Metabolic Analysis and Antibody Function of Stably Expressed rhHER2 McAb Cells

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Abstract

CHO cells which stably express recombinant humanized anti-HER2 monoclonal antibody (rhHER2-mAb) were cultured by feeding glucose.The best condition of cell growth and protein expression was gained through fed-batch culture optimization. The influence of lactate on cell growth and protein expression weredetected. Meanwhile, the concentrations of amino acid and organic acid were measured during the cultures. The binding affinity, anti-tumor activity of rhHER2-mAb in vitro and in vivo were measured and compared with the commercialized HER2 antibody (Herceptin).The growth and metabolism of cells and productivity of rhHER2-mAb were measured under different concentrations of glucose and glutamine during cell culture. The results showed as that: The higher cell density and longer time with high viability were obtained when glucose was fed. However the titer of antibody was almost the same as the control.There was no effect of lactate on cell growth and metabolism, but lactate could reduce the antibody productivity. In addition, there was no significant difference between the rhHER2-mAb and Herceptin in the affinity with HER2 receptor. WST-8 method was used to detect the in vitro anti-tumor activity of this antibody. The results showed that there was no significant different between the rhHER2-mAb and Herceptin in affinity and in vitro anti-tumor activity. The BT-474 xenograft nude mice model was established to detect the anti-tumor activity of this rhHER2-mAb in vivo.

Keywords

Humanized anti-HER2 monoclonal antibody; cell culture; metabolic analysis; activity analysis.

1. Introduction

Breast cancer (BC) is one of the common malignant tumors in women, accounting for 30% of new malignant tumors. Its incidence rate is the first in the incidence of female malignant tumors in the world [1-3], and it is increasing year by year. In recent years, through the study of its molecular mechanism, it was found that HER2/neu, erbB2 (human epidermal growth factor receptor) is selectively overexpressed in breast cancer patients, and its expression level is proportional to the severity of the disease [4]. HER2/neu, erbB2 is a member of the type I transmembrane tyrosine kinase receptor family and is encoded by the proto-oncogene CerbB2 (HER2/neu) and has the activity of a receptor tyrosine kinase (RTK).Its molecular weight is 185 kDa, and it is mainly distributed on the cell membrane and can initiate and induce cell proliferation and growth through its own mutual binding, thereby triggering the proliferation and metastasis of tumors [5-15]. About 25% to 30% of breast cancer patients have HER2 receptor overexpressed in cancer cells. The prognosis of these patients is significantly worse than those with low HER2 expression [16].The recombinant human anti-HER2 monoclonal antibody (rhHER2-mAb) produced by Roche is also known as trastuzumab (trade name: Herceptin) and is the first humanized monoclonal antibodies for HER2/neu gene amplification. It can specifically recognize and act on the extracellular location of HER2, allowing it to leave the cell membrane by the phagocytosis and enter the nucleus, inhibiting its signal transduction, so as to achieve treatment of tumors. It is mainly used for the treatment of human breast

cancer mediated by over-expressed HER2 (human epidermal growth factor) growth and metastasis [17-19], and also for the treatment of other HER2 highly-expressing tumors. Figure 1 shows the molecular structure of herceptin: It is a glycosylated, covalently cross-linked tetramer consisting of two heavy chains and two light chains, where each light chain contains 214 amino acids and each heavy chain contains 450 amino acids. Herceptin's constant and variable zones are the human (IgG1) part (white area), and the hypervariable area is the mouse source part (black area). The molecular weight of the whole antibody was 148.72 kDa, and the isoelectric point was between pH 8.3-9.5. The extinction coefficient is 1.43 [20]. Herceptin was approved for listing in the United States in 1998 [21]. It was marketed in China in 2002 and has achieved exact results in clinical trials and subsequent applications. In 2010, Herceptin, the first FDA-approved clinically-applied solid tumor, had global sales of $5.5 billion. Herceptin's generic research has become a hot topic at home and abroad.

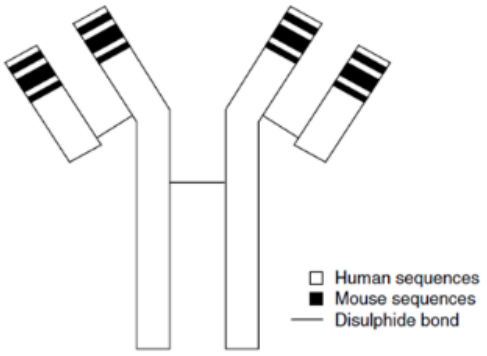

Figure. 1. Herceptin molecular structure

Mammalian cells can use glucose to produce ATP via glycolysis, TCA cycle, and oxidative phosphorylation; under hypoxic conditions, pyruvate turns to lactic acid. However, in most mammalian cell lines, glucose to lactic acid metabolism is not affected by oxygen supply. This phenomenon is called "Wage's effect" or "aerobic glycolysis. “Aerobic glycolysis” is an inefficient metabolic type that produces only 4 mole of ATP per mole of glucose when lactic acid is produced from glucose [22,23]. It has been reported that the more glucose in the medium, the faster its consumption and the more lactic acid it produces. While lactic acid inhibits cell growth and product formation, it also affects the glycosylation structure of proteins [24,25].

Glutamine glycolysis is achieved through the TCA cycle of glutamine. The first step is: glutamic acid is converted to glutamine by glutamines. Glutamine then produces α-ketoglutarate under the action of deaminase or transaminase, a-ketoglutarate and alanine are produced under the action of alanine aminotransferase; Alpha-ketoglutaric acid and aspartic acid are produced by the action of aspartate aminotransferase, and one or two molecules of ammonia are produced in this process [26,27].It is documented that ammonia produced by almost all CHO cells is released from the amino group of glutamine and produces alanine and glutamic acid. Most of the alpha-ketoglutarate is derived from transamination rather than from the action of glutamine dehydrogenase. The rate of decomposition of glutamine depends on pH, temperature, and phosphate concentration [28-31].

Mammalian cells can use amino acids for protein biosynthesis and cell growth, and can theoretically be used for catabolism. During the specific experimental period, the amino acid metabolism was studied by combining the rate of amino acid depletion and its production rate with the C13 labeling experiment.

2. Experimental Method

2.1 Cell Culture and Density and Activity Measurement

2.1.1 Cell Culture

CHO cells stably expressing rhHER2-mAb were seeded in serum-free ProCHO5 medium at the cell inoculumdensity of 5 x 10^5 cells/mL in their logarithmic phase. The cultures were incubated in

disposable 50 ml TubeSpin bioreactors in the presence of 5% $CO_2$and saturated humidity at 37°C with agitation at 180 rpm. Cells were subcultivated every 3-4 days at a seeding density of 3-5 × 10^5 cells ml^{-1}.

2.1.2 Cell Density and Activity Assay

Cells were counted with blood cell counting and the cell viability was determined by trypan blue staining.The count is repeated three times and the average value is taken.Pipette 70 μl trypan blue staining solution and 10 μl cell culture fromTubeSpin bioreactors into the EP tube, shaking well and adding the blood count plate, then observed under the microscope (diluted 8 times). Count cell density and cell viabilityin a good view of the cells.Cell viability = (total cell count - number of dead cells)/total cell count.

2.2 Protein Expression Detection

Collected cell culture then centrifuged at 800×g and time 5 min,the supernatant was separated and stored at −20°Ctill used for ELISA detection. Two-site sandwich ELISAs were carried out to identify and quantify therhHER2-mAb fusion protein in cell culture supernatant.Microtiter plates coated with 100μl ofGoat anti-human IgG-Fc (dilute it to l:1000) were incubated at 4°Covernight. Aspirate each well and wash, repeating the process two times,wash by filling each well with 300μlPBSTwash Buffer.The diluted samples, Herceptin standards and quality control products were added for 1h at 37°Cand each samples was repeated three wells in one test. Aspirate each well and wash by filling each well with 300μlPBST wash buffer three times.Add alkaline phosphatase-conjugated Goat anti-human IgG-Fc was added at 1:1000 dilutions in PBST for test antigens. After an hour at 37°Cand five times washes,add 100μl of coloring fluid to each well and incubate for 15 minutes at room temperature, and then reactions were stopped with stopping solutions (100μl /well). Absorbance was measured at 405nm using a microplate ELISA reader.

2.3 Glucose Assay

Add 100μl of assay reaction mixture into each well of glucose standard, blank control, and test samples to make the total glucose assay volume of 101μl /well. Mix thoroughly and incubate the reaction for 15 minutes at 37 °C, protected from light. Determine the absorbance of each tube at 505nm. Glucose (mmol/L) = sample tube absorbance (A) / calibration tube absorbance (A) × calibration solution concentration. When the test result is greater than 28mmol/L, please dilute with normal saline and measure again. The result is multiplied by the dilution factor.

2.4 Determination of Lactic Acid Content

Add 50μl of enzyme mix and 10μl coloring fluid into each tube of standard, blank control, and test samples which there are 1μl 3nM standard solution, double distilled water, and sample to be tested. Mix thoroughly and incubate the reaction for 10minutes at 37 °C, stop reaction and determine the absorbance at 530nm. Lactate content (mmol/L) = (assay tube absorbance - blank tube absorbance) / (standard tube absorbance - blank tube absorbance) × standard concentration (3mmol/L) × sample dilution before the test.Note: Pre-experiments are needed before batch experiment to determine the absolute OD between 0.1-0.3. If the absolute OD is less than 0.1, the sample concentration must be increased to re-measure; if the absolute OD is greater than 0.3, the sample needs to be diluted.

2.5 Determination of Pyruvate Content

The pyruvate content was determined by colorimetry. The measurement procedure is as follows:

Table 1

	Blank	Standard	Sample
double distilled water(ul)	4		
0.2uMPyruvate standard(ul)		4	

Test sample(ul)			4
Reagent二(ul)	20	20	20
After mixing, bath at 37 ° C for 10 minutes			
Reagent三(ul)	100	100	100

After 5 minutes at room temperature, the absorbance of each tube was measured at 505 nm.

2.6 Determination of Amino Acid Content

The amount of amino acids in the cell supernatant was determined using HPLC. Take 100ul of amino acid mixed standard solution or sample extract, add 1ul of internal standard and 100ul of OPA reagent, react for 2min, then sample can be analyzed. The chromatographic conditions were as follows: UV detection wavelength: 338nm; column temperature: 40 °C; flow rate: 1 mL/min, gradient elution. The gradients used were as follows:

Table 2

time(min)	0	14	25	28	30
B(%)	0	50	100	0	0

2.7 Determination of Organic Acid Content

The amount of organic acid in the cell supernatant was determined using HPLC. The chromatographic conditions were as follows: UV detection wavelength: 215nm; column temperature: 30 °C; flow rate: 1 mL/min, isocratic elution.

2.8 Extracellular Affinity Detection of Anti-HER2 Humanized Monoclonal Antibodies

Dilution the HER-2 Protein to 2.5 μg/mL for detection and the method is the same as the protein expressiondetection.

2.9 The Main Pharmacodynamics Study of Anti-Tumor Effect of Anti-HER2 Humanized Monoclonal Antibody

0. 72mg 17β-estradiol sustained-release tablets implanted subcutaneously in the neck 1 day before inoculation,trypsin digestion the BT-474 cells in logarithmic growth phase and washed with serum-free 1640 medium. The number of viable cells was adjusted to 1×10^{7} cells/mL, mixed with matrigel gel 1:1, and inoculated on the nude mouse breast pad or subcutaneously to establish a tumor model. After tumor cells were inoculated, the long diameter (L) and short diameter (W) of the tumor were measured and the tumor volume was calculated. The formula for calculating the tumor volume (TV) is: $TV = 0.5 \times L \times W^2$. After 2 weeks of tumor cell inoculation, nude mice were randomly divided into 3 groups, 7 in each group, and were dosed as follows: The PBS control group and rhHER2-mAb treatment group received an injection dose of 10 mg/kg; each group was administered intraperitoneally twice a week for 5 weeks.

3. Result

3.1 Effects of Glucose and Lactic Acid on Cell Growth and Protein Expression

3.1.1 Effects of Different Concentrations of Glucose on Cell Growth and Protein Expression

The glucose concentration in the experimental group was 0.5 g/L, 1.0 g/L, 1.5 g/L,and 3.0 g/L,the control group did not add glucose. As we can see from Figure 2, the higher the glucose concentration, the higher the maximum cell density, and after adding 3.0 g/L glucose, the maximum live cell density is 9.0×10^{6} cells/mL. At the same time, the duration of high cell viability was prolonged. After 20 days of cultivation, the experimental groups were still around 65%while the cell viability of the control group was lower than 50%. As shown in Figure 2D, the protein expression level of the control group

was higher than that of the experimental group after addition of glucoseat the 16th day, where the control group had reached 200 mg/L, while the experimental group was between 100 to 150 mg/L. However, after 16 days of culture, the amount of proteinexpression in the experimental group increasedrapidly, and there was no significant difference in protein expression in the end, the expression of protein in each experimental group was about 180 mg/L, and the control group was maintained at 200 mg/L.

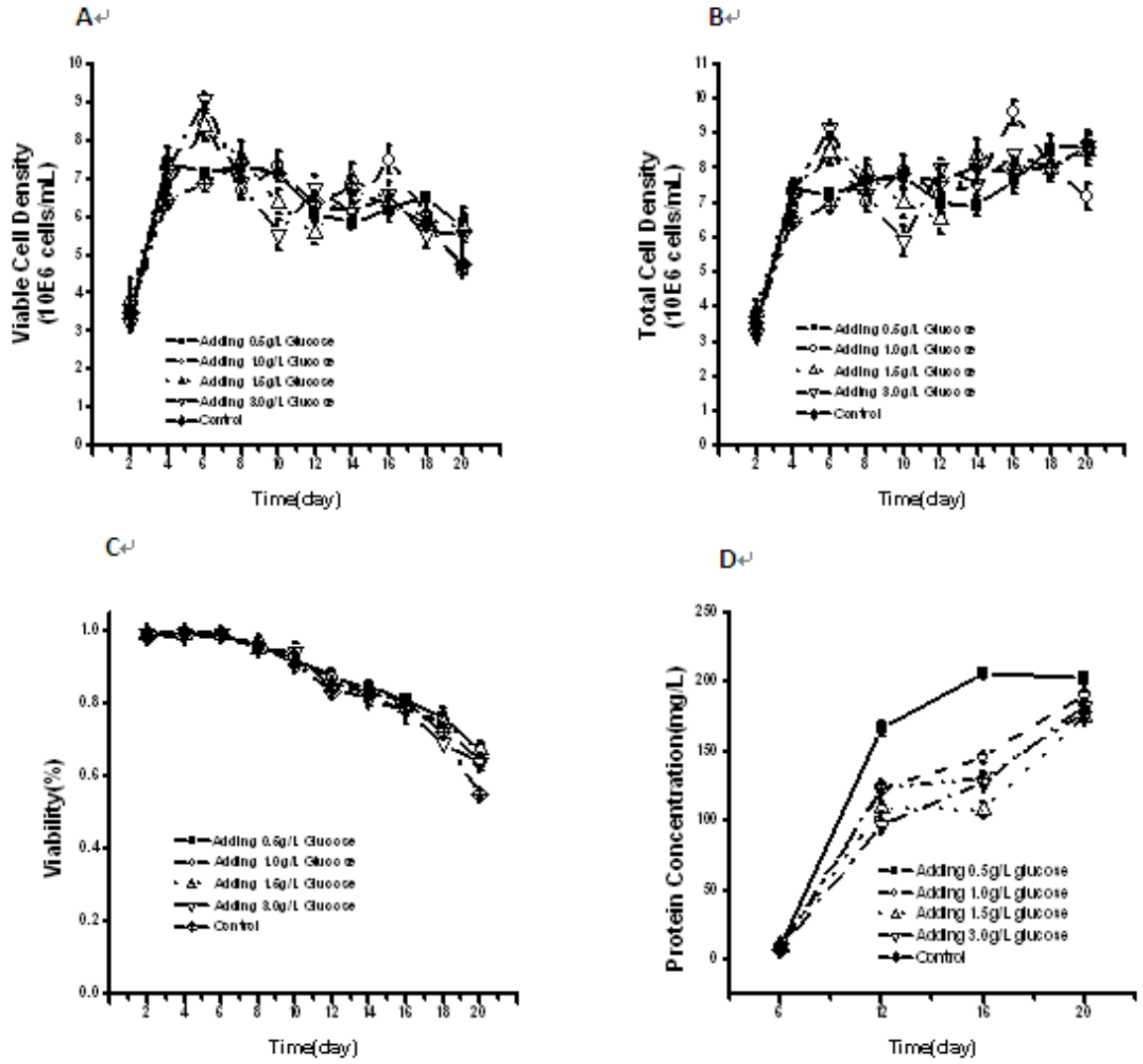

Figure .2. Effect of Glucose on Cell Growth and Protein Expression
(A) Live cell density (B) Total cell density
(C) Cell viability (D) Protein expression

As shown in Fig 3A, the greater the amount of glucose added, the higher the residual glucose content in the cell culture supernatant. And it was calculated that the glucose consumption rate at the later stage of the culture would be decline (data not shown). As can be seen from Figure 3B, the lactic acid content in the culture supernatant of the experimental group and the control group showed the same tendency: first, a large amount of lactic acid was produced at the initial stage, and reached the maximum value on the 4th day, which was approximately 18 mM. With metabolism, lactic acid begins to be consumed, the culture supernatant contains about 6 mM lactic acid at the end of culture. The ammonia and pyruvate content in the culture supernatant of each experimental group was the same as that of the control group: the maximumammonia content was 2.2 mM (see Fig 3C); As for the pyruvate ,the rate of consumption was faster in the first 4 days, subsequently, the pyruvate content

decreased slowly, and at the end of the culture was approximately 0.2 mM in the supernatant (see Figure 3D).

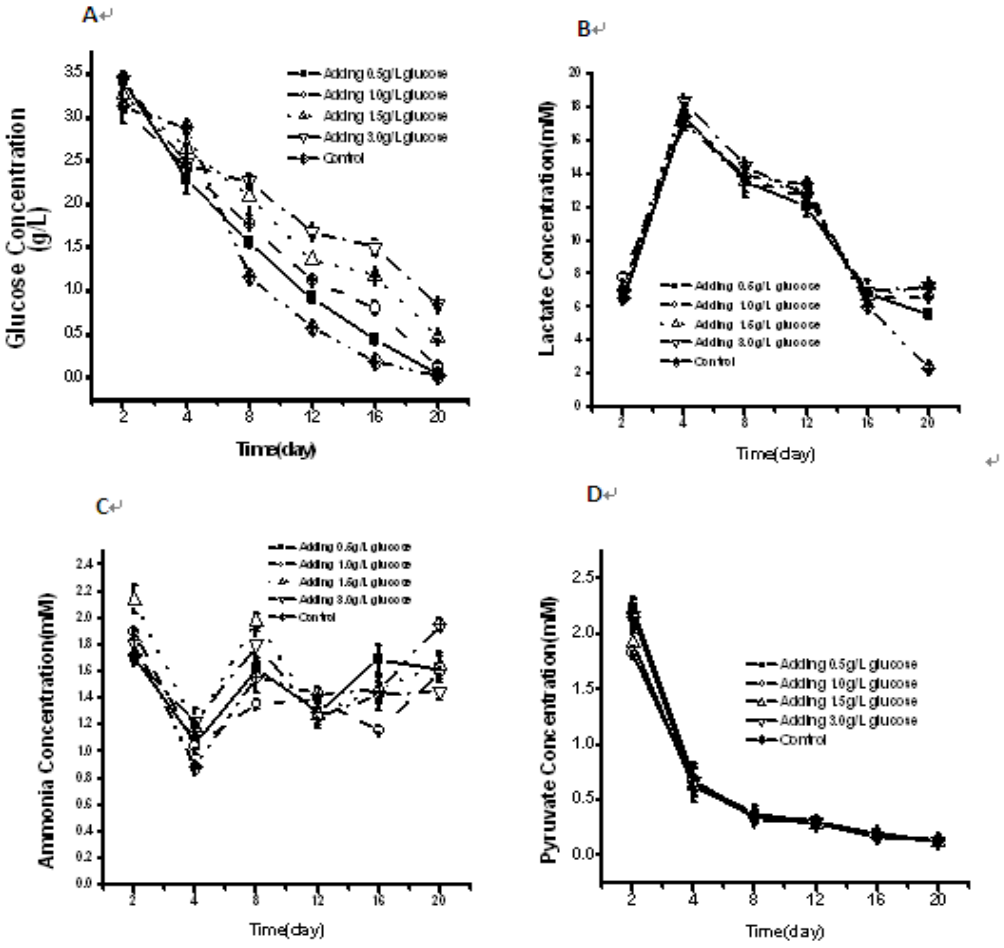

Figure .3. Glucose content, lactate content, ammonia content and pyruvate content in cultured recombinant CHO cells with different glucose concentrations
(A) Glucose content (B) Lactic acid content
(C) Ammonia content (D) Pyruvate content

3.1.2 Effect of Maintaining a Certain Concentration of Glucose on Cell Growth and Protein Expression

In the experimental group, the glucose content in the cell culture supernatant was maintained at least 3 g/L, whilethe control group wasnot added glucose.As shown in Figure 4, there was no significant difference in cell density, cell viability, and protein expression between the experimental group and the control group. The maximum cell density was approximately 8.0×10^6 cells/mL. At the end of the culture, there was also no significant difference in the protein expression between the two, approximately 165 mg/L.

It can be seen from Fig. 5A, The glucose content was significantly higher in the experimental group than the control group due to supplementation of glucose during the experiment, and was still above 3 g/L at the end of the 21-day culture. Correspondingly, sufficient glucose is metabolized to lactic acid, resulting in a large accumulation of lactic acid, and the culture supernatant contains approximately 10 mM in the culture supernatant (see Fig. 5B). Figure 5C shows that the ammonia content in the culture supernatant of the experimental group was lower than that in the control group: the maximum ammonia content in the experimental group was 3.0 mM, while that in the control

group was only about 2.2 mM. The change of pyruvate content in the culture supernatant of the experimental group was not significantly different from the control group (see Figure 5D).

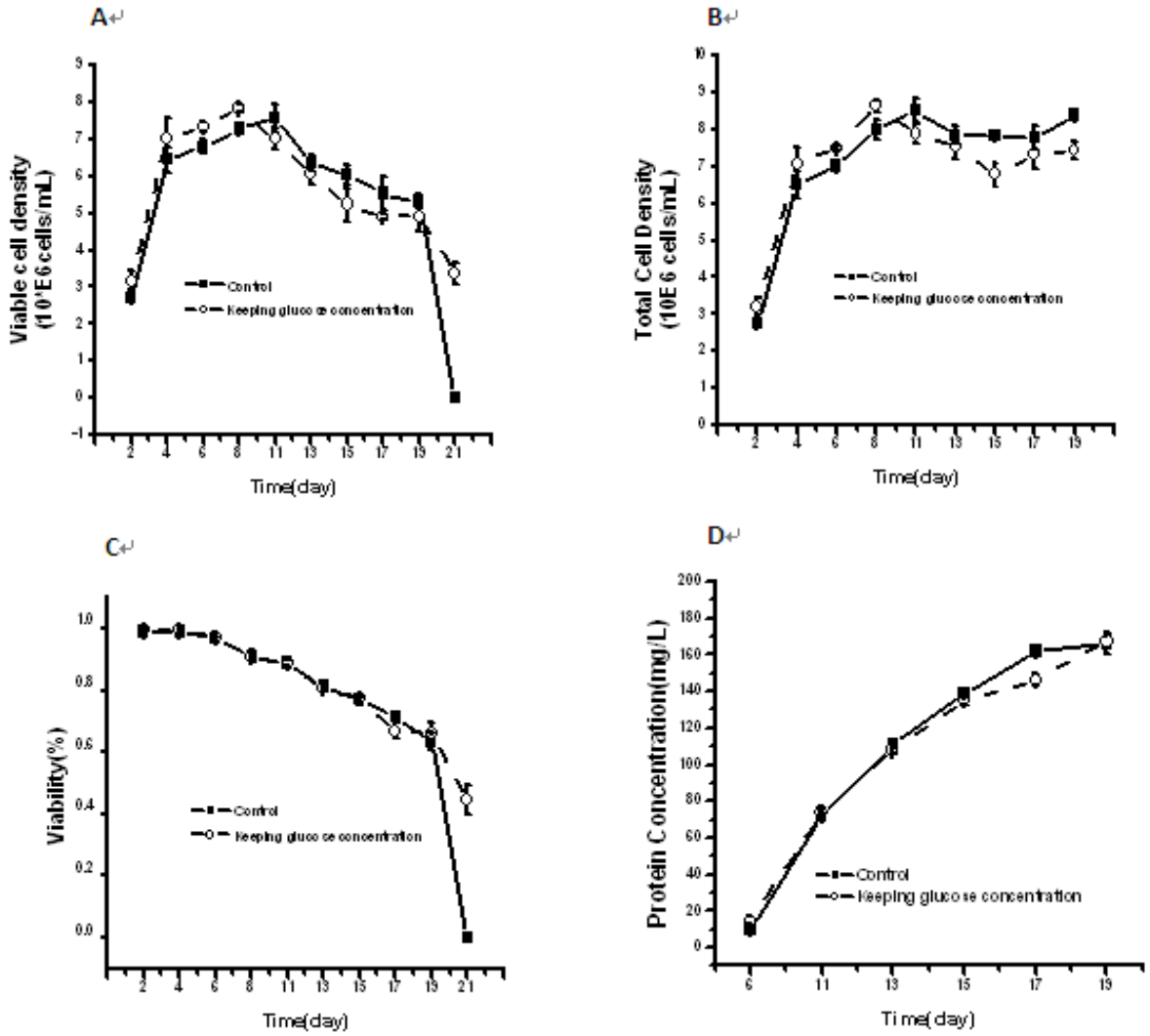

Figure.4. Effect of maintaining a certain glucose concentration on cell growth and protein expression

(A) Live cell density (B) Total cell density

(C) Cell viability (D) Protein expression

3.1.3Effects of Lactic Acid on Cell Growth and Protein Expression

Lactic acid is one of the main metabolic byproducts of animal cell culture. It is mainly derived from the metabolic process of glucose. The metabolism of glutamine and other amino acids can also producea little of lactic acid. Many literatures report that it has important effects on cell growth and protein expression [32-42].

The concentrations of lactic acid in the experimental group were: 0.1 g/L; 0.2 g/L; 0.5 g/L. The control group was no lactic acid. As shown in the figure, after adding different concentrations of lactic acid, the maximum viable cell density was not significantly different from that of the control group, but the viability was maintained longer. As can be seen from Figure 6D, the expression of protein in each experimental group was lower than that of the control group.

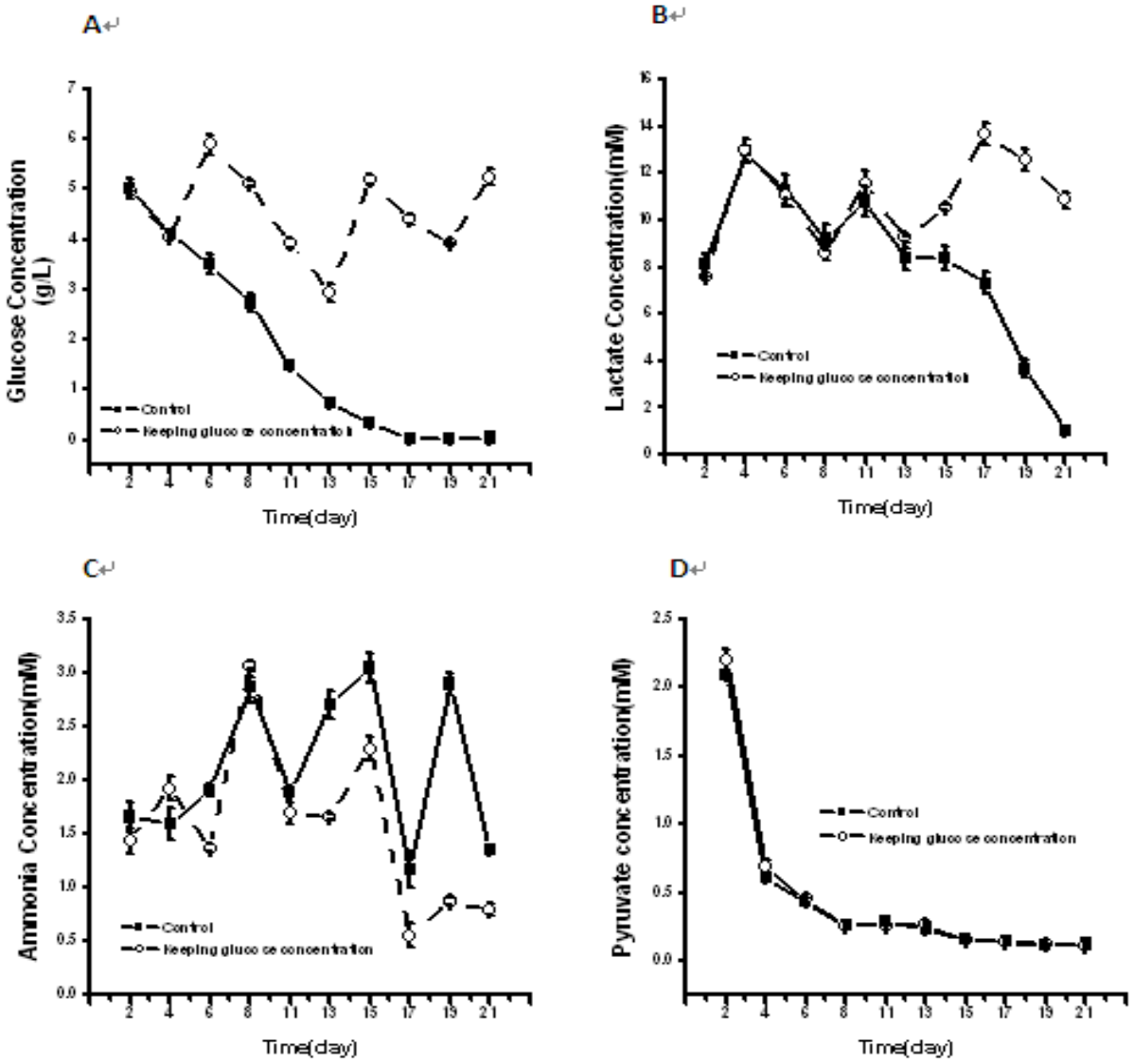

Figure.5. Curves of glucose concentration, lactic acid content, ammonia content and pyruvic acid content during batch culture of recombinant CHO cells maintaining a certain glucose concentration

(A) Glucose concentration (B) Lactic acid concentration

(C) Ammonia concentration (D) Pyruvate concentration

As can be seen from Figure 7, there was no significant difference in glucose concentrations, lactic acid concentrations, ammonia concentrations, pyruvic acid concentrationsbetween the experimental group and the control group.

3.2 Determination of Amino Acids and Organic Acids in Cell Culture Supernatants

3.2.1 Effect of Glucose and Lactic Acid on Amino Acid Utilization in CHO Cell Culture

In the experimental group, 0.5 g/L and 3.0 g/L glucose were added respectively, and no glucose was added to the control group. As shown in Fig. 8, the contents of serine, glycine, and threonine in the supernatants of the experimental group and the control group were similar. Changes in aspartic acid, alanine, tyrosine, and proline contents were similar, but the contents of aspartic acid, tyrosine, and proline in the experimental group were lower than those in the control group while alanine contents greater than the control group. The content of histidine, arginine, methionine, isoleucine, and leucine was similar at the initial stage of culture and the end of culture. However, its greater than that of the experimental group in the stable period. On the 12th day of culture, the histidine content was about 100 nmol in the experimental group and 180 nmol in the control group; the arginine content was about

10 nmol but 20 nmol in the control group; the methionine content was about 12 nmol while the control group was 24 nmol; the isoleucine content was 38 nmol in the experimental group and 48 nmol in the control group; the leucine content was 38 nmol in the experimental group and 53 nmol in the control group.

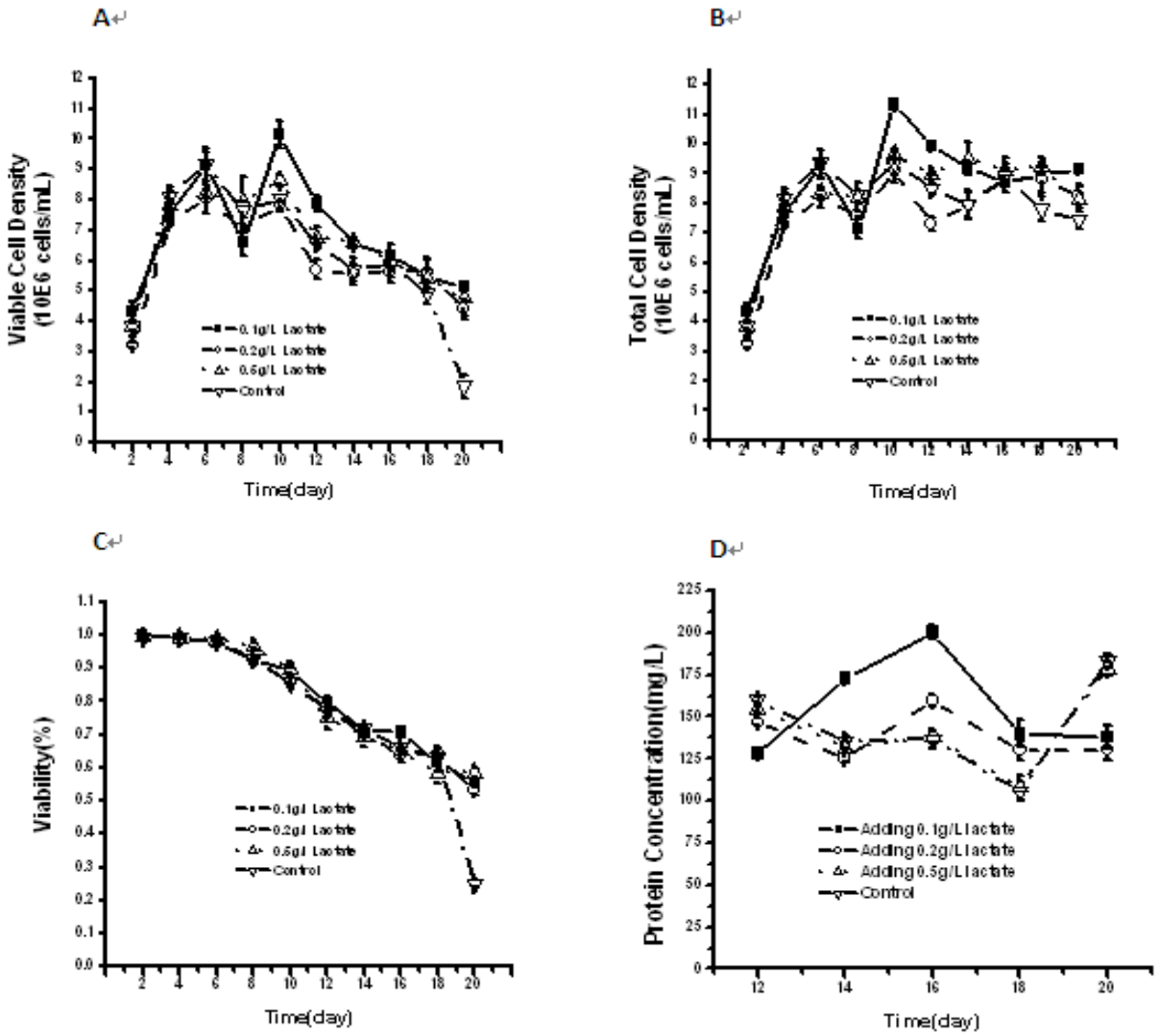

Figure 6. Effect of Lactic Acid on Cell Growth and Protein Expression
(A) Live cell density (B) Total cell density
(C) Cell viability (D) Protein expression

Lactic acid was added to the experimental group and lactic acid was not added to the control group. As shown in Figure 9, only aspartic acid, threonine, alanine, isoleucine, and leucine were detected during CHO cell culture, and there was no significant difference between the experimental and control groups.

3.2.2 Effects of Glucose and Lactic Acid on the Metabolism of Organic Acids in the Culture of CHO Cells

The glucose concentration in the experimental group was 0.5 g/L and 3.0 g/L, respectively. No glucose was added to the control group. As shown in the logarithmic phase and stationary phase, the content of citric acid was 1.0 mg/mL on the 6th day in the control group what was significantly higher than that of the experimental group. At the 12th day of culture, no citric acid was detected in both the control and the experimental groups. The experimental group maintained a certain glucose concentration, and the control group did not perform treatment. It can be seen that the citric acid

content and its change tendency are similar, and the content was 1.0 mg/mL on the second day of culture, and citric acid could not be detected on the 12th day of culture.

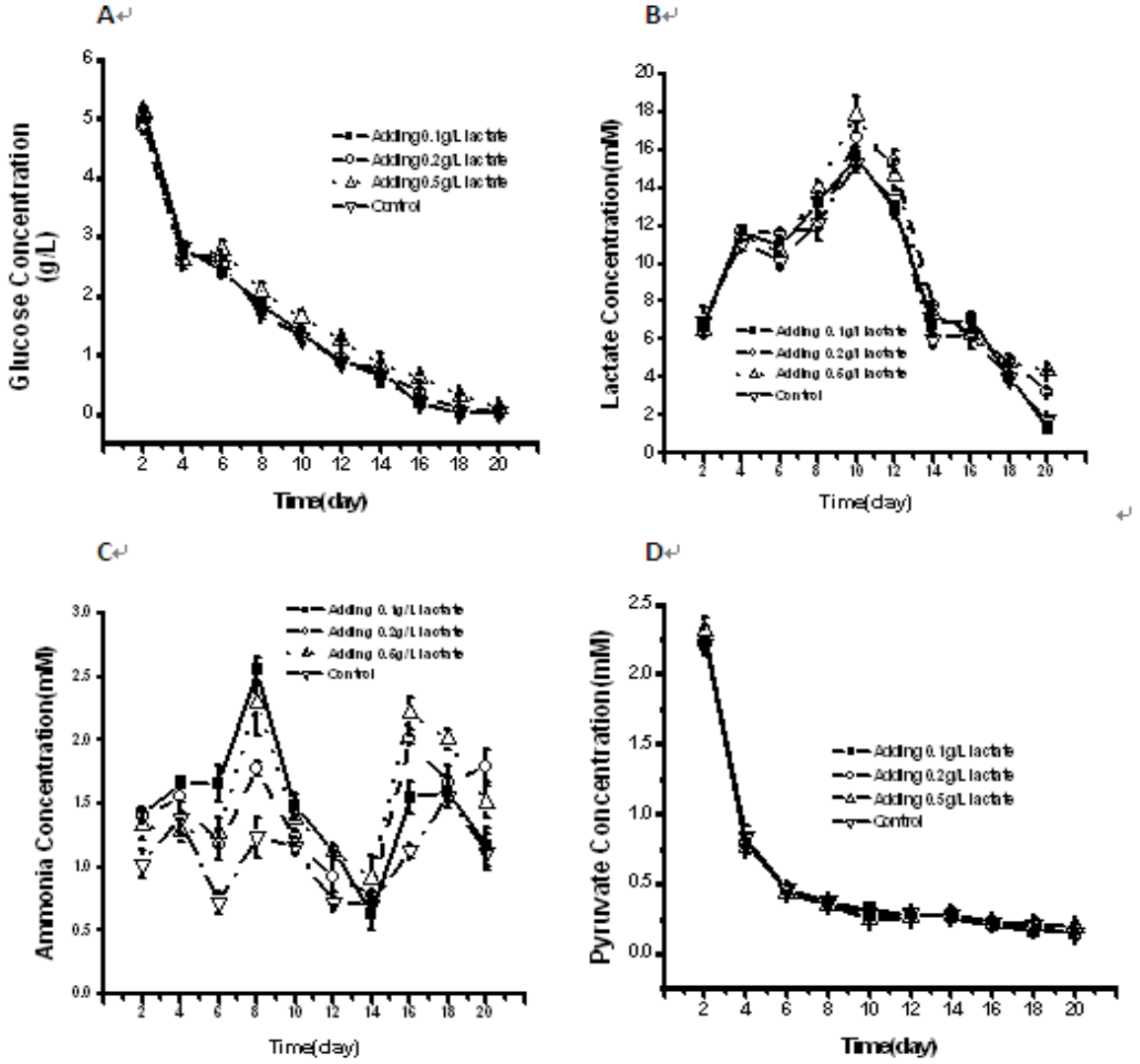

Figure 7. Variation curves of glucose content, lactic acid content, ammonia content and pyruvic acid content in batch culture of recombinant CHO cells with different concentrations of lactic acid

(A) Glucosecontent (B) Lactic acid content

(C) Ammonia content(D) Pyruvate content

Lactic acid was added to the experimental group and not added to the control group. As shown in Fig. 11, the content of α-ketoglutarate, citric acid, and pyruvate in the experimental group and the control group were similar during the cultivation of CHO cells. Among them, The content of α-ketoglutarate was about 0.08 mg/mL on the second day, and it was about 0.14 mg/mL at the end;The content of citric acid was about 0.12 mg/mL at the second day, and that was about 0.50 mg/mL at the end; The pyruvate content was about 0.015 mg/mL on the secondday, and then the content decreased continuouslyalmost no detectable at the end.

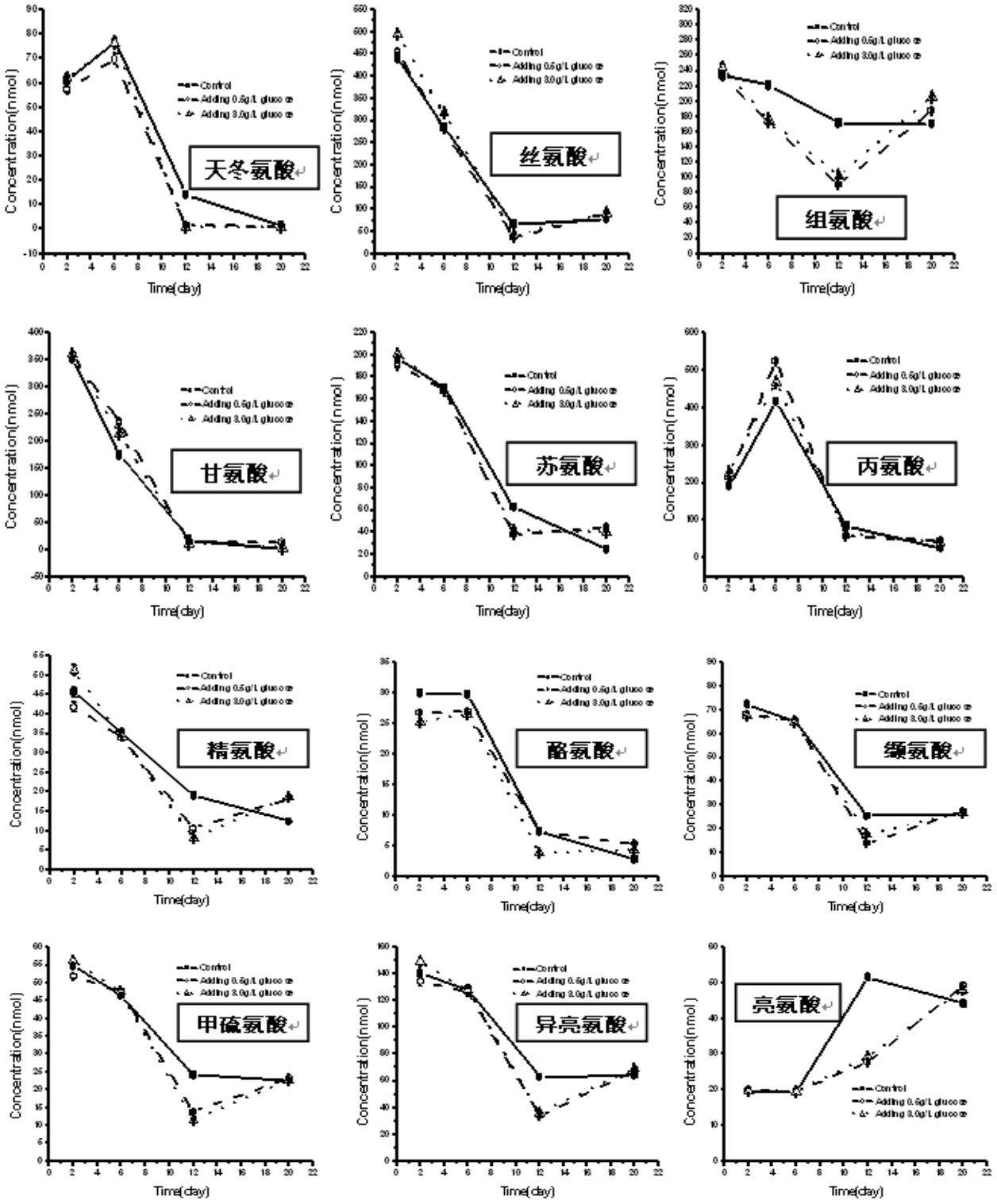

Figure .8. Effect of glucose on amino acid utilization in CHO cell culture

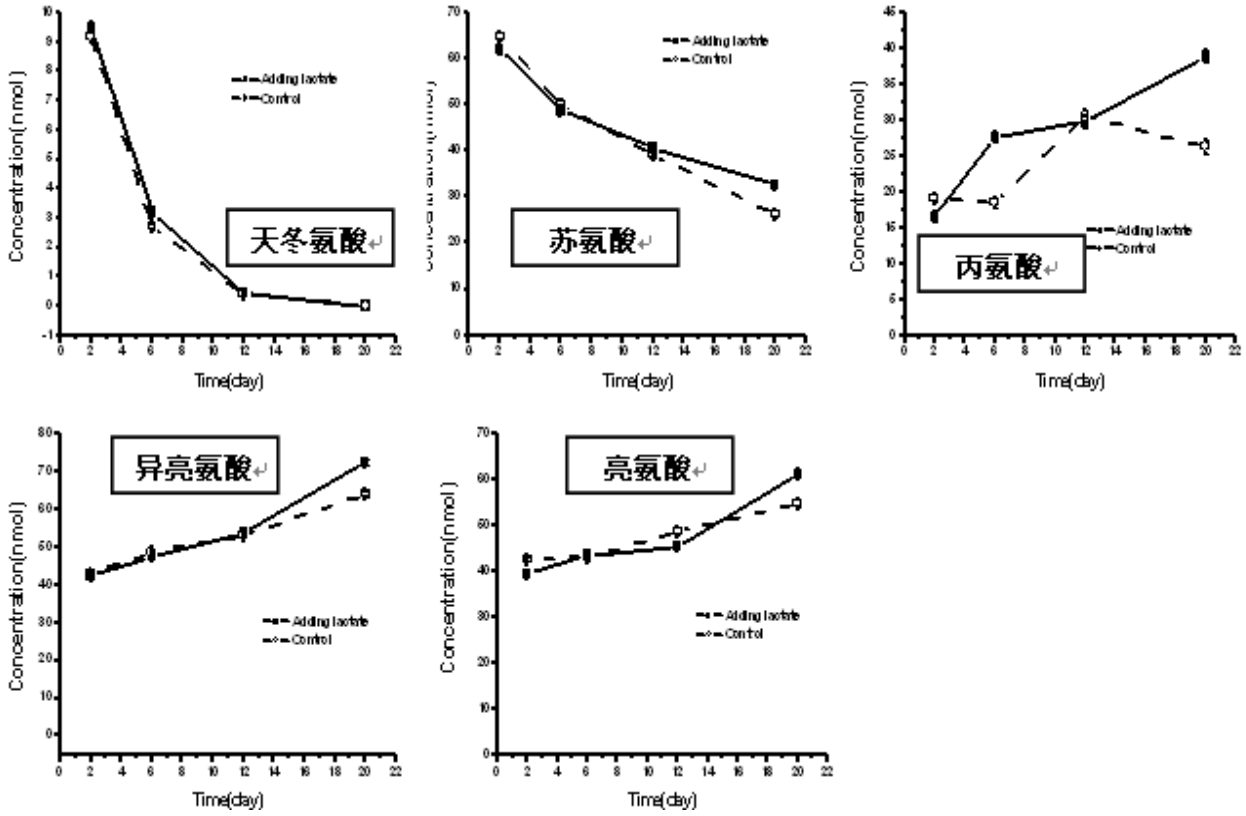

Figure .9. Effect of Lactic Acid on Amino Acid Utilization in CHO Cell Culture

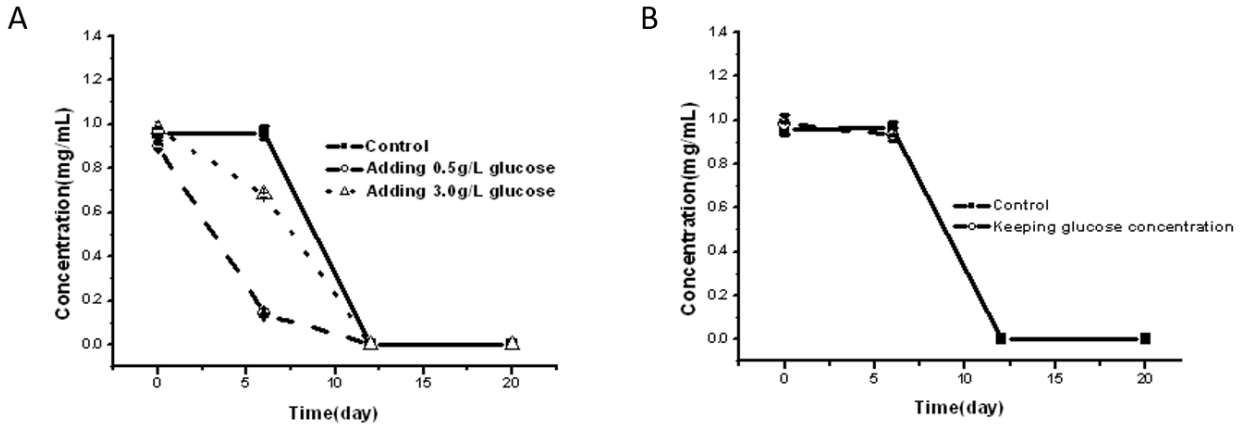

Figure .10. Effect of Glucose Concentration on Citric Acid Metabolism During CHO Cell Culture

3.3 Detection the Extracellular Affinity and Anti-tumor effect of Anti-HER2 Humanized Monoclonal Antibodies

The ELISA method was used to detect the extracellular affinity of anti-HER2 humanized monoclonal antibody for HER2 protein. As can be seen in Figure 12A, the recombinant protein can specifically bind to HER2 protein, and the herceptin standard is used as a control to detect the extracellular affinity of the two proteins. The EC50 of the rhHER2-mAb fusion protein was 0.0423 ± 0.001 nM, 0.0396 ± 0.001 nM; the herceptin standard had an EC50 of 0.0410 ± 0.001 nM. The experimental results show that the two have similarities in extracellular affinity.

At the beginning,WST-8 was used to detect anti-HER2 humanized monoclonal antibodies Anti-HER2 humanized monoclonal antibodies have an inhibitory effect on the growth of HER2 positive BT-474 cells. Next, the BT-474 cells were inoculated on the breast pads. After 3 days, the nude mice all grew tumors with a volume greater than 100 mm3, and the shape was relatively neat. In the rhHER2-mAb-treated group and the PBS-treated group, BT-2474-bearing nude mice were treated continuously for 18 days after inoculation of cells. The tumor volume was significantly reduced in the rhHER2-mAb-treated group, while the tumor volume remained relatively stable in the PBS-treated group. The nude mice of the rhHER2-mAb treated group died after 15 days, while the nude mice of the PBS treated group died after 25 days. For weight of nude mice, there was no significant difference between the rhHER2-mAb treatment group and the PBS treatment group within 24 days of inoculation of cells. However, the rhHER2-mAb treatment group lost weight in the later period, and the survival time was shorter than that of the PBS treatment group.

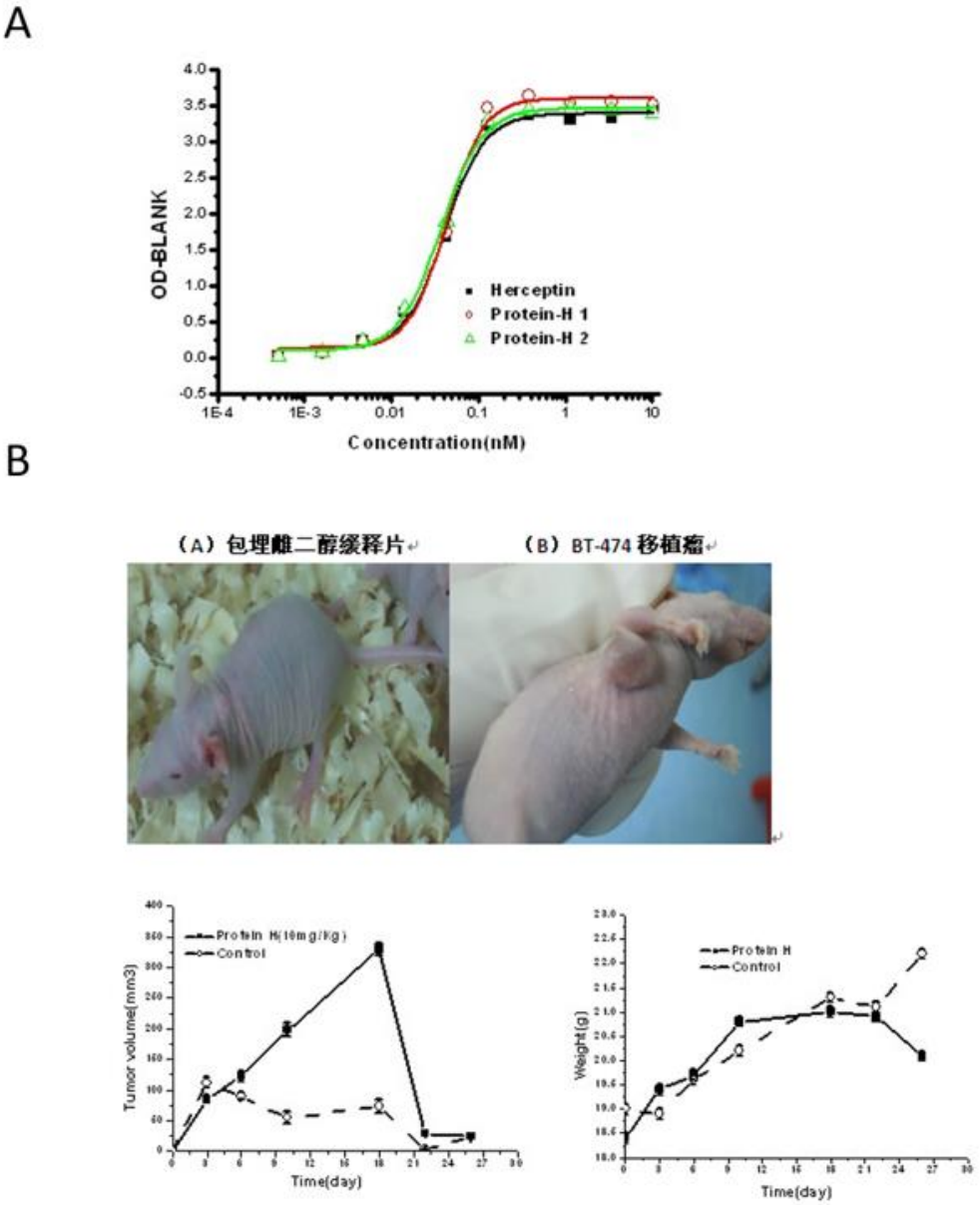

Figure .12. rhHER2-mAb fusion protein Activity Assay and Pharmacodynamic studies
A: rhHER2-mAb fusion protein and Herceptin on HER-2 protein Extracellular affinity comparison
B: Growth curves of BT-474 transplantation tumor and weight changes of nude mice

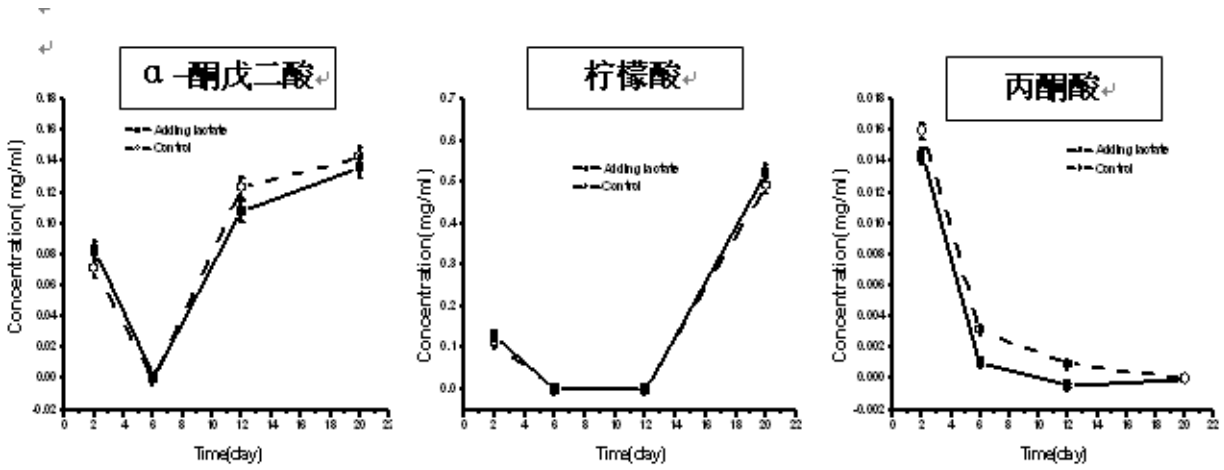

Figure .11. Effect of lactate on the metabolism of organic acids in CHO cell culture

4. Conclusion

Different concentrations of glucose were added during CHO cell culture to observe cell growth and protein expression changes.Among them, the addition of glucose will increase the cell density; the addition of it will contribute to cell protein expression.By studying the effect of lactate, a major metabolic by-product, on cell growth and protein expression, it was found that the addition of lactic acid during cell culture resulted in a decrease in the amount of protein expression.At the same time, the contents of amino acid and organic acid in the culture supernatant were determined and it was found thatafter the addition of glucose, the metabolism of aspartic acid, alanine, tyrosine, proline, and citric acid was affected.

The activity of the recombinant anti-HER2 humanized monoclonal antibody (rhHER2-mAb) expressed in CHO cells was tested. Its extracellular affinity and anti-tumor effects were not significantly different from the standard Herceptin.At the same time, we established a BT474 breast cancer xenograft model to study the anti-tumor effect of this antibody in vivo.In the actual modeling process, nude mice lost weight after 12 days of rhHER2-mAb treatment and died after 15 days;While the PBS-treated group died after 25 days of treatment, the survival time of the nude mice was less than the expected time what can evaluate the anti-tumor effect of rhHER2-mAb in vivo.It may be because of the physiological injury to nude mice caused by the estrogen-sustained-release tablets; the estrogen level is not suitable.

Acknowledgments

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References

[1] Perou C M, Sørlie T, Eisen M B, et al., Molecular portraits of human breast tumours. Nature Publishing Group, 2000. 406: p. 747-752.

[2] Prat APerou C M, Deconstructing the molecular portraits of breast cancer. Molecular Oncology, 2011. 5(1): p. 5-23.

[3] Jemal A, Siegel R, Xu J, et al., Cancer statistics. Wiley Online Library, 2010. 60: p.277-300.

[4] Jemal A, Siegel R, Ward E, et al. Cancer statistics. Wiley Online Library, 2007, 57:43-66.

[5] Czuczmanms, Grillo Lopezaj, Whiteca, et al. Treatment of patients with low-grade B-cell lymphoma with the combination of chimeric anti-CD 20 monoclonal antibody and CHOP chemotherapy[J]. Clin OncoL, 1999, 17(1): 268-276.

[6] Vose JM, Link BK, Grossbard ML, et al. Phase II study of rituximab in combination with CHOP chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma [J]. Clin OncoL, 2001,19 (2):389-397.

[7] Slamon DJ, Leyland-Jones B,Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that over expresses HER2 [J].NEngl J Med, 2001,344(11):783-792.

[8] Atul K. Tandon, Gary M. Clark, Gary C. Chamness, et al. HER-2/neu Oncogene Protein and Prognosis in Breast Cancer[J]. Clin Oncol,1989,7:1120-1128.

[9] Haeri Roh, James Pippin, Jeffrey A. Drebin. Down-regulation of HER2/neu expression Induces apoptosis in human cancer cells that overexpress HER2/neu[J]. Cancer Res,2000,60:560-565.

[10] Kreitman RJ, Stetler- Stevensonm, Wilsonwh, et al. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistanthairy-cell leukemia [J]. N Engl J Med,2001,345(4): 241-247.

[11] Yazici H, Altun M, Alatli, et al. C-erbB-2 gene amplification in na2 sopharyngeal carcinoma. Cancer Invest ,2000,18 (1):6-10.

[12] Liu, X., and Rose, D. Differential expression and regulation of cyclooxygenase-1 and-2 in two human breast cancer cell lines[J]. Cancer Res., 1996,56: 5125-5127.

[13] Nakatsugi S., Ohta T., Kawamori T., et al. Chemoprevention by nimesulide, a selective cyclooxygenase-2inhibitor, of 2-amino-1-methyl-6-phenylimidazo [4, 5, -b]- pyridine (PhIP) - induced mammary gland carcinogenesis in rats [J]. Cancer Res., 2010,91: 886–892.

[14] Krebsb, Rauchenbergerr, Reiefer S, et al. High-throughput generation and engineering of recombinant human antibodies[J]. Immunol Methods,2001, 254(1-2):67-84.

[15] Rosenblummg, Verchraegen CF, Murray JL,et al.Phase I study of 90Y-labeled B723 intraperitoneal administration in patients with ovarian cancer:effect of dose and EDTA coadministration on pharmacokinetics and toxicity[J].Clin Cancer Res,1999, 5(5): 953-961.

[16] Goldenberg M M. Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. Elsevier, 1999, 21:309-318.

[17] Kirchner EM,Gerhards R, Voigtmann R. Sequentail immun-chemotherapy and edrecolomab in the adjuvant therapy of breast cancer: reduction of 17-1A-positive disseminated tumor cells[J]. Ann Oncol,2002,13(7): 1044-1048.

[18] Arteagacl. Trastuzumab, an appropriate first-line single-agent therapy for HER2-overexpressing metastatic breast cancer [J]. Breast Cancer Res, 2003, 5 (2): 96-100.

[19] Montserra E. Rituximab in chronic lymphocytic leukemia[J]. Semin Oncol,2003,30(1)Supply 2: 34-39.

[20] Goldenberg M M. Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. Elsevier, 1999, 21:309-318.

[21] Viani G, Afonso S, Stefano E, et al. Adjuvant trastuzumab in the treatment of her-2-positive early breast cancer: a meta-analysis of published randomized trials. BioMed Central Ltd, 2007, 7:153.

[22] Warburg, O. On the origin of cancer cells. Science (New York,NY) 1956, 123:309-314.

[23] Vander Heiden, M. G., Cantley, L. C., Thompson, C. B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science (New York, NY) 2009, 324:1029-1033.

[24] Ljunggren, J., Häggström, L. Catabolic control of hybridoma cells by glucose and glutamine limited fed batch cultures [J]. Biotechnol. Bioeng,1994, 44: 808-818.

[25] Wheeler,T. J., Hinkle, P. C.,The glucose transporter of mammalian cells. Annu. Rev. Physiol. 1985, 47, 503-517.

[26] Curthoys, NP, Watford, M., Regulation of glutaminase activity and glutamine metabolism. Annu. Rev. Nutr. 1995, 15,133-159.

[27] Street JC, Delort AM, Braddock PS, et al. A 1H/15N n.m.r. study of nitrogen metabolism in cultured mammalian cells[J]. Biochem, 1993, 291:485-492.

[28] Tritsch, G. L., Moore, G. E., Spontaneous decomposition of glutamine in cell culture media. Exp. Cell Res. 1962, 28,360-364.

[29] Ozturk, S. S., Palsson, B. O., Chemical decomposition of glutamine in cell culture media: effect of media type, pH, and serum concentration. Biotechnol. Progr. 1990, 6, 121-128.

[30] Lin A, Agrawal P. Glutamine decomposition in DMEM: Effect of pH and serum concentration. Biotechnol. Lett. 1988, 10: 695-698.

[31] Bray HG, James SP, Raffan IM, et al. The enzymic hydrolysis of glutamine and its spontaneous decomposition in buffer solutions[J]. Biochem, 1949, 44:625-627.

[32] Kim SH, Lee G.M. Down-regulation of lactate dehydrogenase-A by siRNAs for reduced lactic acid formation of Chinese hamster ovary cells producing thrombopoietin. Appl. Microbiol. Biotechnol,2007,74: 152-159.

[33] Zhou, M. et al. Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases [J]. Biotechnol,2011, 153: 27-34.

[34] Chen, K. et al. Engineering of a mammalian cell line for reduction of lactate formation and high monoclonal antibody production[J]. Biotechnol. Bioeng.2001. 72:55-61.

[35] Irani, N. et al. Improvement of the primary metabolism of cell cultures by introducing a new cytoplasmic pyruvate carboxylase reaction [J]. Biotechnol. Bioeng,1999,66:238-246.

[36] Fogolin MB, et al. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells [J]. Biotechnol,2004,109:179-191.

[37] Kim SH, Lee GM. Functional expression of human pyruvate carboxylase for reduced lactic acid formation of Chinese hamster ovary cells (DG44). Appl. Microbiol. Biotechnol, 2007,76:659–665.

[38] Wlaschin KF, Hu WS. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport[J]. Biotechnol,2007, 131:168–176.

[39] Altamirano C, et al. Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine [J]. Biotechnol. Progr,2000,16:69–75.

[40] Altamirano, C. et al. Considerations on the lactate consumption by CHO cells in the presence of galactose [J]. Biotechnol,2006,125: 547–556.

[41] Gagnon M, et al. High-end pH-controlled delivery of glucose effectively suppresses lactate accumulation in CHO fed-batch cultures [J]. Biotechnol. Bioeng,2011,108:1328–1337.

[42] Francesca Zagari, Martin Jordan, Matthieu Stettler. Lactate metabolism shift in CHO cell culture: the role of mitochondrial oxidative activity [J]. New Biotechnology,2012,00(00).