAs, providing viscosity to the vitrification solution at a sufficiently high value to behave as a solid, but without crystallization, is essentially a fluid with mechanical properties of a solid [39-41]. According to the laws of thermodynamics, if a liquid is cooled too fast at cryogenic temperatures (resulting in a drop in temperature > 10,000 C/ min) freezing can be avoided by turning into a highly viscous, amorphous state known as glass or solid glass [40, 42].

An increase in the concentration of cryoprotectants at levels sufficient to prevent the formation of ice at any rate of cooling is theoretically preventing a fall in cellular viability. However, this requires the introduction of much higher concentrations of cryoprotectants than those tolerated by the cells. Chemical toxicity is dependent on time, temperature and concentration, and this approach to vitrification has generally required both the careful formulation of multi-polar cryoprotective blends and their gradual introduction at increasingly lower temperatures. However, osmotic damage is increased by lowering the temperature, and thus any protocol for vitrification using this approach is often a compromise between the chemical toxicity inflicted and the osmotic damage in the cell [46, 47].

This remains a significant challenge for large tissues or, equally, for large volumes of functionally interconnected cells such as cell spheroids, as enough time must be given for CPA to penetrate into the all the cells, including those in the core, and for the core cells to dehydrate sufficiently to avoid intracellular freezing, risking toxic effects during CPA exposure and cooling. In the same way, thawing after vitrification is a critical point either, because high CPAs concentration on medium high concentrations, so equally the high warming rates needed to prevent devitrification and ice re-crystallization for all cells within a sample are difficult to achieve in large volumes [15, 43].

The first process of vitrification was described by Luyet (1939) who tested the water vitrification. Since then several techniques have been studied in order to reduce the amount of vitrification solution, obtain a greater reduction of temperature, and all this without causing major cellular losses [67, 68]. Examples and techniques of vitrification: conventional vitrification, closed pulled straw vitrification, open pulled straw vitrification, flexipet stripping pipettes, vitrification in electronic microscopy grids; direct cover vitrification, cryoloop, spatula vitrification, Needle Immersed Vitrification, solid surface vitrification and cryotop [42, 44-45, 48-53, 62].

However, vitrification has been used to preserve cells of humans and many species of animals with success. Vitrification allows the cryopreservation of small organs. For example, intact rabbit kidneys were cryopreserved by a vitrification process. When cryopreserved rabbit kidneys were implanted in an adult host they developed into functional kidneys with blood flow and no signs of rejection [54-56]. This technique was also adopted as a practical alternative method to the slow freezing of spermatozoa, oocytes, and embryos, both in human and veterinary reproductive medicine, due to the simplicity of its execution, economical profitability, and speed of the preservation procedure [57].

In the culture of organoids, studies were made to compare vitrification with conventional cryopreservation, in some of the better results than conventional methods were observed. In an experiment by Pendergraft et al. (2017) to test drugs in human testicular organoids were vitrified for 7 days at t -196 °C, returning to growth in culture after thawing. They concluded that over 90% of cells in cryopreserved 3D cultures retained viability after thawing and maintained viability for an additional 14 days in culture [58]. Another one, Spurrier et al. (2014) used organoids units were isolated from <3 week-old mouse or human ileum, where they were cryopreserved by either standard snap freezing or vitrification. In the snap freezing protocol, organoids were suspended in cryoprotectant and transferred to -80 °C for storage. The vitrification protocol began with a stepwise increase in cryoprotectant concentration followed by liquid super cooling of the organoids solution to -13oC and nucleation with a metal rod to induce vitrification. Samples were cooled to -80 °C at a controlled rate of -1 °C/min and subsequently plunged into liquid nitrogen for long-term storage (72 h) and thawed in a 37 °C water bath. After cryopreservation, the viability of murine was significantly

higher in the vitrification group (93±2%, mean ± SEM) compared to standard freezing (56±6%) and human cells demonstrated similar viability after vitrification (89±2%) [59].

26. Cryopreservation Organoids Protocols

In literature exist different cryopreservation/thawing protocols to intestinal organoids with a variety of results and methods to evaluate the viability of organoids after thawing. From them can be observed what has been done to maintain the characteristics and viability of intestinal organoids after their preservation (Figure 3).

1: Chandra et al., 2019 [63]:

Species	Organoids	Cryopreservation	Thawing	Results
 40 dogs	-Enteroids -Colonoids -Health and non-health (intestinal inflammatory disease and intestinal tumors).	- It was tested tow differents freezing medium: Recovery cell freezing media (Invitrogen) and 90% FBS + 10% DMSO - They opted to preserve whole canine enteroids and colonoids in liquid nitrogen using the commercial recovery cell freezing media.	- Thawed in cold CMGF-medium, centrifuged at 100g for 5 min at 4 °C, resuspended in thawed Matrigel, and seeded into 24-well plates as	It was not find a difference between the freezing media and the recovery cell freezing media in quality or quantity of both, entereoides and colonoids.

Advantages	The protocol was tested in health and non-health intestinal organoids; It was created a bio-archive of cryopreserved enteroids and colonoids for future use in basic and applied research Tested to types of different freezing protocols.
Disadvantages	It was just compared to the organoids cryopreserved/thawing with organoids in culture using microscopy, but possibilities about mutations or changing in the expression of specific genes were not describe. Even it was not used more significative laboratory testes to check if they really were growing equally, the comparison was empiric. It was can be done about the different cell freezing mediums tested. It did not specify the spets of freezing and thawing protocol, even what kind of freezing they did. Even how much longer the organoids were kept in liquid nitrogen.

2: Töpfer et al., 2019 [8]:

Species	Organoids	Cryopreservation	Thawing	Results
 96 wells	Colonoids	Cryopreservation in plate (96 wells): - 2h post-seeding was replaced with 100 µl Organoid-CPA (20min/37°C), - Gradient-frozen to a final temperature of -80°C using an EF600 gradient freezer, - Transferred to	- Organoids in plate were transfered to -20°C/30 min and 100 µl/ well warm unsupplemented culture medium was added, - Plates were warmed again at 37°C/ 3 min, - Supernatant was replaced with 100 µl/well unsupplemented culture medium, - Incubaded at 37°C with 5% CO_2 / 20 min, - Medium was replaced with 100 µl/ well bovine expansion medium.	It was not find sigmificantly a difference in outgrowth potential and sensitivity to staurosporine toxicity between the freezing colonoids and non-freezing.

Advantages	They suggested it can be used to different kinds of cells/tissue. The freezing/thawing organoids culture can be used in cytotoxicity screenings, bioprinting technology and to establish tissue-specific monolayer testing platforms.
Disadvantages	Organoids were just cryopreserved for a short period (1 day); It was described what concentration of cells/well

3: Han, et al. 2017 [35]:

Species	Organoids	Cryopreservatio	Thawing	Results
 C57BL/6 male mice (8-10 wk age, *n* = 4)	-Enteroids	-Used two medium conditions: ENR or ERN-CV. ENR: left intact or dissociated into single crypt-like colonies using enzyme-free cell dissociation buffer (STEMCELL Technologies Inc.). -Undissociated and dissociated organoids were resuspended in freezing medium, *e.g.*, 10% DMSO and 10% fetal bovine serum or recovery cell culture freezing medium (RCCFM; Invitrogen). - It was used ROCK inhbitor in three conditions: 30 min before freezing in the culture and in freezing medium and after thawing. - Storage in liquid nitrogen for 1-3 months.	- The organoids were quickly thawed, plated and used culture medium + ROCK INH (just for 3 days). - Cultivated by 7 days.	- The undissociated organoids showed better recovery (crypt-villus viseabled) from cryopreservation with 10% DMSO compared with that of dissociated organoids, - The direct addition of Y-27632 into freezing medium during freezing resulted in superior recovery compared with that of untreated organoids, organoids pretreated with Y-27632, or organoids treated with Y-27632 after thawing. - The undissociated organoids wich was added Y-27632 presented superior recovery after freezing/thawing.

Advantages	The quantitative analysis of crypt viability after freezing/thawing was used performed methyl thiazolyl tetrazolium (MTT) assays associated with morphology characteristics. The organoids were stored for a long time. It was tested 3 different methods to use ROCK INH and organoids dissociated or not
Disadvantages	It was described what concentration of cells/well Thawing methodology described superficially It was not checked the expression of specif genes as proliferation and differentiation after freezing/thawing.

Figure 3: Cryopreservation/thawing protocols to maintaining long culture of intestinal organoids in different species and your disadvantages and advantages.

Exist a different protocols of cryopreservation/thawing of intestinal organoids in different species, in all most, the DMSO continues been the most popular which have been proving great results when used in slow freezing protocols. However, some points discussed in this paper is not a routine in the labs of cell culture verify each protocol is the best to the specific specie in culture, even if the optimal freezing temperature related in literature is really the best. Some of them even not check if ice damage did not cause modifications in DNA after freezing/thawing which are a complex mechanism where the cells have a risk of damage in each step.

In one of the papers discussed above was cited the cryopreservation and thawing using plates (cells adhered to a substrate) to replace cryotubes (dispersed cells). Cryopreserving cells in an adherent state can significantly shorten and simplify the post thawing culturing steps, decreasing of cell culture time after theses process. Furthermore, this approach unveils new opportunities for specific in vitro cell-based assays [7]. Like the cryopreservation of dispersed cells, the cryopreservation of adhered cells faces challenges associated with avoiding cell injury, particularly within intermediate freezing temperatures -15°C to -60°C (intracellular ice formation and osmotic injury). But to adhered cells still exist that adjacent cells share cell-cell junctions, such as gap junctions, which allow solution transport between cells and can serve as a path for ice growth leading to sequential [7, 60].

27. Thawing and Devetrification

In addition, the freezing process maybe is not the only one that

causes cell destruction; the recovery process after thawing usually involves de-vitrification that can still induce ice crystal formation. Cryoinjury during the warm processes is thought to result in part from devitrification of the intracellular solution, the formation of new ice nuclei, or growth of the existing crystal in the cytosol upon softening of the glassy matrix of the solidified cell [61].

For a better understanding of cryoinjury during warm, it is necessary to know first what devitrification. It is an act or effect of devitrification which through some substance in vitreo states changes to crystal state. Disvitrification in aqueous cryoprotectant solution has been investigated experimentally and theoretically, to evaluate the stability of vitrification solutions and to estimate the minimum warming rate required (It is a variety with species evolution) to avoid ice formation in the extracellular/intracellular solution [24].

It has been a developed researches line to discover how to avoid devitrification effects. One way to prevent devitrification during the warming process, hydrogels have been used to protect the cryopreserved cells/tissues in a rapid recovery procedure to enhance the viability of cells in cryopreservation [5-6, 25]. Lu and collaborators (2017) combined core-shell hydrogel capsules and cryoprotectant together to protect organoids from both vitrification and devitrification. It was compared with the bulk Matrigel™, the capsules had a much higher recovery of cryo-preserved organoids. While in bulk, just small cell aggregates could grow into organoids after thawing and re-culturing in Matrigel™ (larger ones failed to survive), the recovery efficiency was only around 20%. On the other hand, in capsules even whole organoids could survive and grow after recovery from the cryo-storage, and the cell viability was improved to 80%. In the recovered-cell from the core-shell group was verify even one week before the standing to LGR5-eGFP and CD44. Thus, their results suggested that the core-shell hydrogel structure might suppress the ice formation during cryopreservation; however, it is important to note, they related the alginate shell deformed due to the mechanical force generated during freezing or thawing process, the capsules clearly played a protective role in the cryo-storage of organoids [6].

3. Conclusion

Many variables should be considered (freezing rate, cell types, culture time, cell concentration) that will determine success after thawing. However, it is not a routine to check the viability of cells with all these variables after, and before freezing/thawing, do it can assist to understand which kind of conditions is better to reduce the time expended in culture after this process and the cellular damage. It is known that after freezing there is a great loss of cells, but there has been no quantification of cell death. Therefore, more studies need to be done regarding the membrane permeability of different cell types in different media to optimize freeze protocols. After all, questions surrounding cryopreservation and cell thawing protocols with the least possible damage, so that the cell can return to its primary functions are very strong issues that have been debated for years, including study groups that come this way in cryopreservation as a way to prolong life by running away from imminent death. More information about the physiological mechanisms in the cells when subjected to different concentrations of solutions at different temperatures, can not only assist in the advancement of organoid's technology but, also, in all areas of Regenerative Medicine that the storage of cells and tissue are critical points to their development and clinical application (as the cells banks).

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