Innovative Cryo Enterprises L.L.C.

I.C.E. CUBES #1

Tiny bits of information to help improve your cryopreservation practice

Hello, "ICE Cubes" is the first official e-newsletter of Innovative Cryo Enterprises I.C.E. I am publishing this to provide an ongoing connection to current users and others interested in cryopreservation. This e-letter will be published randomly, possibly on a bi-yearly basis, or as important news comes up. Enjoy!

This e-letter will provide information and advice on how to optimize our (ICE) vitrification system in your laboratory. ***Important:*** This newsletter contains ***suggested*** revisions to some operating protocols so please read through this carefully and contact me if you have any questions. These modifications are only *suggestions* based upon input by current users that have noticed things or have found useful tips that may affect the overall performance of the vitrification system. Please note that most of these ideas may only be observations and not necessarily proven facts. Any major protocol changes are incorporated and the revised protocols are emailed to each and every user. The observations listed here are only *suggested changes* that may eventually lead to a universal protocol change.

In the ongoing pursuit to assist clinics in obtaining high survival and pregnancy rates with a simple, safe, and successful vitrification system we continue striving to learn how to make our system better. There has been quite a bit of input from our users which is very valuable to all of us. There are numerous clinics around the world using the system for vitrification of blastocysts, cleavage-stage embryos, and oocytes. Without regular collection of data it is hard to determine exact numbers and success rates, however, using our system there have been at least 700-1500 babies born from blastocyst vitrification and 50-150 babies from oocyte vitrification. For example: In a multi-center child development study, following ICE-Oocyte/micro-secure (mS)-VTF, Schiewe and coworkers determined that all 11 babies born were healthy, without any anomalies, and experienced normal 1-year old check-up without any developmental issues. We will be providing a summary of data in future peer-reviewed publications. Also, sometime next year we will publish a website with additional information and reference material. Thank you for your support!

Did You Know???

DMSO Toxicity:

There are a number of studies describing cryoprotectant toxicity. These range from chemical studies in cryobiology by Gregory Fahy (G.M. Fahy, The relevance of cryoprotectant 'toxicity' to cryobiology, Cryobiology 23 (1986) 1–13.; G.M. Fahy, T.H. Lilley, H. Linsdell, M.St. John Douglas, H.T.Merriman, Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms, Cryobiology 27 (1990) 247–268.) to more general review articles (Liebermann 2003 Reproductive BioMedicine Online).

Dimethylsulfoxide (DMSO) was and is being used as the primary or one of the primary cryoprotectants because of its rapid passage through cell membranes, despite its toxic potential (Fahy, 1984; Rall, 1987; Fahy, 1990--- Fahy GM, Lilley TH, Linsdell H et al. 1990 Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. Cryobiology 27, 247–268; Mukaida et al., 1998). Toxicity of DMSO (or any other cryoprotectant) is based upon its chemical structure and its ability to interact with water molecules. DMSO is one of the better vitrificants, however it is also one of the more toxic. By contrast, Ethylene glycol and glycerol are among the less toxic cryoprotectants (see Fahy).

Many babies have been born from a variety of cryoprotectants and various freezing/vitrification procedures. They all can work if the procedure is done properly every time. However, this is difficult with some procedures. The standard EG/DMSO system (micro-volume/ultra-rapid cooling vitrification) has the potential to obtain near 100% survival of cells, but this is dependent upon the duration in the cryoprotectant solutions. Too much time and cells die mainly due to toxicity; too little time and not enough dehydration will occur and intracellular ice will cause the demise of the cells. The incubation times range from 1-2 min to 5-15min in equilibration solutions and then generally 60 sec or less in the final vitrification solution prior to plunging in liquid nitrogen. These times vary and are not standardized leading to many of the issues with this system. There seems to be a fine line between duration and concentration in DMSO in order to obtain reproducible success rates.

Our system does not use DMSO for the above reasons. Using less toxic cryoprotectants such as ethylene glycol and glycerol, more time is afforded to perform the vitrification procedure, and these chemicals are overall less likely to induce toxic injury. They allow for more equilibration time in the cryoprotectants prior to vitrification, so standardized times (eg. 5min V1, 5min V2 and, 2min V3; for blastocysts) can be used for adequate equilibration of embryos despite embryo-to-embryo differences. Our research and your clinical success rates together prove that this is a highly effective vitrification system that is easy to learn and use.

Did You Know???

HSA vs.SSS & SPS:

There is a difference between HSA and SSS & SPS. First SSS or SPS is made up of 85% HSA and 15% immunoglobulins. Therefore, SSS/SPS is essentially the same as HSA. One major difference is the concentration of HSA in HSA and SSS/SPS. HSA contains 100mg/ml of HSA, while SSS/SPS contains only 50-60mg/ml HSA. Therefore, 20% SSS/SPS is approximately equal to 10% HSA. The immunoglobulins in SSS/SPS are beneficial to embryo development and therefore good to have around. However, one must be careful to add twice as much SSS/SPS to obtain a similar protein concentration of HSA.

Try This!!!

Serum Concentration and Cryopreservation:

It has been apparent to me for many years, and I am sure others, that serum protein is beneficial to thawed cells, namely oocytes and embryos. Without going into detail, suffice it to say that serum has effects on cellular membranes and aids overall cellular health during the post-thaw recovery process, which may take numerous hours. During slow-cooling of mouse oocytes it was mandatory to have elevated BSA concentrations to maintain cellular integrity and help prevent zona hardening. For human gamete and embryo cryopreservation this also holds true. There is around 20% HSA in our vitrification thaw media for all stages. Based on our own work and input from you the users of the system, there are a few suggested modifications pre- and post-thawing. During the post-thaw period we find it beneficial to have around 10-20% HSA in the culture media. For blastocysts this would mean culturing in 10-20% HSA until transfer. For oocytes and embryos, we suggest using this elevated protein concentration for the first day after thawing, basically from overnight to 24h post-thaw. It has also been discovered that (for oocyte vitrification) from the time of egg collection through culture to the blastocyst stage, it was beneficial to overall development to have at least 10% HSA in all culture media. Keeping this in mind, remember that 10% SSS is actually 5% HSA and you would need to add around 10% HSA to obtain 15% total HSA concentration. One would not want to add 40% SSS to obtain 20% HSA as this would dilute out the media too much and quite possibly do more harm than good. Please contact me for more information regarding this.

Protocol Change!!!

In recent experiments, Schiewe and coworkers tested a reduction in the thawing / rehydration times for our blastocyst protocol. In the current protocol, blastocysts are placed in T2-T5 media for 5min each. The time out of the incubator, from thawing to rehydration lasts around 25-30min. With the general overall impression (I think most of us would agree, although it may be difficult to prove) that it is better

to have embryos in the incubator at 37°C than out on the workbench at room temperature. They conducted experiments with consented, medical discard blastocysts aimed to reduce rehydration processing. This AAB award winning study (Zozula et al, 2012) warmed/rehydrated blastocysts at intervals of 5min, 3min, 1min, or as a negative control directly from T1 to T5 to cause very rapid rehydration and osmotic stress. The results indicated that all blastocysts thawed equally well using the 5min, 3min, and even the 1min protocols (93%). Even more impressive was that >70% survived a direct move from T1 to T5! Keep in mind that too rapid rehydration will cause the cells to swell and burst, so a carefully orchestrated thaw and rehydration protocol is mandatory to optimize survival and future pregnancy rates. Based on these experiment conducted at SCIRS and the fact that other vitrification protocols use a 3min rehydration time, and years of past experience with different rehydration protocols, we believe that 3 min in each thaw dilution is more than adequate and safe, and therefore are changing the step-out protocol for blastocysts to 3min per step. Several labs (at least 2 to date) are currently using this 3min step-out time for blastocysts and have found no difference in survival rates from the 5min protocol. The SCIRS group has thawed more than 200 BLs in 2012 using 3 min intervals, achieving over 98% survival and high clinical pregnancy rates, comparable to their fresh BLETs (see Whitney et al., 2012 ASRM Abstr.#P249). Follow-up studies with eggs and embryos are being conducted, and we hope that the 3min step-out will also be applicable to these stages as well. If you are willing to test this out with other stages please let me know and we can continue to monitor the situation, and hopefully improve these protocols as well.

Good To Know!

Quality Control Test Results:

We recently conducted quality control testing on a batch of blastocyst vitrification media that was 12 months expired! The tests included endotoxin and mouse embryo assay (MEA; actual freezing of mouse blastocysts). The media maintained its sterility, low endotoxin levels, and easily passed the MEA test (100%), with numbers equal to the freshly made media 18 months prior. From this data we are confident that the media you currently use is good (functionally) well past the printed expiration date on the label.

Keep In Mind!!!

Culturing thawed cleavage-stage embryos:

In the past few years at ASRM and other meetings there have been several posters and other data presented on cleavage-stage embryo vitrification. Some of these articles recommended culturing thawed D3 embryos overnight to determine the best one(s) for transfer. Apparently, there have been a few reports of embryo arrest after thawing. These reports used a different vitrification, however it may be wise to use this approach for your first transfers of vitrified D3 embryos to make certain there is no problem with culture. I do not have any other information on these reports but there may have been issues with the vitrification protocol used, or the embryos themselves, or their culture system/media. These may have been one time occurrences so it is difficult to know if there is a real problem or if it is

simply user error. Please refer to the above section on Serum Concentration as a possible aid in preventing this.

ICE Vitrification System:

As you already know our vitrification system (ICE) is a large-volume system that has several marked differences from the more widely-known micro-volume (DMSO/EG) system. Although the basic cryobiological theory behind each system is similar, they are very different. For instance, with our system there is no need to collapse expanded blastocysts prior to vitrification. Also, the more expanded blastocysts with a thinning zona, will survive very well, and thus many current users choose to vitrify mainly on D6 (depending on the actual size and development of the blastocysts). This is in marked contrast to the micro-volume system where a small to medium-sized D5 blastocyst is the preferred stage for vitrification. And, larger more expanded blastocysts need to be collapsed (more often than not) to obtain the best results.

Even though our vitrification system can be used with a large volume of media in a conventional cryo-straw, it can also be used with any of the available micro-volume devices (eg. cryo-loc, cryo-top, micro-Secure, etc.). There is a simple modification to the thaw procedure when using a micro-volume device. The reported results have been very good from both types of devices. We do not regularly suggest using commercial micro-volume devices mainly due to their expense, but would recommend mS-VTF as an effective, non-commercial low-cost/FDA compliant alternative (see Schiewe et al., 2012 ASRM Abstr. #P59). Of course, any device that you feel comfortable with and works well in your lab is the one to use. There are currently several clinics successfully using a variety of devices with our vitrification media. If you have any questions please contact me.

Protocol Tip!!!

Time and Temperature:

As many of you know the protocol for vitrification of oocytes, embryos, and blastocysts is relatively simple. Two things to keep in mind are 1) Time and 2) Temperature. The protocols do not specifically state the minimum time in V3, however since you have 2 or 4 min, depending on stage of the cells to be frozen, it is necessary to rinse/wash the cells completely in V3 before loading into the straw or other device. Do not rush this step, as the V3 is the solution that will vitrify! Take a minimum of 1 min in the V3 prior to plunging in LN2. You can use a timer to help you. Remember, this method is different than the traditional EG/DMSO system, and toxicity concerns are much reduced, so there is no need to rush the procedure. Give those cells plenty of time in V3 and this will reduce the possibility of inadequate dehydration.

In regards to Temperature: The protocol states to dehydrate the cells in V1, V2, and V3 at room temperature or 23C. Adequate dehydration is critical for successful vitrification. If the surface of workbench you are using is metal, it may feel very cold to the touch, as metal acts as a heat-sink. The surface temperature may be well below room temperature and could slow cellular dehydration. It is suggested to either turn on your heating element in your hood to around 26-27C to obtain a surface temperature of 23-25C (not cold to the touch) or elevate the dish containing the cells above the surface

using a stack of papers or plastic tube holder or similar. Basically anything that does not feel cold and will eliminate the "heat-sink" effect of the metal hood. In addition, remember to allow the full time in each step to ensure adequate dehydration and the best outcome.

I thank you for your continued support. We are always trying to improve this already successful system, in hopes of increasing survival and pregnancy rates so that *all* clinics using the system will achieve results similar to their fresh/nonfrozen embryo transfer rates. If you have any questions or comments about this newsletter or how to improve our system please contact me.

Best,

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