한국 공공 제대혈은행의 보관 정책 개선: 장기 보관 및 단기 보관 제대혈의 품질 비교

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Improving Storage Policy in Korean Public Cord Blood Banks: Comparison of Quality between Long-Term and Short-Term Storage of Cord Blood

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Background: The decreased use of cord blood units (CBU) due to improvements in haploidentical transplantation is a financial burden for public cord blood banks. Currently, there is no guidance regarding the length of cryopreservation of CBU in Korean public banks. The relative quality of long-term storage CB (LTCB) and short-term storage CB (STCB) needs to be evaluated to establish a storage policy.

Methods: Thirty-four and thirty-one units of CB cryopreserved for less than one year and up to 14~15.5 years, respectively, in the Busan Gyeongnam Public Cord Blood Bank were assessed. The total nucleated cells (TNCs), CD34+ cell counts, and colony-forming units-granulocyte monocyte (CFU-GM) were examined. The cell viabilities were evaluated by Eosin-Y exclusion staining and 7-aminoactinomycin D flow cytometry. The number of stored Korean public CB units from 2000 to 2016 was determined and categorized according to TNCs.

Results: The post-thawing viability of the STCBs measured by flow cytometry was consistently higher than that of the LTCBs (TNCs, 62.5% vs 57.3%; MNCs, 93.1% vs 88.9%; CD34+ cells 95.7% vs 94.0%). The CD34+ cell viability was significantly higher in STCB ($P=0.03$). The CFU-GM after thawing was higher in STCBs (61.5 ± 23.4 vs 49.9 ± 22.8 [0.95 mm^2] $P=0.05$). Of the 48,161 CB units stored until 2016, Dec, 9,493 (19.7%), which were stored until 2006, had been stored for more than 10 years.

Conclusion: LTCB with a low number of cells ($<0.7 \times 10^9$ cells) should be considered to exclude from storage for therapeutic purposes to improve the storage efficiency. (Korean J Blood Transfus 2020;31:119-130)

Key words: Cord blood, Cryopreservation, CFU-GM, CD34, Viability, Public cord blood bank

Received on May 4, 2020. Revised on July 24, 2020. Accepted on August 4, 2020

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This study was supported by a grant provided by the Korea Centers for Disease Control and Prevention, Republic of Korea (2016-E83004-00).

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Introduction

Cord blood (CB) is an important source of hematopoietic stem cell transplantation and regenerative therapy [1,2]. CB is easier to obtain than bone marrow or peripheral blood stem cells, because CB is a ready-made product. The improvement of procedures for haploidentical stem cell transplantation has resulted in the decreased use of CB [3-5]. This has increased the financial strain on public CB banks and threatens the sustainability of public umbilical CB blood banks [6].

Public blood banks store CB indefinitely for possible clinical use. Hematopoietic stem cells in CB stored for 23.5 years showed functionally efficient recovery [7]. There was no difference of clinical transplant outcomes between CB stored up to 12.2 years and CB stored for a short time in 86 CB transplant recipients [8]. However, a report described that CB bags cryopreserved longer were more vulnerable to breakage [9]. The Japanese CB Bank limits the storage duration of CB to 10 years [10]. With the implementation of the Cord Blood Management and Research Act in 2010, a cut-off of total nucleated cells (TNCs) for freezing of CB was established in Korea [11]. Cryopreservation of CB above 0.7×10^9 TNC began in 2011 July.

A significant correlation between TNCs and hematopoietic colony-forming cells (CFCs) and CFCs of CB units was strongly associated with covariates of post-transplantation survival [12]. Therefore, transplantation clinicians prefer CB with high numbers of TNCs. Currently, there is no regulation for the duration of storage of CB in Korean public banks. The cost of storage for CB has increased. To improve

the policy for CB storage, we presently compared the quality of long-term storage and short-term storage of CB (LTCB and STCB, respectively), and determined the duration of storage and the TNCs of CB already stored in public banks.

Materials and Methods

1. Study population

Sixty-five units of CB donated to a public CB bank (Busan Gyeongnam Public Cord Blood Bank, Korea) were included. Thirty-four units of CB cryopreserved for less than 1 year (STCB) and 31 units of CB stored for up to 14~15.5 years (LTCB) were used to compare the quality of CB. CB stored for less than 1 year was selected because of insufficient TNCs for clinical use. The study was approved by the Institutional Review Board of Dong-A University Hospital (IRB No. 16-191).

2. Preparation of CB units

The CB was processed within 36 hours of collection. All CB units were processed and cryopreserved as published previously [13]. The processing and cryopreservation methods for STCB and LTCB were the same. Briefly, the buffy coats of CB were separated by volume reduction and red blood cell depletion, and a mixture of 3 mL dimethylsulfoxide (CryoSure-DMSO; WAK-Chemie Medical GmbH, Steinbach, Germany) and 3 mL dextran 40 (Dextran-40; Dex Injection, Daihan, Korea) in 24 mL of the mononuclear fraction of CB were added before freezing. A liquid nitrogen system (MVE 1520, HE-190; Chart Industries, Inc., Luxembourg,

Belgium) were used for cryopreservation. Measurements of TNCs, CD34+ cells, and cell viability were performed as described subsequently using aliquots of fresh CB units before processing and following thawing of CB samples. After rapid thawing in a 37°C water bath, the colony-forming unit-granulocyte macrophage (CFU-GM) assay was conducted.

3. TNCs, cell viability, CD34+ cell count, and CFU-GM assay

The complete blood count (CBC) analysis of TNCs differed according to the duration of CB storage. The measurements were done using the SE-9000 device (Sysmex, Kobe, Japan) for LTCB and the Sysmex XE-2100 (Sysmex, Kobe, Japan) for STCB.

Cell viability assays were performed using Eosin-Y exclusion staining and 7-aminoactinomycin D (7-AAD) staining for flow cytometry. CB samples (each 50 µL) were mixed with an equal volume of 2% Eosin-Y dye (Sigma-Aldrich, St. Louis, MO). At least 100 cells were microscopically analyzed for viability in a hemocytometer chamber. Cell viability was expressed as the percentage of living cells counted. The thawed CB samples were prepared according to the manufacturer's instructions using a 7-AAD viability dye contained in a Stem-Kit Reagent (Beckman Coulter Company, Brea, CA). A sequential gating procedure using the International Society of Hematotherapy and Graft Engineering (ISHAGE) method was performed [14]. The cell viability using 7-AAD labeling in each TNCs, mononuclear cells (MNCs), and CD34 cell populations was determined. The percent of post-thawing 7-AAD viability was expressed as a percent of 7-AAD-negative cells

among TNCs, mononuclear cells (MNCs), and CD34 cell populations.

Enumeration of CD34+ cells was performed using the method of the ISHAGE guidelines for CD34+ cell determination by flow cytometry. CD34+ cells in pre-frozen LTCB were detected with ProCOUNT reagent (BD Biosciences, San Jose, CA) and analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter, CA, USA). CD34+ cells of post-thawed LTCB and those of pre-frozen and post-thawed STCB were assayed using Stem-Kit Reagent and NAVIOS flow cytometer (Beckman Coulter, CA, USA). Each protocol was performed according to the manufacturer's instructions.

The CFU-GM assay was performed using the CAMEO-4 miniaturized semi-solid methylcellulose medium (HemoGenix, Colorado Springs, CO). Cryopreserved CB that was rapidly thawed in a 37°C water bath was adjusted to a TNC density of 2×10^6 cells/mL. The total volume prepared was 0.6 mL, including 0.06 mL of adjusted CB cells and 0.54 mL of CAMEO-4 master mix. Using a positive displacement repeater pipette (Eppendorf, Hamburg, Germany), 0.1 mL was dispensed into four replicate wells in a 35 mm Petri dish, each with a growth surface area of 0.95 mm².

4. Collection of information of storage status of public CB in Korea

We searched the number of stored public CB units from 2000 to 2016, and categorized the CB units according to TNC. All information was collected through Korean Cord Blood Information Center.

5. Statistical analysis

The difference of TNCs, CD34+ cell count, CFU-

GM, and cell viability between STCB and LTCB were compared by Student's t-test. Regression analysis between post-thawed CD34+ cell counts and

Table 1. Comparison of TNCs, CD34+ cell count, and CFU-GM in short-term storage CB and long-term storage CB

		Short-term CB (n=34)	Long-term CB (n=31)	P value
TNCs ($\times 10^8$)	Pre-freezing	6.6 $\pm$ 1.0 (4.58~8.17)	5.5 $\pm$ 1.6* (2.74~9.56)	0.001
	Post-thawing	5.1 $\pm$ 0.9 (3.17~6.55)	5.5 $\pm$ 1.4 (3.60~7.32)	0.16
	Recovery rate (%)	77.4 $\pm$ 9.8 (63~118)	102.8 $\pm$ 18.3 (61~159)	<0.0001
CD34+ cells (μ L)	Pre-freezing	64.6 $\pm$ 47.8 (10~266)	64.1 $\pm$ 48.7 (10~238)	0.96
	Post-thawing	45.6 $\pm$ 26.7 (8~123)	45.1 $\pm$ 21.3 (8~104)	0.93
	Recovery rate (%)	77.2 $\pm$ 25.2 (32~158)	88.6 $\pm$ 39.9 (18~236)	0.18
CFU-GM (0.95 mm ²)	Post-thawing	61.5 $\pm$ 23.4 (10~134)	49.9 $\pm$ 22.8 (15~183) [†]	0.05

Data are presented as mean $\pm$ SD (range).

*TNCs were measured by different complete blood cell analyzers.

[†]The P value was calculated after excluding outliers.

Fig. 1. Distribution plots of (A) post-thawed CD34+ cell count ($P=0.93$), (B) post-thawed CFU-GM excluding outliers ($P=0.05$), and (C) recovery rate of CD34+ cell count ($P=0.18$).

post-thawed CFU-GM was performed. A $P < 0.05$ was considered significant. Analysis were conducted using Medcalc computer software (version 17.9.2; Medcalc, Ostend, Belgium).

Results

1. Comparison of TNC, CD34+ cell count, and CFU-GM

TNC, CD34+ cell count, and CFU-GM in STCB and LTCB units are shown in Table 1. The pre-freezing TNC of LTCB units were measured with different CBC analyzers, so the recovery rate of TNC may exceed 100%. The steps for processing of CB and personnel were not changed between STCB and LTCB. The recovery rate of CD34+ cell count after thawing did not differ according to duration of storage, but the recovery rates of LTCB varied. Post-thawed CFU-GM was higher in STCB than that of LTCB, but the statistical significance was marginal ($P=0.05$) (Fig. 1).

2. Comparison of cell viabilities

Eosin-Y exclusion staining and 7-AAD staining for cell viability revealed differences between STCB and LTCB (Table 2). Eosin-Y exclusion staining indicated $>90\%$ viability, except for post-thawed STCB (88.6%). The 7-AAD viability differed according to the gating of TNCs, MNCs, and CD34+ cells (Fig. 2). In post-thawed CB, viability of CD34+ cells in STCB was significantly higher than that of LTCB ($95.7\% \pm 2.8$ vs $94.0\% \pm 3.2$ $P=0.03$).

3. Correlation between post-thawed CD34+ cell count and CFU-GM

A positive correlation between post-thawed CD34+ cell count and post-thawed CFU-GM was observed (correlation coefficient 0.47, 95% CI 0.25~0.63, $P < 0.001$) (Fig. 3). STCB displayed superior correlation ($r=0.57$) to LTCB ($r=0.39$) but the difference was not significant ($P=0.36$).

4. Information of storage status of public CB

Of the 48,161 CB units stored until 2016 (The

Table 2. Cell viabilities measured by Eosin-Y exclusion staining and 7-AAD flow cytometry in short-term storage CB and long-term storage CB

		Short-term CB (n=34)	Long-term CB (n=31)	P value
Eosin-Y (%)	Pre-freezing	90.8 $\pm$ 1.7	97.7 $\pm$ 2.4	<0.0001
	Post-thawing	88.6 $\pm$ 2.7	90.8 $\pm$ 4.5	0.01
7-AAD (%)	Post-thawing			
	TNCs	62.5 $\pm$ 11.5	57.3 $\pm$ 17.0	0.16
	MNCs	93.1 $\pm$ 3.0	88.9 $\pm$ 12.8	0.08
	CD34+ cells	95.7 $\pm$ 2.8	94.0 $\pm$ 3.2	0.03

Data are presented as mean $\pm$ SD (range).

Fig. 2. Viability of TNCs, MNCs, and CD34+ cells determined by flow cytometry using 7-AAD.

Fig. 3. Regression analysis between post-thawed CD34⁺ cell counts and post-thawed CFU-GM. A positive correlation between post-thawed CD34⁺ cell count and post-thawed CFU-GM was observed (correlation coefficient 0.47, 95% CI 0.25~0.63, $P<0.001$) in 65 CB units. The short-term storage CB (A) displayed superior correlation ($r=0.57$) to long-term storage CB (B) ($r=0.39$) but the difference was not significant ($P=0.36$).

search date was October 19, 2017), 10,964 (22.7%) units contained $<0.7 \times 10^9$ cells. All of 10,964 CB units $<0.7 \times 10^9$ cells were stored only until 2011. Since 2012, CBs $<0.7 \times 10^9$ cells were not cryopreserved for therapeutic use. The number of CBs stored for more than 10 years as of 2016 was 9,493 (19.7%).

Discussion

The first CB transplant was performed in 1988. CB has become an important source of hematopoietic stem cell transplantation [15]. As the use of haploidentical stem cell transplantations continues to increase, the number of CB transplants in malignant diseases has decreased since 2011 [5]. The financial sustainability of umbilical CB banks in Korea has been threatened. A cost-effectiveness

analysis for continuous operation of CB banks suggested increasing the pre-freezing TNC level or centralizing blood bank activities [6,16]. We performed this study to improve the storage policy of CB in public CB banks in Korea.

The capacities of hematopoietic stem cells in LTCB have been studied [7,10,17,18]. These studies were conducted on umbilical CB stored for 10 to 23.5 years, and the experiments were performed on approximately 20 CB samples. The data suggested that the efficient preservation of the potency of long-term cryopreserved hematopoietic stem cells of CB. In this study we tested more than 30 CB samples in each group. As far as we know, this represents the largest number of CB samples studies to date, though the storage duration did not exceed 20 years.

The factors that may affect cell viability and clo-

nogenic potential include cryopreservation process, thawing process, and storage duration. A recent report studying 27 CB units has shown that viability of CD45+ and CD34+ cells was not affected by the thawing methods. The thirteen CB units cryopreserved for more than 10 years showed decreased cell viability but CFU was not significantly diminished [19]. In our study, the cryopreservation process did not change after the cord blood bank was started, and the thawing method was equally applied to STCB and LTCB. Though the statistical significance was marginal, the post-thawed CFU-GM was lower in LTCB in our result. In addition, 31 stem cell products stored for 11~19 years (median 15 years) from peripheral blood or bone marrow reportedly deteriorated during long-term storage. TNC recovery, CD34+ cell count, and cell viability decreased with time, but CFU-GM did not [20]. The previous studies have indicated that the potency of LTCB is not affected by cryopreservation. In this study, marginal reduction of clonogenic potential in LTCB was observed. Statistical differences according to cryopreservation may have been observed as more than 30 CB were studied each, depending on the timing of CB storage. As previous studies have reported reductions in cell viability of LTCB, quality control is necessary during cryopreservation.

The CD34+ cell dose and CFU-GM dose are better predictors of neutrophil recovery than the TNC in CB transplantation [12,21]. The positive correlation between CD34+ cell count and CFU-GM after thawing was observed; STCB showed superior correlation to LTCB in our study. These findings are consistent with the previous study of 18 CB units [10]. The correlation coefficient (r) between post-

thawed CD34+ cells and post-thawed CFU-GM was 0.430 in 18 units of CB cryopreserved for 10 years in the previous study and 0.39 in our study. For STCB, the r was 0.706 and 0.57, in the same respective order. In our study, CD34+ cell viability assayed by flow cytometry with 7-AAD after thawing exceeded 94% in both groups. However, CD34+ cell viability was significantly higher in the STCB group. It has been reported that TNC, viable CD45+ and CD34+ cells have a statistically significant correlation with CD34 viability after thawing [22]. In addition, a recent study suggested that CB transplantation with low TNC but high CD34+ cells was associated with enhanced engraftment [23]. These finding indicates the importance of viable CD34+ cells as a relevant factor for hematopoietic cell recovery. Lower post-thawed CFU-GM and lower CD34+ cell viability indicate the negative effect of long-term cryopreservation on the clonogenic potential.

Cell viabilities measured by Eosin-Y exclusion staining were higher in LTCB in the present study. LTCB units were collected from the hospital affiliated with a public CB bank or the nearby hospitals. This enabled quicker delivery from collection to preparation of CB units. However, one study suggested that this dye exclusion staining may not accurately assess the viability upon storage. The viability of CB as measured by Trypan blue dye may be falsely reassuring, even CB units with no CFU growth [24].

The cell dose should be considered concerning the reduction of treatment-related mortality by delayed neutrophil engraftment in CB transplantation [25]. Among CB units with $>2.0 \times 10^9$ cells, 95.7%

had supplied CB for transplantation according to a report from the Japanese Cord Blood Bank Network [26]. Disproportionate use of CB units with high cell count was also observed in Korea. The CB units with below 1.0×10^9 cells had a usage rate of less than 1%. The CB units with cell numbers above 1.1×10^9 and below 1.2×10^9 showed 1.2% usage and CB units with a cell number of 2.0×10^9 or more showed usage rate of 9.8% for therapeutic purpose [27]. Clinical demand of CB with a high cell count and the financial difficulties of public CB bank prompted the increase in the cryopreservation criteria of TNCs [28]. A pre-freezing level of 1.8×10^9 TNCs was suggested as the most cost-effective strategy for public CB banks [6]. In Japan, TNCs before processing is 1.25×10^9 and 17.4% of CB units stored has $>1.25 \times 10^9$ TNCs [29]. In Korea, cryopreservation of cells has been performed since July 2011 in post-processing CB units with $>0.7 \times 10^9$ cells. Considerable low cell count CB units have been stored in public CB banks; in over 10% of the CB units, CB is stored for more than 10 years. Considering that transplantation clinicians tend not to prefer CB units with low cell count and the reduced clonogenic potential of long-term cryopreserved CB, long-term cryopreserved CB with a low number of cells should be considered to exclude from storage for therapeutic purposes. The CB units that are not targeted for hematopoietic stem cell transplantation may be used in the research of regenerative medicine and cell therapy [2,30].

The study has some limitations. First, the CFU-GM assay was not carried out before cryopreservation, so the recovery rate of CFU-GM could not be calculated. Second, additional markers that

could distinguish storage lesion from long-term cryopreservation, such as apoptosis, were not evaluated [31,32]. Nevertheless, apoptotic markers would be used as a rapid evaluation of engrafting potential to predict CFU. All CB units tested in this study performed CFU-GM and it is considered that the effect of cryopreservation was limited. And third, even though the pre-freezing TNCs of LTCB units were measured with different CBC analyzers, the pre-freezing TNCs in LTCB were significantly lower than those of STCB. This may have affected lower post-thawing CFU-GM in LTCB.

In summary, this investigation of the largest number of LTCB to date in Korea revealed lower post-thawing CFU-GM and lower CD34+ cell viability. This suggests that the long-term cryopreservation of CB has a negative effect on the clonogenic potential. LTCB with a low number of cells should be considered to exclude from storage for therapeutic purposes to improve storage efficiency.

Acknowledgments

We are grateful to all laboratory technicians and mothers donated cord blood. Our thanks go to Miri Kang and Sung Sook Cho.

요약

배경: 조직적합성 반일치이식의 증가로 인해 제대혈의 사용이 줄어들어 공공 제대혈 은행의 재정적 부담이 증가하고 있다. 국내 기증 제대혈의 보관 기간에 관한 규정은 없으며, 보관 정책을 설정하려면 장기보관 및 단기보관 제대혈의 품질 평가가 필요하다.

방법: 부산경남 공공 제대혈 은행에서 1년 미만 및 최대 14~15.5년 동안 냉동 보존된 34개 및 31개 제대혈 유닛을 각각 평가 하였다. 총 유핵세포(TNCs), CD34+ 세포 수 및 CFU-GM를 조사 하였다. 세포 생존율은 Eosin-Y 염색 및 7-amino-actinomycin D 유세포 분석에 의해 평가되었다. 2000년부터 2016년 12월까지 보관된 한국 공공 제대혈 단위의 수는 총 유핵세포에 따라 분류하였다.

결과: 유세포 분석에 의해 측정된 단기보관 제대혈제제의 해동 후 생존율은 장기보관 제대혈보다 일관되게 높았다(TNCs, 62.5% vs 57.3%; MNCs, 93.1% vs 88.9%; CD34+ 세포 95.7% vs 94.0%). CD34+ 세포 생존율은 단기보관 제대혈에서 유의하게 더 높았으며($P=0.03$), 해동 후 CFU-GM은 단기보관 제대혈에서 더 높았다(61.5 ± 23.4 vs 49.9 ± 22.8 [0.95 mm^2] $P=0.05$). 2016년 12월까지 보관된 48,161개의 제대혈 유닛 중 2006년까지 보관되었던 9,493 (19.7%)이 10년 이상 경과하였다.

결론: 공공 제대혈은행의 저장 효율을 향상시키기 위해 적은 세포 수($<0.7 \times 10^9$)를 가진 장기 보관 제대혈을 치료적 목적으로 보관하는 것은 배제하여야 할 것이다.

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