Research Article

Efficient CRISPR/Cas9-Mediated BAX Gene Ablation in CHO Cells To Impair Apoptosis and Enhance Recombinant Protein Production

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Background: Despite recent advances in recombinant biotherapeutics production using CHO cells, their productivity remains lower than industrial needs, mainly due to apoptosis.

Objectives: Present study aimed to exploit CRISPR/Cas9 technology to specifically disrupt the BAX gene to attenuate apoptosis in recombinant Chinese hamster′s ovary cells producing erythropoietin.

Materials and Methods: The STRING database was used to identify the key pro-apoptotic genes to be modified by CRISPR/Cas9 technique. The single guide RNAs (sgRNAs) targeting identified gene (BAX) were designed, and CHO cells were then transfected with vectors. Afterward, changes in the expression of the Bax gene and consequent production rates of erythropoietin were investigated in manipulated cells, even in the presence of an apoptosis inducer agent, oleuropein.

Results: BAX disruption significantly prolonged cell viability and increased proliferation rate in manipulated clones (152%, P-value = 0.0002). This strategy reduced the levels of Bax protein expression in manipulated cells by more than 4.3-fold (P-value <0.0001). The Bax-8 manipulated cells displayed higher threshold tolerance to the stress and consequence apoptosis compared to the control group. Also, they exhibited a higher IC50 compared to the control in the presence of oleuropein (5095 μM.ml^{-1} Vs. 2505 μM.ml^{-1}). We found a significant increase in recombinant protein production levels in manipulated cells, even in the presence of 1,000 μM oleuropein compared to the control cell line (p-value=0.0002).

Conclusions: CRISPR/Cas9 assisted BAX gene ablation is promising to improve erythropoietin production in CHO cells via engineering anti-apoptotic genes. Therefore, exploiting genome editing tools such as CRISPR/Cas9 has been proposed to develop host cells that result in a safe, feasible, and robust manufacturing operation with a yield that meets the industrial requirements.

Keywords: Apoptosis, Bax Gene, CRISPR/Cas9, Cell longevity, Gene editing, Oleuropein

1. Background

Protein therapeutics represent one of the most exciting breakthroughs of modern medicine, used in the treatment of an ever-expanding list of diseases and disorders (1). As the demand for improved quantity, purity, and quality in biotherapeutic products continues to increase,

novel strategies for engineering efficient eukaryotic cells become more necessary (2). Both academic and industry research groups continue to develop cost-effective methods for producing heterologous proteins to support preclinical and clinical evaluations of potential therapeutics (3).

Chinese hamster ovary (CHO) cells have been the most commonly used mammalian host for large-scale commercial production of therapeutic proteins (4-6). However, bottlenecks in protein quality and weakness in expression efficiency still remain due to the intrinsic genetic properties of the cell (6). The reason behind CHO cell productivity limitation is apoptosis stemming from cellular stress during protein production (7). Apoptosis can be induced by various stress factors, such as increased osmolality of the medium, oxygen deprivation, and exposure to shear stress (8). Thus, genome engineering and manipulation of CHO cells with the aim of improving the quality and quantity of recombinant products are of great interest to biopharmaceutical companies and the health system.

The prevention of apoptosis in protein-expressing cell lines will therefore increase cell viability, suppress cell death, extend cell culture lifespan and increase the productivity of the target product (9). Optimization of mammalian cell culture technology is essential for the economical production of biopharmaceuticals in bioreactors. Nevertheless, a significant problem faced in bioreactor culture is cell death, which decreases the overall production yield (10). Several areas were identified that could further improve cell culture systems. For instance, it is of considerable value to inhibit apoptosis in culture to extend the time of high cell viability and prolong protein production (11).

The best-known key regulators of the death process are the Bcl-2 family proteins (Bcl-2, Bcl-xL, Bax, and Bak), which constitute a critical intracellular checkpoint of apoptosis cell death within a common death pathway (12, 13). Bax (Bcl-2 associated X protein) is a pro-apoptotic protein and it is essential for mitochondria-mediated apoptosis (14). Bax protein undergoes a conformational change in response to signals that lead to its translocation from the cytosol to the mitochondria and the delivery of pro-apoptotic proteins (10, 14) The result of this phenomenon causes apoptosis or programmed cell death. It can decrease the yield of a bioreactor in the production of recombinant proteins in industry.

Recent studies utilized CRISPR/Cas-based systems for genetic manipulations in humans, animals, and plants (9). CRISPR/Cas9 has proven to be a highly valuable tool for genome editing in many organisms (15). For its specificity, efficacy, and simplicity, the CRISPR/Cas9 system has been called the biggest biotechnological discovery of the century and has opened new possibilities for precise genome editing (16). This system enables targeted genetic modifications in cultured cells, in addition to in total animals and plants. The use of these enzymes in research, medicine, and biotechnology fields occurs from their power to provoke site-specific DNA cleavage in the genome, the repair of which allows high-precision genome editing (17).

The compound oleuropein is the most prominent polyphenol component of olives and leaves (18, 19). This compound has shown cytotoxic, anti-proliferative, apoptotic, and anti-metastatic effects in various cell lines and cancers (18). Oleuropein induces apoptosis by suppression of the PI3K/AKT signaling pathway and through activation of caspases (20).

2. Objectives

The present study aimed to investigate the effect of exploiting the targeted CRISPR/Cas9 technology to manipulate the genome of the erythropoietin producer CHO-K1 cells by disrupting the BAX gene, which encodes a protein involved in the apoptotic pathway, in order to develop the production of recombinant proteins in CHO cell factories. As the CHO cell line is a well-characterized mammalian expression system for apoptosis investigation in research and the biopharmaceutical industry, it was selected to validate our strategy. For this purpose, the expression levels of the Bax gene and the cell survival rate and expression of the Bax gene in manipulated CHO cells were compared to the non-manipulated control group after CRISPR/cas9 assisted specific gene disruption. Moreover, the effect of Bax gene ablation on impairing apoptosis and the consequence of the increased quantity of erythropoietin production was investigated in the presence of different concentrations of apoptosis inducer agent, oleuropein. Cell viability and proliferation rate of the genetically modified and non-manipulated cells both 24 and 48 hours after induction of apoptosis were compared as well as the amount of erythropoietin production in the presence and absence of oleuropein. The CHO cell line was selected because it is a well-characterized

mammalian system for apoptosis investigation in research and the biopharmaceutical industry.

3. Materials and Methods

3.1. Cell Line and Cell Culture
Recombinant Chinese hamster ovary adherent cells (rCHO-K1) produce erythropoietin from the production and research complex of the Pasteur Institute of Iran. The cell line was cultured in DMEM/F-12 medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA).

3.2. STRING Database
String is a database of known and predicted protein-protein interactions (physical and functional). Information relevant to the target gene was transmitted to the STRING server in order to generate the gene network. Experiments, computer models, and publicly available text collections all find their way into the STRING database.

3.3. Design of SgRNA
sgRNAs were designed by the online tool Optimized CRISPR Design (http://crispr.mit.edu/), which takes an input sequence, identifies and ranks suitable target sites. After they were designed, each sgRNA was analyzed using the off-target finder website (http://www.rgenome.net/cas-offinder/) for the bulge type, number, and size mismatch. Several targets were found (**Table 1**).

3.4. Transfection of rCHO-K1 Cells
The rCHO-K1 cell line producing human erythro-poietin was detached from the plates and then transfected with a one μg plasmid (pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro). Transfections were carried out by ScreenFect A plus (Wako, Japan) according to the manufacturer′s instructions. Twenty-four hours post-transfection, cells were treated with different concentrations of puromycin (abmgood, Canada) (1, 2, 3, 4, 5, and 6 μg.mL^{-1}). After successful transfection and antibiotic selection, the next step was to isolate a single clone by serial dilution in DMEM/F-12 (20% FBS).

3.5. DNA Extraction
About 1×10^6 cells were collected by centrifugation. Then, genomic DNA was extracted by DNG-Plus™ reagent according to the manufacturer′s instructions (Sinaclon, Iran).

3.6. PCR Amplifications and Sequencing
PCR amplification was done by master mix (Thermo Scientific, USA) and specific Bax primers. To amplify Bax, we used a specific forward primer 5′-CTACAGGGGTGAGTGTGAGG -3′ and reverse primer 5′- GCTGACTGGCTAAGCATTTGC -3′ to obtain a PCR product of 533base pairs (bps).

3.7. QRT-PCR Analysis
Total RNA was isolated from the transfected and non-transfected CHO-K1 cell pellets collected using Trizol reagent according to the manufacturer′s instruction (Invitrogen). cDNA was synthesized from 700 ng of total RNA using the Viva cDNA synthesis kit (Vivantis, Malaysia) using MMLV-RT and random hexamer primers. For the Bax cDNA amplification, a specific forward primer: 5′-ACTTCA GCCACAAAGATAGTCAC-3′ and reverse primer: 5′-CTAACAAACTGGTGCTCAAGG-3′ were used to obtain a PCR product of 190 base pairs (bps). PCR reactions using specific primers for Chinese hamster GAPDH were carried out for normalization. The forward primer was 5′ CATCACCATCTTCCAGGAGC 3′, and the reverse primer was 5′ CTTGGTTCACACCCATCACA 3′, yielding a PCR product of 194 bp in size.

Table 1. The sequence of sgRNA.

No.	Sequence	Direction	Exon	Mismatch	Position
1	Target 1: GGT CAG CTC AGG TGT ATC CC	3′ → 5′	3	0	200 - 181
2	Target 2: AGC GAG TGC CTC AGG CGA AT	5′ → 3′	3	0	240 - 259
3	Target 3: GGA TAC TAA CTC CCC ACG AG	5′ → 3′	4	0	894 - 913

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3.8. Determination of Cell Growth and Viability

Cells (manipulated and control cell lines) were cultured in a T-25 flask at densities 0.5×10^6 cells/flask. Cell concentrations were made using an improved Neubauer hemocytometer, and viable cells were distinguished from dead cells by the Trypan blue dye exclusion method.

3.9. Induction of Apoptosis and MTT Test

Approximately 5,000 cells of each manipulated cell line and control CHO-K1 cell were seeded in a 96-well plate in triplicate. After twenty-four hours, the medium was replaced with a medium containing various concentrations of oleuropein (500, 1,000, 2,000, 4,000, and 8,000 μM), and cells were incubated at 37 °C for 24 and 48 h. Then, the cells were incubated in 0.5 mg.mL^{-1} solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) at 37 °C for 4 h in the dark. The medium was discarded, and purple formazan crystals were dissolved by adding 150 μL isopropanol per well. In the end, the absorbance of the colored solution was quantified at a wavelength of 570 nm by a microplate reader.

3.10. Western Blot

The total protein of the supernatants from manipulated and non-manipulated CHO-K1 cells were quantified using a BCA Protein Assay kit (Thermo Scientific, USA). All of the samples were loaded on a 12% SDS-PAGE gel (BioRad, Hercules, CA) and transferred to the PVDF membrane (Bio-Rad, USA). Then, the membranes were incubated with Anti-Bax (Cat No: ab32503, Abcam, USA) and anti-beta actin-loading control antibodies (Cat No: ab8227; Abcam, USA). The membranes were washed three times with TBST, and incubated with goat anti-rabbit IgG H&L (HRP) (Cat No: ab6721; Abcam, USA) secondary antibodies. Protein expression was normalized by β-actin.

3.11. Scratch Wound Healing Proliferation Assay

For evaluation of the effect of Bax inhibition on cell proliferation and migration, 1×10^3 cells from manipulated and CHO-K1 cells (control) were seeded into 24-well plates. The cell monolayer was manually scratched using a pipette tip. The cellular debris and non-adherent cells were removed by replacing the fresh media containing 2,000 μM oleuropein. Scratch closure and proliferation cells were monitored at T=0 and T=48h in each well. The difference between edges was calculated by ImageJ software.

3.12. Measurement of Erythropoietin

Manipulated and non-manipulated CHO-K1 adherent cell lines producing recombinant human erythropoietin (EPO) were grown in T-flasks. After one day of culture, the medium consisting of FBS was replaced with a serum-free production medium (with and without 1,000 μM oleuropein) for EPO production. The concentration of EPO secreted into the medium was measured using an EPO ELISA Kit (antibodies-online, Germany) according to the manufacturer′s guidelines at T=0, T=24, T=48, T=72, and T=96.

3.13. Statistical Analysis

All data are shown as mean± SEM. Comparisons between cell lines were analyzed using the Student′s t-test (two groups) or a one-way analysis of variance (ANOVA) by Prism v.8 (GraphPad software). Differences with P values fewer than 0.05 were regarded as statistically significant.

4. Results

4.1. STRING Database Evaluation

The BAX gene was the first identified pro-apoptotic member of the Bcl-2 protein family. Upon initiation of apoptotic signaling, Bax undergoes a conformational shift. Upon induction of apoptosis, BAX becomes mitochondrial membrane-associated. Using the STRING database (https://string-db.org/), the Chinese hamster's BAX apoptotic regulating gene network (Cricetulus griseus) was drawn up. There was a minimum interaction score of 70% (high confidence) and a maximum of 50 genes that could interact (**Fig. 1**). Transfection was done by 400 ng plasmid. After 24 hours of incubation, a concentration of 3 μg.mL^{-1} of antibiotics was chosen for single clone selection. The next step was to isolate a single clone by serial dilution in DMEM/F-12 (20% FBS). Puromycin–resistant cells were detached by trypsin, counted by an improved Neubaer haemocytometer, and serial dilution was done up to reach an individual single cell. After two weeks of incubation in 96 well plate, single clones were identified and transferred to a 24-well plate.
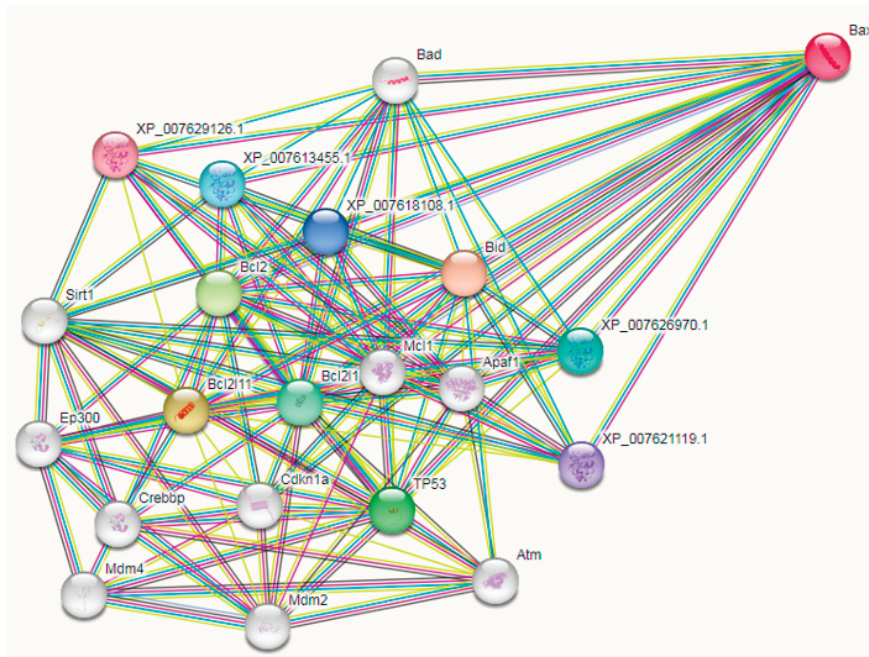
Your input		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	(Homology)	Score
● Bax	Apoptosis regulator BAX (192 aa)									
Predicted Functional Partners:										
● Bid	*BH3 interacting domain death agonist; Counters the protective effect of Bcl-2*				●	●	●	●		0.993
● Bcl2l 11	*bcl-2-like protein 11 isoform X1; BCL2 like 11; Derived by automated computational analysis using gene prediction method: Gnomon. Supportin,...*					●	●	●		0.973
● Bcl2	*Apoptosis regulator bcl-2 isoform x3; Suppresses apoptosis in a variety of cell systems including factor-dependent lymphohematopoietic and neural cells...*					●	●	●	●	0.971
● TP53	*Acts as a tumor suppressor in many tumor types; induces growth arrest or apoptosis depending on the physiological circumstances and cell type...*				●	●	●	●		0.959
● Bcl2l 1	*Bcl-2-like 1 (apoptosis regulator Bcl-X); Bcl-2-like protein 1*				●	●	●	●	●	0.959
● XP_007626970.1	*Cytochrome c-like; Electron carrier protein. The oxidized form of the cytochrome c heme group can accept an electron from the heme group of ...*					●	●	●		0.948
● XP_007613455.1	*Cytochrome c, testis-specific*					●	●	●		0.943
● XP_007618108.1	*Cytochrome c-like*					●	●	●		0.936
● XP_007621119.1	*Cytochrome c, somatic; Derived by automated computational analysis using gene prediction method: Gnomon. Supporting evidence includes similarity ...*					●	●	●		0.936
● XP_007629126.1	*Cytochrome c, somatic; Electron carrier protein. The oxidized form of the cytochrome c heme group can accept an electron from the heme group of ...*					●	●	●		0.936
● XP_007636416.1	*Cytochrome c, somatic; Electron carrier protein. The oxidized form of the cytochrome c heme group can accept an electron from the heme group of ...*					●	●	●		0.936
● XP_007645124.1	*Cytochrome c-like; Derived by automated computational analysis using gene prediction method: Gnomon. Supporting evidence includes similarity to: ...*					●	●	●		0.936
● Apaf1	*Low quality protein: apoptotic protease-activating factor 1 isoform x3; Apoptotic peptidase activating factor 1; The sequence of the model RefSeq ...*				●	●	●	●		0.930
● Bak1	*Bcl-2 homologous antagonist/killer*				●	●	●	●	●	0.925

Figure 1. The results of the STRING analysis of the Bax gene in Cricetulus griseus.

4.2. Sequencing, Alignment, Cell growth and mRNA Expression

One hundred and twenty different individual clones were picked up and sequenced by the dideoxy terminal method. All DNA sequences were aligned with non-manipulated CHO-K1 (control) by Clustal online tool (www.ebi.ac.uk/Tools/msa/clustalo/), and three different manipulated cell lines were selected. Cell growth in the manipulated cell line was monitored by cell counts after 72 h with 500,000 cells at the time 0 in the T-25 flask compared to untransfected cells. **Figure 2** shows the cell count between the control (CHO-K1) and three manipulated cell lines (Bax-8, Bax-18, and Bax-33). The Bax-8 cell line has shown the highest significant difference with control (152%, P-value=0.0002).

mRNA expression levels of the Bax gene in the three manipulated cell lines (Bax 8, Bax-18, and Bax-33) were measured by qRT-PCR. Transcription levels of the target gene were normalized by GAPDH, calculated using the $2^{-\Delta\Delta Ct}$ method, and compared to the wild-type cell line (**Fig. 2**). Using a delta-delta threshold cycle ($\Delta\Delta CT$) method, relative expression was calculated for target genes (Bax-8 cell line). A significantly lower level of Bax mRNA was observed in the Bax-8 cell line compared with the control (20-fold, P-value <0.0001).

4.3. Western Blot, Oleuropein Treatment and Cell Survival Analysis

The Bax gene-reduced expressions due to the site-specific CRISPR/Cas9 disruption were also confirmed by Western blotting and compared to non-manipulated CHO cells (**Fig. 3**). β-actin was used as an internal control for normalizing protein expression. Data presented here showed that CRISPR/Cas9 assisted gene disruption led to the 4.3-fold decline in Bax protein expression in the clone Bax-8 (P-value <0.0001).

For evaluating the resistance to apoptosis, the manipulated and non-manipulated cells were treated with 500, 1,000, 2,000, 4,000, and 8,000 µM.mL^{-1} of oleuropein for 24 and 48 hours. As shown in **Figure 3**, manipulated cells (Bax-8 individual clone) exhibited significant resistance to apoptosis in the presence of an apoptotic agent, oleuropein, after 24- and 48-hours incubation. The results demonstrated that Bax deficiency in the CHO cells could extend the cell′s lifespan and prevent apoptosis in CHO cells. However, it showed that performing CRISPR/Cas9-mediated knockouts

of pro-apoptotic genes could be applied to generate a suitable host for the production of biopharmaceuticals. Increased cell survival was found in the Bax-8 clone (manipulated cell line) compared to the normal CHO-K1 cell line (**Fig. 3**). The IC50 of manipulated cells was higher than control cells (5095 µM.mL^{-1} Vs. 2505 µM.mL^{-1}). Results were shown the IC50 of individual manipulated cells increased after a decrease in the expression of the Bax gene (IC50 has indirect relation with the expression of the Bax gene).

4.4. Proliferation Analysis

The proliferation capabilities and filling of the scratch gap in the presence of 2,000 µM oleuropein were evaluated (**Fig. 4**). With this test, we tried to assess the growth rate of cells in unfavorable conditions and the presence of an apoptotic stimulant. Cell density, cell growth, and filling the gap were also evaluated. These processes were measured at T=0 and T=48 h following the scratch. As shown in **Figure 4**, gap closure and cell proliferation are higher in a manipulated cell line (Bax-8 clone) compared to the control cell line. Moreover, it was found that clone Bax-8 exhibited increased cell proliferation owing to the repression of the Bax gene in manipulated cells (Clone Bax-8), even in the presence of an apoptosis inducer. There was a significant difference in gap closure between the control and Bax-8 clone (P-value=0.0048).

4.5. Masuring the Amount of Erythropoietin

The results were shown that the amount of erythropoietin produced in a manipulated cell line (Bax-8) is higher than in the control cell line (**Fig. 5**). This increase in production is significantly different in the presence of 1,000 µM oleuropein (P-value <0.0001 for the ratio of CHO-K1 Vs. p-value = 0.0002 for the ratio of Bax-8) after 96 hours.

5. Discussion

Biomolecular networks have been constructed for various purposes. One of the most useful, general, and attractive types of networks is the most constitutive-protein association network, which includes all coding genomes in a specific genome and highlights its function (21, 22). To construct a functional communication network for the growth of an organism, evidence of interaction from various sources is considered.
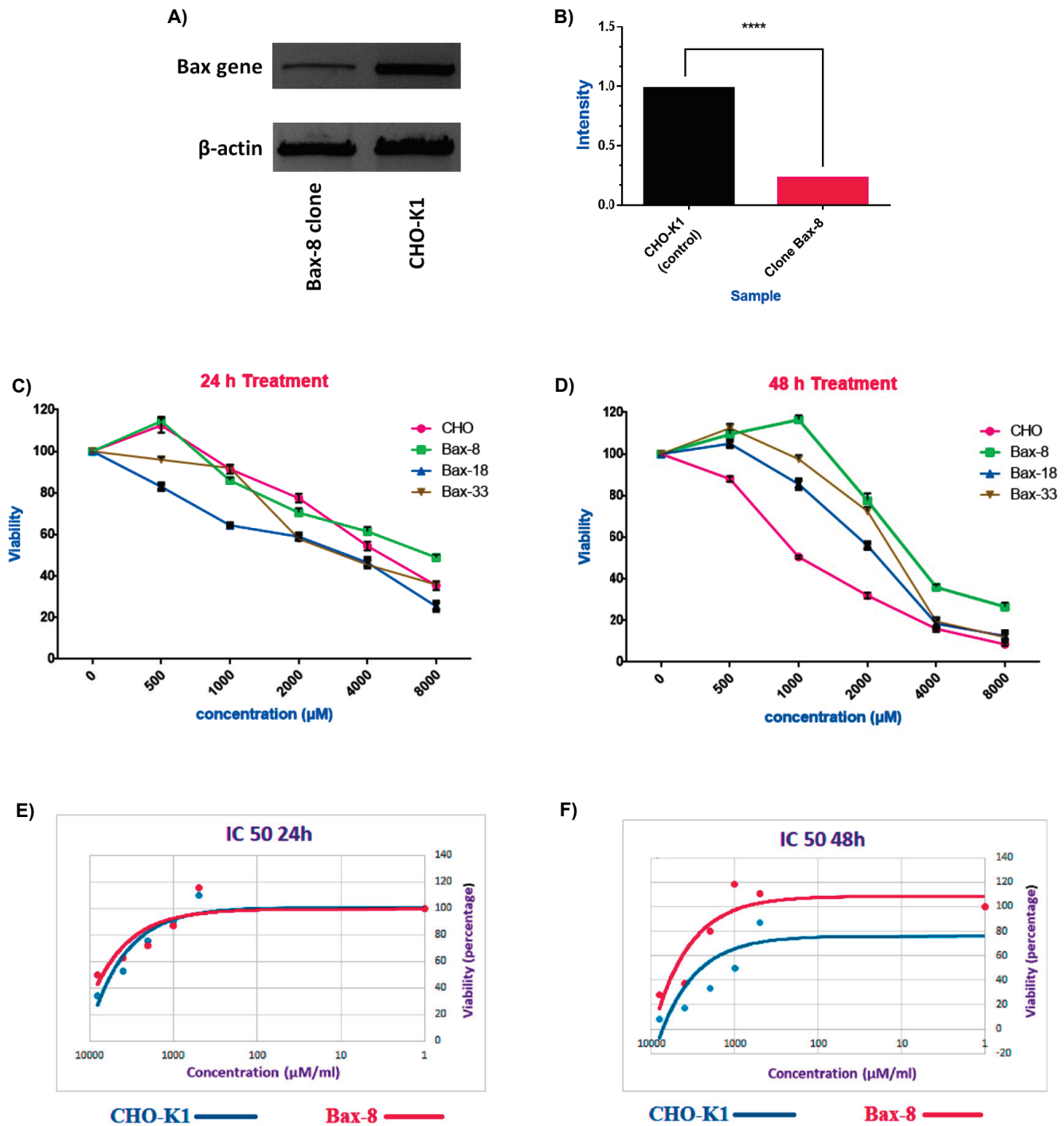
Figure 2. The results of sequence analysis of obtained clones, cell proliferation assay, and mRNA expression. A) The sequence of the non-transfected CHO-K1 (control) and **B)** edited clone (Bax-8 cell line). **C)** Multiple sequence alignment of three single clones (Bax-8, 18, 33) was shown compared to the control CHO-K1 cells. **D)** Bax gene disruption interfered with the cell proliferation of manipulated cell lines compared to the control. **E)** The expression profile of the Bax gene in different manipulated cell lines (Bax-8, Bax-18, and Bax-33) compared with the control cell line.

Figure 3. Evaluation of Bax protein expression and viability of three different manipulated clones. **A**) Compare Bax protein intensity between CHO-K1(control) and clone Bax-8 (individual manipulated cell) (up), and compare β-actin intensity between CHO-K1(control) and clone Bax-8 (down), **B**) Western blot analysis was shown the expression level of Bax protein in CHO-k1 (as control) and manipulated cell (Bax-8). The viability of three different manipulated clones (Bax-8, Bax-18, and Bax-33) and control was assessed in the presence of different concentrations of oleuropein after 24 h **A**) and 48 h **B**). Comparison of the IC50 value Bax-8 clone with control in the presence of 500, 1,000, 2,000, 4,000, and 8,000 µM.mL^{-1} of oleuropein **C**) at T=24 and **D**) at T=48 h.

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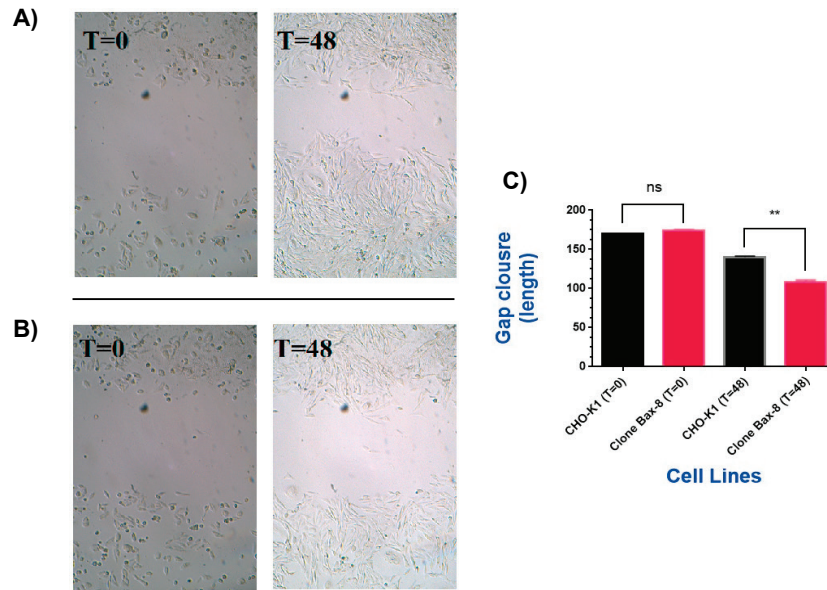**Iran. J. Biotechnol.** April 2023;21(2): e3388

Figure 4. **Scratch wound healing proliferation assay**. **A**) Bax-8 clone in the presence of 2,000 µM oleuropein at T=0 and T=48, **B**) The control cell line in the presence of 2,000 µM oleuropein at T=0 and T=48. **C**) There was a significant difference in gap closure between the control and Bax-8 clone.
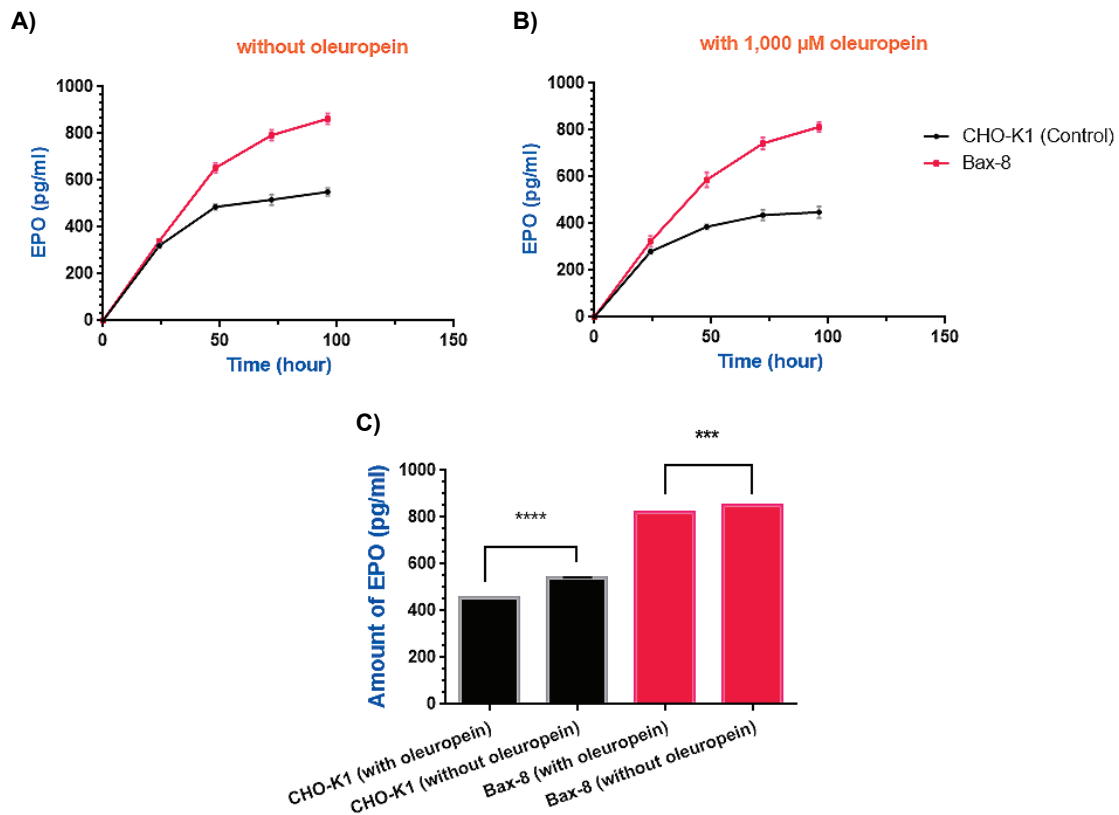

Figure 5. Erythropoietin production. **A**) Comparison between CHO-K1 cell (control) and clone Bax-8 (manipulated cell) in the medium without oleuropein, and **B**) in the medium of 1,000 µM oleuropein. **C**) There was a difference in erythropoietin production in both conditions but less in the manipulated cell line.

The STRING database is one of several online resources dedicated to educational communication networks at the organismal level (21).

So first, through the STRING database, which is a database of known and predicted protein interactions (21). These interactions include direct (physical) and indirect (functional) connections and originate from computational prediction, knowledge transfer between organisms, and interactions collected from other databases. Then, we check the target and its route. Using this database, we surveyed the apoptosis network and did the knocking out of this gene disrupts this network, and as a result, is apoptosis delayed or not.

Although various expression system exists, mammalian cells remain the principal hosts for recombinant protein production. Many biopharmaceuticals are produced commercially in immortalized CHO cells due to their safety for use in humans (23). But, one of the problems in the CHO cell production system is the low-stress tolerance of the cells in bioreactors and an inability to maintain the viability of the culture over an extended period, which affects productivity in the industry. Continuous attempts have been devoted to improving the utility of CHO cells as hosts for commercial recombinant protein production concerning enhancing the protein yield (23). Genome editing is increasingly used to improve resistance to environmental stresses during protein production in the biopharmaceutical industry (22). Because programmed cell death is genetically controlled, in the present study, we exploit CRISPR technology to manipulate the genes involved in apoptosis to prolong cell viability. For this purpose, we first performed a computational analysis of pro-apoptotic genes to find the main regulator to be selected for site-specific gene disruption.

Cell death in bioreactors can occur due to apoptosis and has always caused problems in the large-scale culture of mammalian cells to produce recombinant proteins (11). It is responsible for 80% of cell deaths in the Chinese hamster ovary (CHO). An enormous challenge during the production process is the maintenance of viable cultures. The focus has mainly been on increasing the specific product and extending the culture′s longevity by preventing programmed cell death. It is of considerable value to down-regulate or prevent apoptosis in culture to facilitate extended culture lifespan and increased volumetric production. Genetic engineering strategies, such as overexpression of anti-apoptotic survival genes

or down-regulation of apoptotic effector molecules implemented in some research centers for increasing yields (23).

Many attempts have been done for gene knock-down using small interfering RNAs (siRNA) and microRNA (miRNA), but off-targeting and temporary inactivation of the gene of interest prevented these methods. A recent comparative study showed that CRISPR has far fewer off-target effects than RNAi (24). For example, siRNA inhibition of PDK1 only confers ~32% reduction of mRNA level. And also, a high degree of gene knock-down may require multiple selection rounds (25).

CRISPR/Cas9 systems have demonstrated promise as a versatile tool to potentially make these genomic engineering practices more routine in industrial biotechnology. The cost savings of the new technique is also substantial (9). The advantages of the CRISPR/Cas9 system include its ease of RNA design for new targets, the dependency on a single, constant Cas9 protein, and the ability to address many targets simultaneously with multiple guide RNAs (26). As a tool for optimizing culture yield, this study exploited a CRISPR/Cas9 technology to generate site-specific gene disruptions in the BAX gene, which acts as apoptotic regulators to prolong cell viability in CHO cell line producing erythropoietin. Our data showed that disruption of the BAX gene and consequently enhancing the longevity of the cell culture could be successfully achieved using the CRISPR/Cas9 system. Furthermore, genome editing enables the engineering of cell lines, such as CHO cells, that are widely used to produce therapeutic proteins. Xiong *et al*. found that CRISPRi can be successfully applied to repress Bak and Bax genes and rescue CHO cells from cell stress-induced apoptosis (7). Shen *et al*. enhanced recombinant protein production by CRISPRi in CHO cells without impeding cell growth (27). Although the mechanism CRISPRi occurs at the DNA level, it allows the silencing of genes without permanently knocking out the gene. It may be masked by the unedited cells within the edited cell population.

Gene editing by CRISPR/Cas9 may alter the fitness of edited cell lines. In some cases, genome editing renders a growth advantage. For example, the Bax-8 cell line in our study shows increasing cell proliferation and decreasing Bax gene expression in cascade apoptosis. Vice versa, the Bax-18 cell line shows increased cell proliferation and Bax gene expression. It is due to

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the effect off-target of the CRISPR system on gene manipulation (this is one of the disadvantages of the CRISPR/Cas9 system).

Oleuropein (olive-derived polyphenol with pharmacological properties) showed anticancer, anti-oxidative, and anti-inflammatory effects (28, 29). It reduces cell growth by stimulating extrinsic and intrinsic apoptosis pathways (30). Antognelli *et al.* indicate that oleuropein can induce apoptosis in A549 cells (31). Yan *et al.* indicated that oleuropein effectively inhibited cell viability and induced apoptosis in HepG2 human hepatoma cells (20), and there is no report on its toxicity (29). As our result, treatment of manipulated cell lines with a high concentration of oleuropein for 24 h and 48 h enhanced the number of manipulated cells (Bax-8) comparing the non-manipulated cells.

We showed that CRISPR/Cas9 could repress the expression of the Bax gene in cascade apoptosis and rescue CHO cells from cell stress-stimulated apoptosis. We also used the scratch wound healing proliferation assay to detect cell proliferation in the presence of oleuropein (a stimulant of apoptosis) in cells in high-density conditions. Results showed that cell density was significantly increased by manipulating the Bax gene even in the presence of apoptotic stimuli (152%, P-value=0.0002). The high-level production of recombinant proteins is directly correlated with a high density of cells. The density of cells is closely tied to cell environmental conditions. By manipulating apoptotic genes in unfavorable conditions and in the presence of an apoptotic inducer, recombinant protein-producing cell lines can produce more culture yield. This suggests that the inhibition of apoptosis in the manipulated cells enabled them to retain their cellular activity for a longer time to allow for cell growth and EPO production. These results can help the pharmacological industry and biotechnology that produce more products and decrease the cost of vaccines and recombinant proteins. This is directly related to human and social health.

6. Conclusion

Some of the most critical threats facing the large-scale biomanufacturing and pharmaceutical industries include low efficiency of protein expression and purification, mainly due to the low genetic robustness of the current industrial host cells. Therefore, exploiting alternative genome editing strategies such as CRISPR have been suggested to generate novel variants of existing host cells to improve the most appropriate economic traits that result in a safe, feasible, and vigorous manufacturing operation with a yield that meets the industrial requirements. We successfully found that and rescued CHO cells from cell stress-induced apoptosis. The current study revealed that improved stress tolerance in CHO cells could be obtained by CRISPR/Cas9 mediated repression of the Bax gene, one of the key regulators interacting with the caspase system causing the apoptotic switch. We further confirmed that obtained deferred cascade apoptosis can shorten the development process by improving the host cell′s lifetime and making the scale-up manufacturing process more straightforward. These results can create an opportunity for more efficient, rapid, and feasible production of vaccines or recombinant proteins.

Conflict of Interest
All of the authors declare that they have no conflict of interest.

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