Original Article

Optimization of human interferon beta protein expression in Chinese hamster ovary cells

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Abstract

Background and aims: Human interferon beta-1a (hIFNβ-1a) is a 22.5-kDa glycoprotein used to treat diseases such as multiple sclerosis (MS). Because of appropriate post-translation modifications, protein isolation, and lack of toxicity in Chinese hamster ovary (CHO) cells, we cloned hIFNβ-1a encoding sequence into these cells by recombinant DNA technology to achieve stable expression of this recombinant protein.

Methods: The hIFNβ-1a encoding sequence was designed based on the CHO cells' codon usage and the Gene Bank data, and then syntactically constructed in the pUC57 vector. After confirmation, the synthesized sequence was cloned into the pcDNA3.1 expression vector by using EcoRI and XhoI sites via *Escherichia coli* DH5α competent cells. Then, the recombinant vector pcDNA-hHIFNβ1a was linearized by BglII and transfected into the CHO cells using lipofectamine. The transfected cells were proliferated and screened by gentamicin. Certain concentrations of zinc sulfate, DMSO, and glycerol were used to enhance protein expression. Finally, the recombinant protein expression was qualitatively evaluated using different techniques.

Results: The hIFNβ1a integrity was confirmed by DNA sequencing and specific software. The construction and sub-cloning of hIFNβ1a-pcDNA3.1 in *E. coli* were confirmed by colony-PCR with specific primers and restriction enzyme mapping. The screening of transfected CHO cells was performed using gentamicin. The protein expression was confirmed by RT-PCR, MTT assay, SDS-PAGE, and Western blot. Comparison of the optimized and control samples demonstrated that chemical treatment enhanced the protein expression.

Conclusion: We achieved the stable clones of CHO cells expressing the active form of human interferon beta.

Keywords: hIFNβ-1a, CHO cells, pcDNA3.1 expression vector, lipofectamine, MTT assay

Introduction

Interferons are pleiotropic cytokines with antiviral and immunomodulatory properties that are produced by host cells in response to the invasion of free pathogens, and stimulate the immune system and increase body resistance against pathogens (1). In 1992, Day et al isolated the interferon-beta gene from mouse DNA and amplified it in *Escherichia coli* (2). Today, recombinant beta interferon is produced in two hosts: mammalian and bacterial, known as interferon beta 1a (IFNβ-1a) and 1b (IFNβ-1b), respectively (3). The hIFNβ-1a is composed of 166 amino acids and has a molecular weight of around 22 500 Da.

This product is produced by recombinant DNA technology by inserting the gene responsible for interferon-beta production into the Chinese hamster ovary (CHO) cells (4). Both natural and recombinant interferon beta are glycosylated and bind to the N-terminal of a carbohydrate complex.

Interferon-beta may limit central nervous system (CNS) damage caused by multiple sclerosis (MS) through an unknown mechanism (5). Due to the ability of mammalian cells to produce high-quality proteins with biochemical properties similar to the naturally synthesized protein, a large number of therapeutic recombinant proteins are produced by these cells today.

The hIFNβ-1a can be produced in the expression hosts such as yeasts, filamentous fungi, insect cells, and plant cells, but due to different glycosylation patterns, their uses are also associated with some limitations (6). Therefore, nearly 70% of recombinant therapeutic proteins are produced by CHO cells. CHO cells have properties that make them suitable for the production of recombinant human proteins for clinical use. These cells easily undergo genetic modifications to facilitate foreign DNA entry and expression of large amounts of foreign protein, are capable of producing glycosylated proteins, and performing post-translational modifications required for protein bioactivity; product safety is another key factor that should be considered in selecting a cell host. The host cell should not contain any pathogenic factors, which may eventually be transmitted to the human body. CHO cells have been proven to be safe hosts for drug production. However, the specific efficiency of these cells is lower than prokaryotic hosts. For example, in the production process, they

have been found to be unstable that is a major problem in the biotechnology industry. Also, protein instability is a common problem in the production, purification, and storage of recombinant pharmaceutical products. Besides, molecular aggregation is a major problem in the production of interferon-beta protein due to the hydrophobic nature of its structure.

There are several reports showing the tendency of non-glycosylated IFNβ to aggregate, which prevents its proper crystallization by X-ray diffraction studies. But there is a similar phenomenon in glycosylated IFNβ produced in CHO cells due to increased protein concentration and temperature (7).

There are several strategies for controlling and maintaining stable production of a high amount of protein, including modifications and induction of different mutations in the hIFNβ-1a encoding sequence to increase the binding of RNA polymerase enzymes, but the addition of culture supplements and regulation of culture variables is also used as a simpler method with good efficiency. Improving culture conditions is critical to sustaining biological activity and increasing crop production. Increased protein expression can substantially reduce the cost and time needed to produce recombinant proteins.

Various physical (e.g. temperature (8 (and pH (9)) and chemical methods (e.g. NaCl (10), acetate (11), etc) have been examined to optimize the expression of hIFNβ. Here, we chose three chemicals including dimethyl sulfoxide (DMSO), glycerol, and zinc.

DMSO is used as an additive in cell culture systems that reduces aggregation and cell death and also increases protein production. It stabilizes proteins in their natural conformation and acts as a chemical chaperone. Fiore and Degrassi reported that DMSO inhibits the growth of CHO cells and prevents their apoptosis and phenotypic differentiation. The polar nature of this substance allows it to permeate into the cell membrane without causing significant damage, but its effect on cell function has not been fully elucidated yet (12).

Glycerol is widely used as a protein stabilizer in the pharmaceutical and biotechnology industries. As an osmolyte, it can form hydrogen bonds and also a hydration shell of water molecules surrounding the protein surface. Like DMSO, this compound has also been identified as a molecular chaperone and a mediator of protein folding within cells, and also targets and detects poorly folded proteins for refolding or degradation. Glycerol has been shown to increase IFNβ stability by reducing more than 50% aggregation. It can also prevent IFNβ aggregation after isolation from the cell culture medium (7).

Zinc inhibits cell apoptosis under certain conditions, and also by altering the secondary structure of mRNA, increases its stability or binding the mRNA-stabilizing proteins. Moreover, zinc directly or indirectly inhibits one or more ribonucleases responsible for the destruction of unstable mRNAs (13).

In this study, the interferon-beta gene was cloned into CHO cells, and the cells were treated with zinc sulfate, DMSO, and glycerol to increase protein expression. Finally, expression levels in untreated and chemically treated conditions were evaluated by different techniques.

Materials and Methods
Materials, vectors, and strains
Escherichia coli coli strain DH5α (Novagen, Iran), pcDNA3.1 vector (Novagen, Iran); CHO cell line (Iranian Biological Resource Center, Iran); HDF cell line (Pasteur Institute), DMEM medium (Bioidea, Iran), FBS (Bioidea, Iran); penicillin-streptomycin antibiotic solution (Gibco, USA), gentamicin antibiotic (Gibco, USA); ampicillin (Roche; Germany), trypsin-EDTA (Bioidea, Iran); Taq Polymerase DNA (Fermentase, Lithuania), T4 DNA Ligase (Fermentase, Lithuania), *EcoRI*, XhoI and *BglII* restriction enzymes (Roche; Germany), agarose, glycerol (SiGMA, USA), PBS Tablet, DMSO (Gibco, USA) Lipofectamine-2000 (Invitrogen; Carlsbad, CA), RNX-Plus (Sinaclon, Iran), PVDF membrane (Roche; Germany) and other chemicals (Merck Co., Germany).

Designing and synthesis of hIFNβ-1a coding sequences
HIFNβ-1a coding sequence was designed based on the optimal CHO cell codon usage and gene bank data for hIFNβ-1a gene sequencing (AAC41702), and pcDNA3.1 expression vector sequence was designed using GeneRunner version 6.1.23 Beta and SnapGene 3.2.1 software and synthesized in the *EcoRV* sequence within the pUC57 vector and the sequencing was conducted by Pioneer Company (France).

Production and replication of recombinant expression vector
First, the plasmid pUC57-IFNβ1a and the shuttle vector pcDNA3.1 were amplified after transfection into competent *E. coli* DH5α cells and cultured on the agar plate containing antibiotic ampicillin (100 mg/mL) and incubated at 37°C.

Since the above-mentioned vectors contain ampicillin-resistant genes, recombinant cells were grown to acquire resistance, and then they were screened. The obtained colonies were selected and amplified in the broth medium containing ampicillin and incubated in a shaking incubator at 37°C for 18 hours. After that, the plasmid was extracted using the kit (GeneAll, Korea). The hIFNβ-1a fragment was enzymatically digested and separated from pUC57 using *EcoRI* and *XhoI* restriction enzymes. The pcDNA3.1 vector was separately extracted from DH5α by DNA purification kit (GeneAll, Korea).

The ligation of hIFNβ-1a fragment into the pcDNA3.1 vector was performed by the T4 DNA ligase enzyme and the product was transferred into competent *E. coli* DH5α cells. To screen for target colonies, PCR-PCR was performed using the forward primer 5′-GGTGGTCATAT-GTCTTACAATCTGCTGGGTTTCCTG-3 and the reverse primer 5′-GGTGGTTGCTCTTCCGCAGTTACG-

GAG.

Following the cultivation of appropriate colonies and extraction of plasmid DNA from them, the production of the recombinant pcDNA-hIFNβ1a vector was confirmed by digestion with the *EcoRI* and *XhoI* enzymes.

Finally, the recombinant vector was linearized by *BglII* and after gel purification by kit, the recombinant vector was prepared for transferring into CHO cells. All PCR products and enzymatic digestion were confirmed by 1% agarose gel electrophoresis.

Cloning of the recombinant pcDNA-hIFNβ1a vector into CHO cells

Culture of CHO cells

The CHO cell line was purchased from the Genetic Resource Center in a culture flask. The appropriate medium for the growth of these cells is DMEM containing 10% FBS serum plus 1% penicillin-streptomycin antibiotic solution to prevent infection. The cell culture medium was changed every two days until the cells covered about 80%-90% of the flask surface. At this stage, the cells were passaged by trypsin-EDTA treatment for transferring into the plate and banking. At all stages, cells were counted with Trypan blue staining.

Determination of bactericidal concentration of gentamicin (G418) for screening

Since the pcDNA3.1 plasmid contains a G418 resistance gene, transfected cells, i.e. the cells containing the recombinant vector pcDNA-hIFNβ1a can be isolated by the treatment of G418. For this purpose, the appropriate antibiotic concentration for CHO cells was first determined.

To do this, 5×10^4 CHO cells/well were cultured in a 24-well plate and treated with different concentrations of antibiotics; the minimum concentration, which killed all cells after two weeks, was selected as the optimal concentration.

Transfection of linear recombinant vector into CHO cells

For transfection, 1.2×10^6 CHO cells/well were cultured in a 6-well plate containing 500 µL DMEM, 10% FBS, and 1% penicillin-streptomycin solution and incubated at 37°C, 95%-100% humidity, and 5% CO_2. After the cells reached 80% confluency, their medium was replaced with the serum- and antibiotic-free medium for transfection after 2-4 hours. Next, 2 µg/well of pcDNA-hIFNβ1a and 2 µL of lipofectamine-2000 were separately added into 150 µL of DMEM in the microtubes. Five minutes later, the contents of the two microtubes were combined and incubated at room temperature for 20 minutes. Then the solution containing pcDNA-hIFNβ1a and lipofectamine-2000 was added into the wells.

Two wells containing untransfected CHO cells were considered as negative controls. Plates containing cells were incubated for 6 h with the mentioned conditions, and then the culture medium was collected, and fresh medium containing 5% FBS and 1% penicillin-streptomycin solution was added into the wells.

Confirmation of the entry of the hIFNβ-1a coding sequence into the CHO genome

To confirm the entry of the hIFNβ-1a coding sequence into the CHO genome, screening was performed by the G418 antibody at the determined concentration. Since the transfected cells were resistant to gentamicin antibiotic, transfected CHO cell colonies survived and proliferated after two weeks of the addition of this antibiotic into the culture medium.

Increased expression of hIFNβ-1a protein by chemical additives

In this study, the combination of zinc sulfate, glycerol, and DMSO was used to enhance protein expression. CHO cells were treated with 125 µM zinc sulfate, 2% glycerol, and 1% DMSO for 6 days following transfection with pcDNA3.1-hIFNβ1a. The cell media were daily collected and replaced with fresh medium containing FBS and penicillin/streptomycin, as well as the above-mentioned compounds.

Assessment of interferon Beta mRNA production using RT-PCR

At this stage, the total RNA of 10^6 transfected CHO cells was extracted by the RNX-Plus solution and confirmed by agarose electrophoresis, showing s18 and s28 bands on the gel. Then, cDNA was synthesized by the cDNA synthesis kit (TaKaRa). After that, standard PCR was performed using specific interferon-beta primers and analyzed by agarose electrophoresis.

Assessment of protein expression using SDS-PAGE and western blotting

To evaluate the expression of hIFNβ-1a protein, culture medium containing the secreted recombinant protein and culture medium containing negative control cell after concentration by PMSF at 45°C, 30 µL of each sample was dissolved in 20 µL of sample buffer (10 mL high gel buffer, 5 mL glycerol 100%, 1 g SDS powder, 500 µL bromophenol blue, and 1 mL 2-mercaptoethanol to a final volume of 20 mL) and poured into SDS-PAGE wells and electrophoresed at 80 V.

In the end, the gel was immersed in a Coomassie blue solution (1 g of Coomassie powder, 100 mL of acetic acid, and 500 mL of methanol to a final volume of 1 L) and after being placed in a decoloring solution (100 mL of acetic acid and 300 mL of methanol to a final volume of 1 L), the protein bands were evaluated.

Western blotting was performed in three steps. First, isolation of all proteins in a sample medium by size was performed using SDS-PAGE gel electrophoresis. Then, the proteins isolated on the gel were transferred to the PVDF membrane (Roche) by wet method. Finally, specific identification of the target protein was performed using

human interferon beta-specific monoclonal antibody (Roche) until the band emerged on the membrane.

Activity evaluation of hIFNβ-1a protein by MTT assay

The anti-proliferative property of interferon beta was used for this test. To this end, 8×10^3 human dermal fibroblasts (HDF) cell/well were cultured in a 96-well plate containing 10% DMEM and 10% FBS and incubated at 37°C with 95%-100% humidity and 5% CO_2.

After about 80% confluency, the medium samples containing interferon-beta secreted by CHO cells were added in pairs into the wells in volumes of 0-50 µL and incubated. After 24 hours, 20 µL of MTT solution (5 mg MTT powder in 1 mL PBS) was added into each well and incubated in the dark for 3 h. Then, the wells became completely free of supernatant and 100 µL of DMSO was added into each well and optical absorbance was read by ELISA reader at 570 nm wavelength.

Results

Designing hIFNβ-1a coding sequence

The hIFNβ-1a coding sequence was designed based on the optimal codons of CHO cells and the gene bank data. The signal peptide and Kozak sequences were added to the begining of the sequence. This sequence enhances gene expression in eukaryotic systems by facilitating the binding of ribosomes to mRNA.

At the end of the gene sequence, two stop codon sequences were added so that translation completion and protein synthesis would be accomplished appropriately. *EcoRI* and *XhoI* enzymes were also selected after enzymatic mapping of the gene and vector by the SnapGene software and added to 3' and 5' sequences, respectively (Figure 1). Subsequently, the sequence of the hIFNβ-1a coding sequence was inserted into the pcDNA3.1 vector by bioinformatics software (Figure 2).
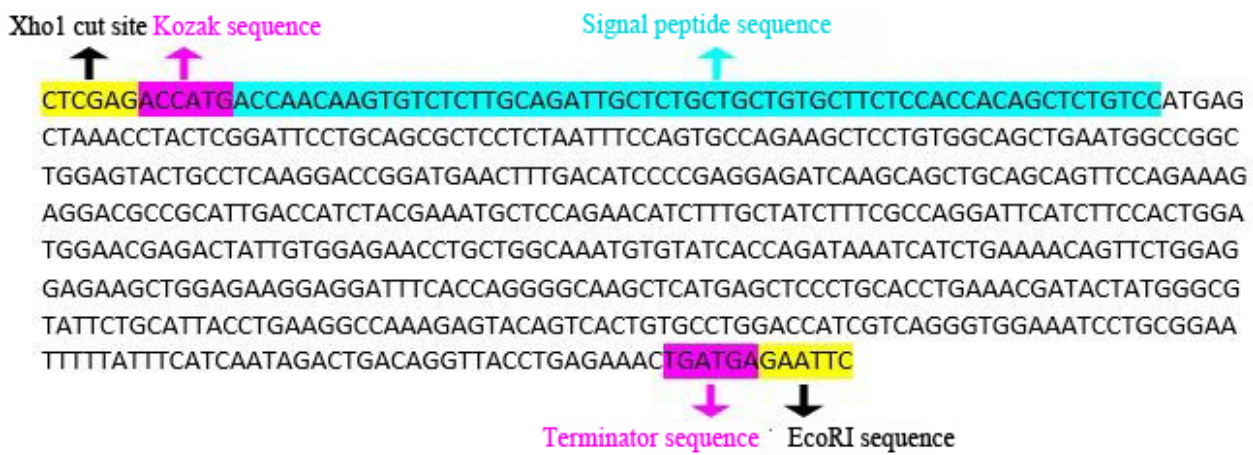
Figure 1. Encoding Sequence of the hIFNβ-1a designed along with the Kozak sequence, stop sequence, cut sites, and secreted signal peptide sequence.
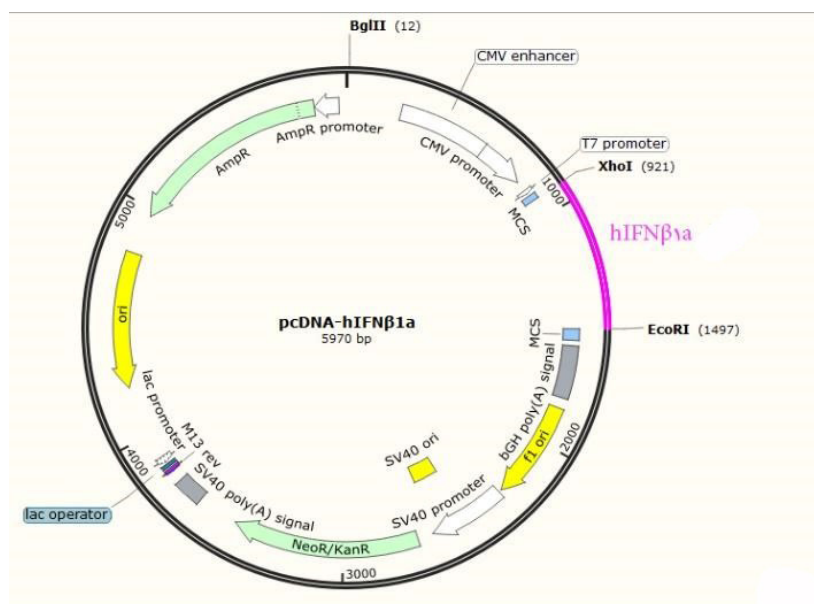
Figure 2. Bioinformatical analysis of hIFNβ-1a encoding sequence entry into pcDNA3.1 plasmid by SnapGene software.

Producing a recombinant expression vector (pcDNA-hIFNβ1a)

First, pUC57-IFNβ1a plasmid was amplified by transfection into *E. coli* competent cells and after extraction, the above plasmid was enzymatically digested by *EcoRI* and *XhoI* enzymes to isolate the hIFNβ-1a coding fragment. After cleavage of the vector with the above enzymes, two separate fragments of 2716 bp (vector) and 576 bp (coding sequence) were expected to be formed.

As shown in Figure 3, the expected fragments appeared as bands (lines 4-9). The 5394-bp pcDNA3.1 plasmid (lanes 1 and 2) is visible on gel electrophoresis.

After purification, the hIFNβ-1a coding sequence fragment was ligated inside the pcDNA3.1 by T4 ligase from the *EcoRI* and *XhoI* enzymes cut sites and transferred to *E. coli* for amplification.

A number of colonies were selected for screening the target colonies and PCR was performed using specific primers, which lead to the appearance of 576-bp fragments on gel electrophoresis (Figure 4).

Transfection of CHO cells with recombinant pcDNA-hIFNβ1a vector and evaluation of expression and function of recombinant hIFNβ-1a protein

The recombinant vector was linearized by *BglII* enzyme and transferred into CHO cells after purification. The screening was performed using 400 ng/mL of antibiotic G418. Untransfected cells were expected to die, and recombinant vector recipient cells were expected to survive and colonize as a result of antibiotic resistance.

Figure 5 shows pre- and post-transfection and finally the formation of cell colonies after the screening, which indicates that the recombinant vector was proliferated by CHO cells.

The cDNA was synthesized from extracted RNAs and RT-PCR was performed using specific primers for the hIFNβ1a sequence, and the product was transferred onto an agarose gel.

The observed bands confirmed the expression of the hIFNβ1a gene at the mRNA level (Figure 6). In addition,

given the sharper band in line 2 (the product under chemical treatment conditions), compared to lines 1 and 3 (untreated), the amount of hIFNβ-1a mRNA production significantly increased in the chemically treated cells compared to the untreated control cells.

Subsequently, the expression of the protein secreted into the cell culture supernatant was evaluated and determined using the tests below:

SDS-PAGE: After pouring the samples into 12.5% polyacrylamide gel wells and performing electrophoresis, the appearance of the 22.5-kD band (lanes 1 and 3) confirmed the production of beta interferon protein; the sharper lane, i.e. lane 3 is related to the sample of transfected CHO cells treated with zinc sulfate, glycerol,
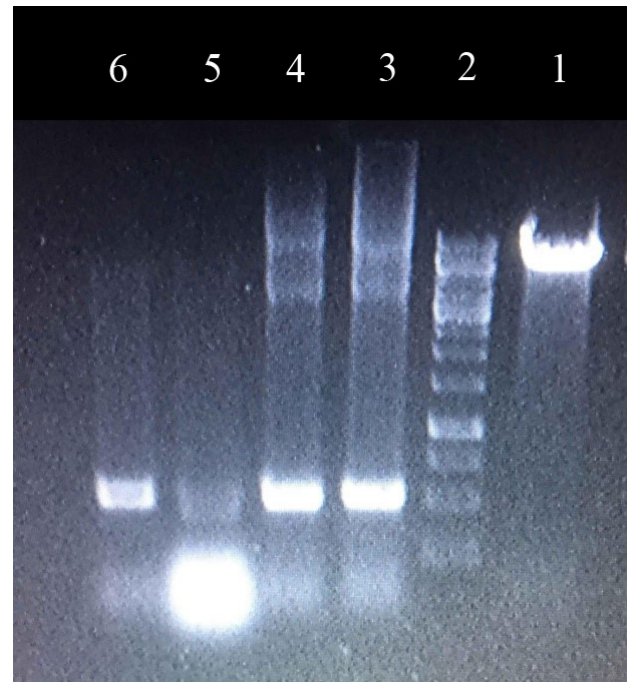
Figure 4. PCR product of the samples extracted from recombinant plasmid pcDNA-hIFNβ1a on agarose gel 1%. Line 1: plasmid pcDNA-hIFNβ1a. Line 2: 1kb DNA ladder line. Lines 3 & 4: Interferon-beta gene bands in the bp576 region.
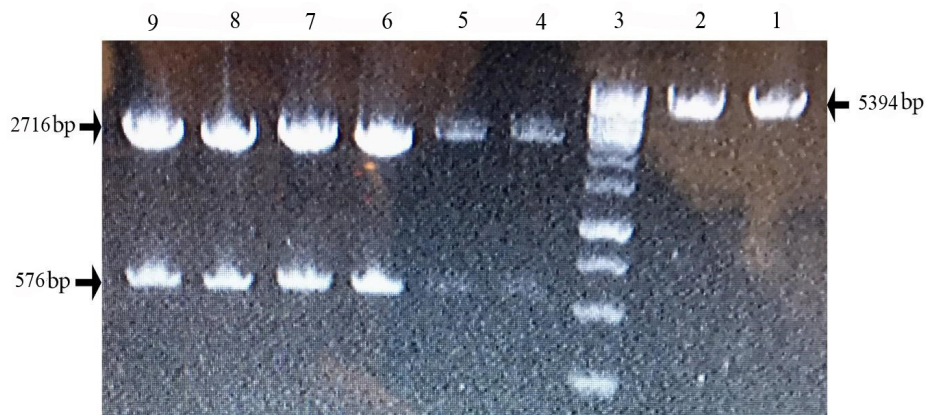
Figure 3. Enzymatic digestion product of pUC57-IFNβ1a and pcDNA3.1 plasmids by *EcoRI* and *XhoI* enzymes. Lines 1 and 2: pcDNA3.1 vector. 3: 1kb DNA ladder. Lines 4-9: plasmid extracts containing bp2716-pUC57 vector body bands and 576 bp-IFNβ1a bands.

C   **B**   **A**
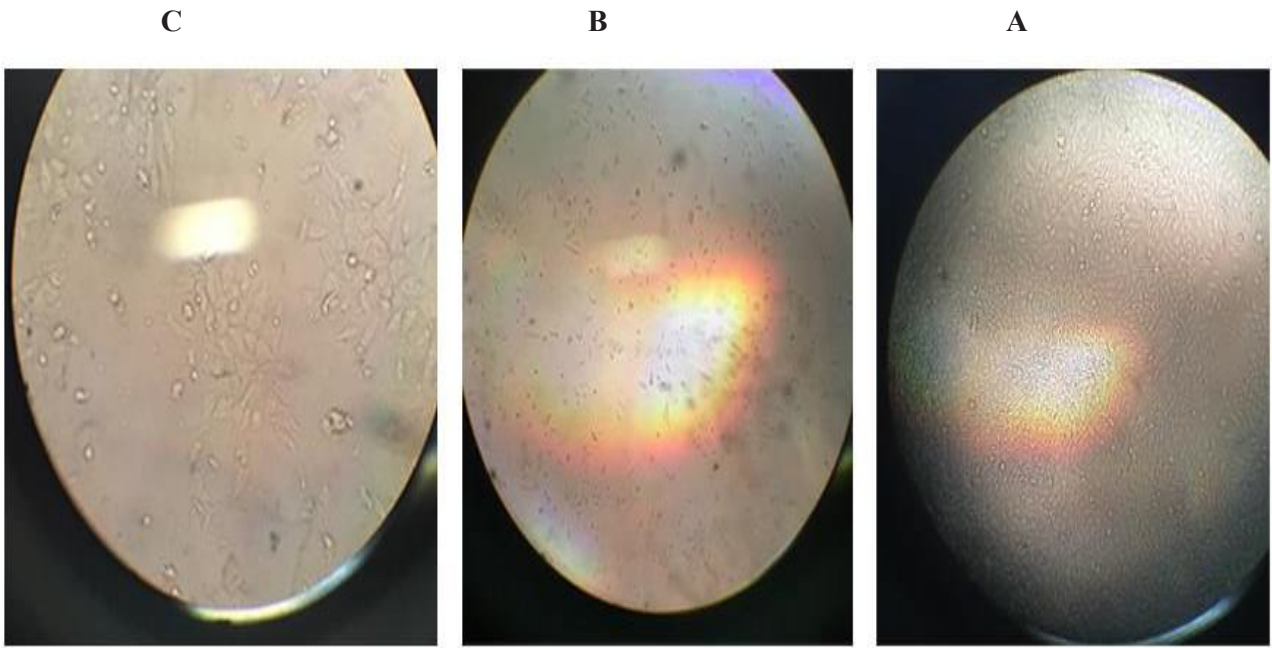
Figure 5. (A): Proliferated CHO cells before transfection. (B) CHO cells 24 hours after transfection. (C) Formation of colonies containing transfected CHO cells.

and DMSO, indicating increased expression compared to the untreated conditions. It should be noted that other bands formed are related to serum proteins. (Figure 7).

Western blotting

At this stage, the samples were symmetrically run on SDS-PAGE and after electrophoresis, the gel was divided into two portions: one portion was stained with Coomassie blue staining solution and then decolored; the second portion was transferred onto the PVDF membrane, and after blocking by 1% BSA solution, it was treated with first
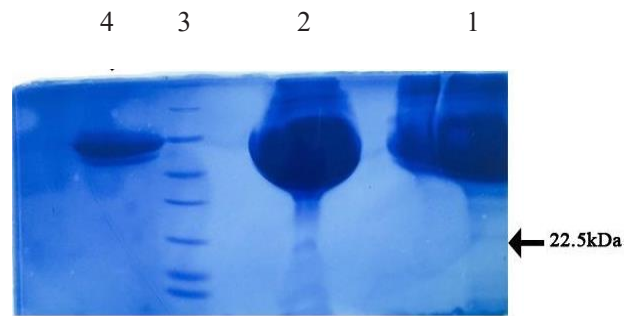
Figure 7. 12.5% Polyacrylamide gel electrophoresis containing the supernatant of transfected cells with and without chemical treatments. Line 1: Supernatant of hIFNβ1a-producing CHO cells without chemical treatment. Line 2: Supernatant of CHO cells producing hIFNβ1a under chemical treatments. Line 3: Unstained protein ladder. Line 4: Negative control
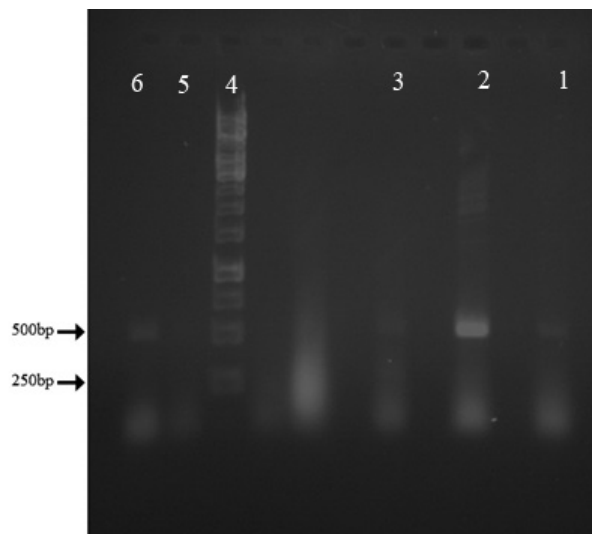
Figure 6. RT-PCR product on agarose gel 1% electrophoresis. Lines 1 and 3: RT-PCR product of untreated transfected samples. Line 2: Samples of transfected cells treated with 125 μM zinc sulfate, 2% glycerol, and 1% DMSO, line 4: 1kb DNA ladder. Line 5: Negative Control. Line 6: Positive Control.

1:1000 antibody (antimouse) and then with the second 1:10000 antibody (specific interferon-beta antibody).

Finally, with the addition of 0.1 g/L DAB, the desired band appeared and the protein expression was confirmed. In Figure 8, the emergence of the band in the 22.5 kDa region results from the binding of the monoclonal antibody to the interferon beta protein and confirms the production of this protein.

Line 2 represents the sample obtained from transfected CHO cells under chemical treatment, which shows a higher expression level compared to line 1, which is related to untreated transfected cells. Moreover, due to the concentration of the protein-containing solution, the serum proteins were also concentrated and the bubble-shape band on top of the gel is related to the serum proteins.

MTT colorimetry: The result of this test was checked by

ELISA reader.

It was expected that with increasing concentration of hIFNβ1a protein added to each well, due to the decreased number of living cells and consequently decreased mitochondrial succinate dehydrogenase enzyme activity, the amount of purple color produced would decrease, resulting in a decrease in the optical absorption of purple color at a wavelength of 490-540 nm. Thus, the diagram obtained with the downward trend in Figure 9 confirmed the production of this protein.

Discussion

Human interferon beta protein is a glycoprotein produced by leukocytes, lymphocytes, fibroblasts, and other immune cells. Nowadays, corticosteroids are also used to treat MS, yet with many side effects. Besides, some cancers, such as pancreatic cancer, and other diseases such as infections of the central nervous system (Clippers), liver (hepatitis C), muscles (myositis), and viral diseases (coronavirus) do not respond well to the available treatments or are associated with side effects. These can be controlled or even treated with a certain concentration of interferon beta1.

The bacterial system is unable to induce glycosylation
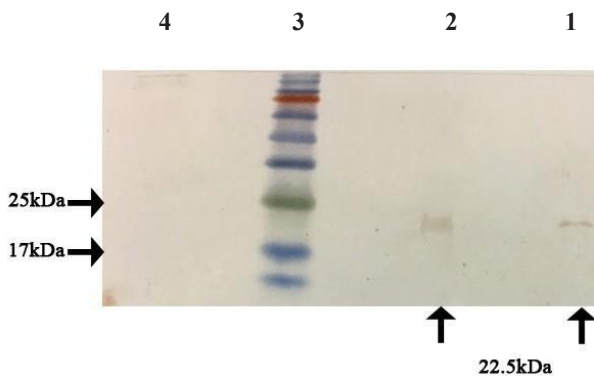
Figure 8. Western blotting analysis of hIFNβ-1a protein expression. Line 1: Sample containing supernatant of cells transfected without chemicals. Line 2: Sample containing the supernatant of transfected cells treated with chemicals. Line 3: Prestained protein ladder. Line 4: Negative Control.
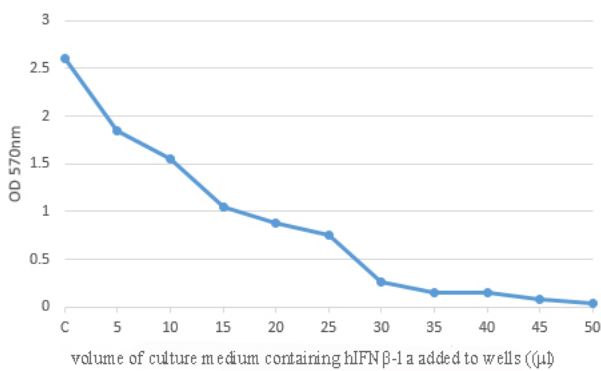
Figure 9. Linear diagram of OD at 570 nm by viable human dermal fibroblasts treated with different volumes (0 to 50 μL) of culture medium containing hIFNβ-1a protein produced by CHO cells.

patterns in proteins, and thus the IFNβ protein produced by this system lacks a glycosylation pattern, and is different from its human variant. This reduces the efficacy of the drug compared to glycosylated form.

Various studies have shown that the production of recombinant human proteins in CHO cells is much higher than that of human cells. Another positive aspect of CHO cells is their immunity, which means that they are resistant to viral infections such as HIV, influenza, herpes, and measles, and unlike human cells, are generally regarded as safe cells (14). Post-translational modifications, glycosylation patterns, and disulfide bonds of proteins produced by this cell line are similar to those of humans, making CHO cells a very suitable cell line for the production of recombinant human proteins (15). Various strategies have been employed to increase the amount of recombinant protein production in CHO cells, such as regulating the culture temperature, adding chemicals, and bioreactor engineering (16). In one study, it was shown that after transfection of CHO cells, the temperature shift from 37°C to 32°C for 3 days increased interferon-gamma production by 40% compared to growth at 32°C, and by 90% compared to growth at 37° C (17). Chemical compounds are also used to stop the cell cycle in the G phase, and thereby increase protein expression, including DMSO and sodium butyrate (3,18). Using molecular chaperones is also an efficient way to increase protein expression in eukaryotic systems, especially CHO cells, by which the protein produced is protected against intracellular proteases. In a study, a protein disulfide isomerase (PDI) sequence was used as a molecular chaperone to protect and increase the levels of two recombinant interleukin-15 proteins and the TNF receptor produced by CHO cells, but PDI did not increase protein secretion (19). It is also important to optimize the production of interferon beta in CHO cells to increase the speed and quality and reduce the cost of drug production. For example, a change in codon preference in the destabilizing region of the end of the interferon beta protein was reported to increase the half-life of the corresponding mRNA, and consequently increase the production of this protein by 2.9 fold (20).

Conclusion

In this study, the CHO cells were used to produce recombinant hIFNβ-1a protein because of the positive characteristics mentioned for this cell line. These cells were able to stably release the recombinant protein into the culture medium. We also used chemicals to increase efficiency, which satisfactorily increased the expression level. Further research should be done to increase protein expression, and particularly the cell stability should be investigated and employed to reduce the cost and time of drug production.

Conflict of Interests

The authors declare that they have no conflict of interests.

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