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Effect of *Vitreoscilla* hemoglobin on recombinant protein expression and energy and redox state of CHO cells

Efecto de la hemoglobina de *Vitreoscilla* en la expresión de proteína recombinante, la energía y el estado redox de las células CHO

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Abstract

Vitreoscilla hemoglobin (VHb) expression confers considerable improvements to microbial cell factories. However, the effect of VHb on animal cells has been scarcely studied. To gain insight about the impact of VHb on aerobic energy metabolism and recombinant protein expression, CHO-K1 cells stably expressing the *green fluorescent protein* (GFP) were obtained and transfected for VHb expression. GFP expression slightly increased, whereas cell viability and the mitochondrial membrane potential were not affected by VHb expression. In contrast, the NAD^+/NADH ratio and ATP cell content tended to decrease, while the $NADP^+$/NADPH ratio increased as a consequence of VHb presence. These results suggest that VHb can improve recombinant protein expression in CHO cells by altering the energy metabolism. This can be useful for the design of robust cell factories for industrial applications.

Keywords: CHO cells; *Vitreoscilla* hemoglobin; GFP; energy metabolism

Resumen

La expresión de la hemoglobina de *Vitreoscilla* (VHb) confiere mejoras considerables a las fábricas celulares microbianas. Sin embargo, el efecto de VHb en células animales se ha estudiado muy poco. Para entender mejor el impacto de VHb en el metabolismo energético aerobio y la expresión de proteína recombinante, se obtuvieron células CHO-K1 que expresan de manera estable la proteína verde fluorescente (GFP, por sus siglas en inglés: *green fluorescent protein*) y se transfectaron para expresar VHb. La expresión de GFP se incrementó ligeramente, mientras que la viabilidad celular y el potencial de membrana mitocondrial no fueron afectados por la expresión de VHb. A diferencia de la disminución en la relación NAD^+/NADH y el contenido de ATP en las células, la proporción de $NADP^+$/NADPH se incrementó como consecuencia de la presencia de VHb. Estos resultados sugieren que VHb puede mejorar la expresión de proteína recombinante en las células CHO alterando el metabolismo energético. Esto puede ser útil para el diseño de fábricas celulares robustas para aplicaciones industriales.

Palabras clave: Células CHO, hemoglobina de *Vitreoscilla*, GFP, metabolismo energético.

1 Introduction

Worldwide sales from biopharmaceuticals produced in Chinese Hamster Ovary (CHO) cells exceed $140 billion per year (Walsh, 2018). Despite remarkable improvements on CHO-based processes during the last years, product titers are still relatively low (5-10 g/L) (Harcum and Lee, 2016). The general knowledge of the physiology of CHO cells under process conditions, and approaches to engineer its metabolism to optimize productivity are still very limited [2]. Constrains of CHO cells for biopharmaceuticals production include poorly understood genomic stability (Wurm and Wurm, 2017), limited growth, low productivity and stress resistance (Fischer *et al*., 2015). Therefore, metabolic engineering strategies to improve CHO cells performance are strongly needed.

A well-established technology to improve microbial processes is the expression of the *Vitreoscilla* hemoglobin (VHb). VHb has a high affinity for molecular oxygen (K_{on} = 78 mM^{-1} s^{-1}), as well as a remarkably high ability to deliver it to other molecules (K_{off} = 5000 s^{-1}), like respiratory terminal oxidases (Zhang *et al*., 2007). VHb expression improves the synthesis of a variety of molecules, as well as cell growth under oxygen limitation (Stark *et al*., 2012). Only a few studies deal with the effect of VHb on cultures under aerobic conditions.

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For instance, VHb expression improved the aerobic cell growth and enzyme production in yeasts like P. pastoris and S. occidentalis, presumably connected with enhanced respiratory activity (Suthar *et al*., 2006; Wang *et al*., 2012; Wu and Fu 2012). VHb expression in CHO cells resulted in an important improvement of recombinant protein (Pendse and Bailey, 1994). A recent study showed that VHb expression can also reduce lactate production and increase growth rate and biomass yields of CHO cells (Juárez *et al*., 2017). Moreover, using an immunofluorescence technique, it was found that VHb is distributed throughout the cytoplasm and intracellular structures in CHO cells (Juárez *et al*., 2017). The above is relevant for the putative function of this microbial globin. In contrast with the large number of studies dealing with VHb applications, little is known about the physiological changes emerging from VHb expression. In the particular case of CHO cells, as far as we know, there are not published studies dealing with metabolic consequences of VHb expression. In the present contribution, VHb is transiently expressed in CHO cells previously modified to stably express GFP. The culture performance was characterized and GFP expression monitored. Physiological parameters that may be affected by the putative action of VHb were measured, like mitochondrial membrane potential, ATP pool, NAD^+/NADH and $NADP^+$/NADPH ratios. The results help to understand the relevance of the energy metabolism altered by VHb expression that result in higher recombinant protein production.

2 Materials and methods

2.1 Cell culture, plasmids and transfection procedure

CHO-K1 cells (ATCCR CCL-61TM) are derived from a subclone of the original cell line (Reinhart *et al*., 2019). This cell line without modifications, with its complete genome annotated and readily available was appropriate for this study. Cells were cultured at 37 °C in a 5% CO_2 atmosphere in F12-K medium (ATCCR 30-2004TM) plus 10% fetal bovine serum (Biowest, Nuaillé, France). For transfection, 2.5 μg of pDNA and 6 μL of Lipofectamine 2000 (Thermo Scientific, CA, USA) were used according to the manufacturer's instructions. The GFP gene (*gfp*) was subcloned from plasmid pVAX1-*gfp* (De La Vega *et al*., 2013) into pcDNA3.1(+) (Thermo Scientific, CA, USA) using the restriction sites *Hind*III and XbaI. The obtained plasmid, pcDNA3.1(+)-*gfp*, was used for insertion of *gfp* into CHO-K1 genome. For transient VHb expression, the plasmid pVAX1-*vgb*, which contains the codon optimized gene for VHb (Juárez *et al*., 2017), was used. In all cases, the heterologous genes are under transcriptional control of the cytomegalovirus promoter (P_{CMV}). Transfection efficiency using pVAX1-GFP as a reporter plasmid and the amounts mentioned above for Lipofectamine 2000 and p-DNA was 62.0 ± 2.5%. This transfection efficiency is higher than in our previous report (Juárez *et al*., 2017), and it was achieved adjusting the cells' amount seeded 24 h before transfection (179,00 cells/well in six well plates). CHO-K1-GFP cultures for VHb transient expression were performed in duplicate. Cell count was carried out using the improved Neubauer hemocytometer and Trypan blue for viability measurement. Each replicate was counted in duplicate Extracellular metabolites, glucose, lactate and glutamine, were measured using an YSI 2700 biochemistry analyzer (YSI Inc.; OH, USA).

2.2 Western blot for GFP and VHb detection

VHb was detected by Western Blot as previously described (Juárez *et al*., 2017). VHb was detected using a monoclonal antibody against VHb from mouse ascitic fluid (Abmart; Shangai, China), and GFP was detected using the antibody Covance MMS-118R (NJ, USA). The assay included a protein extract from cells transfected with pVAX1 as negative control, a protein extract from *E. coli* BL21 expressing VHb as positive control, and the anti-actin antibody (sc-1616; Santa Cruz Biotechnology; TX, USA) as loading control.

2.3 Development of GFP-expressing CHO cells

In order to improve the integration of the *gfp*, plasmids pcDNA3.1(+)-*gfp* and pcDNA3.1(+) backbone (used as control) were linearized using the enzyme SspI. CHO-K1 cell were transfected with the linearized plasmid as described above. The culture medium was changed 24 h post-transfection and 1 mg/mL of Geneticin^R (G-418, Thermo Fisher, CA, USA). This amount of G-418 was added based on previous tests using untransformed cells to define the lethal G-418 concentration. After 72 h, the geneticin concentration was increased to 1.2 mg/mL. The cells were continuously subcultured and media containing selection antibiotic was replaced twice a week. After 3 weeks of treatment (subculture 12), a cell bank was prepared and stored in liquid nitrogen. Thus, CHO-K1-GFP and CHO-K1 cells resistant to Geneticin^R were obtained. During the entire procedures, samples were taken to detect GFP fluorescence using an Axiovert 40 CFL fluorescence microscope (Carl Zeiss; Göttingen, Germany) and images were captured using the ZEN Lite 2.1 software. The polyclonal CHO-K1-GFP cells were semipurified using a Moflo XDP Sorter (Beckman Coulter, Brea, CA, USA) cytometer (Laboratorio Nacional de Citometría, UNAM) (Jayasinghe, 2020). After sorting, the CHO-K1-GFP cells were cultured in F12-K medium supplemented with 20% fetal bovine serum to allow their recovery. Then, cells were subcultured to prepare a cell bank, which was stored in liquid nitrogen. Cell sorting

by flow cytometry has been used to obtain clonal lines (Misaghi *et al*., 2016), however even between clonally-derived populations are significant differences, and only an averaged genomic sequence can be considered (Wurm and Wurm, 2017). Considering the above, for this first approach only a pool of cells expressing GFP were selected from the polyclonal cells selected with G-418.

2.4 Evaluation of GFP expression

GFP fluorescence in cells was measured using the BD FACS Calibur flow cytometer (Becton Dickinson, San José, CA, USA). Cells were treated with trypsin and resuspended in culture medium. Next, they were centrifuged at 700 x g-force for 5 min and washed twice with PBS buffer. Finally, the cell pellet was resuspended in PBS containing 5 mM EDTA. Previously optimized settings to properly visualize the granularity vs. size plot were used to detect the GFP fluorescence of 10,000 cells per run and count the amount of non-fluorescent cells using the FL1 channel. The experiment was performed in biological duplicates.

2.5 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) were measured with the TMRE-Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, UK). Cells were taken 36 h post-transfection, treated with trypsin, centrifuged at 700 x g-force for 5 min and resuspended in 5 mM EDTA PBS. Next, the cells were processed following the instructions of the manufacturer. The kit uses TMRE (tetramethylrhodamine ethyl ester), a positively charged red-orange dye, to label active mitochondria. Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE. TMRE fluorescence was measured using the FL2 channel (orange fluorescence) of the BD FACS Calibur flow cytometer, capturing 10,000 events for the analysis.

2.6 Quantification of ATP, NAD^+/NADH and $NADP^+$/NADPH ratios

Quantitation of ATP, NAD^+/NADH and $NADP^+$/NADPH ratios were performed using the MAK190, MAK037 and MAK038 kits from Sigma Aldrich (Darmstadt, Germany), respectively. A Tecan Infinite M1000 PRO microplate reader (Männendorf, Switzerland) was used to measure the absorbance of the processed samples at 450 nm (for NAD^+/NADH and $NADP^+$/NADPH) or 570 nm (for ATP). Cells were taken 36 h post-transfection, treated with trypsin, centrifuged at 700 x g-force for 5 min and resuspended in 5 mM EDTA PBS. For ATP, 1,250,000 cells per assay were used, while for NAD^+/NADH and $NADP^+$/NADPH, 750,000 and 400,000 cells, respectively, were processed. Cells were then processed following the instructions of the manufacturer for each assay. For ATP quantitation, a calibration curve was set up for each experiment and the total ATP per well is determined by interpolation of the absorbance readings. The reduced cofactors concentrations (in pmol/10^6 cells) are calculated interpolating the corresponding absorbance reading in a calibration curve set up for each experiment.

3 Results and discussion

3.1 Development of CHO-K1 cell constitutively expressing GFP

CHO-K1 were transfected for integrating the antibiotic resistance cassette and *gfp* gene under control of P_{CMV}. Figures 1A and 1B show images of cell samples taken after 3 weeks of culture in presence of G-418 (Geneticin^R). GFP expression was also confirmed by Western-blot (Fig. 1C and 1D). The percentage of fluorescent cells counted in three different fields using fluorescence microscopy was 52 ± 6% (Fig. 1G) and 55.2% by flow cytometry (Fig. 1F). Therefore, it is possible that nearly a half of the cell population integrated only the geneticin resistance cassette into the genome, but not the *gfp* gene. A second possibility is that the *gfp* gene in the non-fluorescent population was integrated in the heterochromatin region. This region is highly condensed, which could prevent gene expression (Gorman *et al*., 2009). To increase the fraction of cells expressing GFP, the cell population was semi purified by cell sorting. The fraction of the cell population expressing GFP increased to *ca.* 81% in the semi-purified stock (Fig.1G) considering basal fluorescence of CHO-K1 cells (Fig. 1E). These cells are named here as CHO-GFP cells, which were transfected with the empty vector pVAX1 or with pVAX1-*vgb* to express VHb under control of P_{CMV}.

Figure 2 shows the results of flow cytometry using non transfected CHO-K1 cells (Fig. 2A and 2B) in which there is only one population with low fluorescence emission, presumably emitted by natural CHO fluorescent molecules. When the CHO-GFP cells were transfected with pVAX1 or pVAX1-*vgb*, the fraction of cells expressing GFP remained above 90% (Fig. 2C and 2E). A *ca.* 7% increase of GFP fluorescence intensity was observed when the cells were transfected with pVAX1-*vgb*, compared with those transfected with the empty plasmid (Fig. 2D and 2F). The number of viable cells per well was similar for cells transfected with pVAX1 (1'645,000 ± 10,000 cells/well) and for those transfected with pVAX1-*vgb* (1'530,000 ± 85,000 cells/well) (Fig. 2G). However the relative mean fluorescence in control cells transfected with pVAX1 represented as 1.00 ± 0.00 A. U., increased to 1.07 ± 0.00 A. U. in cells transfected with pVAX1-*vgb* (Fig. 2H).

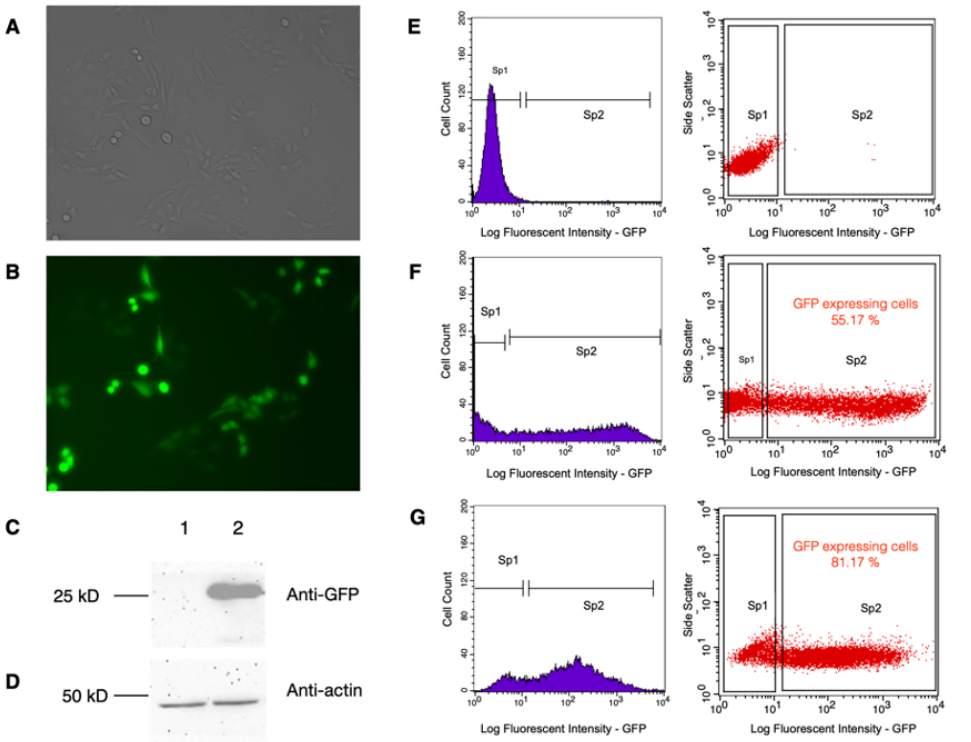

Fig. 1. Fluorescence microscopy of CHO-K1 cells transfected with pCDNA3.1(+)-gfp. Samples correspond to subculture 12 (3 weeks of culture) treated with G-148. The cells were observed in the bright field (A) and exposed to a wavelength of 485 nm for green fluorescence protein (GFP) fluorescence detection (B). Western blot of proteins evaluated with the anti-GFP (C) and anti-actin (D) antibodies: lane 1 protein from CHO-K1 pCDNA 3.1+ cells (negative control), lane 2: protein from CHO-K1 gfp cells. Flow cytometry of CHO-K1 gfp cells: (E) untransfected CHO-K1 cells (basal fluorescence), (F) GFP-expressing cells and (G) semi-purified GFP-expressing cells. Subpopulations SP1 correspond to the fraction of non GFP-expressing cells and SP2 to that of GFP-expressing cells.

Such changes are attributed to the expression of VHb, which was verified by Western blot (Fig. 3). This is in agreement with the increase of the synthesis of diverse products when VHb is expressed in microbial (Stark *et al*., 2015) and CHO cells (Pendse and Bailey, 1994). Pendse and Bailey (1994) reported that VHb expression resulted in an increase of 40-100% of tissue plasminogen activator production. Such increase is substantially higher than that observed in Fig. 2. However, those authors induced VHb expression using 0.1 and 0.5 μM dexamethasone, which should have resulted in a higher expression than that obtained in the present contribution. Even though, authors do not specify the level of expression of VHb using those concentrations of dexamethasone. Nevertheless, VHb induction resulted in a 20-30% decrease of the growth rate and 20% less cells (Pendse and Bailey, 1994). In contrast, in the present study, no changes were observed in the number of viable cells and the stoichiometric parameters evaluated (Supplementary Table S1). This suggests that the VHb expression levels did not cause a strong burden in the present cultures. Furthermore, $Y_{Lac/Glc}$ (Table S1) was similar for cells expressing VHb (1.76 ± 0.05 mol/mol) and for those not expressing this protein (1.75 ± 0.09 mol/mol).

3.2 *Effect of VHb on energy metabolism of CHO-GFP cells*

VHb could act as an enhancer of the aerobic respiration, possibly increasing the activity of the terminal oxidases (Stark *et al*., 2015). Accordingly, it has been demonstrated that the oxidative activity in *E. coli* was higher when VHb-expressing, compared to the non-expressing cells (Jaén *et al*., 2019). Therefore, the ratio of oxidized/reduced cofactors, ATP concentration, and $\Delta\Psi_m$, were studied in VHb-expressing CHO-GFP cells and compared to the non-expressing cells (Fig. 4). Figure 4A shows the median TMRE fluorescence, which is directly proportional to $\Delta\Psi_m$ (Perry *et al*., 2011). Although the TMRE fluorescence in CHO-GFP cells expressing VHb was slightly higher than in the non-expressing cells, the difference was not significant ($p > 0.05$). Therefore, no relevant changes in $\Delta\Psi_m$ were observed.

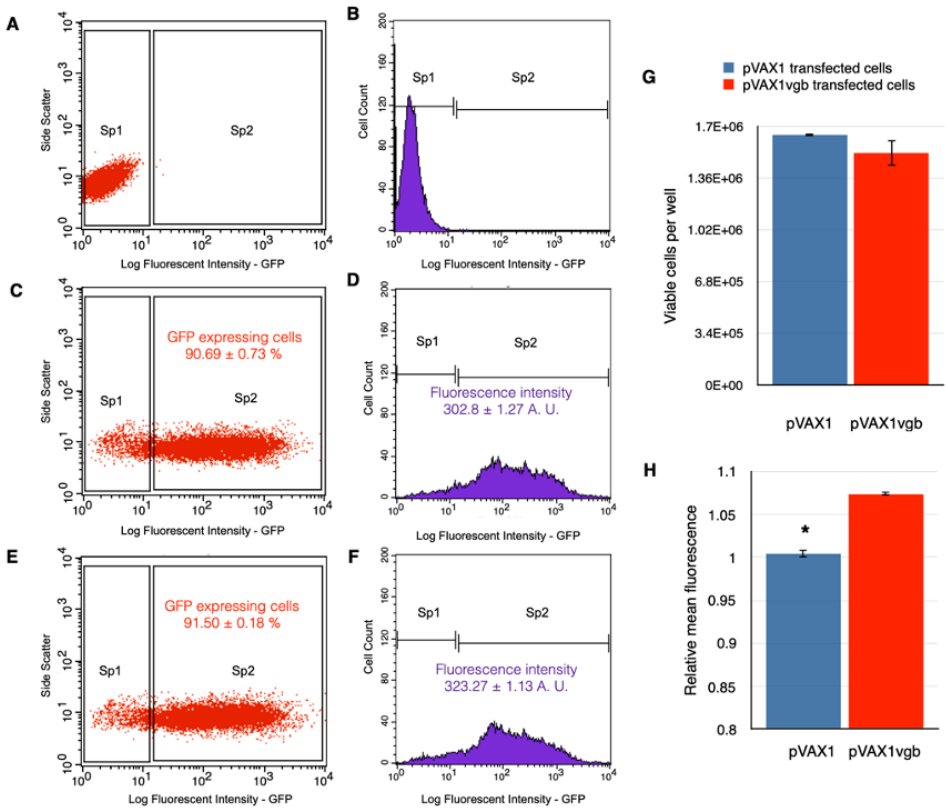

Fig. 2. Effect of Vitreoscilla hemoglobin (VHb) expression on green fluorescence protein (GFP) production in semi-purified CHO-GFP cells. Flow cytometry graphics A, C and E shows log fluorescence intensity-GFP vs side scatter and B, D and F log fluorescent intensity-GFP vs cell count. A and B corresponds to untransfected CHO-K1 cells. C and D corresponds to CHO-GFP cells transfected with the empty plasmid pVAX1. E and F corresponds to CHO-GFP cells transfected with the plasmid pVAX1-*vgb*. (G) Viable cells per well. (H) Relative mean GFP fluorescence, where mean fluorescence from CHO-GFP cells transfected with pVAX-1 was represented as 1.00. Subpopulations SP1 correspond to the fraction of non GFP-expressing cells and SP2 to that of GFP-expressing cells. Data are presented as the mean ± standard error (n = 2). *p < 0.05 was evaluated and significant difference confirmed.

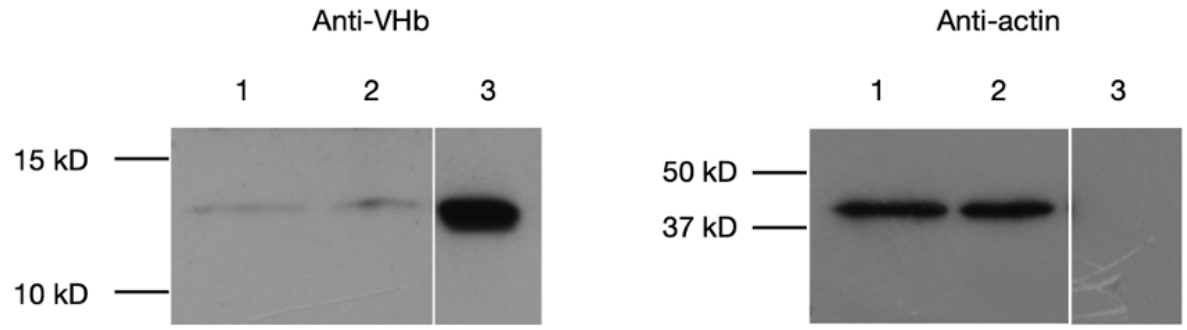

Fig. 3. Western blot of proteins evaluated with the anti-Vitreoscilla hemoglobin (VHb) and anti-actin antibodies. Protein samples of CHO-GFP cells cultures were taken at 24 h (lane 1) and 48 h (lane 2) after transfection with plasmid pVAX1-*vgb*. A protein sample from *Escherichia coli* BL21 expressing VHb from a plasmid was taken as a positive control (lane 3). The positions of molecular size markers (kDa) are shown on the left.

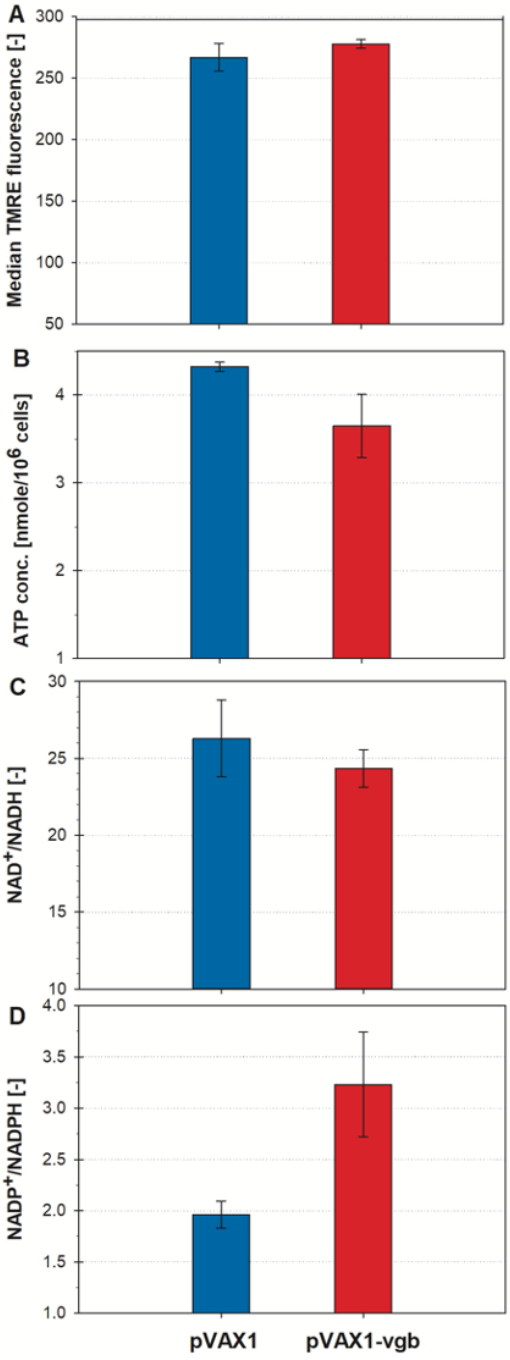

Fig. 4. Effect of *Vitreoscilla* hemoglobin (VHb) expression on the mitochondrial membrane potential measured as TMRE fluorescence (A); intracellular ATP concentration (B); NAD^+/NADH ratio (C) and $NADP^+$/NADPH ratio (D). Blue bars: CHO-GFP cells transfected with empty plasmid pVAX1; red bars: CHO-GFP cells transfected with plasmid pVAX1-*vgb*. Data are presented as the mean ± standard error (n = 2). *p < 0.05 was evaluated and significant difference confirmed.

The intracellular ATP concentration for CHO-GFP expressing VHb was 3.6 ± 0.4 nmol/10^6 cells, whereas for the non-expressing cells was 4.3 ± 0.1 nmol/10^6 cells (Fig. 4B). These values are similar to those previously reported (Pfizenmaier *et al*., 2015). However, in stable CHO cells lines cultured in fed-batch mode leading to hyperosmotic conditions, increased recombinant protein production has been related to a higher ATP pool (Pfizenmaier *et al*., 2016; Becker *et al*., 2019). The conditions of the present study (transient VHb expression, batch culture) may be a reason for the contrasting results. Should more ATP be produced due to VHb expression, it may be readily consumed for higher GFP (Fig. 2H) as well as, VHb expression. This agrees with the unchanged growth rates and biomass yields observed, regardless of VHb expression (Table S1).

The NAD^+/NADH ratio was slightly lower for the cells expressing VHb (24.2 ± 1.2) than for the non-expressing cells (26.3 ± 2.0) (Fig. 4C), which are within the range of the data reported by Ghorbaniaghdam and co-workers (2014). Gupta and co-workers (2017) reported a strong decrease of the NAD^+/NADH ratio in engineered CHO cells with a superior recombinant protein production. However, in the present study a significant difference was not found for the NAD^+/NADH ratio between cells expressing VHb and non-expressing cells. This is in agreement with the similar values found for q_{Lac} and $Y_{Lac/x}$ for both groups of cells. An increase in the production of NADH has been related with improvements in energetic metabolism as well as a decrease in lactate synthesis (Chong *et al*., 2010; Wilkens and Gerdtzen, 2015).

The $NADP^+$/NADPH ratio was higher for the VHb-expressing (3.2 ± 0.5) than from the non-expressing CHO cells (2.0 ± 0.1) (Fig 4D). This is well in agreement with the lower NADPH pool measured in VHb-expressing *E. coli* cells, compared with the non-expressing cells (Stark *et al*., 2012). NADPH provides electrons for biosynthesis and is also required to maintain a reduced intracellular environment (Hosios and Vander Heiden, 2018). In *E. coli* an increase of glucose entering to the pentose phosphate pathway has been reported (Tsai *et al*., 1996). This increase was related with a greater demand of NADPH in VHb expressing cells, possibly as a consequence of increased biosynthesis and decreased TCA flux (Tsai *et al*., 1996). Hence, in CHO cells it would be important to carry out a metabolic flux distribution study in VHb expressing cells to understand its cofactors and metabolites distribution due to the presence of this hemoglobin.

VHb expression has no major effect on the redox state of CHO cells and only marginally increased GFP expression under the conditions evaluated. It is possible that the amount of VHb expressed in CHO-cells was not enough to obtain improvements in the different metabolic parameters measured as well as in GFP production. For this reason, it would be necessary to carry out a dose/response study of VHb in CHO-cells to determine the amount of protein necessary to obtain improvements in metabolic parameters

as well as in recombinant protein production. In the previous study (Juárez *et al*., 2017), VHb expressing cells presented an increase of 60% in their specific cell growth rate as well as 40% less lactate produced per cell with respect to non-expressing cells. In this manner, VHb has the potential to improve the performance of CHO cells in recombinant protein production but further studies will be needed to make the final conclusions.

Conclusions

Transient expression of VHb enhanced GFP production in CHO cells. The increased NADPH regeneration observed in VHb-expressing, compared with non-expressing cells, could be a source of such higher synthetic capacity. A smaller ATP pool in VHb-expressing cells indicates that a larger fraction of energy is used for protein synthesis when VHb is present. This may not be balanced by greater energy generation, as deduced from the unaffected mitochondrial membrane potential and NAD^+/NADH ratio. Therefore, it may be necessary to increase the amount of VHb to improve the observed effects.

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