PACT Web Seminar 9: Question and Answer Session
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Cryopreservation: Troubleshooting
Speaker: Dr. Allison Hubel

Question 1: One of the most common errors associated with the development of cryopreservation strategies is linked to the timing utilized to assess cell viability. Typically, cryopreservation viability assessment is conducted immediately post-thaw (usually within an hour). There are numerous assays that are used to assess, viability. The most common one used is the Trypan Blue dye exclusion assays. Do you know of any other assays that give accurate cell viability assessments?

Answer: I do not recommend, nor do any of my colleagues in this field, using trypan blue dye. You have a wealth of options available to you. If you are interested in fluorescent dyes to determine membrane integrity, there are a huge number of probes. There's propidium iodide, there's fluorescence diacetate, ethidium bromide. There are a wide variety of kits of pre-made probes that are available through Molecular Probes. Now, you still need to understand that determining membrane integrity using a fluorescent dye only gives you a small snapshot on post thaw viability. As the question indicated viability changes with time post thaw and it is unclear what membrane integrity correlates to in terms of functions of the cells. That's why you typically need to use more than one assay. In the hematopoietic stem cell regime, we use colony forming assays and other types of assays that involve more than assessing membrane integrity. So you would really have to look at what cell type you are talking about in order to answer that question more explicitly, but you have identified some very important issues. First of all, the timing of the assay and how you interpret it. You need to be fairly conservative.

Question 2: Trehalose has been shown to have a protective benefit during conventional slow freezing. Do you know if the cell/ice interactions have been characterized in non-planar freezing conditions?

Answer: Speaker indicated that this information is available in a recent manuscript.

Question 3: How in depth will your course go into protocol development?

Answer: We do spend quite a bit of time on it. Some of these slides actually have come from lectures in the short course. We go through each of the steps of the cryopreservation protocol and tell you potential experiments you can do to verify or validate a protocol element that you've developed. So we do go quite a bit into protocol development.

Question 4: Are there any suggestions for storage conditions of post thaw samples prior to post viability testing?

Answer: When a cell has been thawed, you are bringing it back into normal physiological temperatures, and those cells are both nutrient and oxygen deprived. First of all they are in an

environment that is not physiological. They are in the cryopreservation solution. Second is, the cells are oxygen deprived and food deprived and attempting to repair the damage to the cell that has been experienced during freezing. So, the first step is always to remove the cryopreservation solution and the second is to ensure that your samples receive some sort of nutrient. It could be that you restore them to a short-term storage solution or to a tissue culture media or something. These cells are revving up their metabolism they are attempting to repair the damage or the stresses that have been experienced after freezing and thawing and they are in a very fragile state. So, taking good care of them is extremely important.

Question 5: In your first slide you've said that freezing allows for QC testing, is it consistent with regulatory requirements to do lot release testing pre-thaw?

Answer: If I understand the question correctly, what we are talking about here is that certain types of products may require more extensive quality control testing that may take long periods of time. Say you produce a cell therapy and you are going to give it to a patient. If you don't freeze it, you just have whatever liquid storage time available to perform quality control testing and release the product for use. If you have the ability to cryopreserve the cells you then can complete your quality control testing and make sure that your product contains no bacteria, fungus, whatever you are testing for, before it is administered to a patient.

Question 6: Please discuss the effects of prolonged exposure of cryoprotectant pre-freeze; is there a time threshold study?

Answer: Part of that depends upon the additives that you are using. Most people are using a 10% dimethyl sulfoxide (DMSO) solution and DMSO is an organic solvent and cells are an organic entity. That being the case, the DMSO has a well-documented history of what we call biochemical toxicity on the cell. If you take a cell and simply expose it to DMSO and determine viability and different time points, you are going to have decline in viability. The rate at which that viability declines is a function of the temperature, concentration of DMSO, and the cell type. Some cell types are more sensitive than others. You need to determine the kinetics of cell losses for your specific cell product and then engineer your protocol to minimize cell losses associated with exposure to DMSO. Now other cell types, such as red blood cells are exposed to glycerol. Glycerol may result in cell losses because of osmotic stresses. There is not, at least in my knowledge, documentation of a loss in viability with time of glycerol exposure. In other words, glycerol does not result in the same biochemical toxicity as the DMSO. If your solution composition does not contain components that are known to have biochemical toxicity your stability of your cells in that cryopreservation solution should be longer. In other words, you have more time before you put the sample in to your controlled-rate freezer; you have more time that your cells could be exposed to the solution before you start seeing adverse effects. Now remember that most cryopreservation solutions do not contain a lot of nutrients, so there is a limit from the perspective of when are all the cells going to metabolize whenever nutrients are present, that's more the limitation when you are talking about glycerol exposure than you are talking about biochemical toxicity.

Question 7: With your thawed HSC cells, how long is required in culture to return normal membrane function and characteristics?

Answer: That is a wonderful question. I'm not sure I have all the answers. My experience with a variety of cell types is that it can take from 24-48 hours to really see the following things manifest themselves: either the cells would undergo post-thaw apoptosis, so they will be eliminated from the population, or they will undergo their repair process and maintain long term viability. So in other words, in a given population of cells you are going to have cells that are intact that will not survive and you are going to have cells that appear to have fairly shaky membrane permeability but manage to survive. Another factor in assessing post thaw viability has to do with the method of post thaw assessment. DMSO can actually cleave surface receptors of cell types, because it is an organic solvent. It can take up to four hours post thaw for the re-expression of certain surface receptors and that is completely independent of the issue of viability or not. This factor may influence the measurement of cell viability with flow cytometry.

Question 8: Has an optimum time been established to perform assays, particularly CD-34 nucleated cell counts on thawed umbilical cord blood?

Answer: There have been no studies that I know of that have tracked that. I think that the federal government should fund research in that area. I think it's critical.

Question 9: When using transplantation potential for post-thaw assessment, how long does this need to be done *in-vivo*?

Answer: Do you mean how mean serial transplantations? I'd have to defer that kind of question to someone like Dave McKenna who has been through the FDA process more than I have. I know that there are a lot of frustrations when you look at something like taking human hematopoietic stem cells and then determining their transplantation potential. The most common model is the SCID/NOD model. But the simpler reality is that animal cytokines are not the same as human cytokines, so engraftment and expression of the blood cells is limited in animal models, but that's the best we have right now. I know that in general they do require transplantation into SCID/NOD models and perhaps a limited study on serial transplantation, to in other words, take the marrow from mouse 1 and transplant it into mouse 2 to determine that you have a re-population capability. But I can't really speak to what regulatory requirements may be in this matter.

Question 10: Either bone marrow or peripheral blood may be harvested to provide hematopoietic stem cells for autologous transplantation; both however compromise heterogeneous cell population, this may not totally fit into the scheme of this talk, but do you have any suggestions on separating heterogeneous cell populations of cryopreserved cells?

Answer: That is a wonderful question, and you are absolutely right. When we look at a PBSC product or bone marrow product we have a wealth of very complex and interesting cell types that

we all know has a profound influence on the biological activity and yet the freezing response of individual populations within that very heterogeneous population are going to vary. Let's be a little more explicit. Say that we are looking at a bone marrow product, we have mesenchymal cells and we have hematopoietic cells. The freezing response of mesenchymal cells has really been studied by few people and under very limited conditions and that is something that also needs to be studied. The freezing response of hematopoietic cells can vary greatly. Specifically, the freezing response for red blood cells varies dramatically from hematopoietic progenitor cells or CD-34^{+} cells. That may mean future therapies in which we know that different cell populations provide an extremely important benefit for engraftment or any other therapeutic outcome. It may mean separating cells and then turning around freezing them separately, but recombining them again in order to leverage that biological activity to get the biological outcome that you want.

Question 11: How much time post-thaw would you recommend for testing cells so that an accurate measurement can be achieved? Cells will usually be transplanted within an hour after thaw.

Answer: I know and we have to live in the real world, I can't live in the ivory tower of academia forever, because, a lot my work is used in very practical settings. There are two things you can do. First of all, that whenever you do your post-thaw assessments, you do it at that same time point post thaw. This practice helps eliminate one area of variance. Secondly, before you go into clinical use of a protocol, you should do a more in-depth post thaw study. Specifically, you should map out the post thaw viability of your cells for a certain period of time post-thaw and also look at issues of correlating your more long-term assays such as a CFU assay or even LTCIC with what you measure temporally.

In order to make this practical, the simple reality is; I've seen lots of people who do cryopreservation studies and they alter A, B or C (they alter cooling rate, they alter storage conditions) and they measure absolutely no difference in the post-thaw viability. Now what that tells me is their post-thaw assay is not valid or is interpreted incorrectly. If you are starting to see variations in post-thaw viability with the methods that you are using if you alter something, (the cooling rate, the storage temperature), any of those parameters, your assay is probably being sensitive to the changes in the protocol which means you are doing it the right way. If you are measuring everything and it's all the same, all the time no matter what you do to the cells, you have problems. I don't think you have developed the most consummate cryopreservation protocol.

Question 12: What are your thoughts about comparing QC vials frozen alongside product bags? It's not the same freezing environment, so would the QC assay results be valid?

Answer: That is an excellent question. There have been very limited studies. I think out of the University of Nebraska, if I'm remembering correctly, that have looked at the freezing viability of let's say a small 1ml vial compared to a 30, 50, 150ml bag. That study was very lovely and well done and it showed there is a difference. We need more studies to validate the universality of that kind of outcome. The bottom line is in particular when we talk about the viability and functionality of a very rare cell which is what we are talking about in hematopoietic stem cell

products, everyone is struggling with the performance and validation of in-vitro assays, whether the product has been frozen or not. Have we really perfected assays that we can sample from our hematopoietic stem cell products and determine the transplantation potential or the viability or any other important measure? I don't know that that question has really been answered and it's certainly something that's extremely important to the field.

Question 13: Would you recommend beginning a controlled-rate freezing protocol at room temperature or at 4°C?

Answer: My recommendation is to begin at 4°C. That has to do with something that as an engineer I understand and that has to do with heat transfer and thermal mass. When you start out something at 4°C you are probably going to have your sample track closer to the chamber for a longer period of time. Your sample will never totally track with the chamber because of the release of the latent heat of fusion. But as long as you can have your sample track as close as possible to the chamber for at least that initial portion of the controlled-rate freezing that means the consistency of your outcome is improved.

Question 14: Do you recommend post-thaw testing for all product samples or only if a perceived problem occurs? And how soon after freezing would you recommend testing?

Answer: Certainly, if you have a deviation or what you detect as a deviation or a product that has certain questionable attributes pre-freeze, that post-thaw assessment should be important. Part of the issue is that what we are talking about is a fairly open-ending question. In the field of hematopoietic cryopreservation, we have roughly twenty years of experience in this area. All of these things suggest that the post-thaw assessment may not be critical. The simple reality is we are developing products that are different. Specifically, we are developing products that are cultured ex-vivo; we are developing products that may have a different cell number or a different cell composition. When we change the biology and the physics of the freezing process we may change the outcome and it may become very important to do that post-thaw assessment before the samples are actually infused.

If we do the post-thaw assessment, when do we do it? When we thaw the cells, they need a positive environment post thaw. That means they should be infused into a patient as soon as possible or re-established in culture, whatever they are going to be used for at the downstream effort. So we have a limited amount of time to perform a post-thaw assay.

Question 15: Do you have any experience using anti-freezing glycoprotein in complex multi-cell organelles and if so how has it worked?

Answer: Most cryoprotective agents change things in the extracellular environment. They change the amount of ice present, they change the concentration of solution and they also change things if they penetrate the cell, they change things inside the cell as well. Anti-freeze proteins have a different mechanism of action; they actually attach themselves to the ice molecule and alter the way an ice crystal grows. So if you looked—most of you know what a snowflake kind of looks like, and what you would see if the snowflake formed in the presence of anti-freeze proteins is a completely different structure (at least what we see when we look out the window in

Minnesota); anti-freeze proteins alter the way the ice crystal structure is formed and as a result can change the mechanical and chemical environment for the cells and that's the mechanism of action.

Question 16: What cryopreservation media would you recommend for preserving cells for MSC cell therapy application?

Answer: That is an excellent question. The simple reality is there have been very limited studies on MSC cells. We are trying to wait for our turn to get some from PACT so that we could perform some freezing studies. I think there are some good places to begin. A cryopreservation solution has three basic components. The first component is the base of the solution and that is the most of the solution by volume. The second component is a cryoprotective agent. One commonly used cryoprotective agent is glycerol. The third component is a protein. The actual mechanism of action for proteins is as yet unspecified. But it seems to have an activity in some cell types and not others. When we are thinking about those three components we can start there and look at our infusible grade electrolyte solutions that are now pretty conventionally used in HSC preservation such as Normasol-R, Lactated Ringers, Plasmalyte-A, anything that is basically a balanced electrolyte solution. Then, you have to pick cryoprotective agents and right now there are a limited number of studies that are giving us options in terms of what we can use with MSCs. The third element is whether or not protein plays an important role. Those studies haven't been performed as well. Once you formulate your solution you have to pick out the cryopreservation protocol and that hasn't been studied as well. So there are just too many open-ended questions for me to recommend a specific protocol for MSCs, but if you send us some MSCs we will do freezing studies and we'd be overjoyed to work on the development of an MSC protocol because I think it's long overdue.

Question 17: Do you know of any non-toxic medium used for cryopreserving cell therapy and other in-vitro applications?

Answer: We are working on some combinations right now. We have had some very good results using regulatory T-cells. We haven't published it and haven't disclosed it yet, but this would be very, very non-toxic. We are not quite at the point where we are ready to go public on this because we need to have a greater certainty in terms of our post-thaw viability assays.

Question 18: How should preservation of small tissue fragments differ from that of single cell suspensions? Are there any additional considerations to preserve the cells in this tissue?

Answer: You have additional hurdles, but you also have additional opportunities. When you are talking about a tissue you are talking about an organized grouping of cells. Because of that the physical scale of your biological system is different than the scale of an individual cell. Let's say for example, we use a 10 micron diameter of a cell (that's just a general order of magnitude), when you are talking about tissue you are talking about something of a physical scale on the order of millimeters if not centimeters, so because of that you have a much more complex environment. Now because you have a tissue sample, you could pursue other types of options, including options such as vitrification. Vitrification is using very high concentrations of

cryopreservation agents, and not actually freezing, but forming a meta-stable glass that surrounds your tissue and therefore avoid some of the mechanical and chemical stresses that are experienced during freezing. I hate to be so generic about it, but you need to really communicate more details about the tissue and also this is a much more complex system and so it's not something I can really answer over the phone, easily.

Question 19: Will you discuss the benefits of different nutrient solutions used with DMSO as the cryoprotectant media with HPC stored for future transplantations, such as plasmalyte and dextran?

Answer: Not all hematopoietic products are equal. In my experience when you use GCSF stimulation and collect a product with an apheresis machine those cells tend to be more metabolically active than for example, an umbilical cord blood product or a bone marrow product. It's been my experience that you should engineer your cryopreservation solution to have more nutrients as a result. So using a simple electrolyte solution such as Normasol or Plasmalyte-A is an option but you may want to consider supplementing either sugars or nitrogen sources or some other nutrient that the cells may need because your PBSCs are more metabolically active. The other thing you can potentially leverage is handling them at low temperatures for longer periods of time to suppress cell metabolism. In terms of using DMSO, that has been done. The other thing that may be helpful is also buffering the cells. There are strategies you could use to buffer the cells. The simple reality is that you have to use somewhat specialized solutions to buffer under atmospheric conditions when you don't have a 5% CO_2 atmosphere. I hope that I answered your question.

Question 20: What is the maximum of DMSO you can infuse into the patient at one time?

Answer: Zero. I will not support nor will I promote the infusion of DMSO into patients on a regular basis. A colleague of mine just had a graduate student undergo a bone marrow transplant and he experienced the adverse reactions. I know physicians talk about giving antihistamines, and medicating, and all this other stuff, but the patients hate it and there's no reason to infuse DMSO into patients. We are developing a microfluidic device that would allow us to remove it and minimize cell losses and not be infusing DMSO into patients. Give us time to get this farther along so that people do not have to be subjected to the adverse reactions of DMSO. So my answer remains zero. There is absolutely no reason to infuse DMSO into patients, they don't like it and it's not good for them.

Question 21: In your opinion how does high cell concentration such as 1x 10 per ml effect the cryopreservation and freezing environment?

Answer: Very good question. When the water inside the cells becomes a significant fraction of the total water in your system, the presence of high concentrations of cells can influence your post-thaw viability. That's been a known factor for about 30 years. When we talk about hematopoietic stem cell products what we know is that they are collected and processed at a variety of concentrations. In particular PBSCs tend to be collected at very high cell concentrations. What you are getting is a variety of cell types, some of which you don't want to save post-thaw. When you freeze at very high cell concentrations and those cells are destroyed

and release the intracellular contents into the produce, you are going to end up with a very sticky cell suspension. The rule of thumb is do not freeze above a 20% cytocrit, so you have to know the volume of your cell, and the diameter of the cell, which most of you do know because you run culture counters or whatever on your cells and if you get over 20% cytocrit so the volume of the cells is 20% of the total volume of the product you're hitting up against a limit where you will start to see a change in post-thaw viability with cell concentration.

Question 22: Do you have a recommended cell concentration prior to adding cryopreservative?

Answer: No, but we do have a recommended cell concentration once the whole system has been equilibrated; we typically freeze at 30-50 million cells per mil. That is a somewhat low concentration. We have that latitude because we are a research lab. If you are worried about running out of storage space or having enough sample, or any of those issues you certainly can freeze at a multiple of that, just don't go over 20% cytocrit.

Question 23: What is a scientific basis for a rapid warming rate commonly used during thawing? Does a slower, controlled rate of thawing effect cell viability?

Answer: How you thaw is influenced most strongly by how you freeze. That's your initial condition, that's what you are getting your system in. For conventional controlled rate freezing under normal freezing conditions, in other words, something between 0.1 and probably 30 C per minute, thawing the sample rapidly is recommended because any ice crystals that are present will continue to grow and affect the cells. Your cells may have actually formed very, very, very small ice crystals on the inside of them and if they are small enough they will not damage the cells. If you warm slowly, you give time for those ice crystals inside the cells to either form, they may not have formed and now they thermodynamically are able to, or if they formed and they were very, very small and therefore non-damaging, you are giving them time to actually grow and damage the cell. That is the scientific basis for a very fast warming rate. If you are using a different type of protocol, something closer to vitrification, you are going to want a modified warming protocol. That's specialized knowledge and if this person wants me to go into the scientific basis for modified warming protocol for vitrified solutions, I'd be happy to, but that's something that's kind of specialized and esoteric.