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Optimization of a GDNF production method based on Semliki Forest virus vector

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ABSTRACT

Human glial cell line-derived neurotrophic factor (hGDNF) is the most potent dopaminergic factor described so far, and it is therefore considered a promising drug for Parkinson's disease (PD) treatment. However, the production of therapeutic proteins with a high degree of purity and a specific glycosylation pattern is a major challenge that hinders its commercialization. Although a variety of systems can be used for protein production, only a small number of them are suitable to produce clinical-grade proteins. Specifically, the baby hamster kidney cell line (BHK-21) has shown to be an effective system for the expression of high levels of hGDNF, with appropriate post-translational modifications and protein folding. This system, which is based on the electroporation of BHK-21 cells using a Semliki Forest virus (SFV) as expression vector, induces a strong shut-off of host cell protein synthesis that simplify the purification process. However, SFV vector exhibits a temperature-dependent cytopathic effect on host cells, which could limit hGDNF expression. The aim of this study was to improve the expression and purification of hGDNF using a biphasic temperature cultivation protocol that would decrease the cytopathic effect induced by SFV. Here we show that an increase in the temperature from 33°C to 37°C during the "shut-off period", produced a significant improvement in cell survival and hGDNF expression. In consonance, this protocol led to the production of almost 3-fold more hGDNF when compared to the previously described methods. Therefore, a "recovery period" at 37°C before cells are exposed at 33°C is crucial to maintain cell viability and increase hGDNF expression. The protocol described constitutes an efficient and highly scalable method to produce highly pure hGDNF.

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) is one of the most important neurotrophic factors present in the nervous system (Lin et al., 1993, Pöyhönen et al., 2019). Our current knowledge attributes to hGDNF special relevance in the development, maintenance and function of a variety of neurons and glial cells, suggesting an important role in halting the progression of Parkinson's Disease (PD) (Lin et al., 1993, Torres-Ortega et al., 2019). The ability of this neurotrophic factor to increase dopaminergic neuron survival in vitro encouraged several in vivo studies (Garbayo et al., 2011, Garbayo et al., 2016, Herrán et al., 2014, Garbayo et al., 2009, Salvatore et al., 2009) and even clinical trials focused on halting PD progression (Slevin et al., 2007, Whone et al., 2019, Gash et al., 2020). However, the therapeutic potential of hGDNF, as a glycosylated protein, will largely depend on the possibility of administering a protein with a glycosylation pattern similar to the native one (Gupta and Shukla, 2018). For this reason, some clinical studies carried out using the non-glycosylated hGDNF produced in E. coli reported the development of antibodies against the protein, which may represent a safety issue (Slevin et al., 2007, Lang et al., 2006). The production of proteins in mammalian cell lines appears to be more appropriate since they allow the expression of functionally relevant and active proteins, as these systems can provide post-translational modifications (Hunter et al., 2019, Piccinini et al., 2013). To date, despite the

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drawbacks described here, most of the procedures developed for hGDNF expression have used prokaryotic organisms, highlighting E. coli (Lang et al., 2006, Chen et al., 2000, Huang and Ma, 2001).

As an alternative, alphaviruses have been engineered as expression vectors due to their ability to induce the expression of proteins at very high levels in mammalian cells. Among the alphaviruses, those based on Semliki Forest virus (SFV) allow a high degree of expression of topologically diverse proteins (Lundstrom, 2010). More specifically, the electroporation of baby hamster kidney cell line (BHK) with this SFV self-replicating RNA has previously demonstrated to be a high efficiency system for the expression of hGDNF (Ansorena et al., 2013). This system presents the additional advantage that it induces a very strong inhibition of host-cell protein synthesis (shut-off). After the establishment of the shut-off, which takes place between four and eight hours post-electroporation, most protein synthesis becomes vector-specific, facilitating the subsequent purification of heterologous proteins expressed from the SFV vector (Ansorena et al., 2013, Ansorena et al., 2010). Among other factors, the incubation temperature of host cells presents important effects on the level and duration of recombinant protein expression in SFV infected mammalian cell lines, being optimal at 33°C (Schlaeger and Lundstrom, 1998). Moreover, the cytotoxic effect on host cells caused by SFV vectors could be influenced by the temperature employed during incubation (Lundstrom et al., 2003). Several studies have obtained higher productivity in other cell lines using biphasic temperature protocols (Lin et al., 2015, Kaisermayer et al., 2016, Becerra et al., 2012), but until now it had not been studied in our expression system. Therefore, this study investigates the effect of a biphasic temperature culture protocol (37°C-33°C) on the expression of hGDNF using an SFV vector in BHK cells. Our findings demonstrate that the increase of the temperature during the shut-off period from 33°C to 37°C, with the incubation maintained at 33°C during the post-shut-off period, led to a significant improvement in cell viability. In addition, a three-fold increase in hGDNF expression was observed compared to previously established conditions (Ansorena et al., 2013). We have thus improved a method to purify hGDNF by increasing the culture temperature during the shut-off stage. This procedure, which could be scaled up using higher-capacity incubation systems, would allow the production of higher levels of hGDNF, offering an attractive system for the production of this and other recombinant proteins.

2. Material and methods

2.1. Cell lines

Baby hamster kidney (BHK-21) and rat adrenal PC12 cells were purchased from American Type Culture Collection (ATCC). BHK-21 cells were cultured in BHK-21 Glasgow MEM supplemented with 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, 2 mM glutamine, 20 mM HEPES, 100 μg/mL streptomycin and 100 U/mL penicillin (BHK complete). BHK-21 cells were incubated in a humidified atmosphere with 5% CO_2, at 37°C, and kept $\leq$ 24 h before electroporation so they were actively growing on the day of electroporation. Rat PC12 cells were cultured on collagen I-coated plate (Invitrogen, Waltham, MA) in D-MEM supplemented with 5% horse heat-inactivated serum, 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin.

2.2. hGDNF expression by BHK-21 cells transfected with the SFV-hGDNF vector and shut-off analysis

Plasmid pSFV-hGDNF was constructed as described before (Ansorena et al., 2013). The plasmid was linearized with SpeI and used as the template for RNA synthesis using SP6 polymerase (New England Biolabs, Ipswich, MA) as previously described (Liljeström and Garoff, 1995). Briefly, linearized DNA was purified using a PCR purification kit (Qiagen, Germany) and quantified using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Then, 1.5 μg of linearized pSFV-hGDNF DNA were incubated for 1 h at 37°C in SP6 buffer (40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 1 mM DTT and 2 mM spermidine) supplemented with 1 mM (m7G(5′)ppp(5′)G) (New England Biolabs, Ipswich, MA), 2 mM DTT, 1 mM rNTP mix, 4.5 μl of RNAse inhibitor (Promega, Madison, WI), and 0.5 μl of SP6 RNA polymerase (New England Biolabs, Ipswich, MA) in a final volume of 50 μl. This reaction yielded approximately 50 μg of RNA. 25 reactions were performed to obtain 1250 μg of SFV-hGDNF RNA. Each reaction was confirmed by gel electrophoresis. For each electroporation, 25 μg of in vitro synthesized RNA were mixed with 10^7 BHK-21 cells in a volume of 0,8 ml of PBS and electroporated in a 0.4 cm cuvette by giving two consecutive pulses at 850 V and 25 μF in a Bio-Rad electroporator (Hercules, CA). Cells from two electroporations were mixed in 10 ml of BHK-21 complete medium, seeded on a T75 flask (Greiner Bio-One, Kremsmünster, Austria) and incubated at 37°C or 33°C with 5% CO_2. After 8 h (shut-off period) medium was removed, cells were washed twice with 10 ml of PBS (Gibco, Waltham, MA), and 10 ml of BHK-21 complete medium without FBS were added to each flask. Cells were then incubated at 33°C for 24h (post-shut-off period), and then supernatants were collected for Western blot analysis. Quantification of hGDNF present in the supernatants was determined by ELISA (Invitrogen, Waltham, MA) following the manufacturer's instructions.

2.3. Optimization of incubation temperatures in host cells

To study the influence of the incubation temperature on the expression system, electroporated cells were seeded on 6-well plates (1.5 ml/dish) (Corning, Lowell, MA) and incubated at 37°C or 33°C with 5% CO_2. After 8 h (shut-off period) medium was removed, cells were washed twice with 2 ml of PBS, and 2 ml of BHK-21 complete medium without FBS was added to each well. 24 h later (post-shut-off period) supernatants were collected and the quantity of hGDNF expressed was quantified by ELISA. The effect of temperature on cell viability after the shut-off and post shut-off period was also evaluated. Briefly, four 96-well culture plates (Corning) were seeded with 50 μl/dish of electroporated cells and completed up to 200 μl with medium. Plates were incubated at 37°C or 33°C with 5% CO_2. For the plates intended for cell viability analysis after 24h (post-shut-off period), the medium was removed at 8 h, and the cells were washed twice with 200 μl of PBS. Then, 200 μl of BHK-21 complete medium without FBS was added to each well. The viability was determined after shut-off period and post-shut-off period using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) included in the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's recommendations.

2.4. Western blot analysis

SDS-PAGE was performed on NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen, Waltham, MA) under reducing conditions. Proteins were transferred for 2 h onto nitrocellulose membranes (Invitrogen, Waltham, MA) and the membrane was incubated with blocking solution containing 5% non-fat dry milk in PBS for 2 h, followed by incubation with anti-GDNF or anti-insulin like growth factor binding proteins (IGFBPs) 4 or 5 (Santa Cruz Biotechnology, Dallas, TX) diluted 1:2000 in blocking solution overnight at 4 °C. After washing with PBS, a horseradish peroxidase (HRP) conjugated sheep polyclonal antiserum against mouse IgG (GE Healthcare-Amersham, UK) diluted in blocking solution (1:2000) was added for 2 h. Detection was achieved with LumiLight Plus Reagent (Roche, Switzerland). Band densities were analyzed using the software Image Studio Lite 5.2.

2.5. Production and purification of hGDNF

A total of 5.8×10^8 BHK-21 cells were electroporated with 1.25 mg of SFV-hGDNF RNA synthesized in vitro as previously described (50

electroporations of 10^7 BHK-21 cells with 25 ug of RNA each) (Ansorena et al., 2013). Electroporated cells were pooled, resuspended in BHK-21 complete medium, and distributed in 25 flasks of T75 (Greiner Bio-One). After the expression process described in section 2.2, supernatants from all flasks (250 ml) were collected, centrifuged at 1000 g for 5 min to remove cell debris, and filtered through a 0.22 μm filter. 1 ml of SP-SepharoseTM Fast Flow resin (GE Healthcare) was packed into a disposable column (Bio-Rad), which was washed with 1.5 M NaCl in phosphate buffer (PB, 10 mM phosphate pH 7.4) and equilibrated with 150 mM NaCl in PB. The supernatant was transferred through the column and the resin was washed with 10 column volumes (CV) of 150 mM NaCl in PB. Finally, bounded protein was eluted in a single step with 10 CV of 0.5 M NaCl in PB. The presence of the protein was confirmed by western blot and the quantity of purified protein was determined by ELISA. The expression process was assessed under the two experimental conditions investigated (shut-off period: 33°C vs 37°C) and then compared. The purity of the sample was assessed by SDS-PAGE followed by Coomassie blue staining.

2.6. In vitro bioactivity assay

The bioactivity of purified hGDNF was proven by a PC-12 neurite outgrowth assay as previously described (Garbayo et al., 2007). Briefly, PC12 cells were plated on collagen-coated plates at low density (2000 cells/cm^2) and cells were treated 24 h later with 100 ng/ml of hGDNF purified from the supernatant of BHK-21 cells. The neurite outgrowth was analyzed after 10 days of incubation.

2.7. Statistical analysis

All the results were expressed as the mean ± standard deviation (SD). Data obtained from MTS and ELISA assays were statistically analyzed using a Paired-samples t-test by using Graphpad prism Version-6.0 software. The t-test of paired samples was conducted to compare cell survival and hGDNF expression of the biphasic temperature protocol with the conventional protocol. All samples were assayed in triplicate. Values were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Optimization of hGDNF expression in BHK-21 cells after electroporation with SFV-hGDNF RNA vector

SFV vectors can induce a strong shut-off of host cell protein synthesis in mammalian cells, which is one of the major advantages of this system (Berglund et al., 1993). This ability is exploited here to produce hGDNF, since it is the most abundant protein present in the supernatant of transfected cells and can be purified by a simple ion-exchange chromatography. The shut-off allows the inhibition of host-cell proteins such as IGFBP-4 and IGFBP-5, with physical-chemical similarity with hGDNF, and whose subsequent purification would limit the yield of the process (Ansorena et al., 2013, Hsu and Olefsky, 1992). To express hGDNF, we first in vitro transcribed SFV-hGDNF RNA obtaining a total amount of 1250 μg of RNA, which was electroporated into BHK-21 cells (10^7 BHK-21 cells for each electroporation with 25 μg of RNA). Several cell culture parameters can influence the level of expression and the quality of the recombinant protein. In this regard, the cell incubation temperature is an essential factor in the level and duration of recombinant protein expression. In the last few years, many studies have supported the idea that exposure of cell cultures to mild hypothermia during the phase of expression of the specific protein leads to increased productivity. However, in most cases, the molecular mechanisms governing the production of specific proteins under mild hypothermia are currently unknown (Becerra et al., 2012). One study investigated the influence of the incubation temperature (33°C vs 37°C) in two rodent cell lines, BHK-21 and CHO infected with an SFV vector expressing luciferase (Schlaeger and Lundstrom, 1998). The luciferase expression levels were much higher in both cells lines when cultured at 33°C compared to 37°C, with a 10-20-fold increase in luciferase expression at 50 h post-infection (Schlaeger and Lundstrom, 1998). The effect of mild hypothermia on the specific productivity of other proteins such as r-protein or erythropoietin fusion protein (Epo-Fc) in CHO cell culture has also been explored, reporting higher productivity (Kaisermayer et al., 2016, Becerra et al., 2012). For example, the final Epo-Fc concentration was increased around 2.5-fold when the cell culture temperature was decreased from 37°C to 33.5°C (Kaisermayer et al., 2016). As mentioned, our expression system consists of two phases: the shut-off period and the post-shut-off period. In this sense, our group had previously analyzed the levels of hGDNF expression from SFV by testing different time and temperature

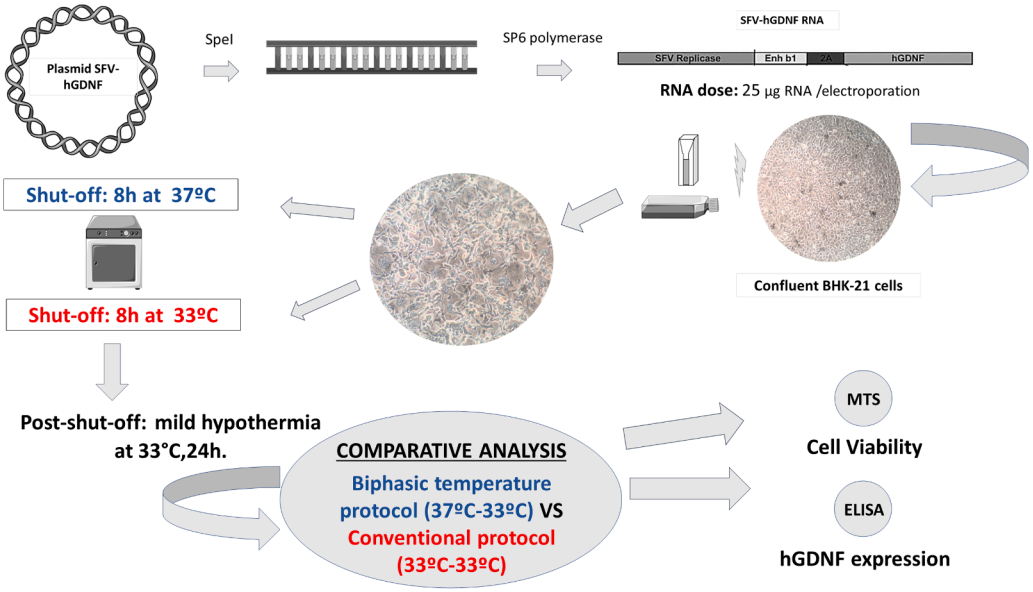

Fig. 1. Schematic representation of the biphasic temperature protocol for hGDNF expression. The plasmid containing SFV-hGDNF was linearized with Spe I and used to transcribe in vitro the vector RNA, using SP6 polymerase. SFV-hGDNF RNA was electroporated into BHK-21 cells. Electroporated cells were incubated at 37°C or 33°C with 5% CO_2. After 8 h (shut-off period), cells were exposed to mild hypothermia at 33°C for 24h (post-shut-off period). Cell viability was determined after the shut-off period and post-shut-off period using MTS. Quantification of hGDNF present in the supernatants was determined by ELISA.

combinations during the shut-off and post-shut-off stages. Moreover, the time required to complete shut-off stage was also determined (Ansorena et al., 2013). The highest hGDNF expression levels were obtained with the combination of an 8 h shut-off period at 33°C followed by a 24 h post-shut-off period at 33°C. Overall, it was concluded that the shut-off period required at least 8 h regardless of the temperature used. The exposure to mild hypothermia during both phases ensured a very high hGDNF expression level with no detection of contaminating proteins. Thus, our expression system seems to be strongly dependent on the incubation temperature of BHK-21 cells after electroporation (Schlaeger and Lundstrom, 1998) and it is known that SFV vector exhibits a temperature-dependent cytopathic effect on host cells (Lundstrom et al., 2003). However, the possibility of using different temperatures for the shut-off and post-shut-off periods was not investigated in that study. The use of a biphasic temperature system has been previously explored by Lin et al. in HEK-293S cells transfected with a green fluorescent protein (GFP) plasmid (Lin et al., 2015). A decrease of temperature up to 33°C, was an effective way to increase protein expression, but only when the transfected culture was allowed to "recover" at 37°C overnight (Lin et al., 2015). Additionally, it is well known that the temperature at which cells are incubated is also one of the most important factors for cell growth, being usually optimal at 37°C (Akram, 2014). Incubations at lower temperatures have been related to poor cell growth (Watanabe and Okada, 1967, Neutelings et al., 2013). In this context, we hypothesized that the performance of shut-off at 37°C might stimulate higher cell viability and also, an enhanced hGDNF expression during the post-shut-off period. Therefore, we decided to test if increasing the shut-off temperature to 37°C could improve hGDNF expression in BHK-21 cells. To evaluate this hypothesis, we studied BHK-21 cell viability and hGDNF expression using the biphasic temperature protocol (37°C for the shut-off period and 33°C for the post-shut-off period) (Fig. 1). The same parameters were analyzed using the conventional protocol in which the whole process is performed at 33°C. Cell viability was evaluated by MTS after shut-off and post-shut-off periods for both protocols showing that cell survival was significantly lower when both periods of incubation were performed at 33°C (Fig. 2). Thus, when the shut-off was performed at 33°C, the absorbance detected by MTS, which is directly proportional to the number of living cells in culture was significantly lower compared to its performance at 37°C (Table 1). hGDNF quantification by ELISA showed that its expression was significantly increased by 20% ($p < 0.05$) when the shut-off period was carried out at 37°C (Table 1). Likewise, the higher expression level achieved with the biphasic temperature protocol (37°C-33°C) was confirmed when performing the same experiment with a larger number of electroporations (50 electroporations) under the two experimental conditions investigated (shut-off period: 33°C vs 37°C) (Table 2). In this higher scale experiment, an average hGDNF expression level of 3.12 ± 1.17 μg/ml was obtained following the biphasic temperature protocol (37°C-33°C), reaching a total amount of 780 μg of GDNF in 250 ml. By contrast, only 1.21 ± 0.14 μg hGDNF/ml and a total amount of 300 μg of

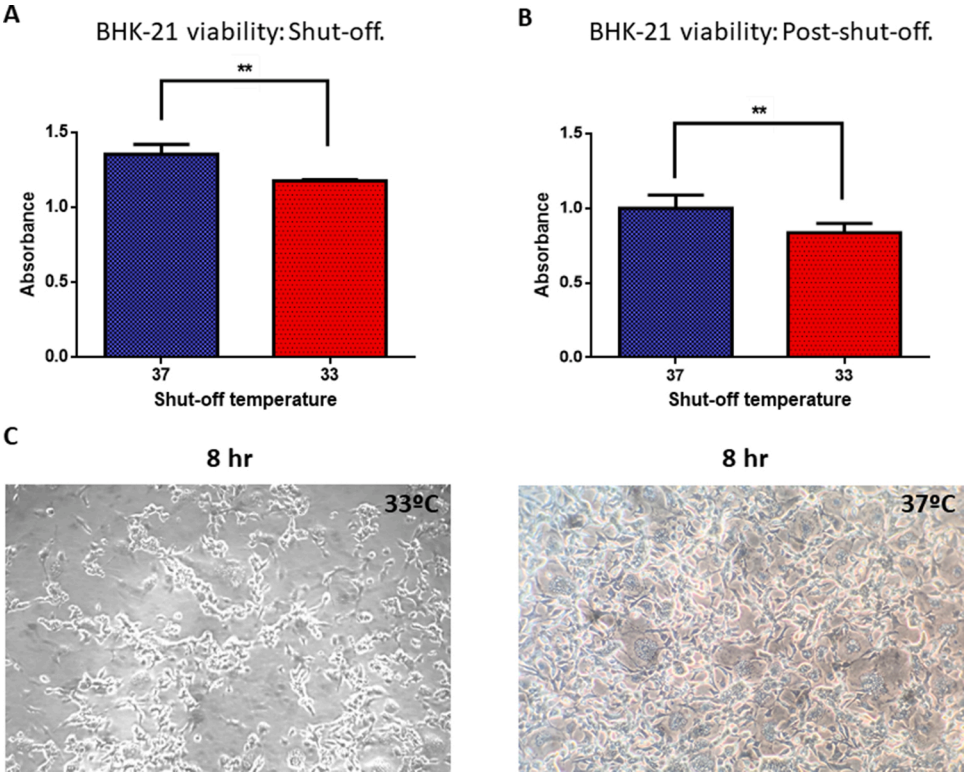

Fig. 2. Analysis of cell viability after the shut-off and post shut-off period. Cells used for the assays were electroporated with SFV-hGDNF RNA and incubated at 37°C or 33°C (N=4) during the shut-off period. A) Cell viability in BHK-21 cells that grew at 37°C (blue bar), as compared with 33°C (red bar) after the shut-off period B) Cell survival in BHK-21 cells that were incubated at 37°C (blue bar), as compared with 33°C (red bar) after the post-shut-off period. Cell survival was significantly increased with biphasic temperature protocol (**, $p<0.01$, Paired t test). The data are shown as the mean ± SD. C) Representative images of cells after shut-off period at 33°C (left) or 37°C (right) taken with a phase-contrast microscope. Magnification: 40x.

Table 1
Optimization of incubation temperature for hGDNF production using SFV system.

Number of electroporations	μg RNA/ electroporation	Shut-off temperature	Post shut-off temperature	μg hGDNF / ml of supernatant	*Absorbance detected in MTS after shut-off	*Absorbance detected in MTS after post shut-off
4	25	37°C	33°C	5.49 ± 1.33	1.35 ± 0,06	0.99 ± 0.09
4	25	33°C	33°C	4.38 ± 0.47	1.17 ± 0.01	0.83 ± 0.06

*Data of absorbance and hGDNF expression were presented as means ± standard deviation (SD).

Table 2
hGDNF expression on a larger scale applying different temperatures during shut-off.

Number of electroporations	μg of RNA/ electroporation	Shut-off temperature	Post shut-off temperature	μg of hGDNFf/ml of supernatant	Total hGDNF protein expression	ml of supernatant
50	25	37°C	33°C	3.12 ± 1.17	780 μg	250
50	25	33°C	33°C	1.21 ± 0.14	300 μg	250
*100	25	33 °C	33°C	————	741 μg	570

*Data extracted from Ansorena et al (Ansorena et al., 2013).

GDNF in 250 ml were obtained using the conventional protocol. According to these results, the new protocol proposed here represents a considerable improvement in hGDNF expression of almost 3-fold compared to previously established conditions (Ansorena et al., 2013) (Table 2).

Accordingly, culturing mammalian cells at low temperature is an established method to maintain cell viability while at the same time inhibiting their growth. This inhibition is associated with G1-phase cell cycle arrest, which has been positively correlated with increased protein productivity (Schlaeger and Lundstrom, 1998, Lin et al., 2015, Kumar et al., 2008, Yoon et al., 2003, Kaufmann et al., 1999). In agreement with that observed with other expression systems, the mild hypothermia achieved at 33°C is suitable to obtain a high expression level during the post-shut-off stage when using the SFV system (Schlaeger and Lundstrom, 1998, Lin et al., 2015, Yoon et al., 2003, Kaufmann et al., 1999). However, in contrast to other studies performed with SFV vectors in BHK-21 cells, the use of low temperature after RNA transfection was not optimal for hGDNF expression (Schlaeger and Lundstrom, 1998, Fernández-Núñez et al., 2015). In our case, maintaining a higher temperature (37°C) during the shut-off period was important to increase hGDNF expression, since it increased cell viability. Although it had been assumed that the same temperature should be applied during the two phases following electroporation, we have shown that this is not optimal. Therefore, our results demonstrate that the conditions used during the initial shut-off period are of great importance for hGDNF expression during the post-shut-off period.

3.2. Shut-off of host cell protein synthesis analysis

As mentioned before, SFV RNA vector replication leads to a strong shut-off of host cell protein synthesis and after a few hours, most protein synthesis becomes vector-specific (Strauss and Strauss, 1994, McInerney et al., 2005, Rivas et al., 2016). To determine the efficiency of this effect in BHK-21 cells under the biphasic temperature culture protocol, the supernatants from 24 h post-shut-off electroporated cells were analyzed by western blot with specific antibodies for cellular proteins IGFBP-4, IGFBP-5 and hGDNF. The presence of these proteins was analyzed because IGFBP-4 and IGFBP-5 are also secreted by this cell line (Ansorena et al., 2013), representing a technical problem for hGDNF purification due to the high physicochemical similarity with hGDNF. The analysis of the supernatants from electroporated cells only showed the presence of hGDNF as previously described by Ansorena et al (Ansorena et al., 2013), indicating that the shut-off had been highly efficient (Fig. 3A).

3.3. hGDNF purification by cation exchange chromatography

The purification strategy of recombinant proteins is crucial, and a chromatographic approach is usually required to meet the purity requirements, which sometimes are higher than 99% in the biopharmaceutical industry (Owczarek et al., 2019). We applied an ion-exchange chromatography, which allowed hGDNF purification in a single chromatographic step. The pH of the sample and buffers used were adjusted to 7.4. The use of an adequate buffer is fundamental for the maintenance of the biological activity of a protein (Ugwu and Apte, 2004) since it will prevent changes in pH that could irreversibly affect its folding, solubility, and function (Ritchie, 2012, Zhang et al., 2013). In the present protocol, we have optimized the pH working conditions, employing a phosphate buffer at pH 7.4 for the purification of hGDNF by ion-exchange chromatography, which has a more physiological pH than the method previously described (Ansorena et al., 2013). Considering the theoretical isoelectric point of our protein (pI=9.4), this pH could allow a greater attraction of hGDNF to the cation exchange column for subsequent elution. All the purified protein was found in the first fraction of elution with 0.5 M NaCl buffer (Fig. 3B). Quantification of purified protein by ELISA showed a total amount of 483 μg of hGDNF, obtained from 250 ml of medium, with a recovery yield of 62%, similar to previous reports (Ansorena et al., 2013). Therefore, pH modification

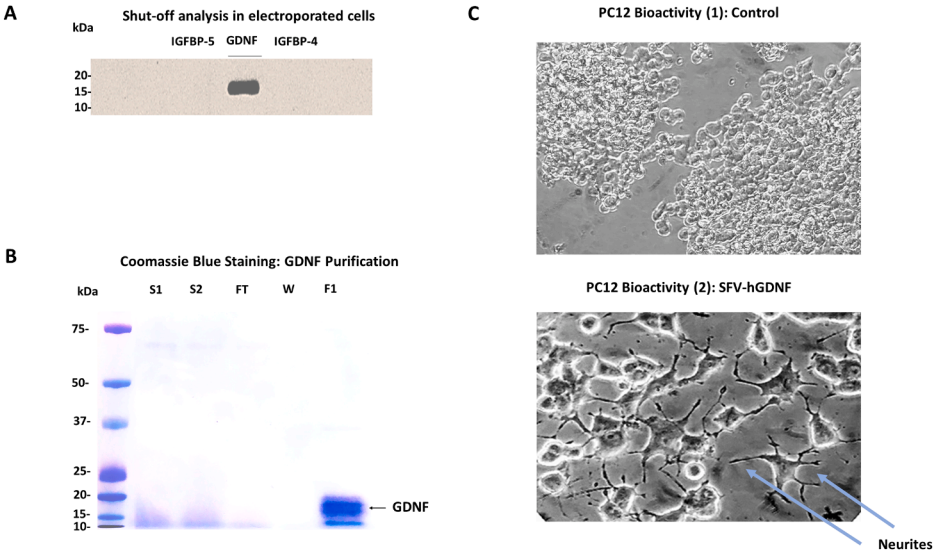

Fig. 3. Analysis of the shut-off efficiency and bioactivity of purified hGDNF. A) Shut-off analysis in electroporated cells: After post-shut-off period, the medium was collected and analyzed by Western blot with antibodies specific for hGDNF, IGFBP-5 and IGFBP-4. B) Coomassie Blue Staining; S1 and S2: supernatant samples; FT: flow-through; W: column wash; F1: eluate with 0.5 M NaCl; C) Analysis of hGDNF activity in vitro. PC12 cells were incubated for 10 days with 100 ng/ml of hGDNF purified from the supernatant of BHK-21 cells transfected with SFV-hGDNF vector. Control cells were incubated without hGDNF. Representative images were taken with a phase-contrast microscope on day 10. Magnification: 20x (1) and 40x (2).

did not affect the final yield of the chromatographic step. The analysis of the elution fractions by SDS-PAGE followed by Coomassie Blue staining showed the presence of several bands at different molecular weights. This complex pattern has been observed previously and corresponds to the different degrees of glycosylation present in hGDNF expressed by BHK-21 cells (Fig. 3B) (Ansorena et al., 2013). Likewise, purity analysis show that it is possible to obtain highly pure hGDNF through a biphasic temperature protocol and the application of a single chromatographic step.

3.4. Activity of purified hGDNF

Having demonstrated that this protocol is highly efficient for the production of glycosylated hGDNF, we next evaluated the bioactivity of the purified protein. Neurite outgrowth assay in the PC12 cell line has been widely used as a model of neuronal differentiation and to evaluate the activity of neurotrophic factors (Chaurasiya et al., 2017). Neurite extension was confirmed in PC12 cells after 10 days of culture (Fig. 3C). These results indicate that recombinant hGDNF produced using the biphasic protocol remains biologically active.

3.5. Future prospects and conclusion

Mammalian cells such as CHO and BHK-21 cells are ideal expression systems to produce recombinant proteins with features closer to their endogenous versions and potentially less immunogenic. However, working with mammalian cell expression systems, increasing protein production with fewer resources and shorter times remains a major challenge (Lalonde and Durocher, 2017). In this case, the key aspect that we used to optimize a previously described method of hGDNF production was the temperature of incubation periods after electroporation. The use of a biphasic temperature protocol (37°C-33°C) led to a considerable (3-fold) improvement in hGDNF expression compared to previously established conditions. The first phase of this culture protocol, in addition to allowing the shut-off of host cell proteins synthesis, is also considered as a recovery period that allows cell viability to be maintained before the cells are exposed to mild hypothermia at 33°C in the second stage. Furthermore, this change allows the expression of a highly pure and bioactive protein, potentially reducing the production costs, since it is possible to produce a greater amount of hGDNF using a smaller number of electroporations and a lower amount of RNA. Even though the quantity of hGDNF produced at a small scale in this study was sufficient for our objective, it would be very interesting to scale-up hGDNF production using this expression system (Núñez et al., 2013, Lundstrom, 2003, Aranda et al., 2014). The ideal situation would be to adapt this method to a process able to handle large volumes of cells with suitable bioreactors. While on a laboratory scale, the SFV vector has been widely used to obtain sufficient amounts of recombinant proteins for different studies, the use of large volumes in bioreactors is mainly limited by the transient expression of the vector due to its cytotoxic character, the inhibition of host cell proteins and RNA-based expression (Casales et al., 2010). In recent years, many studies have focused on the development of vectors with a less cytopathic character. However, the complexity of controlling the duration of the protein expression remains a major limitation (Aranda et al., 2014, Casales et al., 2010, Casales et al., 2008, Schott et al., 2016). On the other hand, cell electroporation is a discontinuous procedure, which could represent a limitation for scaling-up our method. In this regard, recent advances have led to higher throughputs by continuous electroporation using modified microfluidic devices. In fact, continuous cell electroporation has been successfully used to transfer DNA and mRNA into several cell lines (Lissandrello et al., 2020, Yoo et al., 2018, Bhattacharjee et al., 2016) and it could be easily applied to our expression system. With this technology, it would be possible to process larger volumes of cells and to produce higher amounts of hGDNF during shorter times.

To conclude, this study opens up the possibility of using a biphasic protocol that increases cell culture temperature during the shut-off period to improve hGDNF expression. We have shown the importance of a "recovery period" at 37°C to maintain BHK-21 viability before exposing the cells to mild hypothermia at 33°C. Furthermore, our results provide additional important information for the future development of large-scale hGDNF production using SFV vectors in the mammalian cell line BHK-21. Moreover, the system described in this study could be extendable to the expression of different therapeutic proteins.

CRediT authorship contribution statement

Pablo Vicente Torres-Ortega: Validation, Formal analysis, Investigation, Writing - original draft. **Cristian Smerdou:** Conceptualization, Writing - review & editing, Supervision. **Eduardo Ansorena:** Writing - review & editing, Supervision. **María Cristina Ballesteros-Briones:** Investigation. **Eva Martisova:** Investigation. **Elisa Garbayo:** Conceptualization, Writing - review & editing, Supervision. **María J. Blanco-Prieto:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Conflicting Interests

None

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