Bioprocess and Biosystems Engineering (2018) 41:633–640
https://doi.org/10.1007/s00449-018-1898-z

RESEARCH PAPER

Enhanced production of anti-PD1 antibody in CHO cells through transient co-transfection with anti-apoptotic genes *Bcl-x$_L$* and *Mcl-1*

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Received: 15 September 2017 / Accepted: 15 January 2018 / Published online: 25 January 2018

Abstract
Apoptosis has a negative impact on the cell survival state during cell cultivation. To optimize mammalian cell culture for production of biopharmaceuticals, one of the important approaches is to extend cell life through over-expression of anti-apoptotic genes. Here, we reported a cost-effective process to enhance cell survival and production of an antibody through transient co-transfection with anti-apoptotic genes *Bcl-x$_L$* or *Mcl-1* in Chinese hamster ovary (CHO) cells with polyethylenimine (PEI). Under the optimal conditions, it showed reduced levels of apoptosis and improved cell viability after co-transfected with *Bcl-x$_L$* or *Mcl-1*. The overall production yield of the antibody anti-PD1 increased approximately 82% in CHO cells co-transfected with *Bcl-x$_L$*, and 34% in CHO cells co-transfected with *Mcl-1*. This work provides an effective way to increase viability of host cells through delaying apoptosis onset, thus, raise production yield of biopharmaceuticals without the process of generating stable cell lines and subsequent screening.

Keywords Anti-apoptosis · *Bcl-x$_L$* · Chinese hamster ovary cell · *Mcl-1* · Transient gene expression

Introduction

Mammalian cell expression has become a dominant system for recombinant protein production in biopharmaceutical industry, because of its advantages in post-translational modifications and proper protein folding [1]. And Chinese hamster ovary (CHO) cells demonstrated ability of high cell density in growth and relatively safe in clinical applications [2, 3].

Production of biopharmaceuticals in mammalian cell lines have been increased due to improvements in media and optimized bioprocess design [4]. However, much more effort improving productivity is still needed to meet rapidly growing market demand for many biological products. According to the latest updates of the DrugBank, there were over 5028 experimental drugs and 4775 non-redundant protein (i.e., drug target/enzyme/transporter/carrier) sequences linked to these drug entries [5]. Thus, fast development of innovative biological therapeutics would be further appreciated, if the process is capable of producing biopharmaceuticals in a short period with low cost. Transient gene expression (TGE) is gaining popularity as a cost-effective process to produce recombinant protein products [6]. The protein of interest is expressed usually within 16–96 h using TGE technology. And the quality of protein obtained from TGE meets the standard of preclinical assessment [7]. Besides, g/L level of productivity in TGE with CHO cells have recently been reported [8–11]. Even more, TGE of CHO cells at 3 g/L expression level by a kit (ExpiCHO, Invitrogen/Life technologies) was developed and commercially available [12].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00449-018-1898-z) contains supplementary material, which is available to authorized users.

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TGE demonstrated that it is a potential and vital technology that could accelerate the process of screening multiple biotherapeutic candidates, thus, speed up the "Proof of Principal" stage [1].

It would be necessary to optimize mammalian cell culture in TGE for cost-saving production of biopharmaceuticals. One of critical impact factors on productivity would be apoptosis during cell culture process, that is undergoing programmed cell death, when cells are exposed to stresses caused by various factors, including mechanical agitation, nutrient depletion, waste byproduct accumulation, hypoxia and viral infection [13]. It was reported that apoptotic process accounted for approximately 80% cell death during cell culture [14], which affected cell survival state and the quality of protein production [15, 16]. It was also reported that apoptosis was regulated by either activation or suppression of the proteins in *Bcl-2* family, including three groups: anti-apoptotic *Bcl-2*-like proteins (*Bcl-2, Bcl-*x_L, *Mcl-1* and *Bcl-w*), pro-apoptotic *Bax*-like proteins (*Bax, Bak* and *Bok*), and pro-apoptotic BH3-only proteins (*Bad, Bid, Blk*, PUMA, and Noxa) [17, 18].

Several methods had been evaluated to limit the onset of apoptosis and to extend cell life by inhibiting or delaying activation of cell death, which would produce more recombinant proteins for biopharmaceutical applications [19]. One successful approach to extend cell lifetime was overexpressing anti-apoptotic genes to manipulate intracellular biochemistry [13]. It was reported that anti-apoptotic genes could maintain the mitochondria membrane potential, and then prevent the release of cytochrome c during apoptosis [20]. Our previous work suggested that *Bcl-*x_L and *Mcl-1* had significant anti-apoptotic effects (data not shown).

Bcl-x_L was shown to prevent apoptosis in several production host cell lines, including CHO [21–23], BHK [24] and Hybridoma [25, 26] and could be utilized to improve productivity [23, 24, 27–31] through increasing cell viability and reduction of apoptosis. Many previous reports related to overexpressing anti-apoptotic genes were mainly in stable cell lines, which were time- and resource consuming, due to requirement of screening cell lines. *Mcl-1*, another important anti-apoptotic gene, was previously published as it improved productivity in stable transfections with *Mcl-1* by Betenbaugh's group [32]. However, it has not been thoroughly examined for its effects on productivity in TGE before. It was unique that *Mcl-1* did not suppress apoptosis induced by overexpression of either *Bax* or *Bak*-like *Bcl-*x_L, but did bind to them [33]. Additionally, *Mcl-1* was related to maintenance of cell viability but not stimulation of proliferation [34].

Anti-PD1 antibody has been a great successful inhibitor of immune checkpoint, currently being widely used in clinical applications for the treatment of six different tumors [35]. Besides three companies were approved by FDA to sale the antibodies on the market, many other followers are still in various development stages. Optimization of the production process or exploration of new approaches would be particularly interested in biopharmaceutical industry. Here, we described an alternative approach to increase viability of production cells, delay apoptosis onset and enhance transient expression of anti-PD1 antibody through co-transfection with anti-apoptotic genes *Bcl-*x_L or *Mcl-1* without stable cell line screening.

Materials and methods

Cell lines and maintenance

All the cell lines, media, and supplemental components used in this report were from Invitrogen (Carsbad, USA). CHO cells with TGE were cultured in a serum-free CD-CHO medium supplemented with 8 mM GlutaMAX. Cell cultures were maintained in a 37 °C incubator (Thermo Fisher Scientific, Shanghai, China) with 5% CO_2 and shaken at 110 rpm. They were passaged at a seeding density of 5×10^5 cells/mL every 3–4 days. Cell density and cell viability were measured with a trypan blue (1:1, v/v; AMRESCO, Solon, USA) staining method and then counted by the cell counter (Ruiyu Biotech, Shanghai, China). Six-well plates, 24-well plates and 125 mL shake flasks (Corning, New York, USA) were used in this study.

Plasmid construction

Human *Bcl-*x_L (wt) and *Mcl-1* (wt) genes were amplified from NCBI cDNA library by PCR (T100TM Thermal Cycler, BIO-RAD, Hercules, USA). *Bcl-*x_L and *Mcl-1* were then cloned into pcDNA3.1 (–), respectively. The human *Bcl-*x_L (wt) gene was cloned between *Xba*I and *Not*I sites (underlined) using the PCR primers: 5′ GCTCTAGAATG TCTCAGAGCAACCGGGA 3′ and 5′ AAATATGCGGCC GCT.

CATTTCCGACTGAAGAGTGAGCCCAG 3′. The human *Mcl-1* (wt) gene was cloned between *Nhe*I and *Hin-d*III sites (underlined) using the PCR primers 5′ AGCTG GCTAGCATGTTTGGCCTCAAAA.

GAAACG 3′ and 5′ AGCTGCAAGCTTCTATCTTAT TAGATATGCCAAACCAGC 3′. The expression plasmids of anti-PD1 antibody were constructed as described previously [7]. The expression of target proteins was driven by CMV promoter. The constructs were then sequenced to ensure proper insertion direction. All plasmids were extracted by an endo-free maxi-prep kit (D6926, OMEGA Bio-tek, Doraville, USA) according to the manufacturer's instructions and then stored at − 20 °C.

Transfection

Linear polyethylenimine (PEI) (Polysciences, Warrington, USA) (molecular weight of 25 kDa) was prepared in Milli-Q water to concentration of 1 mg/mL as the transfection agent [36]. Cells growing at exponential stage were passaged into fresh culture medium on the day prior to transfection. On the day of transfection, cells were spun down (1000 rpm, 5 min) and re-suspended at density of 3×10^6 cells/mL. All experiments mentioned in this paper, the ratio of DNA/PEI was kept at 1:3. 3 μg/mL DNA was diluted in DMEM (Life technologies, Shanghai, China) at the concentration of 40 ng/μL, and then added three times PEI. After brief vortex, the complexes of DNA and PEI was incubated at room temperature for 10–15 min. Then they were added to the cultures and shaken at 110 rpm. The ratio of light: heavy chains 2:1 was previously used in our Lab and also used in this experiment.

Analysis of apoptosis

The level of apoptosis was assessed using an annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instruction and detected by the flow cytometry BD LSRFortessa (BD Biosciences, San Jose, USA). Cells were gated according to viable, early apoptotic (Annexin-V-FITC), late apoptotic (Annexin-FITC-PI) and dead (PI positive) cell populations. And the error bars represented the standard error of the mean (SEM) in three or more independent experiments.

Quantification of monoclonal antibody production by ELISA

A sandwich enzyme-linked immunosorbent assay (ELISA) [37] was established for quantifying the production of anti-PD1 antibody. First, a 96-well plate was coated with monoclonal anti-human κ chain antibody (Merck Millipore, Shanghai, China) as a capture antibody. Then, add samples harvested from cell culture supernatants. A monoclonal HRP-anti-human IgG antibody (FC specific; Jackson ImmunoResearch, West Grove, USA) was used for detecting enzymatic oxidation reaction with TMB substrate. The signal was assessed by a spectrophotometer at 450 and 630 nm. The data was calculated according to the founded standard curve.

Western blotting

Cells were harvested and lysed in RIPA lysis buffer (137-mM NaCl, 20 mM Tris, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40, 0.5% dexycholate, 0.1% SDS, pH7.4) supplemented with protease inhibitors. The concentration of total protein in clarified lysates was determined by the enhanced BCA protein assay kit (Beyotime, Shanghai, China). Samples were loaded on a 10-well SDS–PAGE (10% or 12%) gel. Then, proteins were transferred to PVDF membrane (Millipore, Darmstadt, Germany). Primary antibodies, including rabbit α/β-Tubulin antibody, rabbit anti-*Bcl-x*$_L$ antibody and rabbit anti-*Mcl-1* antibody (Cell Signaling Technology, Danfoss, USA) were used, and then followed by a HRP-anti-rabbit IgG antibody (Cell Signaling Technology, Danfoss, USA).

Results and discussion

Optimization of TGE parameters

Transfection efficiency was affected mainly by DNA amount, DNA: PEI ratios, and cell density [38]. To determine the optimal conditions of using PEI to transfect CHO-s cell line, we evaluated DNA concentrations of 2.5–4.0 μg/mL, cell densities of $1–3 \times 10^6$ cells/mL and DNA/PEI ratio of 1:2–1:3 (w/w) using pEGFP as a reporter protein. 2 days post transfection, the transfection efficiency was examined by flow cytometry BD LSRFortessa mentioned before with blank plasmid-transfected cells as the negative control.

As shown in supplementary data, the transfection efficiency of DNA: PEI = 1:3 (w/w) was higher than that of DNA: PEI = 1:2 (w/w). At a cell concentration of 3×10^6 cells/mL and DNA: PEI ratio = 1:3 (w/w), over 40% cells were found to express GFP 48 h after transfection when the DNA concentration was either 3.0 or 4.0 μg/mL. It was probable that there was negative affect on cell growth because of overused PEI, thus, we chose optimal transfection conditions as below: transfecting 3×10^6 cells/mL with 3.0 μg/mL DNA at a ratio of DNA: PEI = 1:3 (w/w).

Evaluation of the anti-apoptotic effects of *Bcl-x*$_L$ and *Mcl-1* in CHO cells

Using the optimal transfection conditions as described above, CHO cells were transfected by 3.0 μg/mL of *Bcl-x*$_L$ or *Mcl-1*, respectively, to determine whether over-expression of those two anti-apoptotic genes could affect apoptosis of host cells. The cells transfected with the same amount of null vector pcDNA3.1(–) was used as the control group. After transfection, the percentage of apoptosis was measured at 24, 48 and 72 h, respectively, in three groups (null vector, *Bcl-x*$_L$, *Mcl-1*).

The percentage of cells undergoing apoptosis was shown in Fig. 1a (total apoptosis) and Fig. 1b (late apoptosis). Apoptotic cell number increased with culturing duration, however, as shown in Fig. 1a, the apoptotic cell number in experimental group transfected with apoptotic genes *Bcl-x*$_L$ or *Mcl-1* was considerably fewer than that in the control group. The control group showed 24.7% of the total

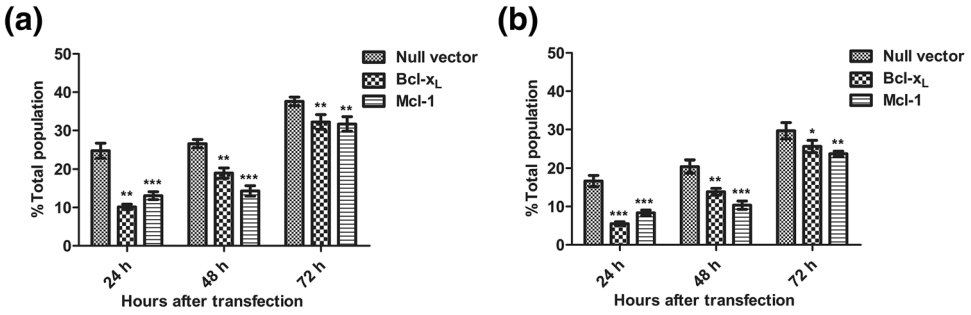

Fig. 1 Comparison of apoptosis with *Bcl-x$_L$* or *Mcl-1* in CHO cells. CHO cells were transfected with a null vector or vector containing *Bcl-x$_L$* or *Mcl-1*, respectively, and were monitored throughout 72 h. **a** Total apoptosis and **b** late stage apoptosis. Error bars represent mean ± SEM for six independent experiments. (*, **, *** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively)

population undergoing apoptosis 24 h post transfection, but only 10.2% and 13.1% were counted in the groups transfected with *Bcl-x$_L$* or *Mcl-1* at the same time, respectively. The gaps between control and experimental groups sustained till a 48-h time point, but narrowed down with further extension of culture time. This might be caused by the loss of external anti-apoptotic genes *Bcl-x$_L$* or *Mcl-1*. Moreover, the group transfected with *Bcl-x$_L$* or *Mcl-1* had higher percentage of FITC-PI-negative cells than the control from 24 to 72 h. There was no significant difference in cell apoptosis between those two experimental groups (*Bcl-x$_L$* and *Mcl-1*).

Late apoptosis was also examined, and the results were shown in Fig. 1b. After transfection, late apoptotic cells in the population transfected with null vector gradually increased from 16.6 to 29.7%, which was higher than that transfected with *Bcl-x$_L$* or *Mcl-1* from 24 to 72 h post transfection. Percentage of cells undergoing late apoptosis was much reduced with transfected with *Bcl-x$_L$*, especially at 24 h after transfection, to 5.5% (Fig. 1b). Likewise, results had been found in cells transfected by another apoptotic gene *Mcl-1* in a range of 8.4–23.7% from 24 to 72 h. Therefore, it was shown that *Bcl-x$_L$* and *Mcl-1* could have positive impact on product yield through delaying apoptosis onset.

Optimization of amount of apoptotic genes in co-transfection

To examine the optimal amount of anti-apoptotic genes *Bcl-x$_L$* and *Mcl-1*, we co-transfected 5–75% (w/w) anti-apoptotic genes in total 3.0 μg/mL DNA with plasmids coding anti-PD1 antibody sequence. The amount of PEI, total DNA concentration and cell density in all groups were kept same.

To confirm expression of anti-apoptotic genes in cells, samples were taken at 48 h after transfection, lysed in RIPA buffer, followed by Western blotting to examine the expression level of the samples with different amount of plasmids coding *Bcl-x$_L$* or *Mcl-1* transfected (Fig. 2c, d). The supernatants were harvested immediately when the cell viability

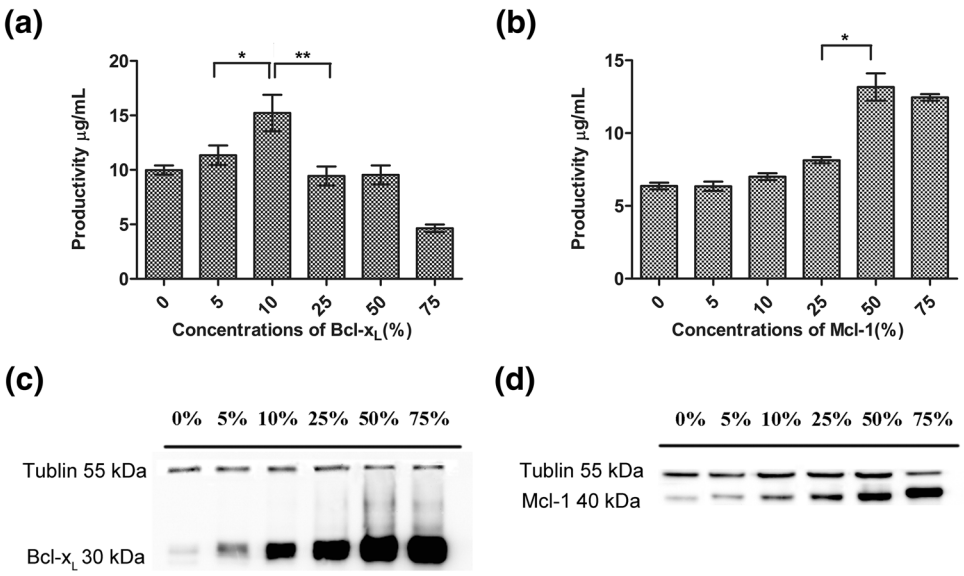

Fig. 2 Assessment of productivity with different percentage of anti-apoptosis genes **a** *Bcl-x$_L$* and **b** *Mcl-1* in co-transfection varied from 5 to 75% (w/w) and expression of **c** *Bcl-x$_L$* and **d** *Mcl-1* by Western blotting. Supernants were taken when cell viability was below 50%. Same amount of cell lysate was taken at 48 h and was loaded in Western blotting. Error bars represent mean ± SEM for three independent experiments. (*, ** indicate $p < 0.05$, $p < 0.01$, respectively)

was below 50% and examined by Elisa assay. As shown in Fig. 2a, the production of anti-PD1 antibody in cells co-transfected with 10% *Bcl-x*$_L$ (w/w) was considerably higher than that in control group, with 15.22 and 9.97 μg/mL, respectively (Student's *t* test, $p = 0.003$). However, the production titer did not increase further in the group over 25% *Bcl-x*$_L$ (w/w) in co-transfection. This was probably because of excessive external gene expression and less-targeted protein plasmid used in co-transfection, which was consistent with a previous study [39]. In the case of group of the cells transfected with *Mcl-1* gene, the result (Fig. 2b) showed that production in cells co-transfected with 50% *Mcl-1* (w/w) increased by approximately two-fold, compared with that in control group (Student's *t* test, $p = 0.017$). There was no further increase with higher percent of *Mcl-1* used.

Assessment of growth, apoptosis and productivity of cells co-transfected with the anti-apoptotic genes *Bcl-x*$_L$ and *Mcl-1*

We co-transfected plasmids coding anti-PD1 antibody with 10% *Bcl-x*$_L$ or 50% *Mcl-1*, respectively, under optimized conditions described above, and productivity of anti-PD1 antibody was assessed. Cells in control group were transfected with 3.0 μg/mL plasmids, in which containing anti-PD1 antibody genes only, and the same amount of PEI. Supernatants were taken every day and analyzed by ELISA. Considered that low cell viability had negative impact on target protein quality, the products were harvested immediately when the cell viability was below 50%. The samples were stored in a −80 °C freezer.

Although cells co-transfected with anti-apoptotic genes reached similar viable densities without statistical significance compared with the control group (Fig. 3a), the cells showed better viability in the group co-transfected with 10% *Bcl-x*$_L$ (w/w) or 50% *Mcl-1* (w/w). Both still maintained over 60% viability on day 5, (66.2 and 77.3% respectively with statistically significant, Student's *t* test, $p = 0.011$, 0.001, respectively). On day 6, the group with anti-apoptotic genes dropped to about 50%, while the control group was only 37.3% on day 5 (Fig. 3b).

To further explore the relationship between the productivity and function of anti-apoptotic genes, we determined the apoptosis of cells in different groups. It showed that cells were at different apoptotic stages including total apoptosis (Fig. 4a) or late apoptosis (Fig. 4b), and cells co-transfected with either *Bcl-x*$_L$ or *Mcl-1* were much lower percentage than that of control group at 24, 48, 72 h after transfection. Compared with the previous studies [39], overall increase of apoptosis with higher cell density initially could be explained by cell contact and more PEI used. In general, toxicity of PEI is relatively low, however, large amount of PEI used in the cell culture system might still affect cell apoptosis [40, 41].

The cumulative productivity of anti-PD1 antibody gradually increased in all three groups (Fig. 5b). The cumulative productivity in the three groups was similar in first 3 days, rising from 0 to 2 μg/mL. From day 4, the cultures that were co-transfected with 10% *Bcl-x*$_L$ or 50% *Mcl-1*

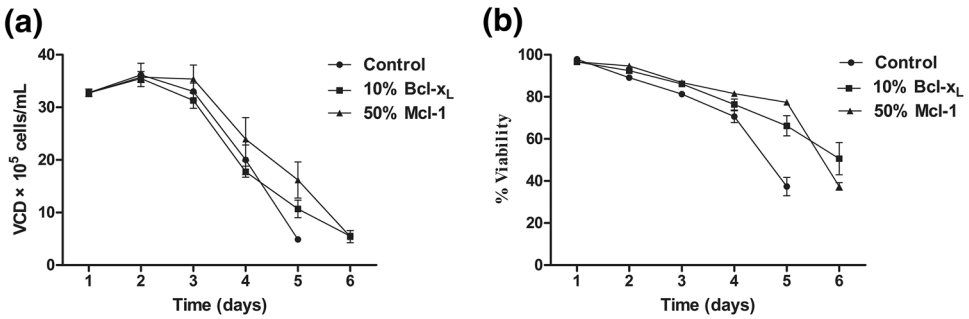

Fig. 3 Assessment of **a** cell density and **b** cell viability with 10% *Bcl-x*$_L$ or 50% *Mcl-1* (w/w) throughout a 6-day cell culture. Error bars represent mean ± SEM for three independent experiments

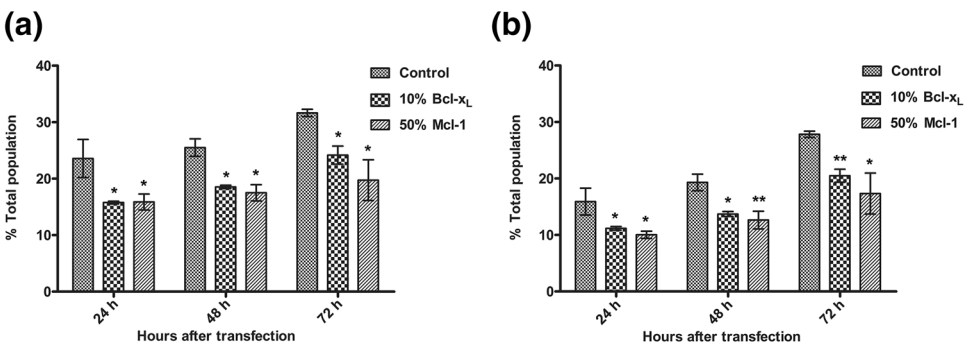

Fig. 4 Assessment of *Bcl-x*$_L$ or *Mcl-1* impact on CHO cells apoptosis. Apoptosis was monitored throughout first 3 days. **a** Total apoptosis and **b** late stage apoptosis. Error bars represent mean ± SEM for three independent experiments. (*, ** indicate $p < 0.05$, $p < 0.01$, respectively)

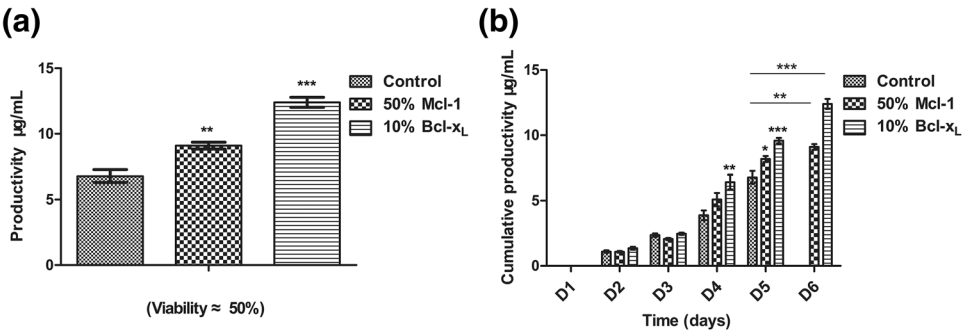

Fig. 5 Productivity Evaluation. **a** Total productivity and **b** cumulative productivity. Samples were taken when cell viability is below 50%. Error bars represent mean ± SEM for three independent experiments. (*, **, *** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively)

showed that anti-PD1 antibody reached 6.4 and 5.1 µg/mL, respectively. However, on the same day, only 3.9 µg/mL of the antibody was produced in the control group. After that, the productivity in the control group increased only till day 5 when it reached harvest point of below 50% cell viability. The titer of the cells transfected by 10% *Bcl-x_L* or 50% *Mcl-1* were increased till day 6 under the same condition and the final expression reached to 12.4 µg/mL (*Bcl-x_L*) and 9.1 µg/mL (*Mcl-1*), respectively, compared with 6.8 µg/mL expression level in the group without anti-apoptotic genes (Fig. 5a). In conclusion, the overall production yield of anti-PD1 antibody increased approximately 82% in CHO cells co-transfected with *Bcl-x_L*, and 34% in CHO cells co-transfected with *Mcl-1* combining results from more than three experiments, which was consistent to previous studies in stable CHO cell lines [32].

To compare anti-apoptotic genes *Bcl-x_L* with *Mcl-1* in applicable prospects, *Bcl-x_L* gene showed more effective in anti-apoptotic effect as well as productivity was significantly increased (Fig. 5). Co-transfection with only 10% *Bcl-x_L* by weight of total plasmid was enough to result similar effect in anti-apoptosis and better product production titer (82 vs 34%) as 50% *Mcl-1*. The differences in promoting productivity might be related to different effects of two apoptotic proteins on apoptosis [23, 42], autophagy [42, 43] and cell cycle [31]. In another word, it might be closely associated with function in metabolism regulation [25, 44].

Two approaches were previously reported to improve cell viability and productivity by co-expressing anti-apoptotic genes: (1) transiently producing target protein in CHO cell that stably expressed anti-apoptotic genes [45], or (2) stable overexpression of anti-apoptotic protein in engineered CHO cell that biopharmaceuticals stably expressed [21, 27, 31, 43, 46]. Reports showed that the engineered cell lines, with *Bcl-x_L* stably expressed, resulted in a 70–270% increase in yield after 14 days in fed-batch culture [45] and *Bcl-x_L* was overexpressed in a CHO cell line that humanized monoclonal antibody titers increased by 80%, compared with parent cell lines [21].

Conclusion

We reported engineered CHO cells to express the monoclonal antibody PD-1 with co-transfection of anti-apoptotic genes to optimize cell cultivation in TGE. Overexpression of either *Bcl-x_L* or *Mcl-1* increased viability of host cells through delaying apoptosis onset. Overall productivity of anti-PD1 antibody increased significantly in CHO cells that were co-transfected with either *Bcl-x_L* or *Mcl-1*. Our data further proved applicability of the apoptotic genes such as *Bcl-x_L* or *Mcl-1* in the antibodies production. This work provides a cost-effective and time-saving method for improving cell survival state and increasing the yields of biopharmaceuticals.

Acknowledgements This project was supported by Natural Science Foundation of Shanghai [Grant No. 15ZR1423800].

Compliance with ethical standards

Conflict of interest All the authors reviewed and agreed to submit this manuscript. The authors declare that they have no conflict of interest.

Research involving human participants and/or animals The study does not contain experiments using animals and human studies.

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