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Araştırma Makalesi / Research Article

Soluble Expression of Human Granulocyte Colony Stimulating Factor (hG-CSF) in *Escherichia coli* Expression System

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Abstract

Human granulocyte colony stimulating factor (hG-CSF) is a hematological growth factor that plays a crucial role in neutrophil production and differentiation. Some foreign biomolecules, especially of human origin, such as hG-CSF, sometimes aggregate because of different factors during expression and create inclusion bodies in *Escherichia coli* (*E. coli*). Refolding process is commonly used to recover these very valuable molecules, but still significant amounts of protein remain unusable. Refolding procedures are frequently costly, time-consuming, and not fully efficient. Therefore, the use of molecular chaperones to improve soluble expression of proteins was evaluated in the study. In this context, hG-CSF was co-expressed with five chaperone plasmid systems (pGro7, pG-KJE8, pG-Tf2, pKJE7, pTf16) to ensure the expression of hG-CSF in soluble form. Among these, the pKJE7 plasmid was found to be the most effective in obtaining hG-CSF in soluble form, yielding 92% purity after Ni-NTA affinity chromatography purification. The total yield of hG-CSF obtained was 1.6 mg per 1 L bacterial culture. The biological activity of the soluble hG-CSF was evaluated in human umbilical vein endothelial cells (HUVECs). A 24-hour interaction of hG-CSF with HUVECs resulted in a significant increase in cell viability at all applied doses, demonstrating its bioactivity. As a result, hG-CSF, which previously aggregated as an inclusion body in the *E. coli* expression system, was correctly folded by co-expression with chaperone proteins were obtained as more efficient and purer.

Keywords: Granulocyte colony stimulating factor, Recombinant proteins, Molecular chaperones, *E. coli.*

Escherichia coli İfade Sisteminde İnsan Granülosit Koloni Uyarıcı Faktörün (hG-CSF) Çözünür İfadesi

Öz

İnsan granülosit koloni uyarıcı faktör (hG-CSF), nötrofil üretimi ve farklılaşmasında çok önemli bir rol oynayan hematolojik bir büyüme faktörüdür. hG-CSF gibi özellikle insan kökenli bazı yabancı biyomoleküller bazen ekspresyon sırasında farklı faktörler nedeniyle bir araya gelir ve *Escherichia coli*'de (*E. coli*) inklüzyon cisimcikleri oluşturur. Bu çok değerli molekülleri geri kazanmak için yeniden katlama işlemi yaygın olarak kullanılır, ancak yine de önemli miktarda protein kullanılamaz durumda kalır. Yeniden katlama prosedürleri sıklıkla maliyetlidir, zaman alıcıdır ve tam olarak verimli değildir. Bu nedenle çalışmada proteinlerin çözünür ifadesini geliştirmek için moleküler şaperonların kullanımı değerlendirildi. Bu bağlamda, hG-CSF'nin çözünür formda ekspresyonunu sağlamak için hG-CSF, beş şaperon plazmit sistemi (pGro7, pG-KJE8, pG-Tf2, pKJE7, pTf16) ile birlikte eksprese edildi. Yapılan deneyler sonucunda, pKJE7 plazmitinin hG-CSF'nin çözünür formda elde edilmesinde en etkili sistem olduğu belirlenmiş ve Ni-NTA afinite kromatografisi ile %92 saflıkta saflaştırılmıştır. Elde edilen hG-CSF'nin toplam verimi, 1 L'lik bakteri kültüründen 1,6 mg olarak hesaplanmıştır. Çözünür formdaki hG-CSF'nin biyolojik aktivitesi, insan göbek kordonu endotel hücreleri (HUVECs) üzerinde değerlendirilmiş ve 24 saatlik etkileşim sonucunda, tüm uygulanan dozlarda hücre canlılığında anlamlı bir artış sağladığı gözlemlenmiştir. Sonuç olarak daha önce *E. coli* ekspresyon sisteminde inklüzyon cisimciği olarak agregasyona uğrayan hG-CSF'nin şaperon proteinleri ile ekspresyonu yapılarak doğru bir şekilde katlanmasıyla daha verimli ve daha saf bir şekilde elde edildi.

Anahtar Kelimeler: Granülosit koloni uyarıcı faktör, Rekombinant proteinler, Moleküler şaperonlar, *E. coli*.

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1. Introduction

Human granulocyte colony stimulating factor (hG-CSF) is an approximately 25 kDa glycoprotein. hG-CSF regulates the differentiation, proliferation, and activation of hematopoietic cells (Basu, Dunn, & Ward, 2002; Roberts, 2005). hG-CSF is a protein especially for the function of neutrophils and neutrophil type immune system. Neutrophil formation can be suppressed by drugs used in cancer treatment and neutropenia occurs. In this case, it may cause serious infections in cancer patients. hG-CSF is widely used in the clinic to support the immune system in people suffering from diseases such as febrile neutropenia, pneumonia, leukemia, diabetic foot infections and HIV and cancer patients receiving chemotherapy (Cerchione, Nappi, & Martinelli, 2021; Coward, Nathavitharana, & Popat, 2012; Nomdedeu et al., 2015; Von Aulock & Hartung, 2002). Lenograstim, Filgrastim and Pegfilgrastim are the best-known forms of hG-CSF. Lenograstim is produced in mammalian cells and is the glycosylated form of hG-CSF. Filgrastim is produced in *E. coli* and is the non-glycosylated form of hG-CSF. Pegfilgrastim is the PEGylated form of hG-CSF that is not in glycolyzed form (Welte, 2014).

The cost-effective production of hG-CSF will benefit the healthcare system and the patient due to the wide range of uses of this protein. *E. coli* is a frequently used host for recombinant protein production due to its advantages such as cheap and fast protein production (Kaplan et al., 2024). However, in *E. coli*, recombinant protein can be produced as insoluble and non-functional aggregates called inclusion bodies (Rosano & Ceccarelli, 2014). In previous studies, the production of hG-CSF protein in *E. coli* resulted in the formation of inclusion bodies. Some additional protein denaturation and refolding steps are required to obtain the bioactive form of hG-CSF from inclusion bodies (V. Babaeipour, Khanchezar, Mofid, & Pesaran Hagi Abbas, 2015; Kim, Lee, Lee, & Oh, 2013; D. V. Rao, M. L. Narasu, & A. K. Rao, 2008; Tiwari et al., 2012; S. Vemula et al., 2015). In addition to these studies, Sima et al. (2020) produced the hG-CSF protein as a fusion with intein in *E. coli* and was able to purify the protein in one step (Sima, Shafiee, & Jahanian-Najafabadi, 2020). Trinh et al (2021) described a method for the production of hG-CSF as non-classical inclusion bodies *in E. coli* (Trinh, Thuoc, & Thao, 2021). It is generally more advantageous to utilize methods that enhance the expression of soluble proteins rather than attempting to refold proteins aggregated in inclusion bodies (Khosrowabadi, Takalloo, Sajedi, & Khajeh, 2018). Co-expression of chaperone proteins with target proteins in *E. coli* can ensure correct folding of the protein and reduce inclusion body formation (Sachsenhauser & Bardwell, 2018). Chaperone proteins include DnaJ, DnaK, GroEL, GrpE, GroES, and trigger factors are widely utilized in *E. coli* (İncir & Kaplan, 2024). The most effective chaperone combinations, GroEL-GroES and DnaK-DnaJ-GrpE, have been shown to accomplish protein

refolding (Jhamb & Sahoo, 2012; Voulgaridou, Mantso, Chlichlia, Panayiotidis, & Pappa, 2013; Yan, Hu, Guan, & Yao, 2012).

In this study, hG-CSF protein was expressed with chaperone proteins to obtain it in soluble form in *E. coli*. The efficiency of five different plasmids encoding chaperone proteins (pGro7, pG-KJE8, pG-Tf2, pKJE7, and pTf16) in the production of hG-CSF protein in soluble form was compared. hG-CSF protein co-expressed with chaperones in *E. coli* was purified by Ni-NTA affinity chromatography and its *in vitro* stimulatory effect on HUVEC was evaluated.

2. Materials and Methods

2.1. Co-expression of hG-CSF Protein with Chaperone Proteins

Co-expression of hG-CSF protein with chaperone proteins was performed as detailed in our previous study (Kaplan, İmamoğlu, & Gökçe, 2021). Briefly, plasmids encoding chaperone proteins (pGro7, pG-KJE8, pG-Tf2, pKJE7, and pTf16) were transferred to *E. coli* BL21 (DE3) cells via heat shock. Cells were grown in Luria-Bertani (LB) medium containing 20µg/mL chloramphenicol. Single colony was taken and grown in LB broth containing 20µg/mL chloramphenicol at 240 rpm 37ºC. Then, the pTOLT-G-CSF plasmid that we cloned in our previous study was transferred to these cells separately via heat shock (Bozkurt, Bilgin, Erden, Turan, & Gökçe, 2019). Cells were spread on LB agar medium containing 20µg/mL chloramphenicol and 100µg/mL ampicillin. A single colony was taken from *E. coli BL21 (DE3)* cells containing chaperone plasmid and pTOLT-G-CSF plasmid and grown overnight in LB medium containing 20µg/mL chloramphenicol and 100µg/mL ampicillin at 37°C and 240 rpm.

2.2. hG-CSF expression analysis and quantification

Culture was inoculated overnight culture in LB medium containing 20 µg/mL chloramphenicol and 100 µg/mL ampicillin. The addition of L-arabinose (0.5 mg/mL) and/or Tetracycline (5 ng/mL) stimulated chaperone protein production. When OD_{600} reached 0.6, 1M IPTG was added to promote the production of hG-CSF protein, and the bacterial culture was cultured for 3 hours at 37°C and 240 rpm. Cells were harvested by centrifugation at 8,000 rpm for 5 minutes at room temperature and resuspended in a lysis buffer ontaining 100 mM NaH_2PO_4-Na_2HPO_4 (pH 8.0), 100 mM NaCl, 100 mM phenylmethylsulfonyl fluoride (PMSF), and 100 mM benzamidine. Lysis was performed on ice using an ultrasonicator (9 cycles, 100% power, Bandelin Sonopuls HD 2070) for 30 minutes.

Following lysis, the supernatant was collected by centrifugation at 12,000 rpm for 15 minutes at 4°C and analyzed using 12% SDS-PAGE (Kaplan et al., 2021).

2.3. Purification of hG-CSF

Protein purification was performed as the protocol detailed in our previous work (Eczacioglu, Ulusu, Gokce, & Lakey, 2022; Kaplan, İmamoğlu, & Gökçe, 2022). Cells were suspended in a lysis solution and sonicated for 60 minutes on ice. The cell lysate was centrifuged (12.000 rpm, 15 minutes). The supernatant was loaded onto a column that contained Ni-NTA agarose resin. The protein was purified using an elution solution containing 300 mM imidazole. Purified hG-CSF protein was analyzed on 12% SDS-PAGE and dialyzed against 1x Phosphate Buffered Saline (PBS) (pH 7.4).

2.4. Investigation of hG-CSF biological activity on HUVEC cell line

The *in vitro* stimulatory effect of hG-CSF on HUVEC was examined using the MTT assay as detailed previously (Bozkurt et al., 2019). Briefly, HUVEC cells seeded in 96-well plates at a concentration of 5×10^4 were exposed to hG-CSF at a concentration range of 15, 31, 62, 125 and 250 ng/mL for 24 h. After incubation, 100 µL of MTT reagent was added to each well and incubated for 4 h. Formazan crystals were dissolved with dimethyl sulfoxide (DMSO) and their absorbance was read at a wavelength of 570 nm. % cell viabilities were calculated compared to the absorbance of control cells not treated with hG-CSF.

3. Findings and Discussion

The majority of hG-CSF expression occurs in inclusion bodies in *E. coli*, and in order to produce its bioactive form, other processes such as protein denaturation and refolding are required (Trinh et al., 2021). Although *E. coli* is widely used in the production of recombinant proteins, it has disadvantages such as protein misfolding and the formation of disordered aggregated proteins as inclusion bodies (Şenol, Kaplan, İmamoğlu, & Gökçe, 2021). Previous studies have demonstrated that co-expressing the target protein with molecular chaperones to reduce inclusion body formation during expression of the recombinant protein increases protein solubility. Chaperone proteins facilitate the proper folding process of the newly synthesized polypeptide and also enable the unwinding and refolding of misfolded proteins (Saibil, 2013). The restricted ability of chaperones in prokaryotic host cells may be the cause of the aggregation of overexpressed heterologous proteins (Francis & Page, 2010). According to some research, using molecular chaperones comprising various

sets of plasmids, such as proteins pGro7, pG-KJE8, pG-Tf2, pKJE7, and pTf16, might increase the soluble synthesis of recombinant proteins (Nazari et al., 2020). To ensure hG-CSF protein expression in soluble form, co-expression was performed with the proteins pGro7, pG-KJE8, pG-Tf2, pKJE7, and pTf16 chaperone plasmid set to realize co-expression with chaperone proteins. **Figure 1** shows the co-expression of hG-CSF protein with chaperone proteins. It was found that co-expression with the pKJE-7 chaperone plasmid, which expresses dnaK (70 kDa), dnaJ (40 kDa), and grpE (22 kDa) proteins from the chaperone protein systems, was much more efficient in producing the hG-CSF protein in soluble form. The heat shock protein family, which includes dnaK, dnaJ, and grpE, is involved in a variety of cellular processes, including translocation across cellular compartments, stability and folding of newly synthesized polypeptide, and aggregate disintegration and unfolding (Mayer & Bukau, 2005).

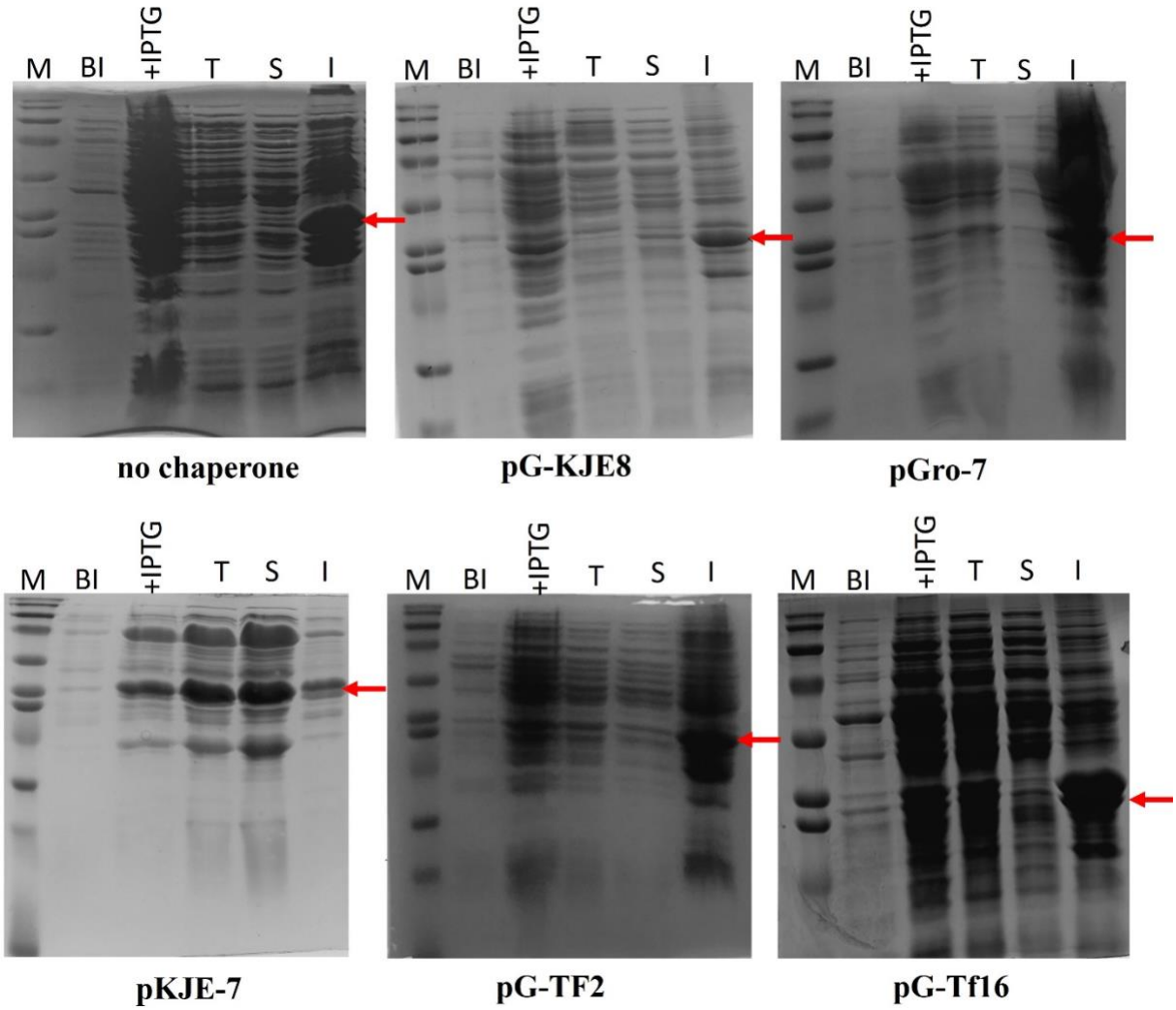

Figure 1. SDS-PAGE analysis of co-expression of hG-CSF protein with the pG-KJE8, pKJE7, pGro7, pTf16 and pG-Tf2 and chaperone plasmids. **BI**: *E. coli BL21 (DE3)* cells before inducing with IPTG, **+IPTG**: *E. coli BL21 (DE3)* cells after inducing with IPTG. **T**, **I** and **S** represent total, insoluble and soluble fractions, respectively.

hG-CSF protein co-expressed with the pKJE-7 chaperone plasmid system was successfully purified using nickel affinity chromatography. SDS-PAGE analysis revealed that hG-CSF protein of approximately 32 kDa was purified to 92% purity, as expected (**Figure 2**). As a result, 1.6 mg hG-CSF protein was obtained from 1L bacterial culture.

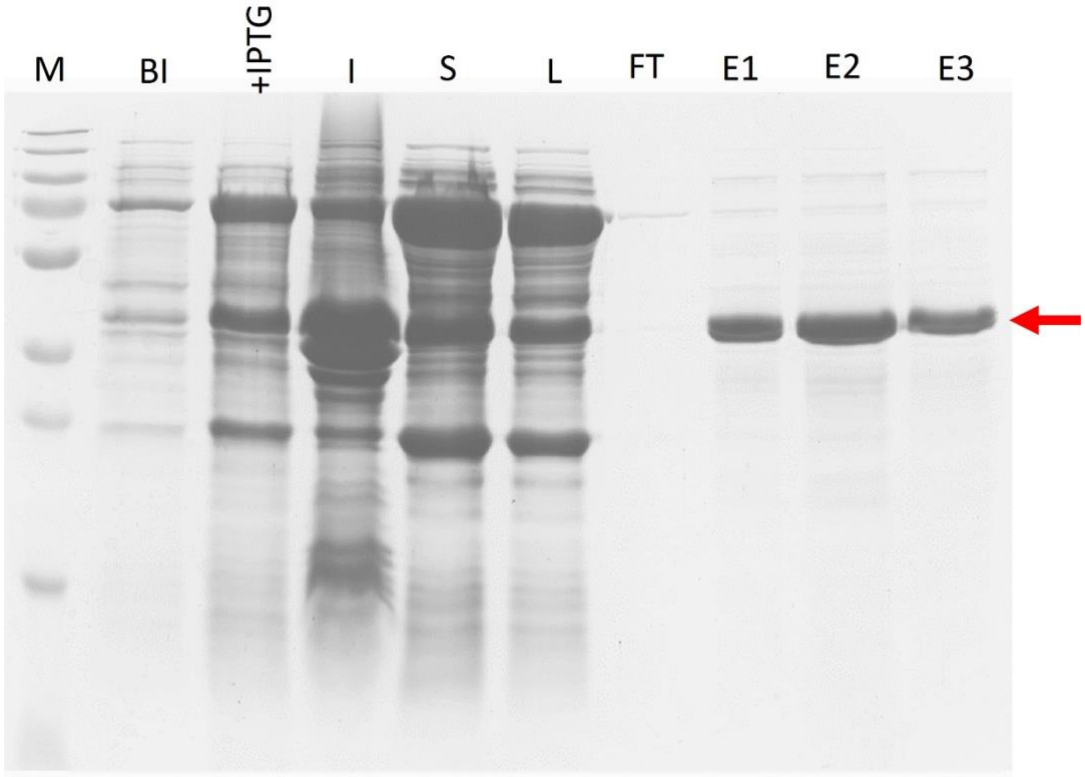

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Figure 2. Analysis of the hG-CSF protein expressed with the pKJE-7 chaperone plasmid system on a 12% SDS-PAGE gel. **M**: protein marker, **BI**: before induction, **+IPTG**: *E. coli BL21 (DE3)* cells after inducing with IPTG. **I**: insoluble fraction **S**: supernatant, **L**: Load, **FT**: Flow-through, **E1**: Elution 1, **E2**: Elution 2, **E3**: Elution 3

hG-CSF contains a cysteine residue at position 17 and forms two disulfide bonds: one between Cys(36) and Cys(42), and another between Cys(64) and Cys(74). The process of refolding is rather complex and requires the addition of suitable redox reagents to facilitate the synthesis of the proper disulfide bonds (Kubota et al., 1990). Numerous research is continuously conducted to improve the production process to lower the cost of hG-CSF products (Valiollah Babaeipour, Khanchezar, Mofid, & Abbas, 2015; Bozkurt et al., 2019; D. V. K. Rao, M. L. Narasu, & A. K. S. B. Rao, 2008; Sandeep Vemula et al., 2015). Vemula et al. revealed a downstream process involving purification using extradimensional chromatography on the production of hG-CSF. Their method involved dissolving inclusion bodies in an 8 M urea solution, followed by refolding the protein in a buffer containing the L-cysteine hydrochloride redox system specific to hG-CSF. They were able to produce active hG-CSF with a purity of over 95% and an 86% maximum refolding efficiency. The production of hG-CSF from inclusion bodies often has the drawbacks of being labor-intensive, expensive, and requiring

a lot of detergent to dissolve the inclusion bodies (Sandeep Vemula et al., 2015). The literature has assessed a variety of strategies that increase soluble expression of hG-CSF. A few of them propose to use a fusion partner to produce protein in soluble form. They produced hG-CSF in soluble form in the cytoplasm of *E. coli* by labeling its C terminus with IgG1 Fc and its N terminus with maltose binding protein (MBP) (Do et al., 2017). Sima et al. used self-cleavable intein to perform a one-step purification process that produced physiologically active recombinant hG-CSF protein (Sima et al., 2020).

Studies performed *in vitro* demonstrated that hG-CSF positively impacted cell survival and motility in HUVECs and exhibited direct stimulatory effects. Bozkurt et al. revealed that rhG-CSF positively impacted HUVEC migration and proliferation in a concentration-dependent manner. Applying different dosages of rhG-CSF to HUVECs induced cell migration through the extracellular matrix, an important step in the angiogenesis process (Bozkurt et al., 2019). Liu et al. also showed that G-CSF affects endothelial cell behavior. In this study, G-CSF was shown to increase the viability and angiogenic ability of injured liver cells, which in turn promoted the proliferation and tubule formation of HUVECs (Liu et al., 2022). In addition, in another study by Mei et al., G-CSF was reported to stimulate migration and tube formation in HUVECs, indicating its direct effect on endothelial cells (Mei et al., 2023). These findings highlight the importance of using HUVECs to assess the biological activity of hG-CSF, as they provide a convenient model to assess its effects on endothelial cell functions such as proliferation, migration, and angiogenesis. In this study, the biological activity of hG-CSF protein was investigated on HUVEC cells. The 24-hour interaction of different concentrations of hG-CSF with HUVEC cells was evaluated in terms of % viability and proliferation results. It was revealed that recombinantly produced hG-CSF protein in soluble form increased HUVEC cell viability at all applied doses (**Figure 3**).

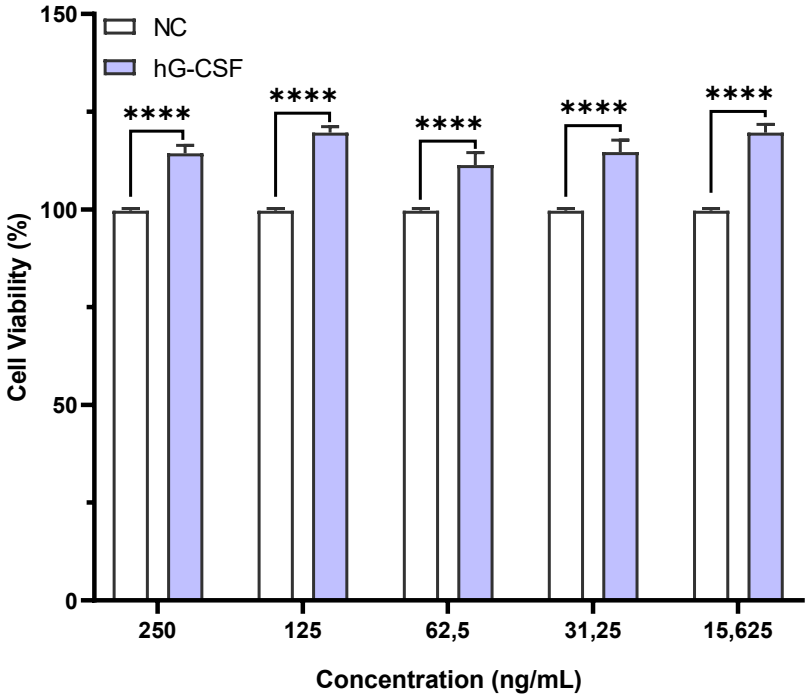

Figure 3. Analysis of hG-CSF on HUVEC cell viability and comparison against negative control (NC)

4. Conclusions and Recommendations

In this study, co-expression of hG-CSF protein with chaperone proteins was performed to produce it in soluble form. We compared the efficacy of different chaperone plasmids on hG-CSF. Co-expression with the pKJE-7 chaperone plasmid expressing dnaK (70 kDa), dnaJ (40 kDa) and grpE (22kDa) proteins was effective in producing hG-CSF in soluble form. It was revealed that hG-CSF protein, obtained in soluble form with approximately 92% purity, increased the proliferation of HUVEC cells. Our findings reveal that the co-expression strategy with chaperone proteins to produce hG-CSF protein in a soluble form in *E. coli* cheaply, quickly and at low cost. Future studies require optimizing the expression conditions such as induction temperature and inducer concentration to further improve the soluble production of hG-CSF in this proposed system. In addition, alternative chaperone combinations and alternative expression strains can be investigated to increase folding efficiency. Furthermore, structural analysis of recombinant hG-CSF, including disulfide bond formation and tertiary structure verification, may reveal more comprehensive analyses of the folding mechanism. Moreover, in this study, the biological activity of recombinant hG-CSF was investigated in HUVEC cells, but testing its biological activity in other relevant cell models or *in vivo* systems may be quite useful to determine its therapeutic potential. Scaling up the production process and developing and optimizing purification strategies should also be important future studies for industrial applications.

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Authors' Contributions

MS: Methodology, Investigation, Writing – review & editing **ÖK**: Methodology, Investigation, Formal Analysis, Writing – review & editing **Rİ**: Methodology, Investigation **İG**: Supervision, Conceptualization, Funding, Writing – review & editing.

Statement of Conflicts of Interest

The authors confirm that this article content has no conflicts of interest.

Statement of Research and Publication Ethics

The authors declare that this study complies with Research and Publication Ethics.

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