

GFc7 as a Smart Growth Nanofactor for ex vivo Expansion and Cryoprotection of Humans' Hematopoietic Stem Cells

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Background: Nowadays, smart synthesized nanostructures have attracted wide attention in the field of stem cell nanotechnology due to their effect on different properties of stem cells.

Methods: GFc7 growth nanofactor was synthesized based on nano-chelating technology as an iron-containing copper chelator nanocomplex. The effect of this nanocomplex on the expansion and differentiation of hematopoietic stem cells (HSCs) as well as its performance as a cryoprotectant was evaluated in the present study.

Results: The results showed that the absolute count of CD34⁺ and CD34⁺CD38⁻ cells on days 4, 7, 10 and 13; the percentage of lactate dehydrogenase enzyme on the same days and CD34⁺CXCR4 population on day 10 were significantly increased when they were treated with GFc7 growth nanofactor in a fetal bovine serum (FBS)-free medium. This medium also led to delayed differentiation in HSCs. One noticeable result was that CD34⁺CD38⁻ cells cultured in an FBS medium were immediately differentiated into CD34⁺CD38⁺ cells, while CD34⁺CD38⁻ cells treated with GFc7 growth nanofactor in FBS medium did not show such an immediate significant differentiation. De-freezing GFc7-treated CD34⁺ cells, which were already frozen according to cord blood bank protocols, showed a higher percentage of cell viability and a larger number of colonies according to colony-forming cell assay as compared to control.

Conclusion: It can be claimed that treating HSCs with GFc7 growth nanofactor leads to quality and quantity improvement of HSCs, both in terms of expansion in vitro and freezing and de-freezing processes.

Keywords: expansion, GFc7, human hematopoietic stem cells, nanocomplex, nano-chelating technology

Introduction

Hematopoietic stem cells (HSCs) were first used more than half a century ago and the United States Food and Drug Administration (FDA) have approved HSCs transplant for use against malignancies.^{1,2} However, the low number of HSCs is the major obstacle to their broader utilization in cell therapy. Despite rapid expansion of HSCs, in vitro studies show that controlling HSCs self-renewal and differentiation has still remained challenging. This problem suggests that additional methods and growth factors for expansion of HSCs are required in vitro studies to tackle this limitation.^{3,4}

It is reported that using frozen HSCs separated from umbilical cord blood (UCB) after 20 years had no negative effect on cell viability and function in

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HSCs transplant. Although the side effects associated with dimethyl sulfoxide (DMSO) are already recognized, it is still being used as the top standard cryoprotectant in cryoprotection used in UCB banking.⁵⁻⁷

Nanotechnology is the synthesis of functional structures on nanoscale, which results in materials with new chemical, biological and physical characteristics as well as novel conductivity, reactivity, functional conformation and smart structures.^{8,9} These smart structures provide proper responses, including anti-oxidative, adhesive, migrating and proliferative ones, to physiochemical stimuli in cells in a particular, controlled way.¹⁰

Nanotechnology helps stem cells utilize their therapeutic potential for the treatment and repair of damaged tissues. The combination of stem cells and nanotechnology has emerged as a new field of study called “stem cell nanotechnology”. This technology has attracted considerable attention since it greatly contributes to improving regenerative medicine and tissue engineering.^{11,12}

Recently, novel nanostructures have been produced by nanochelating technology with the aim of treating various diseases such as cancer and diabetes.^{13,14}

In the previous study, GFc7 growth nanofactor was synthesized based on nanochelating technology by self-assembly method and its effect on expansion of human mesenchymal stem cells (hMSCs) was analyzed from various aspects. The results showed that GFc7 growth nanofactor improved cell proliferation and maintained the pluripotency properties of hMSCs.¹⁵

In the present study, the effect of GFc7 growth nanofactor on the expansion of primitive HSCs (CD34⁺ and CD34⁺CD38⁻ cells) as a well-established marker of human progenitor cells,¹⁶⁻¹⁸ their differentiation in ex vivo culture, cell-protection capacity against oxidative stress and quality and quantity after cryoprotection was evaluated.

Materials and Methods

Materials and Instruments

Materials

GFc7 growth nanofactor was synthesized by Sodour Ahrar Shargh Company [Iran]. Nonessential Amino Acids, GlutaMAX, Penicillin G (100 U/mL), Streptomycin (100 mg/mL) and Phosphate-Buffered Solution (PBS) were purchased from Gibco-Life Technologies [USA]. Fetal Bovine Serum (FBS) was taken from PAA Biotech [Austria], hydrogen peroxide (H₂O₂) from Merck

[Germany] and Dimethyl Sulfoxide (DMSO) and Dextran 40 from Cryosure [Germany]. Propidium Iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 99% (MTT), Stemline[®] II Hematopoietic Stem Cell Expansion Medium, stem cell factor (SCF), Flt-3 ligand (Flt-3L), thrombopoietin (TPO) and Iscove's Modified Dulbecco's Media (IMDM) were purchased from Sigma-Aldrich-St Louis [USA]. All antibodies were purchased from Dako [Denmark]. Standard SYBR Green PCR kit was taken from Fermentas [Germany], ALDEFLUOR[™] Kit and Methylcellulose from stem cell technologies [Canada] and CD34 and CD38 MicroBead Kit from Milteny Biotec [USA]. Hydroxyl ethyl starch (HES) was purchased from Fresenius Kabi [Germany], Sepax processing kit from Biosafe [Switzerland] and Ficoll-Paque PLUS density gradient from Amersham Biosciences [USA].

Instruments

The list of equipment and instruments is as follows:

Cooling Device [Coolmix-210, Biosafe, Switzerland]. Control Rate Freezer [Planer, UK]. Absorbance Micro Plate Readers [ELx800TM; BioTek, USA]. Flow Cytometry (FACS) [Becton Dickinson, USA]. Rotor Gene 6000 instrument [Corbett, Australia]. Sepax [Biosafe, Switzerland]. Inverted Microscopy (Olympus, USA).

GFc7 Growth Nanofactor

GFc7 growth nanofactor is an iron-containing copper chelator nanocomplex. This new growth factor for the expansion of stem cells was synthesized based on a nanotechnology patented in the United States Patent and Trademark Office (USPTO) with the subject of “Chelate Compounds”.¹⁹ The synthesis method is thoroughly explained in the previous study.¹⁵

High-resolution transmission electron microscopy (HRTEM) images of GFc7 growth nanofactor were captured by a transmission electron microscope in the University of Tehran Science and Technology Park according to a standard protocol.²⁰

To specify the porosity of GFc7 nanoparticles, BET (Brunauer, Emmett and Teller) Surface Area Analysis was conducted at Tehran University using Quanta Chrome Instruments, version 2.2. In addition, Dynamic Light Scattering for Nanoparticle Size Analysis was conducted using DLS device – Malvern brand, nano ZS (red badge) ZEN 360 model – at KEFA laboratory, Sharif University of Technology.

Table I Design of Dosages and Study Groups

Groups		Treatments
A	Test	CD34+ cells in an FBS-free medium (with Gfc7)
	Control	CD34+ cells in an FBS-free medium (without Gfc7)
B	Test	CD34+ cells in an FBS medium (with Gfc7)
	Control	CD34+ cells in an FBS medium (without Gfc7)
C	Test	CD34+CD38- cells in an FBS-free medium (with Gfc7)
	Control	CD34+CD38- in an FBS-free medium (without Gfc7)
D	Test	CD34+CD38- in an FBS medium (with Gfc7)
	Control	CD34+CD38- cells in an FBS medium (without Gfc7)

CD34⁺ and CD34⁺CD38⁻ Cells Isolation and Culture

Fresh UCB samples were provided by Iran National Cord Blood Bank (Tehran, Iran).²¹ Mononuclear Cells (MNCs) of UCB samples were separated by Ficoll-Paque PLUS density gradient according to the manufacturer's instruction.²² Afterwards, PI solution was used to evaluate cells viability by FACS analysis. In the next stage, CD34⁺ and CD34⁺CD38⁻ cells were separated from MNCs.

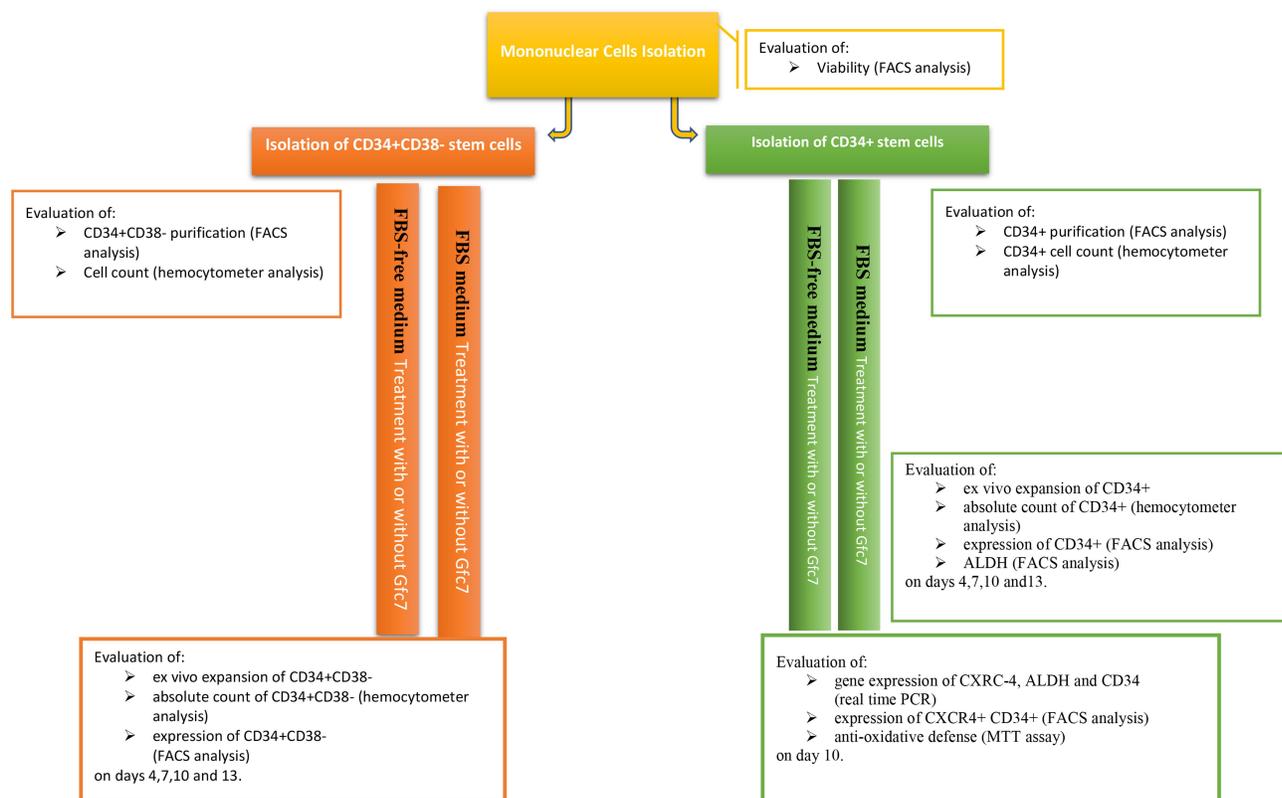
Isolating CD34⁺ Cells

According to MicroBeads manufacturer's instruction, CD34⁺ cells were isolated from MNCs. Magnetic CD34 MicroBeads were used to label CD34⁺ cells. Then, they were loaded and retained onto a MACS[®] Column placed in a MACS Separator, where the unlabeled cells ran through. After removing the MACS[®] Column from the magnetic field, the CD34⁺ cells were separated.^{23,24}

Isolating CD34⁺CD38⁻ Cells

According to MicroBeads manufacturer's instruction, CD34⁺CD38⁻ cells were isolated from MNCs of human UCB via two steps. In the first step, CD34⁺ cells were separated by the procedure explained above. In the second step, magnetic CD38 MicroBeads were used to label the separated CD34⁺ cells in the first step. Then, they were loaded and retained onto a MACS[®] Column placed in a MACS Separator. The CD34⁺CD38⁺ cells remained on the column, while CD34⁺CD38⁻ cells ran through.^{18,25-27}

The cells were labelled by PE - anti-human CD34 antibody and FITC- anti-human CD38 antibody in order to specify the purity of separated cells. The groups were

**Figure 1** Design and time line of the study.

labeled according to Table 1 and the design of the study is shown in Figure 1.

Ex vivo Expansion of CD34⁺ and CD34⁺CD38⁻ Cells

Culturing and expansion of both CD34⁺ and CD34⁺CD38⁻ cells were performed in two ways:

FBS-Free Medium

2×10⁴ cells per well of both types of cell groups (Groups A & C) were cultured in a stem line medium where supplemented with 100 ng/mL SCF, 100 ng/mL Flt-3L, 100 ng/mL TPO and 1% penicillin G and streptomycin with (Group A & C, Test) and without (Group A & C, Control) 0.1 ng/mL GFC7 growth nanofactor (Table 1).

FBS Medium

2×10⁴ cells per well of both types of cell groups (Groups B & D) were cultured in an IMDM medium where supplemented with 10% FBS, 100 ng/mL SCF, 100 ng/mL Flt-3L, 100 ng/mL TPO and 1% penicillin G and streptomycin with (Groups B & D, Test) and without (Groups B & D, Control) 0.1 ng/mL GFC7 growth nanofactor (Table 1).

Three samples were analyzed in this experiment. The cells were cultured in a humidified incubator (37°C - 5% CO₂ - 95% humidity) and were seeded onto 24-well plates in triplicate wells for 13 days.

Absolute Count of CD34⁺ and CD34⁺CD38⁻ Cells

The cells number of CD34⁺ and CD34⁺CD38⁻ was counted by the hemacytometer, and then the population percentage of CD34⁺ and CD34⁺CD38⁻ was determined by FACS analysis. Finally, the absolute counts were obtained using the following formulas:²⁸

“The population percentage of CD34⁺ × the number of cells”

“The population percentage of CD34⁺CD38⁻ × the number of cells”

UCB Preparation Process for Cryoprotection

Red blood cells of UCB were depleted by HES (0.6%), and then the bag was attached to Sepax processing kit under sterile conditions. Afterwards, a cell separator (Sepax) was used to separate HSCs from UCB. Later in the process, the buffy coat was extracted and the UCB bag

was detached. In the freezing stage, DMSO and Dextran 40 were added to the buffy coat, and then it was transferred to a programmable control rate freezer (Planer) to decrease the sample temperature to -100°C. Finally, the samples were stored in a dry nitrogen shipper first and were then transferred to a liquid nitrogen quarantine tank at -135°C until their use.²¹

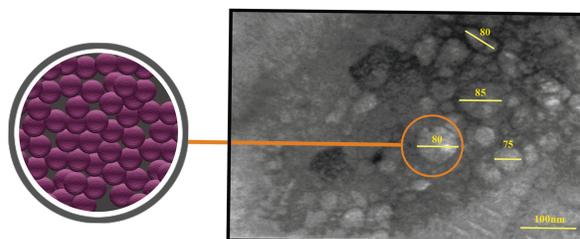
HSCs Recovery After Being Thawed

The samples were thawed by removing them from storage and immediately immersing them with gentle agitation in water at 37°C. After that, their cell viability and colony forming were assessed.²¹

FACS Analysis

FACS analysis was performed according to the protocol in the previous study to evaluate anti-CD34-PE, anti-CD38-FITC

A



B

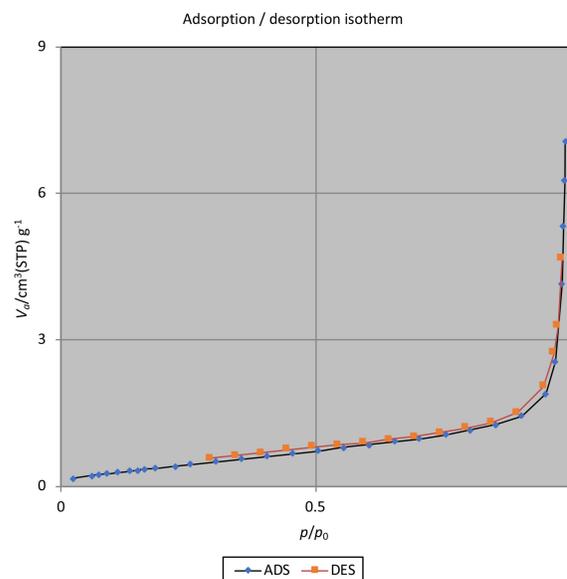


Figure 2 (A) HRTEM image of GFC7 growth nanofactor. (B) BET test results of GFC7 growth nanofactor.

Note: The scale bar is 100 nm and the median size of nanoparticles is 80 nm.

and anti-CXCR4-FITC as well as PI staining. The data were assessed by Win MDI 2.8 software, and the results were illustrated using histograms or dot plots.¹⁵

Aldehyde Dehydrogenase Assay

Aldehyde dehydrogenase (ALDH) assay was carried out in CD34⁺ cells according to the protocol presented in ALDEFLUOR™ Kit. According to this protocol, ALDEFLUOR™ is a reagent used to identify cells which express various levels of ALDH enzyme. The amount of

fluorescent reaction was measured by FACS analysis, and a particular inhibitor of ALDH (diethylaminobenzaldehyde) was utilized to control the background fluorescence.²⁹

Colony-Forming Cell Assay

Clonogenic progenitors from thawed CD34⁺ cells were assayed in MethoCult medium by using 1000 freshly isolated cells. After 14 days of incubation (37°C, 5% CO₂), colonies were counted by bright-field inverted microscope.^{30,31} Different colony-forming units, including

Z-Average(d.nm): 147.9

Pdl: 0.377

Intercept: 0.537

Result quality: Good

Diam.(nm) %Volume Width(nm)

Peak 1: 82.26 38.0 7.871

Peak 2: 111.3 8.3 32.67

Peak 3: 251.0 7.3 74.82

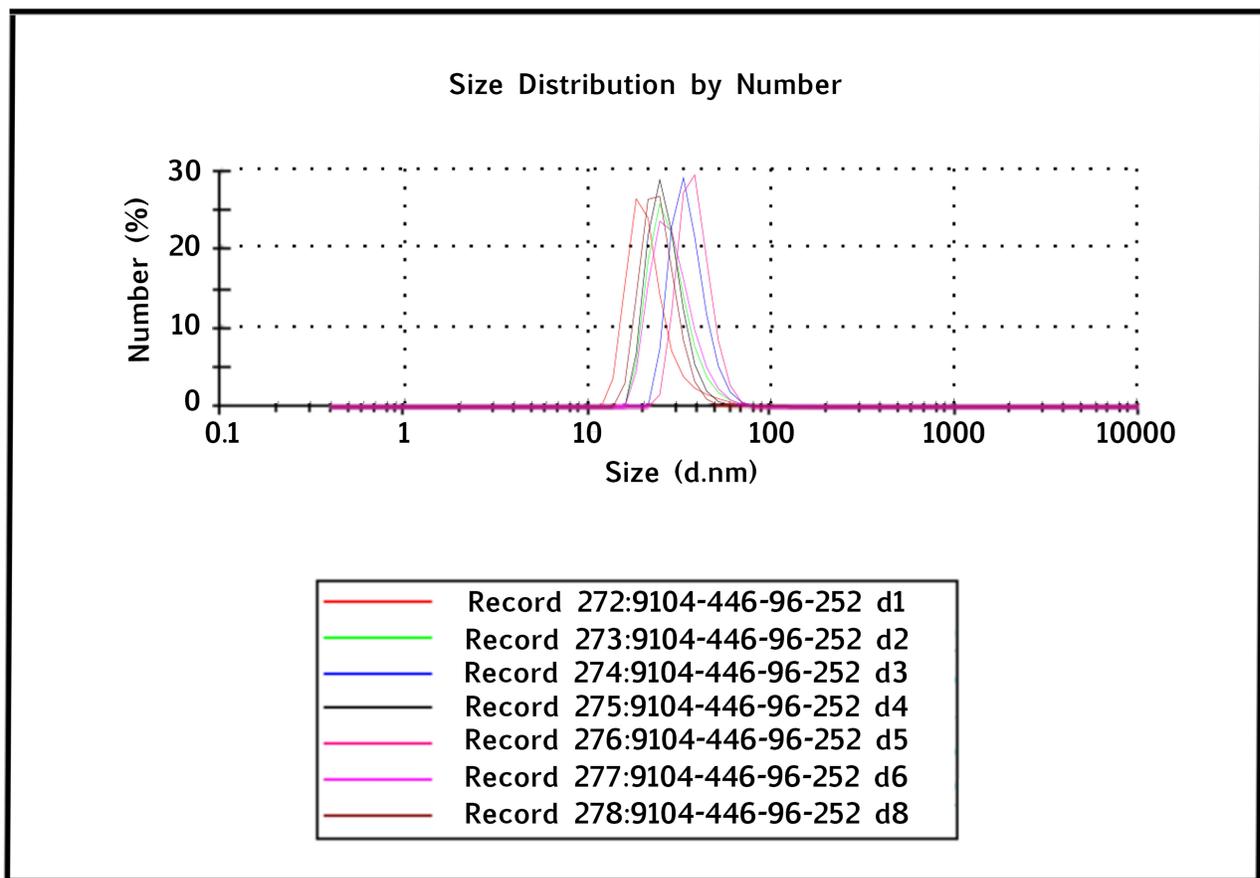


Figure 3 Dynamic light scattering analysis of GfC7 growth nanofactor.

granulocyte-monocyte colony-forming unit (CFU-GM), granulocyte-erythrocyte-macrophage-megakaryocyte colony-forming unit (CFU-GEMM) and erythroid burst-forming unit (BFU-E), were counted using a bright-field microscope.

Real-Time PCR Quantification

Evaluating the gene expressions of CD34⁺ cells was performed 13 days after treatment by real-time PCR analysis, where Trizol was used to extract total RNA, and then the synthesis of cDNA was carried out by M-MuLV Reverse Transcriptase and oligo primers. Gene quantification and real-time PCR quantification (qRT-PCR) analysis was performed using a standard SYBR Green PCR kit by Rotor-Gene 6000 instrument. The details of qRT-PCR analysis are described in the previous study. Rotor-Gene Q software (Corbett) was used for data analysis of the threshold cycle average, and the data were normalized to endogenous controls

(GAPDH) and calibrated to untreated cultured cells as control. Finally, the relative mRNA expression levels were measured according to the $\Delta\Delta C_T$ method.¹⁵

Measuring Cytoprotective Effect of GFc7 Against Oxidative Stress in CD34⁺ Cells

MTT assay was performed in H₂O₂-induced model to measure the cytoprotective effect of GFc7 against oxidative stress in CD34⁺ cells.³² HSCs were plated at a density of 1×10⁴ cells/well in 96-well plates in 100 μ L specific medium. Afterwards, two treatments were conducted to assess the cytoprotective effect of different doses of GFc7 growth nanofactor against oxidative stress in CD34⁺ cells;

- GFc7 growth nanofactor treatment for 10 days following H₂O₂ treatment (5 μ M) for 72 hrs.
- Simultaneous treatment with GFc7 growth nanofactor and H₂O₂ (5 μ M) for 72 hrs.

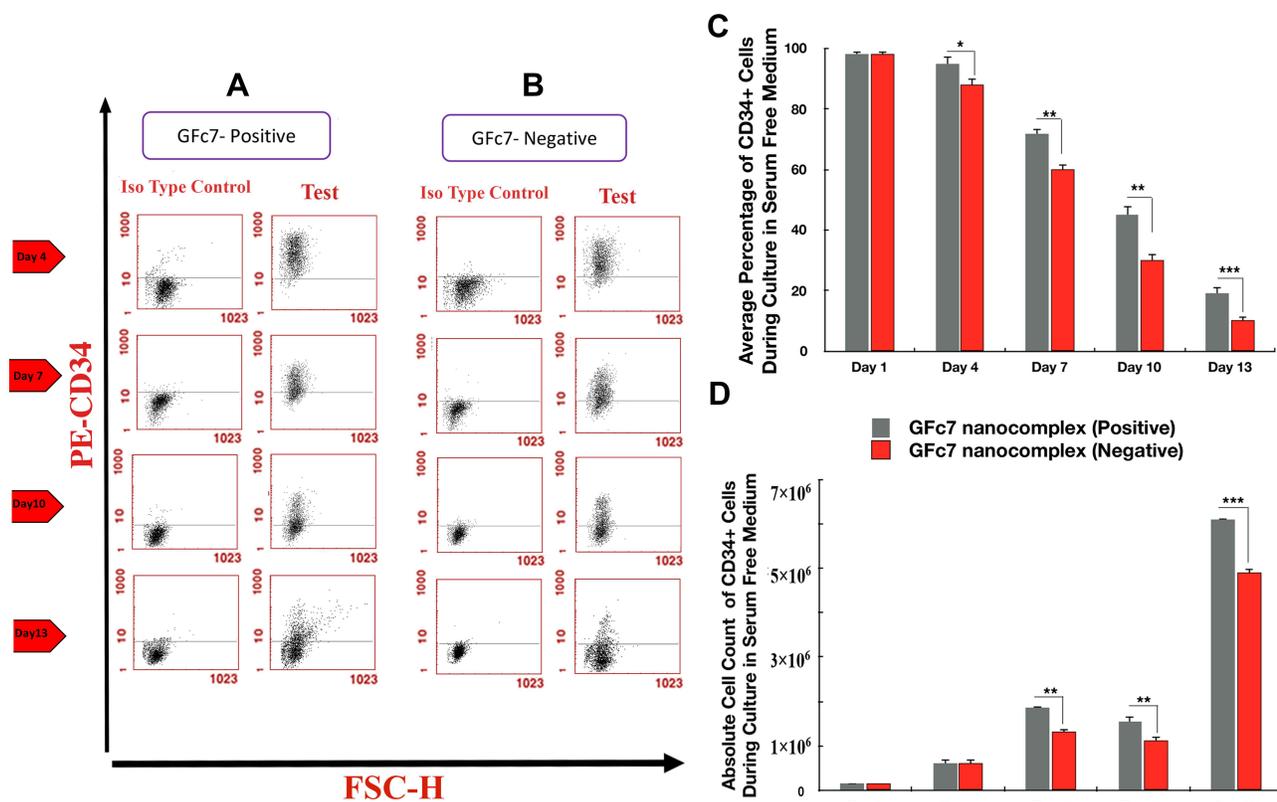


Figure 4 (A) Dot plot diagram of the analysis of CD34⁺ cells treated with GFc7 growth nanofactor in an FBS-free medium (Group A, Test) on days 1, 4, 7, 10 and 13. (B) Dot plot diagram of the analysis of CD34⁺ cells treated without GFc7 growth nanofactor in an FBS-free medium (Group A, Control) on days 1, 4, 7, 10 and 13. (C) A comparison between the percentage of CD34⁺ cells treated with and without GFc7 growth nanofactor (Group A, FBS-free medium). (D) A comparison between the absolute count of CD34⁺ cells treated with and without GFc7 growth nanofactor (Group A, FBS-free medium).

Notes: Cell surface analysis of CD34⁺ cells was done by anti-CD34-PE (Channel FL-2H). Data are expressed as mean±SD. Asterisks show data sets that are significant at different levels: *p<0.05, **p<0.01, ***p<0.001.

In control groups, HSCs were treated with H₂O₂ (control positive) and without any treatments (control negative).

Statistical Analysis

All experiments were conducted in triplicate and the analysis of variance (ANOVA) test was applied to compare multiple groups; the difference was considered significant when the P value was lower than 0.05. All data are shown as mean \pm Standard Deviation (SD). Statistical data analyses were performed by the IBM SPSS Statistics (version 25) software.³³

Results

Characteristics of GfC7 Growth Nanofactor

The TEM image of GfC7 growth nanofactor showed that the size of the nanoparticles was approximately 80 nm (Figure 2A). Moreover, the BET analysis illustrated the porous structure of this nanocomplex (Figure 2B). Moreover, the results of DLS test showed that there was

no significant difference in the size of the molecules compared to the size in TEM image. According to this test, the nanoparticles were approximately 82 nm. Zeta potential was also 147.9 d.nm, proving the molecular stability of GfC7 growth nanofactor (Figure 3).

Purification of CD34⁺ and CD34⁺CD38⁻ Cells

The results of surface markers analysis revealed that 98% \pm 1 of HSCs expressed CD34⁺ and 15% \pm 0.5 of the result cells expressed CD34⁺CD38⁻. The results of MNCs analysis showed that the cell viability of HSCs was 98% \pm 1 (Figure S1).

Effect of GfC7 Growth Nanofactor on CD34⁺ Cells During ex vivo Expansion FBS-Free Medium

The percentages of CD34⁺ cells treated with GfC7 (Group A, Test) were 95% \pm 2 (day 4), 72% \pm 1.6 (day 7), 45% \pm 2

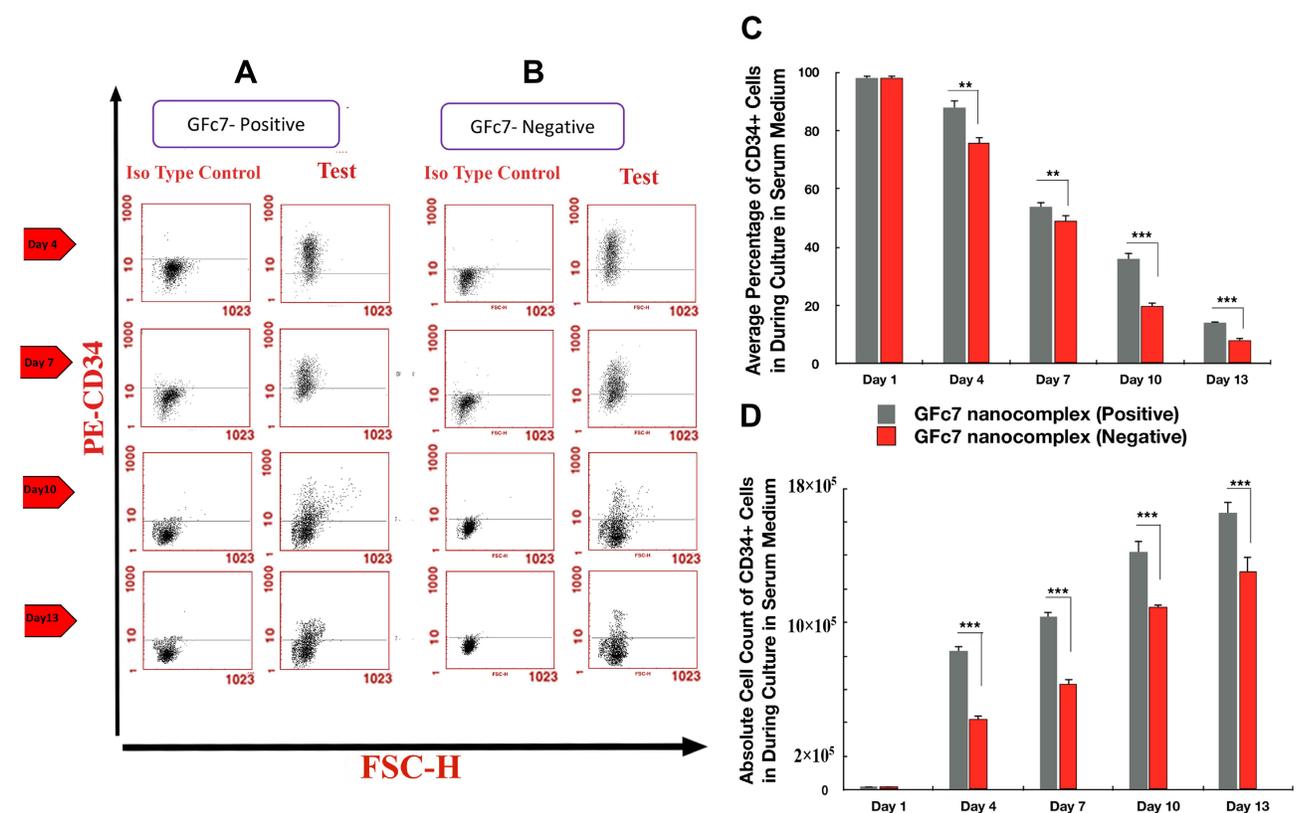


Figure 5 (A) Dot plot diagram of the analysis of CD34⁺ cells treated with GfC7 growth nanofactor in an FBS medium (Group B, Test) on days 1, 4, 7, 10 and 13. (B) Dot plot diagram of the analysis of CD34⁺ cells treated without GfC7 growth nanofactor in an FBS medium (Group B, Control) on days 1, 4, 7, 10 and 13. (C) A comparison between the percentage of CD34⁺ cells treated with and without GfC7 growth nanofactor (Group B, FBS medium). (D) A comparison between the absolute count of CD34⁺ cells treated with and without GfC7 growth nanofactor (Group B, FBS medium).

Notes: Cell surface analysis of CD34⁺ cells was done by anti-CD34-PE (Channel FL-2H). Data are expressed as mean \pm SD. Asterisks show data sets that are significant at different levels: **p<0.01, ***p<0.001.

(day 10) and $19\% \pm 1.5$ (day 13), while the percentages in control (Group A, Control) were $88\% \pm 2.3$, $60\% \pm 1.4$, $30\% \pm 3$ and $10\% \pm 2$ on the same days, respectively. Likewise, the absolute counts of $CD34^+$ cells treated with GfC7 (Group A, Test) were $165 \pm 40 \times 10^3$ (day 1), $620 \pm 95 \times 10^3$ (day 4), $1872 \pm 65 \times 10^3$ (day 7), $1550 \pm 100 \times 10^3$ (day 10) and $6100 \pm 80 \times 10^3$ (day 13), while the absolute counts in control (Group A, Control) were $165 \pm 40 \times 10^3$, $600 \pm 92 \times 10^3$, $1320 \pm 11 \times 10^3$, $1121 \pm 120 \times 10^3$ and $4900 \pm 8 \times 10^3$ on the same days, respectively (Figure 4).

FBS Medium

The percentages of $CD34^+$ cells treated with GfC7 (Group B, Test) were $88\% \pm 2$ (day 4), $54\% \pm 1.6$ (day 7), $36\% \pm 1$ (day 10) and $14\% \pm 8$ (day 13), while the percentages in control (Group B, Test) were $76\% \pm 2.5$, $49\% \pm 1.4$, $20\% \pm 1$ and $8\% \pm 0.5$ on the same days, respectively. Likewise, the absolute counts of $CD34^+$ cells treated with GfC7 (Group B, Test) were $165 \pm 40 \times 10^3$ (day 1), $832 \pm 20 \times 10^3$ (day 4), $1032 \pm 34 \times 10^3$ (day 7), $1420 \pm 14 \times 10^3$ (day 10) and 1652

$\pm 90 \times 10^3$ (day 13), while the absolute counts (Group B, Test) in control were $165 \pm 40 \times 10^3$, $420 \pm 24 \times 10^3$, $630 \pm 11 \times 10^3$, $1092 \pm 70 \times 10^3$ and $1303 \pm 67 \times 10^3$ on the same days, respectively (Figure 5).

Effect of GfC7 Growth Nanofactor on $CD34^+CD38^-$ Cells During ex vivo Expansion FBS-Free Medium

The percentages of $CD34^+CD38^-$ cells treated with GfC7 (Group C, Test) were $54\% \pm 2$ (day 4), $40\% \pm 1.6$ (day 7), $30\% \pm 2$ (day 10) and $10\% \pm 0.6$ (day 13), while the percentages in control (Group C, Control) were $37\% \pm 1.8$, $31\% \pm 1.4$, $18\% \pm 1.2$ and $7\% \pm 0.5$ on the same days, respectively. Likewise, the absolute counts of $CD34^+CD38^-$ cells treated with GfC7 (Group C, Test) were $17 \pm 10 \times 10^3$ (day 1), $413 \pm 98 \times 10^3$ (day 4), $1092 \pm 65 \times 10^3$ (day 7), $1222 \pm 70 \times 10^3$ (day 10) and $915 \pm 85 \times 10^3$ (day 13), while the absolute counts in control (Group C, Control) were $17 \pm 10 \times 10^3$,

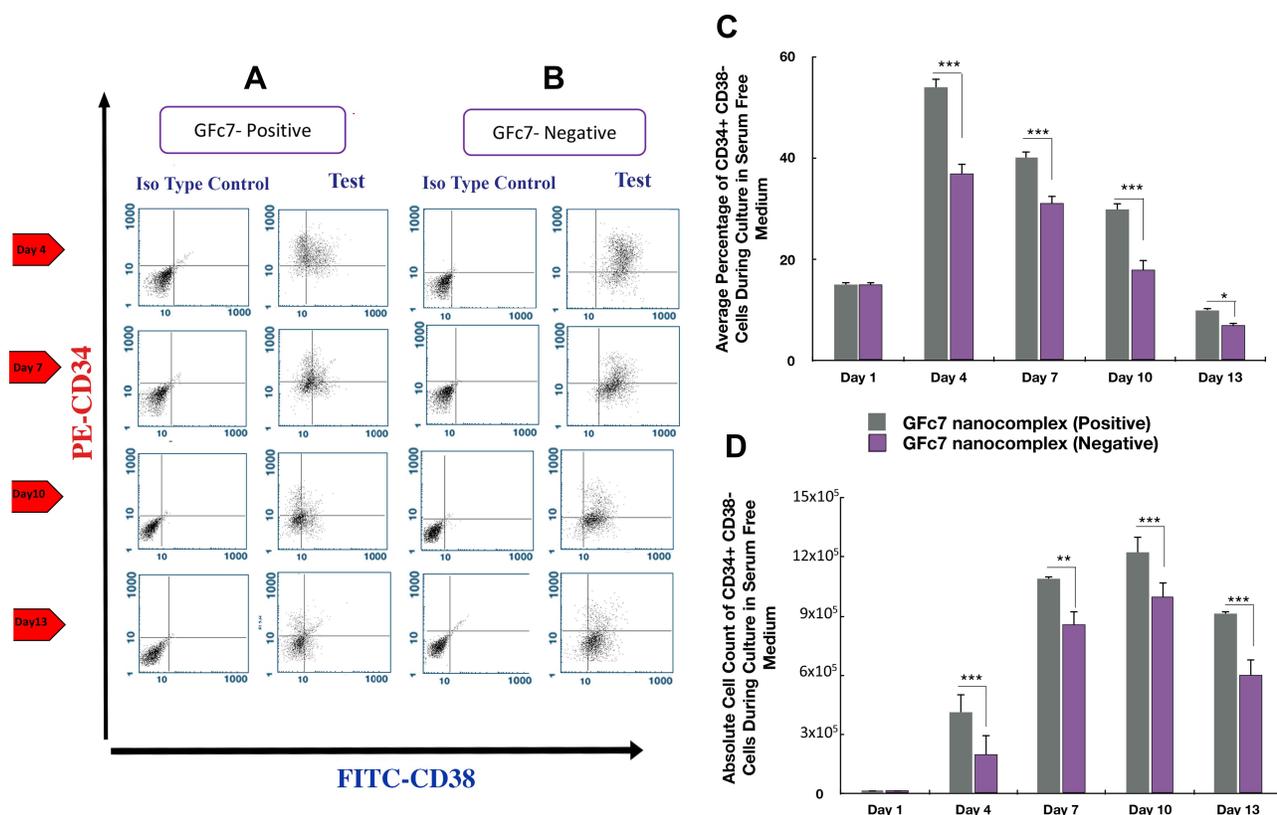


Figure 6 (A) Dot plot diagram of the analysis of $CD34^+CD38^-$ cells treated with GfC7 growth nanofactor in an FBS-free medium (Group C, Test) on days 1, 4, 7, 10 and 13. (B) Dot plot diagram of the analysis of $CD34^+CD38^-$ cells treated without GfC7 growth nanofactor in an FBS-free medium (Group C, Control) on days 1, 4, 7, 10 and 13. (C) A comparison between the percentage of $CD34^+CD38^-$ cells treated with and without GfC7 growth nanofactor (Group C, FBS-free medium). (D) A comparison between the absolute count of $CD34^+CD38^-$ cells treated with and without GfC7 growth nanofactor (Group C, FBS-free medium).

Notes: Cell surface analysis of $CD34^+CD38^-$ cells was done by anti- $CD34$ -PE, $CD38$ -FITC (Channel FL2-H, Channel FL1-H). Data are expressed as mean \pm SD. Asterisks show data sets that are significant at different levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

$200\pm 91\times 10^3$, $860\pm 11\times 10^3$, $100\pm 80\times 10^3$ and $600\pm 7\times 10^3$ on the same days, respectively (Figure 6).

FBS Medium

The percentages of $CD34^+CD38^-$ treated with GfC7 (Group D, Test) were $44\%\pm 2$ (day 4), $30\%\pm 1.4$ (day 7), $24\%\pm 1.9$ (day 10) and $13\%\pm 0.5$ (day 13), while the percentages in control (Group D, Control) were zero on all four days. Likewise, the absolute counts of $CD34+CD38-$ treated with GfC7 (Group D, Test) were $17\pm 10\times 10^3$ (day 1), $430\pm 24\times 10^3$ (day 4), $750\pm 30\times 10^3$ (day 7), $572\pm 70\times 10^3$ (day 10) and $1100\pm 67\times 10^3$ (day 13), while the absolute counts in control (Group D, Control) were zero on all four days (Figure 7).

Effect of GfC7 Growth Nanofactor on CXCR4 Cells During ex vivo Expansion

The percentage of $CXCR4^+CD34^+$ cells treated with GfC7 was $26\%\pm 0.5$ on day 10, while the percentage in control was $16\%\pm 0.3$ on the same day (Figure 8A). Likewise, the absolute count of $CXCR4^+CD34^+$ cells treated with GfC7

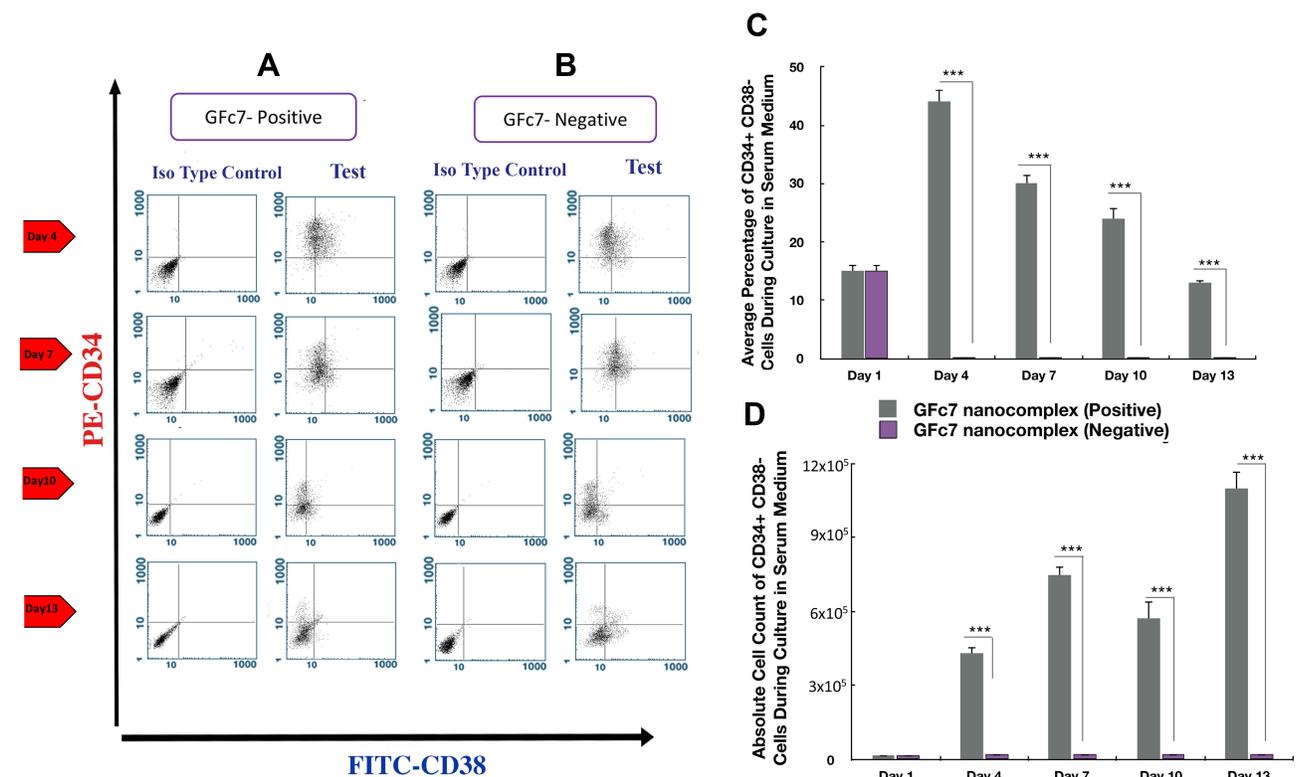
was $2900\pm 50\times 10^3$ on day 10, while the absolute count in control was $1700\pm 30\times 10^3$ on the same day (Figure 8B).

Effect of GfC7 Growth Nanofactor on ALDH During ex vivo Expansion of $CD34^+$ Cells

The percentages of ALDH in $CD34^+$ cells treated with GfC7 were $63\%\pm 2.1$ (day 4), $60\%\pm 1.6$ (day 7), $54\%\pm 2$ (day 10) and $49\%\pm 0.6$ (day 13), while the percentages in control were $48\%\pm 2.3$, $43\%\pm 1.4$, $37\%\pm 1.9$ and $42\%\pm 0.5$ on the same days, respectively (Figure 8C).

Effect of GfC7 Growth Nanofactor on Gene Expression

The gene expressions of CD34, CXCR4 and MGCL in $CD34^+$ cells treated with GfC7 increased by 1.9 ± 0.03 , 2.1 ± 0.3 and 5.1 ± 0.4 , respectively, while the gene expressions in control were 0.1 ± 0.02 , 0.1 ± 0.04 and 0.7 ± 0.05 (Figure 9A).



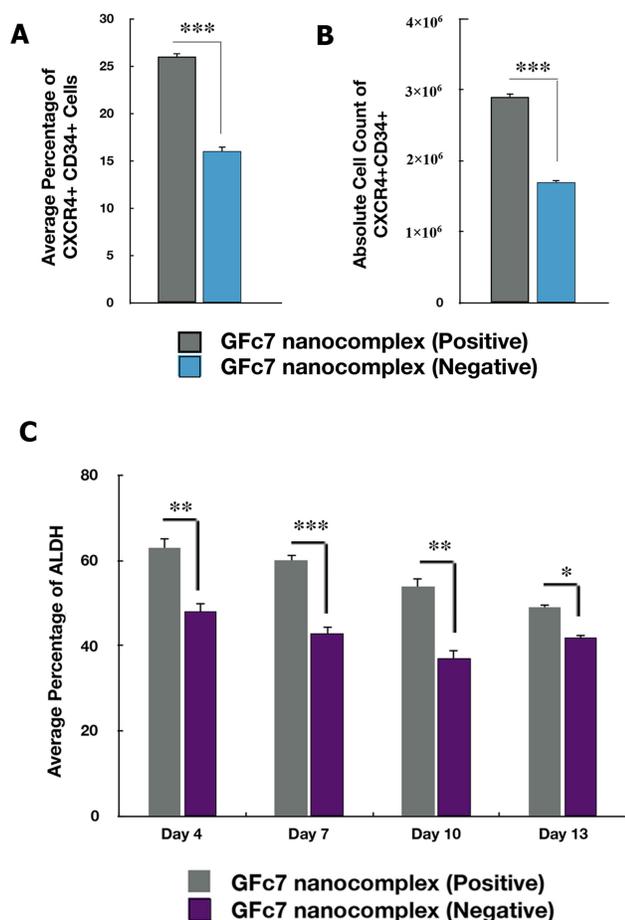


Figure 8 (A) A comparison between the percentage of CXC4⁺ CD34⁺ cells treated with and without GfC7 growth nanofactor. (B) A comparison between the absolute count of CXC4⁺ CD34⁺ cells treated with and without GfC7 growth nanofactor. (C) A comparison between the percentage of ALDH enzyme treated with and without GfC7 growth nanofactor.

Notes: Three samples were analyzed in this experiment. Data are expressed as mean±SD. Asterisks show data sets that are significant at different levels: *p<0.05, **p<0.01, ***p<0.001.

Effect of GfC7 Growth Nanofactor on Protection of CD34⁺ Cells in the Presence of H₂O₂

Assessing the cytoprotective effect of GfC7 against oxidative stress in CD34⁺ cells treated with GfC7 followed by treatment with H₂O₂: The optical densities with different doses of GfC7 were 0.078±0.005 (1000 µg/mL), 0.140±0.004 (100 µg/mL), 0.199±0.006 (10 µg/mL) and 0.102±0.009 (1 µg/mL), while they were 0.226±0.002 in control negative and 0.109±0.009 in control positive (Figure 9B).

Assessing the cytoprotective effect of GfC7 against oxidative stress in CD34⁺ cells treated with GfC7 and H₂O₂ at the same time: The optical densities with different doses of GfC7 were 0.0995±0.005 (1000 µg/mL), 0.207±0.006 (100 µg/mL), 0.126±0.004 (10 µg/mL) and 0.073

±0.006 (1 µg/mL), while they were 0.226±0.002 in control negative and 0.0995±0.009 in control positive (Figure 9C).

The results showed the cytoprotective effect of GfC7 against oxidative stress in CD34⁺ cells at 100 µg/mL and 10 µg/mL doses.

Effect of GfC7 Growth Nanofactor on Recovery of CD34⁺ Cells After Being Thawed

The cell viability of CD34⁺ cells was assessed in two stages (Figure 10);

Immediately After De-Freezing

The percentage of cell viability in GfC7 group was 80% ±3, while it was 61%±2.2 in control (without GfC7).

24 Hrs After De-Freezing

The percentage of cell viability in GfC7 group was 52% ±5, while it was 30%±4.5 in control.

Effect of GfC7 Growth Nanofactor on Colony-Forming Cells of CD34⁺ Cells After Being Thawed

Colony-forming assay was conducted and the number of colonies was counted to evaluate CD34⁺ cells function. This assay was performed in two stages (Table 2):

Immediately After De-Freezing

The numbers of colonies of CFU-GM, CFU-GEMM and BFU-E in GfC7 group were 56±3, 26±1 and 13±1 while they were 60±1, 20±3, 13%±1 in control, respectively.

24 Hrs After De-Freezing

The numbers of colonies of CFU-GM, CFU-GEMM and BFU-E in GfC7 group were 52±2, 22±3 and 15±1, while they were 41±2, 14±2 and 8±2 in control, respectively.

Discussion

HSCs are identified as one of the most well-characterized tissue-specific stem cells with decades of basic research and clinical application, and UCB is the most accessible source to supply this type of stem cells in cell therapy. Adequate number of HSCs is a determining factor in cell therapy. However, the low number of HSCs in UCB unit has restricted the application of these cells to some patients with a specific age and weight range.^{34,35}

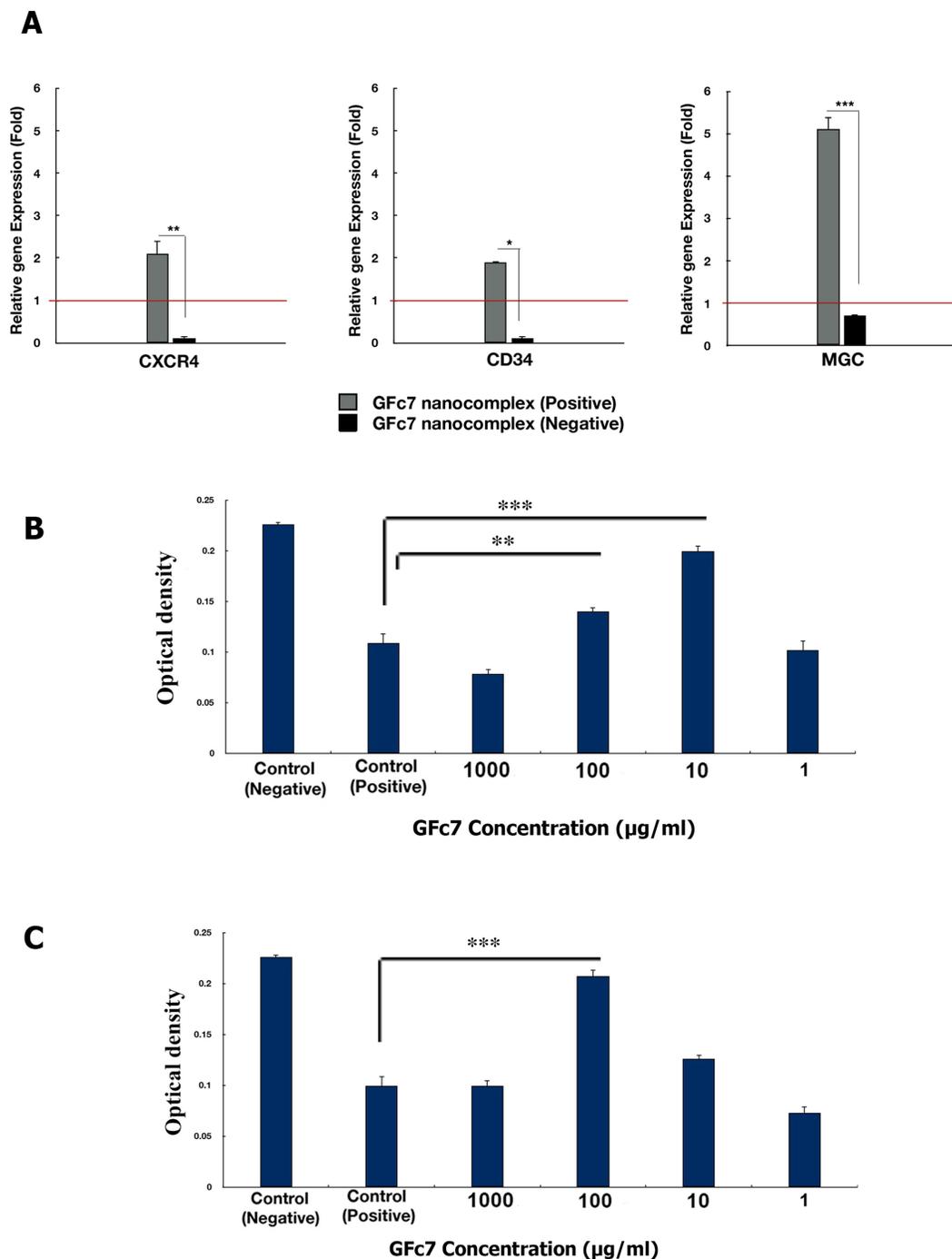


Figure 9 (A) Effect of GfC7 growth nanofactor on the relative gene expression levels of CD34, CXCR4 and ALDH in CD34⁺ cells cultured with and without GfC7 growth nanofactor. **(B)** The MTT cell proliferation assay to analyze the cytoprotective effect of GfC7 against oxidative stress in CD34⁺ cells treated with several doses of GfC7 growth nanofactor followed by treatment with H₂O₂. **(C)** The MTT cell proliferation assay to analyze the cytoprotective effect of GfC7 against oxidative stress in CD34⁺ cells simultaneously treated with several doses of GfC7 growth nanofactor and H₂O₂.

Notes: Data were normalized to GAPDH as the endogenous control gene. The red horizontal line shows the one-fold enrichment cut-off criterion. Three samples were analyzed in this experiment. Data are expressed as mean \pm SD. Asterisks show data sets that are significant at different levels: * p <0.05, ** p <0.01, *** p <0.001.

The in vitro expansion of HSCs by growth factors before transplant is a priority in this field. T Peled claims that copper chelator tetraethylenepentamine can regulate the balance between self-renewal and

differentiation of HSCs when used for expansion.³⁶ Recent studies have shown that the application of nicotinamide as a growth factor expands CD34/CD133⁻ cells in vitro, delays cell differentiation and

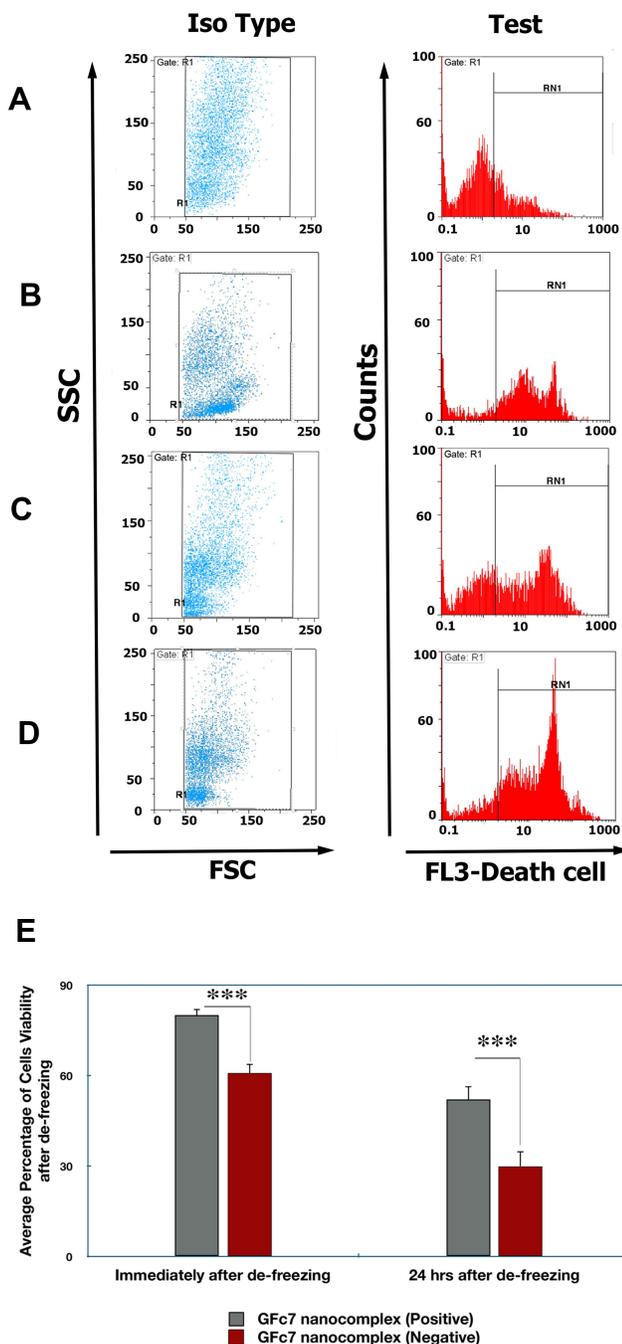


Figure 10 (A) Histogram diagram of the cell viability analysis of GFc7-treated CD34⁺ cells immediately after de-freezing. (B) Histogram diagram of the cell viability analysis of CD34⁺ cells immediately after de-freezing without treatment with GFc7 growth nanofactor. (C) Histogram diagram of the cell viability analysis of GFc7-treated CD34⁺ cells 24 hrs after de-freezing. (D) Histogram diagram of the cell viability analysis of CD34⁺ cells 24 hrs after de-freezing without treatment with GFc7 growth nanofactor. (E) A comparison between the percentage of cell viability of HSCs treated with and without GFc7 growth nanofactor.

Notes: Data are expressed as mean±SD. Asterisks show data sets that are significant at different levels: ***p<0.001.

results in 9-fold increase in cells.³⁷ In another study, it was reported that human and murine expression of PGE2 receptors in HSCs and short-term exposure

Table 2 A Comparison Between the Numbers of the Colonies of CD34⁺ Cells Treated with and without GFc7 Growth Nanofactor Immediately After De-Freezing (A) and 24 Hrs After (B)

A			
	CFU-GM	CFU-GEMM	BFU-E
Treatment with GFc7	56±3	26±1	13±1
Treatment Without GFc7	60±1	20±3	13±1
B			
Treatment with GFc7	52±2	22±3	15±1
Treatment Without GFc7	41±2	14±2	8±2

Notes: Data are expressed as mean±SD. Three samples were analyzed in this experiment.

to PGE2 improved cell viability and expansion of HSCs.³⁸

Recently, various studies have been conducted in the field of nanotechnology to overcome stem cells limitations in cell therapy, such as nanoparticles applied in targeted drug delivery system.^{39,40} Wang Y experimented on liposome-protamine-DNA complex and stated that cell-specific promoters enabled lipid-based nanoparticles to facilitate genes delivery to retina cells.⁴¹ In another study, it was proven that owing to the tumor-homing ability of nano-engineered MSCs, they could be used as vectors to deliver diagnostic and therapeutic nanoparticles into a tumor.⁴²

In the previous study, the positive impact of GFc7 growth nanofactor on hMSCs properties was revealed to be significant in vitro.¹⁵ Therefore, the effect of this growth nanofactor on HSCs expansion and their properties after treatment was evaluated in the present study.

CD34 glycosylated transmembrane protein is the defining characteristic of human HSCs. This marker is involved in progenitor cells maintenance in a phenotypically undifferentiated state.^{43,44} In the current study, the expansion of CD34⁺ after treatment with GFc7 growth nanofactor was evaluated and compared with control (without GFc7). The results showed that the population percentage of CD34⁺ was higher than control.

The absolute count of CD34 in one unit of UCB is 2.07 ±1.31×10⁶, and 170×10³ of CD34 cells per kilogram of the bodyweight is required for transplant.^{21,43} Therefore, it can be claimed that CD34 cells expansion with GFc7 growth nanofactor can provide the proper number of cells required for transplant in an adult weighing over 55 kg.

As CD34 marker alone is not capable of representing actual HSCs, CD34⁺CD38⁻ stem cell population was measured as well. CD34⁺CD38⁻ immunophenotype represents a

rare and highly primitive population of stem cells in UCB⁴⁵ with low HLA-Dr expression in all HSCs studies to date. Because CD34⁺CD38⁻ cells are immediately differentiated into CD34⁺CD38⁺ in the presence of FBS, FBS-free mediums are utilized for maintenance and expansion of these stem cells,⁴⁶ yet these mediums are too costly. As a result, in the second stage of this study, CD34⁺CD38⁻ cells were expanded in FBS medium in the presence of GfC7 growth nanofactor to investigate their differentiation and expansion so as to find a more economical way than FBS-free medium. The results showed that CD34⁺CD38⁻ cells lasted to exist even 13 days after expansion, whereas they were differentiated and transformed to CD34⁺CD38⁺ just 4 days after expansion in control according to dot plot diagram of FACS analysis.

In this study, after treating HSCs with GfC7 growth nanofactor, the percentage of ALDH, as a marker of HSCs, was computed and then compared with control in various days of expansion.^{47,48} The results indicated that the treated cells expressed higher percentage of ALDH than control. Therefore, it could be concluded that the treated cells with GfC7 growth nanofactor possessed higher stemness property.

In one experiment, Asfour I evaluated engraftment after transplant in a number of patients and reached the conclusion that SDF-1/CXCR4 in HSCs plays a vital role in engraftment after transplant.⁴⁹ In another study, it was revealed that the mobilization of CD34⁺CXCR4⁺ cells in acute MI has a positive correlation with the improvement of left ventricular ejection fraction.⁵⁰ As a result, the importance of CD34⁺CXCR4⁺ cells after transplant made us assess the expression of these cells after expansion when treated with GfC7 growth nanofactor, and the results showed that expansion in GfC7 group was 10% higher than that of control.

In the next stage of the current study, the gene expressions of CD34, CXCR4 and MGCL were analyzed, showing that they were higher in GfC7 group than control.

Nowadays, private and public UCB banks are established to provide patients with HSCs by freezing these stem cells.¹ Higher quality and quantity of these stem cells is essential in the freezing and de-freezing process in order to help the patients with the higher performance of these stem cells after transplant.⁵¹

Freezing and de-freezing process naturally reduces stem cells quality due to the presence of free radicals.^{52,53} In one of the stages of the current study before de-freezing, it was revealed that in the presence of H₂O₂, HSCs treated with GfC7 growth nanofactor had higher viability.

The freezing process of HSCs was performed by using the standard protocol of UCB banks to evaluate the quality of HSCs treated with GfC7 growth nanofactor after de-freezing. This evaluation was performed in two stages; a) immediately after de-freezing, b) 24 hrs after de-freezing. The results showed that the treated cells with GfC7 growth nanofactor had higher percentage of cell viability than control in all three stages, as it was also confirmed by colony-forming cell assay (this assay was utilized to study hematopoietic progenitors proliferation and differentiation pattern and investigate their ability to form stem cell colonies in a semisolid medium). This higher cell viability could be attributed to the cytoprotective effect of GfC7 against oxidative stress in CD34⁺ cells.

Conclusion

The results of the present study support the hypothesis that treating HSCs with GfC7 growth nanofactor leads to HSCs quality and quantity improvement in terms of expansion in vitro and freezing and de-freezing process, which consequently improves their efficient performance in the body.

Abbreviations

HSCs, hematopoietic stem cells; SCF, stem cell factor; TPO, thrombopoietin; Flt-3L, Flt-3 ligand; UCB, umbilical cord blood; DMSO, dimethyl sulfoxide; PBS, phosphate buffer solution; H₂O₂, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 99%; hMSCs, human mesenchymal stem cells; FBS, fetal bovine serum; IMDM, Iscove's Modified Dulbecco's Media; HES, hydroxyl ethyl starch; FACS, flow cytometry; HRTEM, high-resolution transmission electron microscopy.

Ethics Approval and Consent to Participate

This study received the ethics approval of Shahid Beheshti University of Medical Sciences, Tehran, Iran, 7808, and the written consent of the patients to participate in the study.

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Disclosure

Dr Mohammad Hassan Nazaran reports a patent US8288587B2 with royalties paid. The authors declare that they have no competing interests.

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