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RESEARCH ARTICLE

Cystine and tyrosine feed reduces oxidative and ER stress in CHO cells

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Abstract

Multi-omics analyses was performed to compare the conditions of adding Tyr and Cystine in CHO cells. The addition of cystine resulted in decreased viability and productivity owing to endoplasmic reticulum (ER) stress and the promotion of ER-associated degradation (ERAD) and apoptosis. In contrast, addition of Tyr suppressed ER stress and apoptosis. This effect could be due to the increase in ubiquinone (Coenzyme Q10) biosynthesized from Tyr. To inhibit apoptosis caused by cystine addition, Tyr was added simultaneously with cystine, which improved growth, viability, and mAb productivity owing to the activation of GSH metabolism, suppression of ER stress and oxidative stress, reduction of ERAD, and activation of the tricarboxylic acid cycle.

KEYWORDS
bioprocessing, CHO, cystine, monoclonal antibody, tyrosine

1 | INTRODUCTION

Antibody drugs have attracted considerable attention recently owing to their high specificity for their targets and fewer side effects.[1] Research on the production process of antibody drugs is important to improve productivity and quality. CHO cells are standard host cells used for antibody drug production. CHO cells are primarily fed-batch cultured, which has significantly improved viable cell density (VCD) and specific productivity (Qp) over the past few decades.[2,3] Processes have been designed to prevent the depletion of essential nutrients and accumulation of lactic acid and ammonia by optimizing the media and feed strategies to achieve high productivity.[4] Most studies have focused on one factor at a time, whereas studies on the interactions of essential components, such as amino acids, are still limited. The supply of amino acids to culture is a significant challenge for large-scale antibody production. Amino acid levels must be controlled to achieve high productivity. However, controlling Cysteine (Cys) and tyrosine (Tyr) is difficult because of their cytotoxicity and low solubility.[5,6]

Depletion of intracellular Cys during monoclonal antibody (mAb) production decreases productivity, cell proliferation, and viability, which may be related to glutathione (GSH) metabolism. GSH plays a significant role in detoxifying reactive oxygen species (ROS) produced in the mitochondria, as well as in modulating disulfide bond formation in the endoplasmic reticulum (ER) and forming conjugates with reactive electrophiles to facilitate their detoxification.[7–9]

As Cys is the limiting substrate of GSH synthesis, depletion of Cys leads to the shortage of GSH. The formation of disulfide bonds is the rate-limiting step in antibody production because many

Abbreviations: ACO, Aconitase; ATF, Activating transcription factor; ATP, Adenosine triphosphate; BER, Base Excision Repair; CHO, Chinese hamster ovary; CHOP, C/EBP homologous protein; Cys, Cysteine; Ddit3, DNA damage-inducible transcript 3; ER, Endoplasmic reticulum; ERAD, Endoplasmic reticulum-associated degradation; Ern1, Endoplasmic reticulum to nucleus signaling 1; Ero1, ER oxidoreductin 1; FAD, Flavin adenine dinucleotide; GADD34, Growth arrest and DNA damage-inducible gene 34; GGT, Glutamyl transferase; Gpx, Glutathione peroxidase; GSH, Glutathione; GSS, GSH synthase; GST, Glutathione-S-transferase; H_2O_2, Hydrogen peroxide; HC, Heavy chain; Hspa5, Heat Shock Protein Family A (Hsp70) Member 5; IgG, Immunoglobulin G; LC, Light chain; LC-MS, liquid chromatography mass spectrometry; mAb, Monoclonal antibody; NADH, Nicotinamide adenine dinucleotide; NER, Nucleotide Excision Repair; P4hb, Prolyl-4-hydroxylase; PC, Pyruvate carboxylase; PDI, Protein disulfide isomerase; PEPCK, Phosphoenolpyruvate carboxykinase; Ppp1r15a, Protein phosphatase 1 regulatory subunit 15A; ROS, Reactive oxygen species; rpm, Rotation per minute; SDH, Succinate dehydrogenase; Sel1l, Suppressor/Enhancer of Lin-12-like; SLC1A5, Solute carrier family 1 member 5; SLC7A11, Solute carrier family 7 member 11; SOD, Superoxide dismutase; Sqstm1, Sequestosome 1; TCA, Tricarboxylic acid; Tyr, Tyrosine; UBXN4, UBX domain-containing protein 4; VCD, Viable cell density; Via, Viability.

disulfide bonds are present in antibodies. Protein disulfide isomerase (PDI) and its family of proteins catalyze disulfide bond formation. PDI catalyzes oxidative folding by transferring electrons to and from the Cys sulfur atom in the nascent polypeptide chain. The reduced PDI is re-oxidized by a flavoprotein, ER oxidoreductin 1 (Ero1). When Ero1 oxidizes PDI, Ero1 receives two electrons from PDI. These two electrons pass through FAD to molecular oxygen (O_2), and the reduced oxygen is converted to hydrogen peroxide (H_2O_2). H_2O_2 is metabolized by glutathione peroxidase (Gpx) using the reducing power of GSH. Therefore, depletion of GSH induces ER stress due to the oxidative stress caused by H_2O_2.[5,10,11] Oxidative stress also activates the amino acid response and decreases tricarboxylic acid (TCA) cycle activity, leading to apoptosis.[12]

Cys dissociates relatively easily under physiological conditions (pH 7.4) to give rise to a thiolate anion, which is toxic to the cells. Therefore, Cys concentration in the medium is usually low.[13] In contrast, cystine, the oxidized derivative of cysteine, is considered a safe source of Cys because it does not dissociate thiol. It is transported into cells by the cystine/glutamate transporter[14] and then reduced to Cys. Cystine and GSH cooperatively regulate the mTORC1 pathway, integrated stress response, and ferroptosis.[15] However, the mechanisms of cys-mediated intracellular stress responses, regulation of cell proliferation, and its other roles are poorly understood.

Tyr is essential for antibody production in CHO cell cultures to maintain specific productivity and to avoid Tyr sequence mutations. Tyr starvation reduces the maximum VCD during the growth phase and causes rapid cell death and a pH decrease during the productive phase.[16,17] Tyr starvation causes autophagic cell death.

Thus, understanding the cellular response to the depletion of medium components and the underlying mechanisms is essential for designing robust bioprocesses and maximizing recombinant protein expression. However, more research is required to determine how each component in the medium increases or decreases cellular stress. An important challenge is to more efficiently protect CHO cells from nutrient depletion and other stressful culture conditions and to delay apoptosis.

In recent years, omics analysis approaches have been actively used to analyze the properties of CHO cells in detail under various conditions, as a useful tool for elucidating intracellular mechanisms.[18] In particular, the analysis of gene expression profiles is useful for understanding intracellular events, and RNA-seq is widely used as a method. Furthermore, recent developments in proteomics analysis technology using liquid chromatography mass spectrometry (LC-MS) have made it possible to obtain protein expression profiles at high depth and throughput.[19]

In this study, transcriptomic and proteomic analyses identified factors that inhibit antibody production related to ER stress, oxidative stress, decreased TCA cycle activity (mitochondrial dysfunction), autophagy, and apoptosis associated with the addition of cystine and Tyr and the interaction between cystine and Tyr.

2 | EXPERIMENTAL SECTION

2.1 | CHO fed-batch culture for omics study

DG44 cells adapted to serum-free suspension culture were transfected with the IgG1 expression vector, and antibody-producing clones were cultured. Fed-batch cultures of CHO cells were conducted in shaker incubators using 125-mL Erlenmeyer flasks. Cultures were performed at 37.0°C, 125 rpm (25 mm orbital shake diameter), 5% CO_2, using a proprietary chemically defined basal medium containing sodium bicarbonate, galactose, L-glutamine, and insulin in a starting volume of 30 mL. For each cell line (clone 1 and clone 2), 15 production culture investigated 5 different feeding conditions with three biological replicate each. The total number of cultures is 30. The viable cell density at the start was inoculated at 0.5×10^6 cells mL^{-1}. Proprietary chemically defined feed medium consisting of amino acids, vitamins, trace elements, and glucose was used as the feed medium. The feed medium was replenished at regular intervals to avoid nutrient depletion during the fed-batch culture and to extend the culture period. To investigate the potential role of Cys and Tyr, stock solutions of cystine and Tyr for each culture condition were prepared (Table 1) and added to the cell culture at day 3, 6, 9 and 12 as for feed medium. The amounts of cystine and Tyr added for each culture condition are also listed in Table 1 as a ratio to the cystine concentration of C:T = 1:0 condition. Feed and stock solutions of cystine and Tyr were added after sampling for measurements at a defined number of culture days.

2.2 | CHO batch culture with coenzyme Q10

Batch cultures were performed using the same IgG-expressing clones (clone 1) as described above. Cultures were performed in shaker incubators using 125 mL Erlenmeyer flasks. Twelve production culture investigated four different conditions with three biological replicate each. The medium used was CD DG44 medium (Thermo Fisher Scientific) with L-glutamine, insulin, and pluronic as the basal medium in a starting volume of 25 mL for three cultures under each condition. Batch cultures were started by adding supplements, as shown in Table 1, and sampling was performed on day 7.

2.3 | Determination of nutrient and metabolite concentration, cell count, cell viability, and titer

The methodology is detailed in the Supplementary Material.

2.4 | Amino acid measurement

The methodology is detailed in the Supplementary Material.

TABLE 1 Fed-batch culture and batch culture condition list.

Fed-Batch culture conditions					
Condition name	Cystine and Tyrosine stock solution conditions	Amount of Cystine to be added in one feed		Amount of Tyrosine to be added in one feed	
		Ratio	Addition amount [μmol]	Ratio	Addition amount [μmol]
C:T = 0:0	–	0.0	0.0	0.0	0.0
C:T = 1:0	L-Cystine [61.8 mM] in pH 11.73	1.0	3.7	0.0	0.0
C:T = 0:3	L-Tyrosine [198.9 mM] in pH 11.73	0.0	0.0	3.2	11.9
C:T = 1:3	L-Cystine [61.8 mM], L-Tyrosine [198.9 mM] in pH 11.73	1.0	3.7	3.2	11.9
C:T = 3:3	L-Cystine [61.8 mM], L-Tyrosine [66.3 mM] in pH 11.73	3.0	11.1	3.2	11.9

Batch culture conditions		
Condition name	Solvent type	Additional concentration
Control	–	–
Tyr	Water	0.80 mM
Ethanol	Ethanol	1.0% Ethanol v/v
Coenzyme Q10	Ethanol	0.012 nM in 1.0% Ethanol v/v

2.5 | Transcriptomic and proteomic profiling of CHO cells

2.5.1 | Sample preparation

The culture samples were sampled and were analyzed with three technical replicates from each culture condition. The culture samples was centrifuged (14000 g × 30 s × 2°C) to remove the supernatant. The remaining pellet was washed with 2 mL of ice-cold phosphate-buffered saline (PBS), centrifuged again (14000 g × 30 sec × 2°C) to remove PBS, and frozen at -80°C until analysis.

2.5.2 | Transcriptomic analysis

Sequencing was performed at Azenta Life Sciences (Chelmsford, MA). A summary of this is provided below: Total RNA was extracted from the cell pellets, and mRNA was enriched by polyA selection. mRNA-based libraries were prepared and sequenced in the paired-end mode using Illumina NGS.

RNA-seq data were trimmed using Fastp version 0.21.0[20] and then aligned to genomic DNA sequences (*Cricetulus griseus* CHOK1GS, Ensembl release 104, accession number GCA_900186095.1) using STAR version 2.7.6a.[21] Transcripts were counted using RSEM version 1.3.3[22] based on alignment results. Differentially expressed genes were identified using edgeR version 3.38.1[23], and the false discovery rate (FDR) threshold was <0.05.

In the analysis of RNA amounts of heavy and light chains in IgG1, the same analysis was performed using the DNA sequence of the expression vector as a reference, and the expression was calculated as a ratio to the total RNA-seq counts using genomic DNA as a reference.

2.5.3 | Protein digestion

The methodology is detailed in the Supplementary Material.

2.5.4 | LC-MS analysis

The methodology is detailed in the Supplementary Material. DIA isolation windows is shown in Table S1.

2.5.5 | Proteome data analysis

Data acquired by LC-MS were analyzed using Scaffold DIA version 3.1.0 (Proteome Software, Oregon, United States). Spectral libraries were created using Scaffold DIA and Prosit[24] based on the amino acid sequences (*C. griseus* CHOK1GS, Ensembl release 104) obtained from Ensembl. The parameters for preparation were set to a charge range of 2–4 and *m/z* range of 495–745. Scaffold DIA searches were performed using the prepared libraries. The set parameters are listed below: Peptide and fragment mass tolerances up to 10 ppm and one missed cleavage were allowed. The charge and length of the peptides were set to 2–4 and 6–127, respectively. The FDR threshold for the peptide search was set to 0.01.

The protein levels of the heavy and light chains were calculated using DIA-NN version 1.8.1[25] from the data acquired by LC/MS. First, the raw files were converted to mzML files using MSconvert version 3.0.20293[26] and then to dia files using DIA-NN. A spectral library was created from the amino acid sequence of IgG1 and then analyzed. DIA-NN parameters were set to a charge range of 2–4, peptide length range of 7–45, precursor ion *m/z* range of 495–745, fragment ion *m/z* range

of 200–1800, and MS1 and MS2 accuracy of 10 ppm.

Pathway analysis was performed using Ingenuity Pathway Analysis software (IPA, QIAGEN).

2.6 | Oxidative stress level analysis

The methodology is detailed in the Supplementary Material.

3 | RESULTS

We first investigated the effect of Cys addition in fed-batch culture. To avoid Cys cytotoxicity, cystine was used as the source of Cys. We conducted comparative experiments with (C:T = 1:0) and without (C:T = 0:0) cystine addition and examined the intracellular events by transcriptomic and proteomic analyses. We identified 18024 transcripts and 10506 proteins (Table S2).

The results of the cystine-supplemented cultures are depicted in Figure 1. Although the viable cell density was partially increased in the cystine-supplemented condition, the addition of cystine decreased viability, titer, and pH in the late phase of the culture, in contrast to previous studies.[5] The decrease in pH in the late phase of the culture could be related to the increase in lactate shown in Supplemtary 1.

Hspa5 (*Bip* and *GRP-78*) and *Ire1a* (*Ern1*) are ER stress markers.[27,28] The addition of cystine markedly increased mRNA expression of them. Furthermore, the expression of the activating transcription factor 3 gene (*Atf3*), induced by the activation of protein kinase R-like ER kinase (PERK) by ER stress,[29] was also markedly elevated. These results indicated that adding cystine to the culture causes ER stress.

To examine the details of the adverse effects of cystine addition, we compared the results of omics analyses (Figure 1). The data on day 14 were omitted because viability could not be maintained until day 14 in the C:T = 1:0 condition.

Slc7a11 encodes the cystine/glutamate transporter gene, which is involved in the uptake of cystine into the cell.[14] *Slc7a11* mRNA expression was suppressed in the late phase of the culture with cystine addition (C:T = 1:0), probably due to the sufficiency of cystine. We examined the mRNA levels of the activating transcription factor 6 gene (*Atf6*), a component of ER-associated proteolysis (ERAD)[30] and the UBX domain-containing protein 4 (UBXN4), also known as UBXD2. UBXN4 is an ER-intrinsic membrane protein that promotes ERAD.[31] Sel1l is involved in the ubiquitin-dependent degradation of misfolded ER proteins in ERAD.[32] Cystine addition significantly induced these mRNAs in the late phase of the culture, indicating that cystine caused ERAD.

Glutamine transporter SLC1A5, an indicator of glutaminolysis,[33] was significantly upregulated on day 12 in the C:T = 1:0 condition. Accordingly, the glutamine concentration under the C:T = 1:0 condition is less than the C:T = 0:0 condition. These results are inconsistent with prior observation that cells with insufficient Cys could not generate α-ketoglutarate from citric acid and relied on glutaminolysis to replenish the TCA cycle intermediate, α-ketoglutaric acid.[5,11]

ER stress-associated ATF4 upregulates Ppp1r15a, also known as GADD34, which promotes apoptosis through proteasome inhibition.[34] DNA damage-inducible gene 3 (*Ddit3*) encodes the transcription factor CHOP, a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors. CHOP is induced by ER stress and mediates apoptosis.[35] The mRNA levels of *Ppp1r15a* and *Ddit3* were significantly elevated in the C:T = 1:0 condition, indicating that the addition of cystine promotes apoptosis.

We compared transcriptomic data sampled in the late phases, such as day 9, 12, and 14. Day 12 had the highest number of differentially expressed genes (Sup. 2). Ingenuity pathway analysis was performed for the day 12 data to determine the pathways activated under the C:T = 1:0 condition (Table S3). Unfolded protein response pathway and ER stress pathway were activated in the cystine-added condition.

The cystine addition experiment indicated the importance of the inhibition of intracellular stress and apoptosis. According to Tnag et al., a sufficient supply of Tyr prevents a decrease in pH during the late phase of culture and suppresses autophagic cell death.[6] We investigated the effects of Tyr addition (Figure 2). In the Tyr addition condition, the cell viability and titer increased slightly, and a pH decrease did not occur. The mRNA levels of ER stress makers such as *Hspa5*, *Ern1*, and *Atf3* did not increase. Ingenuity pathway analysis was performed and the top five pathways with a higher z-score in the C:T = 0:3 condition than in the C:T = 0:0 condition are shown in Table S4. The results suggest that activated oxidative phosphorylation pathway related to cell viability under Tyr-added conditions.

Omics analyses were performed to understand the mechanism of Tyr-mediated viability maintenance (Figure 2). To verify the effect of Tyr addition on autophagy suppression, we examined *P62/ SQSTM1* gene expression levels. The accumulation and decrease of P62/Sqstm1 indicate the inhibition and enhancement of autophagy.[36] Tyr addition did not change the mRNA expression level of P62/ Sqstm1.

In contrast, the mRNA expression level of *Ppp1r15a* decreased under Tyr-added conditions, suggesting that Tyr addition protected cells from apoptosis. To examine oxidative stress-related factors, we investigated the mRNA expression levels of Gpx1 and glutathione synthetase (GSS). Gpx1 plays a significant role in reducing most reactive oxygen species in cells using GSH as a reductant.[37] GSS catalyzes the condensation of γ-glutamylcysteine and glycine to form GSH.[38] Tyr addition did not change the expression levels of *Gpx1* and *Gss* mRNAs, possibly due to the shortage of Cys. In contrast, the protein expression levels of ERO1A and ERO1B increased in the Tyr-added condition, suggesting an increase of H_2O_2. However, viability and pH were maintained. Other response system for oxidative stress should be activated.

Coenzyme Q10, also known as ubiquinone, is known to inhibit oxidative stress and to suppress the generation of intracellular ROS.[39] We hypothesized that coenzyme Q10 synthesized from Tyr might reduce oxidative stress and inhibit apoptosis because coenzyme Q10 is biosynthesized from Tyr in mammalian cells.[40] To test this hypothesis, we investigated the effects of coenzyme Q10 addition in batch cultures (Figure 2). The addition of coenzyme Q10 significantly improved the viability and antibody production compared with the addition of

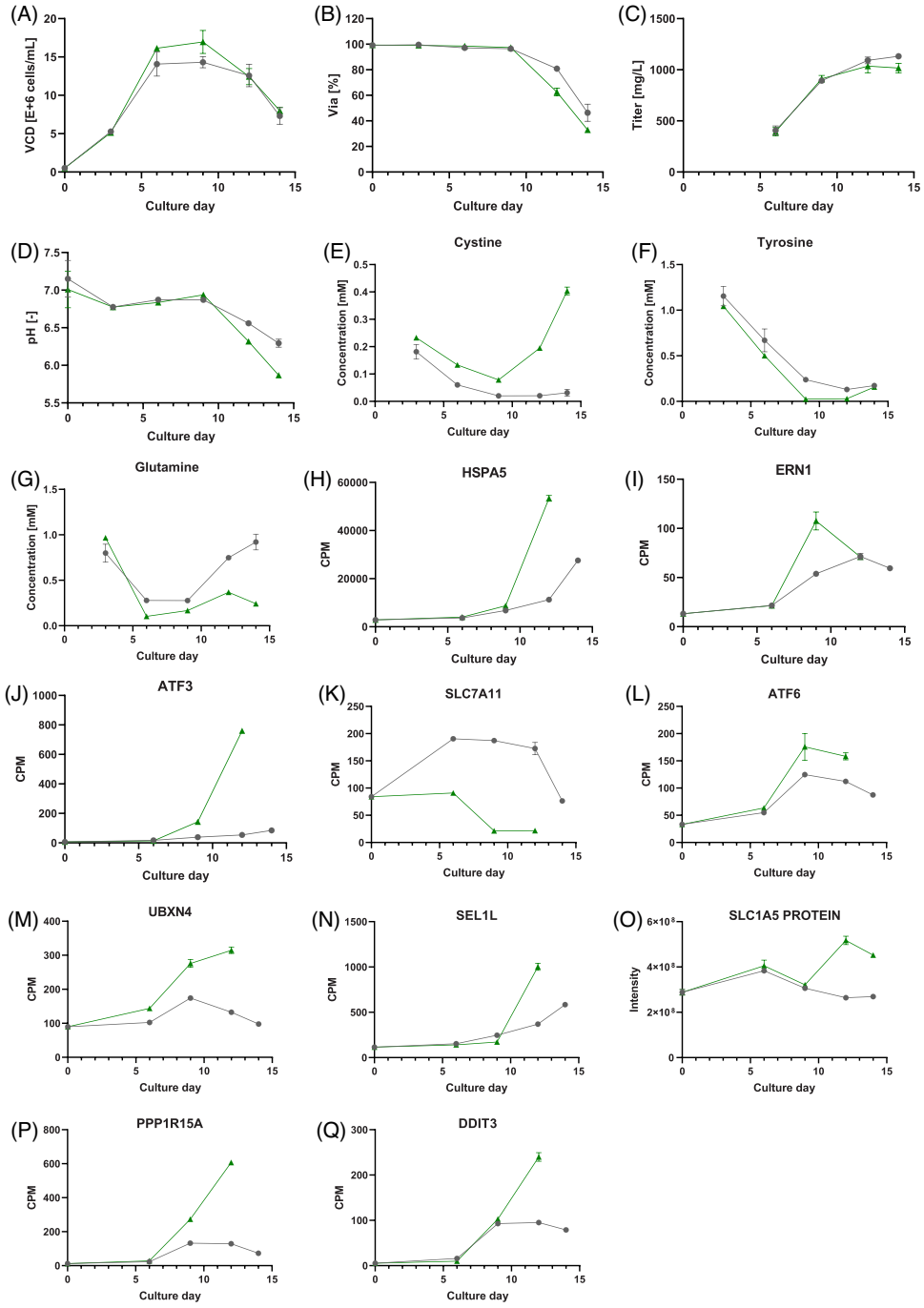

FIGURE 1 The effect of Cys addition in fed-batch culture. (A) VCD profile, (B) Viability profile, (C) Titer profile, (D) pH profile, (E) – (G) time-course plots of cysteine, tyrosine and glutamine, (H) – (Q) time-course plots of the associated RNA transcripts (CPM) and proteins (abundance). Each value represents the mean value ($N = 3$), and error bars represent the standard deviation. Gray (circle dots) and green (triangle dots) indicate C:T = 0:0 and C:T = 1:0 conditions, respectively. All data are from clone 1.

ethanol, the solvent for coenzyme Q10. The effect of Tyr was relatively small at this condition. Tyr concentration in the supernatant of each batch culture condition is shown in Supplementary 3 (A). Tyr concentration was high in the Tyr addition condition. Tyr consumption was marginal in all conditions probably due to the low antibody productivity in batch culture. Supplementary 3 (B) shows the results of oxidative stress level measurements. The oxidative stress level was partially reduced in the Tyr-added condition. Since ethanol, the solvent for coenzyme Q10, affects the measurement, we could not measure the oxidative stress level in the presence of coenzyme Q10. These results partly support the hypothesis that Tyr protect cells from oxidative stress by the production of coenzyme Q10.

To suppress the effect of the decreased reducing power caused by the addition of cystine, Tyr was simultaneously added with cystine

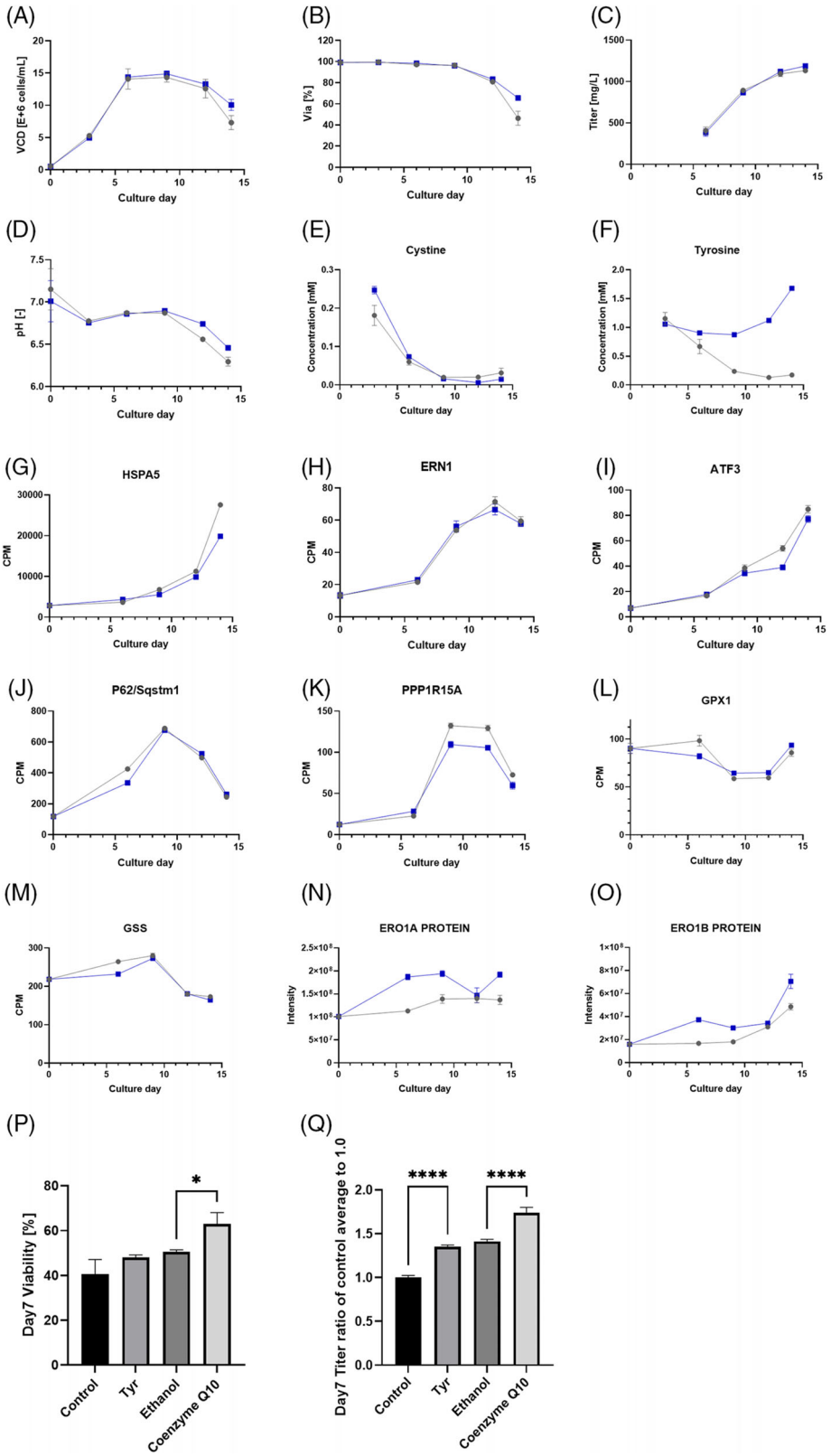

FIGURE 2 The effect of Tyr addition in fed-batch culture. (A) VCD profile, (B) Viability profile, (C) Titer profile, (D) pH profile, (E) – (F) time-course plots of cystine and tyrosine and (G) – (O) time-course plots of the associated RNA transcripts (CPM) and proteins (abundance). Each value represents the mean value ($N = 3$), and error bars represent the standard deviation. Gray (circle dots) and blue (square dots) indicate C:T = 0:0 condition and C:T = 0:3 conditions, respectively. (P) and (Q) Viability and productivity data with coenzyme Q10 addition. Each value represents the mean value ($N = 3$), and error bars represent the standard deviation (*P-value < 0.0274, ****P-value < 0.0001). (P) Viability rate at day 7, (Q) productivity at day 7 (Ratio of average productivity under control condition to 1.0). All data are from clone 1.

(Figure 3). The addition of sufficient Tyr increased the proliferative capacity, maintained viability, and improved productivity. The final productivity was about twice that in the C:T = 1:3 condition as C:T = 0:3 condition and nearly three times higher in the C:T = 3:3 condition. The C:T = 3:3 condition also significantly improved proliferation compared with the C:T = 1:3 condition. In the late phase of the culture, Tyr was barely consumed in the C:T = 0:3 condition. In contrast, most Tyr was consumed in the C:T = 3:3 condition. In the C:T = 1:3 condition, most cystine was consumed and Tyr was partially reduced. These results suggest that Tyr is required for cystine consumption. Without Tyr, cystine would not be adequately consumed.

The expression levels of ER stress markers, *Hspa5*, *Ern1*, and *Atf3*, were lower in the late phase of the culture under the C:T = 1:3 and C:T = 3:3 conditions compared to the C:T = 0:3 condition. Thus, the addition of sufficient amounts of cystine and Tyr suppressed ER stress. Other data on cultural trends with clone 2 are also presented in Supplementary 4. Almost similar culture trends were observed for a clone expressing another IgG1. The effects of cystine and Tyr on CHO cells should be general for antibody producing CHO cells.

Transcriptomic and proteomic analyses were performed to determine the synergistic effects of cystine and Tyr (Figure 3). The expression levels of PDIs and P4hb, a molecular chaperone associated with PDI, increased in the presence of sufficient cystine and Tyr. The intracellular protein levels of HC and LC were significantly increased in the C:T = 3:3 condition compared with C:T = 0:3 (Supplementary 5). Contrary to the enhanced disulfide bond formation, the expression of endoplasmic reticulum oxidase 1s (ERO1s), Ero1a, and Ero1b decreased under these conditions. And the expression of ER stress markers was suppressed. Thus, PDIs and related chaperones should function adequately to fold mAbs and prevent the accumulation of misfolded proteins.

Superoxide dismutase 1 and 2 (SOD1 and SOD2) protect cells from oxidative stress by converting superoxide anion radicals to less harmful hydrogen peroxide in the cytosol and mitochondria, respectively.[41,42] The addition of cystine increased the protein expression levels of SOD1 and SOD2 under the C:T = 1:3 and C:T = 3:3 conditions. The enhanced antibody production should result in the increase of superoxide. Hydrogen peroxide produced by SODs was treated with glutathione peroxidase, using GSH as a reductant. The addition of cystine suppressed oxidative stress caused by antibody production by supplying GSH.

Next, we examined the factors related to GSH metabolism and ERAD. The extramembrane enzymes γ-glutamyltransferase (GGT) and glutathione S-transferase (GST) are associated with GSH metabolism. GGT catalyzes the first step in the degradation of extracellular GSH, the hydrolysis of the γ-glutamyl bond between glutamate and cysteine.[43] GSTM is a GST classified in the μ family that catalyzes the binding of GSH to electrophilic molecules.[44] The results for *Ggt1* in Figure 4 show that under the C:T = 0:3 condition, the Cys supply is insufficient. *Ggt1* mRNA expression was increased to degrade GSH and supply Cys. As the amounts of intracellular Cys in the C:T = 1:3 and C:T = 3:3 conditions were sufficient, *Ggt1* expression was suppressed, and GSH degradation did not proceed. Because the *Gstm* mRNA level was higher in the C:T = 1:3 and C:T = 3:3 conditions than in the C:T = 0:3 condition, intracellular detoxification in the cell was activated. These results suggest that under the C:T = 1:3 and C:T = 3:3 conditions, the GSH concentration was kept high enough for H_2O_2 reduction and cell detoxification. The mRNA levels of ERAD-related genes, *Atf6*、*Ubxn4*, and *Sel1l*, and the protein expression level of SLC1A5, a glutaminolysis indicator, were significantly higher under the C:T = 0:3 condition, and they decreased with an increase in cystine. The higher expression of SLC1A5 under C:T = 0:3 condition is related to the decreased of glutamine (Figure 3). These results indicate that sufficient cystine and Tyr can reduce ERAD and glutaminolysis. Thus, by supplying sufficient amounts of cystine and Tyr, cells can respond appropriately to ER and oxidative stress and suppress ERAD. The expression level of *Slc7a11* was significantly increased only in the C:T = 1:3 condition on day 12. This suggests that the C:T = 1:3 condition falls short of cystine, especially in the late phase of the culture, such as day 12 and beyond.

The effects of cystine and Tyr on the TCA cycle and apoptosis-related factors were also investigated (Figure 4). Oxaloacetate, a key intermediate of the TCA cycle, is synthesized from pyruvate carboxylase (PC). Phosphoenolpyruvate carboxykinase 2 (PEPCK2) converts oxaloacetate into phosphoenolpyruvate. As *Pck2* is under the control of the transcription factor, *Atf3*, AAR and ER stress upregulate *Pck2* expression.[45] In the C:T = 3:3 condition, the expression of the PEPCK2 gene, *Pck2*, was suppressed. In contrast, the expression of the PC gene, Pc, was enhanced under the C:T = 1:3 and C:T = 3:3 conditions. An increase in PEPCK2 under low-cystine conditions causes a decrease in oxaloacetic acid, resulting in decreased TCA cycle activity. In contrast, *Pc* expression increased in the late phase of the culture under the C:T = 1:3 and C:T = 3:3 conditions. Production of oxaloacetate was enhanced, particularly in the C:T = 3:3 condition, indicating activation of the TCA cycle. The key enzymes involved in TCA cycle activation are succinate dehydrogenase (SDH) and aconitase (ACO). SDH, which comprises SDHA and SDHB, oxidizes succinate to fumarate.[46] ACO converts citrate to isocitrate. In particular, aconitase 2 (ACO2) is expressed in the mitochondria and plays an essential role in the TCA cycle. Aconitase has been widely used as a biomarker of oxidative stress and has been suggested to function as an intramitochondrial sensor of redox status, thereby acting as an oxidation-sensitive TCA cycle regulatory enzyme.[47] The protein expression levels of SDHA, SDHB, and ACO2 were higher in the late phase of the culture under the C:T = 1:3 and C:T = 3:3 conditions compared to C:T = 0:3 conditions. Thus, the addition of cystine and Tyr is important for maintaining TCA cycle activity.

The mRNA levels of *Ppp1r15a* and *Ddit3* decreased under the C:T = 1:3 condition and C:T = 3:3 conditions compared to the C:T = 0:3 condition, indicating that apoptosis was suppressed under the C:T = 1:3 and C:T = 3:3 conditions.

Compared to the C:T = 0:3 condition, the C:T = 1:3 and C:T = 3:3 conditions were found to inhibit apoptosis to the same extent from the reduction of cellular stress; however, there was a significant difference in proliferation and productivity between the conditions.

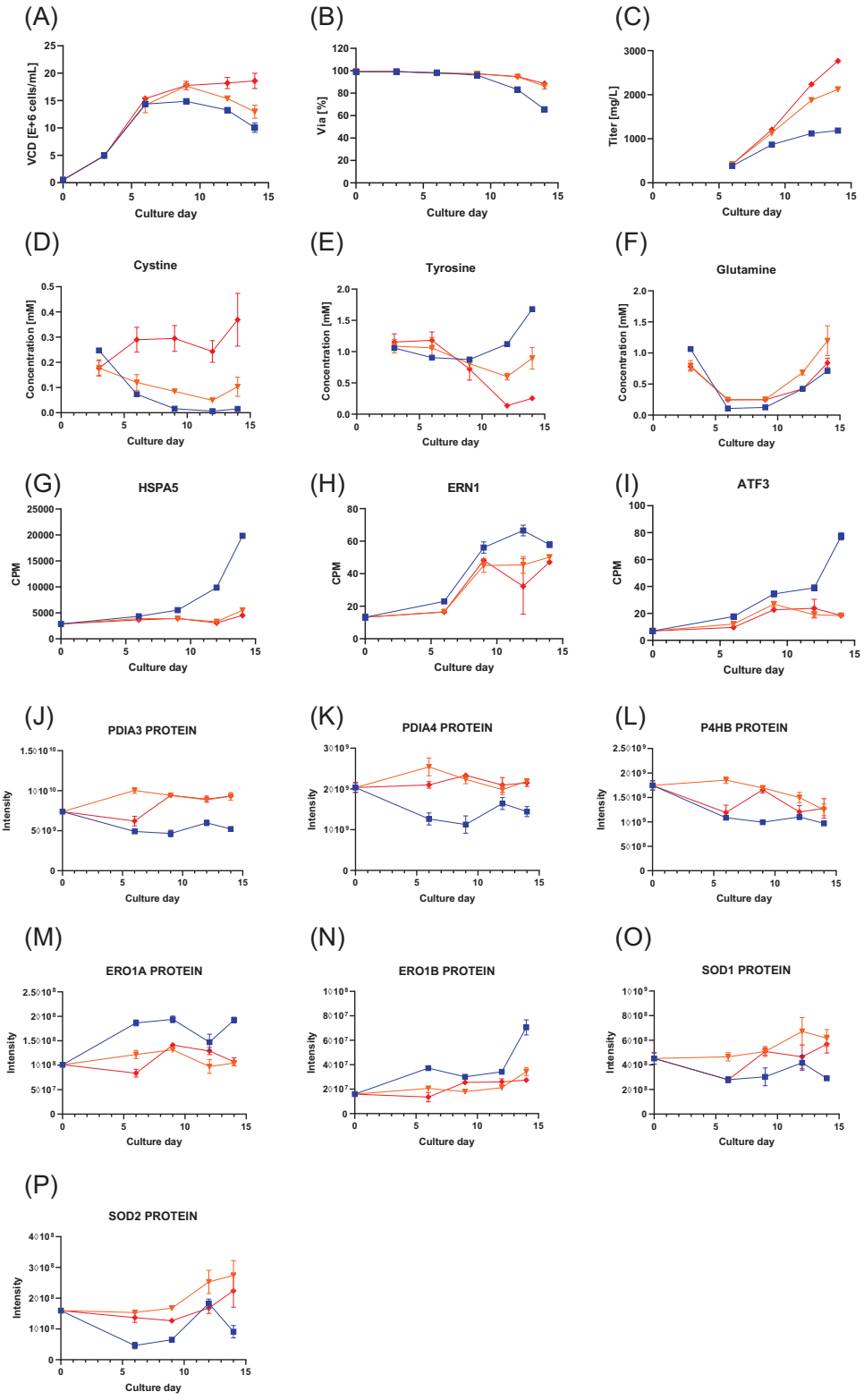

FIGURE 3 The synergistic effects of cystine and Tyr in fed-batch culture. (A) VCD profile, (B) Viability profile, (C) Titer profile, (D) – (F) time-course plots of cysteine, tyrosine and Glutamine, (F) – (O) time-course plots of the associated RNA transcripts (CPM) and proteins (abundance). Each value represents the mean value ($N = 3$), and error bars represent the standard deviation. Blue (square dots), orange (inverted triangular dots), and red (diamond dots) indicate the C:T = 0:3, C:T = 1:3 and C:T = 3:3 conditions, respectively. All data are from clone 1.

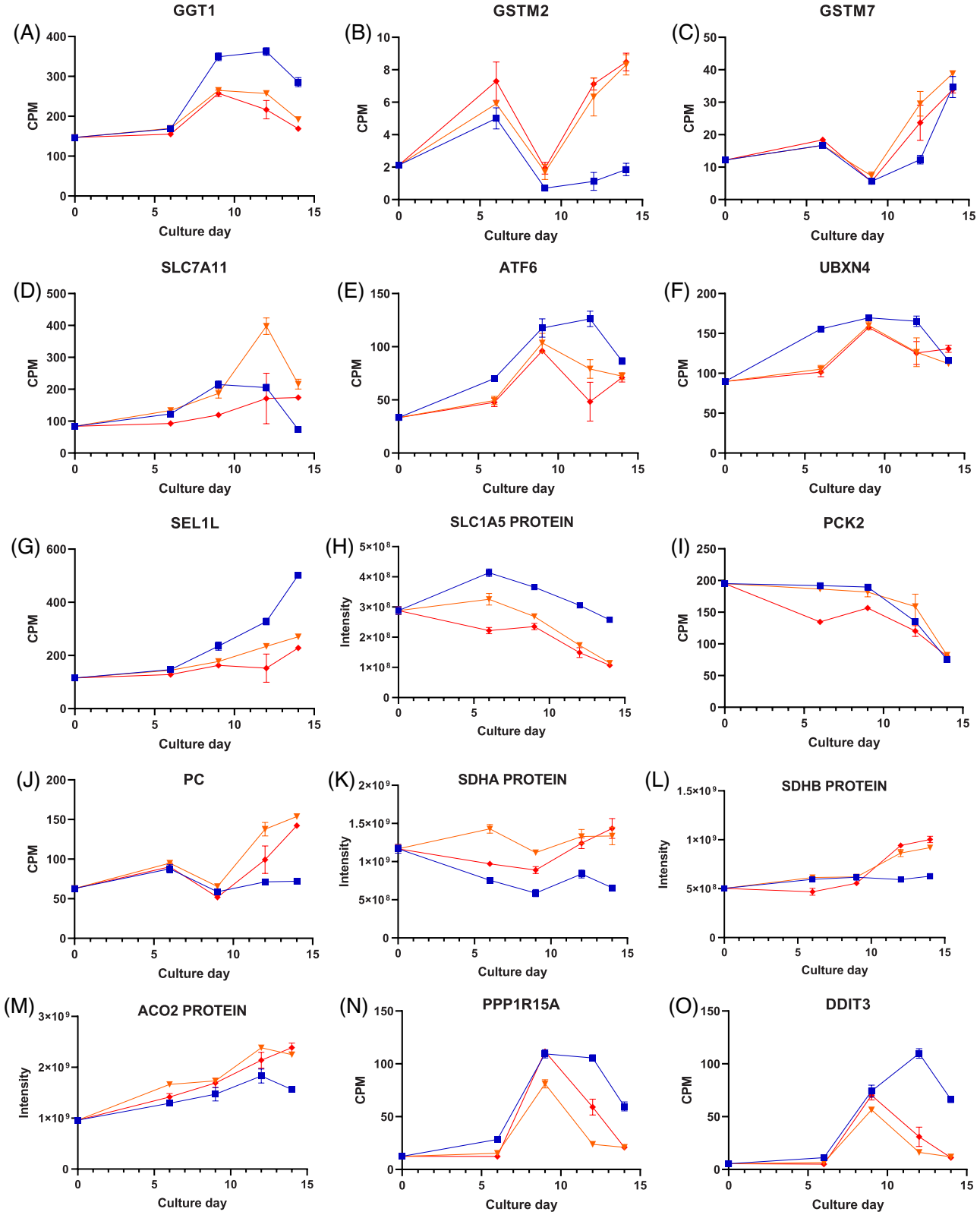

FIGURE 4 The effects of cystine and Tyr on the TCA cycle and apoptosis-related factors. (A) – (O) Time-course plots of the associated RNA transcripts (CPM) and proteins (abundance). Each value represents the mean value ($N = 3$), and error bars represent the standard deviation. Blue (square dots), orange (inverted triangular dots), and red (diamond dots) indicate the C:T = 0:3, C:T = 1:3 and C:T = 3:3 conditions, respectively. All data are from clone 1.

Ingenuity pathway analysis for the day 12 data shows the pathways activated under the C:T = 3:3 condition. The top five pathways with a higher z-score in the C:T = 3:3 condition than in the C:T = 1:3 condition include oxidative phosphorylation pathway, kinetochore metaphase signaling pathway, and pathways related to DNA repair and replication (Table S5).

4 | DISCUSSION

In this study, multi-omics analysis revealed the effect of cystine and Tyr addition on intracellular stress in fed-batch culture for mAb production. The addition of cystine alone causes ER stress, resulting in a decrease in pH, viability rate, and productivity in the late phase of the culture. The negative effect of cystine was likely caused by the consumption of reducing power for the conversion of cystine to Cys.

In contrast, the addition of Tyr alone partially suppressed ER stress and inhibited apoptosis. Although a previous study showed that the addition of Tyr has an inhibitory effect on autophagic cell death,[6] our results showed that the addition of Tyr did not change the mRNA expression level of an autophagy marker, P62/ Sqstm1. The protein expression levels of ERO increased in the Tyr-added condition, which would produce more H_2O_2 upon the oxidation of PDI. In contrast, the expression level of *Gss* did not increase in the Tyr-supplemented condition, indicating that the amount of GSH was sufficient to treat H_2O_2 produced by EROs. These results suggest the existence of another system for coping with oxidative stress. Ubiquinone (coenzyme Q), an antioxidant in the cell, is biosynthesized from Tyr in mammalian cells. As the addition of ubiquinone was as effective as that of Tyr, Tyr might exhibit antioxidant ability through ubiquinone. Although ubiquinone is mainly active in the mitochondria,[48] there are a couple of reports on the occurrence of ubiquinone in the Golgi and ER membranes.[49] UBIAD1 is reported to be a nonmitochondrial ubiquinone forming enzyme.[50]

To suppress the ER stress caused by cystine while satisfying the cellular requirements of Cys, Tyr was added with cystine. The addition of sufficient cystine and Tyr could improve the proliferative capacity, maintain viability, and increase productivity. Significant suppression of ER stress, activation of GSH metabolism, appropriate response to oxidative stress by SOD1 and SOD2, reduction of ERAD, and activation of the TCA cycle were observed. Activation of the TCA cycle can significantly improve cell proliferation and productivity by improving ATP production and energy utilization. The addition of sufficient amounts of cystine and Tyr increased PDI expression. In particular, a marked increase in PDIA3 and PDIA4 expression was observed. Previous studies have shown that PDIA3 and PDIA4 are involved in disulfide bond formation of antibodies in CHO cells.[51]

These omics results on the stress response to cystine addition are generally consistent with the results of Ali et al.[5,12] Ali et al. confirmed an increase in SOD2 with Cys addition, but not an increase in SOD1.[5,12] SOD1 is the predominant SOD in most cells, accounting for 70%–80% of total cellular SOD activity, and is found to be expressed in the cytoplasm.[42] SOD1 expression level was more upregulated in the C:T = 1:3 condition than in the C:T = 3:3 condition, possibly because of the amount of cystine added. This could also be the difference between Cys and cystine additions. However, further studies are required to elucidate the mechanisms of expression regulation.

To investigate the difference in proliferation and productivity between the C:T = 1:3 and C:T = 3:3 conditions, ingenuity pathway analysis was performed for the day 12 data to determine the pathways activated in the C:T = 3:3 condition. Interestingly, three of the top five pathways were found related to DNA repair and replication. The base excision repair (BER) pathway is associated with the repair of DNA damage and strand breaks caused by endogenous and exogenous mutagens. Nucleotide excision repair (NER) enhanced pathway is involved in recognizing and removing local single-stranded DNA damage. The cell cycle control of chromosomal replication is a DNA replication pathway during cell division. In addition, the kinetochore metaphase signaling pathway, which is related to interactions between kinetochores and microtubules, and segregating chromosomes during cell division, was also activated. Thus, under the C:T = 3:3 condition, the cells actively proliferated until day 12.

Corresponding to active proliferation, the most activated pathway was the oxidative phosphorylation pathway. Ubiquinone is required for the transfer of electrons from NADH- or FAD-dependent enzymes to the respiratory Complex III within the inner mitochondrial membrane.[40] There may be a relationship between the effectiveness of the addition of coenzyme Q10, as shown in Figure 2, and the activation of oxidative phosphorylation (Table S3); however, further research is needed to verify this hypothesis.

The relationship between cellular stress and apoptosis caused by the addition of cystine and Tyr can be clarified by omics analysis. Appropriate levels of cystine and Tyr for antibody production in CHO cells were observed. Each factor presented in this study is a potential marker for monitoring stress during bioprocesses. Multi-omics analysis provides a detailed understanding of the biological changes associated with changes in nutrient availability and identifies possible process improvements. This also allowed us to identify additive factors that could replace Tyr in reducing oxidative stress. This multi-omics approach may be applicable to various culture modes such as perfusion cultures. Understanding cellular responses to fluctuations in bioprocess conditions can lead to improved productivity and robust control of bioprocesses.

AUTHOR CONTRIBUTIONS

Yusuke Shibafuji: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing – original draft. Nobuyoshi Nagao: Data curation; Formal analysis; Investigation; Methodology; Software; Writing – review & editing. Masafumi Yohda: Conceptualization; Investigation; Project administration; Supervision; Writing – review & editing.

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Methodology, Project administration, Validation, Visualization, Writing – original draft. N.N.: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. M.Y.: Conceptualization, Investigation, Project administration, Supervision, Writing – review & editing.

CONFLICT OF INTEREST STATEMENT

The authors declare no commercial or financial conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supplementary Material of this article.

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REFERENCES

1. Walsh, G. (2018). Biopharmaceutical benchmarks 2018. *Nature Biotechnology*, *36*(12), 1136–1145.
2. Dinnis, D. M., & James, D. C. (2005). Engineering mammalian cell factories for improved recombinant monoclonal antibody production: Lessons from nature? *Biotechnology and Bioengineering*, *91*(2), 180–189.
3. Lai, T., Yang, Y., & Ng, S. K. (2013). Advances in mammalian cell line development technologies for recombinant protein production. *Pharmaceuticals*, *6*(5), 579–603.
4. Jeon, M. K., Yu, D. Y., & Lee, G. M. (2011). Combinatorial engineering of ldh-a and bcl-2 for reducing lactate production and improving cell growth in dihydrofolate reductase-deficient Chinese hamster ovary cells. *Applied Microbiology and Biotechnology*, *92*(4), 779–790.
5. Ali, A. S., Raju, R., Kshirsagar, R., Ivanov, A. R., Gilbert, A., Zang, L., & Karger, B. L. (2019). Multi-omics study on the impact of cysteine feed level on cell viability and mAb production in a CHO bioprocess. *Biotechnology Journal*, *14*(4), e1800352.
6. Tang, H., Zhang, X., Zhang, W., Fan, L., Wang, H., Tan, W. S., & Zhao, L. (2019). Insight into the roles of tyrosine on rCHO cell performance in fed-batch cultures. *Applied Microbiology and Biotechnology*, *103*(16), 6483–6494.
7. Ketterer, B., Coles, B., & Meyer, D. J. (1983). The role of glutathione in detoxication. *Environmental Health Perspectives*, *49*, 59–69.
8. Chakravarthi, S., & Bulleid, N. J. (2004). Glutathione is required to regulate the formation of native disulfide bonds within proteins entering the secretory pathway. *Journal of Biological Chemistry*, *279*(38), 39872–39879.
9. Ribas, V., García-Ruiz, C., & Fernández-Checa, J. C. (2014). Glutathione and mitochondria. *Frontiers in Pharmacology*, *5*, 151.
10. Chaudhari, N., Talwar, P., Parimisetty, A., Lefebvre d'Hellencourt, C., & Ravanan, P. (2014). A molecular web: Endoplasmic reticulum stress, inflammation, and oxidative stress. *Frontiers in Cellular Neuroscience*, *8*, 213.
11. Zeeshan, H. M., Lee, G. H., Kim, H. R., & Chae, H. J. (2016). Endoplasmic reticulum stress and associated ROS. *International Journal of Molecular Sciences*, *17*(3), 327.
12. Ali, A. S., Chen, R., Raju, R., Kshirsagar, R., Gilbert, A., Zang, L., Karger, B. L., & Ivanov, A. R. (2020). Multi-omics reveals impact of cysteine feed concentration and resulting redox imbalance on cellular energy metabolism and specific productivity in CHO cell bioprocessing. *Biotechnology Journal*, *15*(8), e1900565.
13. Clement, G. E., & Hartz, T. P. (1971). Determination of the microscopic ionization constants. *Journal of Chemical Education*, *48*(6), 395–397.
14. Koppula, P., Zhuang, L., & Gan, B. (2021). Cystine transporter SLC7A11/xCT in cancer: Ferroptosis, nutrient dependency, and cancer therapy. *Protein Cell*, *12*(8), 599–620.
15. Yu, X., & Long, Y. C. (2016). Crosstalk between cystine and glutathione is critical for the regulation of amino acid signaling pathways and ferroptosis. *Scientific Reports*, *6*, 30033.
16. Yu, M., Hu, Z., Pacis, E., Vijayasankaran, N., Shen, A., & Li, F. (2011). Understanding the intracellular effect of enhanced nutrient feeding toward high titer antibody production process. *Biotechnology and Bioengineering*, *108*(5), 1078–1088.
17. Feeney, L., Carvalhal, V., Yu, X. C., Chan, B., Michels, D. A., Wang, Y. J., Shen, A., Ressl, J., Dusel, B., & Laird, M. W. (2013). Eliminating tyrosine sequence variants in CHO cell lines producing recombinant monoclonal antibodies. *Biotechnology and Bioengineering*, *110*(4), 1087–1097.
18. Stolfa, G., Smonskey, M. T., Boniface, R., Hachmann, A. B., Gulde, P., Joshi, A. D., Pierce, A. P., Jacobia, S. J., & Campbell, A. (2018). CHO-omics review: The impact of current and emerging technologies on Chinese hamster ovary based bioproduction. *Biotechnology Journal*, *13*(3), e1700227.
19. Kawashima, Y., Nagai, H., Konno, R., Ishikawa, M., Nakajima, D., Sato, H., Nakamura, R., Furuyashiki, T., & Ohara, O. (2022). Single-shot 10K proteome approach: Over 10,000 protein identifications by data-independent acquisition-based single-shot proteomics with ion mobility spectrometry. *Journal of Proteome Research*, *21*(6), 1418–1427.
20. Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*(17), i884–i890.
21. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21.
22. Li, B., & Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics [Electronic Resource]*, *12*, 323.
23. Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140.
24. Gessulat, S., Schmidt, T., Zolg, D. P., Samaras, P., Schnatbaum, K., Zerweck, J., Knaute, T., Rechenberger, J., Delanghe, B., Huhmer, A., Reimer, U., Ehrlich, H. C., Aiche, S., Kuster, B., & Wilhelm, M. (2019). Prosit: Proteome-wide prediction of peptide tandem mass spectra by deep learning. *Nature Method*, *16*(6), 509–518.
25. Demichev, V., Messner, C. B., Messner, S. I. V., Lilley, K. S., & Ralser, M. (2020). DIA-NN: Neural networks and interference correction enable deep proteome coverage in high throughput. *Nature Method*, *17*(1), 41–44.
26. Kessner, D., Chambers, M., Burke, R., Agus, D., & Mallick, P. (2008). ProteoWizard: Open source software for rapid proteomics tools development. *Bioinformatics*, *24*(21), 2534–2536.
27. Yoshida, H., Haze, K., Yanagi, H., Yura, T., & Mori, K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. *Journal of Biological Chemistry*, *273*(50), 33741–33749.
28. Hetz, C. (2012). The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nature Reviews Molecular Cell Biology*, *13*(2), 89–102.
29. Kilberg, M. S., Pan, Y. X., Chen, H., & Leung-Pineda, V. (2005). NUTRITIONAL CONTROL OF GENE EXPRESSION: How mammalian cells respond to amino acid limitation. *Annual Review of Nutrition*, *25*, 59–85.
30. Hetz, C., Chevet, E., & Harding, H. P. (2013). Targeting the unfolded protein response in disease. *Nature Reviews Drug Discovery*, *12*(9), 703–719.
31. Liang, J., Yin, C., Doong, H., Fang, S., Peterhoff, C., Nixon, R. A., & Monteiro, M. J. (2006). Characterization of erasin (UBXD2): A new ER

protein that promotes ER-associated protein degradation. *Journal of Cell Science*, *119*(Pt 19), 4011–4024.
32. Sun, S., Shi, G., Han, X., Francisco, A. B., Ji, Y., Mendonça, N., Liu, X., Locasale, J. W., Simpson, K. W., Duhamel, G. E., Kersten, S., Yates J. R., 3rd, Long, Q., & Qi, L. (2014). Sel1L is indispensable for mammalian endoplasmic reticulum-associated degradation, endoplasmic reticulum homeostasis, and survival. *Proceedings of the National Academy of Sciences USA*, *111*(5), E582–E591.
33. Zhang, F., & Du, G. (2012). Dysregulated lipid metabolism in cancer. *World Journal of Biological Chemistry*, *3*(8), 167–174.
34. Liu, L., Ito, S., Nishio, N., Sun, Y., Chen, N., Tanaka, Y., & Isobe, K. (2015). GADD34 facilitates cell death resulting from proteasome inhibition. *Anticancer Research*, *35*(10), 5317–5324.
35. Oyadomari, S., & Mori, M. (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death and Differentiation*, *11*(4), 381–389.
36. Bjørkøy, G., Lamark, T., Pankiv, S., Øvervatn, A., Brech, A., & Johansen, T. (2009). Monitoring autophagic degradation of p62/SQSTM1. *Methods in Enzymology*, *452*, 181–197.
37. Faucher, K., Rabinovitch-Chable, H., Barrière, G., Cook-Moreau, J., & Rigaud, M. (2003). Overexpression of cytosolic glutathione peroxidase (GPX1) delays endothelial cell growth and increases resistance to toxic challenges. *Biochimie*, *85*(6), 611–617.
38. Njålsson, R., & Norgren, S. (2005). Physiological and pathological aspects of GSH metabolism. *Acta Paediatrica*, *94*(2), 132–137.
39. Huo, J., Xu, Z., Hosoe, K., Kubo, H., Miyahara, H., Dai, J., Mori, M., Sawashita, J., & Higuchi, K. (2018). Coenzyme Q10 prevents senescence and dysfunction caused by oxidative stress in vascular endothelial cells. *Oxidative Medicine and Cellular Longevity*, *2018*, 3181759.
40. Meganathan, R. (2001). Ubiquinone biosynthesis in microorganisms. *Fems Microbiology Letters*, *203*(2), 131–139.
41. Zelko, I. N., Mariani, T. J., & Folz, R. J. (2002). Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology & Medicine*, *33*(3), 337–349.
42. Fukui, M., & Zhu, B. T. (2010). Mitochondrial superoxide dismutase SOD2, but not cytosolic SOD1, plays a critical role in protection against glutamate-induced oxidative stress and cell death in HT22 neuronal cells. *Free Radical Biology & Medicine*, *48*(6), 821–830.
43. Paolicchi, A., Dominici, S., Pieri, L., Maellaro, E., & Pompella, A. (2002). Glutathione catabolism as a signaling mechanism. *Biochemical Pharmacology*, *64*(5-6), 1027–1035.
44. Xiang, Z., Snouwaert, J. N., Kovarova, M., Nguyen, M., Repenning, P. W., Latour, A. M., Cyphert, J. M., & Koller, B. H. (2014). Mice lacking three loci encoding 14 glutathione transferase genes: A novel tool for assigning function to the GSTP, GSTM, and GSTT families. *Drug Metabolism and Disposition*, *42*(6), 1074–1083.
45. Méndez-Lucas, A., Hyroššová, P., Novellasdemunt, L., Viñals, F., & Perales, J. C. (2014). Mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) is a pro-survival, endoplasmic reticulum (ER) stress response gene involved in tumor cell adaptation to nutrient availability. *Journal of Biological Chemistry*, *289*(32), 22090–22102.
46. Rutter, J., Winge, D. R., & Schiffman, J. D. (2010). Succinate dehydrogenase – Assembly, regulation and role in human disease. *Mitochondrion*, *10*(4), 393–401.
47. Lushchak, O. V., Piroddi, M., Galli, F., & Lushchak, V. I. (2014). Aconitase post-translational modification as a key in linkage between Krebs cycle, iron homeostasis, redox signaling, and metabolism of reactive oxygen species. *Redox Report : Communications in Free Radical Research*, *19*(1), 8–15.
48. Wang, Y., & Hekimi, S. (2016). Understanding ubiquinone. *Trends in Cell Biology*, *26*(5), 367–378.
49. Kalen, A., Appelkvist, E. L., Chojnacki, T., & Dallner, G. (1990). Nonaprenyl-4-hydroxybenzoate transferase, an enzyme involved in ubiquinone biosynthesis, in the endoplasmic reticulum-Golgi system of rat liver. *Journal of Biological Chemistry*, *265*(2), 1158–1164.
50. Mugoni, V., Postel, R., Catanzaro, V., De Luca, E., Turco, E., Digilio, G., Silengo, L., Murphy, M. P., Medana, C., Bakkers, D. Y., & Santoro, M. M. (2013). Ubiad1 is an antioxidant enzyme that regulates eNOS activity by CoQ10 synthesis. *Cell*, *152*(3), 504–518.
51. Komatsu, K., Kumon, K., Arita, M., Onitsuka, M., Omasa, T., & Yohda, M. (2020). Effect of the disulfide isomerase PDIa4 on the antibody production of Chinese hamster ovary cells. *Journal of Bioscience and Bioengineering*, *130*(6), 637–643.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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