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Oxidative stress-alleviating strategies to improve recombinant protein production in CHO cells

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Abstract

Large scale biopharmaceutical production of biologics relies on the overexpression of foreign proteins by cells cultivated in stirred tank bioreactors. It is well recognized

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and documented fact that protein overexpression may impact host cell metabolism and that factors associated with large scale culture, such as the hydrodynamic forces and inhomogeneities within the bioreactors, may promote cellular stress. The metabolic adaptations required to support high level expression of recombinant proteins include increased energy production and improved secretory capacity, which, in turn, can lead to a rise of reactive oxygen species (ROS) generated through the respiration metabolism and the interaction with media components. Oxidative stress is defined as the imbalance between the production of free radicals and the antioxidant response within the cells. Accumulation of intracellular ROS can interfere with the cellular activities and exert cytotoxic effects via the alternation of cellular components. In this context, strategies aiming to alleviate oxidative stress generated during the culture have been developed to improve cell growth, productivity, and reduce product microheterogeneity. In this review, we present a summary of the different approaches used to decrease the oxidative stress in CHO cells, and highlight media development and cell engineering as the main pathways through which ROS levels may be kept under control.

Keywords

Chinese hamster ovary cells; CHO; oxidative stress; cell engineering; antioxidant

1. Introduction

Over the last two decades, with 62 approved recombinant proteins between 2011 and 2016, protein-based pharmaceuticals (biologics or biopharmaceuticals) strategy has proven to be a key player in the development of treatments for complex diseases (Lagassé et al., 2017). Mammalian cells are the preferred host to produce recombinant therapeutics, such as monoclonal antibodies. Their intracellular machinery is capable

of producing post-translationally modified proteins (mainly glycosylated forms) displaying similar modifications to those observed in human cells. Several mammalian cell lines have been used for production of recombinant proteins, including murine myeloma (NS0, Sp2/0) cells, baby hamster kidney (BHK21) cells and human cell lines (HEK293 and HT-1080), however 70% of recombinant therapeutic proteins are produced in Chinese Hamster Ovary cells (CHO) (Dumont et al., 2016; Kim et al., 2012).

For safety and raw material control considerations, biopharmaceutical industries are now using serum free, chemically defined cell culture media. However, the impact of serum depletion with regards to growth performance and culture viability is considerable, and can, in severe case, promote apoptosis (Yao et al., 2017). Both the antioxidant properties of serum and the link between free radicals and cell death are well established, and it is therefore likely that oxidative stress resulting from the serum-depletion is one of the root causes behind the decrease in cell viability (Halliwell, 2003; Lewinska et al., 2007).

Oxidative stress is defined as the imbalance between the production of free radicals and the antioxidant response within the cells (Sies et al., 2017). Reactive oxygen species (ROS) are natural byproducts of aerobic metabolism, however, if their levels become too high, these compounds can impact cell health through their high reactivity towards biological components, including protein, lipids, RNA and DNA (Halliwell, 2006). Protein are particularly vulnerable to oxidative modifications, notably by $OH^{\bullet}$ which can react with all amino acid residues, or $ROO^{\bullet}$ which shows higher affinity for sulfur side-chain amino acids, such as L-cysteine and L-methionine, or aromatic amino acids such as L-tryptophan and L-tyrosine (Cai et al., 2013; Davies, 2016).

When cells are not able to counterbalance these damages, apoptosis is activated to prevent necrosis. Although this phenomenon is a naturally-occurring defense mechanism in organisms, it is not desirable in the context of bioprocesses. In addition to the negative impact on cell culture performance, production of free radicals during the culture can also be detrimental through the recombinant protein microheterogeneity (He et al., 2018; Xu et al., 2014). Recombinant protein microheterogeneity corresponds to all the recombinant protein variants that can be generated during the production process, namely charge variants, size, variants bearing different N-glycosylations, different levels of oxidation on specific amino acids such as tryptophan and methionine, etc… Overall, changes in proteins microheterogeneity may lead to changes in coloration of the recombinant protein of interest. These variants can originate from different post-translational modifications or non-enzymatic reactions (Beyer et al., 2018). Acidic variants of monoclonal antibody (mAb) have previously been shown to correlate with ROS levels, supporting the existence of a link between oxidative stress and recombinant protein microheterogeneity (Mallaney et al., 2014).

In the context of biopharmaceutical production, oxidative stress can be caused by a number of factors, including bioreactor oxygen inhomogeneity, rich cell culture media, high productivity, and waste accumulation. One important process parameter during bioproduction is oxygen level. Maintaining a constant and homogenous oxygen level in the bioreactor is challenging and can be influenced by many parameters, such as gas flow, stirring speed, gas transfer capacity of the fluid and antifoam addition. In large bioreactors, local dO_2 gradients can be formed (Xing et al., 2009). This heterogeneity in oxygenation can result in localized hypoxic condition within the culture, and lead to an increase in the sensitivity of CHO cells to oxidative

stress that is detrimental to cell growth, culture productivity and/or product quality (Lewis et al., 2016; Lin et al., 1993; Lin et al., 1992). On the other hand, hyperoxia can also be a problem in bioprocesses. Restelli and coworkers showed that an excessive dO_2 concentration in the culture impacted the glycosylation profile of recombinant erythropoietin produced in CHO cells (Restelli et al., 2006). The authors hypothesized that high dO2 concentrations promoted ROS production and ROS-dependent protein alteration, and that the energetic cost of detoxification these toxic compounds could lead to a decrease in others critical metabolic processes.

Another potential source of oxidative stress is the elevated energetic demands required by the cell to produce recombinant proteins. The main source of ATP in aerobic organisms is oxidative phosphorylation (OXPHOS), which takes place in the mitochondria. Therefore an increase of metabolic fluxes towards OXPHOS, the TCA cycle, and related pathways is often observed in cells producing recombinant protein (Templeton et al., 2013). However, complex I and III activities within the electron transport chain are the main source of ROS within the cells (Turrens, 2003). An increase in OXPHOS activity is, therefore, likely to generate ROS, among other pathway byproducts.

In addition to the increase of energy requirements, the combination of the normal production of host protein and recombinant protein production can lead to an accumulation of protein in the endoplasmic reticulum (ER) (Mathias et al., 2018). ER is the central location for protein folding and the first step of the secretory pathway. Folding of proteins, in particular disulfide bond formation, requires an oxidative environment and, in certain cases, can induce ROS generation. For example, oxidation of ER oxidoreductin 1 (ERO1) is required in order to exchange its disulfide

bond with its enzymatic partner, the protein disulfide isomerase (PDI). This exchange releases H_2O_2 as a by-product in the ER (Tu et al., 2004). In addition, when the ER is overloaded by unfolded proteins, a protection system called the unfolded protein response (UPR) is activated by a number of transcription factors and can also lead to apoptosis and ROS production (Hetz, 2012). Activation of UPR transcription factors, such as the pro-apoptotic transcription factor C/EBP-homologous protein (CHOP) and the DNA damage-inducible 34 protein (GADD34), coupled to altered calcium homeostasis is accompanied by an increase in ROS. Furthermore, CHOP-dependent activation of GADD34 and ERO1α is reported to promote accumulation of ROS in the ER due to an increase of oxidation events (Marciniak et al., 2004). Moreover, ROS activate inositol-1,4,5trisphosphate receptors (IP3Rs) which release calcium in the cytoplasm. This calcium signaling induces mitochondrial oxidative stress and further ROS production (Cao et al., 2014). Therefore, it is essential that the cells have an efficient secretory machinery to avoid the accumulation of protein in ER and ultimately the overproduction of ROS. This relationship between accumulation of unfolded recombinant protein in the ER and oxidative stress has been evidenced in the biopharmaceutical production of blood coagulation factor VIII (Malhotra et al., 2008).

During cultivation of mammalian cells, and especially in fed batch mode, a number of media components and cell metabolism byproducts are accumulating in the extracellular environment. Some cell culture media components such as vitamins, amino acids, glucose, and even antioxidant compounds such as polyphenolic compounds can generate ROS in the presence of oxygen (Halliwell, 2014; Kelts et al., 2015; Schnellbaecher et al., 2019). Moreover, specific cell metabolism byproducts reported to be growth inhibitors can also lead to oxidative stress (Pereira et al., 2018). For example, phenyllactate, which was identified as a growth inhibitor in CHO cells,

promotes lipid peroxidation in rat cortex cells tissue (Fernandes et al., 2010; Mulukutla et al., 2017). Likewise, methylglyoxal, a byproduct of glucose and amino acid metabolism, has been shown to induce oxidative stress in rat cells (Fukunaga et al., 2005). In addition, this compound has also been shown to lead to increased levels of acidic species of mAbs produced in CHO cells (Chumsae et al., 2013).

Due to this large number of potential sources of oxidative stress during bioprocesses and given the deleterious impact of ROS on process performances, different strategies have been developed over the past few decades to counteract this issue. In particular, the approach consisting in altering cell culture media, mainly via supplementation of the medium with antioxidant molecules, is widely described in literature. The decrease of oxidative stress in response to antioxidant supplementation, occurring through direct ROS scavenging or antioxidant cellular defense activation, has been shown to reduce cell death and the product microheterogeneity, and increase productivity of the process. While cell culture media modification is the currently preferred option in the CHO cell scientific community, the emergence of CHO omics has also opened up new avenues to alleviate oxidative stress by cell engineering.

In this review, we first give an overview of the oxidative stress in mammalian cells by describing the potential sources of ROS during the cultivation as well as the cellular antioxidant defense. Thereafter, we provide a description of the two main strategies found in literature to alleviate oxidative stress: supplementation of media formulations with antioxidant molecules, and cell line engineering.

2. Oxidative stress in mammalian cells

2.1. Sources of reactive species in mammalian cells

2.1.1. Intracellular generation of ROS

ROS refer to a group of molecules derived from oxygen, which, due their oxygen content or the presence of unpaired electrons, display high reactivity towards a large array of biomolecules. Among ROS, the free radical forms, such as the superoxide anion radical, the hydrogen hydroxyl radical, or the peroxyl radical, are more reactive than the corresponding reduced forms, hydrogen peroxide or organic hydroperoxide. In addition to ROS, other radical species such as reactive nitrogen species (RNS), reactive sulfur species (RSS), reactive carbonyl species (RCS), and reactive selenium species can be generated (Sies et al., 2017). The production of ROS and RNS can be enzymatic or non-enzymatic (Dhawan, 2014). Moreover, in the context of bioprocesses, reactive species can be generated intracellularly due to the cell metabolism, or extracellularly in the cell culture media due to deterioration of media components.

The intracellular ROS mainly originate from the respiratory chain in the mitochondria (Turrens et al., 1985). Superoxide anions produced in the mitochondrial matrix will be dismuted into H_2O_2 by the manganese-dependent superoxide dismutase (MnSOD), while those generated in the intramembrane space are converted by the copper-zinc superoxide dismutase (CuZnSOD) (Fukai et al., 2011; Turrens, 2003). Superoxide radicals can also form peroxynitrite after reaction with nitric oxide originating from arginine degradation by nitric oxide synthases in the mitochondria and in the peroxisomes (Ghafourifar et al., 2005; Schrader et al., 2006). In addition, the superoxide anion, can react with H_2O_2 through the Haber-Weiss reaction, to produce the hydroxyl radical $OH^{\bullet}$ which is considered the most deleterious ROS (Nordberg et

al., 2001). Indeed, due to its high reactivity, $OH^{\bullet}$ non-preferentially oxidizes amino acids, DNA, or lipids. The hydroxyl radical can also be generated in presence of transition metal (Fe^{2+} or Cu^{2+}) by the cleavage of H_2O_2 through the Fenton reaction. In contrast to the superoxide anion, H_2O_2 have less reactivity and can diffuse through membranes (D'Autreaux et al., 2007). Due to its diffusion properties, H_2O_2 has an important role in signaling. For example, H_2O_2 modulates the activity of transcription factors such as activator protein-1 (AP-1) involved in cell proliferation, apoptosis, survival, and cell differentiation. It also regulates the activity of the sterol regulatory element binding protein 1 (SREBP1) involved in cholesterol, lipids and fatty acids synthesis (Marinho et al., 2014).

In addition to the mitochondria, ROS can also be produced in other locations within the cell. Beta-oxidation of fatty acids takes place in the mitochondria and in peroxisomes, which contain many types of oxidases such as Acyl-CoA oxidase and xanthine oxidase (Eaton et al., 1996; Schrader et al., 2006). In consequence, ROS generation also occurs in peroxisomes as a product of these enzymatic reactions. Another location for ROS generation is the ER. The lumen of the ER is an oxidative environment which promotes the biochemical reactions required for protein folding (Tu et al., 2004). Formation of disulfide bonds through the ERO1/PDI pathway, releases H_2O_2 in the ER lumen. Moreover, H_2O_2 is generated by NADPH oxidase 4 (NOX4) and by the microsomal monooxygenase system (MNO) in the ER membrane (Brandes et al., 2014; Zangar et al., 2004; Zeeshan et al., 2016). ROS production in the ER can also activate the release of Ca^{2+} in the cytosol. In turn, this signaling cascade leads to a release of cytochrome c from the mitochondria and initiates apoptosis (Cao et al., 2014). This cascade of events shows the relationship between oxidative stress, ER stress, and apoptosis (Malhotra et al., 2007). Moreover, the

recombinant protein produced can itself be a source of reactive species. A large protein with a large number of cysteine will have a higher propensity to accumulate in ER due to the large number of disulphide bonds to be formed. The enzymes responsible for disulfide bond formation, ERO1 and PDI, will therefore generate more H_2O_2. This phenomenon was illustrated in yeast through the comparison of the expression of alpha amylase, a large protein with numerous cysteines but only four disulfide bonds, and human insulin precursor, a small protein with three disulfide bonds (Tyo et al., 2012). Because of the presence of many cysteines in the sequence of alpha amylase, the probability of incorrect disulfide bond formation is increased during protein folding. Along these line, Tyo and colleagues demonstrated that more oxidative and osmotic stress transcription factors were activated upon alpha amylase expression than human insulin precursor expression. Incorrect disulfide bonds can be reduced by PDI thanks to its isomerase activity (Wilkinson et al., 2004). However if the folding rate is too slow, misfolded proteins will accumulate in the ER leading to the unfolded protein response which, in turn, can promote oxidative stress (Malhotra et al., 2007).

2.1.2. Cell culture media-derived ROS

In addition to intracellular sources, medium components can react with oxygen, light, and other components to generate ROS (Grzelak et al., 2000). For example, riboflavin is light sensitive and can generate ROS by photooxidation. Grzelak and coworkers showed in their study that the riboflavin-dependent ROS production is amplified in presence of tryptophan, tyrosine, pyridoxine, and folic acid in medium (Grzelak et al., 2001). Folic acid is also light sensitive in presence of oxygen and can be degraded to 6-formylpterin and pterin-6-carboxylic acid, which, in turn, generate ROS (Gazzali et al., 2016; Juzeniene et al., 2016). Another vitamin, which is easily oxidized in cell

culture media and generates H_2O_2, is ascorbic acid (Long et al., 2009). More generally, thiol compounds present un the media can also be a source of H_2O_2 following autoxidation reactions or interaction with other media components, such as metal ions (Grzelak et al., 2001; Hua Long et al., 2001). For example, reduced glutathione (GSH) can form complexes with copper in a reducing environment which leads to superoxide generation (Speisky et al., 2009). Glucose also reacts with oxygen and metal ions and produces ROS or reactive degradation products like methylglyoxal (Chumsae et al., 2013).

ROS and byproducts generated by media components oxidation can lead to degradation of the overexpressed protein and an increase in product microheterogeneity. For example, a recent study have demonstrated that ROS were generated in the medium at high iron concentration (Xu et al., 2018). By lowering iron concentration, they managed to decrease both free radicals generation and mAb microheterogeneity, especially tryptophan oxidation responsible of mAb coloration levels (Xu et al., 2014). However, as lowering iron led to a decrease of product titer, an adaptation of the process through cell line adaptation and further basal medium modifications were required to restore the original titer.

2.1.3. Antioxidant response in mammalian cells

In order to maintain reactive species at non-deleterious levels, mammalian cells have developed an array of antioxidant systems, including different detoxification enzymes and signaling pathways. One of the major players of this defense is GSH (Figure 1). GSH is a tripeptide (γ-L-glutamyl-L-cysteinyl glycine) which can be oxidized to form glutathione disulfide (GSSG). GSH acts as a ROS scavenger and as the substrate of

detoxification enzymes such as glutathione peroxidases (GPXs) and glutathione-S-transferases (GSTs) (Espinosa-Diez et al., 2015).

Eight GPXs have been identified so far in mammalian cells: GPX1-4 are selenoproteins, while GPX5-8 use cysteines to carry out their enzymatic activity (Brigelius-Flohé et al., 2013). However, some of them, such as GPX5, are tissue-specific and not present in CHO cells. These enzymes use GSH as a reducing substrate, but it has been shown that some of them can use other reducing substrates like thioredoxin or PDI. Their major substrate is H_2O_2, but they can also detoxify peroxynitrite and oxidized chain of lipids (LOOH). Other enzymes such as catalase and peroxiredoxins also detoxify H_2O_2 (Nordberg et al., 2001).

Glutathione-S-transferases conjugate GSH with reactive electrophilic compounds to facilitate their removal from the cell through specific transporters (Salinas et al., 1999). GSSG is returned to its monomeric active form (GSH) through the activity of glutathione reductase and cofactor NADPH.

Two other proteins involved in antioxidant defense are glutaredoxins (Grx) and thioredoxins, which catalyze disulfide bond reductions. Thanks to this function, these proteins are capable of reversing post-translational modifications caused by ROS such as sulfenylation, glutathionylation, and cysteinylation (Hanschmann et al., 2013). Thioredoxins also transfer electrons to peroxiredoxins (Prxs), which are H_2O_2 scavengers, and to methionine sulfoxide reductases which reverse methionine oxidation (Lu et al., 2014). Moreover, thioredoxins are involved in the modulation of some transcription factors activity such as Nuclear Factor-κB (NF-κB) and AP-1 (Schenk et al., 1994). Oxidized thioredoxins are reduced by thioredoxin reductases

using NADPH as an electron donor while oxidized glutaredoxins are reduced by glutathione.

Another important aspect of cellular antioxidant response is the number of signaling pathways involved. In presence of ROS, nuclear factor erythroid 2 (NRF2) dissociates from Kelch-like ECH-associated protein 1 (Keap 1). Free NRF2 will then activate the antioxidant response element (ARE) localized in genes coding for detoxifying enzymes such as glutathione-S-transferase and heme oxygenase-1, which degrades free heme (Gong et al., 2001), but also enzymes involved in the biosynthesis of GSH such as the catalytic and regulatory subunits of the glutamate-cysteine ligase (GCL) (Nguyen et al., 2003). Another example of a transcription factor involved in the antioxidant response is NF-κB. The activation of this transcription factors has been shown to correlate with the expression of antioxidant enzymes, such as superoxide dismutases (MnSOD and ZnCuSOD), GST-pi, and GPX-1, in different tissues (Morgan et al., 2011). However, it has been reported that NF-κB is also involved in the transcription of pro-oxidant enzymes, such as xanthine oxidase/dehydrogenase and inducible nitric oxide synthase. The sterol regulatory element binding proteins 2 (SREBP-2) involved in lipid homeostasis, also contributes to the antioxidant response through the regulation of paraoxonase-2 expression in CHO cells (Gu et al., 2014). This enzyme is a hydrolase with a wide range of substrates, that has been reported to exert antioxidant activity in several tissues, including brain, ovarian carcinoma, and intestinal epithelial cells (Devarajan et al., 2018; Giordano et al., 2011; Précourt et al., 2012).

In the context of biopharmaceutical production, several cellular adaptations to increased ROS generation and oxidative stress have been reported. Templeton et al.

described a correlation between the production of mAb and increased fluxes into the TCA cycle during fed batch cell culture. Their metabolic flux analysis also showed that the ratio of NADPH/$NADP^+$, which is high at the beginning of the process, decreased considerably during the late exponential and stationary phases (Templeton et al., 2013). Moreover, they observed the same effect on the intracellular GSH to GSSG ratio. As NADPH is required to recycle GSSG back to GSH, they suggested that the increased TCA cycle activity, which positively impacted mAb production, was a spontaneous cellular reaction to oxidative stress. Similarly, a rise in activity of the oxidative pentose phosphate pathway could also be a way for the cell to produce NADPH and strengthen its antioxidant defenses. The NADPH/$NADP^+$ ratio is an indicator of the redox state of the cell and is often used as an oxidative stress marker (Blacker et al., 2016). However, there are cellular pathways that do not involve glutathione regeneration and use NADPH as a cofactor, one example is lipid biosynthesis (Lewis et al., 2014). Although oxidative stress will tend to decrease the intracellular NADPH/$NADP^+$ ratio, it can also be impacted by other metabolic adaptations.

3. Medium development approaches to limit oxidative stress

Despite the array of internal antioxidant defense mechanisms, cultivated cells may benefit from extracellular antioxidant activity to reduce production-derived oxidative stress. Antioxidant components are extensively used in chemically defined serum-free media to counterbalance the loss of serum and hydrolysate antioxidant properties (Saito et al., 2003). This section summarizes classes of compounds bearing antioxidant properties that were assessed for culture of CHO cells (see Table 1).

3.1. Thiol compounds

The majority of cellular antioxidant defense systems contain a thiol moiety. The mode of action of these compounds is broad : (i) They can act as a substrate for detoxifying enzymes (e.g. GSH), (ii) they can directly scavenge ROS (e.g. N-acetylcysteine (NAC)), or (iii) they can be involved in metal chelation (e.g. lipoic acid) (Biewenga et al., 1997; Deneke, 2000; Sun, 2010). In addition, they can participate in the thiol/disulfide intra or extracellular redox balance and/or in cell signaling (Deneke, 2000; Go et al., 2013). Studies carried out by Yun and colleagues indicate that the addition of GSH in serum-free media reduces cell death and increase tissue plasminogen activator concentration (Yun et al., 2001). Besides GSH, a large number of thiol compounds with antioxidant properties can be supplemented to cell culture media to support cells defenses against oxidative stress. For instance, NAC is another a thiol antioxidant that is commonly used in CHO cell culture to prevent apoptosis induced by oxidative stress (Lord-Fontaine et al., 1999; Wu et al., 2008; Xue et al., 2015). Some studies have demonstrated that NAC can improve production of recombinant human interferon-β-1a and erythropoietin, especially in combination with a sodium butyrate treatment (Chang et al., 1999; Oh et al., 2005). An alternative to NAC is N-acetylcysteine amine, which has been shown to be less cytotoxic than NAC (Wu et al., 2008). Both compounds act as ROS scavengers and are precursors of cysteine and GSH that can contribute to increasing intracellular GSH content. Through direct interaction with thiol groups, NAC is capable of modulating the activity of signaling molecules, such as transcription factor NF-κB and c-Jun N-terminal kinase (JNK). However, this activity has only be demonstrated in specific cell type and further investigation will be required to gain a better understanding of the range of activities and antioxidant potential of NAC (Zafarullah et al., 2003).

The use of S-sulfocysteine as a replacement for cysteine in cell culture media has recently been shown to improve recombinant protein production by increasing the GSH pool in CHO cells (Hecklau et al., 2016). The use of S-sulfocysteine in feed solutions was hypothesized to reduce the production of hydrogen sulfide by protecting thiol groups against oxidation. In an additional study, the same lab reported that this compound could also lower antibody low molecular weight species and trisulfide bond levels by decreasing free hydrogen sulfide in the medium (Seibel et al., 2017).

Similarly, supplementation of the culture with thiazolidine in feed solutions can help stabilize redox sensitive vitamins and amino acids, and decrease their ROS content in feed. These molecules are obtained by condensation of cysteine with pyruvate or alpha ketoacids. In 1985, the antioxidant properties of pyruvate and alpha ketoacids , such as alpha-ketoglutarate, were highlighted in CHO cells exposed to H_2O_2 (Andrae et al., 1985). Pyruvate has also been shown to scavenge H_2O_2 present in cell culture media, thereby ensuring its stability (Long et al., 2009; McCoy et al., 2015). Addition of thiazolidine molecules during cell culture led to a decrease of cell death at the end of the production process and an increase of a recombinant mAb titer (Kuschelewski et al., 2017). Interestingly, the decrease of oxidative stress is observed upon thiazolidine supplementation appears to occur through an increase of intracellular GSH levels and expression of MnSOD and CuZnSOD.

Sulfur-containing amino acid, taurine, has also been reported to reduce cell death by increasing the GSH content, reducing lipid peroxidation, and reducing catalase and erythrocyte G6PD activity (Gurer et al., 2001). However, it has a low ROS scavenging activity compared to its precursors hypotaurine, s-carboxymethylcysteine, cysteamine, and cysteinesulphinic acid (Aruoma et al., 1988).

Finally, lipoic acid, a fatty acid derivative with antioxidant properties, is another thiol compound tested in the context of CHO cell cultivation. It was used in early chemically defined medium such as Ham's F12 developed for CHO cells (Hamilton et al., 1977). Lipoic acid is known to reduce lipid peroxidation, scavenge ROS, and chelate iron and copper, thereby contributing to a reduction of cell death (Gurer et al., 1999; Maharjan et al., 2016). However due to its insolubility in aqueous solutions, other thiol-containing molecules are generally preferred for medium supplementation.

3.2. Vitamins

Although the main function of vitamins is to act as enzyme cofactors, antioxidant properties have also been highlighted for some of these molecules. Alpha-tocopherol (vitamin E), is well known for its ROS scavenging properties and its ability to counteract lipids peroxidation. However, despite some attempts, this compound is rarely included in cell culture media composition due to its poor solubility in water (Halliwell, 2014). Ascorbic acid (vitamin C), a cofactor of enzymes involved in acetyl CoA metabolism, displays high reactivity towards oxygen, which both confers it with antioxidant potential, but can also be detrimental to the culture if it is not stabilized by other molecules, such as magnesium, selenium, or GSH (Dolińska et al., 2012; Touitou et al., 1996). Vitamin C protects cells from lipid peroxidation, through the regeneration of oxidized vitamin E. However, vitamin C supplementation during the culture does failed to positively impact CHO cell growth as a result of its high instability in culture media (Kurano et al., 1990). The development of more stable derivatives of these molecules was considered to address these solubility and stability issues (Hata et al., 1989). For example, the use of stabilized derivatives like L-ascorbic 2-phosphate can help to decrease cell death and improve recombinant protein

titer (Yun et al., 2001). However, the use of vitamin derivatives comes with a risk of altered antioxidant potential that has to be carefully considered.

3.3. Trace elements and chelators

Trace elements are essential for cell function and survival, as they act as cofactors for key enzymes and play a major roles in cell signaling and metabolism (Arigony et al., 2013; Hamilton et al., 1977). Metal ions may play a pro-oxidant or antioxidant role depending on their concentration. For instance, iron and copper are key players in ROS generation as they are involved in Fenton and Fenton-like reactions, respectively (Fenton, 1894; Pham et al., 2013). Another element, selenium, can, at relatively low concentrations, activate cellular antioxidant defense as it is a cofactor of detoxifying enzymes (Brigelius-Flohé et al., 2013; Fukai et al., 2011). Selenium has been reported to activate antioxidant defense as it is a cofactor of some GSH peroxidases and thioredoxin reductase (Powis et al., 1997; Weiss et al., 1997). In addition, it inhibits H_2O_2-induced TRPM2 channels impacting the Ca^{2+} influx (Naziroglu et al., 2013). However, it has to be used carefully as, at high concentrations, selenium can display pro-oxidant properties and generate ROS (Lee et al., 2012). Concentrations of selenium below 1 µM have been shown to be safe for CHO-K1 cells and are often included in cell culture medium (Zhang et al., 2006; Zwolak, 2015). However, toxic concentrations of selenium have to be determined for each cell line. Moreover, medium composition has to be considered when supplementing cultures with selenium as this compound can interact with other trace elements. Finally, selenocystine supplementation has also been demonstrated to decrease cell death of CHO cells exposed to lead-induced oxidative stress (Aykin-Burns et al., 2006).

To avoid the negative effect of trace element addition and facilitate their uptake by the cells, chelator molecules are usually used. Indeed, the chelation of reactive metal ions, like Fe^{2+} or Cu^{2+}, can help decrease oxidative stress. Addition of components like transferrin, polyamines, and ferric citrate maintains iron in an inert state and promotes its transport into the cells, thereby improving cell viability and recombinant protein production (Bai et al., 2011; Gaboriau et al., 2004; Lovaas, 1997; Wang et al., 2011). Defroxamine and aurintricarboxylic acid in combination with GSH have also been shown to improve CHO cell viability during recombinant tissue plasminogen activator production (Yun et al., 2003).

3.4. (Poly)-Phenolic compounds

Phenolic compounds are well known for their antioxidant properties and their use in the treatment of diseases and aging has been the object of many studies (Mao et al., 2017). The effects of (poly)-phenol derivative addition in CHO cell cultures has been assessed in both academic and industrial research. Epigallocatechin gallate and rutin were used to decrease acidic variants of a mAb produced by CHO cells (Hossler et al., 2015). In addition, baicalein has been shown to decrease cell growth and increase recombinant mAb production (Ha et al., 2017). Baicalein can decrease ROS levels and inhibit the activity of transcription factors involved in the ER stress response by interacting with immunoglobulin protein (BiP) and CHOP. Furthermore, addition of butylated hydroxyanisole (BHA) has been reported to decrease apoptosis and reduce accumulation of blood coagulation factor FVIII in the ER after treatment of CHO cells with sodium butyrate (Malhotra et al., 2008).

The addition of antioxidant molecules to cell culture media with the aim of reducing oxidative stress is an easily implemented and relatively successful approach used in

the industrial sector. However, such supplementations are to be investigated on a case-by-case basis, considering the different modes of action of the potential candidates, the chemistry of culture media, and the selected cell clones or cell lines used in the culture. In particular, potential interactions between the antioxidant supplements and components of the medium is a critical aspect of the optimization of the supplementation process. As they can both positively or negatively impact the culture depending on cases, it is important for such interactions them to be fully characterized, a fact that is often complicated by the non-disclosure of media formulations used in commercial processes. Due to the variability factors associated with supplementation, the use of empirical statistical analysis and high throughput assays is recommended. In bioproduction, it is generally assumed that the cell lines have different historical backgrounds (origin, clone, selection procedure) and, as a direct consequence, display differences in metabolism and sensitivity to oxidative stress (Reinhart et al., 2018). A better understanding of these differences can be obtained through the use of omics techniques, and can help experimenters highlight reactions leading to oxidative stress and adapt antioxidant supplementation to specific cell lines.

4. Use of cell engineering to reduce oxidative stress

Thanks to recent technical advances in genetics, cell engineering can be used to upregulate or downregulate pathways of interest. Considerable effort has been put into developing strategies to reduce apoptosis, with several efforts resulting in increased viability and, indirectly, improved process productivity (Meents et al., 2002). Notably, the overexpression of bcl-2 or bcl-x_L has been successfully used to activate anti-apoptotic pathways in CHO cells (Tey et al., 2000; Zustiak et al., 2014). A similar approach can be considered to relieve oxidative stress by increasing cellular defense

or decreasing ROS-generating cellular activities. As redox reactions are the basis of cellular energy production and protein folding processes, any attempts to reduce these fundamental reactions may negatively impact the production process. For this reason, the strategy aiming to increase the antioxidant defenses is generally seen as preferable for the purpose of recombinant protein production.

The interest in genetic manipulation of the GSH biosynthesis pathway is not new. Already in 1996, Tamura and coworkers overexpressed human glutathione reductase in CHO cells and were able to increase their resistance to oxidative stress (Tamura et al., 1996). Likewise, in 2002, the overexpression of the GCL catalytic subunit was demonstrated to increase the resistance to lead-induced oxidative stress in CHO cells (Fernandes et al., 2002). More recently, in the bioprocess field, a high intracellular concentration of GSH in high producers was observed in a metabolome comparison of CHO cells (Chong et al., 2012). These results were then confirmed by a proteome comparison of high and low producer cultivated in bioreactors using a batch process. This study highlighted an up-regulation of genes related to the GSH pathway (Orellana et al., 2015). Similarly, GSH-related amino acid transporters have been reported to have higher expression during stationary phase, when the specific productivity is higher (Kyriakopoulos et al., 2013). Moreover, it has been shown that GSH plays a role in the maintenance of the redox status within CHO cell ER by preventing formation of non-native disulfide bonds (Chakravarthi et al., 2004). In this context, several studies have examined the biosynthesis and turnover of GSH in CHO cell factories. First, the overexpression of the regulatory subunit of GCL in CHO-K1 cells was observed to promote clone productivity. Interestingly, this study also showed that the overexpression of GCL catalytic subunit does not impact recombinant protein production; suggesting that the intracellular GSH content is not the direct

cause of a higher productivity (Orellana et al., 2017). In another study, it was demonstrated that a partial inhibition of GCL by methionine sulfoximine (MSX) or buthionine sulfoximine (BSO) in GS–CHOK1SV cells leads to an increase in productivity (Feary et al., 2017). The authors hypothesized that partial inhibition of GSH synthesis helps to oxidize the ER environment. This modulation of the redox status of the ER increase the oxidized form of Ero1, thereby promoting activation of PDI enzymes and improving protein folding. Moreover, they suggest that the partial inhibition of GSH synthesis can be a selection method for high producer clones. Although there are still many unknowns concerning the involvement of the GSH synthesis pathway in recombinant protein production in CHO cells, and a certain degree of contradictory results, the aforementioned studies illustrate this pathway's potential.

The expression of other antioxidant enzymes or transcription factors involved in oxidative stress has also been a target of cell engineering approaches. Overexpression of human peroxiredoxin 5 and human MnSOD in CHO cells were reported to lead to a decrease in cell death caused by oxidative stress (Banmeyer et al., 2004; Warner et al., 1993). However, it is worth noting that these studies were performed in CHO cells with the aim of mimicking cancer cell metabolism and growth, not in the context of heterologous protein expression. With regards to transcription factors, ATF4, which is activated during the UPR response and in oxidative stress conditions, was shown to protect fibroblasts against oxidative stress (Harding et al., 2003). Moreover, overexpression of ATF4 or GADD34, an activator of ATF4, were both reported to lead to an increase in titer in several CHO cell lines (Haredy et al., 2013; Ohya et al., 2008; Omasa et al., 2008).

More recently, miRNA have been used to specifically target oxidative stress in CHO cells. In other cell lines, several miRNAs, including miRNA-145, miRNA-451 in erythroid cells and miR-1 and miR-133 in rat cells, have been shown to modulate oxidative (Jadhav et al., 2013). Although the use of miRNA in CHO cells has mostly been focused on the modulation of ER stress or, more generally, apoptosis, the potential of this approach for prevention oxidative stress is garnering more interest. Following depletion of miR-23 using a microRNA sponge, Kelly et al. observed an increase in production of the recombinant protein. In parallel, mitochondrial activity was boosted and production of antioxidative proteins Thioredoxin 1 and peroxiredoxin 6 was increased (Kelly et al., 2015).

So far, due to the long lead times and heavy workloads associated with cell engineering, a limited number of studies have been published on improving the CHO cells chassis by genetic engineering in order to alleviate oxidative stress. However, the engineering of cell line has unquestionably proven its value as supported by the studies reporting its potential to reduced apoptosis and ER stress (Borth et al., 2005; Mohan et al., 2009; Pieper et al., 2017; Prashad et al., 2015). Oxidative stress cell engineering is complex due to the number of pathways involved and their interconnections of these pathways with other cellular functions. Despite this complexity and the long process needed to generate an appropriate clone, the work published so far is promising with regards to the use of cell engineering to alleviate oxidative stress in bioprocesses.

5. Discussion

With the development of high biomass and high productivity CHO bioprocesses, scientists faced a new hurdle in the increase of cellular stresses resulting from boosted

metabolism, higher resource demands (e.g. for dissolved oxygen), and higher waste accumulation. In this context, oxidative stress, which occurs when there is an imbalance between oxidant molecules accumulation and antioxidant response, can become an issue due to its detrimental impact on cell viability, productivity, and the integrity of the recombinant protein being produced. Dissolved oxygen, can be controlled to limit oxidative stress. However, maintaining dissolved oxygen homogeneity in the bioreactor is complicated to study and requires particular attention during scale up as large scale bioreactors often present different geometries to those used during process development. High dO_2 usually has to be maintained in the bioreactor to avoid an oxygen dead zone even if simulation tools are now available to optimize process parameters depending on bioreactor size and dimensions (Dhanasekharan et al., 2005; Koynov et al., 2007).

In light of the challenges encountered in trying to control all aspects of large scale bioreactor processes, the approaches consisting in making the cells and the culture process more resistant to potential sources of oxidative stress have to be considered. Although, increased energy metabolism and a consequent activity of the folding/secretion machinery have been observed or modeled in high producer cell lines, it remains possible to limit ROS byproducts of these cellular processes (Borth et al., 2005; Ghorbaniaghdam et al., 2014; Mathias et al., 2018; Prashad et al., 2015; Templeton et al., 2013). Two of the main strategies considered to date consist in (i) the supplementation of the cell culture media with antioxidant molecules, and (ii) the engineering of metabolic pathways associated to oxidative stress. Supplementation of cell culture media with antioxidant compounds appears to be the easiest and fastest solution from an industrial point-of-view. It requires few changes to the process, and can be implemented at later stages of process development. However, due to the

possible interactions of antioxidant molecules with other cell culture media components and variability between CHO cells lines, considerable time and effort has to be put into component screening to identify the best match for the combination of the cell line, media, and process. For this reason, cell engineering appears as an interesting alternative to supplementation. However, this approach requires early implementation and evaluation during process development, and a change in the cell line will be more complicated to put in place once the first clinical phases have been carried out for a given recombinant protein production process. This strategy remains to be extensively studied in CHO cells as cell engineering research has thus far mainly focused on the modulation of secretory pathways, apoptosis, and the unfolded protein response pathways. However, compelling evidence from microbial strains, such as *Escherichia coli*, *Saccharomyces cerevisiae,* and *Yarrowia lipolytica*, highlight the potential of this approach for controlling oxidative stress and promoting cell growth in cultures (Basak et al., 2012; Davy et al., 2017; Ukibe et al., 2009; Xu et al., 2017).

Most of the studies cited in this review were performed on fed batch processes where the accumulation of waste and media components may be additional sources of oxidative stress. As described above, the supplementation of antioxidants can be used to scavenge ROS produced. However, removal or reduction of unstable components from the cell culture medium, such as ascorbic acid, is an alternative way to reduce reactive oxygen species (Halliwell, 2014). The use of cell engineering to reduce byproduct generation is another option. For example, it has recently been shown that phenyllactate production could be reduced by cell engineering of the phenylalanine-tyrosine catabolic pathway (Mulukutla et al., 2019). Finally, continuous processes are another potential solution to issues arising from the accumulation of waste in the medium. This type of cultivation had gained in popularity in the industry as an

efficient way both increase process yields and better control product quality, however, the ability of this approach to reduce oxidative stress remains to be investigated (Kunert et al., 2016).

To date, both supplementation and cell engineering strategies have focused on decreasing cell death and increasing of productivity, with little consideration going towards product quality. Recently, researchers have started to explore the impact of heightened antioxidant activity on product microheterogeneity. However, the possibility of reducing product microheterogeneity upon oxidative stress engineering in CHO cells remains undocumented.

While media development is likely to remain the dominant strategy for the time being, oxidative stress engineering has shown promising results and offers a credible alternative to support recombinant protein production in CHO cells. Moreover, new tools, such as genome scale models, might provide insight into limitations and potential improvements to these strategies, and may open the door to the use of a combined approach, using both antioxidant supplementation and cell engineering, to control ROS production and oxidative stress, and simultaneously increase productivity and maintain product quality.

Acknowledgements and conflict of interest

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Conflict of interest

V. Chevallier and L. Malphettes are employees of UCB Nordic A/S and UCB Pharma S.A., both of which carry out production activities in the area of interest.

Mikael R Andersen does not have any conflict of interest.

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Table

Table 1 : Antioxidant compounds used in CHO cells to reduce oxidative stress

Component	Way of action described in literature	Effect observed	References
Thiol compounds			
Glutathione	ROS scavenger Substrate for ROS scanvenger enzymes Maintain high mitochondria potential	Decrease cell death Increase titer	(Yun et al., 2001, 2003)
N-acetylcysteine and N-acetylcysteine amine	ROS scavenger Increase of intracellular glutathione pool	Decrease cell death Increase titer Impact on sialylation	(Chang et al., 1999; Ercal et al., 1996; Issels et al., 1988; Oh et al., 2005; Tanel et al., 2007; Wu et al.,

			2008)
S-sulfocysteine	Stabilized cysteine Up regulation of SODs transcription Increase of intracellular glutathione pool	Decrease cell death Increase titer Decrease recombinant protein fragments level Decrease recombinant protein trisulfides level	(Hecklau et al., 2016; Seibel et al., 2017)
Thiazolidine	Stabilization of the cell culture media Up regulation of SODs transcription Increase of intracellular glutathione pool	Decrease cell death Increase titer	(Kuschelewski et al., 2017)

Taurine and it precursors	Increase of intracellular glutathione pool Reduce lipid peroxidation Reduce catalase and erythrocyte G6PD activity Reduce mitochondrial superoxide generation	Decrease cell death	(Aruoma et al., 1988; Gurer et al., 2001; Jong et al., 2012)
Lipoic acid	ROS scavenger Iron and copper chelator	Decrease cell death	(Gurer et al., 1999; Maharjan et al., 2016)
Alpha ketoacids			
Pyruvate	ROS scanvenger	Decrease cell death Decrease recombinant	(Andrae et al., 1985; Kshirsagar et al., 2012; Long et al.,

		protein trisulfides level	2009)
Alpha-ketoglutarate	ROS scanvenger Iron chelator	Decrease cell death	(Andrae et al., 1985; Bayliak et al., 2016)
Vitamins			
Ascorbic acid and derivative	ROS scavenger	Decrease cell death Increase titer	(Yun et al., 2001)
Alpha-tocopherol	Lipid peroxyl radical scavenger	Decrease cell death	(Chepda et al., 1999; Murati et al., 2017)
Trace elements			
Selenium / selenocysteine	Cofactor of antioxidant enzymes Regulate expression of	Decrease cell death	(Aykin-Burns et al., 2006; Gasser et al., 1985; Hamilton et al., 1977; Weiss et

	glutathione peroxidase		al., 1997)
Chelators			
Defroxamine	Iron chelator	Decrease cell death	(Yun et al., 2003)
Aurintricarboxylic acid	Iron chelator	Decrease cell death	(Tabuchi et al., 2010)
Polyamines	Iron chelator	Decrease cell death Increase titer	(Gaboriau et al., 2004; Lovaas, 1997)
Phenolic compounds			
Butylated hydroxyanisole	ROS scavenger Iron chelator	Decrease cell death Increase titer	(Malhotra et al., 2008)
Baicalein	ROS scavenger	Increase titer	(Ha et al., 2017; Hamada et al., 1993)
Epicatechin gallate	ROS scavenger	Decrease recombinant protein	(Hossler et al.,

		charge variants level	2015)
Rutin	ROS scavenger	Decrease recombinant protein charge variants level	(Hossler et al., 2015)

Figure

Figure 1 : Major enzymatic reactions involving glutathione.
Conjugation reactions with an electrophilic donor are catalyzed by glutathione-S-transferases (GST).
Reduction of substrate such as H_2O_2, lipid peroxide (LOOH) and peroxynitrite ($ONOO^-$) are catalyzed by glutathione peroxidases (GPx). Disulfide bonds reduction can be catalyzed by glutaredoxins (Grx). Reduction of GSSG to GSH is catalyzed by the glutathione reductase (GR) and required NADPH.