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Research Article

Recombinant human G-CSF production as a protein based drug candidate for hematology and oncology

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ABSTRACT

The human granulocyte colony stimulating factor (hG-CSF) is a member of the CSF family. The first purpose of this study was production of recombinant human G-CSF (rhG-CSF) which is an important therapeutic protein for angiogenesis based clinical applicaitons. rhG-CSF was produced as inclusion body in the *Escherichia coli* strain BL21 (DE3) pLysE with pTOLT vector system. The rhG-CSF was purified by Ni-NTA agarose afinity chromatography and characterized with SDS-PAGE analysis. The effects of this therapeutic protein on cell viability was measured by MTT assay. Additionally, angiogenic potential of produced rhG-CSF was investigated via HUVEC cell line by *in vitro* scratch assay. As a result, the purified protein induced cell proliferation and the EC50 value of protein based drug candidate was 0.051 mM. Additionally, it was determined that the migration ability of the HUVECs was promoted by rhG-CSF in a concentration-dependent manner by *in vitro* scratch assay.

Keywords: Granulocyte colony stimulating factor, recombinant proteins, angiogenesis, cell proliferation.

Hematoloji ve onkoloji için protein esaslı bir ilaç adayı olarak rekombinant insan G-CSF üretimi

ÖZ

İnsan granülosit koloni uyarıcı faktör (hG-CSF), CSF ailesinin bir üyesidir. Bu çalışmanın temel amacı, anjiyogenez bazlı klinik uygulamalar için önemli bir terapötik protein olan rekombinant insan G-CSF (rhG-CSF)' sinin üretimi olarak belirlenmiştir. rhG-CSF, bir *Escherichia coli* suşu olan BL21 (DE3) pLysE'de pTOLT vektör sistemi ile inklüzyon cisimciği olarak üretilmiştir. rhG-CSF, Ni-NTA agaroz afinite kromatografisi aracılığıyla saflaştırılmış ve SDS-PAGE analizi ile karakterize edilmiştir. Bu terapötik proteinin hücre canlılığı üzerine etkisi MTT testi ile değerlendirilmiştir. Ek olarak, üretilen rhG-CSF'nin anjiyogenik potansiyeli, HUVEC hücre hattı ile gerçekleştirilen *in vitro* çizik analizi ile araştırılmıştır. Sonuçta, saflaştırılmış proteinin hücre proliferasyonunu arttırdığı görülmüş ve protein temelli bu ilaç adayının EC50 değeri, 0.051 mM olarak hesaplanmıştır. Ek olarak, HUVEC hücrelerinin migrasyon yeteneklerinin rhG-CSF ile konsantrasyona bağlı bir şekilde arttığı belirlenmiştir.

Anahtar Kelimeler: Granülosit koloni uyarıcı faktör, rekombinant proteinler, anjiyogenez, hücre proliferasyonu.

1. INTRODUCTION

The human granulocyte colony stimulating factor (hG-CSF), a glycoprotein, is a member of the CSF family.[1–3] It is a cytokine which have many functions such as stimulating of hematopoiesis and production of bone marrow neutrophilic granulocyte colonies.[4] A recombinant form of G-CSF is used to accelerate the treatment success of neutropenic after chemotherapy and allow higher-intensity treatment regimens in some cancer patients in oncology and hematology. Angiogenesis is the new blood vessel formation that occur with proliferation and migration of endothelial cells. These cells form the inner surface of the blood vessels that feed

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the other surrounding cells with oxygen and nutrients. The effect of G-CSF on these cells is rarely investigated and the results are still uncertain.[5] According to this comprehensive application, the rhG-CSF has been expressed in various expression host systems. Three forms of it are suitable for clinical use: a glycosylated form obtained by expression in mammalian cell line (*Chinese Hamster Ovary*) (such as Lenograstim), a non-glycosylated form expressed in an *E. coli* expression system (such as Filgrastim and Nartograstim) and second-generation G-CSF (such as Pegfilgrastim).[6-8] Researches on the cloning, expression and purification of rhG-CSF have been carried out in order to eliminate the limitations of production efficiency and cost. Because in the pharmaceutical industry, the production of bioproducts requires a simple and cost-effective process that includes easier steps with high throughput. Therefore, studies on the expression of recombinant rhG-CSF using various expression hosts such as *P. Pastoris*, [1,10-11] in tobacco BY-2 cells,[10] in *S. cerevisiae* [2] and in *P. fluorescens* [12] by a number of research group have been performed (Table 1).

E. coli is a frequently used host for the production of recombinant protein.[12,13] Additionaly, the advantages of a low-cost and fast high density cultivation, and the availability of several of cloning vectors and engineered host strains enable *E. coli* to provide an average for the high yield and economic production of recombinant proteins.[15,16] Nevertheless the *E. coli* recombinant protein production system has several disadvantages. For instance, overexpression of recombinant proteins in *E. coli* often results in insoluble and nonfunctional proteins, so called inclusion bodies (IBs).[17] Various techniques are used to solve this problem, such as the use of different promoters and host strains, coexpression of chaperones and optimization culture conditions.[13,14] On the other hand, the production of the recombinant protein referred to as inclusion body has not only disadvantages, but also has some advantages.[15,18] The primary advantage is the ease of purification of IBs and it contains high degree purity of target protein.[17] Additionally IBs are often an advantage when the yield of the native protein is low because of extensive proteolysis.[18]

In this study, codon-optimized mature hG-CSF gene was cloned into our patented inducible high-copy pTOLT expression plasmid and rhG-CSF-TolA-III fusion protein was produced as inclusion body in the *E. coli* strain BL21 (DE3) pLysE. In the pTOLT vector, the proteins bind to the C-terminus of TolAIII by a short linker containing the thrombin recognition site. Furthermore it also carries an N-terminal 6xHis-tag for the purpose of purification. TolAIII is small domain that is achieved high product yields as a soluble protein in the cytoplasm of *E. coli*. Thus, pTOLT, in which the TolAIII domain is used as a fusion protein partner, is an expression vector with remarkable properties that enable proteins to be expressed at high levels in recombinant cells in a host cell. Up to 90 mg fusion protein per liter bacterial culture was obtained using pTOLT vector.[19,21] Obviously human G-CSF protein has been recombinantly produced using several prokaryotic expression vectors (Table1), but no recombinant human G-CSF (rhG-CSF) production using the pTOLT vector system. Using this vector system, it is aimed to produce hG-CSF in *E.coli* in higher amount. Because the increased production of recombinant proteins is important to obtain a higher amount of functional product per unit volume and per unit time.

In this context hG-CSF was produced as inclusion body. At first, IBs were isolated, IBs were solubilized and refolded. Finally, the hG-CSF was purified by Ni-NTA agarose afinity chromatography. Then recombinantly produced hG-CSF was tested on human umbilical vein endothelial cells (HUVECs) at different concentrations to understand the effect of G-CSF on endothelial cells. Additionally, the cell migration process which is the crucial step for angiogenesis was investigated by an *in vitro* scratch assay. This assay is an easy, inexpensive and well-developed technique for the investigation of cell migration *in vitro*.[16]

2. MATERIALS AND METHODS

2.1. Codon optimization and Construction of G-CSF expression plasmids

In this work, codon optimization was performed with the aim of the gene expression in desired level in *E. coli*. To improve the expression levels of the hG-CSF gene, the gene sequence was recoded on the basis of the codon preference characteristics of *E. coli* K12 organism, without differentiating the amino acid sequence of the corresponding proteins. Online optimization software [Java Codon Adaptation Tool (JCat)] was used for codon design. The nucleotide sequence of the hG-CSF protein optimized for the *E. coli* organism is given in Figure 1. Plasmid containing the optimized G-CSF coding nucleic acid sequence was purchased from BIOMATIK (Clone ID: S2543-2, Gene ID: L5165K). DNA sequence encoding for G-CSF was amplified using spesific primers which are contained *SacI* and *Kpn*I restriction sites (Table 2). The PCR amplification product was inserted into pTOLT expression vector (Figure 2), that added a N-terminal six-histidine tag, using *SacI* and *Kpn*I restriction sites. *E. coli* DH5α cells which is the host strain for cloning were transformed with the pTOLT-G-CSF plasmid. Transformation mix were spread on LB agar culture plates including ampicillin (0.1 g ml^{-1}) and they were incubated at 37°C overnight. After transformation, plasmids isolated from positive clones were screened by PCR and then analyzed by DNA sequencing.

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Table 1. Recombinant hG-CSF expression systems

Expression host	Expression vector	Soluble/Insoluble Form	rhG-CSF concentration	Referance
E. coli BL21 (DE3)	pET23a	Inclusion body (insoluble)	-	17,18
E. coli BL21 (DE3)	pET22b	Inclusion body (insoluble)	60 µg ml^{-1}	21
E. coli BL21 (DE3)	pET28a	-	-	22
E. coli M15	pQE3	Inclusion body (insoluble)	-	3
P. pastoris strain GS115	pPIC9	Soluble	3.1 mg l^{-1}	23,24
P. pastoris strain SMD1168H	pPICZa	Soluble	50.66 µg ml^{-1}	25
Saccharomyces cerevisiae	pIL20GC	Soluble	232.7 mg l^{-1}	6
Aspergillus niger	pAN56-2M	Soluble	5 mg l^{-1}	1
Pseudomonas fluorescens strain DC454		Soluble	350 mg l^{-1}	26
Tobacco Bright Yellow-2	pMDC83	Soluble	4.19 mg l^{-1}	27
(BY-2) cells	pMDC43	Soluble	17.89 mg l^{-1}	27

Table 2. Oligonucleotides employed to obtain the DNA sequence of HG-CSF by PCR amplification

Primers	Sequence
GCSF *Sac*I TolT sense	TTTTTGAGCTCATGGCAGGCCCTGCT
*GCSF Kpn*I TolT antisense	TTTTTGGTACCTCAGGGTTGGGCCAA

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Wild        ATGGCCGGCCCCGCCACCCAGAGCCCCATGAAGCTGATGGCCCTGCAGCTGCTGCTGTGG    60
Optimize    ATGGCTGGTCCGGCTACCCAGTCTCCGATGAAACTGATGGCTCTGCAGCTGCTGCTGTGG    60
            ***** ** ** ** *******   ** ****** ******** ********************

Wild        CACAGCGCCCTGTGGACCGTGCAGGAGGCCACCCCCCTGGGCCCCGCCAGCAGCCTGCCC    120
Optimize    CACTCTGCTCTGTGGACCGTTCAGGAAGCTACCCCGCTGGGTCCGGCTTCTTCTCTGCCG    120
            ***   ** ************ ***** ** ****** ***** ** **       ******

Wild        CAGAGCTTCCTGCTGAAGTGCCTGGC--AGGTGCGCAAGATCCAGGGCGACGGCGCCGCC    178
Optimize    CAGTCTTTCCTGCTGAAATGCCTGGAACAGGTTCGTAAAATCCAGGGTGACGGTGCTGCT    180
            ***   ************ ********   **** ** ** ******** ****** ** **

Wild        CTGCAGGAGAAGCTGGTGAGCGAGTGCGCCACCTACAAGCTGTGCCACCCCGAGGAGCTG    238
Optimize    CTGCAGGAAAAACTGGTTTCTGAATGCGCTACCTACAAACTGTGCCACCCGGAAGAACTG    240
            ******** ** ******    ** ****** ******** ************ ** ** ***

Wild        GTGCTGCTGGGCCACAGCCTGGGCATCCCCTGGGCCCCCCTGAGCAGCTGCCCCAGCCAG    298
Optimize    GTTCTGCTGGGTCACTCTCTGGGTATCCCGTGGGCTCCGCTGTCTTCTTGCCCGTCTCAG    300
            ** ********* ***    ***** ***** ***** ** ***       ******    ***

Wild        GCCCTGCAGCTGGCCGGCTGCCTGAGCCAGCTGCACAGCGGCCTGTTCCTGTACCAGGGC    358
Optimize    GCTCTGCAGCTGGCTGGTTGCCTGTCTCAGCTGCACTCTGGTCTGTTCCTGTACCAGGGT    360
            ** ************ ** ******    **********    ** ******************

Wild        CTGCTGCAGGCCCTGGAGGGCATCAGCCCCGAGCTGGGCCCCACCCTGGACACCCTGCAG    418
Optimize    CTGCTGCAGGCTCTGGAAGGTATCTCTCCGGAACTGGGTCCGACCCTGGACACCCTGCAG    420
            ************ ***** ** ***    ** ** ***** ** ********************

Wild        CTGGACGTGGCCGACTTCGCCACCACCATCTGGCAGCAGATGGAGGAGCTGGGCATGGCC    478
Optimize    CTGGACGTTGCTGACTTCGCTACCACCATCTGGCAGCAGATGGAAGAACTGGGTATGGCT    480
            ******** ** ********* ************************ ** ***** *****

Wild        CCCGCCCTGCAGCCCACCCAGGGCGCCATGCCCGCCTTCGCCAGCGCCTTCCAGCGCCGC    538
Optimize    CCGGCTCTGCAGCCGACCCAGGGTGCTATGCCGGCTTTCGCTTCTGCTTTCCAGCGTCGT    540
            ** ** ********* ********* ** ***** ** *****     ** ********* **

Wild        GCCGGCGGCGTGCTGGTGGCCAGCCACCTGCAGAGCTTCCTGGAGGTGAGCTACCGCGTG    598
Optimize    GCTGGTGGTGTTCTGGTTGCTTCTCACCTGCAGTCTTTCCTGGAAGTTTCTTACCGTGTT    600
            ** ** ** ** ***** **    **********   ********* **    ***** **

Wild        CTGCGCCACCTGGCCCAGCCC     619
Optimize    CTGCGTCACCTGGCTCAGCCG     621
            ***** ********* ******
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Figure 1. The multiple alignment of optimized and wild-type DNA sequence of G-CSF by using ClustalW2 program.

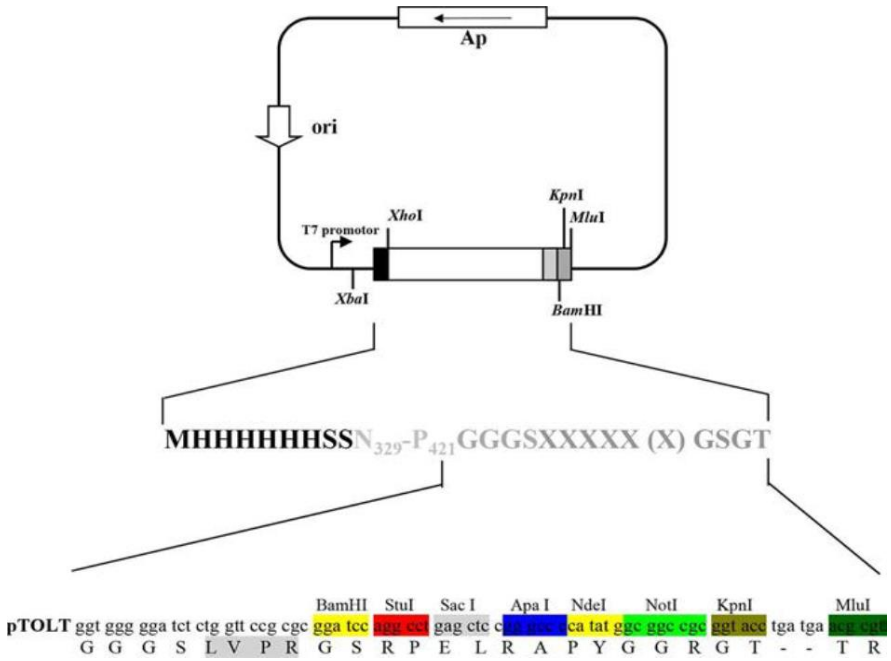

Figure 2. pTOLT cloning region.[13]

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2.2. Protein expression

In this work, the host cell *E. coli* strain BL21 (DE3) pLysE (Novagen, Inc.) was used for rhG-CSF expression. This strain was transformed by recombinant plasmid pTOLT-G-CSF. Transformed cells were spread on several LB agar plates including both 0.1 mg ml^{-1} ampicillin, chloramphenicol (34 µg m l^{-1}) and grown at 37ºC overnight. One colony of BL21(DE3) pLysE recombinant was cultivated in 4ml of selective LB medium and was grown at 37°C, with shaking (240 rpm) for 16 h. This overnight culture was inoculated into 600 ml of selective LB and incubated at 37°C with shaking (240 rpm) until the OD_{600} was 0.6. The expression of recombinant hG-CSF was induced by IPTG and then the cultures were grown for an additional 3 h. The culture was harvested by centrifugation and the cell pellets were resuspended in buffer A (100 mM Tris-HCl buffer (pH 7.0) containing 1 g l^{-1} lysozyme, 1 mM PMSF and 1mM benzamidine. The bacterial cells were lysed using a sonicator (Sonics VCX 130), soluble and insoluble fraction were separated via centrifugation (Vision VS-30000i) at 30.000 rpm for 1 hour at 4°C.

2.3. Refolding and recombinant G-CSF purification

The IBs were resuspended in 10 ml (5 ml g^{-1} wet weight pellet) of buffer A containing 5 mM EDTA, 5 mM DTT, 2 M urea, 2% (w/v) triton X-100 and sonicated four cycle for 5 minutes at 60-65% of the maximum power in a sonicator (Sonics VCX 130) followed by centrifugation (27.000 rpm for 20 min at 4°C). The inclusion body wash step was repeated three times. The washed pellet obtained in previous step was resuspended in 6 mL of buffer A containing 5 mM EDTA and 5 mM DTT. And then resüspansion were shaken on an orbital shaker at 150 rpm at 25°C for 30 min. The washed inclusion bodies were dissolved by adding a freshly prepared buffer B (50 mM Tris-HCl pH 7.0, 5 mM EDTA, 8 M Guanidine-HCl, 5 mM DTT) dropwise with the aid of a pasteur pipette (4 ml g^{-1} wet weight pellet). The solution was incubated at 25°C for 60 min with shaking (150 rpm) then solubilized inclusion bodies were refolded by refolding buffer (100 mM Tris-HCl pH 8.0, 10 mM DTT, 20% gliserol (w/v) and incubated 4 hours at 4°C. Refolded G-CSF was dialyzed against 25 mM, pH 7.8 Tris-HCl buffer containing 100 mM NaCl at 4ºC for 16 hours. Qiagen Ni-NTA metal-affinity chromatographic procedure was used to prufication of soluble recombinant protein carrying N-terminal 6x histidine. Purity of rhG-CSF was determined by 12% SDS-PAGE[28] and visualized by commasie blue. The UV absorbance at 280 nm was used to determine protein concentration.[29]

2.4. Investigation of *in vitro* stimulating effect of rhG-CSF on HUVECs proliferation

HUVECs were cultured in DMEM with 15% FBS. These cells were cryopreserved in liquid nitrogen previously in our animal cell culture laboratory. *In vitro* stimulating effect of G-CSF on HUVECs proliferation was investigated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[30]

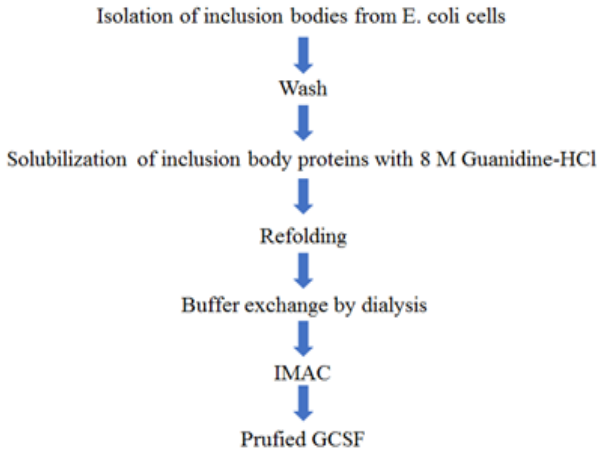

Figure 3. Steps of the refolding protocol and G-CSF prufication.

This test was performed at different concentrations (100-1.62 mM) and cells were left in contact with rhG-CSF for 48 hours. In this experiment, the cells were plated in triplicates and treated for 3 hours with MTT solution (0.5 g l^{-1}). Following the MTT removal after this incubation period, purple formazan products were dissolved completely by DMSO. The absorbance of the colored solution was measured by microplate reader at 570 nm. The results are expressed as the concentration that stimulates growth of cell by 50% against control cells (control group received only media without any rh-G-CSF) EC50 (half maximal effective concentration) and were calculated by a regression analysis using GraphPad Prism 6 software for HUVECs.[31]

2.5. Cell migration assay

In the present study, preliminary investigation was performed to consider the effect of rhG-CSF on HUVECs migration by *in vitro* scratch assay. This test is used widely to measure migration, as it provides a simple and economical installation for modelling angiogenesis. The first step created an artificial wound in a cell monolayer. In this study, for *in vitro* scratch assay, the HUVEC cell suspension was dispensed onto six well cell culture plate. When the cells reached to 90% confluency at this plate, the cell monolayer was scraped with p200 pipette tip to create a scratch. The debris was removed and smoothed the scratched edge by washing the cells with culture medium. Then cells were incubated with rhG-CSF containing media of different concentrations

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(100-1.62 mM) which were tested by MTT assay also. During incubation period (48 hours), cell images were captured by a phase contrast inverted microscope and quantitatively analyzed by image analysis software provided by Olympus. Finally, percent wound healing was calculated and the results were plotted.[32,33]

3. RESULTS AND DISCUSSION

3.1. Codon optimization and Construction of G-CSF expression plasmids

In this work, rare codon-free hG-CSF gene was cloned into pTOLT expression vector. Using the recombinant plasmid containing hG-CSF gene as the template, the DNA sequences of hG-CSF gene were amplified by PCR. After running a 2.0% agarose gel, the amplified fragments were 624 bp, which was consistent with the reported size of hG-CSF (Figure 4) Sanger sequencing analysis verified that the DNA sequences were corrected. Recombinant plasmid containing hG-CSF gene was used to express hG-CSF protein.

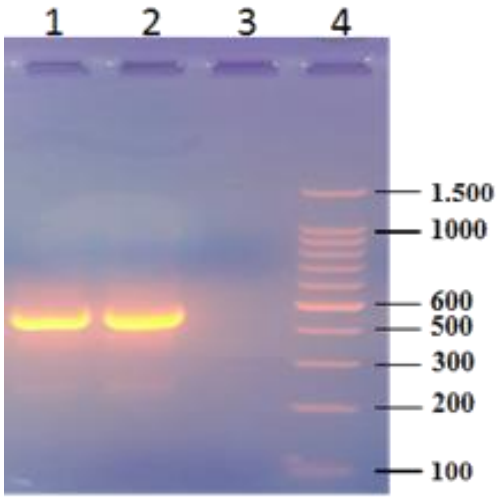

Figure 4. Agarose gel (2.0 %) electrophoresis result showing PCR verification of recombinant plasmid pTOLT-G-CSF after the cloning. Columns 1-2 positive 624 bp product indicates that G-CSF gene fragment correctly inserted into pTOLT system. Column 4 λ-EcoR I/Hind III DNA marker.

3.2. Protein expression, protein refolding and recombinant G-CSFpurification

Recombinant hG-CSF was expressed and purified as described in materials and methods. The molar absorption coefficient and molecular weight of Tol-A-III-G-CSF fusion protein were calculated as 32430 M^{-1} cm^{-1} and 34551.89 Dalton using "ExPASy ProtParam tool", respectively. Afterwards the purified Tol-A-III-G-CSF fusion protein concentration was determined as 1.78 mg ml^{-1} by absorbance at 280 nm using UV spectrophotometer (Varian Cary 50). The recombinant TolAIII-G-CSF expression was confirmed by SDS PAGE. The SDS-PAGE analysis showed that the purified TolAIII-GCSF fusion protein around 34 kDa (Figure 5) (which corresponds with a theoretical molecular weight of 34551.89 kDa).

When the image of SDS-PAGE was examined, it was observed that the protein in the inclusion body form in the pellet obtained after high speed centrifugation was quite pure. Therefore, in this study, it is necessary to optimize these steps in order to minimize protein loss in refolding and purification stage.

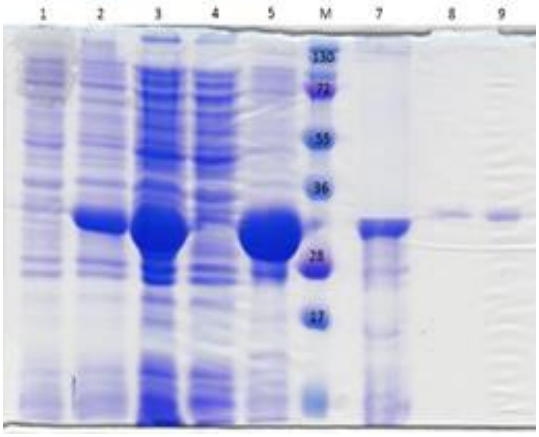

Figure 5. Coomassie-stained SDS-PAGE (12%) analysis 1. Bacterial cell lysates before IPTG addition, 2. Bacterial cell lysates after IPTGaddition 3. total bacterial cell lysate of *E. coli* BL21 (DE3)pLysE [pTOLT-hG-CSF] after sonication, 4. Collected supernatant after centrifugation of the lysate, 5. Collected pellet after centrifugation of the lysate, 6. BioRad dual colour precision plus protein marker 7. Refolded Tol-III-A-hG-CSF fusion protein 8-9. purified Tol-III-A-hG-CSF fusion protein.

3.3. Investigation of *in vitro* stimulating effect of rhG-CSF on HUVECs proliferation

In the present study, HUVECs were cultured until they reached 70%-80% confluency (Figure 6).

Figure 6. Inverted microscope image taken from HUVECs (The scale bars indicated 100 μm for x4 magnification).

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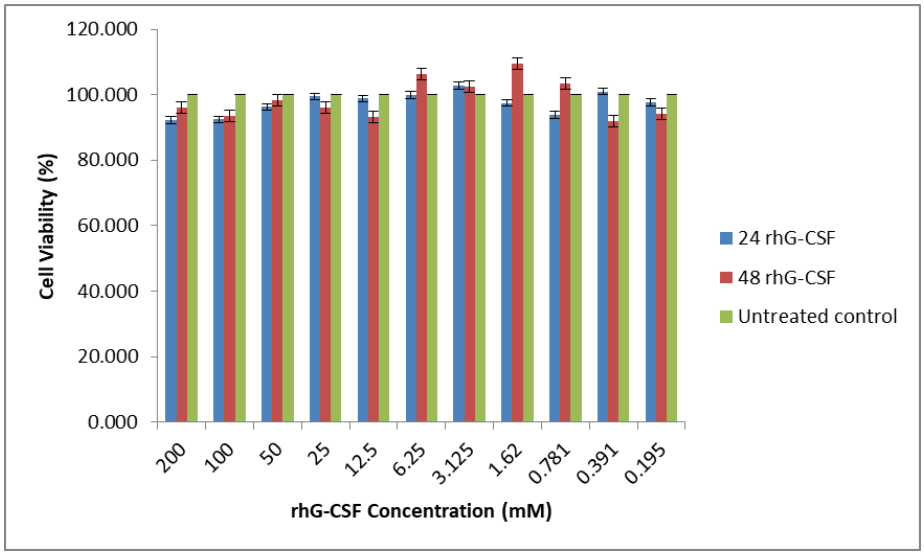

Figure 7. *In vitro* stimulating effect of rhG-CSF on HUVEC cell proliferation.

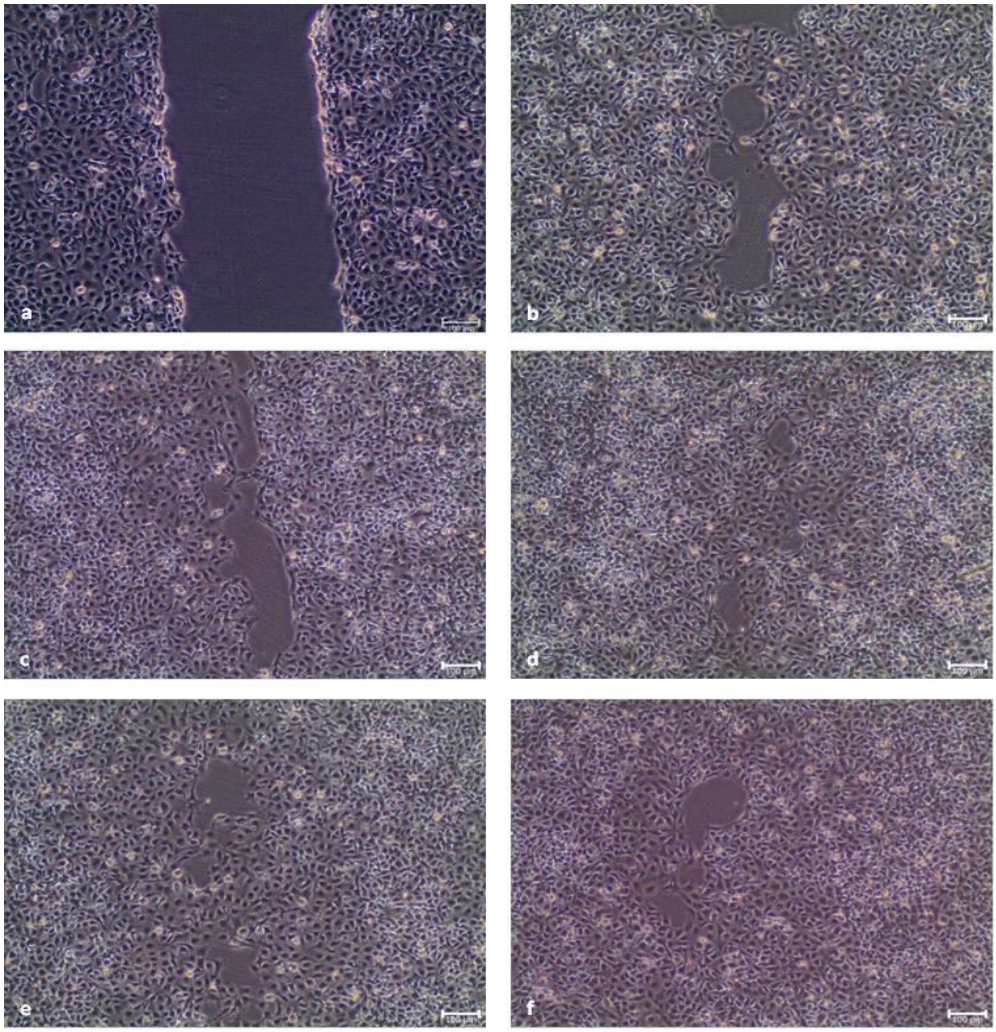

Figure 8. Inverted light microscope images of HUVECs after scratching (a: 0 hour, b-f: 24 hours). The scale bars indicated 100 µm for x4 magnification (b: untreated control, c: HUVECs incubated with 100 mM G-CSF, d: HUVECs incubated with 25 mM G-CSF, e: HUVECs incubated with 6,25 mM G-CSF, f: HUVECs incubated with 1,62 mM G-CSF).

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To investigate the *in vitro* biological activity of rhG-CSF, HUVECs cells were incubated with this therapeutic protein. For this purpose, MTT assay was performed and cell viabilities were calculated to determine effective concentration for cell proliferation. As a result, HUVECs which were incubated with rhG-CSF at 1.62 mM concentration for 48 hours, demonstrated the highest cell viability against negative control (Figure 7). Additionally, the EC50 value of the recombinantly produced rhG-CSF protein was calculated as 0.051 mM.

3.4. Cell migration assay

We examined the migration ability of HUVECs to determine the effect of rhG-CSF treatment on the angiogenesis potential of endothelial cells *in vitro*. For this purpose, *in vitro* scratch assay was carried out with HUVEC cells at four different rhG-CSF concentrations (100-1.62 mM) (Figure 8).

It was clearly observed that following 24 hours of incubation of the rhG-CSF with HUVEC cells, the percent wound healings was calculated as 73.9 %, 86.09 %, 82.07 %, 87.94 % respectively (Figure 4). When this data was compared with untreated control (77.2 %), it was concluded that the migration and wound closure ability of the HUVECs was promoted by rhG-CSF at 24 h in a concentration-dependent manner (25 mM, 6.25 mM, 1.62 mM) and peaked at 1.62 mM (Figure 9).

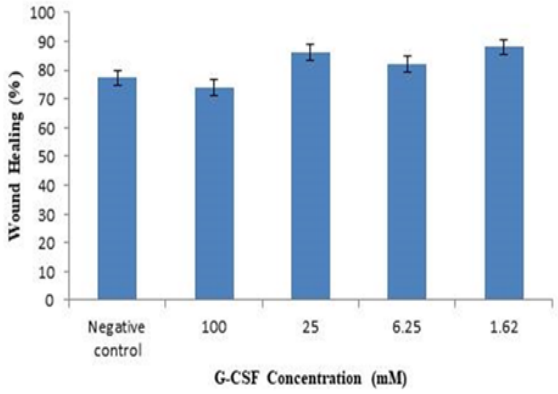

Figure 9. The percent wound healings for HUVECs.

4. CONCLUSIONS

In this study, we have constructed the pTOLT-G-CSF that permits the production of *E. coli* Tol-A-III domain of Tol-A protein and mature peptide of hG-CSF fusion under control of the T7 promoter system. The construct was prepared with the addition of 6xHis-tag which was used for the purification of this fusion protein by a single affinity chromatography step. Recombinant hG-CSF was overexpressed as inclusion body in *E.coli* Bl21pLysE expression host using this construct. Inclusion body formation should not always be considered a disadvantage. Sometimes it can provide a good alternative to successful purification, especially when high purity protein cannot be obtained due to host contaminants. In this case, however, the solubilization and refolding steps should be revised to high recovery of bioactive recombinant protein from the inclusion bodies. Although rhG-CSF production in the present study was at the sufficient level as amount, refolded active form of protein was low. This problem can be solved optimizing refolding condition for better yield. Additionally, the hG-CSF can be separated from the Tol-A-III protein fusion and 6xHis-tag for higher activity, nevertheless this additional process increases the cost of protein production and causes a loss in the amount of protein. In the pharmaceutical industry, the production of bioproducts requires a simple and cost-effective process that includes easier steps with high throughput.

In the early 1990s, *in vitro* studies had demonstrated that rhG-CSF (0.1 µg ml^{-1}) had direct stimulating effects on HUVECs showing a positive effect on the cell viability and motility. Additionally, recent studies had further shown that by local administration of lower doses, rhG-CSF may provide a beneficial environmental conditions for angiogenesis. However, in another *in vitro* study, controversial results were reported. According to these findings, rhG-CSF induced apoptosis and inhibitied cell proliferation when administrated systematically at a relatively higher doses.[15] In the present study, it was observed that the rhG-CSF demonstrated positive effect on proliferation and migration of HUVECs in a concentration dependent manner.[15,33] Therefore, the results we obtained in our study were consistent with the previous findings.

In conclusion, it was observed that when rhG-CSF administrated at true doses (25 mM, 6.25 mM, 1.62 mM) in HUVECs. rhG-CSF stimulated the cell migration through extracellular matrix, which was a crucial process during angiogenesis. Nevertheless, it should be clear that further analysis is needed to assess the angiogenesis mechanism on the basis of rhG-CSF.

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Conflict of interests

Authors declare that there is no a conflict of interest with any person, institute, company, etc.

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