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Engineering of the *Caspase-3* Gene in Recombinant *CHO* Cells Caused More Apoptosis Resistance and enhanced Recombinant Protein Production Than the *BAX* Gene

Amirabbas Rahimi[1,2], Morteza Karimipoor[1], Reza Mahdian[1], Atefeh Alipour[3], Saadi Hosseini[2], Marzieh Mohammadi[1], Hooman Kaghazian[4], Hosein Shahsavarani[5], Mohammad Ali Shokrgozar[2*]

[1]Molecular Medicine Department, Pasteur Institute of Iran, Tehran, Iran; [2]Laboratory of Regenerative Medicine and Biomedical Innovations, National Cell Bank Department, Pasteur Institute of Iran, Tehran, Iran; [3]Department of Nano-Biotechnology, Pasteur Institute of Iran, Tehran, Iran; [4]Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, Tehran, Iran; [5]Department of Cell and Molecular Biology, Shahid Beheshti University, Tehran, Iran

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ABSTRACT

Background: *BAX* and *caspase-3* are essential genes in the apoptotic pathway of cells, promoting the apoptotic cascade through different mechanisms. Inhibition of these genes can increase the longevity of cells in cell culture. This study aimed to compare the effects of CRISPR-Cas9-mediated knockdown of *BAX* and *caspase-3* genes on apoptosis inhibition, cell lifespan, and EPO production in CHO cell lines.
Methods: The *BAX* and *caspase-3* gene expression was evaluated in the rCHO cell lines producing EPO using the CRISPR-Cas9 method. Their anti-apoptotic effects and the level of EPO expression were also compared. In addition, OP as an apoptosis inducer, was introduced to the manipulated cell line to assess the stability and viability of the manipulated cell lines.
Results: The rCHO cells with the manipulated *BAX* gene exhibited a higher cell density than those with the manipulated *caspase-3* gene (152% vs. 142%). Despite the increased cell density in the cells with the *BAX* gene manipulation, EPO production was higher in the cells with the manipulated *caspase-3* gene (1.58-fold increase in the *BAX*-manipulated cells compared to a 1.70-fold increase in the *caspase-3*-manipulated cells).
Conclusion: Our observations suggest that the downregulation of the *BAX* and *caspase-3* genes using the CRISPR method, inhibits apoptosis and enhances the yield of recombinant EPO, even in the presence of an apoptosis inducer. Additionally, reduction of *caspase-3* expression was proved to be more effective than *BAX* in extending the lifespan of cells and producing heterologous recombinant proteins. ***DOI: 10.61882/ibj.4934***

Keywords: Apoptosis, *BAX*, *Caspase-3,* CRISPR-associated protein 9

Corresponding Author: Mohammad Ali Shokrgozar
Laboratory of Regenerative Medicine and Biomedical Innovations, National Cell Bank Department, Pasteur Institute of Iran, Tehran, Iran;
Tel./Fax: (+98-21) 66953311; E-mail: shokrgozar1967@gmail.com; ORCID ID: 0000-0001-5123-4155

INTRODUCTION

Recombinant Chinese hamster ovary cells are widely used for the production of therapeutic proteins in human[1,2]. However, apoptosis is a major challenge in the industrial cultivation of these cells, as it leads to cell death, resulting in low yields of recombinant protein production[3] and decreased product quality[1]. Preventing apoptosis through gene

List of Abbreviations:
BCL-2: B-cell lymphoma-2; **CHO:** Chinese hamster ovary; **CRISPR-Cas9:** clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9; **EPO:** erythropoietin; **MTT:** 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; **OP:** oleuropein; **rCHO**: recombinant Chinese hamster ovary

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engineering could extend culture periods and increase product yields[4].

Apoptosis is mediated by specific proteins in cells. *BAX* plays a crucial role in the regulation of apoptosis, while *caspases* are recognized as the executioners of this process, inducing morphological changes that are characteristic of apoptosis[5,6]. One strategy to improve recombinant protein production yield in industrial cells is to disrupt the function of pro-apoptotic genes using genome manipulation tools, which can prolong lifespan of the cell[7]. Among the various genome-editing tools, the CRISPR-Cas system has effectively been utilized in CHO cells[8-11].

In our previous studies, we manipulated the rCHO cell line producing EPO using CRISPR-Cas9 to knockdown the *BAX* and *caspase-3* genes, resulting in the establishment of two stable clones[12,13]. Herein, we employed OP as a small molecule model to evaluate the effects of apoptosis inducers on the manipulated cell and assess the resistance of the manipulated cell line to apoptosis (Fig. 1). The present study aimed to compare the effects of manipulating the *BAX* and *caspase-3* genes on the growth kinetics of the rCHO cell line, as well as the expression levels of the heterologous recombinant protein, to establish a stable cell line.

MATERIALS AND METHODS

Cell culture

The adherent rCHO cell line producing human EPO was a gift from the Production and Research Complex of the Pasteur Institute of Iran. The manipulated stable cell lines with indel formation in *BAX* (*BAX*^{Mut} cells) and *caspase-3* (*caspase-3*^{Mut} cells) were grown in a DMEM-F12 medium supplemented with FBS (10%; Gibco, USA) and penicillin/streptomycin (1%; Sigma-Aldrich, USA) in an atmosphere containing 5% CO_2 at 37 °C. Unmanipulated cells were used as control. *BAX*^{Mut}, *caspase-3*^{Mut}, and control rCHO cells (5×10^5) were cultivated in T-25 flasks and incubated for 72 h without refreshing the culture medium. Then, viable cell density was calculated by differentiating the live from the dead cells using the trypan blue dye exclusion method and counting the cells with an improved Neubauer hemocytometer[12].

Cell morphology

The *BAX*^{Mut} and *caspase-3*^{Mut} cells were fixed using 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 20 minutes. The cells were then washed twice with PBS (BIO-IDEA, Iran), stained with Wright-Giemsa stain (Sigma-Aldrich) for 10 minutes, and washed again three times with PBS. Finally, the morphology of the cells was examined under a light inverted microscope (ZEISS, Germany).

Extraction and evaluation of DNA quality

The *BAX*^{Mut}, *caspase-3*^{Mut}, and control cell lines were treated with 4,000 µM of OP (DroHerb, China). After 72 hours, the genomic DNA was isolated using a modified salting-out method[14] and analyzed for fragmentation by loading it onto a 1% agarose gel (Sinaclon, Iran).

Scratch assay

The *BAX*^{Mut}, *caspase-3*^{Mut}, and control cells were seeded in 24-well plates. After manually scratching a cell monolayer, the medium was replaced with fresh media containing 2,000 µM of OP. The closure of the scratch and cell proliferation were monitored using ImageJ software for 48 hours.

MTT assay

BAX^{Mut}, *caspase-3*^{Mut}, and control cells (5×10^3) were seeded in the 96-well plates in triplicate. Subsequently, the cells were treated with 500, 1,000, 2,000, 4,000, and 8,000 µM of OP for 24 and 48 h. All the cells were incubated with 0.5 mg/ml of MTT solution in the dark at 37 °C for 4 h. After incubation, the formazan crystals were dissolved by adding 150 µl of isopropanol per well. The absorbance of the resulting colored solution was then measured at a wavelength of 570 nm using a microplate reader[12,13,15]. Finally, the toxic effects of OP were evaluated by determining the IC_{50} values on the manipulated cells.

Apoptosis assay

BAX^{Mut}, *caspase-3*^{Mut}, and control cells (100×10^3) were incubated separately with 2,000 µM of OP and 2.5% DMSO as the control of the apoptosis inducer, for 48 hours. After incubation, the cells were harvested, centrifuged at 300 ×g for 5 min and washed twice with cold PBS. Each cell line was then resuspended in 100 µl of Annexin V binding buffer. Subsequently, 3 µl of each of phycoerythrin and 7-AAD viability staining solution were added, and the samples were gently vortexed before incubating in the dark at room temperature for 15 min. Finally, 400 µL of Annexin V binding buffer was added to each tube. The apoptotic rate of the cells was analyzed using flow cytometry (CyFlow®/Germany), which requires a minimum of 20,000 cells for accurate analysis.

Measurement of EPO concentration

The *BAX*^{Mut}, *caspase-3*^{Mut}, and control cell lines (1.5×10^5) were grown in T-flasks. After one day, the medium was replaced with a serum-free production medium, at the presence or absence of 1,000 µM of OP. The secretion of EPO in the supernatant was measured by the EPO ELISA Kit (Antibodies-Online GmbH, Germany) according to the manufacturer's

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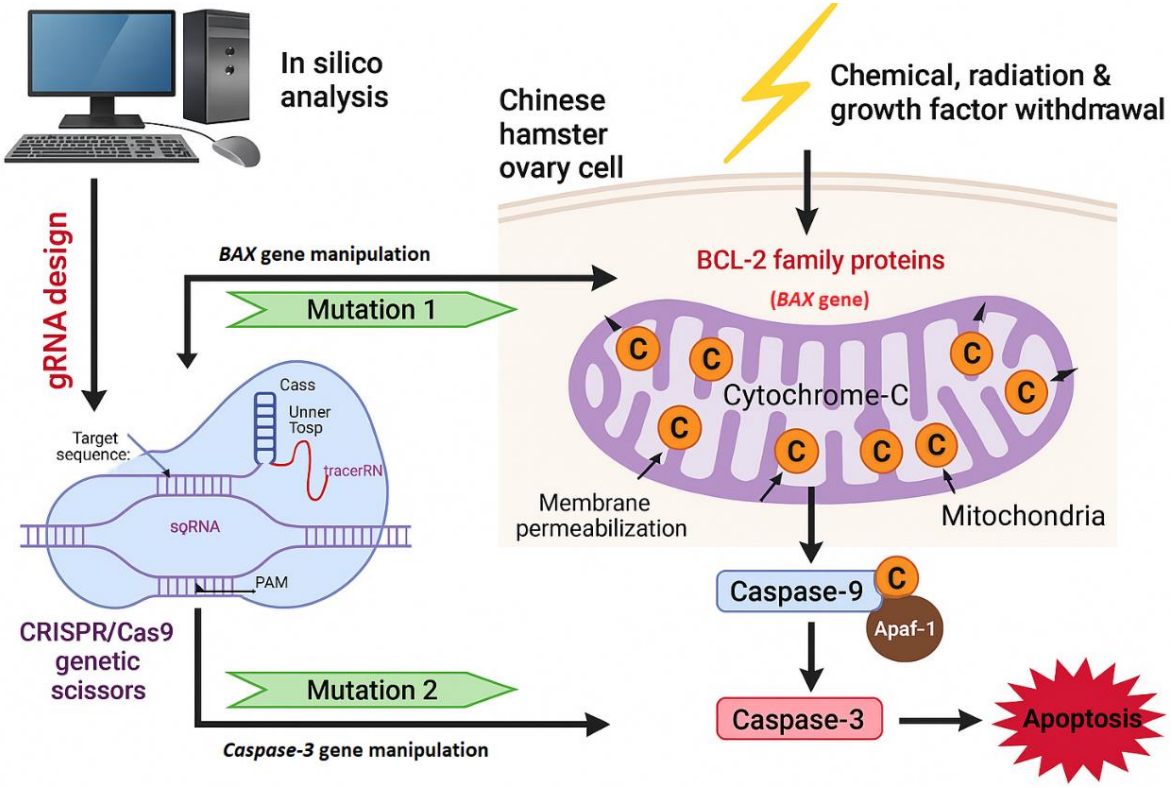

Fig. 1. Schematic illustration of manipulating the *BAX* and *caspase-3* genes using the CRISPR-Cas9 system and the events occurring in the apoptosis pathway.

guidelines at the following time points: 0, 24, 48, 72, and 96 hours[12].

Study of growth curve

BAX^{Mut}, *caspase-3*^{Mut}, and control were cultured separately in triplicate in six-well plates, with and without OP (2,000 µM). Then, the cells were counted after staining with trypan blue at 24, 48, 72, and 92 hours.

Statistical analysis

The data were analyzed using the student's t-test for two study groups or one-way analysis of variance (ANOVA) for more than two groups by GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA). Results with $p < 0.05$ were considered statistically significant.

RESULTS

From our previous research, we selected two EPO-producing CHO cells with modified *BAX* and *caspase-3* genes, designated as *BAX*^{Mut} and *caspase-3*^{Mut}. These modified cell lines express lower levels of *BAX* and *caspase-3* compared to the control[12,13]. The *BAX* mRNA expression was observed to be 20-fold lower than the control ($p < 0.0001$), and the *Caspase-3* mRNA expression was found to be 2.7-fold lower than the control ($p < 0.0005$).

Mutated cells affected the growth of the cells

The growth of *BAX*^{Mut}, *caspase-3*^{Mut}, and control cells was evaluated by counting the cells in T-25 flasks at different time points. Figure 2 illustrates the cell density of the control compared to the mutated cell lines after 72 h of incubation. The cell densities of *BAX*^{Mut} and *caspase-3*^{Mut} demonstrated significant differences from the control and even from each other (152% for *BAX*^{Mut} [$p = 0.0002$] vs. 142% for *caspase-3*^{Mut} [$p < 0.0017$]).

Scratch test results

In the cell culture media containing 2,000 µM of OP, the proliferation rate and gap closure in the scratch test showed that the proliferation rate of *caspase-3*^{Mut} cells is more than that of the *BAX*^{Mut} cells (Fig. 2B). Evaluation of morphological changes after 72 hours, without refreshing the medium and using Wright-Giemsa staining, showed that *caspase-3*^{Mut} and *BAX*^{Mut} cells displayed less nuclear condensation, decreased cell fragmentation, fewer apoptotic bodies and cytoplasmic blebs as well as less separation from other cells[16] compared to the control cells (Fig. 2C).

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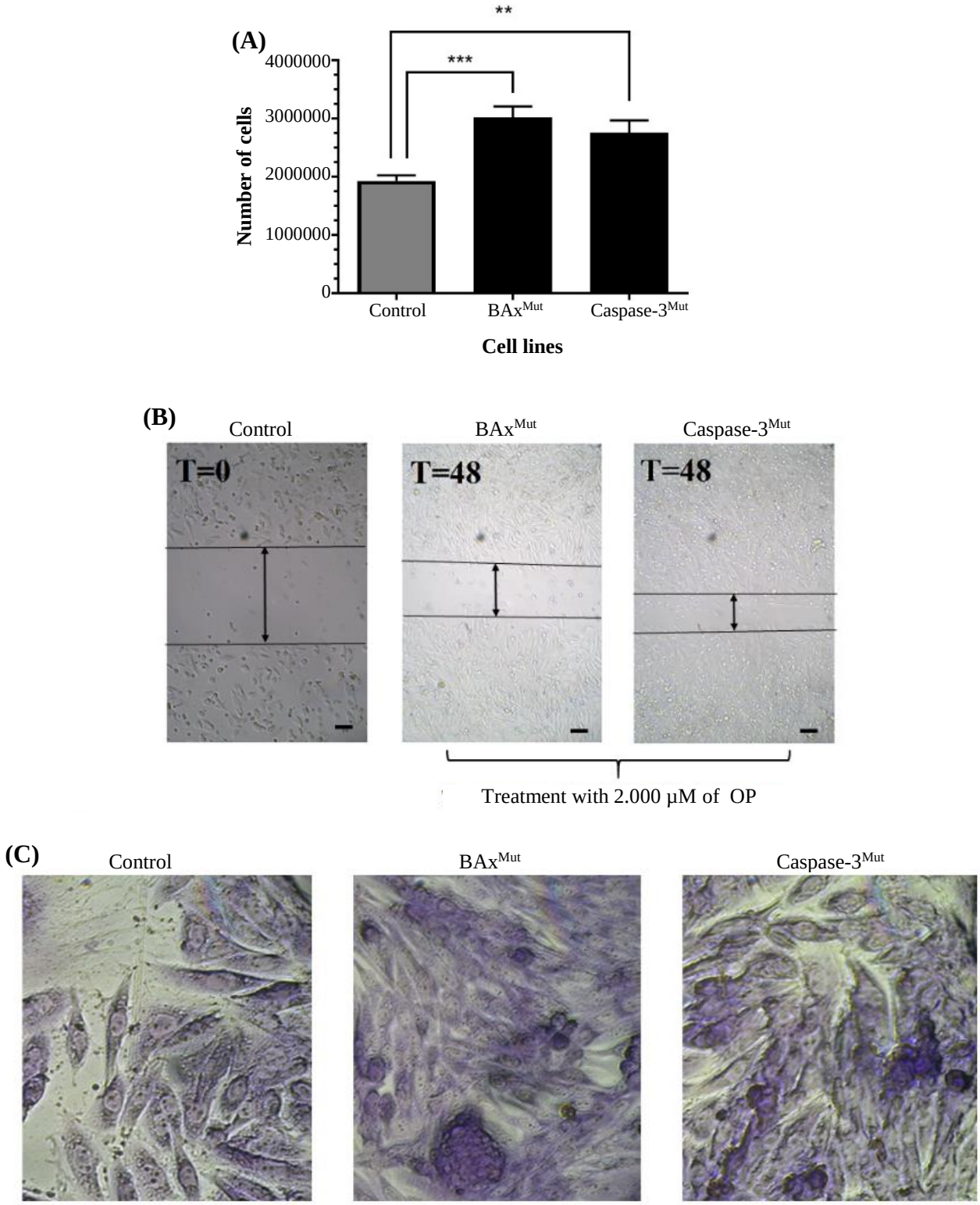

Fig. 2. Assessment of proliferation rate and cell morphology. (A) Proliferation rate of mutated cell lines was compared to that of unmanipulated cells (152% for *BAX* [$p = 0.0002$] and 142% for *caspase-3* [$p < 0.0017$]). No significant difference in cell density was observed in *BAX*^{Mut} and *caspase-3*^{Mut} cells without OP. ($^{**}p \leq 0.01$ and $^{***}p \leq 0.001$). (B) Evaluation of the proliferation rate and gap filling after treatment with 2,000 µM of OP indicates that the *caspase-3*^{Mut} cells fills the gap faster than the *BAX*^{Mut} cells (scale bar = 50 µM). (C) Morphological evaluation of cells after 72 hours without medium refreshment was conducted using Wright-Giemsa staining. The results showed that the cells with manipulated *BAX*^{Mut} and *caspase-3*^{Mut} genes exhibited less nuclear and cytoplasmic condensation compared to control cells.

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Cell viability and IC_{50} value

The *BAX*^{Mut} and *caspase-3*^{Mut} cells were incubated with different concentrations (500, 1,000, 2,000, 4,000, and 8,000 µM) of OP for 24 and 48 hours, to evaluate their apoptosis resistance (Fig. 3A and 3B). Both cells showed a significant resistance to apoptosis. The results displayed that the deficiency of *BAX* and *caspase-3* in the rCHO cells reduced apoptosis and extended the lifespan of *BAX*^{Mut} and *caspase-3*^{Mut} cells (Fig. 3C-3J). Following 24 and 48 hours of incubation with the mentioned concentrations of OP, the IC_{50} values for *BAX*^{Mut} and *caspase-3*^{Mut} cells were compared (Fig. 3K-3L). The findings revealed that the IC_{50} of the mutated cells increased with the downregulation of *BAX* and *caspase-3* expression, indicating an inverse correlation between IC_{50} and the *BAX* and *caspase-3* expression level[12,13]. After 24 and 48 hours of treatment with OP, the IC_{50} for *caspase-3*^{Mut} cells was higher than that for *BAX*^{Mut} cells. The IC_{50} of *caspase-3*^{Mut} cells was 7271 µM, whereas that of *BAX*^{Mut} cells was 6986 µM after 24 hours of incubation with OP. After 48 hours of incubation, the IC_{50} values were 5,742 µM and 5,100 µM for *caspase-3*^{Mut} and for *BAX*^{Mut} cells, respectively.

Manipulated cells showed different EPO production

The quantity of *EPO* in *BAX*^{Mut} and *caspase-3*^{Mut} cells was higher than that in control cells, and these mutant cells were less affected by environmental conditions (Fig. 4A and 4B). After 96 hours of incubation with and without 1,000 µM OP, a significant difference was observed in the amount of EPO production. The increase in EPO production was higher in the *caspase-3*^{Mut} cells compared to the *BAX*^{Mut} cells in the absence of OP (867 pg in the *BAX*^{Mut} cells vs. 925 pg in *caspase-3*^{Mut} cells; $p = 0.0007$). Both the *BAX*^{Mut} and *caspase-3*^{Mut} cells showed greater resistance to apoptosis induced by OP, enabling them to survive longer and produce more EPO after 96 h (778 pg in the *BAX*^{Mut} cells vs. 900 pg in *caspase-3*^{Mut} cells; $p < 0.0001$; Fig. 4C).

Apoptosis decreased in the manipulated cells

A flowcytometry technique was utilized to monitor apoptosis in *BAX*^{Mut} and *caspase-3*^{Mut} cells. The results confirmed a reduction in the expression of *BAX* and *caspase-3* genes, indicating decreased apoptosis in the manipulated cells after treatment with 2,000 µM of OP and 2.5% DMSO, as apoptosis inducers (Fig. 5A-5D). Specifically, the percentage of early and late apoptosis in the manipulated cell lines decreased to 7.3% in *BAX*^{Mut} cells and 4.86% in *Caspase-3*^{Mut} cells, as compared to 12.49% in cells treated with 2.5% DMSO and 15.25% in those treated with 2,000 µM of OP. We observed significant morphological alterations, including loss of normal shape and morphology, shrinkage, and nuclear condensation, especially in the unmanipulated cells treated with 2,000 µM of OP for 48 hours. In contrast, these morphological features were less apparent in the manipulated *BAX*^{Mut} and *caspase-3*^{Mut} cells (Fig. 5E-5H).

Cell growth curve and DNA analysis results

In the analysis of the manipulation of the *BAX*^{Mut} and *caspase-3*^{Mut} genes to draw the growth curve, a high density of cells was observed when the *caspase-3* gene was mutated after 96 hours of incubation with 2,000 µM of OP. This result indicated that cells with manipulated *caspase-3* gene showed higher density (Fig. 5I-5J). Furthermore, the investigation of nuclear DNA revealed that both *BAX*^{Mut} and *caspase-3*^{Mut} DNAs exhibited less fragmentation compared to the control cells (Fig. 5K).

DISCUSSION

CHO cell lines are used in the biopharmaceutical industry for the production of recombinant proteins[17]. However, cellular tension-induced apoptosis can lead to reduced protein yields[18,19], which affects viable cell density and duration of cell culture, ultimately lowering the yields of recombinant protein[3]. Various strategies and techniques have been implemented to improve protein yields, extend the lifespan of cell cultures, and increase viable cell density[20]. Genetic engineering techniques are designed to enhance protein production, prolong cell culture life, and create stable proteins[21].

Apoptosis is triggered by genes such as *BAX*, *BAK*, *BID*, and *caspases*[22-26]. The *BAX* gene promotes apoptosis by releasing cytochrome C[25,27-29] and activating the APAF-1 protein in response to environmental stressors[24]. This process is an essential step in the activation of the apoptosis pathway[23]. *Caspase-3*[24] and *caspase-6*[30] play crucial roles in DNA fragmentation, leading to the formation of apoptosomes, activation of downstream caspases, and programmed cell death[24,28].

Studies have shown that manipulating genes associated with apoptosis through CRISPR interference can result in increased cell density[18], enhanced recombinant protein production[31], reduced apoptosis[32], improved viability of CHO cells via RNA interference repression[5], and a high quantity and quality of CHO products using CRISPR-Cas9[33]. *BAX*, a gene with strong apoptosis-promoting capacity, can be manipulated to prevent apoptosis progression in the intrinsic pathway[4,22] and regulate *caspase* activity[23,34,35]. Modifications of the BCL-2 pathway have been shown to enhance culture performance[36], resulting in increased erythropoietin produced by CHO

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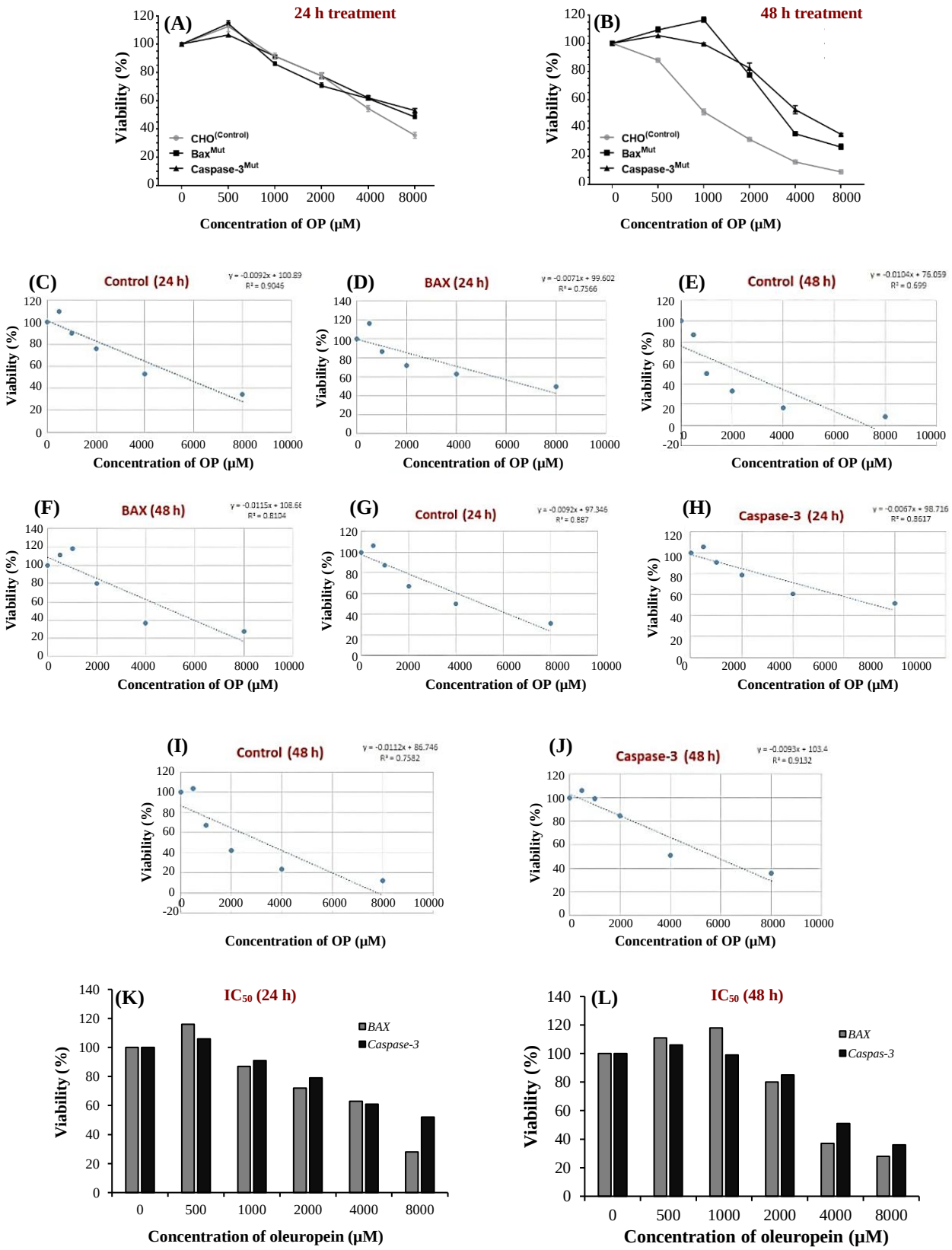

Fig. 3. Viability of the mutated and control cell lines measured after 24 (A) and 48 (B) hours of treatment with OP. The results of IC_{50} values at the presence of OP after 24 and 48 hours in the *BAX*^{Mut} (C-F) and the *caspase-3*^{Mut} (G-J) cells The IC_{50} values for *BAX*^{Mut} and *caspase-3*^{Mut} cells after 24 (K) and 48 (L) hours of incubation with OP showed significant differences in viability ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$).

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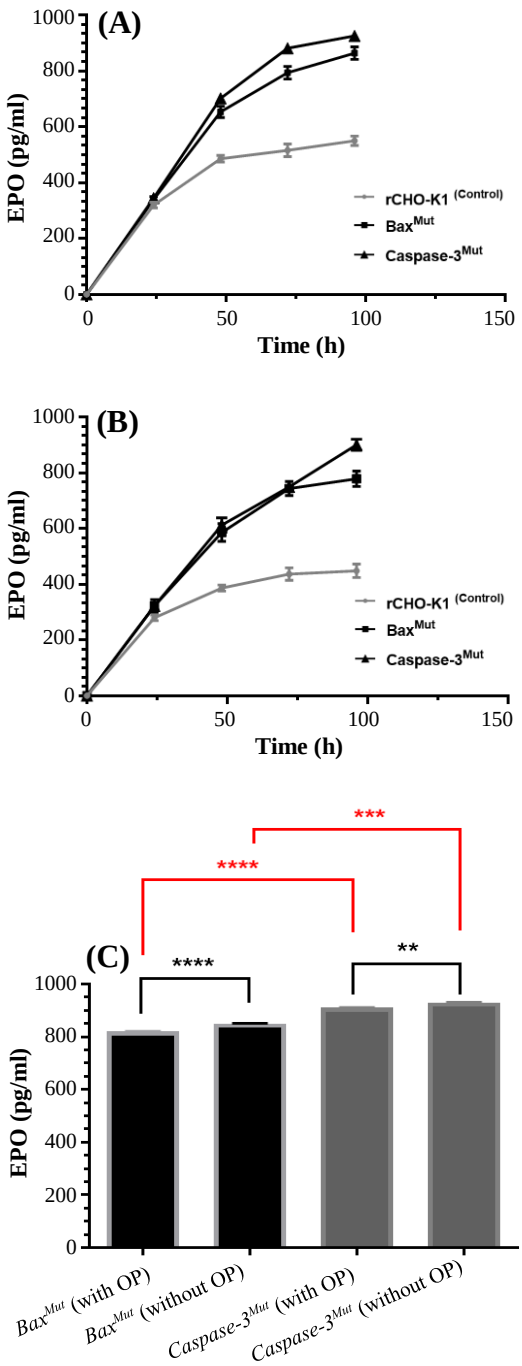

Fig. 4. EPO production in (A) *BAX*^{Mut} and *caspase-3*^{Mut} cell lines without OP and (B) medium containing 1,000 µM of OP. The amount of EPO was reduced in the cell line with the edited *caspase-3* gene compared to the cell line with the edited *BAX* gene ($^{**}p \leq 0.01$, *** = $p \leq 0.001$ and $^{****}p \leq 0.0001$).

cells and decreased expression of the mutated *BAX*^{Mut} gene in the mutated cells[13]. *Caspase-3* has a crucial role in apoptosis[20], affecting cell density and viability as much as 40%[32]. Research indicates that the knockdown of *caspase-3* in CHO cells can increase cell density up to 40%. Mutated rCHO-K1 cells exhibited a 1.42-fold increase in density and a 2.7-fold decrease in *caspase-3*^{Mut} mRNA expression[12]. These mutated cells demonstrated longer growth periods than control, likely due to the impact of the *caspase-3* gene on apoptosis, as this gene is located at the end of the apoptosis pathway.

In this study, we evaluated the effects of manipulating the *BAX* and *caspase-3* genes on recombinant production yield and cell density. The results indicated that downregulating *caspase-3* had a greater impact on suppressing apoptosis compared to downregulating *BAX*. Moreover, in the presence of an apoptosis inducer (OP), cell density increased, EPO production enhanced, and greater resistance to apoptosis occured. Additionally, it was noted that the *caspase-3*^{Mut} cells exhibited a higher IC_{50} than *BAX*^{Mut} cells with increasing the concentration of OP[13].

Understanding the functional relationship between the *caspase-3* and *BAX* genes is crucial for understanding apoptosis and regulating cell fate. While both proteins have distinct roles, research suggests that manipulating the *caspase-3* gene may be more effective in rCHO cells than *BAX* gene in apoptosis. This hypothesis could be further examined using antisense and small interfering RNA techniques targeting both *BAX* and *caspase-3* genes. Exploring these interactions would be essential for optimizing viability, survival, and productivity of producer cells in pharmaceutical and biotechnological applications. Although apoptosis was considered irreversible[37], recent research has shown that blocking specific genes can increase cell survival in response to weak apoptotic signals[18]. The activation of *caspase-3*, along with *caspase-6* and *-7*, presents a critical step in the execution phase of apoptosis, which is more significant than the initiation phase. Inactivating late genes, such as *caspase-3* and *-7*, is an important target for regulating and modifying the apoptotic cascade. In other words, cells in which the *caspase-3* gene has been manipulated exhibit less sensitivity to environmental changes. According to nuclear DNA analysis, *BAX*^{Mut} and *caspase-3*^{Mut} cells exhibit less DNA damage than the control cells. DNA fragmentation acts as a biochemical "point of no return", irreversibly destroying genomic integrity. Caspase-activated DNase cleaves DNA at internucleosomal junctions, preventing cellular recovery and ensuring the completion of apoptosis. Altogether, it can be concluded that CRISPR-mediated editing of the *caspase-3* gene in rCHO cells offers enhanced resistance to apoptosis and increases recombinant protein production compared to *BAX* gene manipulation.

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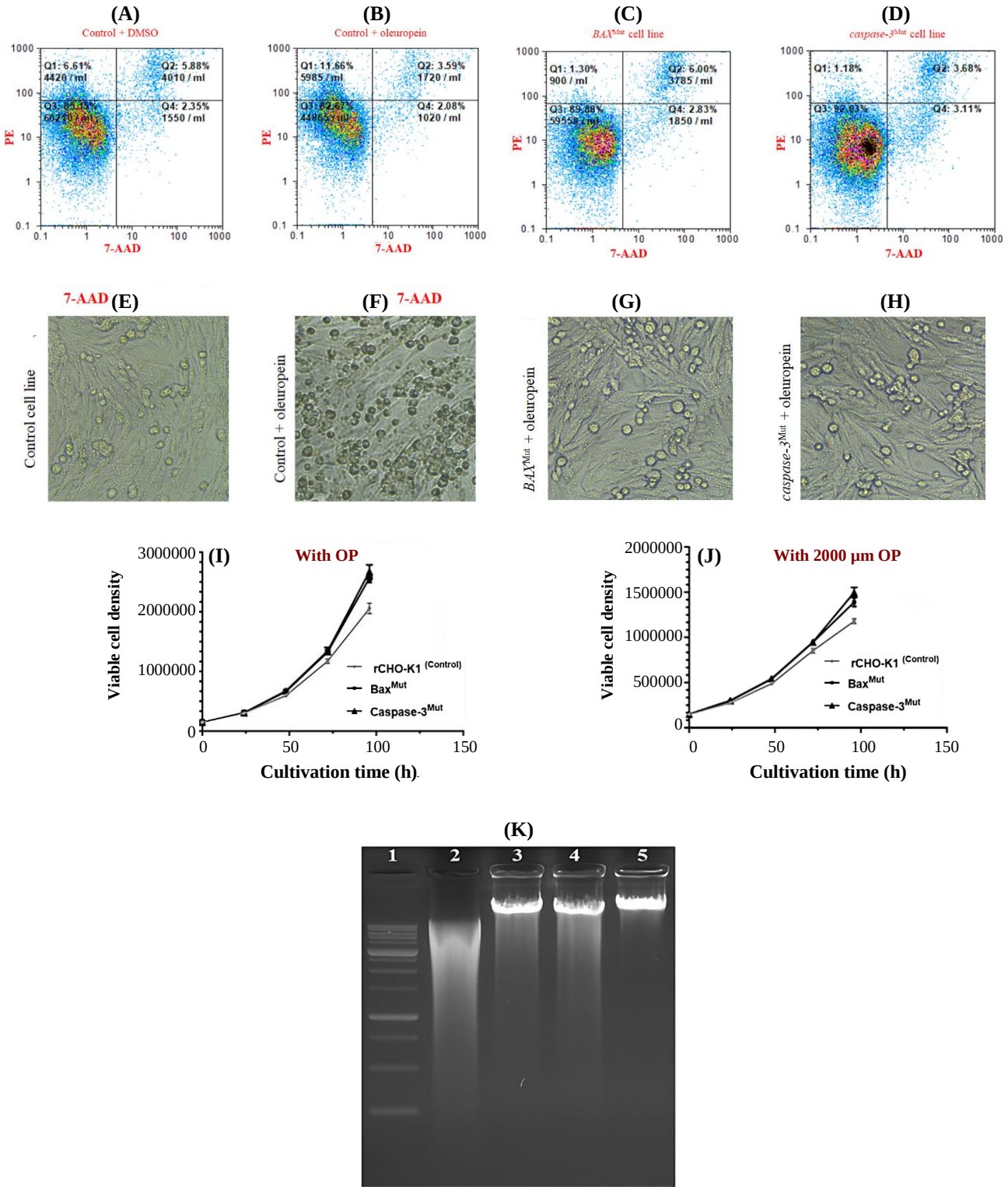

Fig. 5. Flow cytometric, cell morphology, and genomic DNA analysis: (**A**) Control + DMSO (2.5%), (**B**) control + OP (2,000 µM), (**C**) *BAX*^{Mut} cell line + OP, and (**D**) *caspase-3*^{Mut} cell line + OP. Changes were observed in morphology between manipulated and unmanipulated cells following a 48-hour treatment with 2,000 µM OP: (**E**) control cell line without OP, **F**) control + OP (2,000 µM), (**G**) *BAX*^{Mut} cell line + OP (2,000 µM), and (**H**) *caspase-3*^{Mut} cell line + OP (2,000 µM). Growth curve evaluation: the growth curves of the control and mutant cells at 24, 48, 72, and 96 hours, without OP (**I**) and with OP (2,000 µM; **J**). Genomic DNA fragmentation analysis(**K**) from left, lane 1: 1 kb size marker, lane 2: control + 4,000 µM OP, lane 3: *BAX*^{Mut} cell line + 4,000 µM OP, lane 4: *caspase-3*^{Mut} + 4,000 µM OP, and lane 5: control cell line without OP.

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CONCLUSION

This study demonstrated that editing the *caspase-3* and *BAX* genes using the CRISPR-Cas9 tool in rCHO cells producing EPO downregulated their expression, leading to enhanced resistance to apoptosis and increased cell longevity. Although both gene manipulations improved cell survival and protein productivity under apoptotic stress induced by OP, the downregulation of *caspase-3* expression was more pronounced than that of *BAX* in augmenting cell viability and recombinant EPO yield. In comparison to *BAX* mutants, *caspase-3* manipulated showed decreased apoptotic features and increased rates of proliferation, according to morphological evaluations and visibility tests. These findings highlight the critical role of *caspase-3* in the apoptosis pathway and suggest that the knockout of caspase-3 using CRISPR-Cas9 is a more favorable option than *BAX* suppression for enhancing CHO cell line stability and productivity in biopharmaceutical manufacturing. This approach presents a strategy for creating robust cell factories with improved culture longevity and higher yields of recombinant proteins, which are of industrial importance for the large-scale production of therapeutic proteins.

DECLARATIONS

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Ethical approval

All the experimental procedures in this study were approved by the Research Ethics Committee of the Pasteur Institute of Iran, Tehran (ethical code: IR.PII.REC.1401.001).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contribution

AR: performed research, participated in data collection and analysis, and wrote the manuscript; MK: designed the study, supervised, coordinated the experimental work, gave the final approval of the manuscript, wrote the manuscript, and analyzed data; RM: analyzed data; AA, SH, MM, and HK: performed research and participated in data collection and analysis; HS: designed the study, supervised, coordinated the experimental work, gave the final approval of the manuscript, and wrote the manuscript; MS: designed the study, supervised, coordinated the experimental work, and gave the final approval of the manuscript.

Data availability

All relevant data can be found within the manuscript.

Competing interests

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

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Supplementary information

The online version does not contain supplementary material.

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