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Transient warming events and cryogenic storage of cord blood mononuclear cells for stem cell transplantation

Wpływ krótkotrwałego oddziaływania temperatury pokojowej na zamrożone komórki jednojądrzaste z krwi pępowinowej przeznaczone do transplantacji

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Abstract

Background: *The term "Transient Warming Events" (TWE) has been used to describe the effects of brief periods of exposure of cryopreserved cells to ambient temperature in the course of retrieving and returning outgoing samples from the same cane. This study was designed to evaluate the effect of multiple TWEs on cryogenically preserved umbilical cord blood units (UCB) at the New Jersey Community Blood Centre using established assays of cell recovery, viability, and clonogenicity.*

Materials and methods: *The UCBs were collected and processed according to the laboratory SOPs from consenting donors. Each UCB was split into two 25 mL samples: an experimental and a control sample. Both samples were frozen using a Controlled Rate Freezer and then stored below –180°C in the vapour phase of a liquid nitrogen (LN_2) freezer. The experimental samples were exposed 10 times to room temperature until the sample reached a target temperature. We tested target temperatures of –110°C and –80°C. Finally, both experiment and control samples were simultaneously thawed in a 37°C dry bath. Each sample was tested for Total Nucleated Cell (TNC) count, CD34+ cell count, cell viability, and ability to generate Colony Forming Units (CFU) in a standardized assay.*

Results: *When UCB units were each exposed to 10 TWE to a target temperature of –110°C, analysis of cell counts showed a 1.5% loss of TNC, 3.5% loss of CD34+ cells, and 2% drop in CFU, with no loss of viability. None of the values between experimental and control was statistically significant. When UCB units were exposed to 10 TWE to a target temperature of –80°C, the differences between experimental and control samples in the TNC, CD34+ cell count and viability were also not statistically significant. The numbers of CFUs in experimental samples was decreased by 8% compared to control, and reached statistical significance. In the New Jersey Community Blood Centre, removal of a UCB from cryogenic storage takes less than a minute to execute, typical of other UCB storage centres with well planned and executed sample retrieval protocols. It would take three times as long for a 25 ml UCB to reach a target temperature of –110°C, and five times as long to reach a target temperature of –80°C. We found no significant degradation of cell function after repeated TWE to target temperatures as high as –110°C.*

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Conclusions: *The results of this study demonstrate that the TWE that UCB units experience in day-to-day sample retrieval activities are not detrimental to the samples that are returned repeatedly to cryogenic storage at the New Jersey Community Blood Centre. This should encourage other centres to conduct their own TWE studies to evaluate the effects of TWEs with each type of cryopreserved tissue and associated methods of storage and retrieval, before considering investing in highly expensive cryostorage units that are designed to minimize TWEs.*

Key words: cord blood, cryopreservation, transient warming events

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Streszczenie

Wstęp: *Określenie* Transient Warming Events *(TWEs) oznacza krótkie okresy ekspozycji zamrożonych komórek na temperaturę otoczenia, w trakcie rutynowych czynności laboratoryjnych. Celem pracy była ocena wpływu wielokrotnych TWEs na wybrane parametry zamrożonych jednostek krwi pępowinowej (UCB) przechowywanych w* New Jersey Community Blood Center *(NJCBC). Wykorzystano znane metody oceny i odzysku komórek, ich żywotności i zdolności klonogennych.*

Materiał i metody: *Jednostki UCB pobierano za zgodą dawców i poddawano preparatyce zgodnie z obowiązującymi procedurami (SOP,* standard operating procedures*). Każdą z nich dzielono na dwie 25-mililitrowe próbki, mrożono w urządzeniu do kontrolowanego zamrażania i przechowywano < –180°C. Próbki badane poddawano 10-krotnej ekspozycji na temperaturę pokojową, aż do uzyskania temperatur docelowych (–110°C , –80°C). Próbki kontrolne przechowywano w stałej temperaturze poniżej –180°C. Następnie próbki badane i kontrolne rozmrażano równocześnie w urządzeniu do suchego rozmrażania w temperaturze 37°C. W każdej próbce oceniano całkowitą liczbę komórek jądrzastych (TNC,* total nucleated cell count*), liczbę komórek CD34+, żywotność komórek oraz liczbę jednostek tworzących kolonie (CFU,* colony forming units*).*

Wyniki: *W próbkach poddanych 10-krotnemu działaniu temperatury pokojowej aż do osiągnięcia docelowej temperatury –110°C stwierdzono 1,5-procentowe zmniejszenie TNC, 3,5-procentowe zmniejszenie liczby komórek CD34+ oraz 2-procentowe zmniejszenie całkowitej liczby CFU. Nie zaobserwowano strat w żywotności komórek. Różnice między próbkami badanymi a kontrolnymi w zakresie badanych parametrów nie były statystycznie istotne.*

Różnice pomiędzy próbkami badanymi poddanymi 10-krotnemu działaniu temperatury pokojowej, aż do osiągnięcia temperatury –80°C a próbkami kontrolnymi również nie były statystycznie istotne pod względem liczby TNC, liczby komórek CD34+ i żywotności; całkowita liczba CFU w próbkach badanych była niższa o 8%, co było statystycznie istotne. W NJCBC jednorazowa ekspozycja jednostek UCB na temperaturę pokojową nie przekracza 1 min, a uzyskanie temperatury –110°C trwa około 3,5 minuty.

Wnioski: *Wyniki naszych badań wykazują, że wielokrotna, kontrolowana ekspozycja zamrożonych jednostek UCB w trakcie rutynowych czynności laboratoryjnych, nie wywiera szkodliwego wpływu na jakość biologiczną preparatów. Uzyskane przez nas wyniki powinny zachęcić inne ośrodki do prowadzenia własnych badań nad wpływem TWE na jakość poszczególnych rodzajów komórek przechowywanych w stanie zamrożenia oraz stosowanych metod zamrażania i rozmrażania. Dopiero wówczas można podejmować decyzje o zakupie wysoce specjalistycznego sprzętu chłodniczego z zamiarem obniżenia liczby TWE.*

Słowa kluczowe: krew pępowinowa, mrożenie, krótkie okresy ekspozycji na temperaturę pokojową

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Umbilical Cord Blood (UCB) is an attractive source of Haematopoietic Progenitor Cells (HPC). Its many advantages over bone marrow-derived HSCs include its almost immediate availability, low cost, and reduced HLA matching stringency [1, 2]. If prospectively tested and HLA confirmed, they can be delivered within 24–48 hours to any transplant centre in the world. Recent information indicates that UCBs are now chosen more often than Bone Marrow HSCs, and have become the second most popular source of HPC for required patient haematological reconstitution.

Current qualification criteria are based on minimum Nucleated Cell dose/kg [3], CD 34+ cell dose/ kg [4], and cell viability. Transplant physicians are making the decision based on the data collected before freezing and presented by processing laboratory.

In many instances transplant centres report a significant discrepancy between pre-freezing and post-thaw results of UCB units delivered. There may be many reasons behind low cell recovery and cell viability after thawing. Cryopreservation is the best method of long-term storage for HPC; however, storage conditions and sample handling can play a significant role in final cell quality.

It is postulated that Transient Warming Events (TWE) may have an adverse effect on cell quality. This phenomenon was first introduced by Dobrila et al. ("Transient Warming Events and Cell Viability of Placental/Umbilical Cord Blood" presented at the *ISHAGE,* Quebec, Canada and the *5th Tokyo International Symposium on Cord Blood Transplantation* in Tokyo, Japan. The results of these studies have been published online http://www.thermogenesis.com/CMSFiles/Pdf/Clinical/bmtposter.pdf.

The authors suggest that TWE is associated with the Glass Transition temperature (Tg) and that multiple exposures to TWE significantly affect cell viability and function. Bellow Tg temperature, molecules lose their mobility [5], which means that no damage to the cells should be observed. To minimize damage to the cell structure, samples should be stored approximately 20°C below the Tg [5]. According to some studies Tg is observed between –128 and –143°C [6]. Some other studies by Velikov [7] and Giovambattista et al. [8] suggest that true Tg occurs at around –103–108°C.

Unfortunately, in traditional freezers, cells are exposed multiple times (5–6) to ambient temperature when transferred from one freezer to another.

1. Transferring UCBs from a Controlled Rate Freezer to a Quarantine Freezer
2. Transferring UCBs from a Quarantine Freezer to a Permanent Storage Freezer
3. Removing a segment for HLA confirmatory typing
4. Transferring UCBs from the freezer to an LN_2 dry shipper
5. Transferring UCBs from a dry shipper to a temporary freezer at a Transplant Centre
6. Thawing the unit.

In addition, units that are stored in racks shared by multiple units can be exposed to TWE multiple times when other samples are accessed.

We decided to evaluate the effect of such events and calculate the potential risk of multiple exposures on in vitro cell quality after thawing.

Material and methods

Collection and processing

Eighteen Umbilical Cord Blood units (UCB) were collected according to our laboratory's SOP, with appropriate NMDP donor consent. Blood was collected into 250 mL collection bags (Baxter, IL) containing 35 mL of CPD. After UCB qualification, a calculated volume of Hydroxy Ethyl Starch (HESPAN; Braun Medical Inc. CA) was added to the collection bag that was then sterile docked (TCD B40, Haemonetics Corp. MA) with Processing Kit 530 (Biosafe, Switzerland). Cell separation was done using a Sepax AS-210 (Biosafe, Switzerland). Following cell separation (35–45 min) the volume of each unit was adjusted to 40 mL using autologous plasma. 10 mL of 55% DMSO and 5% Dextran 40 (CryoSure-Dex40, WAK-Chemie Medical GmbH, Germany) was then aspirated to a syringe, connected to a processing bag and slowly added (0.5 mL/minute) using an 11 Plus syringe pump (Harvard Apparatus, MA). Each sample was then split into two 25 mL portions, transferred to a freezing container (Pall Medical, NY), placed in metal canisters, and then frozen using a Controlled Rate Freezer (CryoMed, Thermo Electron Corp., OH). When the samples reached –90°C, the bags were transferred to a V3000 Isotherm LN_2 freezer (Custom BioGenic System, MI) and then stored for 3–8 months below –180°C in the vapour phase of liquid nitrogen.

Bags designated for –110°C (10 UCB pairs) or –80°C (8 UCB pairs) were placed in one rack (A) and the control pair samples in another rack (B). One of the cassettes in rack A was opened and a thermocouple was placed on the surface of the UCB. Just before rack A was removed from storage to

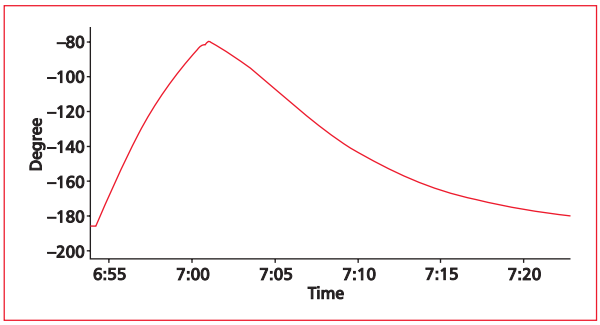

Figure 1. Warming and freezing curve of one event; sample warmed to –80°C. Similar graphs were obtained in the course of study for every sample used in the project and exposed 10 times to the ambient temperature

Rycina 1. Krzywa ogrzania i ponownego zamrażania preparatow do temperatury –80°C. Podobne wykresy uzyskano dla każdego preparatu w trakcie eksperymentu podlegajacego 10-krotnej ekspozycji na temperaturę otoczenia

a room temperature, the thermocouple was connected to an Omegaette HH306 data logger (Omega, CT) and activated to observe and record temperature changes. Each UCB in rack A remained at room temperature until the temperature of the UCB reached the target temperature, at which point rack A was promptly returned to the LN_2 vapour freezer. It took approximately 3.5 minutes for the surface of the cryopreserved UCB to reach –110°C, whereas to reach –80°C it took approximately 6.5 minutes. One of the samples from each UCB was exposed to ambient temperature until the temperature of the samples reached either –110°C or –80°C and was then returned to the LN_2 freezer.

Data representing one of ten TWEs when samples in rack A were warmed to –80°C is shown in Figure 1. Control samples in rack B remained in the vapour phase of LN throughout the whole experiment. This procedure was repeated 10 times every 24 hours on the same 18 samples before all 36 samples were thawed in a 37°C dry bath CytoTherm D1 (CytoTherm, NJ). Samples were thawed in pairs, one pair at a time. Once thawed, the samples were sampled and assessed for Total Nucleated Cell (TNC) count, CD34+ cell count, cell viability, and ability to generate Colony Forming Units (CFU).

Testing

Whole Blood Cell (WBC) count was performed using Sysmex XE-2100 (Sysmex, Japan). The TNC was calculated by multiplying the WBC by the UCB total volume.

The CD34+ cell assay and cell viability were obtained using dual platform ISHAGE protocol and FACScalibur (BD Bioscience, CA) flow cytometer. Monoclonal antibodies used in the CD34+ assay include anti-CD34 and anti-CD45. The cell viability was established using 7-Amino-actinomycin D (7-AAD) . The CFU assay was performed using MethoCult GF H4434 (Stem Cell Technologies, Canada). Twenty-five thousand Nucleated Cells were seeded on each of three dishes. Then they were incubated at 37°C and 95% humidity for 14 days. After 14 days three types of colonies were counted: BFU-E, CFU-GM and CFU-GEMM.

Statistics

Paired student's test was used to show whether there was a statistical significant difference between the test set of data and the control set of data.

Results

The first set of experiments when samples in rack A were warmed to –110°C showed small differences between tested and controlled samples. Average time to warm to —110°C was 3.5 minutes. The TNC was reduced by 1.5%. The total number of CD34+ cells was 3.5% below the control, and total number of CFU's was only 2% lower. Viability of the cells tested the same. All differences were not statistically significant.

Because of the lack of significant effects of TWE in the first set of experiments, the experiments were repeated, only this time the samples were warmed to -80°C. This process took on average 6.5 minutes. This time three out of four measured parameters were not statistically different. The only difference was observed in Total CFU, which was 8.5% lower in samples exposed to TWE when compared to their own controls stored in the vapour phase of LN_2 during the whole study. Closer analysis of different forms of colonies showed that the only difference was observed in numbers of BFU-E population. The other colonies of CFU-GM and CFU-GEMM remained not statistically significant.

Discussion

There is no question that the handling of biological tissue like UCB cells for the long term in LN_2 freezers may have some effect on post-thawing cell quality and consequently have an impact on post-transplantation bone marrow reconstitution. Tran-

Table 1. Quality results for 10 products following 10 exposures to ambient temperatures until the sample reached –110°C. Results presented as Mean ± 1 Standard Deviation

Tabela 1. Parametry jakości dla 10 preparatów po 10 ekspozycjach na temperaturę otoczenia, do momentu ogrzania preparatu do –110°C. Wyniki przedstawiono jako średnią ± 1 odchylenie standardowe

Final temperature –110°C N = 10	Control UCB not expose TWE	UCB expose 10 times to TWE	Student's *t* test
TNC (× 10^6 cells)	Range 579–793 Mean 649.95 ± 74.23	Range 562–799 Mean 640.50 ± 71.74	0.41 ns
CD34 (× 10^6 cells)	Range 1.01–3.65 Mean 1.92 ± 0.84	Range 0.83–3.71 Mean 1.85 ± 0.94	0.73 ns
Viability (%)	Range 73–90 Mean 82.40 ± 5.62	Range 73–91 Mean 62.40 ± 5.62	0.53 ns
Total CFU (× 10^6 cells)*	Range 0.180–0.867 Mean 0.449 ± 0.249	Range 0.158–0.895 Mean 0.441 ± 0.253	0.61 ns

NS — not statistically significant; *n = 9: CFU analysis was available only for 9 samples

Table 2. Quality results for 8 products following 10 exposures to ambient temperatures until the sample reached –80°C. Results presented as Mean ± 1 Standard Deviation

Tabela 2. Parametry jakości dla 8 preparatow po 10 ekspozycjach na temperaturę otoczenia do momentu ogrzania preparatu do –80°C . Wyniki przedstawiono jako średnią ± 1 odchylenie standardowe

Final Temperature –80°C n = 8	Control UCB Not Exposed to TWE	UCB Exposed 10 times to TWE	Student's t-test
TNC (× 10^6 cells)	Range 295–646 Mean 474.83 ± 131.07	Range 303–656 Mean 481.78 ± 130.51	0.54 (NS)
CD34 (× 10^6 cells)	Range 0.71–3.25 Mean 1.32 ± 0.83	Range 0.69–3.17 Mean 1.29 ± 0.80	0.55 (NS)
Viability %	Range 73–90 Mean 89.88 ± 6.75	Range 83–90 Mean 91.25 ± 5.65	0.11 (NS)
Total CFU (× 10^6 cells)	Range 0.157–0.819 Mean 0.366 ± 0.211	Range 0.109–0.657 Mean 0.308 ± 0.191	($p < 0.05$)

NS — not statistically significant

sient warming events are associated with morphological changes of ice at the so-called glass transition temperature. Many studies suggest that this is observed around –135°C; however, there are other studies suggesting that the Tg happened at around –108°C [7, 8]. The change of ice property at the Tg temperature can potentially affect cell viability. The multiple exposures that occur in every laboratory can accelerate this process. There has been little published data to determine whether multiple excursions through the Tg temperature have an effect on cells that can be considered relevant to their desired functional therapeutic efficacy.

Our first step was to test the hypothesis that multiple events of transient warming could reveal symptoms of adverse reaction. Two identical samples were prepared from one unit and stored in the vapour phase of LN_2 for at least three months. One sample was then repeatedly exposed to room temperature to allow the sample surface warm to —110°C.

After 10 events, both control and test samples were thawed simultaneously and evaluated.

To our surprise we did not observe any evidence of cell deterioration. Small changes in all tested parameters were not statistically significant. This very important observation leads us to the conclusion that T*g* may occur at a higher temperature than –135°C. Based on the studies of Velikov [7] and Giovambattist [8], T*g* is observed at –108°C. This could potentially explain why we did not observe any changes in cell quality in our first study. To address this question, we tested these same parameters using this same protocol but warming our experimental UCBs to -80°C. The second set of stu-

dies required longer time exposure (about 6.5 min) to warm up the surface of the sample to –80°C.

This time the changes in TNC and Total CD34+ as well as in cell viability were not statistically significant. However, a significant drop in total CFUs of about 8.5% was observed. The results of these two experiments suggest that the real T*g* is either lower than –135°C or that excursion of the cells through the T*g* range does not significantly affect any of the parameters measured in this study. Irrespective of the explanation, our data indicates that we can put quite reasonable limitations on the time and number of transfers of UCB in and out of the LN_2 cryopreservation during normal daily operations of our cord blood bank without causing damage to the cells.

Well organized and planned storage and appropriate training of laboratory employees at the New Jersey Community Blood Centre results in exposure of UCB to ambient temperatures for much less than 3.5 minutes. In our laboratory we try to reduce the time when UCB units are exposed to room temperature, and most of the manipulations, like taking a segment of the bag or inspecting the ID before shipping the unit to transplant centre, are carried inside the vapour LN_2 freezer. From our experience, each manipulation takes less than one minute, which, based on our study, warms the sample to –160 ± 5°C.

In summary, we believe that it is important to properly train laboratory personnel how to handle cryopreserved samples and make them understand the consequences of TWE. Multiple warm up above the T*g* temperature will affect cell quality and may cause a lack of engraftment after transplantation. Although our TWE experiments to -80°C demonstrated that even excursions through the T*g* had little, if any effect on sample integrity, based on documented studies of glass transition temperatures it is nonetheless wise to avoid multiple excursions around a temperature of approximately –108°C. This is clearly not a significant issue for our facility. Our study results should encourage other centres to conduct their own TWE studies to evaluate the effects of TWEs with each type of cryopreserved tissue and associated methods of storage and retrieval, before considering investing in highly expensive cryostorage units that are designed to minimize TWEs.

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