

Regulation of cell growth and apoptosis through lactate dehydrogenase C over-expression in Chinese hamster ovary cells

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Abstract Lactate has long been credited as a by-product, which jeopardizes cell growth and productivity when accumulated over a certain concentration during the manufacturing process of therapeutic recombinant proteins by Chinese hamster ovary (CHO) cells. A number of efforts to decrease the lactate concentration have been developed; however, the accumulation of lactate is still a critical issue by the late stage of fed-batch culture. Therefore, a lactate-tolerant cell line was developed through over-expression of lactate dehydrogenase C (LDH-C). In fed-batch culture, sodium lactate or sodium pyruvate was supplemented into the culture medium to simulate the environment of lactate accumulation, and LDH-C over-expression increased the highest viable cell density by over 30 and 50 %, respectively, on day 5, meanwhile the viability was also improved significantly since day 5 compared with that of the control. The percentages of cells suffering early and late apoptosis decreased by 3.2 to 12.5 and 2.0 to 4.3 %, respectively, from day 6 onwards in the fed-batch culture when 40 mM sodium pyruvate was added compared to the control. The results were confirmed by mitochondrial

membrane potential assay. In addition, the expression of cleaved caspases 3 and 7 decreased in cells over-expressing LDH-C, suggesting the mitochondrial pathway was involved in the LDH-C regulated anti-apoptosis. In conclusion, a novel cell line with higher lactate tolerance, lowered lactate production, and alleviated apoptosis response was developed by over-expression of LDH-C, which may potentially represent an efficient and labor-saving approach in generating recombinant proteins.

Keywords Lactate · Lactate dehydrogenase C · Chinese hamster ovary cells · Sodium pyruvate · Apoptosis

Introduction

Chinese hamster ovary cells are the predominant cell line employed for the production of recombinant protein in the past several decades (Jayapal et al. 2007). The yield of recombinant protein is determined by the integral viable cell density and the protein productivity (Wurm 2004). The former parameter is easily endangered by many by-products generated during the fermentation of CHO cells, such as lactate (Lao and Toth 1997) and ammonia (Hansen and Emborg 1994).

Lactate is generated in the glycolytic pathway, which originates from pyruvate through the catalytic reaction of lactate dehydrogenase (LDH). The side effects of lactate are brought about by lactate itself or base added to neutralize the lactate in bioreactor, which inevitably elevates the osmolality (Cruz et al. 2000). Lactate accumulation may impair cell growth and give rise to loss in productivity (Wagner 1997). Besides, lactate also induces apoptosis in mammalian cells (Jaskille et al. 2004).

Efforts have been made to alleviate the adverse effect of lactate, which typically resort to the decrease of lactate

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concentration. Medium or process optimization is one way to produce less lactate and sometimes may even force cells to consume lactate (Gagnon et al. 2011; Altamirano et al. 2000; Ma et al. 2009; Wong et al. 2005). However, this strategy is often constricted to the specific producer, and the formulation and process need modification for an alternative producer.

Alternatively, genetic engineering is a promising strategy to overcome the problems brought by lactate. These efforts include engineering of GLUT5 transporter to employ fructose as the main carbon source (Wlaschin and Hu 2007), *Bcl-2* to redirect the fate of pyruvate into the mitochondrial oxidation pathway (Templeton et al. 2014), GLUT1 RNAi to decrease glucose consumption flux (Paredes et al. 1999), malate dehydrogenase II to increase the concentration of NADH (Chong et al. 2010), human pyruvate carboxylase to accelerate the flux into tricarboxylic acid (TCA) cycle (Kim and Lee 2007a), and lactate dehydrogenase-A RNAi to block lactate formation (Kim and Lee 2007b). Besides, engineering of two or three combinational genes has been employed, such as the combination of lactate dehydrogenase-A and *Bcl-2* (Jeon et al. 2011), lactate dehydrogenase and pyruvate dehydrogenase kinase (Zhou et al. 2011), alanine aminotransferase, and taurine transporter (Tabuchi and Sugiyama 2013), as well as the combination of E1B-19K, Aven, and a mutant of XIAP (Dorai et al. 2009). These efforts decrease the accumulation rate of lactate to some extent; however, lactate will finally accumulate to a cell injurious concentration in the late phase of fed-batch culture. Therefore, a cell line which is capable of surviving high concentration of lactate is demanding.

LDH-C is a subtype of lactate dehydrogenase, which is generally expressed in animal sperm and preferably converts lactate to pyruvate (Koslowski et al. 2002). Study indicates that LDH-C is upregulated during the late phase of fed-batch culture, which is typically accompanied by lactate consumption (Szperalski et al. 2011). In this study, we developed a novel cell line capable of tolerating higher levels of lactate by lactate dehydrogenase C (LDH-C) over-expression and investigated the impact of LDH-C on cell growth and cell apoptosis.

Material and methods

Construction of expression vector

The mRNA of LDH-C gene (GenBank, NM_002301.4) was isolated from human *SK-BR-3* cells (ATCC, Manassas, VA) according to the instructions of NucleoSpin RNA extraction kit (Macherey-Nagel, Düren, Germany); then the cDNA was obtained through reverse transcription PCR. Human LDH-C gene was amplified using the forward primer 5'-cccaagcttgccaccatgtcaactgtcaaggagca and reverse primer 5'-ccgctcgagtgtaaaatattagatccttttgaatat, respectively, with *Xho*I

and *Hind*III (Takara, Dalian, China) restriction sites for subsequent cloning into vector pcDNA3.0 (Invitrogen, Carlsbad, CA). PCR products were separated on 1 % agarose gels, and the gene of interest was recovered following the instructions of DNA gel extraction kit (Sangon Biotech, Shanghai, China); then the target fragment was cloned into pGEM-T vector (Promega, Madison, WI).

The plasmids were extracted with plasmid DNA extraction kit (Sangon Biotech). They were sequenced and cloned into the expression vector pcDNA3.0 to acquire the expression vector pcDNA3.0-LDH-C.

Construction of cell line over-expressing LDH-C

To generate a CHO cell line over-expressing LDH-C, pcDNA3.0-LDH-C was transfected into CHO-K1 cells (ATCC CCL-61) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

The transfectants were cultivated in 1640/DMEM (50:50) medium containing 10 % fetal bovine serum (FBS, Gibco, Carlsbad, CA). Selection was carried out with 0.8 mg/mL G418 (Invitrogen), and positive clones were isolated by the limiting dilution method.

All established cell lines were adapted to serum-free suspension culture in shake flasks with a working volume of 30 mL supplemented with 4 mM glutamine and 0.8 mg/mL G418 (Jing et al. 2014).

Real-time PCR

Real-time PCR was performed on a PCR amplifier (Prism 7500, Applied Biosystems, Carlsbad, CA) to evaluate the level of LDH-C mRNA in stable transfectants. Specific primers (Sangon Biotech) for human LDH-C were designed as follows: forward, 5'-attgtcacagcaggtgcaaggcagcaggag; reverse, 5'-caggactataatggactatggcaggaatgattga. Forty cycles of amplification were run for 15 s at 94 °C and 60 s at 60 °C. Beta-actin was used as the control (Chusainow et al. 2010).

Before the real-time PCR, the expression of LDH-C gene was evaluated primarily. The conditions of real-time PCR were applied except that the amplification cycle was set as 25. Then, the PCR product was analyzed on 1 % agarose gel.

Cell culture

Human breast cancer *SK-BR-3* cells (ATCC) were cultured in 1640/DMEM (Gibco) containing 10 % FBS. CHO-K1 cells (CCL-61, ATCC) were passaged every 4 days to a density of 5.0×10^5 cells/mL and maintained in shake flasks at 37 °C and 150 rpm in a 5 % CO₂ humidified environment.

Fed-batch culture of cells was conducted in shake flasks with the initial cell concentration of 5.0×10^5 cells/mL in a proprietary serum-free medium CHOM-B01 (Jing et al.

2014). After culturing for 72 h, 3 % of a proprietary supplemental medium CHOM-S01 was supplemented. Meanwhile, glucose concentration in the culture broth was assayed and replenished to 2.5 g/L daily by adding 20 % concentrated glucose solution since day 3 without further notification (Jing et al. 2014). In addition, 1 mM glutamine (Gibco) was supplemented every other day since day 3. Approximately 0.5 mL of culture suspension was sampled daily from the flasks for cell counting and other analysis. In sodium lactate or sodium pyruvate feeding experiments, 40 mM sodium lactate (Sigma, St. Louis, MO) or 40 mM sodium pyruvate (Sigma) was added to the culture on day 3.

Determination of LDH enzyme activity

Cells of 1.0×10^7 were collected and centrifuged at 1000 rpm for 5 min at 4 °C. The supernatant was discarded and the sediment was washed twice with phosphate-buffered saline (PBS) solution followed by ultrasound treatment for 10×3 s with a rest of 5 s during each interval. Then, the resulting suspension was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was harvested to obtain the crude enzyme solution, and was subjected to protein concentration detection by BCA assay (Beyotime, Nantong, China).

Enzyme activity was detected based on the literature with some modifications (Janke et al. 2010). Activity of lactate dehydrogenase (LDH) which catalyzed lactic acid to pyruvate was measured in potassium phosphate buffer (pH 7.6), containing 70 mM sodium lactate and 2 mM NAD^+ (Sangon Biotech) at 25 °C. The activity of LDH enzyme which catalyzed pyruvate to lactic acid was routinely measured in potassium phosphate buffer (pH 7.0), containing 2 mM pyruvate and 0.2 mM NADH at 25 °C. Variation in absorbance at 340 nm ($\Delta A/\text{min}$) was recorded and converted to the specific enzyme activity based on the formula below. Of note, in the experiment above, total reaction volume was 1 mL with 20 μL crude enzyme solution spiked, and the optical path length was 1 cm.

$$\text{Specific enzyme activity} = \frac{\Delta A/\text{min} \times \text{dilution}}{0.1244 \times \text{protein concentration}}$$

Apoptosis assay by flow cytometry

Apoptosis was determined by Alexa Fluor 488 Annexin V/dead cell apoptosis kit (Invitrogen), and analysis was conducted following the manufacturer's instructions. Briefly, 1.0×10^5 cells were collected and washed twice with PBS after centrifuging at 1000 rpm for 5 min, then re-suspended in 500 μL binding buffer. Five microliters propidium iodide (PI) and 1 μL annexin V were added, and cells were incubated at room temperature for 15 min protected from light; then,

samples were analyzed with flow cytometry. All samples were run in triplicate.

Mitochondrial membrane potential assay

Mitochondrial membrane potential was detected with the mitochondrial membrane potential assay kit (Beyotime). In brief, 5.0×10^5 cells were centrifuged and re-suspended in 0.5 mL CHOM-B01 medium containing 0.5 mL JC-1 staining solution. After incubation at 37 °C for 20 min, the cells were washed twice with JC-1 staining buffer, and the fluorescence was read at 488 nm (red) and 525 nm (green), respectively. The mitochondrial membrane potential was shown as the ratio of red/green fluorescence intensity (Li et al. 2009).

Western blot analysis

Cells of 1.0×10^6 were treated with phenylmethanesulfonyl fluoride containing sodium dodecyl sulfate lysis buffer (Beyotime) and were subjected to separation on 12 % polyacrylamide gels followed by transference onto nitrocellulose membranes (Millipore, Bedford, MA) for proteins less than 20 kDa or polyvinylidene fluoride (Millipore) for proteins greater than 20 kDa.

Membranes were blocked overnight at 4 °C in tris-buffered saline spiked with 5 % bovine serum albumin. Next, proteins on the membrane were blotted with the corresponding primary antibodies (anti-rabbit caspase 3 antibody and anti-rabbit caspase 7 antibody, Cell Signaling Technology, Beverly, MA) for 1 h at room temperature, followed by incubation with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (Santa Cruz, Dallas, TX). Signals were detected using an enhanced chemiluminescence kit (Perkin-Elmer Life Sciences, Waltham, UK) according to the manufacturer's instructions, and β -actin was served as the loading control.

Analytical method

Cell density was determined using a hemacytometer, and viable cells were distinguished from the dead cells using the trypan blue exclusion method. Concentrations of lactic acid and glucose in the supernatant were assayed with the corresponding detection kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions.

Statistical analysis

The statistical analysis was carried out using the SPSS program (IBM, Armonk, NY). Data obtained from three independent experiments were presented as mean \pm standard deviation (SD) and *p* values were calculated according to the pair-

sample Student's *t* test. A *p* value less than 0.05 was considered statistically significant.

Results

Construction of cell line over-expressing LDH-C

Expression vector pcDNA3.0-LDH-C was constructed and transfected into Chinese hamster ovary cells using Lipofectamine 2000; then, the G418-resistant cell pool was obtained and clones were isolated by limiting dilution method. Twenty-two clones were picked from the pool with LDH-C, and ten of these were LDH-C positive followed by gel electrophoresis analysis (data not shown). Clones #6 and #20 showed relatively higher expression of exogenous human LDH-C gene than the other clones. In addition, ten clones were picked from the pcDNA3.0-transfected cell pool, and clones #3 and #4 were selected randomly as the negative controls. Clones were identified at three levels: mRNA level, cell growth level, and enzyme activity level. At the mRNA level, clones were analyzed by real-time PCR. The cell pool transfected with pcDNA3.0-LDH-C was set as a positive control (PC). The LDH-C expression levels of clones #6 and #20 increased approximately 13-fold and 10-fold, respectively, compared with those in the positive control group (Fig. 1a). LDH-C-positive clones #6 and #20 were used for further research. At the cell growth level, viable cell density (VCD) of LDH-C-positive clones #6 and #20 was significantly improved compared with that of the controls on day 4 ($p < 0.05$). The VCD of LDH-C-positive clones #6 and #20 were 6.8×10^6 and 6.0×10^6 cells/mL on day 4, higher than that of pcDNA3.0 #4 and #3 (Fig. 1b). We selected LDH-C #6 and pcDNA3.0 #4 for LDH enzyme activity analysis. At the LDH enzyme activity level, the LDH-C-positive clone #6 demonstrated significantly higher LDH-specific enzyme activity (lactate to pyruvate direction) in contrast with that of the control, while the enzyme activity of the reverse reaction showed no significant difference (Fig. 1c). In conclusion, considering the performance of clones at the three mentioned levels, the LDH-positive clone #6 was employed in the following study and vector transfected clone #4 was used as the control.

Growth and lactate profile of LDH-C over-expressed cell line

The effect of LDH-C over-expression was examined primarily in a typical fed-batch culture in shake flasks. Significantly higher VCD was achieved on days 2 and 3 in LDH-C #6 compared with that of the control ($p < 0.05$); however, the difference of VCD was not significant ($p > 0.05$) after day 3 (Fig. 2a). In addition, the difference in cell viability was not

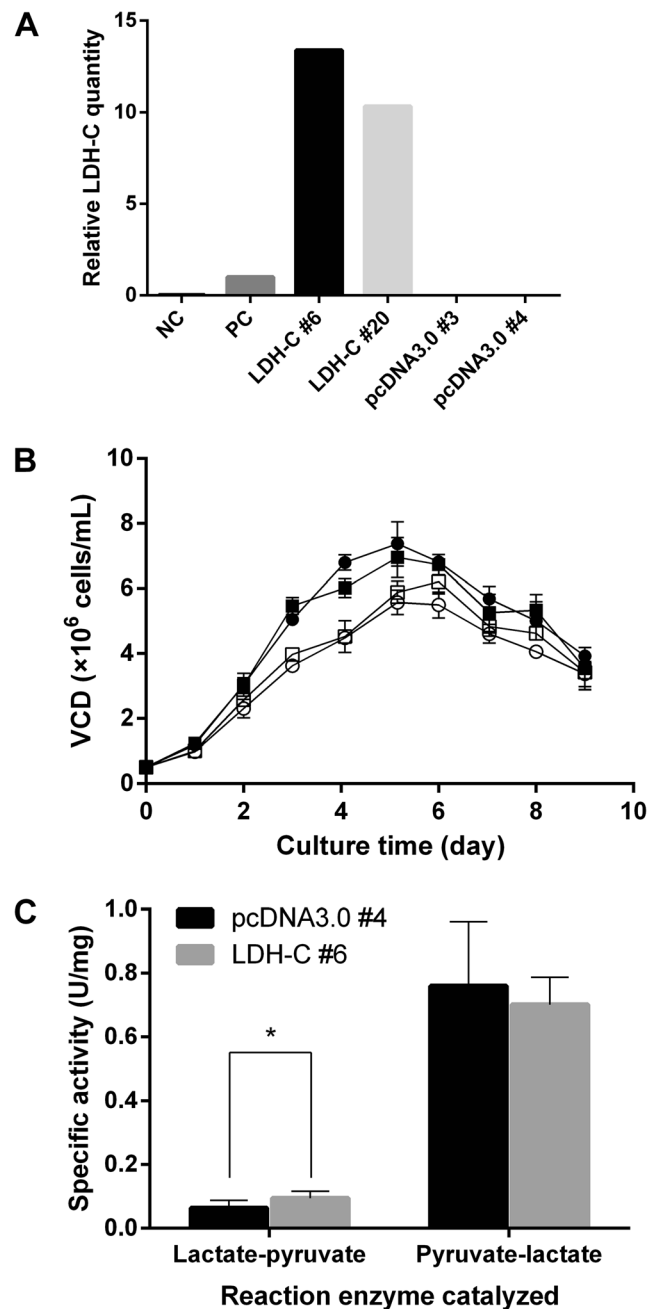


Fig. 1 Confirmation of the clones with LDH-C over-expression at the level of mRNA, cell growth, and enzyme activity. **a** Real-time PCR. Negative control (NC) indicates CHO cells with empty pcDNA3.0, and positive control (PC) indicates pcDNA3.0-LDH-C-transfected CHO cell pool. **b** Viable cell density (VCD) in fed-batch (filled circle: LDH-C #6, filled square: LDH-C #20, empty circle: pcDNA3.0 #3, empty square: pcDNA3.0 #4). **c** Specific LDH enzyme activity (from lactate to pyruvate and from pyruvate to lactate) in fed-batch with 40 mM sodium pyruvate feeding on day 3. Error bars represent standard deviation determined in triplicate experiments. * $p < 0.05$

significant between LDH-C #6 and control (Fig. 2b). LDH-C, a subtype of LDH, is an enzyme closely related to the metabolism of lactate. The profile of lactate in the culture medium was measured to investigate the effect of LDH-C over-

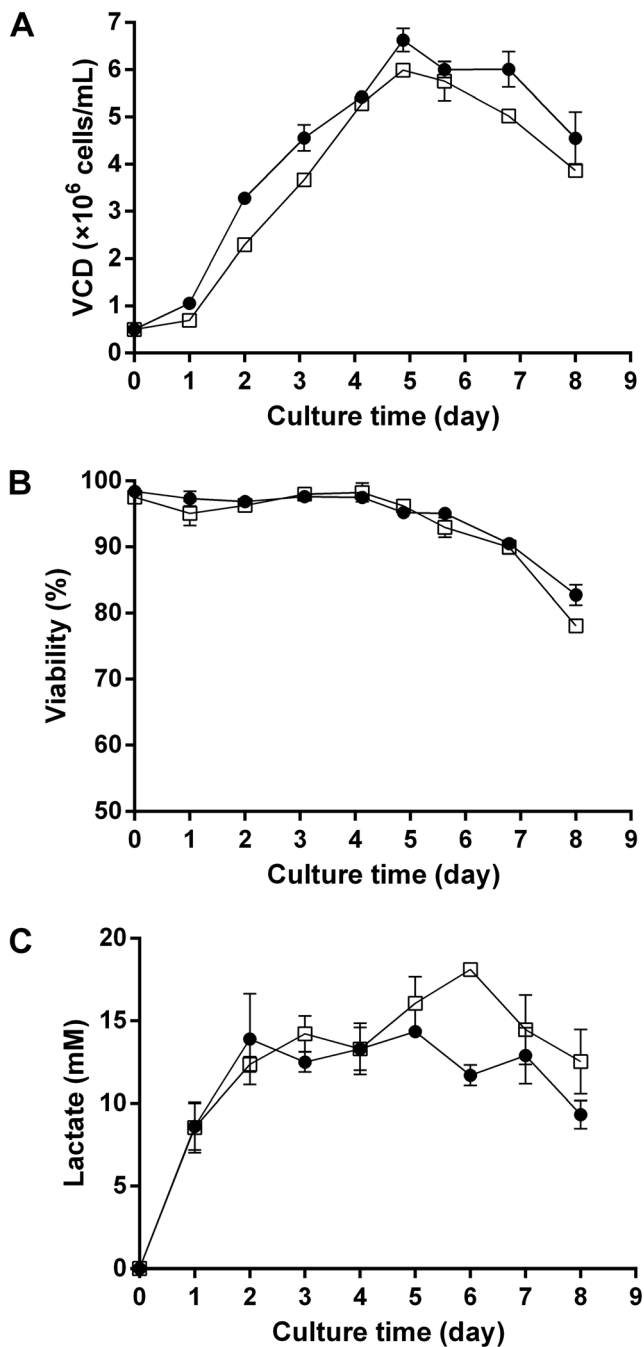


Fig. 2 Growth and lactate profile of LDH-C over-expressed cell line. **a** Growth curve. **b** Viability. **c** Lactate concentration. Filled circle: LDH-C #6, empty square: pcDNA3.0 #4. Error bars represent standard deviation determined in triplicate experiments

expression on the metabolism of lactate. In the typical fed-batch, lactate accumulated from the beginning of the fed-batch culture to day 3 in the process of culture; then, the concentration of lactate maintained at about 15 mM, and the highest lactate concentration was no more than 20 mM (Fig. 2c). However, the trend in lactate showed no significant difference after LDH-C over-expression compared with that of the control ($p > 0.05$).

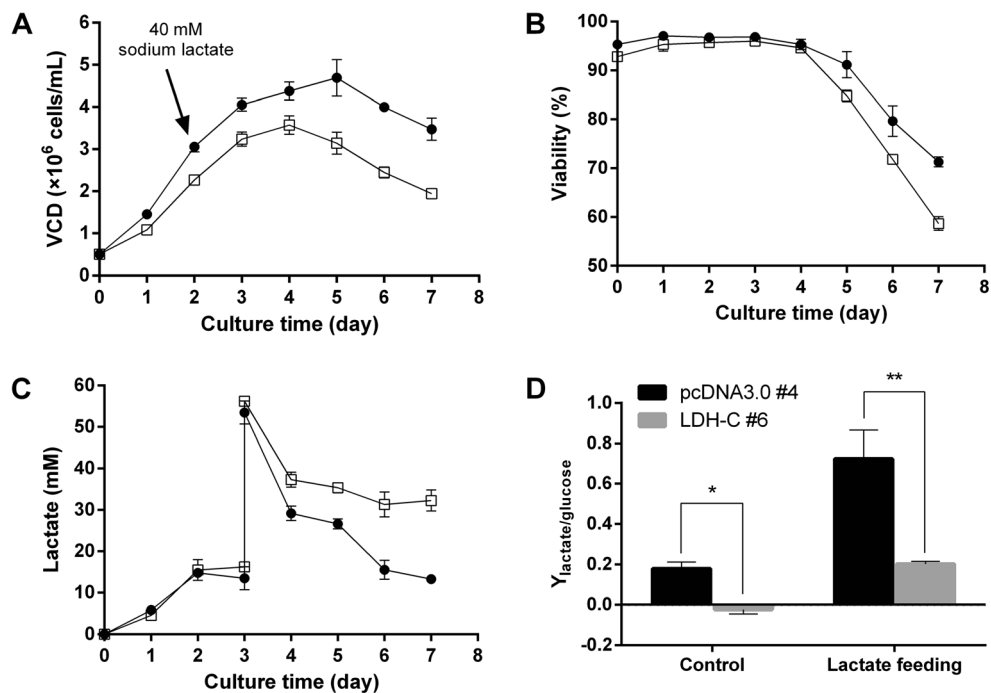
Growth and lactate profile of LDH-C over-expressed cell line with sodium lactate feeding

Generally speaking, a lactate concentration less than 20 mM does not impair the cell growth, while a lactate concentration greater than 40 mM is harmful to cell growth and productivity (Wagner 1997). In the high-density culture of CHO cells in bioreactors, the peak lactate concentration was frequently greater than 40 mM (Gagnon et al. 2011). In view of the lactate consumption function of LDH-C as indicated in Fig. 1, we simulated the environment of high concentration of 40 mM lactate by feeding sodium lactate on day 3 directly into the medium. Significantly higher VCD was achieved in clone LDH-C #6 than that of the control from day 1 to day 7 ($p < 0.05$), and the highest VCD was 4.69×10^6 cells/mL, 31.4 % higher than that of the control group, which was 3.57×10^6 cells/mL (Fig. 3a). A similar trend in viability was also observed in clone LDH-C #6. On day 5, the cell viability of LDH-C #6 was significantly higher than that of the control ($p < 0.05$), and the trend continued until the end of fed-batch (Fig. 3b). The concentration of lactate was about 55 mM after the feeding of lactate on day 3, which was consistent with the lactate concentration in bioreactors. Then, cells began to consume lactate and lactate concentration declined gradually in both groups. On day 7, lactate concentration of LDH-C #6 was 12.9 mM, while the lactate concentration in the control group was 30.4 mM. From day 3 to day 7, 40.0 mM lactate was consumed in LDH-C #6, and 25.8 mM was consumed in the control group (Fig. 3c). The lactate concentration of LDH-C #6 was significantly lower than that of the control from day 4 to day 7 ($p < 0.05$). To evaluate the impact of lactate supplementation on the metabolism of lactate, the yield from glucose to lactate ($Y_{\text{lactate/glucose}}$), which was an indicator of lactate formation rate, was calculated from day 3 to day 7. $Y_{\text{lactate/glucose}}$ was significantly lower in cells over-expressing LDH-C with or without lactate feeding (Fig. 3d). After lactate supplementation, 10.9 % glucose was converted to lactate after LDH-C over-expression, while that of the control was 37.5 %, indicating much less lactate was converted from glucose.

Growth and lactate profile of LDH-C over-expressed cell line with sodium pyruvate feeding

LDH catalyzes the inter-conversion of lactate and pyruvate, as it converts NAD^+ to NADH and back. Sodium pyruvate was supplemented into the medium on day 3 to assay the impact to cell growth. LDH-C over-expression enabled the highest VCD to be 8.50×10^6 cells/mL, 56.5 % higher than that of the control, which was 5.43×10^6 cells/mL (Fig. 4a). In addition, a trend of significantly ($p < 0.05$) higher VCD and viability were achieved in LDH-C #6 than that of the control from day 1 to day 9 and from day 7 to day 9, respectively (Fig. 4a,

Fig. 3 Growth and lactate profile of LDH-C over-expressed cell line in the condition of 40 mM sodium lactate feeding. **a** Growth curve. **b** Viability. **c** Lactate concentration. **d** Yield from glucose to lactate. Sodium lactate of 40 mM was added on day 3. Filled circle: LDH-C #6, empty square: pcDNA3.0 #4. Error bars represent standard deviation determined in triplicate experiments. * $p < 0.05$, ** $p < 0.01$



4b). Notably, on day 9, the viability of LDH-C #6 was 78.4 %, 19.0 % higher than that of the control. After sodium pyruvate feeding, the level of lactate was almost unchanged on day 4; then, the lactate concentration of LDH-C #6 and control increased gradually. On day 9, the level of lactate was 40.5 mM for LDH-C #6 and 7.4 mM lower than 33.1 mM of the control (Fig. 4c). The lactate concentration of LDH-C #6 was significantly lower than that of the control from days 6 to 9 ($p < 0.05$).

Effect of LDH-C over-expression on apoptosis

Apoptosis is the dominant factor leading to cell death during the late stage of fed-batch culture. Under lactate stress, with sodium lactate or sodium pyruvate feeding, LDH-C over-expression improved the cell viability as indicated in the results above. Cell apoptosis was analyzed in the fed-batch with 40 mM sodium pyruvate feeding from day 6 to day 8.

First, annexin V and PI were used to detect the early and late cell apoptosis. Cells stained by Annexin V alone implied that cells undergoing early apoptosis, and cells stained by both annexin V and PI indicated late apoptosis. Cells were exposed to 40 mM sodium pyruvate since day 3, and there was a significant apoptosis rate decrease between cells treated with experimental groups and the control group from day 6 to day 8 (Fig. 5a–c). The early apoptosis rates of LDH-C #6 were 5.3, 6.5, and 9.6 %, respectively, from day 6 to day 8 and 3.2, 12.0, and 12.5 %, respectively, lower than those of the control (Fig. 5a). During this period, the late apoptosis rates of LDH-C #6 were 5.0, 6.3, and 8.2 %, respectively, decreased

by 4.3, 2.0, and 2.6 %, respectively, compared with those of the control (Fig. 5b). Then, apoptosis rate was calculated by adding the early and late apoptosis rate together, which was the percentage of cells undergoing apoptosis in the gated cells (Fig. 5c). After LDH-C over-expression, apoptosis rates were 10.3, 12.8, and 17.8 %, respectively, which were 7.5, 14.0, and 15.0 % lower than those of the control.

Then, apoptosis was confirmed by a mitochondrial membrane potential (MMP) assay. JC-1 was used for MMP detection. In the case of high MMP, JC-1 exists in aggregate form, which emits red fluorescence, and non-aggregated JC-1 emits green fluorescence. The ratio of red to green fluorescence reflects the energy state of cells and is considered as an indicator of apoptosis. The cells over-expressing LDH-C showed increased MMP, and the difference was significant compared with that of the control from day 6 to day 8 (Fig. 5d).

The decrease in mitochondrial energy state triggers the activation of downstream caspases. Caspase 3 and caspase 7 were widely used as apoptosis marker (Han et al. 2010), which were detected by Western blot, and β -actin was set as the internal standard (Fig. 6). The quantity of cleaved caspase 3 was lower than that of the control from day 6 to day 8. The expression of cleaved caspase 7 increased from day 6 to day 8, and almost no cleaved caspases were detected in cells over-expressing LDH-C, while both of these two form caspases were detected in the control cells. It was apparent that LDH-C over-expression decreased the amount of cleaved caspases 3 and 7 on days 6, 7, and 8.

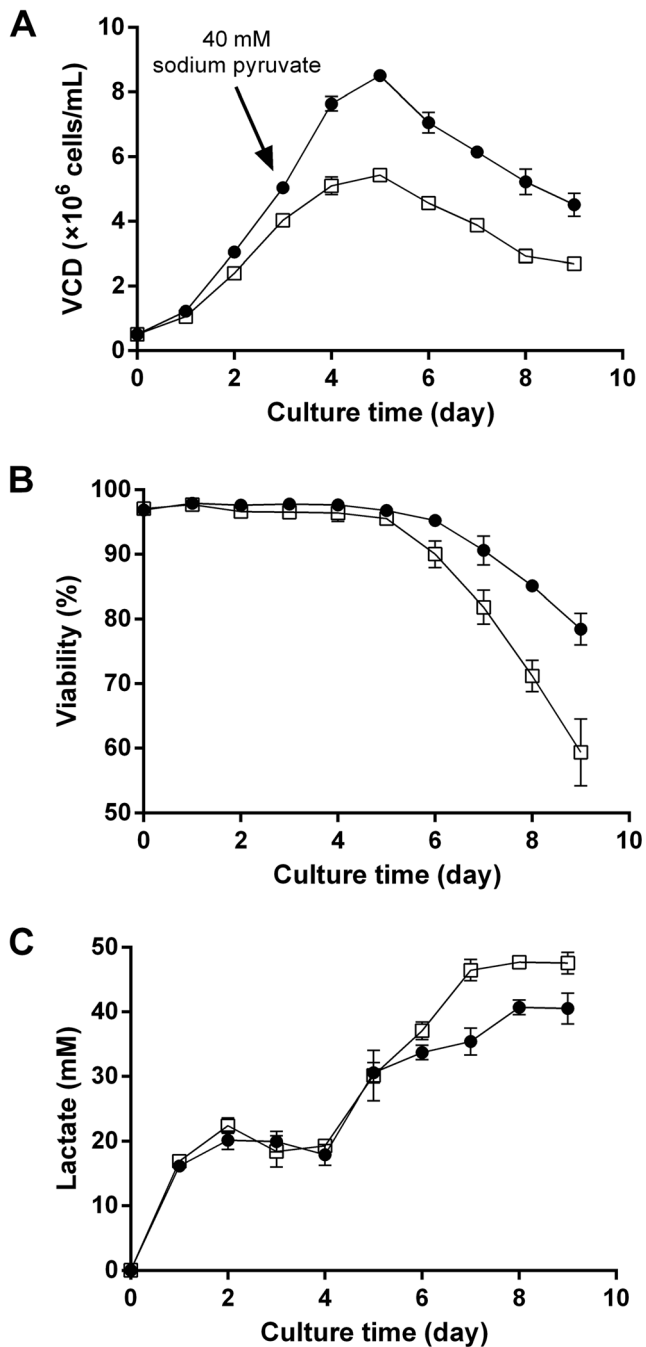


Fig. 4 Growth and lactate profile of LDH-C over-expressed cell line in the condition of 40 mM sodium pyruvate feeding. **a** Growth curve. **b** Viability. **c** Lactate concentration. Sodium pyruvate of 40 mM was added on day 3. *Filled circle*: LDH-C #6, *empty square*: pcDNA3.0 #4. *Error bars* represent standard deviation determined in triplicate experiments

Discussion

Lactate is an inhibitory product accumulated in culture medium of CHO cells, which hampers cell growth and reduces the yield of recombinant proteins (Lao and Toth 1997). To address lactate accumulation, we developed a genetic engineering

method by over-expression of LDH-C. In the situation of high lactate, LDH-C over-expression prompts cell growth and inhibits apoptosis, eventually improves integral viable cell concentration.

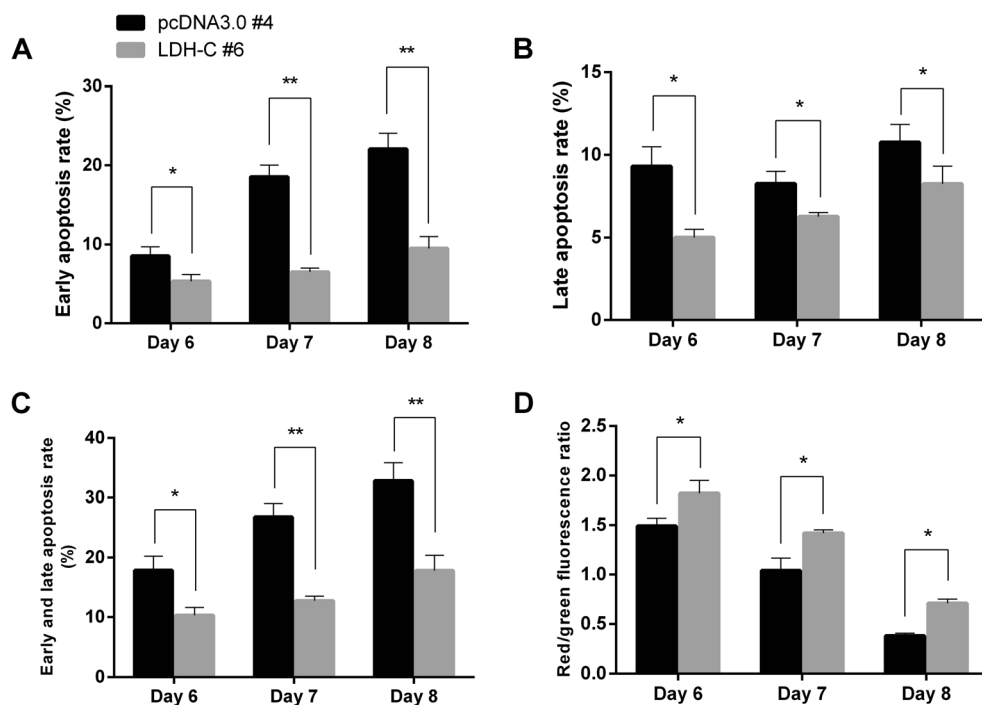
A cell line with LDH-C over-expression was constructed successfully. A PCR assay at the mRNA level indicated that LDH-C was expressed, and further assay of the enzyme activity confirmed the ectopic expression of human LDH. The enzyme activity from lactate to pyruvate increased significantly, while the activity of the reverse reaction was not significantly changed. This implies that LDH-C consumes lactate rather than blocks the formation of lactate.

In the previously developed typical fed-batch culture, LDH-C over-expression improved the viable cell density at the early phase of cell culture; however, it failed to affect the VCD at the late period of the exponential and stationary phase. As shown in the study, LDH-C converts lactate to pyruvate. Although LDH-C over-expression improved the viable cell density in the condition of lactate lower than 20 mM, better improvement might be achieved if higher concentration of lactate was applied considering that lactate functioned as the substrate of LDH-C. Generally, lactate concentration lower than 20 mM is harmless to cell growth or productivity, while lactate concentration exceeding 40 mM inhibits cell growth, and lactate concentration between 20 and 40 mM impairs productivity (Wagner 1997). To evaluate the performance of LDH-C at the lactate concentration injurious to cell growth, we fed sodium lactate directly or sodium pyruvate to make the final concentration of lactate greater than 40 mM.

In the situation of 40 mM sodium lactate feeding on day 3, the lactate concentration was raised up to 40 mM due to the lactate previously formed. Over-expression of LDH-C elevated the viable cell density and improved viability. Moreover, the lactate concentration decreased faster than that of the control after lactate supplementation and finally made the lactate level lower than 20 mM. This reveals that LDH-C accelerates the consumption of lactate in the condition of high lactate concentration. In contrast, during the normal fed-batch without lactate feeding, no significant growth and viability differences were observed, and the lactate concentrations of the two groups were no more than 20 mM. Lactate concentration lower than 20 mM may be insufficient to fully activate LDH-C.

Pyruvate is the product of LDH-C. In the experiment with 40 mM sodium pyruvate on day 3, a similar phenomenon was observed with regard to viable cell density and viability. Of note, lactate amassed up to 40 mM, and LDH-C over-expression also decreased the concentration of lactate significantly on the late phase of fed-batch. On day 5, the viable cell density of LDH-C over-expressed cells was much higher than that supplementing 40 mM sodium lactate. Pyruvate can be decarboxylated to form acetyl CoA, which is one of the key molecules in various biological reactions (Ozden et al. 2014). Additionally, pyruvate is an anti-oxidant protecting cells

Fig. 5 Apoptosis analyzed by flow cytometry and fluorescence microplate reader in the fed-batch supplemented with 40 mM sodium pyruvate from day 6 to day 8. **a** Early apoptosis rate. **b** Late apoptosis rate. **c** The sum of early and late apoptosis rate. **d** Mitochondrial membrane potential determined by JC-1. Error bars represent standard deviation determined in triplicate experiments. * $p < 0.05$, ** $p < 0.01$



against the lethal effects of hydrogen peroxide (Andrae et al. 1985). These may serve as explanations for the enhanced cell density compared with that of lactate feeding.

In addition to improved cell growth of LDH-C over-expression under lactate stress, viability was also augmented, in the case of 40 mM sodium pyruvate feeding for example. Further analysis indicated alleviation in apoptosis, increase in mitochondrial membrane potential, and decrease in cleaved caspases 3 and 7. This implied that the mitochondrial pathway participated in LDH-C inhibited apoptosis, i.e., LDH-C elevated mitochondrial membrane potential, which inhibited the activation of caspase 3 and caspase 7, and finally blocked the process of apoptosis to a certain extent. Increased lactate production lowers the mitochondrial membrane potential, an indicator of apoptosis (Tiefenthaler et al. 2001). In the situation of 40 mM sodium pyruvate feeding, inhibition of apoptosis may derive from the decrease in lactate concentration through the function of LDH-C.

Lactate represents an undesirable product in the culture of mammalian cells (Wagner 1997; Cruz et al. 2000; Jaskille et al. 2004), and many solutions are proposed to overcome the problem (Gagnon et al. 2011; Altamirano et al. 2000; Ma et al. 2009; Wong et al. 2005). A genetic engineering strategy is almost a method once and for all, which takes less effort and time. LDH-C engineering is an effective way to improve cell growth and shows no significant side effect to CHO cells, such as metabolic burden. LDH-C over-expression steers glucose to the more efficient pathway-TCA cycle, rather than the by-pass

pathway of lactate production; thus, cell line with LDH-C over-expression demonstrates the characteristic of energy high-efficiency. Furthermore, engineering of LDH-C saves time and energy to construct a CHO cell line for the further production of recombinant proteins.

In conclusion, a novel cell line with higher lactate tolerance, lowered lactate production, and alleviated apoptosis response was developed by engineering of LDH-C. In the situation of high concentration of lactate, this cell line shows preferable cell growth and attenuated apoptosis, demonstrating the potential of LDH-C

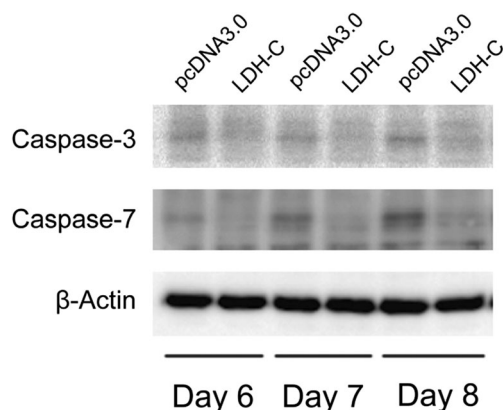


Fig. 6 Western blot analysis of total intracellular proteins in fed-batch culture supplemented 40 mM sodium pyruvate. Proteins were probed with caspase 3 (cleaved) and caspase 7 (cleaved) antibody. β -actin was used as the internal control

engineering for superior host cell line with better therapeutic protein yield and quality of product.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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