# Metabolic Analysis of rhHER2-mAb in CHO Cells and Its Antitumor Effect in vitro

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## Abstract

**CHO cells which stably express recombinant humanized anti-HER2 monoclonal antibody (rhHER2-mAb) were cultured by the different concentration of DMSO and pH adjustment, to optimize the cell growth process. Detection the changes of the amino acid and organic acid in the process of the whole cell culture, comparing antitumor effects of this antibody protein and HER2 antibody drug (Herceptin) in vivo at the same time. We measured cell growth, protein expression, and metabolism during each batch of culture by adding different concentrations of DMSO and pH adjustment during cell culture. The results showed that: although DMSO had no effect on cell growth, the antibody titer nearly doubled with adding of DMSO. When DMSO was added to the medium, lactate and ammonia production decreased, aspartic acid, valine, and citric acid concentration increased during the culture, while there was no significant difference in pyruvate concentration and glucose concentration. When the pH of culture environment was constant, the cell density increased to 8.5×106cells/mL, while the control group was 7.5×106cells/mL. Glucose had been completely consumed at day13, while the control at day17. The maximum of lactate concentration was 18mM when the pH was constant, while the control was 13mM. There was no significant difference in the antibody productivity and pyruvate concentration. Meanwhile, there was an increase of 150nmol alanine and decrease of 25nmol isoleucine and 10nmol methionine were founded. In addition, the WST-8 method was used to detect the in vitro antitumor activity of this antibody. For SK-BR-3 and BT-474 cells, which were the HER2 positive cell lines, the maximum inhibition rate were (22.5±2.0) % and (72.3±2.0) %, and ED50 were 0.18 ± 0.02 ng/mL and 0.19 ± 0.02 ng/mL, respectively. And the rhHER2-mAb had no effect on the HER2 negative cell line MCF-7 cell. The results showed that there was no significant differences between the rhHER2-mAb and Herceptin in affinity and in vitro antitumor activity.**

## Keywords

**Humanized anti-HER2 monoclonal antibody; CHO cells; metabolic analysis.**

## 1. Introduction

Breast cancer (BC) is one of the most common malignant tumors in women, accounting for 30% of the new malignant tumors. The incidence of breast cancer in the world is the first in female malignant tumors [1-3]. In recent years, the research on the molecular level of breast cancer has attracted more and more attention. It has been found that the most important factor leading to breast cancer production is the overexpression of human epidermal growth factor receptor 2 (HER2). HER2 is one of the members of the epidermal growth factor receptor family (EGFR) and is capable of binding to ligands to form dimers that change their conformation, resulting in the phosphorylation of intracellular receptor tyrosine kinases and that promotes cell proliferation and differentiation. At

present, Herceptin, as the earliest humanized monoclonal antibody against HER2, has achieved excellent efficacy in the treatment of breast cancer among many anti-tumor drugs targeting HER2.

In the process of expression of recombinant proteins by mammalian cells, the host cells used to express the recombinant protein are mainly CHO cells, NSO cells, BHK cells, and COS cells. Among them, the CHO cell line is one of the most popular mammalian cell lines currently used in biopharmaceuticals, with the following advantages: with accurate post-transcriptional modification, the proteins expressed in molecular structure, physical and chemical properties and biological functions that are close to natural higher biological protein molecules [4]; It has the ability of stable integration of exogenous genes and efficient amplification [5,6]. In the future, CHO cells will continue to be the preferred expression system for the production of recombinant protein drugs, are increasingly used in the production of antibody drugs [7, 8].

The metabolic group is a new concept that emerges with the continuous development of life science research, mainly referring to all metabolites of an organism or cell. Currently, there are three main directions with the study of cell metabolism: metabolic control analysis, metabolic flux analysis, and metabolic network analysis.

Metabolic Flux Analysis (MFA) is a commonly used quantitative analysis method in metabolic engineering. The intracellular flux distribution can be calculated by using the stoichiometric model of the main reaction and the mass conservation relationship of the metabolites in the cell. It does not need to consider the complicated kinetic process and reduces the difficulty of calculation. MFA has become a powerful method for analyzing intracellular metabolic fluxes in living cells in the past two decades.

The purpose of this study was to obtain culture conditions that highly express rhHER2-mAb by optimizing the addition process, and to further detect changes in amino acid and organic acid during cell culture, providing a necessary theory for further research and optimization of CHO cell culture process. At the same time, the corresponding theoretical basis is provided for the production and application of the drug by studying the anti-tumor effect in vivo of rhHER2-mAb.

## 2. Materials and methods

### 2.1 Cell culture

For the suspension cell culture, CHO cells which stably express rhHER2-mAb were cultivated in ProCHO5 medium at a density of $0.5\times10^6$cells/mL. Cells were maintained as a suspension in a 50-mL Tubespin containing 10 mL medium in 5% CO2 air at 37°C using a shaking incubator with a rotation speed of 180 rpm. For adherent cell culture, cells were incubated in T25 flasks containing 5mL medium in 5% CO2 air at 37°C, cells were passaged in their logarithmic growth phase. In addition, the supernatant and pellet was stored at -20°C. Trypan blue stain was used to identify viable cells, which were counted with a cell counter.

### 2.2 Enzyme-linked immunosorbent assay (ELISA)

The sample was centrifuged at 1000 rpm for 5 min, and the supernatant were stored at -20°C. After the culture period was over, the content of rhHER2-mAb fusion protein in the culture supernatant was determined by ELISA. The OD value was measured at 405 nm, and the standard curve was made by the Herceptin standard to calculate the content of rhHER2-mAb fusion protein. Three replicates were made for each sample dilution gradient and averaged.

### 2.3 Effects of DMSO on cell growth and protein expression

CHO cells which stably express rhHER2-mAb were cultivated in ProCHO5 medium at a density of $0.5\times10^6$cells/mL. It was changed to 31° C after 4 days of culture, and DMSO with different concentration was added: 0.1%; 0.5%; 1.0%; 1.5%; 2.0%. Samples were taken every two days to determine cell growth, cell viability, protein expression and the content of glucose, lactic acid, ammonia and pyruvate in cell culture supernatants.

### 2.4 Effects of pH on cell growth and protein expression

CHO cells which stably express rhHER2-mAb were cultivated in ProCHO5 medium at a density of $0.5\times10^6$cells/mL. After 48 h, the pH was adjusted with $NaHCO_3$ and cultured at 31° C after 4 days. Samples were taken every two days to determine cell growth, cell viability, protein expression and the content of glucose, lactic acid, ammonia and pyruvate in cell culture supernatants.

### 2.5 Determination of amino acids and organic acids in cell culture supernatants

The sample was centrifuged at 200g for 5 min, and the supernatant was stored at -80°C. For amino acid detection, the amino acid mixture standard solution or sample extraction solution was 100ul, add 1ul of internal standard and 100ul OPA reagent, then analyze after 2min. HPLC conditions: 338nm of UV detection; 40°C of column temperature; flow rate: 1 mL/min, gradient elution. HPLC conditions for organic acid detection: 215 nm of UV detection; 30°C of column temperature; flow rate: 1 mL/min, gradient elution.

### 2.6 Anti-tumor effect of anti-HER2 humanized monoclonal antibody in vitro

BT-474, SK-BR-3, and MCF-7 cells were cultured for 2 h in RPMI 1640 medium (10 % FBS) in 5% CO2 air at 37°C. The purified anti-HER2 humanized monoclonal antibody and Herceptin standards were diluted with RPMI 1640 medium and added to the cell culture medium at different concentrations, 50μl/well. The cells were cultured for 16-18 h, so that the cell abundance reached 80% or more, and then WST-8 was added at 20μl/well for 4 h. The OD value was measured at 450 nm and the reference wavelength was 630 nm.

## 3. Results

### 3.1 Effects of DMSO on cell growth and protein expression

The concentration of DMSO in the experimental group was 0.1%; 0.5%; 1.0%; 1.5%; 2.0%; DMSO was not added in the control group. After DMSO was added, the maximum viable cell density reached by the experimental group was not significantly different from that of the control group as shown in Figure 1. With the increase of the concentration of DMSO added, the viability of cells was maintained longer than 80% and longer than that of the control group. After 16 days of culture, the viability of cells in the experimental group with DMSO concentration of 2% was rapidly reduced to 0, the control group is still about 40%. In addition, with the addition of DMSO, protein expression gradually increased. Among them, the expression level of the control group protein was 117 mg/L, and when the DMSO concentration was 2%, the protein expression level reached 180 mg/L.

As shown in Figure 2, glucose consumption was similar between the experimental group and the control group during the whole cell culture process. The change of lactic acid content in the cell culture supernatant was as follows: In the early stage of culture, lactic acid was generated in large quantities and reached its maximum value on the $4^{th}$ day, which was about 15mM. Subsequently, metabolic drift occurred and the state of lactic acid was changed to consumption. And the higher the concentration of DMSO, the less lactic acid in the culture. The change of ammonia content in the cell culture supernatant was as follows: the ammonia content increased rapidly at 4 days after culture, reaching a maximum of 3.8mM, and then the content decreased. Compared with the control group, ammonia content in the cell culture supernatant of the experimental group decreased rapidly after adding DMSO on the $4^{th}$ day, and the higher the concentration of DMSO, the less ammonia in the culture. Finally, the changes of pyruvate content in the experimental group and the control group were similar.

### 3.2 Effects of pH on cell growth and protein expression

Due to a large amount of lactic acid produced during the rapid growth of cells, the acidification of the culture environment will affect the growth of cells and the expression of proteins. In the experimental group, NaHCO3 was used for pH adjustment on the $3^{rd}$ day to maintain its pH constant. As shown in Fig 3, the maximum cell density reached in the experimental group with constant pH was $8.5\times10^6$ cells/mL, while that in the control group was $7.5\times10^6$ cells/mL. However, at 19 days, the

viability of the control group was still about 60%; the experimental group has been reduced to 38%. In addition, there was no significant difference in the effect of maintaining a constant pH environment on protein expression, all of which were about 165 mg/L.

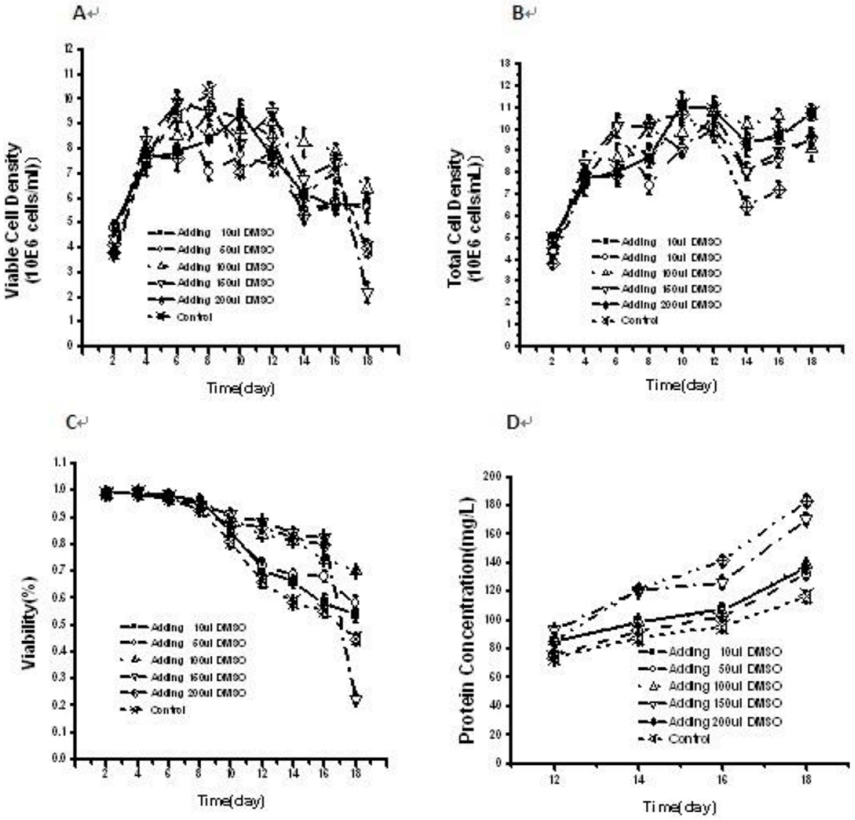


Figure.1 Effect of DMSO on cell growth and protein expression
A: Live cell density; B: Total cell density; C: Cell viability D: Protein expression.

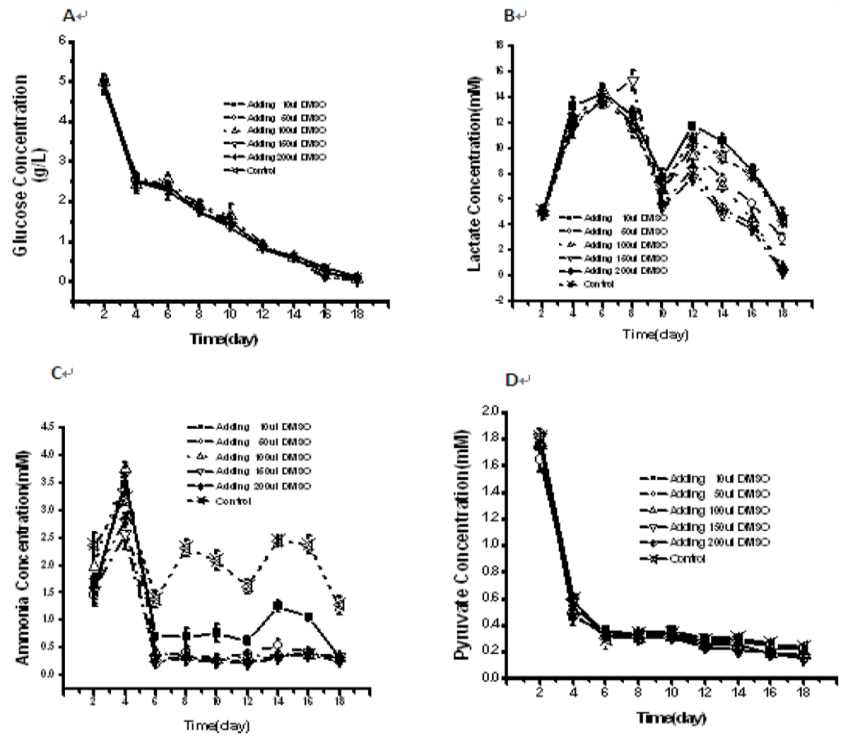


Fig.2 The curve of glucose, lactic acid, ammonia and pyruvic acid content during the culture of recombinant CHO cells with different concentrations of DMSO
A: Glucose content; B: Lactic acid content; C: Ammonia content; D: Pyruvate content.

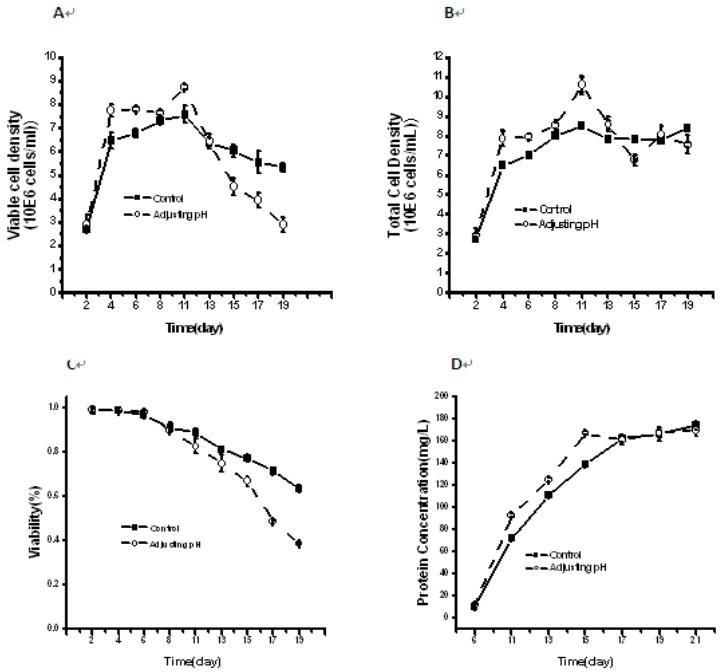


Figure.3 Effect of pH on Cell Growth and Protein Expression
A: Live cell density; B: Total cell density; C: Cell viability; D: Protein expression.

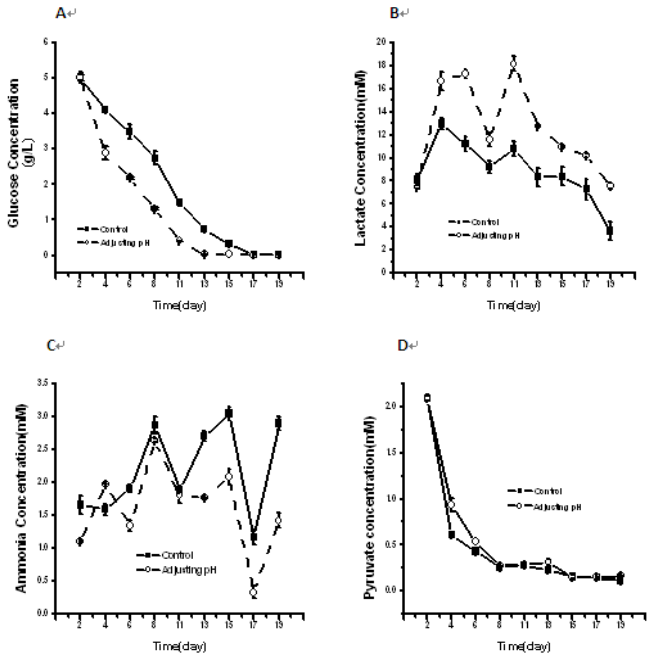


Fig.4 The curves of glucose, lactic acid, ammonia and pyruvic acid content during culture of recombinant CHO cells at constant pH
A: Glucose content; B: Lactic acid content; C: Ammonia content; D: Pyruvate content.

As can be seen from the figure 4, the glucose residue in the culture supernatant of the experimental group with a constant pH is significantly lower than that of the control group. At the same time, the lactate content in the culture supernatant of the experimental group is higher than the control group, with a maximum of 18mM, whereas the control group only 13mM, the ammonia content in the supernatant is also higher than the control group. However, the changes of pyruvate content in the experimental group and the control group were similar.

### 3.3 Effect of DMSO on amino acid utilization in CHO cell culture

200ul DMSO was added to the experimental group and no DMSO was added to the control group. As shown in Figure 5, the contents and trends of serine, glycine, threonine, alanine, tyrosine, methionine, isoleucine, and leucine in the culture supernatant of the experimental and control groups were similar. In addition, the content of aspartic acid and valine in the experimental group was significantly higher than that in the control group. In the period of decay, the content of histidine in the experimental group was less than that in the control group, and the experimental group was 50nmol, while the control group still had 180nmol. The concentration of arginine in the experimental group was higher than that in the control group during the period of stability and decline, the experimental group was 35nmol on the 12$^{th}$ day, and 19nmol in the control group.

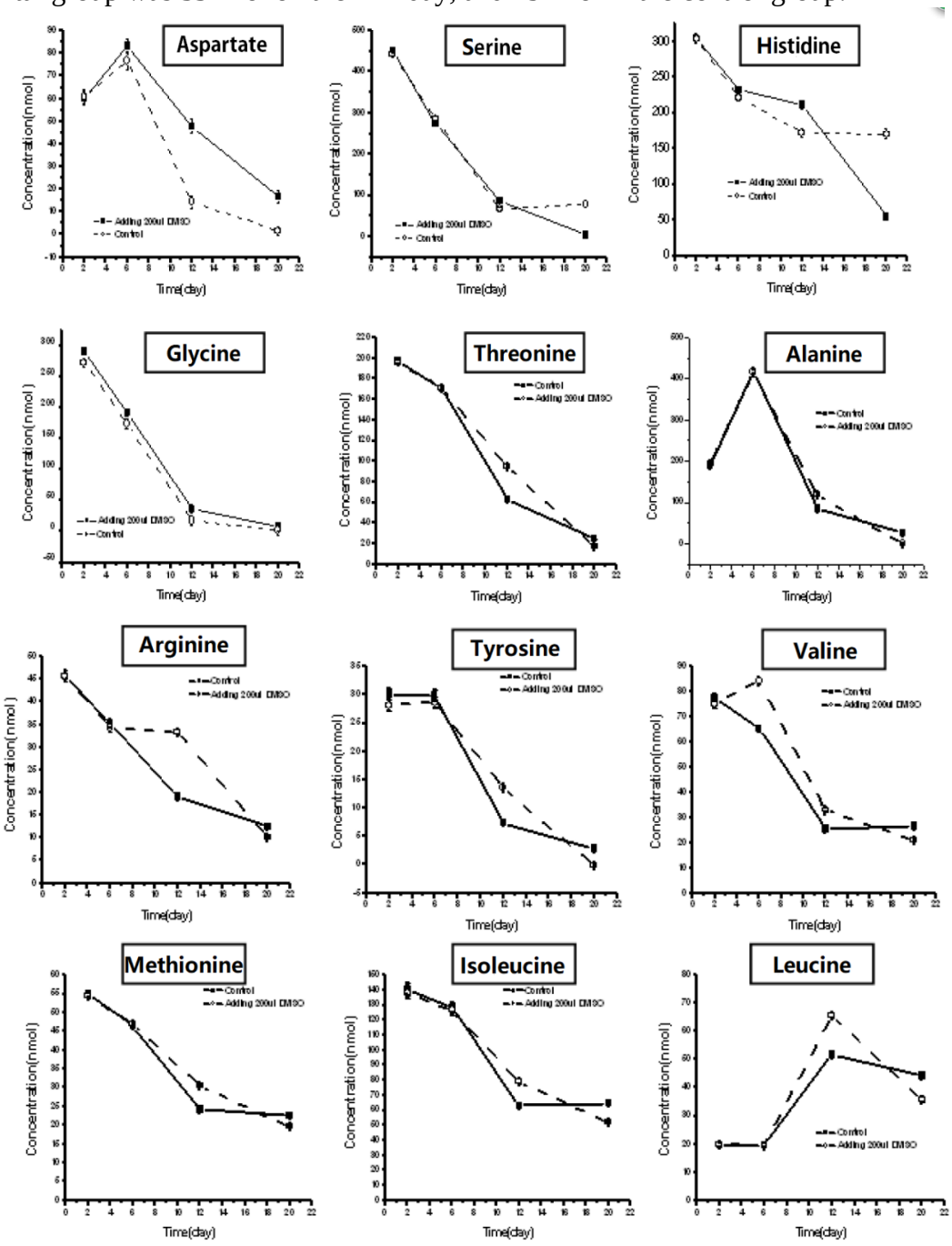


Figure.5 Effect of DMSO on amino acid utilization in CHO cell culture

### 3.4 Effect of pH on amino acid utilization in CHO cell culture

As shown in Fig 6, the pH of the experimental group was kept constant, and the control group was not treated. In the CHO cell culture process, the contents and trends of aspartic acid, serine, glycine,

threonine, arginine, proline, and tyrosine in the culture supernatant of the experimental and control groups were similar. The alanine content in the experimental group was greater than that in the control group during the whole culture process; methionine and isoleucine were similar in the initial stage of culture and at the end of the culture, while the methionine and isoleucine in the control group was greater than that of the experimental group. The histidine content in the control group slowly decreased, and the content was 170nmol at the end of the culture, the histidine in the experimental group decreased first and then increased, however, and the content was 230nmol at the end of the culture.

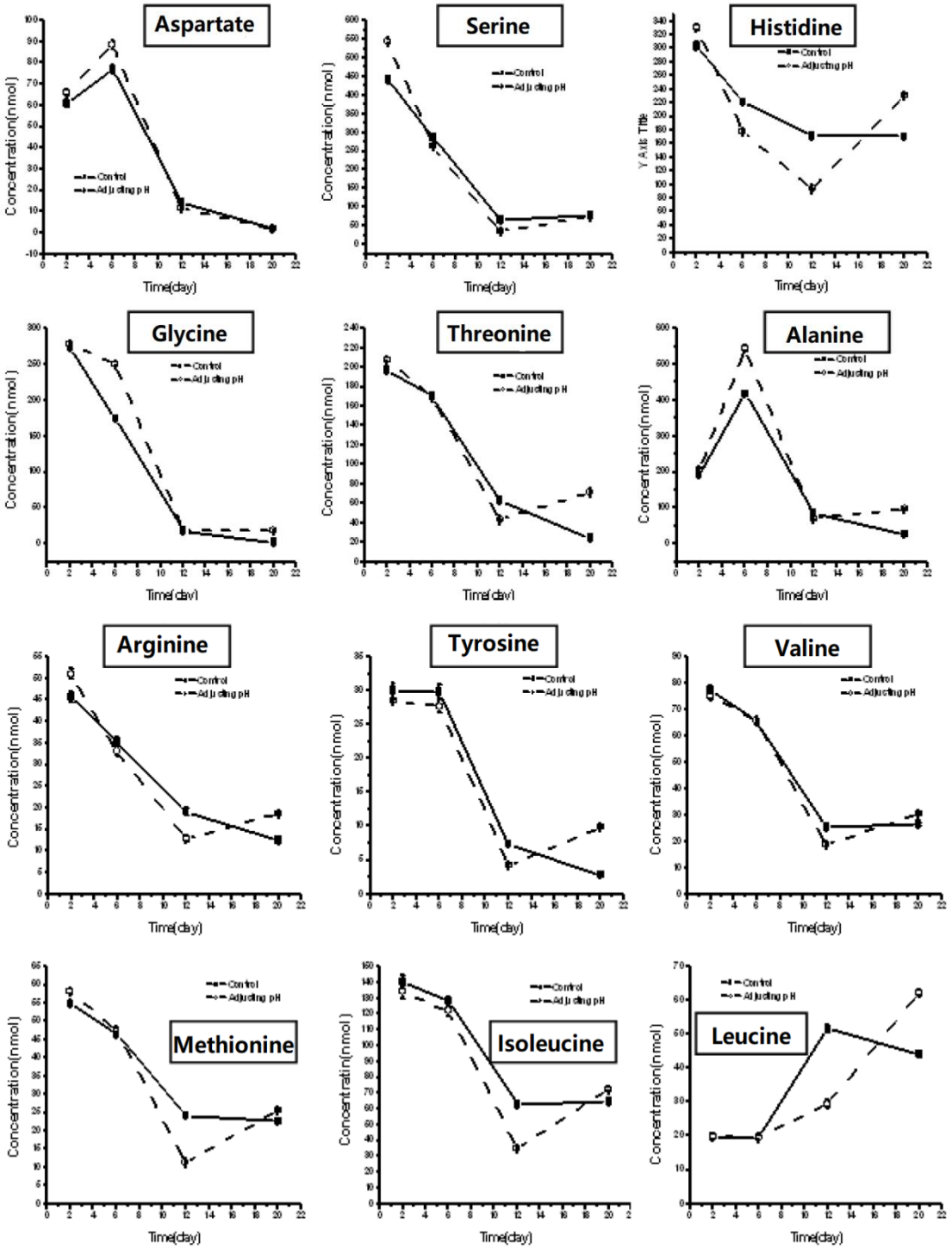


Figure.6 Effect of maintaining constant pH on amino acid utilization during CHO cell culture

### 3.5 Effect of DMSO on the metabolism of organic acids in CHO cell culture

200ul DMSO was added to the experimental group and was not added to the control group. Alpha-ketoglutarate and pyruvate were not detected during the experiment. The citric acid content change curve in figure 7. It can be seen that the content in the control group is significantly smaller than the experimental group in the logarithmic growth phase and the stable phase. On the 6th day, the control group content is 1.0 mg/mL, and the experimental group is 1.5 mg/mL, and citric acid was not detected in the control and experimental groups at 12 days of culture.

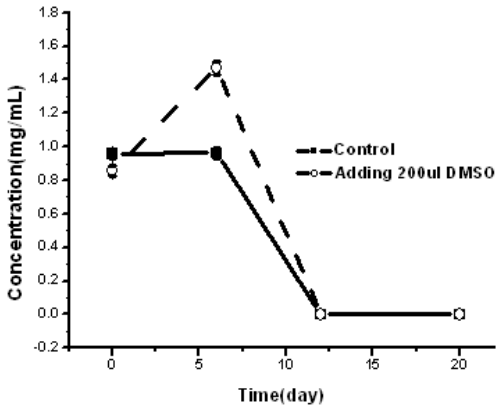


Figure.7 Effect of DMSO on citric acid metabolism during CHO cell culture

### 3.6 Effect of pH on metabolism of organic acids in CHO cell culture

The experimental group maintained a constant pH environment and the control group was not treated. Alpha-ketoglutarate and pyruvate were not detected during the experiment. It can be seen that the content and its variation trend are similar in figure 8. The content of the citric acid on 3rd day is about 0.8mg/mL, and the citric acid has not been detected for 12 days.

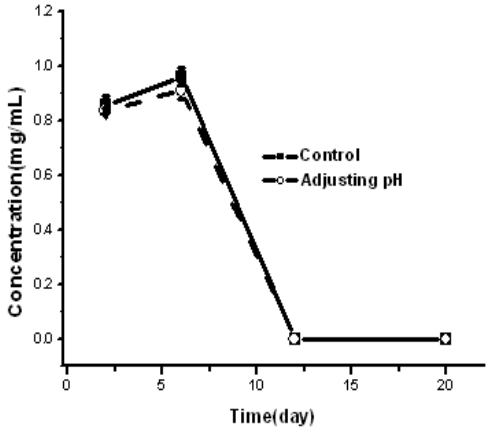


Figure.8 Effect of constant pH on citric acid metabolism during CHO cell culture

### 3.7 Antitumor effect of anti-HER2 humanized monoclonal antibody in vitro

we used the WST-8 method to detect the growth inhibitory effects of anti-HER2 humanized monoclonal antibodies on BT-474 cells (HER2-positive), SK-BR-3 cells, and MCF-7 cells (HER2-negative). The antibody can inhibit the growth of SK-BR-3 cells and BT-474 cells and has no effect on MCF-7 cells in figure 9. The maximum inhibitory rate of rhHER2-mAb fusion protein against SK-BR-3 cells was (22.5±2.0) %, and the ED 50 was 0.18±0.02 ng/mL. The maximum inhibition rate of Herceptin on SK-BR-3 cells was (20.8±2.0) %, ED 50 is 0.10 ± 0.01 ng/mL. The maximum inhibition rate of rhHER2-mAb fusion protein on BT-474 cells was (72.3±2.0) %, and the ED 50 was 0.19±0.02 ng/mL. The maximum inhibition rate of Herceptin on BT-474 cells was (74.4±2.0) %. The ED 50 is 0.16 ± 0.02 ng/mL. The rhHER2-mAb fusion protein and Herceptin had no effect on MCF-7 cells at

the same time, and the results showed that there was no significant difference in their biological activity.

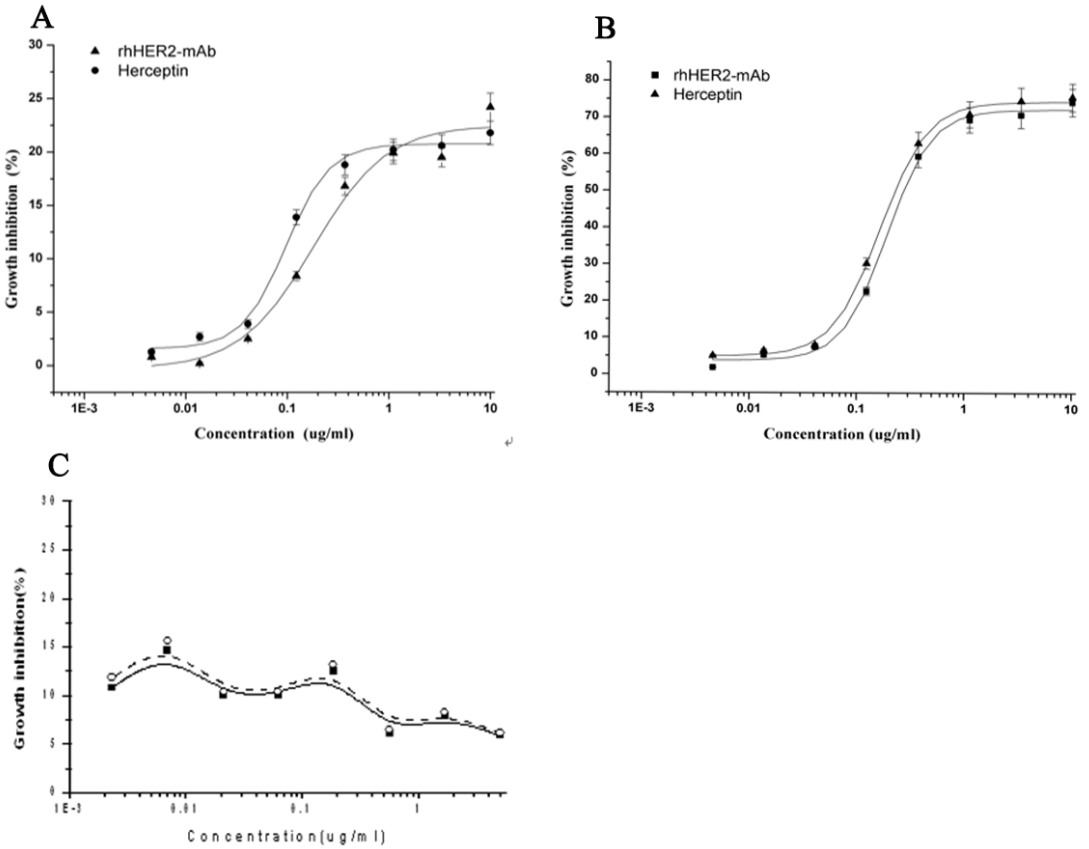


Figure.9 Comparison of rhHER2-mAb fusion protein and Herceptin activity against different breast cancer cells
A: SK-BR-3 cells; B: BT-474 cells; C: MCF-7 cells.

## 4. Conclusion

DMSO is a stimulating reagent for cell culture during cell culture, some studies have shown that DMSO can increase protein expression [9-15]. Our results verified this, and when DMSO was added to 2%, the protein expression was higher than that of the control group. The content of lactic acid and ammonia in the culture supernatant after adding DMSO will be reduced compared with the control group at the same time, and the reduction of the main metabolic byproducts is more conducive to protein expression. In addition, DMSO has a certain inhibitory effect on growth and a certain improvement in protein expression. The effect of DMSO on growth is not significant, but it can increase the expression of foreign protein by nearly one-fold in experimental results. From the metabolism, it can be seen that after the addition of DMSO, the lactic acid and ammonia content of metabolic byproducts will decrease, and the citric acid content will increase significantly. The reduction of metabolic byproducts may also have a positive effect on protein expression.

Maintaining a relatively constant pH can increase the cell density, decrease the glucose content in the supernatant, and increase the lactic acid content. Shows that the cultivation of the pH constant environment more conducive to the growth of the cells, glucose will quickly use up, accordingly produce lactic acid and ammonia in great quantities, it also may be the reason for the protein expression were not significantly increased. In addition, the WST-8 method was used to detect the anti-tumor effect of anti-HER2 humanized monoclonal antibodies in vitro in this study. The results showed that there was no significant different between the rhHER2-mAb and Herceptin in affinity and in vitro anti-tumor activity.

Metabolic flux analysis is important for the understanding of various metabolites and metabolic pathways in large-scale culture of CHO cells. Therefore, in-depth research and exploration should be continued, and new detection and analysis techniques should be fully applied to comprehensively determine and analyze the specific changes in process variables and how they affect cell growth and protein expression, thus for large-scale cell culture and improve the protein expression with better theoretical support

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