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Glycosylation-related genes in NS0 cells are insensitive to moderately elevated ammonium concentrations

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Abstract

NS0 and Chinese hamster ovary (CHO) cell lines are used to produce recombinant proteins for human therapeutics; however, ammonium accumulation can negatively impact cell growth, recombinant protein production, and protein glycosylation. To improve product quality and decrease costs, the relationship between ammonium and protein glycosylation needs to be elucidated. While ammonium has been shown to adversely affect glycosylation-related gene expression in CHO cells, NS0 studies have not been performed. Therefore, this study sought to determine if glycosylation in NS0 cells were ammonium-sensitive at the gene expression level. Using a DNA microarray that contained mouse glycosylation-related and housekeeping genes, the of these genes was analysed in response to various culture conditions – elevated ammonium, elevated salt, and elevated ammonium with proline. Surprisingly, no significant differences in gene expression levels were observed between the control and these conditions. Further, the elevated ammonium cultures were analysed using real-time quantitative reverse transcriptase PCR (qRT-PCR) for key glycosylation genes, and the qRT-PCR results corroborated the DNA microarray results, demonstrating that NS0 cells are ammonium-insensitive at the gene expression level. Since NS0 are known to have elevated nucleotide sugar pools under ammonium stress, and none of the genes directly responsible for these metabolic pools were changed, consequently cellular control at the translational or substrate-level must be responsible for the universally observed decreased glycosylation quality under elevated ammonium.

Keywords

DNA microarrays; biopharmaceuticals; glycosylation; mammalian cells; CHO cells

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1. Introduction

Mammalian cells are increasingly being used to produce glycoproteins for human clinical therapies. The extent of glycosylation – or the addition of sugar groups to proteins – impacts the effectiveness of treatments, which makes protein glycosylation quality a top priority (Butler, 2006; Butler and Meneses-Acosta, 2012; Hudak and Bertozzi, 2014; Jenkins et al., 1996; Lingg et al., 2012; Ozturk, 1992). The culture environment, in which ammonia and ammonium ions accumulate, dictates cell growth rates, recombinant protein production, and protein glycosylation quality (Berlec and Strukelj, 2013; Butler, 2006; Chen and Harcum, 2005; deZengotita et al., 2002; Gawlitze et al., 1999; Hansen and Emborg, 1994; Jenkins, 1996; Jenkins et al., 1996; Kim et al., 2013; Miller et al., 1988; Quek et al., 2010; Reuveny et al., 1986; Schneider et al., 1997; Yang and Butler, 2000a). Ammonia is mainly produced in cell cultures due to L-glutamine metabolism. A secondary source of ammonia in cell culture is the spontaneous degradation and deamination of nitrogen-containing entities, such as L-glutamine, where several substitutions have been investigated (Genzel et al., 2005; Hong et al., 2010; Yao et al., 2012). Ammonium levels can reach 10 mM and higher in end-stage cultures, but negative effects have been observed at levels as low as 2 mM ammonium (Andersen and Goochee, 1995; Andersen et al., 1994; Kim et al., 2013; Ozturk, 1992; Wahrheit et al., 2014; Wong et al., 2005). Specifically, elevated ammonium levels reduce protein glycosylation quality, by decreasing terminal sialylation levels as well as both the tetraantennary and tetrasialylated oligosaccharide structures, while increasing molecular heterogeneity (Baker et al., 2001; Chen and Harcum, 2005; Hossler et al., 2009; Seo et al., 2014; Yang and Butler, 2000b, 2002). All of these factors lower the efficacy of therapeutic glycoproteins and having to remove these isoforms increases costs (Jenkins et al., 1996).

Interestingly, in NS0 cells elevated ammonium has been shown to increase intracellular nucleotide sugar pools, which serve as precursors for many glycosylation reactions (Burleigh et al., 2011; Ryll, 1994; Valley et al., 1999). Additionally, when cells are supplemented with glycosylation precursors, such as N-acetylmannosamine and galactose, glycosylation was not significantly increased, despite increased nucleotide sugar pools (Baker et al., 2001; Clark et al., 2005; Gu and Wang, 1998); however, when N-acetylmannosamine and galactose were combined with uridine or cytidine, glycosylation was significantly increased (Wong et al., 2010). On the other hand, glycoengineering, which involves cloning additional copies of genes encoding important glycosylation enzymes, has contributed to improved protein glycosylation and product quality (Lee et al., 2012; Onitsuka et al., 2012). Early work cloned additional copies of the two genes encoding important glycosylation enzymes – sialyltransferase and galactosyltransferase – and produced modest improvements in protein glycosylation (Weikert et al.). And recently, genes have been added that increase resistance to sialidase, a degradation enzyme (Werner et al., 2012). Unfortunately, this strategy does not address the relationship between culture conditions and cell metabolism, and therefore insights into how ammonium and other culture byproducts influence cell behaviour – specifically the activity of genes governing glycosylation – would complement glycoengineering tools and enable the development of even better strategies to optimize culture conditions for recombinant protein production.

This research sought to expand our limited understanding of protein glycosylation control, by investigating how elevated ammonium levels influence glycosylation at the gene expression level. To this end, an Affymetrix-platform DNA microarray was employed. The DNA microarray contained known mouse glycosylation-related nucleotide sequences and numerous housekeeping genes for mouse, the host organism of the NS0 cell line. Elevated ammonium is known to significantly decrease protein sialylation in NS0 cells (Baker et al., 2001), and the sialyltransferase gene has been observed to be ammonium-sensitive in Chinese hamster ovary (CHO) cells (Chen and Harcum, 2006), so altered gene expression was expected in NS0 cells, as well. Additionally, both CHO and NS0 cell lines are used to manufacture recombinant glycoproteins for clinical therapies and can be cultured in defined media in suspension cultures (Gatti et al., 2007). Initially, DNA microarrays were used to analyse the gene expression activity of NS0 cells exposed to four different culture conditions: control, elevated ammonium, elevated salt as an osmolarity control, and elevated ammonium with a proline addition, as proline had been observed to reduce the negative effects of ammonium on product quality for CHO cells. Subsequently, real-time qRT-PCR was used as an orthogonal analysis method to confirm the results observed for the elevated ammonium culture condition using a newer media formulation.

2. Materials and Methods

The NS0 cells (ECACC 85110503) for the DNA microarray studies were donated to Clemson University by Merck, Inc, and originally from the European Collection of Cell Cultures. The NS0 cells for the real-time qRT-PCR studies were purchased from Sigma (Catalog no. 03061601 — ECACC 85110503), where Sigma is the official US ECACC distributor. NS0 cells are a mouse myeloma cell line with lymphoblast morphology, non-secreting clone, and cholesterol auxotroph (Whitford, 2003). The NS0 cell lines used in these studies do not express a recombinant protein and represent the host strain or the fusion partner for generation of hybridomas. For the DNA microarray studies, an animal-derived protein-free media, HyQ ADCF-Mab (Hyclone) (first-generation media), was used and supplemented with 5 mM glutamine (Hyclone) and LS-250 cholesterol supplement (4 mL per L media; Hyclone) in 1000-mL spinner flasks with 100 rpm agitation. For the cultures analysed by real-time qRT-PCR, a second-generation ADCF-Mab media (Thermo Hyclone) was used with a LS-1000 cholesterol supplement (1 mL per L media; Hyclone) in 250-mL spinner flasks with 60 rpm agitation. Both studies cultured the cells in humidified incubators at 37°C and 5% CO₂. A common seed culture was used to inoculate the control and treatment cultures, which were then cultured in parallel. Biological triplicates were used for each condition examined. The ammonium, salt, and proline stock solutions (all 5000 mM) were prepared in Milli-Q water and added to the fresh media prior to the addition of cells. Milli-Q water was added to the control and treatment cultures to normalize culture volumes and media concentrations (1 to 6 mL per L). For initial growth studies, the culture conditions were basal media (control), and basal media supplemented with either 10 mM NH₄Cl (ammonium), 10 mM NaCl (salt), or 10 mM NH₄Cl and 20 mM proline (proline). For the DNA microarray studies, the culture conditions were basal media (control), and basal media supplemented with either 5 mM NH₄Cl (ammonium), 5 mM NaCl (salt), or 5 mM NH₄Cl and 10 mM proline (proline). Osmolality of the media was measured using the freezing

point method (Schmelzer et al., 2000). The 5 mM additions increased osmolality by approximately 30 mOsm/kg, or only approximately 10%. Cell concentrations and viabilities were determined by the Trypan blue exclusion method. Glucose was measured using a Blood Glucose Analyzer (Hemacue). For the real-time qRT-PCR studies, the control and elevated ammonium (5 mM) conditions were investigated. Cell concentrations were quantified using the Scepter 2.0 Handheld Automated Cell Counter (Millipore). Glucose and lactate were quantified using a Yellow Springs Instruments (Yellow Springs, OH) 2900 Biochemistry Analyzer.

2.1. RNA isolation

Cells were harvested by centrifugation at 3000 g for 5 min at 4°C in order to obtain total RNA. The supernatant and pellets were saved at -20°C and -80°C, respectively. Total RNA was isolated from the pellet using the RNeasy-Midi™ kits (Ambion), except the DNase treatment was increased to 2 µl of DNase and 1 hour. The total RNA was further purified using the RNeasy Qiagen kit. RNA concentrations were determined using a Ribogreen RNA quantification kit (Molecular Probes).

2.2. DNA microarray analysis

Ten micrograms of total RNA (1 µg/µl) per sample was sent to the Consortium for Functional Genomics for gene expression analysis on the GLYCOv3 Chip. The Affymetrix-platform and data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Barrett et al., 2013) and are accessible through GEO Platform accession GPL11096 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL11096>. The GLYCOv3 Chip Mouse Gene List can also be found at <http://www.functionalglycomics.org/static/consortium/resources/resourcecoree.shtml> (accessed April 10, 2014). These Glyco-Gene Chips are available to academic researchers through a proposal process. The GLYCOv3 Chip contains 952 mouse glycosylation-related probes, including probes for the sialyltransferase, galactosyltransferase, sialidase, and fucosyltransferase genes. All 14 of the genes (including the two housekeeping genes), previously investigated in CHO cells under elevated ammonium by qRT-PCR, were on GLYCOv3 Chip (Aebi et al., 2000). Biological triplicates for each condition were analysed for a total of 12 DNA microarrays. Briefly, upon arrival of the total RNA, the Consortium for Functional Genomics staff assessed the RNA quality using a Bioanalyzer (Agilent). Then, the RNA was reverse transcribed in the presence of the fluorescently-labeled dNTP analogues. The labeled RNA was then hybridized to the GLYCOv3 Chip, including the GeneChip Eukaryotic Hybridization Controls, Bacteriophage Controls, and GC Controls (Affymetrix). The GeneChip Eukaryotic Hybridization Controls contains a mixture of four biotin-labeled cRNA controls with staggered concentrations. The Bacteriophage Controls contains four bacteriophage P1 oligonucleotides, and the GC Control contains 44 random and specific GC oligonucleotides that provide titrated fluorescent signals. These controls provide calibrated intensities that are used to confirm hybridization and chip integrity. These controls are used to determine the "Present" and "Absent" calls for a gene. Additionally, there are 104 housekeeping mouse gene controls on the chip as probes. The GLYCOv3 Chips were scanned with a confocal scanner, which calculated the signal intensities associated with each oligonucleotide on the GLYCOv3 chip into image files viewable by researchers. The data was reviewed for quality

control at the Consortium for Functional Genomics, where the data files were processed and returned as .CEL files. GeneSpring® imported the .CEL data, and each gene was then normalized to the total intensity of the chip. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Barrett et al., 2013) and are accessible through GEO Series accession number GSE28920 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28920>).

2.3. Real-time qRT-PCR analysis

The cDNA was synthesized using 1 µg of total RNA from each sample as template using Retroscript Kit (Life Technologies). Real-time qRT-PCR was performed with Quantitect SYBR green PCR Kit (Qiagen) using primers designed for five glycosylation-related genes: solute carrier family 35 (CMP-sialic acid transporter) [Slc35a1], ST3 beta-galactoside alpha-2,3-sialyltransferase 3 [St3gal3], ST3 beta-galactoside alpha-2,3-sialyltransferase 4 [St3gal4], ST3 beta-galactoside alpha-2,3-sialyltransferase 6 [St3gal6], β (1,4)-galactosyltransferase [B4galt1], and two endogenous control genes: Actin, beta (Actb) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) in a Rotor-Gene 3000 Real-Time Thermal Cycler (Qiagen). Primer sequences are listed in Table 1. Relative gene expression levels were calculated using 2^{-C_T} method with Gapdh as an internal standard (Livak and Schmittgen, 2001). Both Gapdh and Actb did not change throughout the study (ANOVA, $p > 0.05$), as expected.

2.4. Statistical analysis

For the DNA microarray data, a Welch ANOVA test using a Benjamini and Hochberg false discovery rate correction was used for the multiple comparisons ($p = 0.05$, $p = 0.10$, and $p = 0.99$) with Tukey Post-Hoc tests. ANOVA analysis was used to analyse the effect of ammonium on cell densities and growth rates ($p = 0.05$). For real-time qRT-PCR, a student's two-tailed t-test was employed to determine the significant differences between groups, with $p = 0.05$ being considered as statistically different.

3. Results

To study the impact of elevated ammonium on gene expression using a DNA microarray, NS0 cells were cultured under four conditions: normal (control), elevated ammonium, elevated salt, and elevated ammonium with proline. The elevated salt treatment provided an osmolality control, while the proline-addition cultures were included because previous elevated ammonium studies in CHO cells showed that a proline-addition could reduce ammonium-induced growth inhibition and restore some protein glycosylation (Chen and Harcum, 2005). Initially, NS0 were cultured under 10 mM ammonium, 10 mM salt, 10 mM ammonium with 20 mM proline; however, the growth rates of the ammonium-stressed cultures (with and without proline) were only approximately one-fifth of the control culture, while the salt-stressed culture growth rate was only approximately one-third of the control culture (data not shown). These very different growth rates were unquestionably too great to allow for meaningful gene expression comparisons, as samples would have to be taken at different culture ages to obtain similar cell densities, and the cells would be in very different growth phases. Interpretation of data from cultures with multiple growth rate and cell

densities differences would have required significantly more samples than were available due to the high cost of DNA microarrays. Thus, cell growth rates were examined at 5 mM ammonium, 5 mM salt, and 5 mM ammonium with 10 mM proline. Previous studies have shown that ammonium levels as low as 2 mM can negatively impact glycosylation, thus a 5 mM ammonium treatment was deemed to have the characteristics representative of elevated ammonium (Andersen and Goochee, 1995; Andersen et al., 1994). The growth profiles for NS0 cells cultured under 5 mM ammonium, 5 mM salt, and 5 mM ammonium with 10 mM proline are shown in Figure 1 for the control and treated cultures shown as the average of triplicates with 95% confidence intervals. The ANOVA analysis of the growth rates and final cell densities indicated that the culture treatment was a significant factor. Further post hoc testing using paired *t-tests* showed no differences for growth rates between the control and treated cultures ($p > 0.05$); however, the paired *t-tests* did indicate that the final cell densities were different ($p < 0.05$). The final cell densities for the control, 5 mM ammonium, and 5 mM ammonium/10 mM proline cultures were all different from each other; however, the final cell densities for salt cultures were not different from the control cultures ($p > 0.05$), and were not different from 5 mM ammonium cultures ($p > 0.05$). The final cell densities for salt cultures were also different from the 5 mM ammonium/10 mM proline cultures ($p < 0.05$). These profiles and statistical analysis indicate that the culture conditions were very similar, such that growth rate differences would not be a contributing factor in any gene expression differences that might be identified and the final cell densities would only be a minor factor.

For the DNA microarray experiments, the 90-h time point (indicated by a vertical line in Figure 1) was selected for RNA isolation and DNA microarray analysis to identify genes sensitive to ammonium in the exponentially growing cultures. The gene expression analysis was performed at the 90-h time point to balance several experimental constraints. The cultures needed to be in the same growth phase and at similar cell densities, where mid-exponential growth was desired. The cell density needed to be sufficiently in order to obtain enough total RNA from a single sample (50 mL). And, exponential growth after sampling needed to be verifiable after sampling, at least another 12 hours of growth possible in the media. Additionally, due to the cost of DNA microarrays, only 12 DNA microarrays were available to the project from the Scripps Institute. Thus, for four culture conditions to be sampled in triplicate, the experiment was limited to one time point. As shown in Figure 1, all cultures continued to grow exponentially for at least another 12 hours. GeneSpring® was used to analyse the gene expression levels using a Welch ANOVA test with a Benjamini and Hochberg false discovery rate correction for the four conditions, where each chip was normalized to total intensity. Initially, the p-value cut-off was set to $p < 0.05$; however, at this level no genes were determined to be significantly different. Due to the high levels of noise commonly observed in biological systems, the p-value cut-off was increased to $p < 0.10$ and the data re-examined; however, even at this level no genes were identified as being significantly different between the conditions. In order to better understand why the ANOVA analysis was not identifying any significant genes, the p-value was increased to $p < 0.99$, and all genes passed, as expected. The gene with the lowest p-value had a p-value of $p = 0.964$, which indicates no significant difference in gene expression levels between the four conditions examined, when gene intensities were normalized to the total chip intensity. This

means that none of the glycosylation genes had different gene expression levels between any of the four conditions.

Further analysis of the DNA microarray data demonstrated that the Affymetrix titrated controls had the expected dynamic range, indicating the gene chips were responsive to sample-level changes. It was also determined that noise was not the reason for the high p-values as shown in Figure 2 by the high-level of reproducibility of the biological replicates and low noise to signal ratio. Figure 2 only shows the gene expression levels for the twelve glycosylation and two housekeeping genes previously examined for CHO cells, as a graph of all 952 unchanged genes would be unreadable (Chen and Harcum, 2006). Table 2 includes these selected genes with official *Mus musculus* gene names, abbreviations, and Genbank accession number on the GLYCOv3 DNA microarray and the CHO cell (*Cricetulus griseus*) homolog, common name, and GenBank accession number. The gene expression levels for the NS0 cell culture conditions are connected between the conditions only to improve visualization. The standard error bars for each condition and gene are shown (and are barely visible for some genes). These very small standard error bars support that there are “no significant difference” due to the underlying behaviour of the biological system, and not due to a high signal-to-noise ratio in the data. Note: the Fut4 and Neu2 genes were identified as “Absent”, not expressed, under these conditions in NS0 cells. These two genes are only shown to highlight the range of magnitude for gene expression and because these are the homologs of the CHO genes previously reported to be sensitive to ammonium. At the other extreme are the housekeeping genes, which are commonly among the mostly highly expressed genes within an organism. These gene expression data for these glycosylation and housekeeping genes demonstrate the well-distributed dynamic range of gene expression levels within the study and detected by the DNA microarrays. Thus, as further shown in Figure 2, no glycosylation-related genes had significantly different gene expression levels between the four conditions examined using the DNA microarray for the NS0 cultures.

As DNA microarrays are not sensitive to small gene expression changes, and to confirm these surprising gene expression results, the control and elevated ammonium conditions of the experiment were repeated and the gene expression changes were analysed using real-time qRT-PCR, an orthogonal and more sensitive gene expression quantification method (Canales et al., 2006; Provenano and Mocellin, 2007; Schefe et al., 2006; Wurmbach, 2009). For the real-time qRT-PCR experimental repeats, several minor changes were necessary. Specifically, the protein-free, animal-free media originally used for the microarray experiments was no longer available, so the next generation of that media was used. The LS-1000 cholesterol supplement was used instead, since the previous LS-250 supplement was unavailable. The culture volume was decreased from 1000-mL to 250-mL, since less total RNA is needed to perform real-time qRT-PCR analysis. New NS0 cells were purchased from the European Collection of Cell Cultures via Sigma. The second-generation media and LS-1000 supplement resulted in higher growth rates and higher final cell concentration for both the control and elevated ammonium cultures. Figure 3 shows the cell growth profiles for the control and elevated ammonium cultures as the average of triplicates with 95% confidence intervals. As with the DNA microarray experiment, the control NS0

cultures achieved higher final cell concentration than the ammonium-stressed cultures. Three different time points during the exponential growth phase were selected for RNA harvesting: 63-h, 66-h, and 72-h. These time points were chosen to obtain similar cell densities as the NS0 cultures harvested for the DNA microarray study to allow for more appropriate comparisons.

The real-time qRT-PCR analysis examined five glycosylation and two housekeeping genes: Slc35a1, B4galt1, St3gal3, St3gal4, and St3gal6, where Actb and Gapdh served as housekeeping genes for quantification. These five glycosylation genes were selected because these genes represent key control reactions that significantly impact a biopharmaceuticals efficacy (Damiani et al., 2009; Zhang et al., 1998). Additionally, St3gal4 and B4galt1 gene expression was sensitive to ammonium for CHO cells (Chen and Harcum, 2006), thus these two genes were thought to be more likely to have detectable changes in NS0 than genes that were insensitive to ammonium in CHO cells. The Slc35a1 gene product is a membrane protein responsible for nucleotide sugar transport into the Golgi (Bill et al., 1998). The B4galt1 gene product catalyses the linkage of a galactose onto N-acetylglucosamine on a glycoprotein (Kotani et al., 2001). The product of the St3gal3, St3gal4, and St3gal6 genes catalyse the addition of a sialic acid on the terminal galactose of a glycoprotein, which increases the efficacy of many therapeutic glycoproteins (Bill et al., 1998; Butler and Meneses-Acosta, 2012). The *M. musculus* primer sequences for these seven genes can be found in Table 1.

The normalized gene expression levels for Slc35a1, B4galt1, St3gal3, St3gal4, and St3gal6 are shown in Figure 4 normalized to Gapdh of the time 63-h control cultures. Additionally, there was no statistical difference between the control and ammonium cultures at any time point for the five-glycosylation genes, and these gene expression levels did not change across the nine hours examined ($p > 0.05$). To highlight that these gene expression levels were not different, Table 3 lists the p-values for the paired t-test p-values for each time point. Additionally, the relative expression levels of the ST3gal3, ST3gal4, and ST3gal6 genes real-time qRT-PCR was consistent with the relative levels observed by DNA microarrays, where the expression levels were ST3gal4 > ST3gal3 >> ST3gal6. Thus, real-time qRT-PCR analysis demonstrates that NS0 cells are not sensitive to 5 mM ammonium with respect to these five glycosylation-related genes. And, the real-time qRT-PCR results confirm the DNA microarray results as accurate, where the DNA microarray analysis demonstrated that none of the known glycosylation-related genes were sensitive in NS0 cells at 5 mM ammonium.

4. Discussion

Gene expression studies have been conducted on both NS0 and hybridoma cells lines under hyperosmotic pressure (Gao et al., 2004; Shen and Sharfstein, 2006). Wu et al. (2004) examined antibody production in NS0 cells, while Shen and Sharfstein (2006) investigated gene expression changes in OKT3 cells, a hybridoma line, due to hyperosmolality (Shen and Sharfstein, 2006; Wu et al., 2004). Wu et al. (2004) focused their analysis on the cell physiology, thus providing few specific gene details; however, they did report that three carbohydrate genes were up-regulated while one carbohydrate gene was down-regulated.

Shen and Sharfstein (2006) used the Affymetrix Gene Chip MOE430A, which contains over 14,000 genes and 4000 EST probe sets. The GLYCOv3 and MOE430A Chips have 482 genes in common. Of these, only two of the in-common genes were identified as sensitive to hyperosmolality in OKT3 cells (glucosamine-6-phosphate deaminase, and hexokinase 1, GenBank accession nos. **NM_011937** and **NM_010438**, respectively). If the responses were proportional to the stress introduced, then the much lower osmotic level used in this NS0 cell study was unlikely to cause changes in gene expression that were significant enough to detect, as these genes only changed 2-fold under the hyperosmotic stress.

The NS0 cells used in this study did not produce a recombinant protein. A study that compared non-producing (parental) and producing (monoclonal antibody) strains of NS0 cells using DNA microarrays identified 104 genes with differential gene expression (Khoo et al., 2007). The functional annotation of these genes determined that the differentially expressed genes were involved in protein synthesis, lipid metabolism, and cell cycle regulation; however, none of these affected genes were glycosylation-related. The absence of glycosylation-related genes sensitive to ammonium in the current study cannot be entirely attributed to the non-producing status of the strain; however, it is recognized that any genetic modifications could influence the cells' response to elevated ammonium. Consequently, the current study provides a baseline for future quantification of the effects of elevated ammonium on recombinant NS0 cells.

Sodium butyrate is a cell culture media additive that has been used to increase glycoprotein productivity, yet also affects protein glycosylation; however with variable outcomes (Chen et al., 2011; Chung et al., 2001; Crowell et al., 2008; Etchevarry and Ryll, 1998; Hong et al., 2011; Lee et al., 2014; Liu et al., 2014; Mohan and Lee, 2009; Sung and Lee, 2005; Yee et al., 2008). Sodium butyrate influences both the quantity and quality of the recombinant protein product due to its various effects on metabolism, apoptosis, nucleotide sugar pools, fatty acid oxidation, and vesicle transport activity, though some of these changes depend on cell line and protein produced (Hossler et al., 2009; Sunley and Butler, 2010; Yee et al., 2008). The effects of sodium butyrate on global gene expression was first examined for both CHO and NS0 cells using two CHO cell and mouse DNA microarrays developed in-house (Gatti et al., 2007). The CHO and NS0 cells were studied with both the cross-species DNA microarray hybridization and the species-specific DNA microarrays. These studies were conducted to corroborate physiology and gene expression observations (Gatti et al., 2007; Korke et al., 2004; Wlaschin et al., 2005). Interestingly, the gene expression analysis did not identify any glycosylation-related genes with significant expression changes due to sodium butyrate in the CHO or NS0 cell lines (Gatti et al., 2007). Later, Yee et al. 2008 compared the genomic and proteomic responses of CHO and hybridoma cells to a sodium butyrate treatment. Although they reported several glycosylation-related genes were affected by sodium butyrate for both the CHO and hybridoma cells, none of these genes were identified. Additionally, no glycosylation-related proteins were reported to have been effected by the sodium butyrate treatment (Yee et al., 2008). More recently, 52 glycosylation-related gene expression levels for recombinant CHO cells were quantified using real-time qRT-PCR under sodium butyrate (Lee et al., 2014). Several biosynthetic genes were identified to have slightly higher levels under the sodium butyrate treatment; however, the poorer observed

glycoprotein quality was attributed to increased sialidase (Neu1) gene expression levels and lower B4gal2 and St3gal3 gene expression levels, both critical sialic acid biosynthesis genes (Lee et al., 2014). These three studies demonstrate the power of coupling DNA microarray analysis and real-time qRT-PCR to focus on a critical question, such as glycosylation effectors, where DNA microarrays can be used to screen for classes of gene expression changes, and then the higher sensitivity of real-time qRT-PCR can be used to quantify smaller detectable changes to better understand the underlying mechanism. In this study, the real-time qRT-PCR analysis was used to determine if small changes in key glycosylation genes was responsible for the decreased glycoprotein quality commonly observed under elevated ammonium. Instead, the real-time qRT-PCR analysis confirmed the non-significant gene expression changes observed by the DNA microarrays analysis were accurate.

The detrimental effects of ammonium on cell growth, protein productivity, and protein glycosylation has resulted in studies to reduce the major source of ammonium build-up - glutamine in the medium. An early study controlled glutamine in the medium using a fed-batch technique; however, reported decreased protein glycosylation quality (Wong et al., 2005). Unfortunately, this ground-breaking approach selected target glucose and glutamine levels that were most likely too low, in retrospect (Young, 2013). Other research groups have replaced the glutamine in the medium with alternative sources concurrent with reducing the levels in CHO cell cultures with improved results or adapted cells to grow in glutamine-free media (Kim et al., 2013; Taschwer et al., 2012). And more recently, three different TCA cycle intermediates, α -ketoglutarate, citric acid, and succinic acid, were examined for recombinant CHO cells to reduced ammonia accumulation in the culture medium (Ha and Lee, 2014). The α -ketoglutarate replacement decreased the growth rate, but also increased sialylation and decrease ammonium accumulation. The observed increased sialylation was correlated with increased ST3gal and decreased Neu enzyme activity (Ha and Lee, 2014). Interestingly, the relative abundance of the Neu1, Neu2, and Neu3 genes shown in Kim et al., 2013 are significantly different than the relevant abundance of the Neu1, Neu2, and Neu3 proteins shown in Ha and Lee, 2014 for the control cultures, albeit the studies are from the same laboratory with different CHO cell host cell lines. These results demonstrate that gene expression and protein levels are not always proportional. Taken together, the gene expression results for NS0 cells and the glutamine substitution outcomes for CHO cells suggest that enzyme or substrate-level control is likely a very significant factor with respect to ammonium effects on protein glycosylation in NS0 cells.

5. Conclusions

Sialyltransferase gene expression was anticipated to be significantly different between the control and elevated ammonium cultures for the NS0 cells (Chen and Harcum, 2006), since previous NS0 cell culture experiments observed elevated intracellular nucleotide sugar pools in NS0 cells compared to CHO cells (Baker et al., 2001). Also, it was anticipated that other glycosylation genes would be identified as ammonium-sensitive by the NS0 cell experiments, since ammonium adversely affects protein glycosylation. However, in this study, it was demonstrated that glycosylation genes NS0 cells are insensitive to 5 mM ammonium, 5 mM salt, and 5 mM ammonium with 10 mM proline treatments at the gene

expression levels as detected by DNA microarrays and the five glycosylation genes: Slc35a1, B4galt1, St3gal3, St3gal4, and St3gal6, are insensitive to 5 mM ammonium at the gene expression as measured by real-time qRT-PCR. Thus, the further studies should focus on translational and substrate-level control as the mechanism by which elevated ammonium impacts protein glycosylation changes in NS0 cells.

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Abbreviations

Actb	Actin, beta
B4galt1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
CHO	cells Chinese hamster ovary cells
Dpm1	dolichol-phosphate (beta-D) mannosyltransferase 1
Fut4	fucosyltransferase 4
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Gnlda1	glucosamine-6-phosphate deaminase 1
Gnpnat1	glucosamine-phosphate N-acetyltransferase 1
Gpi1	glucose phosphate isomerase 1
Mgat1	mannoside acetylglucosaminyltransferase 1
Neu2	neuraminidase 2
real-time qRT-PCR	real-time quantitative reverse transcriptase PCR
Slc35a1	solute carrier family 35 (CMP-sialic acid transporter), member 1
Slc35a2	solute carrier family 35 (UDP-galactose transporter), member A2
St3gal3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3
St3gal4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4
St3gal6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6
Ugp2	UDP-glucose pyrophosphorylase 2

Highlights

- All glycosylation genes insensitive to ammonium as analysed by DNA microarray.
- Five glycosylation genes insensitive to ammonium as analysed by real-time qRT-PCR.
- Translational or substrate-level control must be responsible for the decreased glycosylation.

Figure 1. Growth profiles for NS0 cells cultured under control, elevated ammonium, elevated salt, and elevated ammonium with proline conditions in a first-generation HyQ ADCF-Mab media with LS-250 cholesterol

Control (•), elevated ammonium (▪), elevated salt (▲) and elevated ammonium with proline (◆) cultures. RNA samples were taken at 90-h for gene expression analysis by DNA microarrays as shown by the vertical line. Error bars represent 95% confidence intervals.

Figure 2. Gene expression levels for 12 genes key glycosylation and two housekeeping genes in NS0 cells cultured under control, elevated ammonium, elevated salt, and elevated ammonium with proline conditions in a first-generation HyQ ADCF-Mab media with LS-250 cholesterol. Data points between the culture conditions are connected to assist with visualization only. The Fut4 and Neu2 genes were “Absent”, i.e., not expressed, under these conditions. Abbreviations are listed in Table 2. Standard error bars are shown.

Figure 3. Growth profiles for NS0 cells cultured under control and elevated ammonium conditions in a second-generation ADCF-MAb Media with LS-1000 cholesterol
Control (•) and ammonium (▪) cultures. RNA samples for real-time qRT-PCR were taken at 63-h, 66-h, and 72-h for gene expression analysis as shown by the vertical lines. Error bars represent 95% confidence intervals.

Figure 4. Gene expression levels for Slc35a1, B4galT1, St3gal3, St3gal4, and St3gal6 for NS0 cells cultured under control and elevated ammonium conditions in a second-generation ADCF-MAB Media with LS-1000 cholesterol

The normalized gene expression levels for the glycosylation genes A) Slc35a1, B) B4galT1, C) St3gal3, D) St3gal4, and E) St3gal6 assessed by real-time qRT-PCR. Gene expression levels were analysed at 63-h, 66-h, and 72-h, and were normalized to the 63-h time point of the Gapdh gene expression of the control. Control –dark grey bars; Ammonium –light grey bars. Error bars represent 95% confidence intervals.

Table 1
***M. musculus* primer sequences used for qRT-PCR analysis of elevated ammonium for NS0 cultures**

Gene	Forward Primer Sequence	Reverse Primer Sequence
Actb	5'- GGT GTG ATG GTG GGA ATG G -3'	5'- TCT CCA TGT CGT CCC AGT TG -3'
B4galt1	5' TGT CTC TCC TCA CAA GGT GG 3	5' TGG TGT CTC CAG CCT GAT TG 3'
Gapdh	5'- GCA AAT TCA ACG GCA CAG TC -3'	5'- ATA CTC AGC ACC GGC CTC AC -3'
Slc35a1	5'- TCT ACA CGT CAG TGG TGG TG -3'	5'- AGC TCC CAG TGC AAA TGA AAG -3'
St3gal3	5' CTG TGA TGA AGT GGC AGT CG 3'	5' CTC TCG CTG GAT GTT GTG TG 3'
St3gal4	5'- GAG TGA TAA GAA GCG GGT GC -3'	5'- GCT TGT CTG CTG CAA TCT CC -3'
St3gal6	5' CCC AAA CAC CCT ACA ACA GG 3'	5' ACA TGG TGG CAT TCC CGT AG 3'

Table 2

Selected *M. musculus* and *Cricetus griseus* glycosylation and housekeeping genes listed with abbreviation, official full name, and Genbank accession number.

This Study: <i>Mus musculus</i>			<i>Cricetus griseus</i>	
Abbreviation	Official Full Name	GenBank Accession Number on GLYCOv3	Gene Name (from Chen and Harcum, 2006)	GenBank Accession Number
Actb	Actin, beta	M12481	Actin	U20114.1
B4gal1	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	NM_022305.2	β (1,4)-Galactosyltransferase (β (1,4)-GT)	AF318896.1
Dpm1	dolichol-phosphate (beta-D) mannosyltransferase 1	NM_010072.2	Dolicholphosphate mannosyl synthase (DPMS)	AF121895.1
Fut4 ^a	fucosyltransferase 4	NM_010242	α (1,3)-Fucosyltransferase (α (1,3)-FT)	AF090449
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	M32599	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	X52123.1
Gnpd1	glucosamine-6-phosphate deaminase 1	NM_011937	Glucosamine-6-phosphate isomerase <i>Mesocricetus auratus</i> (G6PI)	X94699.1
Gnpnat1	glucosamine-phosphate N-acetyltransferase 1	NM_019425	UDP-GlcNAc:UDP N-acetyl-D-glucosamine-1-phosphate transferase (UDP-NAG)	U09453.1
Gpi1	glucose phosphate isomerase 1	NM_008155	Glucose phosphate isomerase (GPI)	Z37977.1
Mgat1	mannoside acetylglucosaminyltransferase 1	NM_010794.2	N-acetylglucosaminyltransferase (NAGAT)	U65791
Neu2 ^a	neuraminidase 2	NM_015750	Sialidase	U06143.1
Slc35a1	solute carrier family 35 (CMP-sialic acid transporter), member 1	NM_011895	CMP-sialic acid transporter (CMP-SiaT)	Y12074.1
Slc35a2	solute carrier family 35 (UDP-galactose transporter), member A2	AB027147	UDP-galactose transporter (UDP-GalT)	AF299335.1
St3gal3 ^b	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	NM_009176.2	NA	NA
St3gal4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	NM_009178.2	Gal(β -1,3/4)-GlcNAc- α (2,3)-sialyltransferase IV (α (2,3)-ST)	AY266675.1
St3gal6 ^{a,b}	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	NM_018784	NA	NA
Ugp2	UDP-glucose pyrophosphorylase 2	AF424698	UDP-glucose pyrophosphorylase (UDP-GPP)	AF004368.1

^a Fut4, Neu2, and St3gal6 were "Absent", i.e., not expressed as assessed by DNA microarray under the condition studied.

^b St3gal3 and St3gal6 are only shown quantitatively by real time qRT-PCR

NA – Not Applicable: Gene was not examined in Chen and Harcum, 2006.

Table 3

The t-tests between the control and ammonium-treated cultures for the five genes determined that the gene expression levels were not different ($p > 0.05$). The p-values are listed for each pairs for the three time points examined, which correspond to the error bars shown in Figure 4.

Gene	Significance level (p-values)		
	63-h	66-h	72-h
B4galt1	0.89	0.33	0.26
Slc35a1	0.89	0.16	0.51
St3gal3	0.33	0.60	0.37
St3gal4	0.62	0.85	0.96
St3gal6	0.97	0.85	0.87