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Original Article

Evaluation of DNA Damage and Repair In *In Vitro* Expanded Cord Blood CD 34 Positive Hematopoietic Stem Cell

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ABSTRACT

Background and Aims: The occurrence of single and double-strand breaks of DNA damage is the major obstacle for proliferation under various environmental factors and, if not repaired, can result in many consequences, including mutation, cell death, and others. So, the present study was conducted to evaluate the damage of DNA and the expression status of DNA repair system genes before and after stem cell proliferation.

Materials and Methods: The MACS method isolated the umbilical cord blood hematopoietic stem cells (UCB-HSCs). In order to investigate cell death, the study of Annexin V/PI was done by flow cytometry. Comet assay made observation and identification of DNA breaks, and the expression of genes normally involved in the repair of DNA breaks was evaluated by real-time polymerase chain reaction.

Results: The average number of stem cells increased by 1.9-fold after three days of proliferation. The apoptotic percentage of cells was negligible (less than 0.2%), and the purity of the $CD34^+$ cells was reduced by about one-third in three days (67%). By examining the expression of DNA repair genes, including KU70, KU80, RAD51, and XRCC1, their increased fold change was not significant. In a microscopic examination of stem cells in the comet assay, there was no significant difference between DNA damage before (1.33% ± 0.31) and after (2.08% ± 0.92) replication.

Conclusion: In our investigation**,** neither DNA damage nor changes in the DNA break repair were observed. However, further studies are required to clarify the DNA break repair by recruiting more UCB-HSCs samples.

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Introduction

Umbilical cord blood hematopoietic stem cell (UCB-HSCs) is one of the richest sources for hematopoietic stem cell transplantation, bone marrow, and peripheral blood. Some advantages include the richness of HSCs, absent donor attrition, relatively less human leukocyte antigen restriction, and ease of collection and storage, making UCB-HSCs a preferable source in most clinical trials [1, 2]. Due to the low number of HSCs for transplantation, protocols have been optimized to enable maximum UCB-HSCs expansion, including co-culture systems, using cytokines such as stem cell factor (SCF), thrombopoietin, fms-related tyrosine kinase 3 ligand (Flt3-ligand), small molecules, three-dimensional matrix scaffold, and over-expression of transcription factors [3].

The efficiency and safety of these methods were questionable owing to the susceptibility of HSCs to endogenous and exogenous sources of DNA damage caused by both the internal and external stresses on the cells. These stresses range from replication stresses (meiotic and mitotic) and genotoxicity effects of byproducts of normal oxidative metabolism like reactive oxygen species (ROS) on DNA to damaging chemicals and ionizing radiation. Continuous exposure of UCB-HSCs to these stresses within the process of in vitro hyper-proliferation can inevitably lead to the accumulation of DNA damage and, consequently, aging and dysfunction of HSCs [4].

DNA damages can be changes in nucleotides like deamination, alkylation; generation of abase sites; and breaks which comprise single strand breaks (SSBs) and double strand breaks (DSBs). Single and double-strand DNA breaks are the most important threats to genome instability [5]. XRCC1, as a DNA binding scaffold protein in the repair of SSBs, is also important in DSBs repair (Alt-NHEJ) [6, 7]. G0 phase HSCs employ a non-homologous end joining (NHEJ) pathway for repairing DSBs while as they enter the cell cycle in the presence of SCF, *in vitro* culture, their mechanism switches to homologous recombination; although, NHEJ is functional throughout the cell cycle [8]. KU70 and Ku80 are key factors in the NHEJ pathway, and RAD51 is one of the main proteins in the homologous recombination process [9, 10]. UCB-HSCs, like other cells, are equipped with DNA damage response (DDR) pathways, which normally ensure the integrity and stability of DNA content in a cell. The role of these pathways is largely unexplored in human HSCs compared to murine HSCs [11-13]. Since HSCs are hyper-sensitive to DNA damage, impairment of DDR pathways could lead to precipitous differentiation and p-53-dependent apoptosis [14]. Since DNA repair proteins are related to apoptosis, such as KU70, that prevent Bax-induced cell death, we also assessed the apoptosis status of cells after cell culture.

Numerous studies designed some experiments to evaluate the quality of UCB-stem cells after expansion [15]; however, there was not enough effort to measure the quality of the DNA content of resultant cells. We aimed to assess the quality of DNA and the accuracy of the DNA repair system after the expansion of UCB-HSCs. In this study, in order to examine the quality of DNA and the accuracy of the DNA repair system of UCB-HSCs, the DNA breaks and gene expression of main DNA break repair proteins (KU70, KU80, RAD51, and

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XRCC1) were evaluated via single cell gel electrophoresis comet assay and real-time polymerase chain reaction (RT-PCR) methods before and after three days of UCB-HSCs culture.

Material and Methods

Stem MACS medium, SCF, thrombopoietin (Miltenyi Biotec, Germany), gel red (Pars Tous Biotechnology, Iran), low melting point agarose and normal melting point agarose, sodium dodecyl sulphate, trypan blue, $MgCL_2$, Glacial Acetic Acid (Sigma-Aldrich, UK), NaCl, Triton X-100, Na_2EDTA Tris base, Tris-HCL, (Merck, Germany), cDNA synthesis kit (Thermos Fisher, UK), RiboX solution (RiboX kit, Neo Biotech, France), Master Mix (Primerdesign Ltd, UK).

Human cord blood (n = 5) was obtained from Iranian Blood Transfusion Organization and diluted in Hydroxyethyl starch (HES). All procedures are performed in accordance with the ethical standards of the University/ Regional Research Ethics Committee. Informed consent was obtained from all individuals. Mononuclear cells (MNCs) were collected after separation on Ficoll-Paque and washed with phosphate buffer saline (PBS). CD34 cells were enriched with magnetic-activated cell sorting (MACS) system in less than 24 hours and immediately used for the experiment. Then, these cells were counted and their viability tested by trypan blue in which sample cells were obtained, mixed with an equal volume of 0.4% trypan blue, and then counted on a hemocytometer slide (improved Neubauer chamber) under the light microscope (Zeiss, Germany) with ×10 objective lens. Samples with a greater than 95% viability were considered for the following analysis. The expression of the CD34 marker was evaluated by flow cytometry and cells plated at 1×10^4 cells /well in a 48-well plate and cultured for three days in a xeno-free medium, Stem MACS medium, supplemented with 100 ng/ml of each one of the recombinant SCF, Flt3-ligand, and thrombopoietin; and 1% penicillin-streptomycin mixture at 37 °C with 5% CO_2. At the end of the cell separation and three days of cell culture, $CD34^+$ UCB-derived HSCs were counted. Their purity was determined utilizing flow cytometry analysis (BD FACSCalibur™, BD Biosciences, USA) after incubation of cells with phycoerythrin-conjugated anti-CD34 (CD34-PE), and the viability of cells was measured via a dye exclusion test (trypan blue) that was performed on day 0, and apoptotic condition of $CD34^+$ cells analyzed by annexin V/PI flow cytometry on day 3 of cell culture. The morphology of cells was analyzed before and after cell culture.

Comet Assay

The alkaline version of the comet assay was carried out on 1×10^5 cells/µl $CD34^+$ HSCs before and after three days of cell culture. Slides were coated with normal melting point agarose (0. 5%) and added with 50 µ of a mixture including cells and low melting point agarose (0.5%). Slides covered by coverslips and placed at 4 °C for one hour. Then, after removing the coverslips, slides submersed in lysis buffer (NaCl, 2.5 M, Tris base, 10 mM, Na EDTA 0.1 M, 1% Triton and 10% dimethyl sulfoxide (DMSO)) at 4 °C for one hour. In order to unwind the DNA, slides immersed in a freshly prepared alkaline solution (0.3 M NaOH and 1mM EDTA, pH>13 (Merck) in a horizontal gel electrophoresis tank for 40 minutes at 4 °C. Electrophoresis was performed in the same buffer at 0.75 volt/cm for 30 minutes. Subsequently, slides

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were washed three times with neutralization buffer (400 mM Tris buffer, pH 7.5) and kept in the dark for 5 minutes. Finally, slides were rinsed in absolute ethanol for 5 minutes and air dried. The air-dried slides were stained with ethidium bromide solution 20 µ (0.02 mg/mL) and covered with coverslips. Slides were analyzed at a magnification of 200× using a Nikon E800 fluorescent microscope (Japan) equipped with a 546–516 wavelength band and a barrier filter (590 nm) attached to a charge-couple device camera. As control positive, $CD34^+$ UC-derived HSCs were treated with vincristine for 2 hours on day 3 of cell culture. The experiments were performed on five cord blood stem cells and repeated in three independent experiments; first, $CD34^+$ cells before cell culture; the same cells on the third day of culture; and the positive control group. Two slides were selected in each group, and the number of 200 cells per slide was counted by visual scoring by an independent observer. Cells were classified from grades 0 to 4 in which grade 0 accounted for intact cells with bright heads and absence of tail, grade 1 for partial DNA tail, grades 2 and 3 with the subsequent gradual increase in the extent of DNA migration, and lastly, grade 4 with little head and long, diffused tail. The final data was calculated according to the equation described earlier by Mozdarani et al. [16].

Quantitative real-time PCR (qRT-PCR)

According to the manufacturer's instructions, the total RNA of UCB-derived $CD34^+$ cells from five different UCB samples were extracted on days 0 and 3 of cell culture (RiboX kit, Neo Biotech, France). Quantity and purity of extracted RNA were controlled via A260/A280 absorbance ratios using a bio-photometer (Eppendorf, Hamburg, Germany), and its quality was assessed by agarose gel electrophoresis. cDNA was synthesized using a high-capacity reverse transcription kit (Thermo Fisher, UK). The following genes were selected for PCR: *KU70*, *KU80*, *RAD51*, and *XRCC1*. Primers were designed using the NCBI database and quantitative PCR was performed in Applied Biosystems 7300 real-time PCR system using Precision FAST qPCR Master Mix (Primerdesign Ltd, UK). Calculation of gene expression was determined with the 2-ΔΔCT method using *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as the housekeeping gene. The data was triplicated and statistically compared using the GraphPad Prism program.

Statistical analysis

We compared values before and after cell culture and expressed them as the mean ± standard deviation (SD). The data were analyzed using a t-test. The results of flow cytometry were analyzed by Flowjo 7.5.5 software (FlowJo, USA). Differences were considered statistically significant at $p < 0.05$.

Results

***Ex-vivo* expansion of UCB-derived $CD34^+$ cells**

Laboratory expansion cultures were initiated with 1×10^4 $CD34^+$ cells derived from separate umbilical cord blood units. After three days of culture in Stem MACS medium, 30×10^4 cells were generated, representing, on average, 1.9 folds increase in stem cells. So, the results in figures 1 (a, b) and 2 (a, b) show that the proportion of CD34 marker on HSCs was 97.7% and 67.4% on days 0 and 3, respectively. 3.2 flow cytometry assessment expression and viability of UCB-derived $CD34^+$ cells.

The viability data of cells assessed by trypan blue on day 0 showed that the survival rate of HSCs was

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high. On average, only 0.2% of dead cells were observed, which is trivial. To determine the frequency of apoptotic cells on third day of cell culture, the numbers of 5×10^4 cell/µl of cultured samples were stained with propidium iodide (PI) and annexin V. Viable cells are annexin VNEG/PINEG, early apoptotic cells annexin V+/PINEG, and necrotic cells annexin V+/PI+.

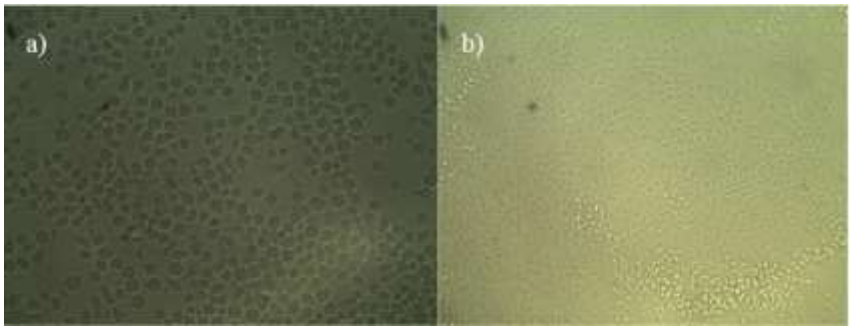


Fig. 1. Morphological and expansion view of $CD34^+$ HSCs. a) A relatively uniform, small, and round shape cells demonstrating features of intact, healthy CD34 HSCs on day 0 (magnification × 400). Approximately 70×10^4 cells/µl was used for cell culture. b) HSCs on day 3 of cell culture are characterized with tails, and compacted colonies representing expansion and division of cells (magnification ×200) on average, 130×10^4 cells/µl yielded on the third day. HSCs= Hematopoietic stem cells

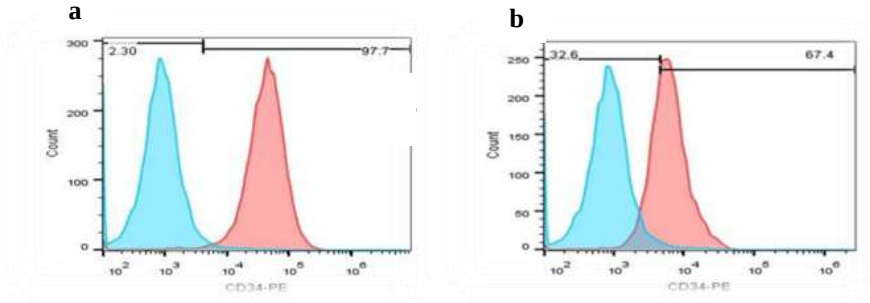


Fig. 2. The expression of cell surface marker CD34 on umbilical cord blood hematopoietic stem cells. The number of cells used in each analysis was about 5×10^4 cells/µl. a) 97.7% of cells expressed CD34 marker and 2.3% were CD 34 ^{neg}. before cell culture. b) The proportion of cells expressing CD34 fell on day 3 to 67.4%, and the remaining 32.6% were CD 34 ^{neg}. The results of flow cytometry were analyzed by Flowjo 7.5.5 software.

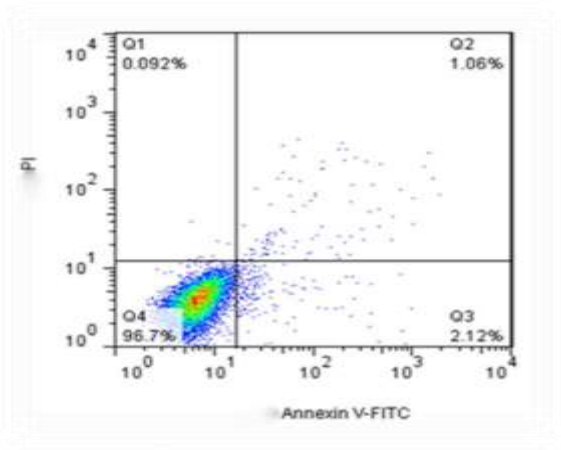


Fig. 3. Apoptotic conditions of $CD34^+$ hematopoietic stem cells on day 3. The majority of cells were viable and non-apoptotic (96.7%) compared to early apoptotic cells (2.12%) and necrotic cells (1.06%). A population of 5×10^4 cells/µl was used.

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Early apoptotic cells translocate phosphatidyl serine from the inner cell membrane to the outer cell membrane and result in the attachment of annexin-V to this marker; as a result, annexin-v is regarded as a marker of apoptotic cells. As shown in figure 3, cells on the third day of culture contained 96.7% live cells, 2.12% apoptotic cells, and 1.06% necrotic cells. In addition, the morphological view of $CD34^+$ cells before and after culture showed intact cells in both groups and is presented in figure 1 (a and b).

DNA break repair genes expression of UCB-derived $CD34^+$ cells before and after culture

Expression levels of four DNA repair genes included *KU70*, *KU80*, *RAD51*, and *XRCC1* plus *GAPDH* as reference gene was measured by qRT-PCR. RAD51 was chosen to represent homologous when *KU70* and *KU80* were involved in the NHEJ pathway. *XRCC1* is a candidate from the single-strand break repair pathway. Although the expression of all damage repair genes had upregulated on day three compared with day 0, these rises were not significant in the genes: *RAD51* (3.46-fold; p = 0.43), *KU70* (2.97-fold; p = 0.06), *KU80* (1.82-fold; p = 0.31), *XRCC1* (2.06-fold; p = 0.21). Results are shown in figure 4 (a, b, c, and d). These data suggest that the expansion of UCB-derived $CD34^+$ cells within three days would not undermine the ability of these cells to utilize DDR pathways related to DNA breaks.

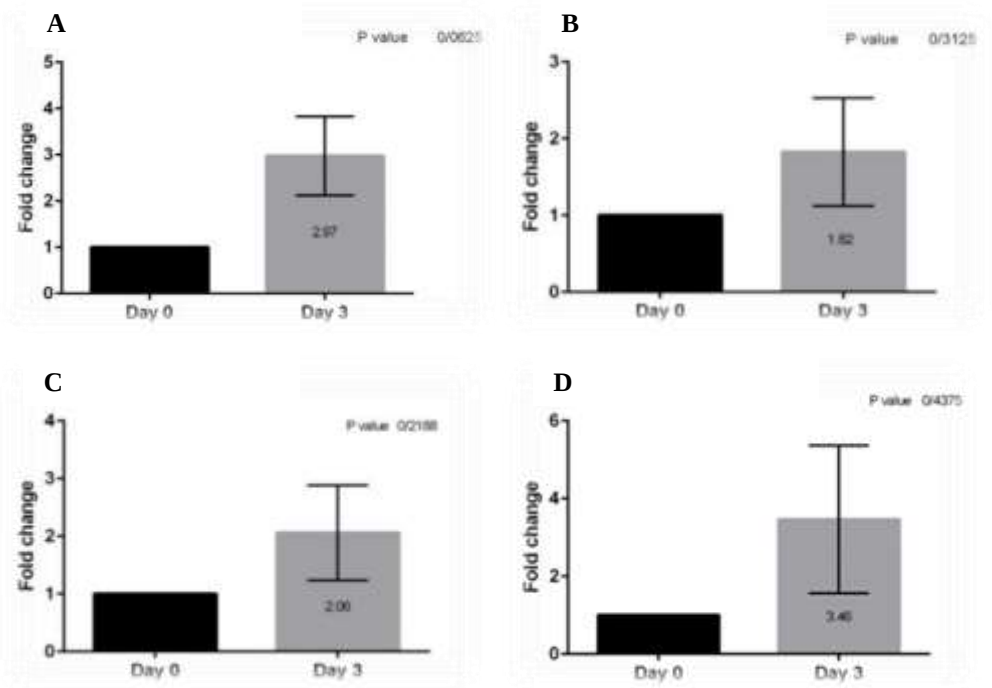


Fig. 4. Expression levels of DNA damage repair genes in $CD34^+$ umbilical cord blood stem cells. DNA damage genes measurement showed an increase in the expression of all genes after three days (n=5). Fold differences in mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method relative to day 0 of cell culture and normalized to the reference gene *GAPDH*. Approximately 15×10^4 cells/µl $CD34^+$ cells were used in each extraction, obtained from 5 cord blood bags. The results correspond to means ± SD from 3 independent experiments. A) *KU70* had 2.97 fold rise in gene expression (p =0.0625). B) Increase in the expression of *KU80* was 1.82 (p = 0.3125). C) On day 3, a 2.06-fold increase was seen in the expression of *XRCC1* (p = 0.2188). D) Although the expression of *RAD51* rose 3.46 times, this increase was not significant (p = 0.4375). Individual data are presented with mean ± SD of at least three RT-PCR experiments and analyzed via GraphPad Prism program (GraphPad Software Inc, USA) and student's t-test.

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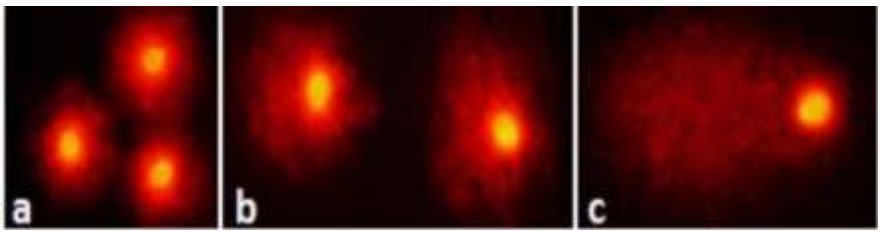


Fig. 5. $CD34^+$ Cord blood-hematopoietic stem cells processed by single-cell gel electrophoresis (comet assay) and stained with gel red, viewed by fluorescence microscopy (Original magnification ×400). (a) cells with no DNA damage (grade 0), (b) cells with low-grade DNA damage (grade 1-2), and (c) A cell with highly damaged DNA (grade 4).

Comet assay analysis of UCB-derived $CD34^+$ cells before and after culture

UCB-derived HSCs expressing CD34 antigen before cell culture were compared to the same cells on day third of cell culture for their functional DNA repair capacity using alkaline single-cell gel electrophoresis (alkaline comet assay). Figure 5 (a, b) presents no DNA tail in test groups, accounting for the absence of both single -and double-stranded DNA breaks. It was found no difference between cells before and after cell culture. As control positive, $CD34^+$ CB-derived HSCs were co-cultured *in vitro* with vincristine for 2 hours on day third of cell culture, there was moderate DNA damage based on the increase in the amount of DNA in the tail of Comet assay (figure 5 c). For each group in each repetition, 400 cells (200 cells in two slides) were counted, and the percent of DNA damage was 1.33 ± 0.31 in HSCs on day 0 culture and 2.08 ± 0.92 on the third day and 218.4 ± 2.14 in HSCs treated with vincristine as control positive group. According to the results, there was no significant difference between HSCs before and after cell culture, while in the positive control group, we see a remarkable increase in DNA damage.

Discussion

Expansion of UCB-HSCs in a laboratory environment can change stem cells' phenotypic and genotypic features and bring about some complications for future clinical applications [17]. In this regard, most of the efforts have focused on improving the numbers and efficacy of UCB-derived $CD34^+$ cells. In the present study, we investigated *in vitro* expansion, SSBs, and DSBs as well as the expression of DNA repair genes of UCB-derived $CD34^+$ cells before and after three days of cell culture. Naturally, the process of aging of hematopoietic stem cells occurs as someone gets. This aging phenomenon leads to limited expansion capacity combined with the skewing in the differentiation of cells which eventually contributes to limited HSCs self-renewal and a surge in hematologic and non-hematologic diseases in older adults [18, 19]. This study found that the numbers of UCB-derived $CD34^+$ cells increased three days after cell culture, although the purity of $CD34^+$ cells on the third day reduced to 67.4% compared to 97.7 on day 0, which implies differentiation and decrease in the self-renewal potential of products. $CD34^+$ cord blood cells were cultured for 14 days it was

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found 61 ± 27.7 folds rise in $CD34^+$ cells along with a 1.2% ± 0.5% reduction in purity of $CD34^+$ cells [15]. Our study showed a 1.9-fold rise in stem cell numbers after three days of cell culture without significant changes in the proportion of viable and apoptotic cells, which was assessed by flow cytometric analysis of $CD34^+$ cells using annexin V/PI. According to the achieved data, 96.7% live cells, 2.12% apoptotic cells, and 1.06% necrotic cells are concordant with other studies and showed cell viability of higher than 90% [15]. The apoptosis rate of *in vitro* expanded $CD34^+$ cord blood cells could be ameliorated in co-culture with mesenchymal stem cells [20]. It has been shown that $CD34^+$ cells have higher levels of anti-apoptotic proteins than $CD34^-$ lymphocytes; thus, they have higher resistance of $CD34^+$ [21] to apoptosis [21]. This pattern could be regarded as a confirmatory sign of the phenotypical healthiness of resultant cells combined with intact and normal morphological characteristics of these cells on both days 0 and third. Normal cells are characterized by relatively uniform, small, and round-shaped cells, while apoptotic features include shrinkage, irregularity, cytoplasmic blebbing, nuclear condensation, and fragmentation [22].

We also studied the integrity and stability of UCB $CD34^+$ cells by assessment of DNA breaks and repair systems. Although there was a generally slight increase in the expression of DNA repair genes explored, they were not statistically significant. The expression of double-strand break repair specific genes, including KU70, KU80, and RAD51 increased 2.97, 1.82, and 3.45 fold, respectively. Although these increases are insignificant, they indicate the fidelity of the DNA double-strand break repair system. Also, XRCC1, as one of the main proteins of the single-strand break repair pathway, had a 2.06-fold increase in expression in the day third of culture compared to day 0. An increase in the expression of these genes accounts for the functionality and efficiency of genes involved in the repair of DNA breaks which are critical for the survival of cells. The lack of significant difference between cells before and after three days of culture implies the integration of DNA content. It seems the damage of DNA is in balance with its repair capacity; otherwise, we will see a significant rise in apoptosis; for example, *KU70* or *KU80* inactivation suppresses cell growth and induce apoptosis [8, 23]. However, the assessment of the cell cycle could be helpful because some DNA repair factors are under cell cycle control. For instance, it has been proved that RAD51 preserves G2/M transition in mouse embryonic stem cells or the expression of DNA damage response genes is lower in G0 HSCs than proliferating HSCs [23]; there was no need to perform this test as our goal was determining the DNA healthiness of produced cells.

Consistent with the RT-PCR results of selected DNA repair proteins, analysis of DNA breaks with comet assay revealed intact cells without significant deleterious signs of DNA damage. The rate of DNA damage in $CD34^+$ UCB cells was 1.33% on day 0 of cell culture compared to 2.08% on day third of cell culture. As control positive, $CD34^+$ UCB-derived cells treated with

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vincristine for 2 hours on day 3 of cell culture showed elevated comet tail lengths (218.4% DNA damage), a marker of DNA damage, in a study by [24]. DNA damage of $CD34^+$ and $CD133^+$ cells derived from UCBs on days 0, 7, 14, and 21 of *ex vivo* expansion were measured by comet assay. While results exhibited less than a 5.0% DNA damage rate on days 7 and 14, the DNA damage rate increased to 28.2% on day 21 compared to day 0. They concluded that the optimal harvest time of cord blood cells after *in vitro* culture is within 14 days because the rate of DNA damage rises significantly after 14 days [24]. Some authors used cytogenetic analyses such as G-bonding karyotype and telomerase reverse transcriptase activity to confirm the safety of *in vitro* expanded $CD34^+$ cord blood cells, revealing no genetic abnormalities in days 6th and 14th cell culture [15].

Contrary to our study, Dircio-Maldonado et al. suggested that expanded cells undergo some functional and genomic changes that undermine their expansion potential compared to non-manipulated cells. In their study, they compared *in vitro* expanded UCB-HSCs (7 days cell culture) with their fresh counterpart by performing hematopoietic colony assay, long-term culture-initiating cells, morphologic analysis, and molecular studies (microarrays and Real time-PCR) [17]. Hermeto et al. suggest using standardized genomic tests such as comet assay for MSC transplantations to assess the safety of clonal expansion of these cells [25]. Another study offered using screening tests like comet assay due to a rise in the DNA damage of MSCs during *in vitro* cultivation evaluated by comet assay [26].

Our result demonstrates the wholesomeness of $CD34^+$ UC-derived HSCs after *in vitro* cell culture, which implies the integrity of genomic content, DDR pathways, and viability of cultivated cells. However, since this study used a single ex vivo culture strategy (serum-free liquid culture supplemented with a combination of cytokines) and three-day cell culture, our results need to be taken with caution concerning the DNA break repair and gene expression of the stem cell population compared to the generated stem cells in different culture conditions (e.g., co-culture, in the presence of small molecules) and using longer cell culture (7 to 11 days). These observations might have clinical implications in UCB transplantation as expanded cells retain genomic integrity.

Conclusion

Differences in the viability and DNA repair capacity of $CD34^+$ cord blood cells were insignificant before and after *in vitro* expansion, despite increases in the number of $CD34^+$ cells. Further studies are needed with larger sample sizes to clarify the DNA break repair of UCB-HSCs. Furthermore, using longer time cell culture (7 to 11 days) may be recommended.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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