Pharmaceutical BIOPROCESSING

Glutamine substitution: the role it can play to enhance therapeutic protein production

The biopharmaceutical market is driven by the steady increase in demand for therapeutic proteins produced in mammalian cells. Glutamine is a main nitrogen source and also a main energy source with glucose in mammalian cell cultures for therapeutic protein production. As a result of glutamine metabolism and the natural decomposition of glutamine, ammonia, which is known to negatively affect cell growth, protein production and sialylation of recombinant glycoprotein, is necessarily accumulated in a culture medium. This review highlights the current strategies and achievements in overcoming the negative effect of ammonia through the glutamine substitution by less ammoniagenic substrates, such as glutamate, pyruvate and α-ketoglutarate.

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Since the first approval of the human tissue plasminogen activator (tPA) produced in mammalian cells in 1987, the biopharmaceutical market has dramatically increased. The annual global market of biopharmaceuticals was estimated at US$ 199.7 billion in 2013 and was predicted to reach US$ 497.9 billion in 2020 [99].

Although a variety of alternative expression systems, including those using microorganisms and insect cells, are available, Chinese hamster ovary (CHO) cells are the most widely used for the commercial production of therapeutic proteins because the CHO-derived glycoprotein quality is compatible with humans and has the desired post-translational modification including glycosylation [1,2]. Currently, six of the top eight therapeutic proteins are manufactured in CHO cells [100].

To meet the increasing needs of the biopharmaceutical market, more than a 100-fold yield improvement of product titers in CHO cells has been achieved over the past two decades, largely due to the development of the cell culture medium as well as the optimization of feeding strategies [3,4]. Nevertheless, the demands of the ever-increasing highly competitive market, particularly for biosimilars, still require a higher production yield and quality of therapeutic proteins in CHO cells.

Glutamine, which plays important roles in the metabolism of cell growth and cell survival, is a major nitrogen source and also an energy source in mammalian cell cultures [5,6]. However, ammonia is necessarily accumulated as a result of glutamine metabolism and natural decomposition of glutamine [7]. Accumulation of ammonia during CHO cell cultures is a concern because of its negative effect on cell growth and glycoprotein production [8,9]. Ammonia is also known to negatively affect product quality including glycosylation patterns. Ammonia concentration as low as 2 mM was found to significantly reduce the terminal sialylation of the glycoproteins [10,11]. The reduction of ammonia accumulation in CHO cell cultures to achieve improved therapeutic proteins has been an ongoing challenge in mammalian cell culture technology.

In this review, we summarize the current strategies and achievements in overcoming the negative effect of ammonia with an emphasis on the glutamine substitution by less ammoniagenic substrates for improved therapeutic proteins.

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Key terms

Therapeutic protein: Protein-based therapeutics has advantages such as highly specific functions, reduction of the side effects and no immune response.

Product quality: Product quality of glycoproteins is determined by several factors such as glycosylation, aggregation, fragmentation and disulfide bond formation.

Ammonia accumulation in culture medium

Mammalian cells utilize glutamine as a nitrogen source and as a key anaplerotic source for biosynthesis. Therefore, glutamine, together with glucose, is a main energy source for mammalian cell cultures [12–14]. Glutamine is the most abundant amino acid in the cell culture medium (2–8 mM) [15,16]. Glutamine, however, is consumed rapidly in mammalian cell cultures because of its diverse metabolic functions within the cells [17].

As shown in Figure 1, glutamine is catabolized mainly in the mitochondrial matrix with the initial removal of the amido group of glutamine to yield glutamate. This reaction is regulated by glutaminase and releases an ammonia molecule. The amido group is also utilized as an amino group donor for purine and pyrimidine biosynthesis and activated sugar formation, but to a much lower extent [18]. Next, the α-amino group is removed by glutamate dehydrogenase, leading to α-ketoglutarate. This reaction also releases a second ammonia molecule. After that, the α-ketoglutarate enters the citric acid cycle to generate energy and produce numerous intermediates for biosynthesis [19,20].

Glutamine is naturally decomposed in the cell culture medium because it is unstable in an aqueous solution state. Glutamine is degraded irreversibly into the pyrrolidonecarboxylic acid and ammonia in cell culture conditions. In addition, this reaction is highly dependent on the culture environment, such as temperature and pH [7]. Pyrrolidonecarboxylic acid

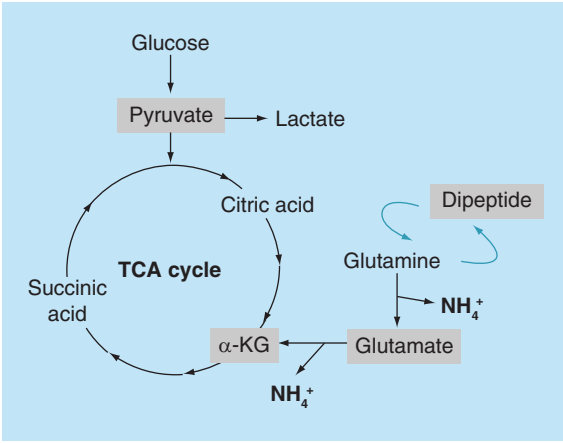

Figure 1. The glutamine metabolism pathway.
Glutamine substitutes used in mammalian cell cultures are shaded.

at a high concentration (20 mM) is not toxic to the cells [21], but ammonia concentration at a low concentration (4.3 mM) can have negative effects on cell growth and glycoprotein production.

Negative effects of ammonia on cell growth & glycoprotein production

Ammonia, which is necessarily accumulated as a result of glutamine metabolism and natural decomposition of glutamine, is a toxic by-product accumulated during mammalian cell cultures [9,22,23]. Negative effects of elevated ammonia in mammalian cell cultures separate into three categories: cell growth, protein production and glycosylation, as summarized in Table 1.

The inhibitory effect of ammonia on cell growth is well documented in several mammalian cell cultures. Ammonia had an inhibitory effect on specific growth rate in hybridoma [24], monkey epithelial cell line (BSC-1) [25] and baby hamster kidney cell line (BHK21) cultures [26]. When ammonia was added to the culture of recombinant CHO (rCHO) cells producing the Fc-fusion protein, ammonia inhibited specific growth rate and decreased cell viability in a dose-dependent manner (Figure 2A & 2B). The maximum viable cell concentration obtained in the culture with 20 mM ammonia addition was approximately 65% of that obtained in the control culture. The inhibitory effect of ammonia on specific growth rate and maximum cell concentration was also observed in many other CHO cell lines, though the degree of growth inhibition by ammonia appears to depend on rCHO cell lines and culture conditions [11,27,28].

The detrimental effect of ammonia on protein production has also been well documented in several mammalian cell cultures. In human fibroblast cultures, 4 mM ammonia addition decreased the interferon-beta (IFN-β) production from 2600 units/ml to 50 units/ml [29]. In hybridoma cell cultures, 5 mM ammonia addition decreased antibody production from 56 μg/ml to 1 μg/ml [30]. In rCHO cell cultures, 7.5 mM ammonia addition decreased tPA production from 85 ng/ml to 45 ng/ml [31]. As shown in Figure 2C, ammonia decreased Fc-fusion protein production in rCHO cell cultures in a dose-dependent manner. The maximum Fc-fusion protein concentration obtained in the culture with 20 mM ammonia addition was approximately 61% of that obtained in the control culture. As shown in Figure 2D, the specific productivity (q_p), which was calculated based on the data collected during the exponential phase of growth, was not affected significantly by ammonia addition ($p > 0.05$). In contrast, the time integral of viable cell concentration during the culture decreased in a dose-dependent manner. Thus, the decreased maximum Fc-fusion

Table 1. Ammonia toxicity on cell growth, product concentration and glycosylation in mammalian cell cultures.

Category	Host	Product	Ammonia addition (mM)	Effect	Ref.
Growth	BSC-1	–	2	30% reduction of growth rate	[25]
	BHK21	–	3	75% reduction of growth rate	[26]
	Hybridoma	IgG	6.7	50% reduction of growth rate	[24]
	CHO	–	8	50% reduction of specific growth rate	[27]
	CHO	EPO	20	25% reduction of final cell yield	[11]
Product concentration	Human fibroblast	IFN-β	4	2600 units/ml → 50 units/ml	[29]
	Hybridoma	Antibody	5	56 μg/ml → 1 μg/ml	[30]
	CHO	tPA	7.5	85 ng/ml → 45 ng/ml	[31]
Glycosylation	CHO	mPL-I	3 and 9	Inhibition of *N*-linked glycosylation	[45]
	CHO	G-CSF	10	Reduction of sialylation	[10]
	CHO	TNFR-IgG	13	Reduction of terminal galactosylation and sialylation	[59]
	CHO	EPO	10	Reduction of sialylation	[10]
	CHO	EPO	30	Reduction of sialylation	[59]
	CHO	tPA	10	Reduction of α2,3-ST, β1,4-GT and CMP-SAT	[65]

BSC: Monkey epithelial cell line; CHO: Chinese hamster ovary; CMP-SAT: Cytidine monophosphate-sialic acid transporter; EPO: Erythropoietin; G-CSF: Granulocyte colony-stimulating factor; mPL: Mouse placental lactogen-1; tPA: Tissue plasminogen activator; TNFR: Tumor necrosis factor receptor.

protein concentration by ammonia addition was mainly due to the reduced cell growth. Many reports have shown that ammonia addition in the range of 4 mM to 10 mM did not significantly affect q_p in hybridoma [24,32] and rCHO cell cultures [33], which supports the idea that decreased protein production in mammalian cell cultures by ammonia addition is mainly due to reduced cell growth.

Decreased cell growth and cell viability in the cultures with ammonia addition are thought to be due to ammonia-induced apoptotic cell death [34,35]. Ammonia addition in the range of five to 10 mM induced apoptosis in C6 glioma cell cultures [36], BHK cells cultures [37] and gastric surface mucous cell cultures [35]. Ammonia addition in mammalian cell cultures may generate the signals that trigger the sequence of events leading to apoptosis [38,39]. Disturbance of intracellular pH caused by ammonia addition induced the complex interplay of nitric oxide, protein kinase C and nuclear factor-kappa B leading to apoptosis in C6 glioma cell cultures [36]. Ammonia addition also induced the release of cytochrome c into the cytosolic fraction, thereby activating the caspase cascade in gastric surface mucous cell cultures [35,40].

Ammonia-induced apoptosis in CHO cells has not been investigated extensively. When ammonia formation was reduced by substitution of glutamine by a dipeptide composed of alanine and glutamine in a culture medium, the apoptosis of rCHO cells expressing anti-CD20 chimeric antibody was reduced [34]. To determine the effect of ammonia addition on apoptosis of rCHO cells, the expression levels of cleaved caspase-3 and 7 of rCHO cells in the cultures with and without the addition of 10 mM ammonia shown in Figure 2 were analyzed by Western blot analysis. As shown in Figure 3, the addition of 10 mM ammonia to the cultures increased the expression levels of cleaved caspase-3 and 7 which are important markers of apoptosis on the late culture period, which confirms that ammonia addition induces apoptosis of CHO cells.

Recently, ammonia derived from glutamine has been identified as an autophagy-stimulating factor [41,42]. Autophagy, which is known as a programed cell death type 2, plays an important role in the maintenance of intracellular homeostasis via regulation of the cellular response to a variable metabolic stress [43,44]. As shown in Figure 3, addition of 10 mM ammonia to the cultures induced conversion of LC3–1 to LC3–2 form, which is an autophagy marker. Thus, ammonia addition induced apoptosis as well as autophagy in rCHO cell cultures.

The detrimental effect of ammonia on the glycosylation of recombinant protein has also been reported in rCHO cell cultures [10,45]. Glycosylation is one of the most critical factors in determining protein quality and can affect the efficacy, biological activity, solubility and

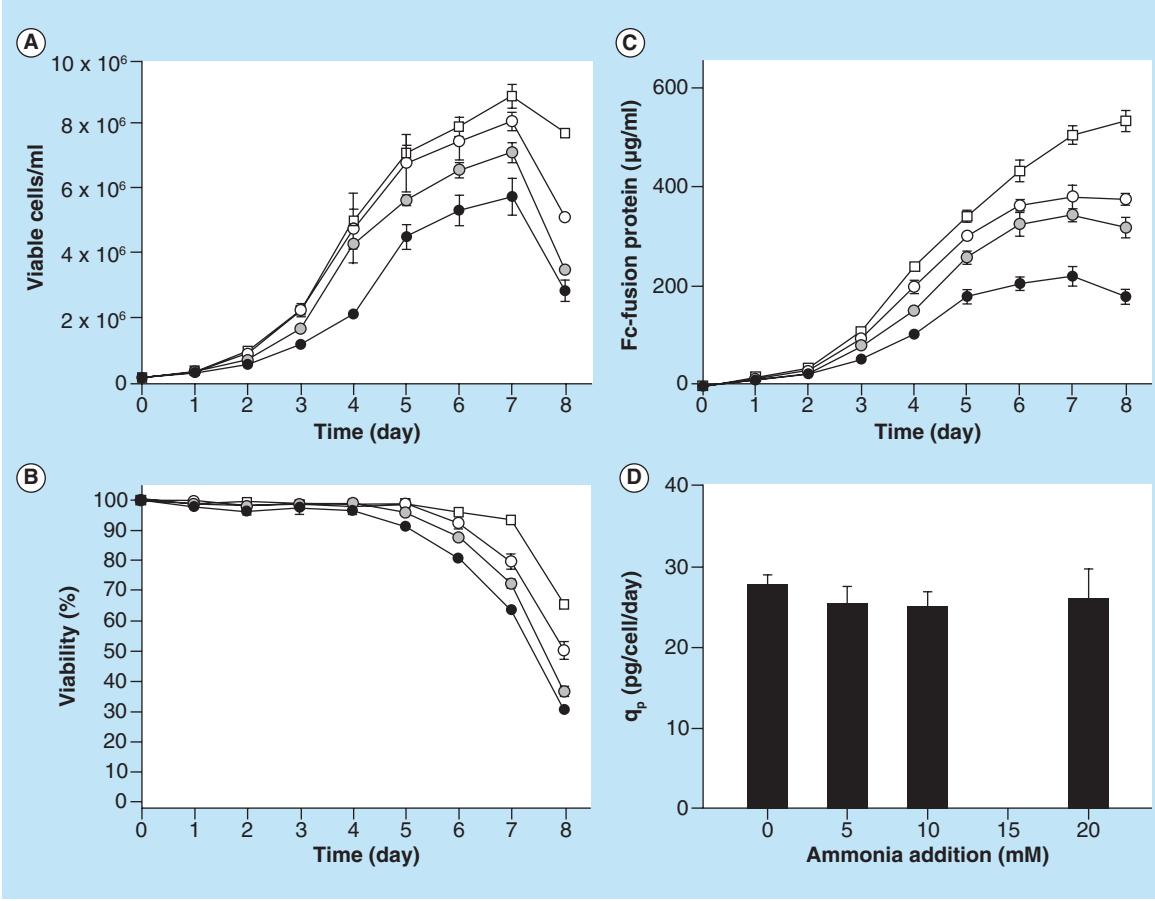

Figure 2. Culture profiles of (A) cell growth; (B) viability; (C) Fc-fusion protein concentration and (D) specific productivity, q_p. rCHO cells producing an Fc-fusion protein were inoculated at 2.0 × 10^5 cells/ml in SFM4CHO (HyClone, Logan, UT) supplemented with 4 mM glutamine (HyClone) and 300 nM methotrexate (Sigma-Aldrich, MO, USA). Cultures were performed in SFM4CHO with 4 mM glutamine without ammonia (open square), with 5 mM ammonia (open circle), with 10 mM ammonia (gray circle) and with 20 mM ammonia addition (closed circle) in 125 ml Erlenmeyer flasks with a working volume of 50 ml. The flasks were incubated on a climo-shaker at 110 rpm, in 5% CO_2/air mixture and 85% humidified at 37°C. The error bars represent the standard deviations calculated from two independent experiments.

in vivo half-life of the therapeutic glycoproteins [46,47]. Glycosylation is affected by numerous culture parameters such as temperature [48,49], dissolved oxygen [50], culture pH [49,51], culture mode [52–54] and chemical supplements [55–57]. Among them, accumulated ammonia is known to negatively affect the glycosylation profiles of therapeutic glycoprotein, which results in decreased *in vivo* efficacy [58].

N-glycosylation of mouse placental lactogen-1 (mPL-1) expressed by CHO cells was inhibited by increasing concentrations of ammonia (three and 9 mM). In addition, inhibition of glycosylation by ammonia was dependent on the extracellular pH [45]. O-glycosylation of granulocyte colony-stimulating factor (G-CSF) produced from CHO cells was inhibited by ammonia concentrations ranging from 0 to 10 mM. In particular, the sialic acid in the α2,6 linkage of *N*-acetylgalactosamine was reduced by the addition of ammonia concentrations as low as 2 mM [10]. The inhibitory effect of ammonia addition on galactosylation and sialylation of tumor necrosis factor-immunoglobulin G (TNFR-IgG) produced from CHO cells was observed [59]. The amount of the galactosylation and sialylation on TNFR-IgG correlated in a dose dependent manner with the ammonia concentration. As the ammonia concentration increased from 1 to 15 mM, terminal galactosylation and sialylation of TNFR-IgG were decreased by over 40%. An inhibitory effect of ammonia addition on the glycosylation of erythropoietin (EPO) produced from CHO cells was also observed [11,60]. The effect of added ammonia on EPO glycosylation was related to a decrease in sialylation of all glycans and a decrease in the proportion of the O-linked glycan. Accordingly, the detrimental effect of ammonia on the glycosylation of recombinant protein has been well received in rCHO

cell cultures. Nevertheless, the cellular mechanism of detrimental effects of ammonia on the glycosylation of recombinant proteins in CHO cell cultures has not yet been clearly understood.

Ammonia increases the intracellular pH of endoplasmic reticulum and Golgi compartments [9,61]. In aqueous solution, ammonia is linked in a pH-dependent equilibrium because the protonation and deprotonation reactions of ammonia are performed very fast in intracellular organelles [9]. Ammonia in the culture media induces acidification of the cytoplasm and alkalinization of the interior of the organelles, including mitochondria, as a result of the ammonia diffusion cycle between the organelles and cytoplasm [62].

Increased intracellular pH disturbs the balance of the nucleotide sugar pools, and thereby reduces cell growth and inhibits the glycosylation by affecting the activities of the enzymes involved in the glycosylation [63,64]. Increased intracellular pH inhibited glycosyltransferase activity, such as α2,6-sialyltransferase [10], α2,3-sialyltransferase (α2,3-ST) and β1,4-galactosyltransferase (β1,4-GT) [59], whereas it increased glycosidase activity such as galactosidase [59] and sialidase [57].

Ammonia affects the glycosylation-related gene expression level as well as enzyme activities. Among 12 glycosylation-related genes evaluated by quantitative real time PCR, α2,3-sialyltransferase, the expression levels of β1,4-galactosyltransferase and CMP-sialic acid transporter (*CMP-SAT*) decreased in the cultures with ammonia addition [65]. With available genomic sequences of CHO cells [66], the effect of elevated ammonia on the glycosylation related genes is better understood. There are approximately 300 genes associated with glycosylation including glycan synthesis, nucleotide sugar transport and degradation in CHO cells [66]. Recently, the changes in mRNA expression levels of 52 *N*-glycosylation related genes, which were categorized as nucleotide sugar synthesis, nucleotide sugar transporter, *N*-glycan chain extension, galactosylation, sialylation, fucosylation and *N*-glycan degradation, in CHO cells that produce the Fc-fusion protein with addition of 10 mM ammonia to the cultures were determined using a NanoString nCounter analysis system [67]. Among them, thirteen genes (*gale, nans, gpi, man2a1, b4galt5, b4galt7, st3gal2, st3gal5, glb1, hexa, hexb, neu1* and *neu3*) were upregulated over 1.5-fold in the culture with ammonia addition. In particular, the mRNA expression level of *neu1* and *neu3*, which are the genes involved in the sialylation degradation, is upregulated over twofold. Likewise, the protein expression levels of *neu1* and *neu3* also increased in the cultures with ammonia addition. Furthermore, transient transfection of *neu-1* or *neu3*-targeted siRNAs significantly improved the sialic acid content of the Fc-fusion protein in the culture with ammonia addition. Thus, ammonia reduced the glycosylation, particularly sialylation, by affecting both glycosylation-related gene expression levels and enzymatic activities.

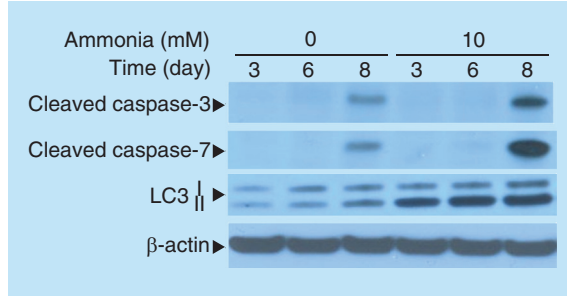

Figure 3. Western blots of cleaved caspase-3/7 and LC3 during cultures in SFM4CHO with or without 10 mM ammonia addition. The antibodies used for analysis were anti-cleaved caspase-3, anti-cleaved caspase-7, anti-LC3 (all from Cell Signaling Technology, MA, USA), and anti-β-actin (Sigma-Aldrich). β-actin was used as a loading control. Culture conditions are described in Figure 2.

Glutamine substitution by lesser ammonia producing substrates

In order to reduce the accumulation of ammonia during cultures, various strategies have been attempted. Among them, substitution of glutamine by less ammoniagenic substrates or stable dipeptides is probably the easiest one to be implemented from the point of view of the bioprocess. A number of articles dealing with glutamine substitution for a reduction of ammonia accumulation in mammalian cell cultures are summarized in Table 2.

Since ammonia is accumulated as a result of glutamine metabolism and natural decomposition of glutamine, substitution of glutamine by less ammoniagenic substrates such as glutamate, α-ketoglutarate, tricarboxylic acid (TCA) cycle intermediates shown in Figure 1 dramatically reduces ammonia accumulation in mammalian cell cultures.

In 1958, glutamate was first used as a substitute for glutamine in HeLa cell cultures for poliovirus production [68]. Glutamate with alanine supplementation replaced glutamine in mouse L-929 cell cultures [69]. Glutamine was also replaced by either glutamate or α-ketoglutarate in McCoy cell cultures, which resulted in approximately a 70% reduction of ammonia accumulation [70]. Glutamine substitution by glutamate significantly decreased ammonia accumulation in murine hybridoma cell cultures as well. However, cell growth and monoclonal antibody production were reduced by approximately 30% by glutamine substitution [71]. In human embryonic kidney 293 (HEK293) cell cultures

Table 2. Glutamine substitution strategies for reduction of ammonia accumulation in mammalian cell cultures.

Host	Product	Glutamine substitution by	Effect	Ref.
HeLa	Poliovirus	Glutamate	Maximal yield of poliovirus production	[68]
HeLa	–	Glutamate	Growth improvement	[69]
Vero, McCoy, BHK	–	Either glutamate and alpha-ketoglutarate	70% reduction of ammonia accumulation	[70]
Hybridoma	IgG1	Glutamate	Reduction of ammonia accumulation (<0.5 mM)	[71]
CHO	tPA	Glutamate	Reduction of ammonia and lactate, increase of tPA production rate	[73]
CHO	tPA	Glutamate	Higher cell concentration, lower production of byproducts	[75]
CHO	tPA	Glutamate	Reduction of ammonia and lactate, increase of tPA production rate	[74]
HEK293	Adenovirus	Glutamate	Reduction of ammonia accumulation about tenfold, 1.8-fold increase of volumetric productivity	[72]
CHO	rIgG	Glutamate	Reduction of ammonia accumulation (<2 mM), 1.7-fold increase of q_p and rIgG titer, increase of galactosylation	[33]
CHO	Fc-fusion protein	α-ketoglutarate	Reduction of ammonia accumulation (<3 mM), 2.7-fold increase of q_p and 1.3-fold increase of titer, increase of sialylation	[76]
CHO, BHK, MDCK	Influenza vaccine	Pyruvate	Reduction of ammonia (<0.4 mM) and lactate (<18.6 mM) accumulation	[77]
Hybridoma	IgG	Ala-Gln or Gly-Gln	Reduction of ammonia accumulation (<3.5 mM), 14% increase of cell yield	[81]
CHO	tPA	GlutaMAX™-1 (dipeptide of Ala-Gln)	Improvement of galactosylation	[82]
CHO	IgG	Ala-Gln	Reduction of ammonia about twofold, twofold increase of IgG titer	[34]

BHK: Baby hamster kidney; CHO: Chinese hamster ovary; HeLa: Human epithelial carcinoma; MDCK: Madin-Darby canine kidney; tPA: Tissue plasminogen activator.

for recombinant adenovirus production, glutamine substitution by glutamate significantly reduced ammonia accumulation and resulted in a 1.8-fold increase in adenovirus volumetric productivity [72].

Glutamine substitution by less ammoniagenic substrates has been also applied to rCHO cell cultures for recombinant protein production. Glutamine was successfully replaced by glutamate in an rCHO cell culture for tPA production [73]. Glutamine substitution by glutamate along with glucose substitution by galactose significantly reduced undesirable ammonia and lactate production and increased the cell density and tPA production in batch cultures [73]. It also decreased formation of by-products and increased culture duration and tPA production in fed-batch cultures [74]. Dual substitution of glutamine and glucose by galactose and glutamate, respectively, further improved cell growth and tPA production in continuous cultures, while reducing ammonia and lactate concentrations [75].

Glutamate was successfully used as a glutamine substitute in rCHO cell culture for recombinant IgG (rIgG) production [33]. Ammonia concentration in a glutamate-based medium did not exceed 2 mM, which is only one third of that in a glutamine-based medium. In addition, a 1.7-fold increase in rIgG titer and q_p, along with favorable galactosylation of rIgG, was achieved by substitution of glutamine by glutamate. Glutamine substitution by glutamate resulted in a 10% increase in the proportion of galactosylated glycans, GlF and G2F.

Glutamate is metabolized to α-ketoglutarate, which is a key intermediate for replenishing and circulating the TCA cycle while producing ammonia. Thus, substitution of glutamate by TCA cycle intermediates

is expected to further reduce the ammonia accumulation during the cultures. When TCA cycle intermediates (citric acid, succinic acid and α-ketoglutarate), along with glutamate were used as a substitute for glutamine with two different rCHO cell lines producing Fc-fusion protein, α-ketoglutarate produced the best production performance [76]. The replacement of glutamine by α-ketoglutarate reduced cell growth. However, it increased culture longevity and q_p, which resulted in a 1.3-fold increase in the maximum product concentration. Furthermore, the sialic acid content of Fc-fusion protein in an α-ketoglutarate-based medium was higher than that in a glutamine-based medium, most likely due to the lower ammonia concentration. The final ammonia concentration in the α-ketoglutarate-based medium was less than one fourth of that in the glutamine-based medium. As shown in Figure 4, α-ketoglutarate also replaced the glutamine successfully in rCHO cell cultures for antibody production. The replacement of glutamine by α-ketoglutarate resulted in a better performance for cell viability, culture duration and antibody production.

For industrial use of α-ketoglutarate as a glutamine substitute, a relatively long lag phase with α-ketoglutarate can be a concern. The lag phase can be shortened significantly using cells adapted to grow in an α-ketoglutarate medium [76]. Alternatively, a low level of glutamine may be supplemented in an α-ketoglutarate based medium to reduce the lag phase of cell growth. In batch cultures of rCHO cells for Fc-fusion protein production, supplementation of 0.5 mM glutamine in an α-ketoglutarate based medium improved cell growth and the final Fc-fusion protein concentration (Figure 5A & 5B), though it slightly increased ammonia concentration (Figure 5C). In addition, α-ketoglutarate is affordable for large-scale cultures, though it is a little more expensive than glutamine.

Pyruvate, which is an important metabolite for energy generation in mammalian cell cultures, also replaced glutamine successfully. Substitution of glutamine by pyruvate provided a significant reduction of ammonia as well as lactate without suppression of growth in MDCK, BHK21 and CHO cell cultures for influenza vaccine production [77]. In particular, MDCK cells grew well without ammonia and lactate accumulation even at very low concentrations of pyruvate (1 mM).

To overcome the chemical decomposition of glutamine in the medium, glutamine can be replaced by stable derivatives such as dipeptides. In dipeptides, the α–amino group of glutamine is chemically bound, and thus dipeptides are more resistant to the natural decomposition by intra or extracellular peptidase in aqueous solution state than glutamine [9,22]. Furthermore, the solubility of dipeptides can be higher than that of glutamine [34,78]. However, the high cost for chemical synthesis of dipeptide can be a concern for use in large-scale cultures [22,34]. A cost-effective production process of dipeptides using microbial fermentation made the use of dipeptides more affordable [79,80].

Key terms

By-products: Lactate and ammonia, which are two major toxic by-products produced from glucose and glutamine, respectively, are necessarily accumulated during mammalian cell cultures.

Fed-batch culture: Operational technique in cell culture where depleting nutrients are fed to the bioreactor.

Continuous culture: Operational technique in cell culture where fresh medium is continuously fed to the bioreactor and culture supernatant is continuously removed from the bioreactor.

Dipeptides, such as alanyl-glutamine (Ala-Gln) and glycyl-glutamine (Gly-Gln), were used as a substitute of glutamine in murine hybridoma cell cultures [81]. The final cell density in a Gly-Gln supplemented medium was 14% higher than in glutamine only. The accumulation of ammonia and lactate was significantly decreased by substituting glutamine for dipeptides. However, monoclonal antibody production was similar among glutamine, Ala-Gln and Gly-Gln supplemented medium. The dipeptide Ala-Gln, was used as a glutamine substitution in CHO cell cultures for tPA production [82]. Ala-Gln supplementation reduced ammonia accumulation and enhanced galactosylation of tPA. Ala-Gln was also used as a glutamine substitute in CHO cell cultures for monoclonal antibody production [34]. Although Ala-Gln suppressed cell growth, aglutamine substitution by Ala-Gln increased monoclonal antibody productivity and decreased apoptotic cell death.

Other strategies to reduce ammonia formation

Glutamine and glucose concentrations in typical cell culture media are much higher than concentrations required by cells for energy metabolism and growth. The excessive level of glutamine and glucose increases an unnecessary consumption rate of these nutrients and result in the accumulation of toxic waste products, such as ammonia and lactate, during the cultures [83]. The excessive accumulation of ammonia due to unnecessarily metabolized glutamine in mammalian cell cultures can be reduced by controlled addition of glutamine and/or glutamine replacement [84,85].

Ammonia formation was reduced by approximately 40% in MDCK cell cultures by controlling the glutamine and glucose concentration below 1.0 mM [24]. In batch and continuous cultures of BHK cells, ammonia

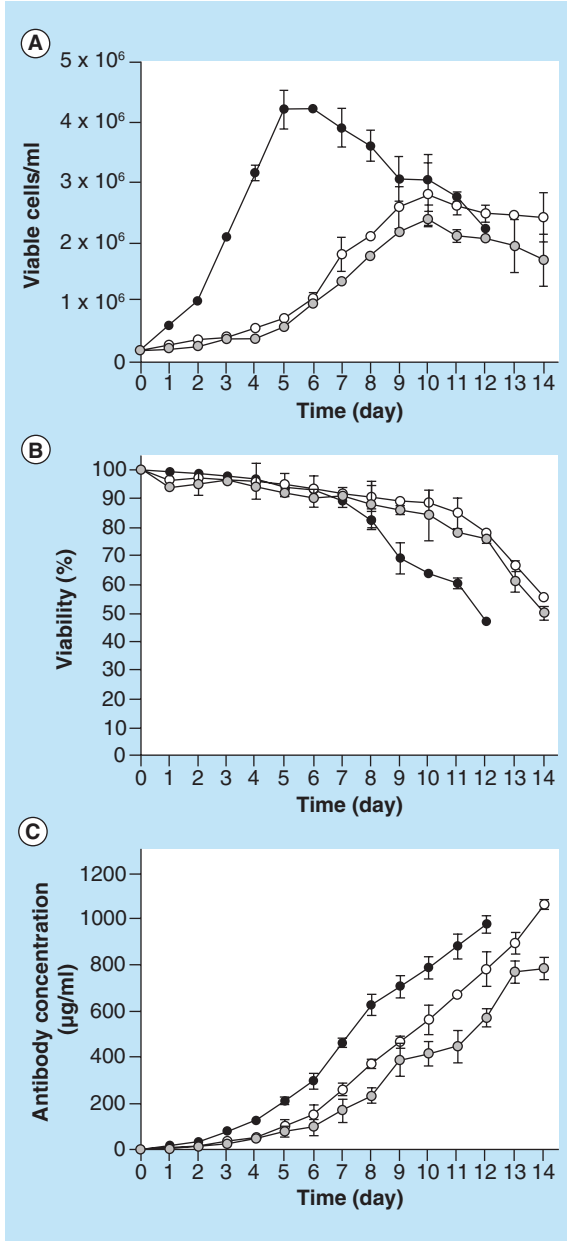

Figure 4. Culture profiles of (A) cell growth; (B) viability; and (C) antibody concentration. Cultures were performed in SFM4CHO with 4 mM glutamine (closed circle), with 4 mM α-ketoglutarate (open circle) and without any supplements (gray circle). Culture conditions are described in Figure 2.

formation was significantly reduced by maintaining the glutamine concentration at less than 1.0 mM [86]. In HEK293 cell cultures for adenovirus production, glutamine concentration was maintained at a level as low as 0.1 mM with a concentrated feed medium using online sampling coupled with automatic feedback control of glutamine [83]. As a result, virus titers in low-glutamine fed-batch cultures with significant reduction of ammonia accumulation were approximately tenfold higher than those in a normal batch culture. The feedback algorithm was also used to maintain the glutamine level at 0.3 mM in fed-batch cultures of rCHO cells for interferon gamma (IFN-γ) production [85]. As expected, this low glutamine fed-batch strategy reduced ammonia and lactate accumulation and enabled up to a tenfold increase in IFN-γ yield, while maintaining both the *N*-glycosylation macro- and microheterogeneity of IFN-γ. However, a very low glutamine concentration (< 0.1 mM) decreased the sialylation and increased the heterogeneity of IFN-γ.

To reduce the ammonia formation in fed-batch cultures for tPA production, a glutamine-containing dipeptide, pyruvate, glutamate and wheat gluten hydrolysate was used as a substitute for glutamine in a two-step feed glutamine replacement process where the cells were initially cultivated in a glutamine containing medium to establish cell growth followed by feeding with a glutamine substitute [84]. This feed glutamine replacement process not only reduced the ammonia concentration by over 45% but also almost doubled the tPA titer without compromising the tPA quality, including glycosylation patterns.

To reduce ammonia formation during the cultures, cells that can grow in a glutamine-free medium or produce less ammonia can be selected by high throughput screening and adaptation. CHO-K1 cells that can grow in the glutamine-free medium were selected using fluorescence-activated cell sorting FACS or magnetic activated cell sorting [87]. The selected cells showed similar or even better growth and viability profiles compared with parental CHO-K1 cells. Likewise, the same strategy was successfully applied to rCHO cells producing an erythropoietin-Fc fusion protein (CHO-EPO-Fc) [88]. The produced EPO-Fcs had comparable quality to those produced by the parental CHO-EPO-Fc in glutamine containing medium, with only minor effects on EPO antennarity.

Genetic manipulation of the metabolic pathway in mammalian cells can reduce ammonia formation. Glutamine synthetase (GS) which catalyzes the conversion of glutamine and ammonia to form glutamine is the most widely engineered target in mammalian cells [89,90]. Transformed cells with the GS gene can grow in glutamate instead of a glutamine supplemented medium with a significant reduction of ammonia accumulation.

Murine hybridoma cells transfected with the GS gene grew in the glutamine-free culture medium without cell growth reduction, while not producing ammonia [91,92]. The CHO-K1 cell line transformed with GS was also constructed and, then the GS gene was amplified with methionine sulfoximine, which is a GS inhibitor. The specific ammonia production rate of the resulting CHO cells was about one-fourth of

that of parental CHO-K1 cells [93]. Co-expression of GS and recombinant protein was attempted in CHO cells [94]. GS expression allowed the cells to grow better in a glutamate based medium without reduction of growth and viability. Moreover, the accumulation of ammonia dramatically decreased from 2.1 to 0.7 mM in glutamate based medium and the expression of recombinant protein increased by approximately 18%.

Other genetic manipulation targets are carbamoyl phosphatate synthetase I (CPS I) and ornithine transcarbamoylase (OTC), which are involved in the urea cycle. The urea cycle is an enzyme system for the elimination of ammonia via conversion of ammonia to urea [95]. CPS I and OTC, which are the first and second steps in the urea cycle, were introduced into CHO cells to reduce the ammonia accumulation during the cultures [96]. The resulting co-engineered cells showed decreased ammonia accumulation and a better cell growth compared with parental cells. CPS I and OTC were also introduced into rCHO cells producing tPA (OTC-tPA-CHO). The biological activity of tPA produced from OTC-tPA-CHO cells was higher than that of any reporting CHO cells [96].

Strategies using an ion exchange resin [97], electrodialysis [98] and hydrophobic membrane [71] are also possible to reduce ammonia accumulation during the cultures, but they are not covered in this review.

Conclusion & future perspective

For production of glycoprotein in mammalian cell cultures, ammonia accumulation resulting from glutamine catabolism and natural decomposition is a concern because of its negative effects on cell growth, protein production and glycosylation. Among various strategies for reduction of ammonia, glutamine substitution by fewer ammonia producing substrates such as glutamate, pyruvate and α-ketoglutarate is quite effective and can be easily implemented in the bioprocess. Excessive accumulation of ammonia due to unnecessarily metabolized glutamine can also be reduced by controlled addition of nutrients such as glutamine and glucose in fed-batch cultures. Alternatively, with increasing information on the ammonia effects on cells at the molecular level, it would be possible to genetically engineer cells to produce less ammonia and/or to be more robust in regard to cell growth and glycoprotein production at elevated ammonia concentrations.

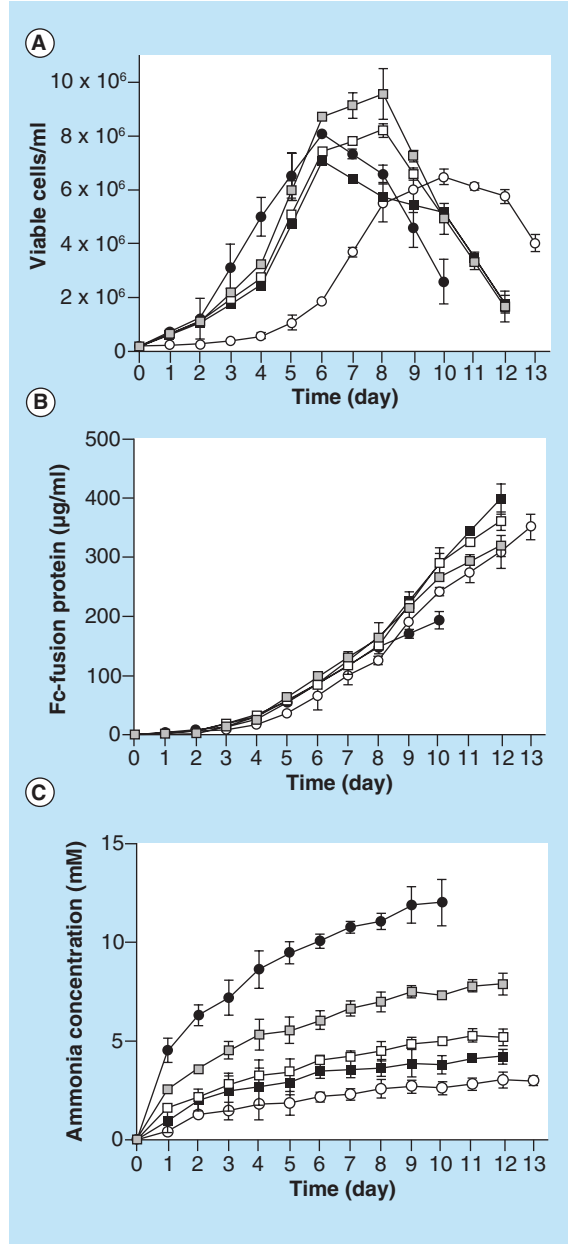

Figure 5. Culture profiles of (A) cell growth; (B) Fc-fusion protein concentration; and (C) ammonia concentration. Cultures were performed in SFM4CHO with 4 mM glutamine (closed circle), with 4 mM α-ketoglutarate (open circle), with 0.5 mM glutamine + 4 mM α-ketoglutarate (closed square), with 1 mM glutamine + 4 mM α-ketoglutarate (open square) and with 2 mM glutamine + 4 mM α-ketoglutarate (gray square). Culture conditions are described in Figure 2.

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Executive summary

Background & glutamine metabolism

- Glutamine is a main energy source in mammalian cell cultures for therapeutic protein production. As a result of glutamine metabolism and natural decomposition of glutamine, toxic ammonia is necessarily accumulated in a culture medium. Thus, a strategy to overcome the negative effects of ammonia needs to be developed.

Ammonia toxicity

- Ammonia is known to negatively affect cell growth, protein production and glycosylation of recombinant glycoprotein.

Strategies for reducing the accumulation of ammonia in a culture medium

- Among various strategies, substitution of glutamine by fewer ammoniagenic substrates is probably the easiest one to be implemented from the viewpoint of bioprocess aspects.
- Glutamine substitution: glutamate, pyruvate, α-ketoglutarate and dipeptides effectively substitute glutamine in mammalian cell cultures.
- Nutrient control: glutamine-limited or glutamine replacement fed-batch cultures reduce ammonia formation.
- Genetic manipulation: the urea cycle and glutamine synthetase genes were introduced to the mammalian cells for reducing ammonia formation and efficiently using glutamine, respectively.

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