Iranian Journal of Pharmaceutical Research
Volume 21 · Issue 1 · e130236

OPEN PEER REVIEW

Targeting Caspase-3 Gene in rCHO Cell Line by CRISPR/Cas9 Editing Tool and Its Effect on Protein Production in Manipulated Cell Line

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SUBMITTED	REVISED	ACCEPTED	DOI
30 Jul 2022	19 Nov 2022	12 Dec 2022	10.5812/ijpr-130236

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1 Original submission

4 comments · 9 Aug 2022 – 13 Aug 2022

Referee 1 Reviewer

MAJOR REVISION 9 Aug 2022

1. Please state the type of study, sample size, sampling strategy, and date and country of the study in the abstract.
2. What is your type of study? Is it an experimental study, cross-sectional study, cohort study, case-control study, clinical trial, systematic review, or …? Please mention it in the methods section.
3. The source of the data is unclear. How did you select the sample, how many samples did you select, and what were the inclusion and exclusion criteria?
4. Did you have any inclusion and exclusion criteria? Please describe them and then state how many samples were included and excluded according to these criteria.
5. What was your sample size formula? What is your expected power? Please mention these in the main text.
6. The code and date of ethical approval should be mentioned in the main text. Ethical considerations should also be described in more detail.
7. Please mention the city, country, and date of the study in the methods section.
8. How many observers did you have? If you had more than one observer, you must mention an agreement index, such as the kappa coefficient, in the methods section.
9. Did you check the normality assumption? Please mention it. It seems that some variables do not have a normal distribution, and you must have used a nonparametric method.
10. In the case of non-normal data, the median and IQR better represent the location and dispersion of the data.
11. There are some spelling and grammatical errors in the text. Please correct them.
12. Most of the article's references are out of date (2019 or later). Please use more up-to-date references and arrange the references according to the journal's format.
13. The results must be presented in a table with the mean and SD. A plot alone is not enough.
14. Please present the demographic variables (such as sex and age) of each database in a table by group, compare the groups, and then proceed to the final results.

Referee 2 Reviewer

MAJOR REVISION 11 Aug 2022

The authors are kindly requested to answer the following questions/concerns:

1- The whole manuscript needs serious English revision. Most probably, there are some typos or missing words in the title. The title in its current structure is a bit vague.

2- CHO-k1 is an adherent subclone of the original CHO cell line that is certainly genetically different from CHO-S, the ideal CHO variant used for pharmaceutical production. Results obtained from a manipulated CHO-k1 can't be extrapolated to CHO-S, and as CHO-k1 is not normally used in the pharmaceutical industry, the significance and rationale of such an investigation on the CHO-k1 platform must be explained.

3- One of the major drawbacks of CRISPR systems is their likely off-target modifications. These modifications can affect protein folding, glycosylation, and other factors affecting protein activity. The authors should prove that protein activity (in this case, EPO activity) is unaltered by determining its potency in an EPO-responsive cell line (similar to UT-7) in a cell-based proliferation assay.

4- The main triggers of apoptosis in the pharmaceutical industry are high cell density and hypoxic conditions. These triggers must be simulated in order to check the efficiency of CHO manipulation. Triggering apoptosis using oleuropein is not a proper way to conduct such an appraisal.

5- All industrial cell culturing worldwide is performed using chemically defined media such as ProCHO (Lonza). Using animal-derived FBS, which is not chemically defined, in the culture can introduce variation in protein production, unless the authors state that all FBS used in the investigation is from the same production batch.

Referee 3 Reviewer

MAJOR REVISION 13 Aug 2022

The manuscript contains valuable information. In this study, the authors used CRISPR/Cas9 technology to knock down the caspase-3 gene in a cell line because this gene plays a role in regulating apoptosis. However, the authors should address the following comments:
1. Several regulators play a role in apoptosis. Why did the authors decide to knock down the caspase-3 gene?
2. How can the authors demonstrate that their CRISPR/Cas9 system did not knock down other sites?
3. This manuscript suffers from poor methodology. The authors should explain each section of the methods in detail. Each method section should be supported by at least one reference.
4. This manuscript suffers from a poor Discussion section. In the Discussion section, the authors have to explain the challenges, concerns, and limitations of CRISPR/Cas technology.
5. In the Introduction section, the authors should clearly state the novelty of this study compared with other similar studies.
6. It is suggested that the authors provide a schematic figure illustrating the process of knocking down the caspase-3 gene in the cells.
7. The quality of English should be improved.

Associate Editor

MAJOR REVISION 13 Aug 2022

According to the reviewers' comments, the manuscript entitled "Targeted caspase-3 gene editing of CRISPR/Cas9-Mediated: evaluation in induced CHO cell line by oleuropein" can be published after major revisions.

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Aug 15, 2022

Dear Dr. Soraya Shahhosseini,

Editor-in-Chief, Iranian Journal of Pharmaceutical research

We would like to express our deep appreciation to you and the reviewers for providing us with the insight and direction needed to complete our submitted manuscript under the title of "**Targeting caspase-3 gene in rCHO cell line by CRISPR/Cas9 editing tool and its effect on protein production**" by Amirabbas Rahimi, Morteza Karimipoor, Reza Mahdian, Atefeh Alipour, Saadi Hosseini, Hooman Kaghazian, Abdolrahim Abbasi, Hosein Shahsavarani and Mohammad Ali Shokrgozar.

Please kindly find the revised version of our manuscript in which the revised parts are indicated in red color based upon the replies to invaluable comments of reviewers. We have carefully considered your invaluable comments and questions for preparing a revised manuscript.

However, to some extent, we would like to emphasize the response to your questions and major comments in this reply sheet as follows. We hope our responses to your comments meet your expectations.

Please let us know if you evaluate that this revision is mature enough to be accepted for publication in the internationally renowned Iranian Journal of Pharmaceutical research.

Thanks in advance,

Mohammadali Shokrgozar, PhD

Professor

National Cell Bank

Pasteur institute of Iran

Reviewer 1:

The manuscript contains valuable information. In this study, the authors used CRISPR/Cas9 technology to knock down the caspase-3 gene in a cell line, because this gene has a role in apoptosis as a regulator. However, the authors should address the following comments as follows:

Comment #1:

1. Several regulators have a role in apoptosis. Why do the authors decide to knock down the caspase-3 gene?

Reply to comment #1:

We do appreciate your time in reviewing our work and your precious comments.

We agree with the reviewer that many genes are involved in the apoptosis pathway; for this reason, we performed in silico screening approach in order to find regulator genes in the apoptosis cascade. we tried to select genes that are at the end of the apoptosis cascade. As shown in Fig1 has a significant effect on the cell life cycle. The advantage of caspase-3 inhibition is that paths of apoptosis signaling converge on executioner proteins. Therefore, targeting caspase-3 at the end of the cascade has superiority (3).

Comment #2 :

2. How the authors can demonstrate that their CRISPR/Cas9 system did not knock down other sites?

Reply to comment #2:

Though gene editing approaches including CRISPR/CAS9 tool almost contained off-target binding but various software has been written to evaluate guide RNAs using a different algorithm and remove guides with a high possibility to have off-targets. Here, we have used an off-target finder tool and have selected top-ranked guide RNA expected to have no off-target. In the experimental phase, our preliminary goal was to investigate the effect of construct on Caspase-3 gene expression and viability of the cell. Of course, further investigation such as checking any possible off-targets by next-generation sequencing methods should be used before the implementation of the cell line in the industry.

Comment #3 :

3. This manuscript suffers from poor methodology. The authors should explain each section of the methods in detail. Each method section should be supported with at least one reference.

Reply to comment #3 :

One of the journal's policies is word count, because of this reason, we explained the methods in a minimum of the word. Anyway, the methods were described in more detail in the revised version of the manuscript.

Comment #4 :

4. This manuscript suffers from poor Discussion. In the section of Discussion, the authors have to explain the challenges, concerns, and limitations of CRISPR/Cas technology.

Reply to comment #4 :

Thank you very much for your time and invaluable suggestions. A paragraph was added to the discussion according to the reviewer's comment and highlighted.

Comment #5 :

5. In the section of the Introduction, the authors should clearly mention the novelty of this study over other similar studies.

Reply to comment #5 :

We regret there were problems with less emphasis on the novelty of this project. We would like to inform you that the novelty of our finding has been added to the manuscript and discussed in detail in the introduction and discussion part.

For selecting the best candidate amongst proapoptotic genes, we first used the STRING database (https://string-db.org/) to analyze protein interactions in the apoptotic regulating gene network of the Chinese hamster (Cricetulus griseus) and caspase-3 was drawn up as the top regulator. Thus the caspase-3 gene that was found to be a plausible target for anti-apoptosis engineering was knocked out via CRISPR/Cas9 technology. The idea behind this approach is that cells exhibit higher viabilities in the culture's late stages, which can improve the overall yield. Though there was a report for using CRISPRi for engineering the caspase-3 gene in CHO cells, we tried to permanently knock out this gene and validate our hypothesis in the presence of an apoptotic agent, oleuropein.

We think that our attempt to find one of the top regulator genes responsible for apoptosis to be knocked out by CRISPR/Cas 9 will result in prolonged cell survival and productivity during bioprocess conditions even in the presence of high waste accumulation and nutrient depletion that induce apoptosis. Recently, researchers at R&D department of Novo Nordisk Foundation (Denmark) have reported downregulation of apoptotic gene's endogenous expression led to increased cell yield using the CRISPRi technique (Xiong 2019), but this technique is temporary. Thus we attempt to knock out the gene involved in the apoptosis pathway and obtain a stable cell line in which the apoptosis pathway gene, caspase-3, is **permanently** inactivated.

Comment #6 :

6. The authors are suggested to provide a schematic figure illustrating the process of knocking down the Caspase-3 gene through the cells.

Reply to comment #6:

The following figure was added to the manuscript in the Experimental section.

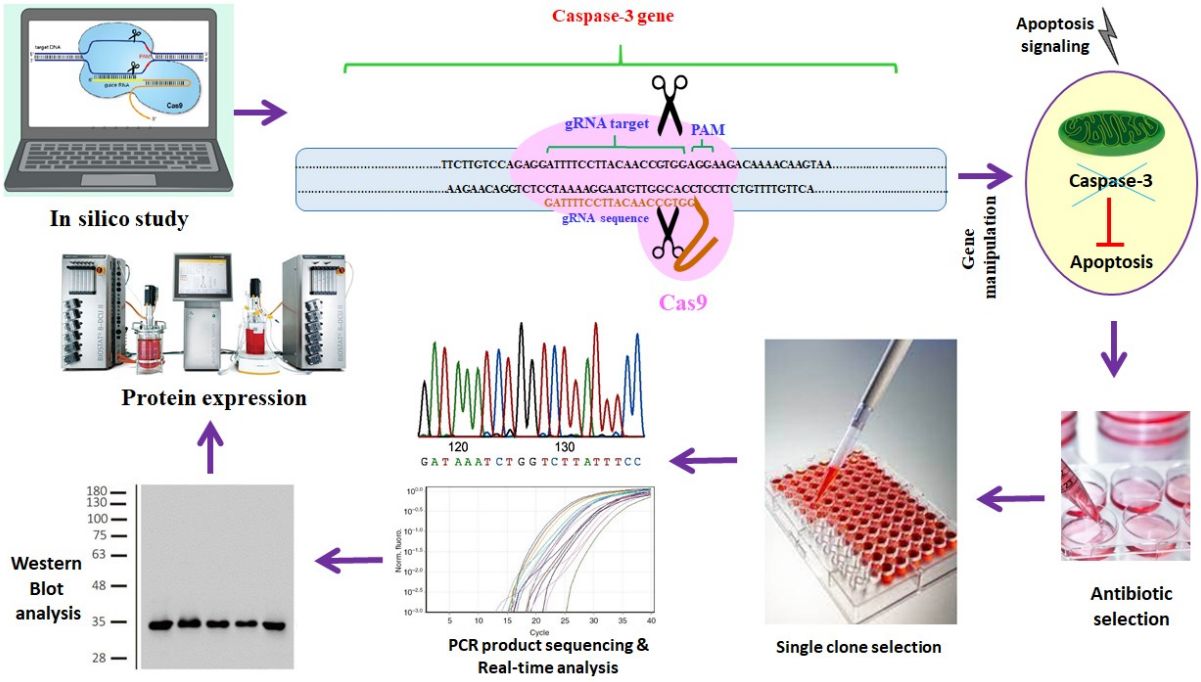


Figure 1) A schematic illustration of gene manipulation for increasing the yield of the cell line.

Comment #7 :

7. The quality of English should be improved.

Reply to comment #7

We do apologize for the problems with the English in our submitted manuscript. We would like to inform you that our manuscript has been further carefully revised by a native professional English language editing service to correct the grammatical mistakes and improve its readability.

Reviewer 2:

The authors are kindly requested to answer the following questions/concerns:

Response to comment #1 of reviewer 2:

1. Please write the type of study, sample size, sampling strategy and date and country of study in abstract.

The type of study is an original study, and this project was done in Iran. Other questions are not applicable.

Response to comment #2 of reviewer 2:

2. What is your type of study? Experimental study, Cross sectional, cohort, case-control, clinical trial, systematic review or … please mentioned it in method section.

Not applicable.

Response to comment #3 of reviewer 2:

3. The source of data is unclear. How did you select sample, how many sample did you select, what was the inclusion and exclusion criteria.

We only had one cell line, a gift from the Pasteur institute of Iran. Other questions are not applicable.

4. Did have any you inclusion and exclusion criteria? Please speak about it and then tell about how many samples include and exclude according to these criteria

5. What was your sample size formula? What is your expected power? please mention in main text.

6. The Code and date of ethical approval should be mentioned in main text. Also ethical considerations should be mentioned with more detail.

7. Please mention the city, country and date of study in method section

8. How many observers did you have? if you had more than one observer, you must mention agreement index like kappa coefficient (write in method section)

9. Did you check normal assumption? Please mention it. It seems that some variables have not normal distribution and you must have used nonparametric method

10. In the case of non-normal data, Median and IQR better present location and dispersion of data

11. There are some spelling and grammatical errors in the text. Please correct them

12. Most of article references are out of date (2019 or upper) . Please use more up to date references and also arrange references according to journal format

13. The result must come to the table with mean and SD. just plot not enough

14. please present the demographical variables (like sex and age)of each data base in table according to each groups and then compare it then go to the final result

Overall, I think most of the reviewer's comments are unrelated to our study and not applicable to our manuscript.

Reviewer 3:

The authors are kindly requested to answer the following questions/concerns:

Comment #1 :

1- The whole manuscript needs serious English revision. Most probably, there are some mistypings or missed words in the title. The title in the current structure is a bit vague.

Reply to comment #1

We do appreciate your time in reviewing our work and your precious comments. According to your suggestion, the manuscript has been carefully revised

followed by checking a professional language editing service to improve the grammar and readability. And the title was changed as follows:

“Targeting caspase-3 gene in rCHO cell line by CRISPR/Cas9 editing tool and its effect on protein production”

Comment #2 :

2- CHO-k1 is an adherent subclone of the original CHO cell line that for sure genetically different from the CHO-S; the ideal CHO variant which is used for pharmaceutical production. Results obtained from a manipulated CHO-k1 can’t be extrapolated to CHO-S; and as CHO-k1 is not normally used in pharma industry, the significance and rationale of such investigation on CHO-k1 platform must be explained.

Replyto comment #2:

Thank you for your comment. To investigate the knockdown of the caspase-3 gene by CRISPR/Cas9 tool, we tried to rCHO cell line for the following reason:

A) The adherent cell was more amenable and easier to transfect the constructs. B) the cell line we used expresses EPO as a heterologous protein. Therefore, we were able to determine the effect of the Caspase-3 gene edition on the yield of EPO recombinant protein.

We agree with the reviewer that the manipulation should finally be investigated on industrial cell lines. But in this study, our aim was to find the effect of caspase-3 knockdown on the viability of CHO cells on a lab scale.

In order to clarify, we would like to explain this cell line is a gift from the Pasteur institute of Iran.

Comment #3 :

3- One of the major drawbacks of CRISPR systems is their likely off target modifications. These modifications can affect protein folding, glycosylation and other factors affecting protein activity. Author should prove the unaltered protein activity (in this case EPO) determining its potency in an EPO responsive cell line (similar to UT-7) in a cell-based proliferation assay.

Replyto comment #3:

One of the disadvantages of the CRISPR/Cas9 system is off-target. Of course, there are some ways to reduce this defect. For this reason, it is necessary to be precise in designing the gRNAs. Also, for the production of recombinant products, the other genes that are effective in cell survival should be checked and investigated, and then the product should be tested completely to ensure its function of the product. Our initial evaluation was only the manipulation of genes involved in the apoptosis pathway on the laboratory scale. In addition, the yield of the EPO was evaluated and it was confirmed that the amount of EPO has been increased in the edited cell line. Other characteristics of EPO such as glycosylation and biological activity was not in the scope of this study.

Comment #4 :

4- The main trigger of apoptosis in pharma industry is high cell density and hypoxic conditions. These triggers must be simulated in order to check the efficiency of CHO manipulation. Triggering apoptosis using oleuropein is not proper way for such appraisal.

Replyto comment #4:

Thank you very much for your time and invaluable suggestions. The main aim of the study was to investigate the knockdown of the caspase-3 gene on cell viability on a lab scale and the study did not scale up in a bioreactor for the production of recombinant proteins on an industrial scale in a genetically modified CHO cell line, more studies are needed (both on the producer cell lines and the products).

For evaluating the impact of caspase-3 knockdown on the inhibition of apoptosis, we used oleuropein, a substance with known apoptotic function in tumor cells. Oleuropein remarkably reduces the viability of CHO cells and decreases the number of CHO cells by inhibiting the rate of cell proliferation and inducing cell apoptosis. According to the literature, oleuropein blocks the cell cycle at G_1 to S phase transition, causing an increase in the number of cells in the G0/G_1 phase. Also, we have some experience with this compound and it has been available in our lab. Therefore, in the absence of apoptotic inducers such as hypoxia in cell culture, we used oleuropein for induction of apoptosis.

And also use scratch wound healing proliferation assay to evaluate cell density and apoptosis. To determine the effect of caspase-3 inhibition on cell proliferation, 1×10^3 cells from edited and control CHO-K1 cells were seeded into 24-well plates in DMEM/F-12 and supplemented with 10% FBS. The cells' monolayer was manually

scratched using a yellow pipette tip. The cellular debris and non-adherent cells were removed by replacing the fresh media containing 4,000 µM oleuropein (according to its IC50 dose). Scratch closure and cell proliferation were monitored at T=24 and T=48h in each well. The difference between edges was calculated by ImageJ software.

As shown in figure 1, gap closure and cell proliferation are higher in a manipulated cell line (clone-62) compared to the control cell line. The result was shown that repression of caspase-3 increases cell proliferation in manipulated clone (clone-62) in the presence of an apoptosis inducer.

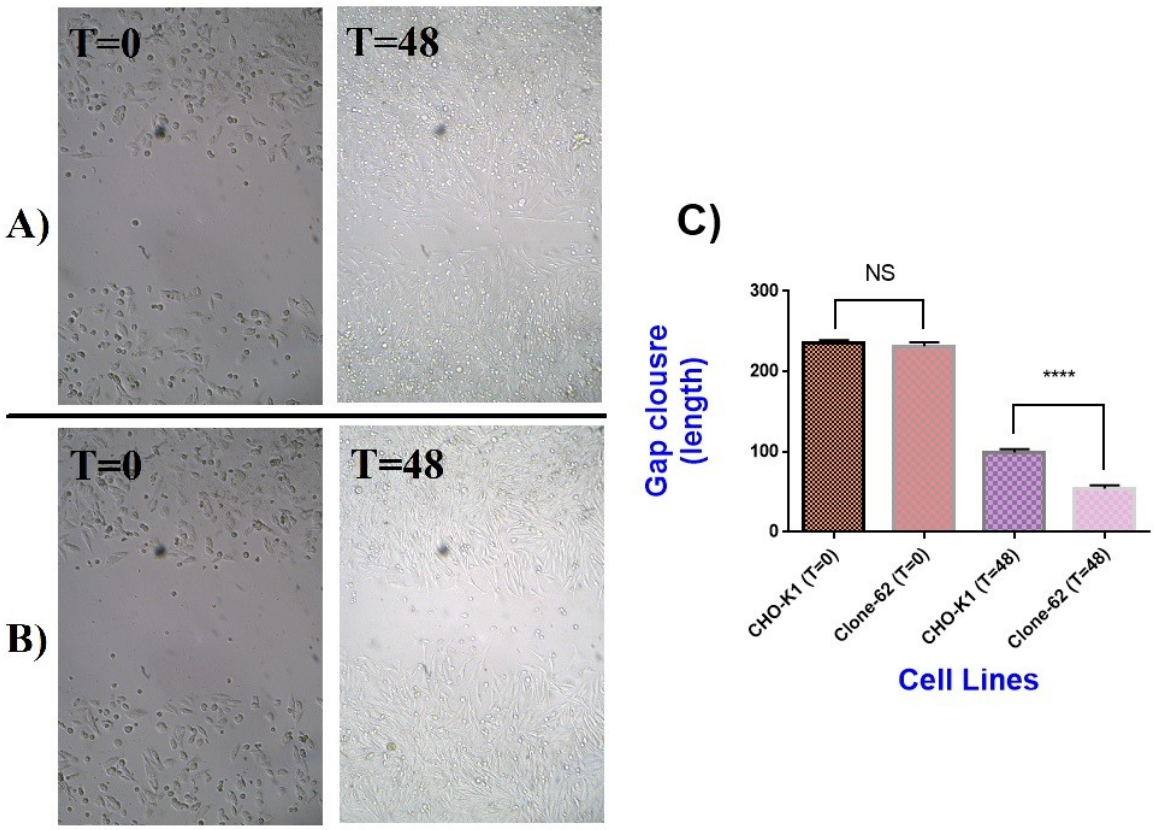


Figure 1) Scratch wound healing proliferation assay. A) The manipulated cell line (clone-62) in the presence of 4,000 µM/ml oleuropein at T=0 and T=48, B) The control cell line in the presence of 4,000 µM/ml oleuropein at T=0 and T=48. C)

There was shown a significant difference in gap closure between the control and clone-62 (P-value <0.0001).

Comment #5 :

All the industrial cell culturing worldwide is used by chemically defined media such as ProCHO (Lonza). Using animal derived FBS in the culture which is not chemically defined can make variation in protein production, unless the authors state that all FBS used in the investigation are from the same production batch.

Replyto comment #5:

Thank you very much for your invaluable comment. FBS in the cell culture can make variations and challenges in recombinant protein production (especially in the purification step). One of the main hurdles in the culture of CHO cells is the inability to maintain viable cells in cultures over an extended period of time. The rapid decline in viability at the end of the culture is exacerbated by the absence of serum. The adherent and suspended CHO cell lines have been used in different genome editing studies. Our main goal of this study was to find the efficiency of caspase-3 gene editing in adherent CHO cell lines on a lab scale, which is more available and easy to transfect and manipulate. For the prevention of variation in protein production, the FBS used in all treatments were from the same batch and the tests were repeated in triplicate.

2 Revision 1

1 comment · 11 Dec 2022

Associate Editor

ACCEPT 11 Dec 2022

The manuscript can be accepted for publication in IJPR.