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PERSPECTIVE

PRODUCTION AND CRYOPRESERVATION OF 3D CULTURES

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Abstract

Three-dimensional (3D) culture systems, which include spheroids (SPs), provide a unique platform for studying complex biological processes in vivo and for enhancing the capabilities of in vitro test systems. Their uniqueness lies in the 3D organization of cells and in the reproduction of complex intercellular interactions, similar to those in native tissues and organs. These "mini-organs" can be used for fundamental research, tissue-engineering constructs, development of preclinical models for testing pharmacological drugs, etc. Important and current issues regarding SPs involve improving methods for their production and cryopreservation. Solving these issues will expand the range and effectiveness of their use in tissue engineering. Here, we describe the authors' research and experience on factors influencing the formation of SPs, which can enhance the understanding of their correct application and standardization. A crucial aspect of this review is the information on applying theoretical approaches based on physico-mathematical calculations to improve the quality of existing cryopreservation protocols for SPs.

Keywords: cryopreservation, spheroid, parameters affecting the spheroid formation, theoretical models.

INTRODUCTION

The limitations and imperfections of traditional models (animal models and 2D cultures) have stimulated the development of and search for effective test systems (1, 2). Specifically, research results in animals often cannot be extrapolated to humans due to species-specific differences and innate differences (e.g., in the immune system). Additionally, pathogens can mutate and adapt to animal models (2).

The use of 2D cultures as test systems does not allow one to reproduce crucial aspects such as the complex microenvironment and sophisticated communication network of biochemical and mechanical signals between cells and the intercellular matrix, similar to those in the organism (3, 4, 5, 6). 2D models cannot replicate processes that are critical for assessing tumor progression, chemoresistance and treatment response (7). The transplanted cells, that are typically introduced into solution as monodisperse cells, quickly die or fail to sufficiently localize at the injection site. This limits the anticipated therapeutic effect (such as for injuries) to just a few days (8). The use of 2D cultures creates significant differences compared to conditions and processes in living organisms,

specifically in terms of the specialized functions of cell division and migration, gene and protein expression, signal transmission and cell sensitivity to various stimuli (7).

Therefore, 3D cell models are an essential addition and alternative to traditional in vivo models and 2D cultures (3, 4, 5, 6). It is believed that 3D models can bridge the gap between in vitro and in vivo studies. Intercellular interactions, secretion of trophic factors and increased viability are considered as key advantages of 3D cultures compared to 2D cultures (9).

Currently, there are several types of 3D models: SPs, organoids, 3D printing, and organ-on-a-chip. All these models are not ideal, they have their advantages and disadvantages, which have been detailed by some authors (2, 10). The use of these model systems is individualized and depends on specific experimental tasks. Their general advantage over 2D cultures lies in their approximation to native tissues and in the reduction in the number of experimental artifacts (2, 10).

The most complex and closest to the body of all 3D models are organoids (7). They perfectly imitate the physical and chemical conditions of a specific tissue. Unlike SPs, organoids have a more organized cellular architecture when cultured on specific matrices, resulting in a structured arrangement of cells. They are widely used in in vitro modeling, including the development of organ-on-a-chip models (2). How are organoids obtained? From what types of cells are they formed? These organotypic cultures (formations) arise from tissue-specific adult stem cells (ASC), embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) (8). Today, significant progress has been achieved in obtaining organoids from cells of various origins. For instance, several studies have investigated organoids derived from human intestine, lungs, kidneys, brain and liver cells, as well as from primary cancers of the colon, prostate, and pancreas (1, 11, 12). However, one of the main challenges lies in their reproducibility (2), which is a significant limitation for the potential applications of organoid 3D cultures. Another significant disadvantage of organoid cultures is their inability to accurately reproduce the complex nature of cancer metastasis involving the affliction of several organs. During the cultivation of organoid explants, random changes in their subpopulation composition can occur (some cells may migrate away from the explant, while others may die off, etc.). All of the above complicates the standardization of such cultures and reduces the reproducibility of the experimental results obtained (2, 7, 10).

3D printing is used to improve reproducibility, to eliminate variability in organoid sizes and to increase the scale of their production (2). However, the limitations of this model in replicating microscopic vessels and reduced cell viability restrict the use of 3D printing as a test system.

Considering these issues, cellular SPs are a useful alternative to organoids, as their production can be more effectively standardized by taking into account the specific features of their structure and formation.

However, despite numerous studies on the production and application of 3D cultures, there are still some unresolved issues related to their mass production, high-throughput screening of drugs and effective low-temperature storage technologies.

The viability of complex 3D cultures is an important problem for their production and low-temperature storage. Their viability depends on the diffusion of nutrients into the inner layers of SPs (13, 14). Numerous platforms and various equipments for cultivating SPs of different sizes and empirically selected cryopreservation protocols elaborated for single cells do not give basic answers about the causes of the hypoxic core formation (region of hypoxia with limitation of natural oxygen diffusion) and its effect on the viability and metabolism of SPs (15). In this regard, the elucidation of major causes that will allow improvements in the quality of SP derivation and low-temperature storage is an urgent objective of current cellular technologies (9, 16, 17).

It is known that multicellular objects can be obtained from both healthy and diseased donors (including those suffering from cancer) and that they can mimic the architecture and physiology of the organs from which they were isolated (11). They can also be obtained from different cells (9, 16, 17, 18, 19).

Features of the intercellular adhesion-based 3D culture structure and formation as well as advantages of 3D cultures over 2D ones are described in many publications (1, 3, 4, 5, 6, 12, 20, 21, 22, 23, 24, 25, 26, 27). Moreover, many parameters should be taken into account when obtaining robust three-dimensional objects. For

example, numerous publications (4, 6, 15, 28, 29, 30, 31, 32) have stressed the importance of volume, diameter, external morphology, transparency (spheroid compaction indicator), growth kinetics (including growth delay) and invasive potential of SPs (Fig. 1).

Let us consider the factors that affect the above parameters and the viability of 3D objects during their production.

FACTORS AND PARAMETERS AFFECTING SP FORMATION

Penetration of compounds into SPs

The penetration of nutrients into SP cells is an important factor affecting the formation of SPs (33). It is known that SPs become larger during cultivation and that the number of cells and the volume of the intercellular matrix (ICM) in them change (6, 15, 34, 35). ICM is usually fibrous, viscoelastic, and cell-adhesive. The ICM composition includes substances that can affect its hydrophilic characteristics, such as proteoglycans and glycosaminoglycans (34, 35). The ICM accumulation during SP cultivation and its influence on cell functions and diffusion of compounds through cellular membranes are debatable issues. For example, having constructed a physical/mathematical model of the osmotic behavior of SPs in cryosolutions, Moiseiev et al. calculated the permeability coefficients for water and DMSO molecules in SPs from L929 cells (35). These authors revealed that the extension of SP cultivation time (from 7 to 21 days) led to an increase in SP size (from 60 to 150-200 μm) and to a decrease in the permeability of water and DMSO molecules in SPs. When analyzing the permeability of cells in 100 μm SPs (100 cells) depending on their position, Moisieiev et al. found a 3.2-fold drop in DMSO permeability coefficients for cells of the inner layer compared to cells of the outer layer (35). At the same time, on the contrary, the energy-consuming processes [energy activation (EA)], which are necessary for the transport of compounds through the cell membrane, were enhanced when cultivation was prolonged. This phenomenon appears to be logical. Such changes were attributed to an enlarged size of SPs, resulting in the increased number and density of cells and matrix amount in SPs (34, 35). Similar assumptions were published by Kim et al. (6) who studied the effect of intercellular matrix on SP fusion. These authors showed that ICM volume increased during SP cultivation, which, in their opinion, slowed down SP fusion. However, in another study (12, 15), the authors came to different conclusions. Thus, when SPs were enlarged from 15,000 to 30,000 and 60,000 cells per SP, their packing density in the inner layers and the ICM amount were reduced. At the same time, the number of cavities detected with hematoxylin and eosin staining increased throughout the SP structure. The outer layer of cells appeared to be more densely packed compared to the inner layer of the SPs for all three sizes (15,000, 30,000, or 60,000 cells per SP). Regarding ICM accumulation in SPs during cultivation, it was discovered that the synthesis of ICM proteins (laminin, elastin, type 1 collagen and fibronectin) was enhanced (15). However, cell-ICM interactions in SPs, which affect SP functional potential, were weakened as SPs became bigger (36). The above results on ICM changes during SP cultivation remain a debatable issue and depend, as described previously, on some parameters (type of cells, number of cells).

The effect of SP size on viability and hypoxic core formation is also a matter of debate. It was proven that SP enlargement diminished cell metabolism and enhanced cell apoptosis (15). Insufficient oxygenation of cells of the inner layer is one of the hypotheses to explain this phenomenon. However, the results of a study (15), which investigated the effects of oxygen tension on hypoxic core formation and cell functions in SPs created from mesenchymal stem cells (MSCs), contradict this opinion. The authors measured the oxygen tension for MSC SPs of different sizes (15,000, 30,000, or 60,000 cells) and, based on mathematical calculations, found that, regardless of size, the oxygen tension varied by <10% from the outer layer to the centre of MSC SPs. The researchers believed that this indicated a reduced packing density in the centre of MSC SPs as the SPs became larger which, as a result, facilitated the transport of oxygen and nutrients to the centre of MSC SPs. These authors associated such changes with an adaptive decrease in the density of matrix deposition and packing with an increase in SP diameter, which prevented hypoxic core formation in SPs (15). However, despite a slight change in oxygen tension in the inner layers of SPs, their cellular metabolism, in particular glucose absorption, significantly declined in line with an increase in the number of cells and in

the size of spheroids. These findings were in agreement with other studies (34, 35, 36). In addition, in the Murphy et al. study (15) the initiation of cell apoptosis in SPs with an increase in the number of cells and without a hypoxic core was also noteworthy. This indicated that other ways of apoptosis initiation, which are not related to hypoxic nuclei or cell density, exist in 3D aggregates. Therefore, based on the above, the functioning of an SP depends more on its size than on the hypoxic core formation, and the area of the hypoxic core depends on the number of cells in an SP, as shown by Murphy et al. (15). Thus, these authors registered a significant increase in the hypoxic core area when the number of cells in MSC SPs increased to 250,000.

In the Murphy et al. study (15) there was no correlation between the size of SPs, which were derived from cancer lines, and cell packing density. This indicates that SP formation depends on the species specificity of cells. That is, there are no unequivocal "hypoxia and metabolic activity - SP size" relationships; each case is individual and multifactorial.

Species specificity of cells

Di Caprio et al. (12) proved that SPs could be heterogeneous in composition, which makes them similar to the tissue structure. Thus, Zinn et al. (18) demonstrated successful SP development using different human cells (HH, HepG2, Hep3B, SNU-1079, and HuCCT1) in combination with three supporting cell types (HSC, HDF, and HUVEC). When the SP production protocol was adhered to, highly viable, non-aggregating spherical SPs (with a diameter of 200–400 μm) were formed. However, depending on the type of cells, the SP yield varied (18). The species-specific cryosensitivity of cells, specifically the physicochemical properties of membranes of different cells, was shown by Kovalenko et al. (37). These authors explained such discrepancies by differences in the viscosity of membrane lipids in cells of different origins. For example, the viscosity of the lipid phase of rat erythrocyte membranes was significantly lower than that of rabbit erythrocyte membranes, which, in turn, may be a cause of rapid penetration of diols and amides into rat erythrocytes (37). Similar discrepancies can occur in different types of cells in SPs. Numerous studies demonstrated the effects of the cell type on their ability to form SPs as well as on SP formation rate and morphology (38). Ferrari et al. (39) reported that SP size depended on the cell line. Murphy et al. (15) showed that the content of intercellular adhesion proteins and the oxygen gradient in SPs also varied among different types of cells.

Special attention is drawn to coculture models, which can reproduce interactions of different types of cells. It is believed that such cocultures will help to clarify the effect of treatment on tumor growth, vascularization, metastasis, and response to chemotherapy (16).

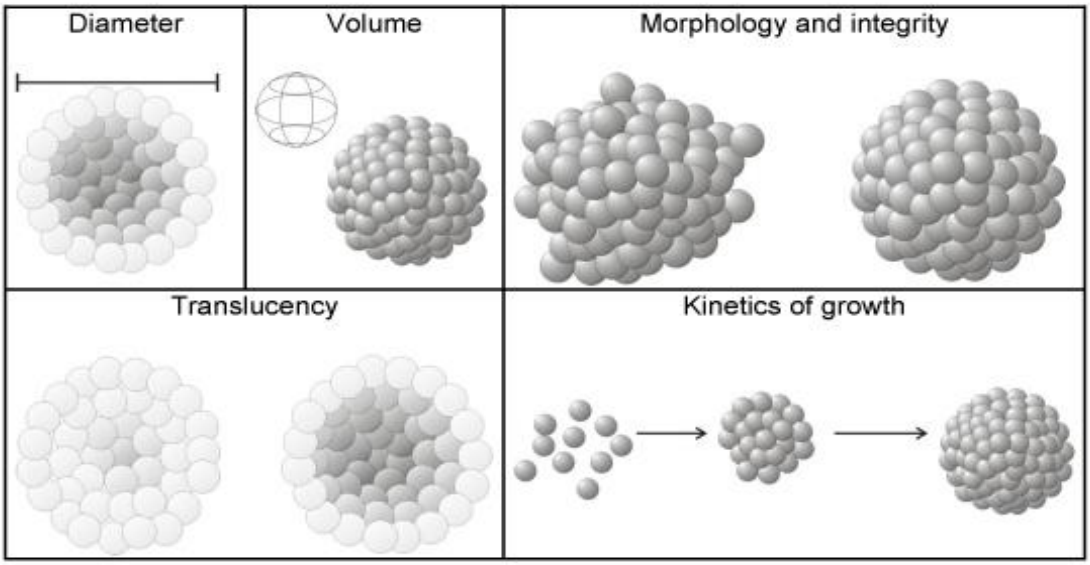

Figure 1. Properties of spheroids that can be assessed using label-free brightfield and phase-contrast microscopy

Porter et al. (40) presented an in vitro coculture model based on immortalized insulin-producing beta cell lines and human endothelial cells in 3D SPs, the goal of which was to mimic islet morphology and to develop a standardized cell model for in vitro diabetes studies. It was found that the metabolic activity was higher in this coculture compared to monoculture, and glucose-stimulated insulin release increased more than 20-fold. In the Brylka et al. study (41) of the interdependent interactions of mesenchymal stromal cells (MSCs) with squamous cell carcinoma (SCC-040 cells) in 3D SPs, the genetic expression of Akt pathway proteins (mTOR, Erk1/2, and S6 p70 kinase) changed in the early stages of tumor development, which may indicate a turning point in tumor progression.

Mechanical stress

Delarue et al. (42) showed that physical properties such as local pressure or local tissue stiffness, could also be important parameters in the regulation of tissue growth. In particular, some genes in *Drosophila* embryos were shown to be mechano-sensitive, i.e., their expression was considerably modified when the pressure in the tissue changed or when the tissue was subjected to mechanical stress. Cell division and death (apoptosis) may also depend on local stress in the tissue (42). This stress dependence provides a vital feedback mechanism for tissue growth that is important in determining the final tissue morphology. This issue also concerns SPs. Cheng et al. (43), Dolega et al. (44) and Delarue et al. (45) proved that, in addition to well-studied parameters of the microenvironment such as hypoxia and angiogenesis, the growth of multicellular objects (tumors) was affected by mechanical stress. In particular, Helmlinger et al. (46) suggested that mechanical stress could affect the phenotype of cells in tumors and, as a result, their proliferation and apoptosis. Multi-cellular spheroids (MCSs) were cultured in porous agarose gels of different stiffness and it was shown that the stiffer the gel was, the smaller the MCSs became. A similar conclusion was drawn by Dolega et al. (44), who developed a microfluidic device for MCS formation in elastic permeable capsules. Delarue et al. (45) demonstrated that SPs from different cell lines subjected to compressive mechanical stress grew more slowly. A sequence of events starting with a decrease in cell volume, followed by reversible induction of a proliferation inhibitor (p27 Kip1) from the center to the periphery of the SP, led to a reversible decline in proliferation. At the same time, the response to mechanical stress was quantitatively the same in different types of cells, despite significantly variable cultivation conditions (47). From the Dolega et al. study (44), it is known that active and passive mechanical forces, which arise as a result of growth, control the tumor spread and the formation of new metastases.

Impact of the cultural environment composition and derivation methods on SP formation

Many studies have focused on how the composition of cultivation media and methods affect the properties of cells in SPs (12, 18, 27, 48, 49, 50, 51, 52, 53, 54). For example, Ryu et al. (55) showed that medium composition and culture method were critical in controlling the phenotypes of patient-derived breast cancer cells (PDBCCs). Sachs et al. (11) developed cultivation protocols that allowed for the generation and long-term reproduction of 3D epithelial organoids. In contrast to the cultivation method developed by Ootani et al. in 2009 (56), adult stem cell cultures lack mesenchymal cells and usually require niche factors, such as mitogenic epidermal growth factor (EGF), Wnt agonist R-spondin, transforming growth factor beta (TGF-β), and noggin as well as extracellular matrix surrogate basement membrane extract (BME or Matrigel). Depending on the species and organ, the following additional supplements are common: Wnt-3A and fibroblast growth factor (FGF) 10, activin receptor-like kinase (ALK) inhibitor A83-01, p38 mitogen-activated protein kinase inhibitor SB202190, and nicotinamide (57, 58, 59).

It should be noted that SP formation mechanisms also depend on the numerous production methods available and on the choice of appropriate biomaterials (12, 18, 19, 27, 48, 49, 50, 51, 52, 53, 54). All these methods have advantages and disadvantages, which are well described in many publications. However, today, the effect of size on SP viability is an important problem in SP production. Thus, large and small SPs appeared to respond differently to the same stimuli. In particular, Cheng et al. (43) demonstrated the effect of SP diameter on the proliferation, differentiation, and cytotoxicity of cells in SPs. It has also been proven that diameter is one of the main criteria for selecting

SPs for effective screening of effectiveness and cytotoxicity of drugs. Therefore, given the challenge and importance of the production of SPs with controlled dimensions, methods that ensure the controlled formation of a large number of viable cell spheroids of identical diameter drew special attention (18, 28, 29, 38, 43). Thus, Han et al. (38), proposed to use a two-phase aqueous system of polyethylene glycol and dextran with a controlled density to create SPs of the same size. This method significantly reduced the manipulation time of SPs, minimized errors, and boosted the reliability and efficiency of SP production. Schmitz et al. (60) showed that not only the number of cells but also platforms for SP production played an important role in the hypoxia evolvement. They found that hypoxia was detected earlier when ultralow adhesive plates were used in comparison with the hanging-drop method (60). This study highlighted that not only the cell type, passage, or SP diameter were crucial parameters for investigating 3D cell aggregates, but also that the actual fabrication platform critically affected the final state of the SPs. Unified platforms for SP cultivation and formation will contribute to the generation of comparable data by different research teams and increase the scope of useful information for 3D culture production.

SP CRYOPRESERVATION

Effect of SP diameter on the efficiency of cryopreservation

The standardization of SPs as cryopreserved products that can be used immediately after thawing for various laboratory studies is a logical development for their use. Therefore, today, attention is focused on experiments that aim to optimize the cryopreservation process, based on the selection of SP size, composition of cryopreserved solutions, cooling rate, etc. (61). Numerous researchers have highlighted issues concerning the quality of cryopreserved SPs with uncontrollable dimensions (4, 6, 29, 31, 33, 43, 62). In particular, Shajib et al. (63) sought to establish an optimal SP size from two widely used cancer cell lines, C4-2B prostate cancer cells and MCF-7 breast cancer cells, and an optimal cryopreservation medium. C4-2B and MCF-7 cells were assembled into tumor SPs using a microwell platform; then they were cultured for 48 h. This cultivation method allows one to effectively mass produce SP tumors of the same size, which helps to optimize the SP formation process. Tumor SPs assembled from 100 cells each were found to yield highly uniform tissue, and these SPs were more viable than those assembled from 200 or 400 cells in the initial cryopreservation experiments. Fresh and cryopreserved C4-2B or MCF-7 tumor SPs responded similarly to the test drug (docetaxel). Park et al. (64) discovered that microcardial SPs with a diameter of 100 μm, which were generated from CD71+ hESC and hiPSC-CMs, showed significantly higher survival both under hypoxic conditions for 7 days and when frozen, compared to single hiPSC-CMs or larger aggregates of hiPSC-CMs (over 300 μm). In addition, a post-thaw survival assay showed that 80% of frozen SPs (smaller than 300 μm in diameter) remained viable after 6-month cryostorage. These authors also suggested that the conspicuous expression of connexin 43 protein inside microcardiac SPs (due to the proximal intracellular contact of hiPSC-CMs) to maintain their integrity seemed to be the main mechanism for better survival under a hypoxic environment and upon freezing.

Cell plating concentration is another important factor influencing SP size (65). For example, Decarli et al. (66) showed that the SP diameter typically ranged from 50 to 800 μm. Gaskell et al. (29) found that, when SPs were formed from C3A hepatocarcinoma cells on a non-adhesive surface, the optimal initial concentration was 750 cells per 100 μL of medium. Wang et al. (67), using gastric cancer cell line HGC-27, showed that the SP diameter growth over time depended on cell plating concentration. The SP size was proportional to the initial number of cells. Thus, at a plating concentration of 300 cells per well, the SP diameter on cultivation day 4 was 400 μm (67). Zheng et al. (30) evaluated the effect of cell plating concentration (1×10^5, 2×10^5, 2.5×10^5, 5×10^5 cells/mL) and of KnockOut™ serum concentration (0, 5, 10, 15%) on the number and size of SPs derived from human dental pulp progenitor stem cells (DPPCs). The results showed that plating concentrations > 2×10^5 cells/cm² and high concentrations of serum (10–15%) led to a fusion of SPs. Pinto et al. (4, 31) gave examples of the effects of plating concentration of different cells on SP formation. Tan et al. (31) modified the number of pluripotent cardiomyocytes (hiPSC-CMs) per SP

and outlined the principle of determining an optimal cell concentration for SP formation. This is related to two competing factors: reduced supply of oxygen and enhanced three-dimensional adhesion of cells due to an increase in the number of cells per SP. Therefore, taking into account these factors, the optimal concentration of cells per SP maximizes the beneficial effects of the microenvironment of three-dimensional structures. Bozhok et al. (65) tested three concentrations of L929 cells (1×10^5, 2×10^5, and 5×10^5 cells/mL) and found that the plating concentration of 2×10^5 cells/mL was optimal for the formation of viable SPs under anti-adhesive conditions. Murphy et al. (15) also demonstrated that the diffusion of nutrients diminished with an increase in the diameter of SPs, leading to a decrease in their metabolic and proliferative potentials and a boost in apoptotic processes.

Current approaches to SP cryopreservation

Current studies on the selection of SP cryopreservation methods are empirical and lack a thorough theoretical approach. It was experimentally established that it was advisable to use a penetrating cryoprotectant, DMSO, at a concentration of 5–15%, for cryopreservation of SPs (68, 69, 70, 71, 72). In several studies (68, 73, 74, 75, 76, 77), attention was paid to reducing DMSO concentration upon cryopreservation in order to abate its toxic effect and to develop serum-free media for thawing in order to optimize the use of SPs in clinical settings. Ma et al. (78) investigated the effect of DMSO concentration (3, 5, 7, 8, 10, 15, 20%) combined with the cooling rate of 1°C/min on viability of neurospheres of different diameters obtained from hippocampus. DMSO at 8% was optimal for 80–100 μm SPs, while 7% was the optimal concentration for 30–50 μm SPs. Dong et al. (73) determined the optimal concentration of DMSO (5%) for cryopreservation of SPs from mesenchymal stromal cells isolated from compact bone-derived mesenchymal stromal cells (BM-MSCs). It was shown that, upon freezing, this concentration did not affect stemness and osteogenic capacity of CB-MSC SPs, raising the possibility of using cryopreserved SPs for bone tissue engineering immediately after thawing without osteogenic induction.

There is a growing interest in chemical compounds and materials that can mitigate cooling-induced injuries, in particular those that are caused by crystallization and apoptosis initiation. Induced nucleation is known to be beneficial for the viability of many cell types after thawing (79, 80). In the Gao et al. studies (81, 82), it was shown that inoculation of soluble ice nuclei (pollen polysaccharides) to prevent supercooling and mitigate intracellular crystallization improved recovery of A549 (human alveolar basal adenocarcinoma) and SW480 (frozen/thawed adenocarcinoma) SPs. A combination of this strategy and of pre-incubation with proline upon freezing in microwell plates increased total recovery of SPs from 40 to 70%, which was shown to be associated with a decrease in the amount of reactive oxygen species and in a rise in F-actin polymerization.

Bissoyi et al. (83) showed that cryopreservation of HepG2 hepatocyte SPs in a standard DMSO solution supplemented with polyampholytes significantly improved viability of cells after thawing, as such cells had more intact membranes. These authors suggested that post-thawing recovery of cells might be associated with preservation of actin polymerization. In addition, after thawing, SPs cryopreserved in standard polyampholyte-containing DMSO solution had a toxicological response to a chemotherapeutic drug (doxorubicin) which was similar to that of native (unfrozen) SPs. It was previously reported that polyampholytes functioned extracellularly in cell monolayers and enhanced dehydration and ion flux control, preventing osmotic shock (84). This may be a key advantage when multicomponent cryoprotectants, including inhibitors of intracellular ice recrystallization, are used for cryopreservation of multicellular structures.

A solution containing human serum albumin (HSA) and N-acetylcysteine proved to be more efficient for cryopreservation of hepatocyte SPs for maintaining increased viability, reducing ammonia levels, enhancing urea secretion, and boosting albumin synthesis compared to a medium with 10% fetal calf serum (74). The effectiveness of using a serum-free medium (commercial cryopreservation medium CryoStor® CS10) for cryopreservation of MSC SPs was also demonstrated (85). Analysis of viability, SP surface morphology, and expression of stem cell markers showed that these parameters were relatively higher after one freeze/thaw cycle in CS10 compared to traditional medium based on Dulbecco's

modified medium with 20% fetal serum and 10% DMSO.

Theoretical approaches to the elucidation of SP formation and of cryopreservation features

Taking into account the importance of cryopreservation parameters in this review, we would like to focus on approaches for selecting effective protocols of SP production and of cryopreservation. At present, there are effective, theoretical calculation-based methods of determining some parameters of 3D cultures. For example, through calculations, Decarli et al. (66) determined the cost of reagents and the amount of obtained SPs. This study provided insight into the quality, reproducibility and cost of implementing 3D models. It was proven that mathematical modelling was an effective approach to the prediction of biological processes and analysis of complex physiological interactions (86, 87). Murphy et al. (15) used a physical/mathematical approach to determine the oxygen tension depending on the radius within SPs. Many discussions concern the effect of mechanical stress on cell growth in the inner layers of SPs. Owing to physical/mathematical calculations, Dolega et al. (44) proposed a model that predicts how the resulting volume modulus of an aggregate and the hydraulic diffusion of leaking intercellular fluid change under the influence of the stress inside the aggregate. Delarue et al. (42) presented theoretical calculations of the response of SPs to changes in external pressure. In this study, it was shown that cell density increased in the SP center 5 min after the pressure spike, but did not change significantly near the SP surface. These authors explained these findings by cell polarization. Based on the experimental results, they proposed a theory that takes into account the dependence of cell division and apoptosis rates on local stress, cell polarity, and active stress generated by cells. Ruske et al. (88) used both analytical arguments and three-dimensional modelling and revealed that proliferation gradients resulted in fluxes and activity gradients and that they both could align the axes of cell orientation within aggregates. Depending on the environmental conditions and on the internal properties of a tissue, the authors identified three different modes of alignment: SPs where all cells were aligned radially, SPs where all cells were aligned tangentially to the surface throughout the aggregate, and SPs with an angular orientation of cells close to the surface and radial alignment in the core. Their research allowed not only drawing conclusions about the dynamic parameters of cells but also elucidating new mechanisms for controlling the alignment of cells inside aggregates, which could affect mechanical properties and invasive potentials of tumors. The study of Amereh et al. (89) introduced a concept that shed light on the relationship between mechanical stress and

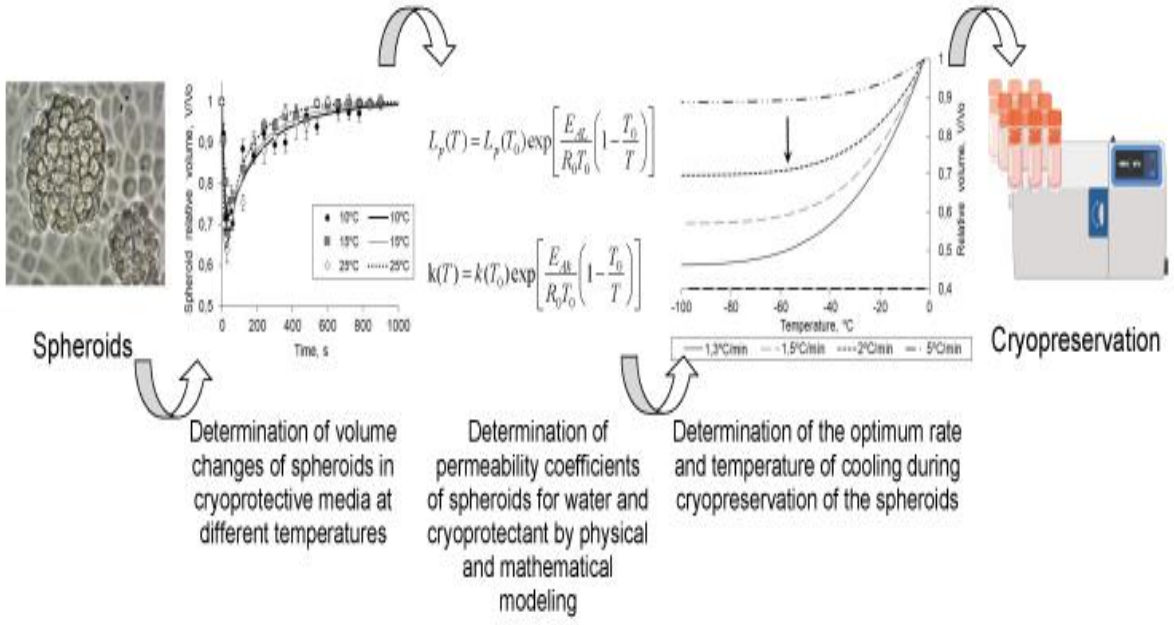

Figure 2. Algorithm for determining the optimal cryopreservation modes for spheroids based on physico-mathematical modeling.

biological responses in tumors. Based on theoretical calculations, the authors elucidated the role of nutrient gradients as one of the key factors influencing tumor progression, its biomechanics, deformation, asymmetric remodeling and stress distribution.

It is known that theoretical modelling has a huge impact in cryobiology as well (90). Theoretical calculations in cryobiology are believed to advance our understanding of the mechanisms underlying cell damage caused by low temperatures and to aid in improving cryopreservation protocols (87). For instance, there are some contemporary studies that represent methods of mathematical modeling related to heat and mass exchange processes during cryopreservation. Models span many spatial and time scales, ranging from cellular to tissue levels (91). Modeling membrane transport has long been utilized for the development of cryoprotective media, aiming at preventing excessive changes in cell volume and at minimizing protocol duration and cryoprotectant toxicity (92, 93, 94). Studies are underway to develop a more comprehensive framework for modeling tissue transport (95). Hence, a mathematical model of water and dissolved substances transport was developed for whole islets of the hamster pancreas. This model allows the preservation of their structural integrity and the optimization of cryopreservation protocols (96).

Olver et al. (97), using physico-mathematical approaches, presented an algorithm for calculating osmotic and cytotoxic damage to sea urchin oocytes during cryopreservation. Specifically, these calculations aimed at determining the optimal DMSO saturation time (ranging from 2 to 30 min), temperature (1.7°C, 10°C or 20°C) and concentration. These authors suggest that the proposed model, based on physico-mathematical calculations, enhances cell viability and elucidates important mechanisms of cell damage.

However, despite a rather extensive implementation of theoretical calculations for three-dimensional objects, few effective protocols of SP cryopreservation are based on theoretical calculations. For example, in Tarusin et al. study (98), theoretical calculations were used to improve the quality of cryopreservation of encapsulated isolated cells. Moisieiev et al. (34, 35) determined the cryopreservation parameters for SPs, which were obtained from L929 cells under anti-adhesive conditions at different timepoints of cultivation. Having analyzed the osmotic behavior of SPs in cryoprotectant solution using physical/mathematical simulations of mass transfer processes, the authors determined the time of exposure to cryoprotectant at different temperatures of cryosolution, temperature, and cooling rate (Fig. 2). The authors treated the SP as a whole entity and considered its integral characteristics, which determine the time parameters of mass exchange between the SP and the medium, as analogues to the permeability coefficients of individual cell membranes. In several studies (34, 35, 61), it was theoretically determined that the time of exposure of SPs to 1 M DMSO was 10 times shorter than that of the standard protocol (10-15 min). The authors established that the time of exposure of SPs to 1 M DMSO was significantly shortened when the temperature was raised from 10 to 25°C. At the same time, the permeability coefficients for water and DMSO molecules increased, while, on the contrary, the energy consumption for compound penetration decreased. The feasibility of the calculations used in that study was proven by the authors in practice (61). Thus, due to the implementation of the theoretically calculated mode, the number of viable, functional, and full-featured SPs significantly increased compared to the standard modes (61, 99). The authors claimed that the proposed approach for determining the optimal time of saturation with a penetrating cryoprotectant could be used for SPs of any diameter, derived from any cells at any cultivation period, and at any cryosolution temperature (34, 35). Calculations can serve as an alternative to routine experiments and studies aimed at reducing the toxic effects of DMSO by lowering its concentration in the cryoprotective medium. In the study of Gordiyenko et al. (100) the usefulness of the aforementioned theoretical approach in determining the optimal cryopreservation protocol was confirmed for SPs derived from another cell type - mesenchymal stem cells.

CONCLUSIONS

Theoretical approaches and simulations based on physical and mathematical calculations for 3D cultures can become an alternative to routine studies and contribute to the improved

production, cryopreservation, and efficiency of 3D cultures in cellular biotechnologies.

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