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Limiting Factors Analysis in Validation of a CryoShipper & a CryoExtra Freezer for Cryo-samples Related to the Abu Dhabi Bone Marrow Transplantation Program

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Abstract: *Introduction:* CryoShipper and CryoFreezer tanks were designed to safely transport and store biological material at cryogenic temperatures. An adverse warming event may occur if the CryoShipper is tilted during transit if temperature and proper levels of liquid nitrogen (LN2) vapors are not maintained. Storing cryogenic hematopoietic stem cell samples for a short or long time requires validating all pieces of equipment employed in a bone marrow transplant program. *Objective:* To determine the limitations of the *MVE CryoShipper CX* and the *Thermo Fisher CryoExtra™ Cryogenic tank* for the Abu Dhabi Bone Marrow Transplantation Program. *Methods:* The MVE *CryoShipper CX* was weighted and primed with LN2, and the excess was removed. After stabilizing the temperature, the shipper was tilted onto its side for 24, 72, and 96 hours. The temperature was monitored and recorded at 15 minutes intervals using a *Datalogger*. After returning to the correct upright position, the dry shipper temperature was also observed for five days after refilling with LN2 to evaluate the secure timing of a shipment. For an LN2 filled *CryoExtra* tank, dual control of the LN2 level and the temperature was done using manual measurement and automatic display. Later, vials and a bag from three healthy donors' frozen white blood cell buffy coat samples were kept inside and defrosted daily to measure total mononuclear cell counts CD45+/7AAD cells viability by flow cytometry for five days. *Results:* The *CryoShipper* maintained cryogenic temperatures below -150°C for the entire duration of each analysis. A maximum temperature of -182.7°C was reached during the 24-hour tilt experiment from the temperature probe. The *CryoExtra 0140* freezer temperature was always between -184°C / -194.2°C, and LN2 levels coincided in both measuring methods during the time slot. Stored and defrosted cells keep their % of viability and absolute number over the expected reference range and compare mean+/-standard deviation (SD) between them without assessing the statistical difference with $p \geq 0.05$. The defrosted cells' mean viability of 83.12%, SD±9.04, and a mean of 1,781 cell/µL, SD±1,215. *Conclusion:* Our modern dry shipper controls much of the vapor within the shipper. The *CryoShipper* can withstand being tilted during transit for 96 hours without risks of an adverse warming event. The *CryoExtra 0140* tank its performance was under the established parameters. Both types of cryogenic devices can be used for the safe cryopreservation of cell samples keeping all the security measures and controls as advised.

Keywords: Cryogenic, Control Rate Freezers, Low-Temperature Storage, Safety, Thermal Risk, Validation, Liquid Nitrogen

1. Introduction

Cells useful for regenerative medicine have shown demonstrable potential for treating many life-threatening diseases. Many centers do hematopoietic stem cells (HSC) transplantation and other cell therapies worldwide. [1] Every time more Universities, biomanufacturing facilities, biotech, and pharmaceutical companies, are introduced into this field, customized cell products for allogeneic and autologous medicines have specific requirements; this has imposed significant challenges on the shipping logistics and supply chain of this kind of cellular products. [2] The released cells' storage, transport, and shipment for regenerative medicine, particularly related to HSC transplantation, must follow strict guidelines. Stem cells are classified as biological drugs by the US Food and Drug Administration (FDA) and other regulatory agencies worldwide. [3, 4]

The Abu Dhabi Bone Marrow Transplantation (AD-BMT®) Program started in 2020 as an initiative of the Abu Dhabi Stem Cells Center (ADSCC) [5-7] to solve an old health problem regarding this type of advanced treatment of hematological malignancies in the United Arab Emirates (UAE).

CryoShippers were designed to transport biological material at cryogenic temperatures safely. However, an adverse warming event may occur if the shipper is tilted during transit. [8] Therefore, correct handling of the shippers is critical. Our laboratory was equipped with an *MVE CryoShipper model CX* belonging to the MVE Vapor Shipper Series. It is designed to transport biological samples safely at cryogenic (-150°C or colder) temperatures, utilizing the *"Advanced QWick Charge Technology"* to charge the vapor shipper in two hours. Manufactured from durable, lightweight aluminum, the vapor shippers employ a hydrophobic compound that absorbs the liquid nitrogen (LN2) to ensure dry, spill-free vapor-phase shipping. [9] These containers can be used to ship HSC samples with a "non-hazardous" classification throughout the world, thus reducing costs and helping to assure sample viability. [10] The insulation material surrounds a central chamber that holds the cryopreserved product in vials or bags. The vessel is charged by filling the interior compartment with LN2, allowing time for absorption. The remaining liquid can be emptied before use, rendering it a "dry" shipper.

On the other hand, the *CryoExtra™ High-Efficiency Cryogenic Storage Model 0140* tank [11] was also selected for long-term storage of LN2 frozen HSC. The storage capacity of 40,600 vials / 1,020 blood bags makes the *CryoExtra Model 0140* suitable for long-term cryopreservation of HSC, as these pieces of equipment have provided outstanding sample protection for scientific research, with uniform cryogenic temperatures throughout the vessel. Automated temperature monitoring and microprocessor-based LN2 level control provide peace of mind for our valuable samples. All vessels can accommodate both vapor- and liquid-phase storage. Nevertheless, for our purpose, we used the vapors phase. Therefore, the freezing process of stem cells required a very well controlled temperature rate to warrant good recovery of living cells; we used the *Thermo Scientific Controller Freezer Model 7453*. [12] According to the Good Laboratory Practices, [13] both devices required different steps of validations before their use for shipment and storage of this type of stem cells in the AD-BMT® Program. This study aimed to evaluate the limiting factors in the *MVE CryoShipper* for its tilt validation and its capacity to keep the temperature for at least seven days before starting its use for frozen HSC shipments from our laboratory to different health care centers in the city. It also evaluated the *CryoExtra 0140 freezer* capacity to support its cryogenic performance.

2. Materials and Methods

2.1. Donors' Cell Samples

Three healthy members of our staff acted as voluntary healthy peripheral blood donors for obtaining the white blood cells (WBC) buffy coat after signing an informed consent as per the Helsinki Declaration for this experimental work using human cells. [14]

2.2. Charging the Shipper at Its First Use

One to three days before transporting the cells, we charged the CryoShipper with LN2;[15] attaching a transfer hose to the LN2 supply liquid valve, we removed the lids and placed the hose inside. After turning the supply tank valve on, the two CryoShipper were filled to the top of the metal reservoir, then replaced the cover. Refilling with LN2 the shipper was done every 30 minutes, so after periodically checking the LN2 level and adding more liquid, LN2 was allowed to be absorbed into the CryoShipper. The CryoShipper absorbs LN2 until saturated (12-24 hours to fully charge). Excess LN2 was finally removed to make the CryoShipper dry and ready for validation as a carrier of samples. All LN2 manipulation was performed following the recommended procedures for safe operations handling and environmental protection. [16]

2.3. MVE CryoShipper Validation

We followed the Standard Operating Procedure (SOP) for the validation (ADSCC SOP.SCL.GEN.012.1.0). The MVE CryoShipper was primed for more than 2 hours with LN2 excess liquid removed to do an initial tilt validation. The initial weight was recorded, and after a short delay to stabilize the temperature, the shipper was tilted onto its side for 24 hours, 72 hours, and 96 hours. The temperature was monitored and recorded at 15 minutes intervals using the *LIBERO CE V.8.13 Datalogger*. [17] Finally, the CryoShipper was returned to the correct upright position and allowed to stabilize. We continued with the proof validation before use if the tilt validation was correct. It was done before the shipment of any cryopreserved samples. The dry shipper weight was recorded before filling and after filling, and a report was made using our CryoShipper validation form with the following data:

Weight:
a) Before Filling = ------- Kgs.
b) After Filling = -------- Kgs.
Passing Criteria – Weight range should be about 6-9 Kg.
Filling Details:
a) Fill Date & Time = -------
b) Temperature before filling = --------

Then we placed the Datalogger cryogenic probe inside the dry shipper in the middle and closed the lid. After filling, the temperature was monitored every 24 hours for seven days. After that, the temperature logger was downloaded and attached to a Report. We inspect the Shipper for any leakage and frosts on it every day.

2.4. Thermo Fisher CryoExtra™ High-Efficiency Cryogenic Storage Model 0140 Tanks Validation

On the other hand, the *Thermo Fisher CryoExtra 0140* cryogenic freezer tank was also evaluated by two separate validation experiments measuring the temperature using a cryogenic thermocouple for five days and comparing it with the temperature shown in the display. In addition, checking the LN2 level manually with a level meter ruler for five days and comparing it with the one shown on the instrument control unit screen, and evaluating its performance regarding cell viability and cell concentration with five different frozen buffy coats samples in the same period.

The WBC buffy coats from three healthy donors were separated from venipuncture collected peripheral blood in a 250 mL blood bag with Anticoagulant Citrate Dextrose Solution A (ACD-A), using a refrigerated centrifuge at the speed of 555 x g for 15 minutes and adjusted to a final cell concentration range between 5.0-5.5x10^6 per mL to be frozen according to our SOP. In brief, we prepared a cryoprotective solution at 4°C with an icebox help, using the Plasmalyte-A at 30% as the diluent, the dimethyl sulfoxide (DMSO) at 20% to later dilute at a final 10% when added to the cells suspension, in the same way, Human Serum Albumin (HSA) initial concentration was set at 8% to obtain 4% of final HSA, and the ACD-A at 10% to get a 5% final concentration. Then the cryopreserving solution was added at a 1:1 ratio (vol/vol) very slowly drop by drop to the cell's suspension and aliquoted into 5 x 1.8 mL Nunc® Cryobank Vials (Thermo Scientific), and a 250 mL blood bag with a maximum exposure time of cells to DMSO less than 30 minutes before being introduced into the Thermo Scientific Controller Freezer Model 7453. The Pre-Set Profile #4 was used for a freezing rate of -1°C per minute till -90°C and then quickly transferred to the CryoExtra tank LN2 vapor phase at -134°C. For defrosting, the frozen samples were swiftly taken from the CryoExtra tank and put into a 37°C water bath until the first signs of melting, then centrifuged to remove the DMSO and resuspended in the same buffered salt solution without DMSO. [18] Another different relevant program for Vials (Program 1) was also tested as per the operational manual of the Thermo-Scientific Control Rate Freezer. The graphs of programs were evaluated and attached to the report by the operator.

A *Beckman Coulter Navios Flow Cytometer* (Brea CA, USA) was used to measure the isolated CD45+ cell's viability by the 7-aminoactinomycin (7AAD) vital staining method [19] and a *Beckman Coulter Automatic Hematology Analyzer DHX900* (Brea CA, USA) to count total WBC and calculate MNC concentrations fulfilling ISO standards. [20]

2.5. Statistical Analysis

The *Shapiro-Wilks* test was performed to determine the normality distribution of variables. The paired *T-Student* test compared CD45+/7AAD cells viability and absolute counts daily before and after freezing/defrosting the cryostored samples for five consecutive days, always using the GraphPad Prime 9 software v.8 (La Jolla CA, USA). [21] A $p \leq 0.05$ was considered a significant difference.

3. Results

3.1. MVE Cryoshipper Tilt Validation

CryoShipper registered data for the tilt validation were as follows: the initial before-filling weight was 11.8 Kg, and after a short delay to stabilize temperature, the weight was 17.7 Kg. When the dry shipper was tilted onto its side for 24 hours, 72 hours, and 96 hours and the temperature was recorded at 15 minutes intervals, the results are shown in Table 1.

Table 1. *Recorded temperatures after 24, 48, and 96 hours of keeping a dry shipper tilted.*

Time interval (Hours)	Average temperature (◦C)	Maximum temperature (◦C)	Minimum Temperature (◦C)
24	-166.35	-182.7	-150.0
72	-165.25	-181.5	-149.0
96	-165.30	-180.6	-150.0

Table 2. *Recorded temperatures and liquid nitrogen levels inside the CryoExtra 0140 freezer.*

Days	Readings			
	Temperature range (Minimum, Maximum)		Liquid Nitrogen Level	
	Manual (°C)	Display (°C)	Manual (mm)	Display (mm)
1	-194.6, -187.0	-194.6, -187.0	230	230
2	-194.2, -184.0	-194.2, -184.0	225	225
3	-194.2, -184.0	-194.2, -184.0	220	220
4	-194.2, -184.0	-194.2, -184.0	210	210
5	-194.2, -184.0	-194.2, -184.0	200	200

3.2. Thermo Fisher CryoExtra 0140 Tank Temperature and LN2 Validation

On the other hand, the CryoExtra's internal temperature range was always between -187°C for the above of the tank and -194.2°C for the bottom for five consecutive days, and the level of LN2 indicated a complete coincidence between manual measurements and the Control Unit automatic display readings as shown in Table 2.

The three donors' cells' viability and concentration evaluated simultaneously during five days indicated good results, without statistical differences before and after defrosting, as shown in Table 3.

Table 3. *CD45+/7AAD cell viability and total mononuclear cells count before and after storage in a Thermo-Fisher CryoExtra 0140 freezer.*

Donor Sample/ 5 Days	Vial/Bag No.	Before Storing (Non-frozen)			After Retrieving (Defrosted)		
		CD45+/7AAD Cells		Total MNC	CD45+/7AAD Cells		Total MNC
		Viability (%)	Count (cell/µL)	cell/ µL	Viability (%)	Count (cell/µL)	cell/ µL
No. 1	V.1	93.02	6,808	7,319	94.09	3,644	3,873
	V.2	93.02	6,808	7,319	94.04	2,951	3,138
	V.3	93.02	6,808	7,319	85.76	4,172	4,865
	V.4	93.02	6,808	7,319	76.32	2,449	3,209
	B.1	93.02	6,808	7,319	74.50	2,548	3,420
No. 2	V.1	85.49	1,055	1,234	92.17	1,577	1,711
	V.2	85.49	1,055	1,234	95.74	1,190	1,243
	V.3	85.49	1,055	1,234	89.63	2,084	2,325
	V.4	85.49	1,055	1,234	89.17	939	1,053
	B.2	85.49	1,055	1,234	88.84	979	1,102
No. 3	V.1	78.24	2,527	3,230	92.28	502	544
	V.2	78.24	2,527	3,230	40.99	956	2,332
	V.3	78.24	2,527	3,230	66.30	1,326	2,000
	V.4	78.24	2,527	3,230	84.79	669	789
	B.3	78.24	2,527	3,230	82.16	737	897
X+/-SD		85.58+/-7.39	3,463+/-2,989	3,928+/-3,102	83.12+/-9.04	1,781+/-1,215	2,167+/-1,332
p					0.6122	0.2785	0.2566

Legend: MNC: Mononuclear cells; 7AAD: 7-AminoActinomycin D; SD: Means' Standard Deviation.
Reference Ranges after defrosted: Temperature: -150 to -196°C; LN2 Level: 180 to 240 mm; $CD45^{+}$/7ADD Cells Viability: 70-100 %; MNC count: Not less than 50% of the values before storing.

3.3. Visual Inspections of the Cryogenic Recipients

After retrieving/defrosting, vials and bags were inspected to detect any leakage and cracks: bag (No. 1) was accidentally punched out after retrieving, but the WBC sample inside was recovered and tested.

4. Discussion

Validation is a mandatory procedure in Good Laboratory Practices [20, 22] for the quality of cryopreserved products and any HSC transplantation Programs' competencies and certifications. Other authors have carried out the validation in MVE CryioShipper with good results. [23] The MVE CX CryoShipper maintained cryogenic temperatures below -150°C for each analysis. The maximum temperature of -182.7°C was reached during the 24-hour tilt experiment from the temperature probe positioned within the shipper, closest to the lid. Therefore, the CryoShipper can withstand being tilted during transit for more than 8 hours during shipment of HSC because an adverse warming event would not occur to the sample within. The neck of the shipper is also comparably narrow, and because nitrogen vapor is heavier than air, much of the moisture will remain within the shipper. Nevertheless, to minimize the risk of adverse warming events in the event of a tilt during transit, the use of the MVE CX CryoShipper in the correct position would be advised. The temperatures were within range, and the comparison is compliant. We recommend not using the CryoShipper as a dry-shipper if the temperature is above -150°C and/or the fully charged LN2 weight device is below 16.5 Kg, indicating loss of LN2. The expected range of proper use has been stated as:

The temperature of LN2 CryoShipper vapor transport: ≤ -150°C. Fully charged CryoShipper weight for vapor transport (with lid): Small 17.7 kg.

Therefore, our CryoShipper levels were within range, and the comparison is compliant. The MVE CryoShipper can withstand being tilted during transit for 96 hours without causing an adverse warming event, enough time for a national and international shipment. [4, 24]

The CryoExtra 0140 freezer tanks, the CD45+/7AAD viabilities/absolute counts, and MNC counts are all within range for bags. In all frozen samples, on some days, cells' viability after defrosting was even higher than before the freezer storage. The absolute number of the CD45+/7AAD cells always diminished, but statistical differences in those cells' viabilities%/counts and MNC absolute counts were not found. Particularly for vials, only two vials of sample No. 3 (V.2, V.3) had lower viability than we expected by the established range. Nevertheless, those results were not considered a failure of the CryoExtra freezer because, unfortunately, a non-planned Flow Cytometer maintenance moment delayed the viability measurement. Still, the viability of the following day's V.4

sample was good. The corrective action taken was to perform more samples for the validation, considering these two days' lower results in the viability measurement. As a negative control, viabilities and counts were also checked by storing at temperatures of 2-8°C and -80°C for 24 hours to check the temperature storage effect and its proved cells were not viable. Despite bag No. 1 being broken after defrosting, the cells sample was recovered and run, and the viability was excellent and compliant. The corrective action was to order new bags from another manufacturer with the best quality standard to assure no other casualties due to the quality of any pack.

5. Conclusion

We concluded that the evaluation of the limiting factor for the validation objectives was fulfilled because our modern dry shipper controlled much of the vapor within the shipper. The CryoShipper can withstand being tilted during transit for 96 hours without risks of an adverse warming event, and the *CryoExtra 0140* tank performance was under the established parameters. Both types of cryogenic devices can be used for the safe cryopreservation of HSC samples keeping all the security measures and controls as advised. Nevertheless, further evaluation will be done systematically as per the validation's procedures, and we always recommend performing more sample run if a doubtful result appear.

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