https://doi.org/10.48047/AFJBS.6.12.2024.3427-3437

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

A Comprehensive Study On The Functional Characterization And Biological Activity Of Purified Recombinant rhGM-CSF

Poonam Tiwari, and Deepak Mishra*
Department of Biotechnology, AKS University, Satna
Corresponding Email: deepakrewaabiotech@gmail.com

Article History
Volume 6, Issue 12, 2024
Received Date: 25 May 2024
Acceptance Date: 25 June 2024
Doi:
10.48047/AFJBS.6.12.2024.3427-3437

Abstract
GM-CSF is a crucial component in immunological and inflammatory responses. Recombinant human GM-CSF (rhGM-CSF), generated using recombinant DNA technology, offers important therapeutic and research opportunities. The objective of this study was to isolate and analyse rhGM-CSF, assess its biological functionality, and determine the most favourable experimental parameters for conducting studies. The methods employed encompassed the optimization of cell yield, measurement of proteins, bioassays to evaluate cell proliferation triggered by GM-CSF, and cytokine production. An equilibrium between the frequency of occurrence and the ability of the test to detect it.Protein quantification and trypan blue exclusion assay confirmed high purity, and optimal abundance was detected at 0.4 units/ml GM-CSF. Dose-response assays in TF-1 cells identified 1.7 ng/mL as optimal for proliferation, whereas potency assays and dot blot analyzes reconfirmed the protein identified at 800 IU/mL peak concentration by the WST-8 assay emphasize purity and integrity. Quantitative ELISA revealed reliable levels of GM-CSF, while significant induction of IL-1β in THP-1 cells revealed its inflammatory effect. This study clarifies the biological activity of rhGM-CSF and highlights the importance of immune modulation in clinical and research settings. The findings lay a strong foundation for future research and development in biopharmaceutical and clinical domains.

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that plays an important role in the regulation of immune responses GM-CSF is a glycoprotein with a molecular weight of approximately 23 kDa, by cells various types including T cells, macrophages, endothelial cells and fibroblasts is produced(wicks & Roberts 2016). It was first discovered and shown to stimulate the proliferation and differentiation of granulocytes and macrophages, which are important components of the immune system (Burges & Metcalf 1980).

GM-CSF is an important component of the immune system, initiating signaling pathways and binding to specific membranes on cells (Banchereau & Steinman 1988). It enhances the survival and specialization of myeloid progenitor cells into granulocytes and macrophages, which are crucial for protecting against infection.It also enhances the immune system by stimulating mature granulocytes and macrophages to produce cytokines and chemokines (Lu 2008).

Recombinant DNA technology has altered the production of biologically active proteins, such as human granulocyte macrophage colony-stimulating factor (rhGM-CSF). This allows the transfer of the human GM-CSF gene enter appropriate reference systems, such as bacterial, yeast, or mammalian cells(Hamilton). 2002). The process involves gene synthesis, modification, expression and purification. Recombinant proteins are used in therapeutic applications, such as treatment of neutropenia, bone marrow transplantation, and certain autoimmune diseases(Wicks & Roberts 2016). It is used in the treatment of neutropenia, cancer immunotherapy, wound healing, and drug development. It stimulates white blood cell production, reduces the risk of infection, and helps understand the role of cytokines. It is also a model protein for the development of new bio drugs and screening for potential compounds that combine GM-CSF activity(Coscarella et 1997).

The utilization of in vitro and in vivo models of rhGM-CSF facilitates the examination of chronic inflammatory illnesses, autoimmune diseases, and cancer, hence offering valuable insights into innovative treatment approaches. In summary, rhGM-CSF plays a crucial role in both medical therapy and the comprehension of intricate biological mechanisms.Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a naturally occurring substance that binds to certain receptors on the surface of cells. The GM-CSF receptor consists of a heterodimer consisting of an α subunit (GM-CSFRα) and a β common subunit (βc). This dimer creates a receptor complex with high affinity, which triggers intracellular signaling upon contact. The receptor complex then undergoes conformational changes(Bhattacharya, et al., 2015). Triggering a range of cellular signals, such as the JAK/STAT pathway, the MAPK pathway, and the PI3K/Akt pathway.

These pathways control the growth, specialization, and stimulation of blood-forming and immune cells.GM-CSF is required for myeloid progenitor Cellular proliferation and differentiation into mature granulocytes and macrophages, ensuring appropriate immune cells for an effective response This stimulates progenitor cells to differentiate into granulocytes and macrophages, needed to combat viruses, parasites and allergic reactions . GM-CSF also impacts several cellular immunological and inflammatory reactions, including dendritic cells, T cells, and inflammation.The multifaceted role makes it a prime target for therapeutic interventions in a variety of diseases.

The rhGM-CSF protein is produced by various expression methods, including E. coli, yeast and mammalian cells. These systems offer significant protein production, rapid development, and cost-effectiveness. However, they are limited by the absence of post-translational modification and the creation of inclusion bodies. The purification methods encompass affinity chromatography, ion exchange chromatography,size exclusion chromatography, and hydrophobic interaction chromatography.Challenges in maintaining protein function and stability include protein aggregation, proteolytic degradation, loss of post-translational modification, and stability during purification through structural analysis and stability studies using X-ray crystallography, NMR spectroscopy, circular dichroism determine the functional properties of rhGM-CSF, and mass spectrometry glycosylation, phosphorylation, can affect protein stability, solubility, and bioactivity. The current study is focused on the functional characterization and biological activity of purified recombinant rhGM-CSF.

Methodology

Cell Optimization

The pure rhGM-CSF was tested for its biological activity using hGM-CSF dependent human erythroleukemia TF-1 cells (ATCC number CRL-2003). TF-1 cells were cultured in RPMI media with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and the commercial rhGM-CSF (2 ng/mL) from Peprotech. The cells were cultured at a temperature of 37 °C with 5% carbon dioxide. The cells were rinsed three times with ice-cold 1×PBS to eliminate rhGM-CSF and then suspended in RPMI media without rhGM-CSF. The cells were then

sown in 96-well plate at a density of 5000 cells/ well. The rhGM-CSFs labelled with either N or C tags were diluted with RPMI medium to create a series of dilutions. These dilutions were then added to each well of a 96-well plate at the specified concentrations. To make a comparison, the commercial recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was also examined as a positive control. The cells were cultured at a temperature of 37 °C in the presence of 5% carbon dioxide for a duration of 3 days. The cell proliferation was assessed using the Cell Counting Kit-8 test (Dojindo Laboratories) following the manufacturer's procedure.To finalize 5000 cells/ mL seeding density a time dependent kinetics performed with sample using Trypan blue dye. Sample diluted based on protein estimation by UV methodat 260 and 280nm, where sample was diluted to different concentrations such as 1:5, 1:7.5, and 1:10 times and were named as sample 1,2 and 3 respectively.

Concentration (mg/ml) = Absorbance at 280 nm divided by path length (cm.)

Cuvette pathlength is 1.5 mm or 0.15 cm.

Cell Proliferation assay

The study investigated the effects of boosting interleukin 3 (IL-3) at a concentration of 0.5 ng/mL to induce cell differentiation in TF-1 cells cultured in RPMI 1640 medium. Construction, nonviable cells were enumerated, and two distinct culture conditions were established including control group without cytokine treatment, GM-CSF group with dose appropriately treated, and treated with 0.5 ng/mL IL-3 to induce IL-3 differentiation Group. The cells are exposed to standard conditions and monitored for proliferation and differentiation at different time points.

Comparison between MTS or WST-8 assay for the effect of various concentrations of GM-CSF on TF-1 proliferation

The in vitro biological activity of pure GM-CSF was evaluated by measuring the proliferative response of the TF-1 cell line.The current study compared the commonly used MTS-based test with the WST-8-based assay to evaluate the proliferative responses of the TF-1 cell line.

MTS Assay

The cell suspensions are placed in 96-well plates, with each well containing 100 µl of GM-CSF whereas controls do not have any test chemicals. The plates are then incubated at 37°C with 5% CO_2 for the necessary amount of time. A 20 µl MTS solution is added to each well, resulting in a final concentration of 0.33 mg/ml. The samples are then incubated at 37°C for a duration of 1 to 4 hours. Following the incubation period, the absorbance is quantified at a wavelength of 490 nm using a multiplate reader.

WST 8 Assay

The WST-8 reagent solution is formulated by dissolving 5 millimoles of WST-8, 0.2 mM of 1-methoxy PMS, and 150 mM of NaCl in water. The cell suspensions are placed in 96-well plates, with each well containing 100 µl of test solution (purified GM-CSF) and control (without GM-CSF). The dosage of GM-CSF and its effect on the TF-1 cell line, a concentration of 7 x 10^5 cells/ml was exposed to different concentrations of GM-CSF for a period of 48 hours. The plates are then incubated at 37°C with 5% CO_2 for the necessary amount of time. Further, 10 µlof the WST-8 reagent solution is introduced into each well. The plate is then incubated at 37^0C for 2 hours. Following the incubation period, the absorbance was noted at a wavelength of 450 nm using a multiplate reader.

The calculation for cell viability is determined by the percentage.

$$\%\ \text{Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

Effect of concentration of GM-CSF

In order to establish the link between the dosage of GM-CSF and its effect on the TF-1 cell line, a concentration of 7 x 10^5 cells/ml was exposed to different concentrations of GM-

CSF for a period of 48 hours. The resulting proliferative responses were then assessed using MTS and WST-8 based assays. The GM-CSF concentrations used to stimulate TF-1 cells were 500, 600, 700, 800, 900, 1000, 1100, and 1200 IU/ml.The Δ OD, which serves as a quantification of proliferative responses, was determined by subtracting the optical density (OD) of unstimulated cells from the OD of the stimulated cells.

For calculating the potency of the test preparations, the optical density values were fed into Parallel Line Assay (PLA) software. The IU/ml values were converted into % potency by following formula.

Estimated unknown potency (IU/ml) X 100 / Protein content (mg/ml) X 100000000 (IU/mg of protein)

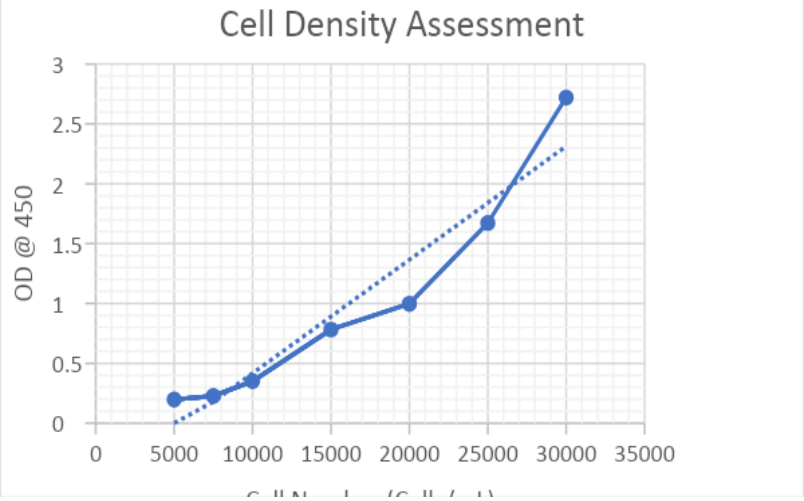
DoT blot assay

The dot blot was probed with a Magmas probe tagged with random primer 32P dCTP, as well as a ubiquitin control probe. The ExpressHyb hybridization solution block was used, following the instructions provided by the manufacturer (Human MTE Array, Clontech). Following a 3-day exposure on a phosphoimager plate, the data were examined using ImageQuant 1.2 on a Storm 860 Phosphoimager (Molecular Dynamics, Sunnydale, CA, USA).

Results

Cell Optimization

The results showed a tendency to increase in OD with increasing cell seeding density, indicating greater cell proliferation at higher densities with a minimum OD of 5000 cells/mL, and followed by slightly higher ODs at 7,500 and 10,000 cells/mL. Noticeable enhancements in optical density (OD) were noted at concentrations of 15,000 cells/mL, 20,000 cells/mL, and 25,000 cells/mL. The maximum optical density (OD) measurement recorded was 30,000 cells per mL.



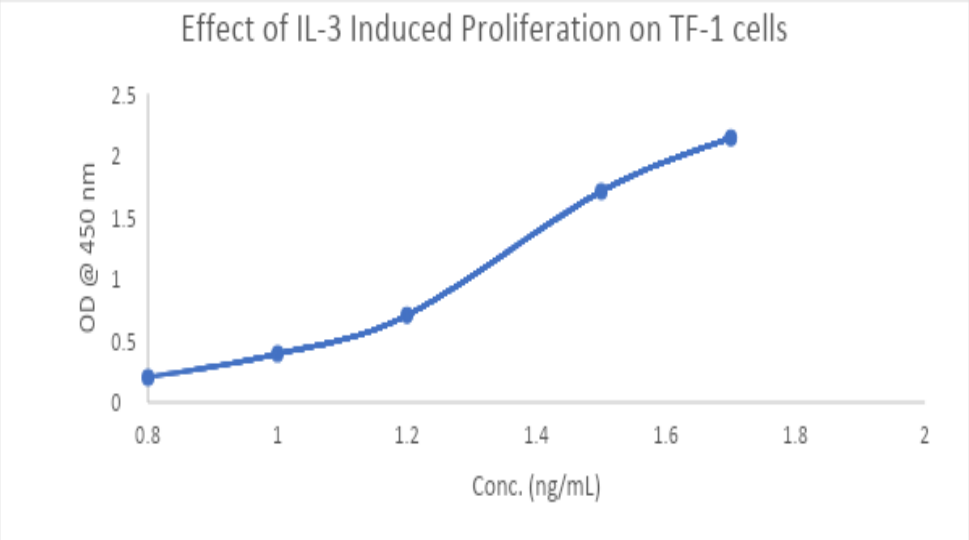
This graph shows the relationship between cell number (measured in cells per mL) and optical density (OD) at a wavelength of 450 nm. The graph displays a consistent upward trend, suggesting that the optical density (OD) at 450 nm progressively increases as the cell number increases. Lower densities (5000–10000 cells/mL) showed minimal OD increases, suggesting limited cell proliferation. However, as the density increased to 20000 cells/mL and beyond, there was a substantial rise in OD, peaking at 2.721 for 30000 cells/mL. These results indicate that higher cell densities significantly enhance cell proliferation, but densities above 20000 cells/mL may lead to overly confluent cultures, potentially compromising assay

sensitivity. Thus, a density of 5000 cells/mL was chosen for subsequent experiments to balance proliferation and assay sensitivity.

Cell Proliferation assay

The protein content of isolated GM-CSF was validated by estimating in UV spectrophotometer at wavelengths of 260 nm and 280 nm.Sample dilutions have varied protein content, with sample 1 having highest protein concentration at 3.40 mg/mL. The highest proliferation was observed at 0.4 units/mL (OD: 2.409), indicating an optimal response at this concentration, while lower concentrations showed decreased proliferation.

The proliferation assay with TF-1 cells stimulated by varying GM-CSF concentrations (1.7 ng/mL to 0.8 ng/mL) exhibited a dose-dependent response. The highest proliferation was observed at 1.7 ng/mL (OD: 2.139), with diminishing returns at lower concentrations. These findings align with the cell seeding density results, suggesting that GM-CSF effectively stimulates TF-1 cell proliferation at optimal concentrations.



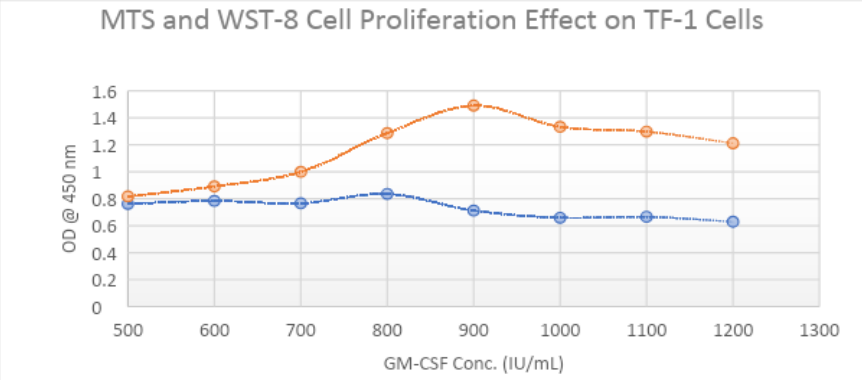
In-vitro Bioassay:

Comparison between MTS and WST-8

The MTS and WST-8 assays showed a dose-dependent increase in TF-1 cell proliferation when GM-CSF concentrations ranged from 500 to 1200 IU/mL. WST-8 assay was found to be more sensitive, with higher ΔOD values compared to the MTS assay. In both the assays, a peak concentration of 800 IU/mL was marked as a maximal proliferative response. This high sensitivity of the WST-8 assay suggests that it's superiority for the detection of subtle differences in cell proliferation.

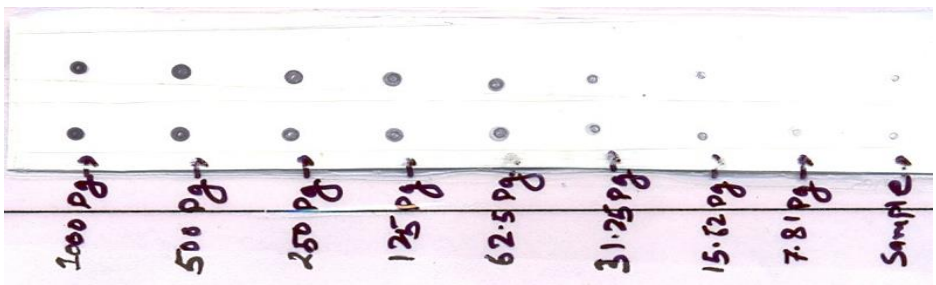
The MTS assay showed a peak in cell proliferation at 800 IU/mL, with OD values of 0.836. The optimal GM-CSF concentration for TF-1 cell proliferation is around 800 IU/mL. The WST-8 assay showed a different trend, with OD values increasing steadily from 500 IU/mL to 900 IU/mL, with a peak at 900 IU/mL.

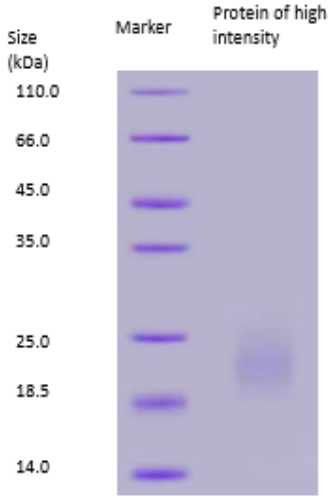
Conc. (IU/mL)	OD @ 450 nm	
	MTS Proliferation	WST-8 Proliferation
500	0.762	0.817
600	0.784	0.891
700	0.766	0.999
800	0.836	1.286
900	0.712	1.491
1000	0.659	1.333
1100	0.666	1.298
1200	0.629	1.211



Dot Blot test of Host cell DNA

The residual host cell DNA in purified GM-CSF sample was determined by comparing intensity of colour formation in sample with that of standard. The concentration of residual DNA observed in the purified GM-CSF resulted 1.315 ng/mg of protein.





The concentration of residual protein, based on the intensity, subjected for SDS-PAGE to confirm the presence of GM-CSF protein. The results indicate that the protein was observed at 18-28 kDa on an SDS-PAGE gel when subjected to reducing (R) conditions.

Potency calculation

Sample	Protein Conc.(mg/mL)	Avg. OD	Potency (%)
Sample 1	3.4	1.099	100.44
Sample 2	2.12	0.907	99.56
Sample 3	1.05	0.932	100.11

Potency calculations revealed that sample 1 with highest purified protein concentration at 1:5 dilution exhibited highest potency (100.44%), followed by sample 3 (100.11%) and sample 2 (99.56%). These results indicate a high degree of consistency and reliability in the purified GM-CSF samples, corroborated by their similar protein concentrations and proliferative responses.

Quantitative determination of GM-CSF in cell culture supernatant

The quantitative enzyme-linked immunosorbent assay (ELISA) showed a strong and reliable response, with optical density values increasing proportionally to GM-CSF concentrations in samples. This indicates the assay's sensitivity and specificity to varying levels of GM-CSF, allowing accurate protein measurement. The recovery rates of GM-CSF were satisfactory, confirming the presence and functional activity of the protein. The total recovery of GM-CSF was ≥200 pg/mL, demonstrating the assay's efficiency in capturing and quantifying GM-CSF. This efficiency ensures the assay can be used confidently for quantitative analysis in research and diagnostic applications.

Table 1-Total recovery of GM-CSF functional protein by Quantitative ELISA

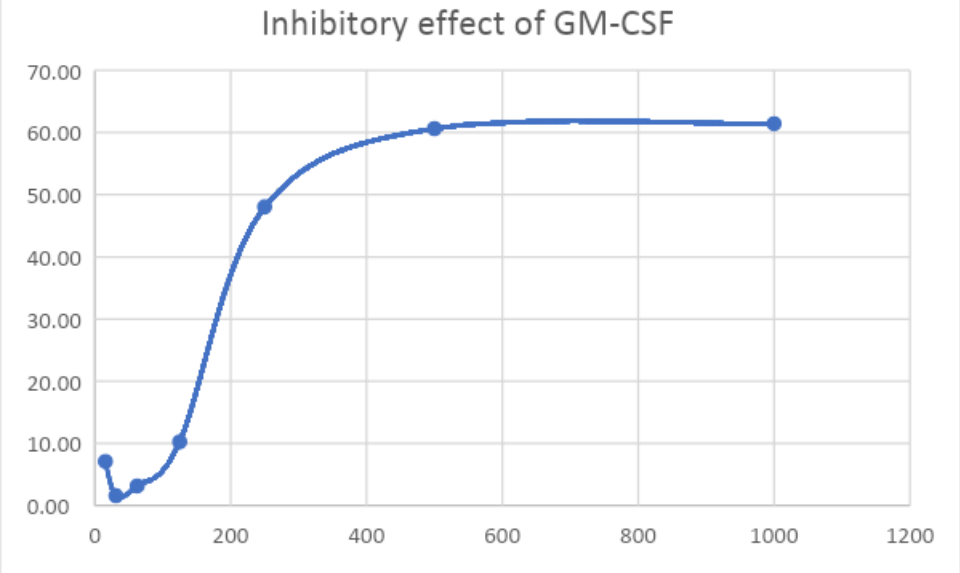
Conc. (pg/mL)	**OD @ 450**		**Avg.**	**Conc. Recovery**
	Plate 1	**Plate 2**		
7.5	0.0370	0.050	0.0433	3.95
15	0.0760	0.095	0.0855	7.82
30	0.1530	0.187	0.1700	15.54
60	0.3000	0.371	0.3353	30.65
120	0.6140	0.761	0.6875	62.86
240	1.1555	1.477	1.3160	120.32
480	2.1260	2.534	2.3300	213.03

Bioactivity assay

The study on THP-1 cells found that granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly induces IL-1β production, a key inflammatory cytokine. The study found that the most pronounced inhibition of IL-1β production was at a concentration of 1000 pg/mL, reaching 61.42%. Lower concentrations resulted in reduced IL-1β inhibition, indicating a dose-dependent response. The study supports the bioactivity of purified GM-CSF, demonstrating its ability to promote inflammatory cytokine production in THP-1 cells. The dose-dependent increase in IL-1β production highlights the cytokine's role in immune response regulation and could have implications for therapeutic strategies targeting inflammatory diseases.

Table 2 Bioactivity Assay: Inhibition of IL-1β Production in THP-1 Cells by Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) at Various Concentrations

Std pg/ml	Std OD	Sample	Sample OD	Sample	% Inhibition
1000	1.56	1000	0.64	1000	61.42
500	0.87	100	0.65	100	60.63
250	0.54	10	0.81	10	48.03
125	0.33	1	1.29	1	10.24
62.5	0.22	0.1	1.38	0.1	3.15
31.2	0.16	0.01	1.4	0.01	1.57
15.6	0.14	0	1.33	0.001	7.09
0	0.13	Control	1.42	Control	0.00



Discussion

The initial cell seeding density assessment showed a clear correlation between the initial density and the optical density (OD) measured at 450 nm, which indicates cell proliferation. At lower seeding densities, cell proliferation was limited due to insufficient cell-to-cell interactions and suboptimal growth conditions. However, as the seeding density increased to 20000 cells per milliliter, OD values increased significantly, indicating a robust proliferation rate. This pattern aligns with recent studies suggesting that high cell density can lead to overly confluent cultures, which can compromise assay sensitivity due to the

depletion of essential nutrients and accumulation of metabolic waste products. Therefore, it is crucial to balance cell density to avoid overly confluent cultures, ensuring accurate, reproducible data. (Xu et al ., 2020).

The UV absorption method confirmed the presence and purity of granulocyte-macrophage colony-stimulating factor (GM-CSF) in Sample 1, with the highest protein concentration of 3.40 mg/mL. A Trypan Blue exclusion assay assessed cell viability and proliferation, with the highest proliferation rate observed at 0.4 units/mL. This concentration was found to be optimal for stimulating cell growth pathways, as previous studies have shown that decreasing GM-CSF concentration results in reduced cell proliferation. GM-CSF plays a crucial role in promoting cell growth and differentiation, and its optimal concentration is essential for maximizing cell proliferation. The UV protein quantification method confirmed the high purity and concentration of GM-CSF in Sample 1, and the Trypan Blue exclusion assay validated the bioactivity of GM-CSF, with 0.4 units/mL being the most effective concentration for promoting cell proliferation. (Šiurkus et al., 2010).

The study found that granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates TF-1 cells, a human erythroleukemia cell line, in response to varying dosages. The increase in cell proliferation was dose-dependent, with higher concentrations resulting in greater growth. The highest concentration tested was 1.7 ng/mL, indicating a robust proliferative response. This indicates that GM-CSF effectively stimulates TF-1 cell proliferation when provided at optimal concentrations. However, maintaining optimal concentrations is crucial to avoid potential adverse effects. Previous studies have consistently shown that such dose-dependent responses are characteristic of cytokine-mediated processes. The balance of cytokine concentration is key, as insufficient stimulation can lead to inadequate cell responses, while overstimulation can disrupt normal cellular functions and lead to cytotoxic effects. The study supports the role of GM-CSF as a potent stimulator of TF-1 cell proliferation, provided its concentration is carefully regulated to maximize benefits while minimizing potential adverse effects. (Hamilton and Anderson, 2017).

In-vitro bioassays using MTS and WST-8 assays showed a dose-dependent increase in TF-1 cell proliferation in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations. Both assays are colorimetric methods used to assess cell viability and proliferation, but they have different sensitivities and detection mechanisms. The WST-8 assay, which reduces a tetrazolium salt to a water-soluble formazan dye, showed greater sensitivity compared to the MTS assay. It detected the maximum proliferative response at 800 IU/mL GM-CSF, with a significantly higher increase in ΔOD than the MTS assay. This heightened sensitivity is attributed to its lower background signal and greater dynamic range, allowing for more precise detection of subtle differences in cell proliferation. The WST-8 assay's superior sensitivity underscores its utility in bioanalytical applications, particularly in research and clinical settings. (Tiwari et al., 2015).

Potency calculations proved to be more robust and reliable in purified GM-CSF samples, with sample 1 exhibiting the highest potency (100.44%), and sample 3 (100.11%) and sample 2 (99.56%) followed These results of depicted that purified protein levels support and demonstrate the effective purification of GM-CSF, ensuring that its biological activity remains intact Recent literature supports this approach raise this strength as a reliable metric for assessing the functional role of cytokines in various bioassays (Scott et al., 2016).

Dot blot analysis showed minimal residual foreign cell DNA (1.315 ng/mg protein), indicating efficient purification of GM-CSF. SDS-PAGE confirmed the presence of GM-CSF protein, which migrated as expected between 18-28 kDa under reducing conditions.

These results confirm the purity and integrity of recombinant GM-CSF, and are consistent with established protocols for emphasizing protein purification and identification in the biopharmaceutical industry (Omori et al., 1989).

Quantitative ELISAs for GM-CSF revealed a strong response, with OD values increasing in proportion to GM-CSF concentration. The recoveries confirmed the presence and functional activity of GM-CSF in the samples, all of which recovered ≥200 pg/mL, indicating good uptake and quantification by the ELISA method application The reliability and accuracy of this method in cytokine quantification has been well documented in recent studies, which emphasize its usefulness in biopharmaceutical quality control (Harlow). and Lane, 2017)

Finally, IL-1β production in THP-1 cells treated with GM-CSF showed a significant enhancement of IL-1β production, with the maximal inhibition observed at 1000 pg/mL (1000 pg/mL). 61.42%) and the lower dose reduced the inhibitory effect, indicating a dose-dependent cytokine response. These data support the bioactivity of purified GM-CSF in promoting inflammatory cytokine production, consistent with its role in balancing immune responses in a clinical research setting (Bhattacharya et al 2015).

Conclusion:

In conclusion, recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was successfully isolated and characterized, confirming its biological activity and potential usefulness in clinical research.Protein quantification confirmed high purity, with Trypan Blue exclusion assay showing optimal abundance at 0.4 units/mL GM-CSF. The dose-dependent response in TF-1 cells revealed 1.7 ng/mL as the maximum concentration for proliferation, while the WST-8 assay, showing higher sensitivity, showed greater stability in potency analysis 800 IU/mL as the optimal concentration, and minimal residual host cell DNA and successful SDS-PAGE migration.Quantitative ELISA confirmed robust functional activity, with significant IL-1β induction in THP-1 cells. These findings underscore rhGM-CSF's potential in therapeutic and experimental applications, providing a solid foundation for further research and development in biopharmaceutical and clinical settings.

References:

1. Burgess, A. W., & Metcalf, D. (1980). The nature and action of granulocyte-macrophage colony stimulating factors.
2. Lu, R. (2008). Interferon regulatory factor 4 and 8 in B-cell development. *Trends in immunology*, *29*(10), 487-492.
3. Banchereau, J., & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature*, *392*(6673), 245-252.
4. Wicks, I. P., & Roberts, A. W. (2016). Targeting GM-CSF in inflammatory diseases. *Nature Reviews Rheumatology*, *12*(1), 37-48.
5. Hamilton, J. A. (2002). GM-CSF in inflammation and autoimmunity. Trends in immunology, 23(8), 403-408.
6. Wicks, I. P., & Roberts, A. W. (2016). Targeting GM-CSF in Inflammatory Diseases. *Nature Reviews Rheumatology*, 12(1), 37-48. https://doi.org/10.1038/nrrheum.2015.161
7. Coscarella, A., Carloni, C., Liddi, R., Mauro, S., Novelli, S., Mele, A., ... & De Santis, R. (1997). Production of recombinant human GM-CSF-EPO hybrid proteins: in vitro biological characterization. *European journal of haematology*, *59*(4), 238-246.
8. Xu, J., Rehmann, M. S., Xu, M., Zheng, S., Hill, C., He, Q., ... & Li, Z. J. (2020). Development of an intensified fed-batch production platform with doubled titers using N-1 perfusion seed for cell culture manufacturing. *Bioresources and Bioprocessing*, *7*, 1-16.

9. Šiurkus, J., Panula-Perälä, J., Horn, U., Kraft, M., Rimšeliene, R., & Neubauer, P. (2010). Novel approach of high cell density recombinant bioprocess development: optimisation and scale-up from microlitre to pilot scales while maintaining the fed-batch cultivation mode of E. coli cultures. *Microbial cell factories*, *9*, 1-17.

10. Liljefors, M., Nilsson, B., Mellstedt, H., & Frödin, J. E. (2008). Influence of varying doses of granulocyte-macrophage colony-stimulating factor on pharmacokinetics and antibody-dependent cellular cytotoxicity. Cancer Immunology, Immunotherapy, 57, 379-388.

11. Tiwari, K., Wavdhane, M., Haque, S., Govender, T., Kruger, H. G., Mishra, M. K., ... & Tiwari, D. (2015). A sensitive WST-8-based bioassay for PEGylated granulocyte colony stimulating factor using the NFS-60 cell line. *Pharmaceutical Biology*, *53*(6), 849-854.
12. Zhu, S., Li, W., Liu, J., Chen, C. H., Liao, Q., Xu, P., ... & Wei, W. (2016). Genome-scale deletion screening of human long non-coding RNAs using a paired-guide RNA CRISPR–Cas9 library. *Nature biotechnology*, *34*(12), 1279-1286.
13. Bhattacharya, P., Budnick, I., Singh, M., Thiruppathi, M., Alharshawi, K., Elshabrawy, H., ... & Prabhakar, B. S. (2015). Dual role of GM-CSF as a pro-inflammatory and a regulatory cytokine: implications for immune therapy. Journal of Interferon & Cytokine Research, 35(8), 585-599.
14. Omori, F., Okamura, S., Takaku, F., & Niho, Y. (1989). Measurement of human G-CSF by enzyme-linked immunosorbent assay using monoclonal antibody. *Research in experimental medicine*, *189*, 163-171.