

Time from cord blood collection to processing and temperature influence the quality of mononuclear cell products isolated using a density-gradient protocol

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Background: For clinical cord blood (CB) transplantation, CB is processed using a standard hydroxyethyl starch protocol generally within 48 h of collection at room temperature. However, for tissue stem cell research, mononuclear cells (MNCs) were isolated from CB using a Ficoll-Paque density-gradient method. Here we report the effect of storage temperature and time from CB collection to processing on the cord blood mononuclear cells (CB-MNCs) isolated using a density-gradient method.

Methods: We processed CB using a Ficoll-Paque density-gradient method to collect the cells in the MNC layer. Cells were analyzed using an automatic blood cell counter, and CD34⁺ cells were counted according to the ISHAGE method.

Results: The recovery rate of viable MNCs in the CB-MNC layer was inversely related to the time from collection to processing of CB samples. However, recoveries of total nucleated cell and CD34⁺ were not affected by the time from collection to processing. The percentage of neutrophil contamination in the MNC layer increased significantly with increasing time from CB collection to processing ($n=100$, $p<0.0001$). Furthermore, CB stored at low temperatures had significantly less neutrophil contamination in the MNC layer than those stored at room temperature for 30 h after CB collection.

Discussion: Storage temperature and time from collection to processing influence the composition of CB-MNCs products processed using a Ficoll-Paque density-gradient method.

Keywords: cord blood (CB), mononuclear cells (MNCs), cord blood (CB) processing, density-gradient method, storage, viability, recovery

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Introduction

Cord blood (CB) cells are of interest not only as a source of hematopoietic stem cells but also tissue stem cells, which have potential therapeutic applications in regenerative medicine. The National Research Cord Blood Stem Cell Resource Bank (<http://scb.ims.u-tokyo.ac.jp/>), the first stem cell bank established in the world, began in 2003 as part of a project to realize the potential of regenerative medicine. It is organized by the Ministry of Education, Culture, Sports, Science and Technology in Japan. The research CB banks isolated cord blood nucleated cells (CB-NCs), CD34⁺ cells, and

mononuclear cells (CB-MNCs) from nonconforming CBs for clinical use, and supplies CB in research-use for domestic researchers via Riken Bioresource Center. In this project, we first processed CB-NCs using a hydroxyethyl starch (HES) centrifugation method, as used clinically for CBB, and provided the frozen CB-NCs units to researchers. However, when frozen CB-NCs units are thawed by the ordinary thawing method, such as by mixing with a large volume of medium, aggregation often occurs due to a large quantity of residual neutrophils and red blood cells. In this situation, general researchers find it difficult to process and con-

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tinue the culture of frozen CB-NCs. In addition, many researchers in the field of regenerative medicine have reported that CB-MNCs contain a potential source for regeneration^{1)~4)}. Therefore, since 2008, we have started to release CB-MNCs products for researchers. CB-MNCs are processed by the well-known density-gradient method based on differences in cell size and density. To assure the quality of CB-MNC products for research use, we set the following quality criteria: 1) informed consent from the mother, 2) negative for infection and genetic background, 3) initiation of CB processing within 36 h of CB collection in obstetrics, 4) neutrophil contamination in the CB-MNCs products of less than 20%, and 5) more than 1×10^7 of MNC per tube. In this setting, we found that neutrophil contamination often exceeded 20%. Here, to resolve the problem of neutrophil contamination, we investigated potential factors, including time and temperature, which may be critical to the excess of neutrophil contamination.

CB stored at room temperature (RT) is preferred to CB stored at a low temperature because platelets in CB units may aggregate at low temperatures^{5)~8)}. Guidelines for the store period of CB samples differ. According to the guidelines (<http://www.factwebsite.org/Standards/>) of many countries except Japan, CB samples should be processed within 48 h of collection in obstetrics, whereas guidelines released by the Japan Cord Blood Bank Network suggest that CB samples be processed within 24 h (<https://www.j-cord.gr.jp/ja/bank/technical.html>). However, the effects of storage time and temperature on the isolation of CB-MNCs using a density-gradient method are not fully understood. Here we show that time from collection to processing and storage temperature influence the quality of CB-MNC products processed using a Ficoll-Paque density-gradient method.

Materials and Methods

CB collection, store and transportation to IMSUT-CRC

The study for Research Cord Blood Stem Cell Resource Bank Project was approved by the Ethical Committee of the Institute of Medical Science, The University of Tokyo (IMSUT), Japan, and by the Tokyo Cord Blood Bank (Tokyo CBB). Informed consent was obtained from the mothers involved in this project. CB was collected in collection bag (Kawasumi, KBS-200CA 8, Japan) and stored at RT (around 20°C) in a plastic

store box setting in a delivery room of obstetrics until the CB units were picked up at around 8:30 am every day. Therefore, some CB units collected in the prior day were stored overnight (up to 24 hours), and others collected in the early morning (until CB units were picked up) were stored shortly in the obstetrics. The collected CB units in the obstetrics were transported to Tokyo CBB at RT by the courier every morning. It almost constantly took less than one hours to transport CB from obstetrics to Tokyo CBB. CB units were determined to be conforming or not for transplantation in Tokyo CBB. The nonconforming CB units were anonymized in the Tokyo CBB and subsequently transported to the research cord blood stem cell bank, namely, IMSUT-Cell Resource Center (IMSUT-CRC). The transportation was carried by the courier or immediately transported by the staff of IMSUT-CRC or Tokyo CBB. During transportation, CB bags were placed at RT in foam polystyrene boxes with cushions. After reception of the bags, the bags were preserved at 15°C until processing.

CB processing

One hundred CB units were processed into MNCs using the bioclean cabinet installed in the IMSUT-CRC. The time from CB collection to processing was defined as the total time elapsed since CB collection at obstetrics and evaluation of conformity at Tokyo CBB until the initiation of CB processing into MNCs at IMSUT-CRC.

After 0.5-ml pre-processed CB sample was drawn for the testing, the CB was diluted to the appropriate volume (up to the multiples volume of 30 ml) with saline. Then 30 ml of CB was poured onto filters inside 50 ml-LeucoSep tubes (Greiner bio-one GmbH, Frickenhausen, Germany) that had been pre-filled with 15 ml of Ficoll-Paque PLUS (GE Healthcare UK Ltd, Buckinghamshire, UK). Then, the tubes were centrifuged at $1,000 \times g$ at 20°C for 20 min. After centrifugation, the MNC layer was collected and washed twice with 30 ml of PBS. The cell suspension was adjusted to 10 ml with PBS, and 0.5 ml of the post-processing sample was used to calculate cell numbers and differential.

To estimate the effect of storage temperature, we divided CB units into four equal parts, respectively. One group was initiated to be processed at 12 h after CB collection, whereas the remaining three were stored for 30 h elapsed since CB collection to processing at 4°C, 15°C, and RT, respectively.

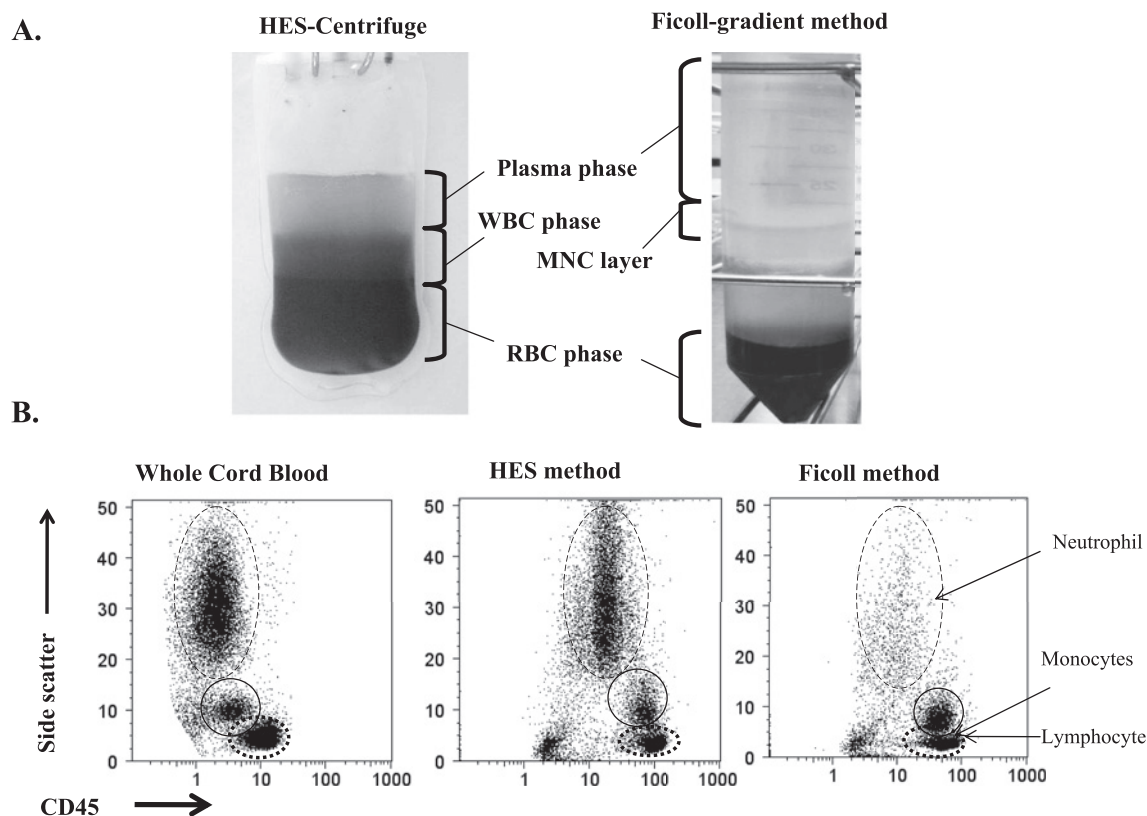


Fig. 1 Comparison of the processing in cord blood

A: Differential display of CB cells and MNCs isolated using a buffy coat layer HES centrifugation method and using a Ficoll-Paque (F/P) density-gradient method. The HES centrifugation method shows a cloudy broad band and the F/P density-gradient method shows a relatively sharp white band. B: Flow cytometric analysis after CB processing using HES centrifugation and density-gradient methods. The X-axis shows CD45 expression, and the Y-axis shows side-scatter. Neutrophils in the CB-MNC layer were reduced in samples processed using the density-gradient method.

NC count and differential white blood cell count

Differential white blood cells (WBC) were counted using an automated hemocytometer XE-2100 (Sysmex Corporation, Kobe, Japan). Total NCs include total WBC and NRBC. MNCs are defined as the sum of lymphocytes and monocytes and the proportion of MNCs is calculated as the sum of them in the TNCs.

CD34⁺ cell count and viability analysis

We measured CD34⁺ cells following the ISHAGE single platform method. We stained and analyzed the pre- and post-processed samples using CD45 FITC- and CD34 PE-conjugated antibodies (BD, Franklin Lakes, NJ) and Via-Probe 7-Amino-Actinomycin D (7-AAD) (BD) in TruCount tube (BD). After lysis of RBC by a PharmLyse reagent (BD), we analyzed cells using a flow cytometry FACS Calibur (BD). For cell viability analyses, viability was defined as the proportion of CD45⁺ 7-AAD⁻ viable WBCs within the total population of CD45⁺ cells.

Recovery rate of TNCs, MNCs and CD34⁺ cells was defined as the percentage that the post-processing cell number was divided by the pre-processing cell number.

Statistical analysis

Data are presented as the mean $\pm$ SD or the median with range. Comparisons between two groups were performed using the Student T test, and Pearson correlation coefficients were calculated to evaluate correlations between groups using by JMP software (version 6.0.2, Cary, NC) and R software, respectively, and *p* values less than 0.05 were considered to be significant.

Results

The HES centrifugation method isolates a cloudy broad band and the density-gradient method isolates a MNC layer which is relatively sharp and white band (Fig. 1.A). According to the flow cytometric analysis of CD45/side scatter after CB processing, neutrophils in

the CB-MNC layer were reduced in samples processed by the density-gradient method (Fig. 1.B).

Effect of time from collection to processing on sample quality

To evaluate the effect of time from collection to processing on sample quality, we examined recovery rates of NCs, MNCs, and CD34⁺ cells using a density-gradient protocol. Median collection volume was 81.0 ml (range 60.0 ml to 133.7 ml), including 28 ml of CPD, and median concentration of NCs before processing was $0.96 \times 10^7/\text{ml}$ (range 0.60×10^7 to $1.7 \times 10^7/\text{ml}$). Median time from CB collection to processing was 14.0 h (range; 6-38 h). Recovery rate of MNCs before and after processing was $71.3 \pm 10.3\%$. This rate decreased significantly with time ($p = 0.0006$; Fig. 2.A). Interestingly, the percentage of neutrophils in the MNC layer increased significantly in proportion to the time from collection to processing ($p < 0.0001$; Fig. 2.B). No significant difference was found in the recovery rates of NCs (Fig. 2.C) or CD34⁺ cells over time (Fig. 2.D).

The viability of CD45⁺ WBCs before processing decreased significantly with increasing time from collection to processing ($p = 0.0085$; Fig. 2.E), although the viability was relatively kept high (mean $\pm$ SD $96.3 \pm 2.6\%$) in this study.

Effect of storage temperature on sample quality

To evaluate the effect of processing time and storage temperature on neutrophil contamination, we divided CB units into 4 samples of equal volume. One sample was immediately processed, and the remaining three were stored at 4°C, 15°C, or RT (25°C) for further processing. As expected, neutrophil contamination increased with increasing storage temperature ($20 \pm 11\%$ at 4°C, $27 \pm 12\%$ at 15°C, and $46 \pm 15\%$ at RT; Table I). Samples processed immediately (within 12 h) contained less neutrophil contamination ($10 \pm 4\%$) than those stored for 30 h at any temperature. Samples processed immediately and samples stored at 4°C had significantly lower neutrophil contamination than those stored at RT. There was no significant difference in the recovery rate of NCs in samples stored at the various temperatures.

Because CB units in obstetrics were stored for various time and at uncontrolled temperature, we introduced the refrigerator to store the collected CB units at 10°C in the storage room of the obstetrics. Contamination of neutrophils in the MNC layer (Fig. 2.F) stored at 10°C ($n=85$) was significantly decreased compared

with that stored at RT ($p < 0.0001$) in the obstetrics. The CB units stored at RT are the same units as those in Fig. 2.B.

Discussion

Most CB banks have now adopted to the HES method⁽⁹⁾⁽¹⁰⁾ within 48 h of collection in obstetrics according to the guidelines of CB processing for CB banking in many other countries. The HES method is sufficient for obtaining stem cells from a limited volume of CB, but generally most hematopoietic progenitor cells are present in the MNC fraction⁽¹¹⁾⁽¹²⁾. Furthermore, tissue-specific stem cells can be found in the MNC population of CBs isolated using a density-gradient method in the regenerative experiments^(1~4), except the special population such as very small ES like cells⁽¹³⁾.

In the present study, we found that prolonged time from CB collection to processing at RT resulted in a significant increase of neutrophil contamination in the MNC layer using a density-gradient protocol. In addition, prolonged time from collection to processing CB resulted in a decrease in recovery rate of MNC (lymphocytes and monocytes), although the final TNC numbers were not affected. These results suggest that prolonged time from CB collection to processing decreases neutrophil density, whereas the density of lymphocytes and monocytes increases after CB processing. Therefore, the final product seems same, as far as the TNC are manually or automatically counted without WBC differentials. Although obtaining mesenchymal stem cells from CB seemed more difficult than we expected⁽¹⁴⁾⁽¹⁵⁾, our results show that prolonged time from collection to processing and preserved temperature might affect the composition of CB samples. To our knowledge, this is the first report that clearly describes the relationship between the time from collection to processing and the composition of CB samples isolated using a density-gradient method. Our results also suggest that these phenomena might occur in other blood- and bone marrow-derived MNCs isolated using a density-gradient method. Although a change in cellular density or the alteration of cell metabolites may explain neutrophil contamination in the MNC layer, the mechanism of this contamination is still unknown.

We also found that CB storage at lower temperatures prevented an increase in neutrophil contamination in the MNC layer when time from collection to processing was prolonged. Some researchers have re-

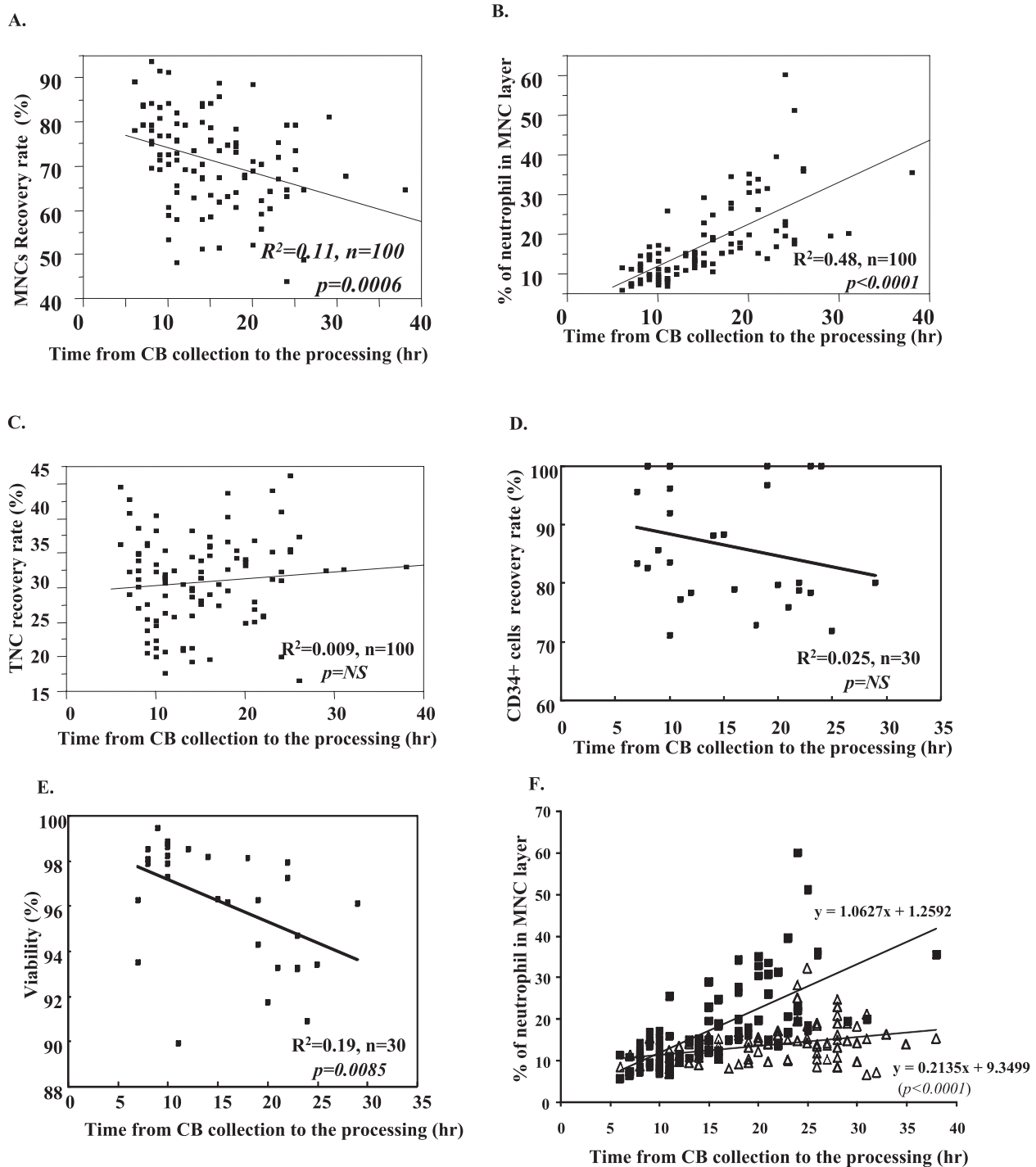


Fig. 2 Influences of time from collection to processing and temperature on the composition of MNC layer

A: MNC recovery rate. The recovery rate of CB-MNCs after F/P density-gradient processing decreased significantly with time from CB collection to processing (in hours; $p=0.0006$). B: The percentage of neutrophils in the MNC layer. The percentage of neutrophils in the CB-MNC layer (neutrophil contamination) increased significantly in proportion to time from CB collection to processing ($p<0.0001$). C: Recovery rate of NCs. The recovery rate of NCs in the CB-MNC layer showed no significant change according to the indicated time from CB collection to processing ($p=NS$). D: Recovery rate of absolute numbers of CD34⁺ cells. CD34⁺ cells in the CB-MNC layer were counted using a flow cytometry-based ISHAGE method. The recovery rate of CD34⁺ cells in the CB-MNC layer showed no significant decline according to the indicated time from collection to processing ($p=NS$). E: Viability of white blood cells after processing using an F/P density-gradient method. Viability is the percentage of 7-AAD⁻ viable cells within the total CD45⁺ white blood cell population ($p=0.0085$). F: The percentage of neutrophils in the MNC layer. ■ indicates CB units stored at RT in obstetrics ($n=100$) and △ indicates CB units stored under temperature control at 10°C in obstetrics ($n=85$) ($p<0.0001$).

Table 1 Effects of time and temperature on CB-MNC processing.

Time after collection	Immediately processed*	At 30 h Stored temperature		
		4°C	15°C	RT (25°C)
In MNC layer (%)				
Monocytes	18 ± 7	15 ± 8	15 ± 6	10 ± 3
Lymphocytes	72 ± 11	66 ± 18	57 ± 8	45 ± 8
Neutrophils	10 ± 4 ¹	20 ± 11 ¹	27 ± 12	46 ± 15 ¹

*CB units, which were transported faster to the processing facility (IMSUT-CRC) and were immediately processed into MNCs within 12 hours after collection. Data are shown as mean ± SD. n = 4, ¹p < 0.05.

ported that storage of platelets at 4°C inhibits cytokine accumulation and bacterial growth¹⁶⁾. Maintaining platelet function is not important for processing CB. In our hand, we did not find any de novo aggregation in CB samples stored at 4°C overnight and returned to RT just before processing (data not shown). Generally, RT is thought to have wide range such as 4 to 25°C, but our results suggest the need to store CB at lower temperature and process it immediately. Furthermore, when we set up a refrigerator in the storage room of obstetrics to keep the collected CB units at 10°C, contamination of neutrophils seemed to be prevented in the CB-MNC layer, as tested by the Ficoll density method, when compared with samples stored at RT (Fig. 2F). This may suggest that a lower fixed temperature (not broad range, RT) to store CB units in obstetrics is better for the fresh storage of CB. Time from collection to processing in clinical CBB requires re-evaluation.

In conclusion, storage temperature and time from collection to processing influences the quality of CB-MNCs products processed using a Ficoll-Paque density-gradient method.

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Disclosure of Interest

We have no disclosure of interests.

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臍帯血単核球分離における採取後経過時間と保管温度条件の影響の検討

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要旨：

近年、臍帯血は造血幹細胞のみならず組織幹細胞ソースとしても注目されている。臨床使用の臍帯血処理方法である HES 遠心分離法による有核細胞の分離について、海外のガイドラインにおいては採取から細胞処理までの時間は 48 時間以内、室温での保管および搬送がよいとされている。一方、組織幹細胞用の細胞処理として多くの論文で採用されているフィコール法による単核球の分離についての検討報告はない。本研究では、臍帯血採取後の経過時間や温度条件が、フィコール法による単核球分離に及ぼす影響について検討した。その結果、採取から細胞処理開始までの経過時間（採取後経過時間）によって有核細胞、CD34 陽性細胞の回収率に有意差は認めないものの、採取後経過時間が長いほど有意に単核球回収率および細胞生存率の低下を認めた。特に、フィコール処理後の単核球層の好中球混入率は、採取後の時間経過に伴い有意に増加した。さらに、臍帯血の保管温度の検討において、採取後 30 時間でのフィコール処理後単核球層の好中球混入率は、比較的低温で保管した場合のほうが室温保管に比べ有意に低かった。今回の検討において、経過時間や温度等の保管条件が、臍帯血単核球処理後の細胞組成に影響を及ぼすことが分かり、採取後の臍帯血管理状態の重要性が再確認された。

キーワード：

臍帯血、単核球、分離方法、フィコール法、保管温度、細胞生存率、回収率