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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Development of recombinant human granulocyte colony-stimulating factor (nartograstim) production process in *Escherichia coli* compatible with industrial scale and with no antibiotics in the culture medium

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Abstract

The granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine that has important clinical applications for treating neutropenia. Nartograstim is a recombinant variant of human G-CSF. Nartograstim has been produced in *Escherichia coli* as inclusion bodies (IB) and presents higher stability and biological activity than the wild type of human G-CSF because of its mutations. We developed a production process of nartograstim in a 10-L bioreactor using auto-induction or chemically defined medium. After cell lysis, centrifugation, IB washing, and IB solubilization, the following three refolding methods were evaluated: diafiltration, dialysis, and direct dilution in two refolding buffers. Western blot and SDS-PAGE confirmed the identity of 18.8-kDa bands as nartograstim in both cultures. The auto-induction medium produced 1.17 g/L and chemically defined medium produced 0.95 g/L. The dilution method yielded the highest percentage of refolding (99%). After refolding, many contaminant proteins precipitated during pH adjustment to 5.2, increasing purity from 50 to 78%. After applying the supernatant to cation exchange chromatography (CEC), nartograstim recovery was low and the purity was 87%. However, when the refolding solution was applied to anion exchange chromatography followed by CEC, 91%–98% purity and 2.2% recovery were obtained. The purification process described in this work can be used to obtain nartograstim with high purity, structural integrity, and the expected biological activity.

Key points

- *Few papers report the final recovery of the purification process from inclusion bodies.*
- *The process developed led to high purity and reasonable recovery compared to literature.*
- *Nartograstim biological activity was demonstrated in mice using a neutropenia model.*

Keywords G-CSF · Bioreactor cultivation · Inclusion bodies · Refolding · Purification · Recovery

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Introduction

The granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine that stimulates and regulates the proliferation, survival, level, and differentiation of neutrophils (Clark and Kamen 1987) and neutrophils bactericidal activity, chemotaxis, and phagocytosis (Weisbart and Golde 1989). It has been widely used as a therapeutic agent for treating neutropenia in oncology patients and as a prophylactic drug for improving the immune system of immunocompromised patients, such as HIV-infected individuals (Hübel and Engert 2003). The recombinant human G-CSF (rhG-CSF) is part of the Exceptional Drugs Program in Brazil, which acquires

expensive drugs using public funds and makes them available for the entire population. The results of using rhG-CSF to treat oncology patients show a decrease in the use of antibiotics, a reduction in hospital-related costs, and an increase in patient survival rates (Hübel and Engert 2003; Welte et al. 1996). Filgrastim is a non-glycosylated variant of rhG-CSF produced in *E. coli* with a sequence of amino acids identical to the wild type of human G-CSF. Nartograstim is a variant of rhG-CSF produced in *E. coli*, also non-glycosylated, that has five mutation points, T1A, L3T, G4Y, P5R, and C17S, which increase the stability and half-life of the molecule, allowing for lower-dose administration (Tanaka et al. 1997).

Nartograstim was already obtained from *E. coli* inclusion bodies (IBs) at a small scale in our laboratory and, after refolding and purification, showed biological activity similar to the commercial version of filgrastim called granulokine (Gomes et al. 2012). However, the plasmid used for expressing the nartograstim gene, pAE-6His, is unstable for the production of recombinant proteins in higher cell density cultures (Silva et al. 2007). Therefore, pAR-KanI, a stable expression vector that allows for the tight control of protein synthesis, was constructed and applied to the production of rhG-CSF (Eguia et al. 2018). pAR-KanI is a high-copy-number plasmid that contains a *par* locus sequence to ensure its partitioning to daughter cells during cell division, thus improving plasmid stability (Eguia et al. 2018; Liu et al. 2005; Ptacin et al. 2010). It was also shown that pAR-KanI was stable in shaker flask cultures even without the addition of antibiotics to the medium (Eguia et al. 2018).

Antibiotic resistance genes in the expression vector and antibiotics in the culture medium are used to select and maintain plasmids in bacterial cells during cell division. Despite being expensive (Vidal et al. 2008), to the best of our knowledge, all of the articles published about rhG-CSF production in *E. coli* describe processes that use antibiotics in the culture medium (Babaeipour et al. 2015; Babaeipour et al. 2017; Kim et al. 2014; Rao et al. 2008; Toghraie et al. 2019; Vemula et al. 2015b).

There is only one biopharmaceutical rhG-CSF drug commercialized in Brazil that is produced locally. The pegylated version of rhG-CSF is among the 20 top-selling biopharmaceutical products in the world (Walsh 2018). It is very important to establish a national production process to obtain rhG-CSF at large scale due to its essential role in the treatment of neutropenia and the prevention of clinical complications in cancer and AIDS patients. Moreover, it could reduce the public investment required to acquire this biopharmaceutical and benefit Brazilian public health programs. The production of nartograstim is an attractive alternative to filgrastim due to its higher stability and longer half-life. It should also be a cheaper alternative to pegfilgrastim since, given its higher stability, nartograstim production does not require the additional steps of pegylation. Consequently, this study is the first step toward the development of scalable nartograstim production and purification processes. This work compared complex and chemically defined culture media in a 10-L bioreactor, demonstrated that the pAR-KanI plasmid can be employed for bioreactor cultivation, established refolding and purification processes, and evaluated the structural integrity and biological activity of the purified nartograstim that was produced.

Materials and methods

Bacterial strain

Escherichia coli BL21 (DE3) STAR pLysS (Invitrogen) was transformed with *pAR-KanI/rhg-csf (nartograstim)*. The plasmid pAR-KanI is a derivative of the high-copy-number plasmid pAE in which a *par* locus was inserted and its ampicillin resistance was replaced by a kanamycin (Kan) resistance gene (Eguia et al. 2018). The *par* locus is a sequence related to ensuring plasmid segregation in low-copy-number plasmids, resulting in a high-copy-number plasmid which is stable in cell cultures even without the addition of antibiotics (Eguia et al. 2018). The producer clone is also resistant to chloramphenicol (Clo), which is the selection marker of the plasmid pLysS that encodes the lysozyme gene of the host strain. The procedures for expression vector construction and cell bank preparation were described by Eguia et al. (2018). The accession number of the nartograstim gene is NM_000759.3 (Kuga et al. 1989).

Inoculum preparation

The inoculum culture used to seed the bioreactor with the auto-induction medium was prepared in shaker flasks as described by Eguia et al. (2018). Briefly, 50 μL of the frozen cell bank was inoculated into a 500-mL flask containing 100 mL of the auto-induction medium without lactose. This medium contained 5 g/L glycerol, 0.5 g/L glucose, 5 g/L yeast extract, 10 g/L phytone, 3.4 g/L KH_2PO_4, 2.7 g/L NH_4Cl, 0.7 g/L Na_2SO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 14.1 mg/L EDTA, 2.5 mg/L $CoCl_2 \cdot 6H_2O$, 15.0 mg/L $MnCl_2 \cdot 4H_2O$, 1.5 mg/L $CuCl_2 \cdot 2H_2O$, 3.0 mg/L H_3BO_3, 2.1 mg/L $Na_2MoO_4 \cdot 2H_2O$, 33.8 mg/L $Zn(CH_3COO)_2 \cdot 2H_2O$, 100.8 mg/L Fe(III)citrate, 45 mg/L thiamine hydrochloride, 50 mg/L kanamycin sulfate, and 30 μg/mL chloramphenicol. The culture was incubated at 36 °C and 250 rpm and was used to inoculate the bioreactor with an initial optical density (OD) of approximately 0.1 at 600 nm.

To select *E. coli* clones capable of growing in chemically defined medium, a new cell bank was prepared from a colony isolated from plates of M9 minimal medium containing 50 μg/mL Kan and 30 μg/mL Clo, and cultivated in HDF medium (Korz et al. 1995) with Kan and Clo (HDF/KanClo), which

contained 20 g/L glycerol, 13.3 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.2 g/L $MgSO_4{\cdot}7H_2O$, 1.7 g/L citric acid, 14.1 mg/L EDTA, 2.5 mg/L $CoCl_2{\cdot}6H_2O$, 15.0 mg/L $MnCl_2{\cdot}4H_2O$, 1.5 mg/L $CuCl_2{\cdot}2H_2O$, 3.0 mg/L H_3BO_3, 2.1 mg/L $Na_2MoO_4{\cdot}2H_2O$, 33.8 mg/L $Zn(CH_3COO)_2{\cdot}2H_2O$, 100.8 mg/L Fe(III)citrate, 45 mg/L thiamine hydrochloride, 50 mg/L kanamycin sulfate, and 30 μg/mL chloramphenicol. Next, a 500-mL flask containing 100 mL HDF/KanClo was seeded with 100 μL of the frozen cell bank, incubated at 36 °C and 250 rpm, and used to inoculate the bioreactor as described above.

Bioreactor cultivation

Two batch cultures were performed in an auto-induction medium that was free of animal-origin components (Campani et al. 2016) and one fed-batch culture was performed in the HDF/KanClo medium. The composition of the auto-induction medium for bioreactor batch cultivation was the same as that used for inoculum preparation, but it contained 60 g/L glycerol, 10 g/L glucose, and 20 g/L lactose.

For the bioreactor initial batch phase, the composition of the HDF/KanClo medium contained 40 g/L glycerol. The feeding medium was composed of 800 g/L glycerol, 20 g/L $MgSO_4{\cdot}7H_2O$, 13 mg/L EDTA, 4 mg/L $CoCl_2{\cdot}6H_2O$, 23.5 mg/L $MnCl_2{\cdot}4H_2O$, 2.3 mg/L $CuCl_2{\cdot}2H_2O$, 4.7 mg/L H_3BO_3, 4 mg/L $Na_2MoO_4{\cdot}2H_2O$, 16 mg/L $Zn(CH_3COO)_2{\cdot}2H_2O$, 40 mg/L Fe(III)citrate, 45 mg/L thiamine hydrochloride, 50 mg/L kanamycin sulfate, and 30 μg/mL chloramphenicol.

For all cultures, the starting volume was 7 L in a 10 L BioStat C-Plus bioreactor (Sartorius, Germany). The cultivations were performed at 30 °C, the pH was controlled at 6.8 by addition of NH_4OH 25% (v/v), and the dissolved oxygen tension at 30% by a cascade controlling the stirring speed from 150 to 1000 rpm and the addition of pure oxygen to the inlet gas from 0 to 100%. In the auto-induction medium, when glucose is exhausted, lactose is consumed as a carbon source and induces nartograstim synthesis. In HDF/KanClo medium, the induction was achieved by adding 0.5 mM IPTG and 20 g/L of lactose to the culture when the biomass reached optical density (OD) at 600 nm 130, which is about 50 g/L. The cells were harvested by centrifugation at 6817*g* for 30 min and frozen at − 20 °C.

Kinetic parameters

Specific growth rate (μ) was determined by Eq. (1):

$$\mu = \left(\frac{1}{Cx}\right) \times \frac{dCx}{dt} \tag{1}$$

where Cx is the cell concentration (g/L) at cultivation time *t* (h). During the exponential growth phase, the angular coefficient of the curve Ln (Cx) versus time was equal to the maximum specific growth rate (μ_{max}).

Yield factor on glycerol ($Y_{CX/S}$) was given by Eq. (2):

$$Y_{Cx/S} = \frac{C_x - C_{x0}}{S_0 - S} \tag{2}$$

where Cx_0 is the cell concentration (g/L) at the beginning of the culture, S_0 is the initial concentration of glycerol (g/L), and S is the concentration of glycerol at the same time as Cx was sampled.

Equation (3) (Horta et al. 2012) was employed to calculate the feeding flow rate during fed-batch culture:

$$\text{Flow rate}\ (L/h) = \left(\frac{\mu_{set}}{Y_{Cx/S}} + m\right) \cdot \left(\frac{C_{Xi} \cdot V_i}{S_f}\right) \cdot e^{\mu_{set} \cdot (t - t_i)} \tag{3}$$

where $Y_{Cx/S}$ is the yield factor on glycerol ($g_{biomass}/g_{glycerol\ consumed}$), *m* is the maintenance coefficient (0.025 $g_{biomass}/g_{glycerol\ consumed}.h$), μ_{set} is the desired specific growth rate (h^{-1}) during the feeding, V_i and C_{xi} are volume and cell concentration of culture at the beginning of feeding, S_f is the concentration of glycerol in the feeding medium, *t* is the time at any instant, and t_i is the time at the beginning of the feeding.

Inclusion body isolation

The frozen cells (100 g) were suspended in 1 L of lysis buffer (20 mM Tris pH 8.0, 50 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, and 1 mM PMSF) and disrupted in a high-pressure continuous homogenizer (APV 60 Homogenizer, APV Gaulin, USA) at 500 bar through a close loop for 8 min with a flow rate of 1 L/min, which represents 8 cycles of cell disruption. The disruption temperature was maintained below 12 °C using a jacketed reservoir in the inlet and a heat exchanger at the outlet of the homogenizer with the chiller temperature set to 4 °C. After centrifugation (17,817*g*, 1 h), the IBs were sequentially washed with 1 to 4 M urea and solubilized with 8 M urea, 5 mM β-mercaptoethanol, and 20 mM Tris buffer pH 8.0, for 24 h at 25 °C. Three refolding methods were evaluated: refolding by diafiltration (Supplementary Material), refolding by dialysis (Supplementary Material), and refolding by direct dilution (described below). Different purification methods were evaluated to define the final purification process (Fig. S1 and S2).

Refolding by direct dilution

Two buffers were evaluated for refolding by direct dilution: Tris/arginine buffer (Fig. S1), composed of 20 mM Tris pH 8.0, 2 mM EDTA, 0.1% Triton X-100, 10% glycerol, and 500 mM arginine; and Tris/glycerol buffer (Fig. S2), composed of 20 mM Tris pH 8.0, 2 mM EDTA, 0.1% Triton

X-100, and 10% glycerol. Solubilized IBs (200 mL or 50 mL) were dropped on 1 L of each refolding buffer with a flow rate of 1 mL/min under stirring. The refolded product was centrifuged at 17,817*g* for 30 min at 4 °C, and the supernatant was analyzed.

Refolding efficiency

The percent of refolding was calculated by Eq. (4):

$$\text{Refolding}\ (\%) = \left(\frac{V\text{sup} \times C\text{sup}}{V\text{solub} \times C\text{solub}}\right) \times 100 \quad (4)$$

where V_{sup} and C_{sup} are the supernatant volume (mL) and protein concentration (mg/mL) in the supernatant after centrifugation. V_{solub} and C_{solub} are volume (mL) and protein concentration (mg/mL) of inclusion bodies solubilized in 8 M urea.

Chromatographic steps

Initially, cation exchange chromatography (SP-Sepharose) was the sole chromatographic step evaluated using the material refolded by dialysis, diafiltration, or direct dilution with Tris/arginine buffer as illustrated in Fig. S1. Next, the purification process was performed using the material refolded by direct dilution with Tris/glycerol buffer as illustrated in Fig. S2. The purification of nartograstim from auto-induction culture was compared to its purification from HDF culture, and the Q-Sepharose followed by SP-Sepharose sequence for nartograstim purification from HDF culture was evaluated. The step-by-step development of the purification process is described in the Supplementary Material. The following is a description of the final purification process.

Post-refolding pH adjustment and loading into cation exchange chromatography

To identify the highest pH at which nartograstim is adsorbed to cation exchange chromatography in SP-Sepharose FF, samples refolded by direct dilution in Tris/glycerol buffer were adjusted to pH 4.5, 4.7, 5.0, 5.2, and 5.5, incubated overnight at 4 °C and centrifuged at 17,817*g* for 30 min (Fig. S2). Pellets and supernatants were analyzed by SDS-PAGE. The supernatant of each pH was filtered with a 0.45 μm membrane and applied to a SP-Sepharose column (10 mL, XK16/20, 5 mL/min of flow rate), which was previously equilibrated with 20-mM sodium acetate buffer with 50 mM NaCl, pH 4.5, 4.7, 5.0, 5.2, and 5.5. The elution was performed with NaCl 100 mM and 500 mM in 20 mM Tris buffer, pH 8.0 (Fig. S2). All chromatography buffers were filtered with a 0.45 μm membrane. After the loading condition was defined, the following two nartograstim purification processes were compared: the first using the cells produced in the auto-induction medium and the second using the cells produced in the HDF medium. The sole chromatographic step used for both processes was performed with a 30 mL SP-Sepharose column (XK16/20, 5 mL/min of flow rate).

Anion exchange chromatography followed by cation exchange chromatography

The step of anion exchange chromatography was added to the purification process to improve yield and purity. Before chromatography, refolded samples were adjusted to pH 5.2, stored overnight at 4 °C, and centrifuged at 17,817*g* for 30 min at 4 °C to remove the precipitated material. The supernatant was diluted in 30 mM Tris buffer with 50 mM NaCl, pH 8.0, filtered with a 0.45 μm membrane, and loaded into a Q-Sepharose FF column (30 mL, XK 16/40) at a 5 mL/min flow rate. All chromatography buffers were also filtered with a 0.45 μm membrane. The column was equilibrated with 30 mM Tris buffer, pH 8.0, with 50 mM NaCl. After loading the sample, the column was washed with 10 CV of equilibrium buffer. The elution was performed in 5CV of 350 mM NaCl in 30 mM Tris buffer, pH 8.0 (Fig. 1).

The Q-Sepharose elution fraction was adjusted to pH 5.0 with acetic acid and the sample was diluted with distilled water to reach the conductivity of the SP-Sepharose binding buffer. An SP-Sepharose column (30 mL, XK 16/20) was equilibrated with 20 mM sodium acetate buffer, pH 5.0, with 50 mM NaCl at a flow rate of 5 mL/min. After loading the sample, the column was washed with 10 CV of equilibrium buffer. The elution was performed in two steps: with 5 CV of 20 mM acetate buffer, pH 5.0, with 350 mM NaCl, and 2 CV of 20 mM acetate buffer, pH 5.0, with 1 M NaCl (Fig. 1).

Analytical methods

Cell viability of the seed batch was performed by plating serial dilutions (10^4 to 10^8) of the cultures in LB-agar. Plates were incubated at 37 °C for 16 h and colony counting was performed.

Cell growth was monitored by OD on a HITACHI U-5100 spectrophotometer using a suitable dilution of the culture. A correlation curve was constructed to convert OD to dry cell weight (DCW): 1 unit of OD at 600 nm equals 0.38 g/L of DCW.

Plasmid stability was evaluated by comparing the number of colonies growing in LB-agar plates with antibiotics and the number of colonies growing in LB-agar plates without antibiotics.

Protein concentration was measured by the bicinchoninic acid method using the BCA protein assay kit (Novagen). Bovine serum albumin (Sigma) was used as the standard.

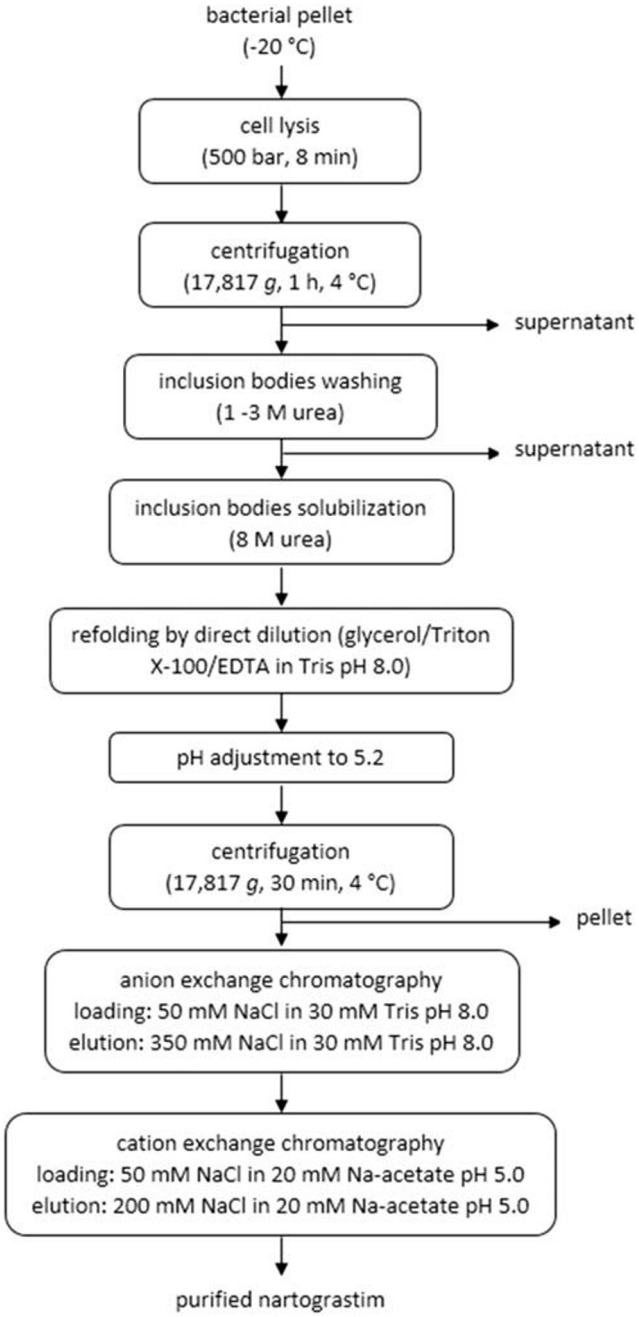

Fig. 1 Final purification process of nartograstim

The relative purity of nartograstim was determined by the densitometry of the SDS-PAGE (15% or 20% gel) bands. After being distained, the gels were scanned in a calibrated densitometer (GS-800, BioRad), and the intensity of protein bands was analyzed using Quantity One 4.6.3 software (BioRad). Equation (5) was employed to calculate the relative purity of the nartograstim. Bacterial pellets taken from 1 mL of culture adjusted to OD of 10 (3.8 g/L DCW) were resuspended in 8 M urea. Purification samples were analyzed by applying 15 μg of total protein to each lane. Resuspended pellets and samples from the purification process (15 μL) were heated to 95 °C for 5 min with 5 μL of sample buffer (Laemmli 1970). The nartograstim concentration was estimated by using Eq. (6).

$$\text{Relative purity}\ (\%) = \frac{\text{Nartograstim band intensity}}{\sum\text{intensity of all bands in lane}} \times 100 \quad (5)$$

$$\text{Nartograstim}\ (\mu g/mL) = \frac{\text{Relative purity}\ (\%) \times \text{protein}\ (\mu g/mL)}{100} \quad (6)$$

To evaluate the nartograstim identity, Western blotting was performed with anti-rhG-CSF mouse serum obtained by Gomes et al. (2012). The secondary antibody was anti-mouse IgG conjugated with peroxidase (Sigma). Detection was performed by using a chemiluminescent substrate (ECL®–GE Healthcare).

Circular dichroism

The secondary structure of nartograstim was analyzed by circular dichroism (CD) spectroscopy, using a JASCO J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). Samples were dialyzed in 10-mM sodium phosphate buffer, pH 7.4, and the measurements were recorded from 190 to 260 nm, using intervals of 0.1 nm in a 0.1 cm path length cell. The CD spectrum was the product of five measurements at 20 °C. The content of the secondary structure was estimated by using the DichroWeb server (Whitmore and Wallace 2008) and the CDSSTR algorithm (Compton and Johnson 1986).

Biological activity

The in vivo biological activity of nartograstim was evaluated by using an induced neutropenia murine model in four groups of eight C57BL/6 female mice. The neutropenia was induced in three groups by inoculating the mice with 4.5 μg of cyclophosphamide diluted in sterile phosphate-buffered saline, pH 7.4 (PBS). During a 4-day period, two of the experimental groups were treated daily, one group with 1.5 μg of nartograstim and the other with 1.5 μg of filgrastim (Granulokine, Roche), both diluted in sterile PBS. The cyclophosphamide group received sterile PBS only for 4 days. Neutropenia was not induced in the fourth group (control), and the animals were injected daily with sterile PBS for 5 days. On the 6th day, mice were anesthetized with an intraperitoneal administration of 10 mg/kg xylazine and 100 mg/kg ketamine and blood samples were obtained by a puncture in the axillary plexus. This protocol was approved by the Instituto Butantan Animal Care and Use Committee (protocol n° 853/11). It is in accordance with standards outlined by Brazilian laws regarding the use of animals for experiments

and with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA).

The blood was transferred to microtubes containing 10% ethylenediaminetetraacetic acid (EDTA), using 50 μL EDTA/mL of whole blood, and the complete blood count was performed. Smears were prepared on glass slides and stained by using the May-Grünwald-Giemsa method modified by Rosenfeld (Santos et al. 2016). The differential leukocyte counts were performed on blood smears prepared immediately after sample collection using a light microscope (magnification × 40). The neutrophil numbers were expressed as a percentage of the total number of white blood cells (WBC) quantified using the automatic blood cell analyzer ABC Vet (HORIBA®, UK).

Statistical analysis

First, data obtained for wet WBC and red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), plaques (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were analyzed to verify if they presented normal distribution. Then, data with normal distribution were analyzed by one-way ANOVA and Dunnett tests. Data that did not display normal distribution were analyzed by Dunn's test. Differences were considered statistically significant when $p < 0.05$.

Results

Bioreactor cultivation

Cultures were performed with auto-induction and chemically defined media. The auto-induction medium resulted in relatively high *E. coli* cell densities in simple batches. The maximum specific growth rate was 0.3 h^{-1} in this medium, and the stationary phase was reached after 30 h of cultivation, when the biomass was 19 g L^{-1} DCW (Fig. 2a). The consumption of glycerol and lactose started when glucose was exhausted after 18 h of cultivation (Fig. 2a). The plasmid pAR-KanI presented high stability before and after induction (100% kanamycin-resistant colonies at 31 h), even without adding antibiotics to the medium, endorsing our previous results in flask cultures (Eguia et al. 2018). SDS-PAGE and Western blotting of samples showed that the nartograstim band of 18.8 kDa appeared after 25 h of cultivation (Fig. 2c). Acetate production remained below the detection level (data not shown). The relative percentage of nartograstim band was 4% at the end of cultivation, representing a final yield of 1.17 g/L. The total wet biomass obtained after centrifugation was 292 g, representing 28 mg of nartograstim per gram of wet cells. These data showed that nartograstim can be produced satisfactorily at a large scale by using pAR-KanI as an expression vector and the auto-induction medium.

The cultivation using a chemically defined medium was performed in fed-batch mode and a high cell density of *E. coli* was reached. The feeding started at 20 h of cultivation, when initial glycerol had been consumed and the biomass had reached 17 g L^{-1} DCW (Fig. 2b). After 32 h of cultivation, the OD reached 164 (62 g L^{-1} DCW) and the culture was induced with a mixture of lactose and IPTG (Fig. 2b). Acetate production remained ≤ 0.4 g/L (Fig. 2b). The maximum specific growth rate of 0.29 h^{-1} was reached during the batch phase, and the specific growth rate, reached during the fed-batch phase, was 0.19 h^{-1}. The yield factor on glycerol was 0.54 $g_{biomass}/g_{consumed\ glycerol}$. The stability of pAR-KanI dropped to 17% at the end of the cultivation process even though the culture was carried out with kanamycin. The metabolic burden was likely too high to maintain both the high-copy-number plasmid replication and the protein synthesis in a chemically defined medium, because the bacteria must synthesize all macromolecules solely from glucose and ammonium. Although nartograstim 18.8-kDa bands could not be clearly observed in SDS-PAGE (Fig. 2d), the Western blot confirmed its production after 34 h of cultivation, 2 h after induction (Fig. 2d). The relative percentage of nartograstim in the cell lysate was 9%, resulting in a final yield of 0.95 g/L. After centrifugation, 2100 g of wet biomass was obtained, representing an estimated production of 4 mg of nartograstim per gram of wet cells. Cells were stored at − 20 °C until use.

Inclusion bodies isolation

One hundred grams of biomass was suspended in 1 L of lysis buffer and disrupted in a continuous high-pressure homogenizer. After centrifugation, the supernatant was discharged; IBs were washed with increasing urea concentrations aiming to remove contaminant proteins and solubilized in a buffer with 8 M urea. Nartograstim bands on SDS-PAGE were clearly observed in samples of cell lysis and 8 M urea only (Fig. 3, top); however, Western blot showed increasing amounts of nartograstim bands in the supernatant with 4 M urea (Fig. 3, bottom) and, therefore, this last wash was eliminated from the purification process. Nonetheless, it is important to point out that the nartograstim loss in the washing steps was very low because it was detected by Western blot only (Fig. 3, bottom) and not by Coomassie staining (Fig. 3, top).

Comparison of refolding methods

Different refolding methods were evaluated (Table 1). The diafiltration accomplished to remove urea was performed by maintaining a constant volume of sample in the reservoir. However, most of the nartograstim returned to its insoluble form after the urea removal; only 3.1% of the molecule

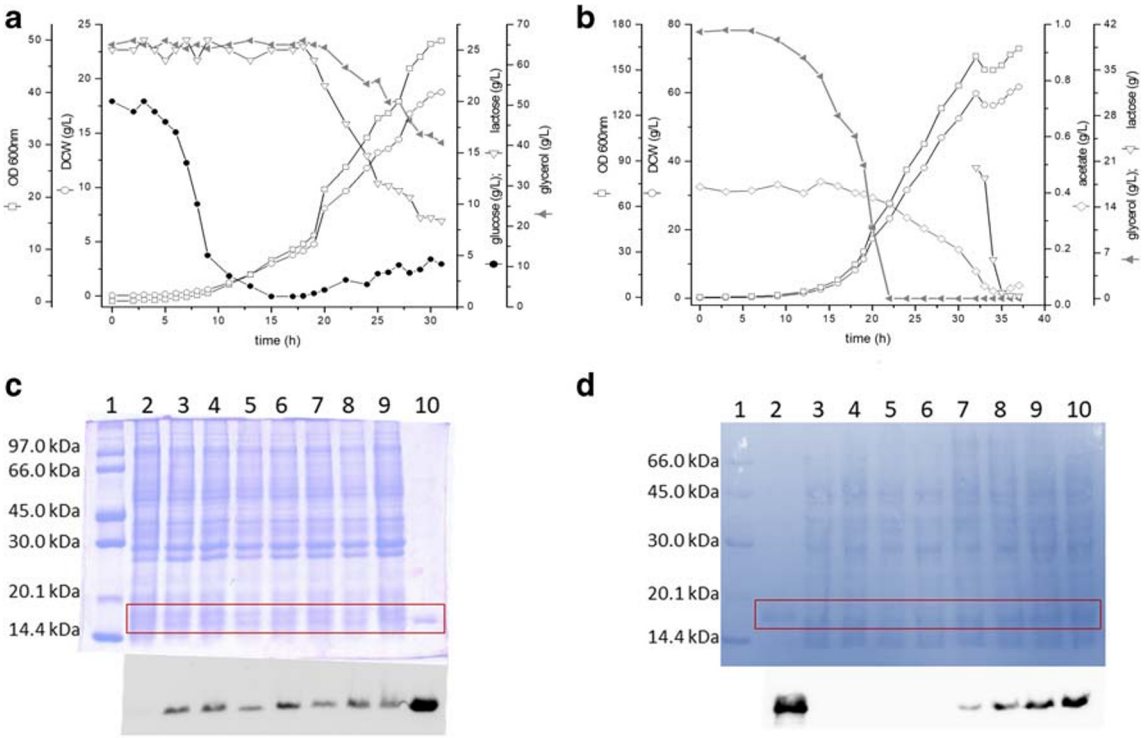

Fig. 2 *E. coli* BL21 (DE3) STAR pLysS cultures for nartograstim production, using two different conditions: **a** auto-induction batch and **b** chemically defined medium with glycerol and combined induction with 0.5 mM of IPTG and 20 g/L of lactose, fed batch. Acetate (diamonds), OD 600 nm (squares), dry cell weight (open circles), lactose or glycerol (triangles), and glucose (solid circles). SDS-PAGE (15% gel, top) and Western blotting (bottom) of culture samples from **c** auto-induction and **d** chemically defined media. In **c**: (1) molecular marker, (2) 18 h of cultivation, (3–9) 25 h to 31 h of cultivation, (10) nartograstim standard. In **d**: (1) molecular marker, (2) nartograstim standard, (3–4) 28 h and 30 h of cultivation, (5–10) 32 h to 37 h of cultivation. Nartograstim expected band size is indicated by the red box

remained in the soluble fraction post-centrifugation. Neither was the refolding by dialysis effective in refolding nartograstim since barely 2.4% of the protein remained in the soluble fraction. These results suggest that the nartograstim concentration may influence the refolding process in these experiments.

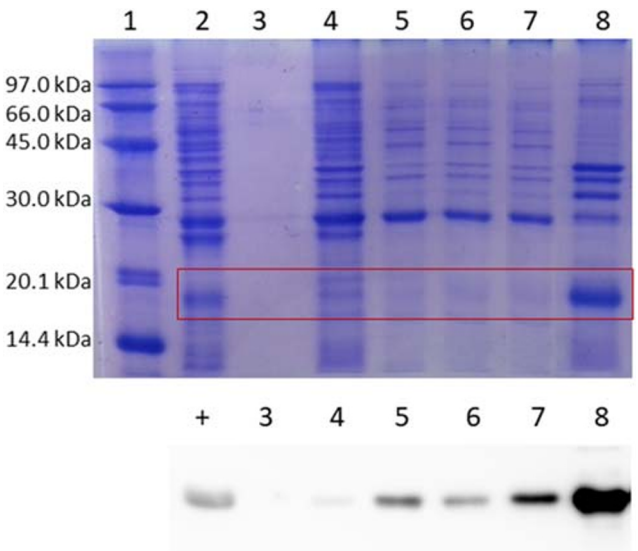

Fig. 3 SDS-PAGE (15% gel, top) and Western blotting (bottom) of the inclusion body recovery samples from the auto-induction medium. (1) Molecular marker; (2) cell lysate; (3) supernatant of cell lysis; (4–7) supernatant of IB washing steps with 1, 2, 3, and 4 M urea, respectively; (8) IB solubilized with 8 M urea; (+) nartograstim standard. Nartograstim expected band size is indicated by the red box.

To evaluate the influence of nartograstim concentration on the efficiency of refolding by dialysis, an 8-M urea sample was diluted to between 11 and 378 μg/mL before performing dialysis. Precipitation was observed in all of the concentrations evaluated. However, a higher refolding efficiency was achieved with lower protein concentration, probably because the molecules have fewer opportunities to interact with each other through hydrophobic regions or to form disulfide bonds, leading to aggregation and precipitation. The highest refolding

Table 1 Efficiency of rhG-CSF refolding by different methods

Refolding method	% refolding
Diafiltration 5 kDa	3.1
Dialysis	2.4
Dialysis with previous sample dilution (11 μg/mL)	96
Dialysis with previous sample dilution (23 μg/mL)	80
Direct dilution in Tris/arginine buffer	33
Direct dilution in Tris/glycerol buffer	99

efficiency (96%) was obtained with 11 μg/mL protein, followed by 80% with 23 μg/mL protein (Table 1).

Refolding by direct dilution with Tris/arginine buffer was more efficient than by diafiltration and dialysis with concentrated samples. It reached 33% of refolding when the protein concentration was approximately 40 μg/mL. However, its refolding efficiency was still lower than that of dialysis with diluted samples (Table 1).

Direct dilution with Tris/glycerol buffer was highly efficient, achieving 99% refolding (Table 1). Since there was no pellet at all, this method presented no loss of nartograstim post-centrifugation of the diluted material. It also allowed for the refolding of the highest protein concentration, 550 μg/mL. Hence, refolding by direct dilution with Tris/glycerol buffer solubilized 24 times higher protein concentration than refolding by dialysis, which solubilized, at best, 11–23 μg/mL, and 14 times higher protein concentration than refolding by direct dilution with Tris/arginine buffer. Therefore, direct dilution with Tris/glycerol buffer was selected as the refolding method to be used for the purification process.

Post-refolding pH adjustment and loading into cation exchange chromatography

To evaluate the highest pH for loading nartograstim into SP-Sepharose, samples of nartograstim refolded by direct dilution with Tris/glycerol buffer were adjusted to pH 4.5, 4.7, 5.0, 5.2, and 5.5. They were incubated overnight at 4 °C and centrifuged (Fig. S2). Pellets and supernatants were analyzed by SDS-PAGE. The pH adjustment removed contaminant proteins of approximately 30–45 kDa in the pellet, while most of the nartograstim remained in the supernatant (Fig. 4). As a result, the nartograstim purity in the supernatant increased to 78% at pH 5.2 (Fig. 4, lane 8). Therefore, pH adjustment before chromatography was included in the purification process (Fig. 1).

The supernatant from each pH tested was applied to a pre-equilibrated SP-Sepharose column and elution was performed with 100 and 500 mM NaCl in 20 mM Tris buffer, pH 8.0 (Fig. S2). The highest pH that permitted binding nartograstim to the resin was pH 5.0, consequently decreasing the adsorption of contaminant proteins.

Purification from cells produced in auto-induction and chemically defined media

After defining the most effective pH for loading nartograstim into SP-Sepharose (Fig. S3), the established methods of nartograstim purification were evaluated by using 25 g of biomass obtained with both auto-induction and HDF medium. To this end, the following purification conditions were employed: refolding by direct dilution in Tris/glycerol buffer (50 mL of material solubilized in 8 M of urea diluted in 1 L of buffer), pH adjusted to 5.0, cation exchange in SP-Sepharose equilibrated at pH 5.0, and elution with 100 mM NaCl also at pH 5.0. In both cases, the recovery of nartograstim was less than 0.5%, yielding less than 50 μg of nartograstim per gram of wet cells, and its final purity was approximately 80% (Table 2).

Chromatography in Q-Sepharose followed by SP-Sepharose

SP-Sepharose chromatography resulted in the recovery of low amounts of nartograstim and purity below the required value (> 90%). Therefore, an anion exchange chromatography in Q-Sepharose FF was included in the purification process. Considering the results found in Q-Sepharose chromatography (Fig. S4), the elution was performed directly with 350 mM NaCl in 30 mM Tris buffer, pH 8.0, eliminating the steps with 100 and 200 mM NaCl. This increased the recovery and purity to 53.2% and 87.3%, respectively (Table 3). The second chromatography (SP-Sepharose) resulted in 6.2% recovery and 91.8% purity (Table 3). Although a lower final purity was achieved than in the former process (Fig. S4), the global recovery was higher: 2.2% as compared to 0.2%, thereby increasing the recovery from 24 μg/g to 0.3 mg/g of nartograstim per wet cells. Taking into account all of

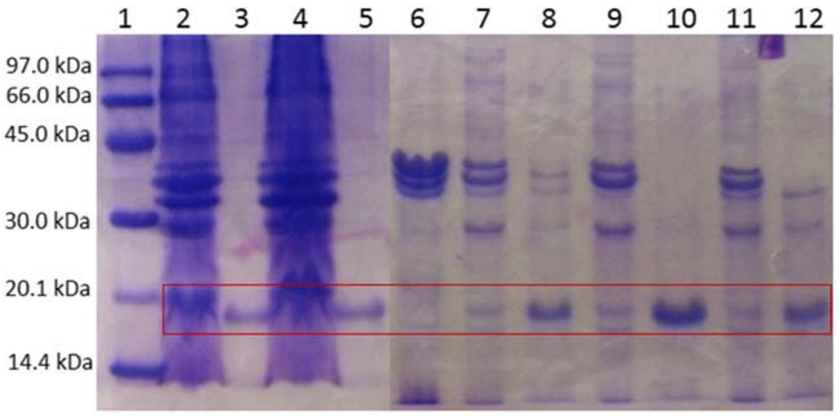

Fig. 4 SDS-PAGE (15% gel) of samples taken after pH adjustment of refolded nartograstim: (2, 4, 6, 8, and 10) pellets from pH 4.5, 4.7, 5.0, 5.2, and 5.5; (3, 5, 7, 9, and 11) supernatants from pH 4.5, 4.7, 5.0, 5.2, and 5.5. Nartograstim expected band size is indicated by the red box

Table 2 Comparison of purification steps of nartograstim (using one chromatographic step) from biomass of cultures in auto-induction and HDF medium

Fraction	Total protein (mg)		Relative purity (%)		Nartograstim (mg)		Yield ($mg_{nartograstim}/g_{wet\ cells}$)		Step recovery (%)		Total recovery (%)	
	AI	HDF	AI	HDF	AI	HDF	AI	HDF	AI	HDF	AI	HDF
Homogenate	5036	4575	7.4	8.8	372	403	14.9	16.1	100	100	100	100
Solubilized	624	1120	48	28	315	304	12.6	12.2	81	78	84	78
Refolded	623	966	51	32	301	307	12.0	12.3	105	98	80	76
pH adjusted	603	810	70	49	422	394	16.9	15.8	134	129	113	98
Fraction SP-sepharose 100 mM	1.62	0.8	78	79	1.0	0.6	0.04	0.02	0.3	0.1	0.25	0.15

the purification steps studied, this was considered the best purification strategy (Fig. 1).

Circular dichroism

Eluted fractions were concentrated and dialyzed against a 10-mM phosphate buffer, pH 7.4, and the secondary structure of the purified nartograstim was analyzed. The circular dichroism spectra, 185 nm to 260 nm, were composed predominantly of alpha-helix (Fig. 5), as expected from previously published data (Lu et al. 1989). The deconvolution of spectra by the CDSSTR algorithm showed the putative secondary structure of nartograstim, which was obtained by using both complex and chemically defined media and purified through one or two chromatographic steps, all in accordance with other findings (Gomes et al. 2012; Vemula et al. 2015b).

Biological activity

The biological activity of nartograstim was evaluated by using a murine model of induced neutropenia. One group of mice received filgrastim treatment (granulokine), a second group received nartograstim treatment, and a third group received cyclophosphamide and PBS instead of treatment. In the fourth group, neutropenia was not induced and mice received only PBS (control). After the experiment, blood was collected and slides were prepared for the differential count of leucocytes. An automatic blood cell analyzer was employed to measure the hematological parameters. The data obtained for RBC, HGB, HCT, and PLT showed normal distribution, while MCV, MCH, and MCHC data did not display normal distribution.

Treatment of mice with nartograstim, or with a commercial filgrastim (granulokine), significantly increased the WBC count and neutrophil percentage when compared to the control groups (Fig. 6). As expected, cyclophosphamide administration induced neutropenia due to its myelotoxicity (Fig. 6b). Despite neutropenia, it is important to emphasize that the total number of leucocytes was not significantly reduced (Fig. 6a), because lymphocytes, as part of WBC, were not affected by cyclophosphamide, neither in bone marrow nor in lymphoid organs. Since the half-life of lymphocytes (weeks or months) is longer than the half-life of neutrophils (approximately 6 h) in the bloodstream, lymphocytes kept circulating and became the predominant cells in the blood (Babior and Golde, 2011). Hence, the WBC count remained the same before and after cyclophosphamide treatment. When neutropenic-induced mice were treated with nartograstim or filgrastim, this situation was reversed (data not shown). Consequently, the WBC count is augmented due to the increase of neutrophils (Fig. 6a).

There were no statistically significant differences in all other hematological parameters analyzed among the groups (data not shown), which means that the treatment with either nartograstim or filgrastim did not affect other hematological parameters. Moreover, the in vivo biological activity of nartograstim was statistically indistinguishable from that of

Table 3 Purification of nartograstim using two chromatographic steps

Fraction	Total protein (mg)	Relative purity (%)	Nartograstim (mg)	Yield ($mg_{nartograstim}/g_{wet\ cells}$)	Step recovery (%)	Total recovery (%)
Homogenate	5565	5.5	306	12.2	100	100
Solubilized (8 M urea)	372	58.2	216	8.6	70.7	70.7
Refolded (Tris/glycerol buffer)	284	73.5	209	8.4	96.5	68.2
Fraction Q-Sepharose 350 mM	127	87.3	111	4.4	53.2	36.3
Fraction SP-sepharose 350 mM	7.2	91.8	6.6	0.3	6.2	2.2

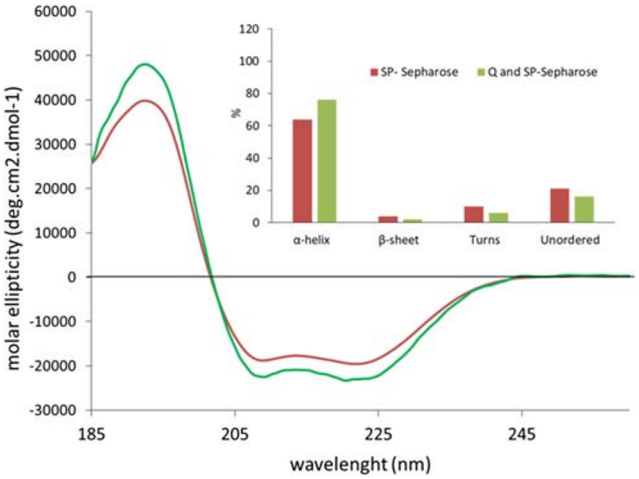

Fig. 5 Circular dichroism (CD) spectra of nartograstim purified by cation exchange chromatography from the batch culture with auto-induction medium (red curve) and by anion and cation exchange chromatography from the fed-batch culture with HDF medium (green curve). The inset shows the percentage of each secondary structure estimated by the deconvolution of spectra using the CDSSTR algorithm

commercial filgrastim, both being capable of increasing WBC count and neutrophils after five days of treatment.

Discussion

The novel plasmid pAR-KanI has been previously shown to be very stable in flasks, even without the addition of antibiotics to the culture medium (Eguia et al. 2018). Based on this fact, this work aimed to evaluate whether the novel plasmid pAR-KanI could be employed to produce and purify nartograstim using methods compatible with industrial-scale production. The culture media evaluated here had been previously employed for other authors to cultivate recombinant *E. coli*. Vélez et al. (2014) compared cultures in fed-batch mode with chemically defined medium induced by IPTG to cultures in batch mode with auto-induction medium induced by lactose, reporting that the second process yielded higher biomass and protein production. Campani et al. (2016) used the auto-induction medium in batch mode to obtain PspA4Pro protein in a bioreactor, reporting 30 g/L of dry biomass. Figueiredo et al. (2017) evaluated chemically defined and auto-induction media to produce PspA4Pro and showed that the auto-induction medium presented a higher specific growth rate, higher productivity, and higher specific production (mg PspA4Pro/g cells). In contrast, the chemically defined medium displayed higher absolute protein production and plasmid stability (Figueiredo et al. 2017). Except for the plasmid stability, the results of this work are in accordance with the aforementioned data, thereby demonstrating the feasibility of both cultivation methods for the industrial scale.

The expression vector pAR-KanI is derived from a high-copy-number plasmid. It requires more energy from cells for replication than low-copy plasmids such as pET vectors. Consequently, the limited nutrient amount of the chemically defined medium is unfavorable to pAR-KanI stability. This is likely due to the metabolic burden that occurs during induction in which cells spend part of their energy to synthesize the recombinant protein (Goyal et al. 2009; Kilikian et al. 2000; Silva et al. 2012). Neither can they maintain gene replication and plasmid partition to daughter cells during division (Ow et al. 2006; Wang et al. 2006), thus reducing cell growth and producing plasmid instability during induction.

The auto-induction medium offers some advantages for recombinant protein production in *E. coli*. First, it promotes rapid cell growth so that batches reach relatively high cell density. Second, it is a much simpler bioreactor operation mode than a fed-batch. Third, because lactose is present at the beginning of cultivation and starts to be consumed when

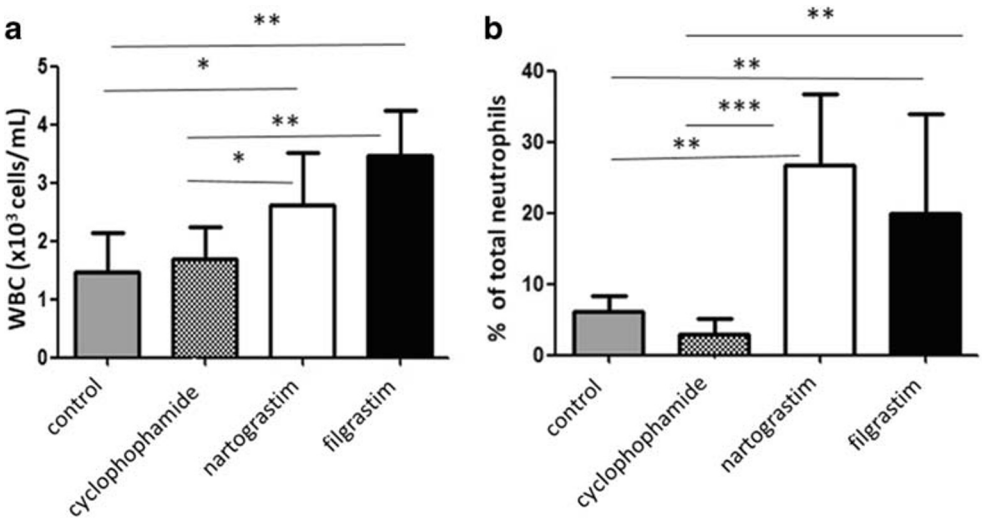

Fig. 6 Nartograstim biological activity. **a** concentration of white cells of blood (WBC) and **b** percentage of neutrophils in the four experimental groups indicated in abscissas ($n = 8$ in each group). Mean and standard deviation are shown for each sample. The statistical significance was analyzed by a one-way ANOVA, Dunnett test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$

glucose is exhausted, the auto-induction medium does not require the definition of the exact moment at which to add the inducer. Studier (2005) showed that cultures of recombinant *E. coli* in the auto-induction medium enabled high protein production without the need to monitor for the induction moment. Specific to this work, the fourth advantage of using an auto-induction medium is the nartograstim production without any antibiotic in the culture medium. Nartograstim has already been produced in shaker flask cultures without the addition of antibiotics to the auto-induction medium (Eguia et al. 2018). This study demonstrates that nartograstim can be also obtained in a bioreactor with similar culture conditions. Nevertheless, nartograstim per gram of cells is still lower than that obtained in flasks; thus, new strategies should be further evaluated to increase the production, for example, by adding lactose after the initial amount is exhausted. In the literature, there are articles showing the use of complex medium LB with an antibiotic in batch culture to obtain rhG-CSF (Rao et al. 2008; Vemula et al. 2015b). Antibiotic is an expensive reagent and may lead to resistance. Therefore, it must be eliminated from the final product during recombinant protein production for therapeutic use (Vidal et al. 2008). To the best of our knowledge, this study is the first to use the auto-induction medium to produce rhG-CSF in a bioreactor without antibiotics. Due to the absence of antibiotics and IPTG, this strategy could decrease the cost of rhG-CSF production. Moreover, a final yield of 1.17 g/L of nartograstim was obtained. This is much higher than the 1.2 mg/L obtained at the laboratory scale described in the literature (Peymanfar et al. 2016). Values higher than 10 g/L were reported for rhG-CSF (Babaeipour et al. 2017; Khalilzadeh et al. 2008). This demonstrates the possibility of increasing nartograstim production.

Chemically defined medium is commonly used to reach high cell densities of recombinant *E. coli* in a bioreactor. This medium has been used specifically to obtain rhG-CSF in fed-batch, always together with the appropriate antibiotic as a selection marker (Babaeipour et al. 2015; Babaeipour et al. 2017; Dasari et al. 2008). In this work, glycerol was selected as the carbon source of the chemically defined medium to avoid acetate production. Indeed, acetate concentration was lower than 0.4 g/L throughout the cultivation, which is below previously reported inhibitory concentrations (Han and Eiteman 2019). Korz et al. (1995) observed lower acetate formation with glycerol than with glucose as the carbon source. Glycerol has been used as a carbon source instead of glucose with satisfactory results because it is less acidogenic than glucose (Carvalho et al. 2012; Xiao et al. 2018). Therefore, using the fed-batch strategy with a chemically defined medium, which is cheaper than an auto-induction medium (Cardoso et al. 2020), would compensate for the need for antibiotics with higher cell and protein concentration. Moreover, the fed-batch has successfully been used for the industrial production of pharmaceutical products (Kopp et al. 2019).

The maximum specific growth rate was 0.3 h^{-1} in both of the media evaluated in the study. Interestingly, Rao et al. (2008) cultured *E. coli* in a 3-L fed-batch reactor to obtain rhG-CSF using a complex medium with ampicillin and employed a feeding rate of 0.3 h^{-1} to guarantee low acetate production and avoid plasmid loss.

Previous data showed that protein synthesis was slower with lactose than with IPTG (Eguia et al. 2018). This is likely because the lac operon must be derepressed before lactose enters into the cell and induces protein synthesis (Einsfeldt et al. 2011). This study confirmed that the induction of protein synthesis is slower with lactose, which explains the absence of nartograstim in the first samples taken from the auto-induction cultures. However, protein yield can be higher with lactose, which is low cost, acts as a carbon source, and does not inhibit cell growth (da Silva et al. 2013). Many researchers have been using lactose instead of IPTG as an inducer with promising results (Campani et al. 2016; Hausjell et al. 2020; Horta et al. 2012; Vélez et al. 2014). Nonetheless, it is clear that both cultivation strategies leave room for the optimization of nartograstim synthesis.

There are several methods to refold recombinant proteins, and four of them were applied to refold rhG-CSF: (1) dialysis (Gomes et al. 2012; Vanz et al. 2008); (2) diafiltration (Dasari et al. 2008); (3) direct dilution (Babaeipour et al. 2015; Chen et al. 2008; Kim et al. 2014; Rao et al. 2008; Vemula et al. 2015a, 2015b); and (4) chromatography (Wang et al. 2009). In this work, dialysis was useful for determining the maximum protein concentration allowed for reaching the highest refolding percentage, approximately 20 μg/mL. Zhao et al. (2014) also showed that lower protein concentration resulted in higher rhG-CSF refolding efficiency and specific activity. Hence, perhaps diafiltration would have been more effective than dialysis if it had been performed in different conditions, such as the use of 20 μg/mL of protein concentration, or if it had been carried out against Tris/glycerol buffer and 550 μg/mL protein, which was reached by direct dilution with this buffer.

Direct dilution is the most commonly used method to refold rhG-CSF obtained at any production scale. However, it is mostly applied on a large scale due to several factors. First, it does not require a membrane so it is cheaper than diafiltration. Second, direct dilution only requires control of the flow rate, which is easier than diafiltration, which requires both the control of the flow rate and the transmembrane pressure. Finally, there is no risk of membrane fouling and loss of protein due to membrane polarization. Chen et al. (2008) employed direct dilution in 0.75 M arginine and reported 65% of refolding. Vemula et al. (2015b) defined a refolding buffer with 10% glycerol as the best condition to refold rhG-CSF, but the refolding percentage was not reported. In this study, direct dilution in Tris/arginine buffer yielded 33% refolding. Arginine is a stabilizer agent in concentrations between 0.5

and 2.0 M. It is employed for refolding proteins by dilution and dialysis because it was hypothesized that guanidine groups of arginine interact with tryptophan residues of proteins to suppress protein aggregation (Tsumoto et al. 2004). Indeed, it was demonstrated that arginine acts to suppress the aggregation of G-CSF caused by intermolecular interactions between hydrophobic regions (Chen et al. 2008). There were aggregates in the presence of 1.0 M arginine (Chen et al. 2008), which could explain why only 33% refolding in the buffer with 0.5 M arginine was reached. The dilution method was applied to refold rhBMP-2 (bone morphogenetic protein-2) with 20 μg/mL of protein concentration, which is lower than was used in this research. One study obtained 60% refolding (Vallejo et al. 2002) and another did not report the refolding efficiency (Gieseler et al. 2017).

Direct dilution in Tris/glycerol buffer yielded near 100% refolding efficiency even when diluting 50 or 200 mL of solubilized material in 8 M urea. Glycerol is also a stabilizer agent employed for refolding by direct dilution (Tsumoto et al. 2004). It was proposed that glycerol interacts with the hydrophobic regions of proteins, forming an amphiphilic interface between the hydrophobic protein surface and the polar solvent, thus avoiding protein aggregation (Vagenende et al. 2009). The optimal protein concentration to refold nartograstim by dialysis proposed by this study is 20 μg/mL, similar to the rhBMP-2 concentration proposed by Vallejo et al. (2002) and Gieseler et al. (2017). Nonetheless, refolding by the dilution of 200 mL of solubilized material in the Tris/glycerol buffer produced a much higher protein concentration, 550 μg/mL, at the end of the refolding process. However, when this protein concentration was reached, the urea concentration remained between 1.0 M and 1.5 M, which interfered in the ion exchange chromatography thereafter. Therefore, it was necessary to decrease the volume of solubilized material from 200 to 50 mL to refold nartograstim in 1 L Tris/glycerol buffer and reach the < 0.4 M urea needed to perform the chromatography. Zhao et al. (2014) compared the efficacy of the dilution and diafiltration methods for refolding rhG-CSF and observed a higher refolding percentage with direct dilution. These authors also evaluated protein concentrations of between 100 and 1000 μg/mL and achieved better-refolding results with lower protein concentrations. In contrast, Kim et al. (2014) determined that a protein concentration of 1 mg/mL must be reached during dilution.

Kim et al. (2013) adjusted pH to 5.5, 6.5, and 7.5 during rhG-CSF purification to evaluate contaminant protein removal. Similarly, samples were adjusted to between pH 4.5 and 5.5 in this work, and precipitation was observed in all of the samples.

Several chromatographic methods have been proposed to purify rhG-CSF after refolding: hydrophobic interaction (Wang and Geng 2012), molecular exclusion (Babaeipour et al. 2015), and ion exchange. Generally, an additional step of chromatography is used to achieve adequate purity (Babaeipour et al. 2015; Dasari et al. 2008; Kim et al. 2014; Rao et al. 2008). Gomes et al. (2012) only employed cation exchange in SP-Sepharose and rhG-CSF was eluted with 100 mM NaCl in 20 mM phosphate buffer, pH 8.0, but the authors reported neither protein recovery nor its final purity. Rao et al. (2008) also employed SP-Sepharose as the sole chromatographic step and they reported that rhG-CSF was eluted with 100 mM Tris buffer, pH 8.0. These authors reported low recovery, without detailing the actual percentage, due to the aggregation that resulted from the contact between protein and elution buffer, which would have activated the residue of free cysteine (Cys_{17}) and produced intra-and-intermolecular-formation of disulfide bonds. It is worth emphasizing that this kind of aggregation does not occur with nartograstim since nartograstim has a serine instead of a cysteine at position 17. It is also important to highlight that the number of purification steps affects production costs. Nevertheless, additional steps must be included if the purity requirement is not reached.

There is a deficit of information in the literature about the recovery of recombinant proteins obtained from IBs. The recovery of active proteins is low when they are obtained from IBs, and conventional methods such as dialysis and dilution are used for refolding (Yamaguchi and Miyazaki 2014). One exception is the work of Gieseler et al. that reported a 2% global recovery of active proteins and protein purity higher than 90%. These results were obtained by using cation exchange chromatography to purify rhBMP-2 from IBs (Gieseler et al. 2017). In this work, cation exchange chromatography was used to purify nartograstim from the material obtained by different refolding methods. However, all of the chromatography performed resulted in low recovery. Considering only the IBs that were refolded by direct dilution in Tris/glycerol buffer, the highest global recovery and purity achieved after two steps of chromatography were 2.2% and 91.8%, respectively. These results are similar to those reported for rhBMP-2 (Gieseler et al. 2017).

The biological activity in vivo shows that nartograstim increased the production of neutrophils, thereby increasing the WBC count. No difference was observed between nartograstim and the commercial filgrastim granulokine, which has been frequently used to treat neutropenia. These results are similar to those previously reported in a preclinical study with non-human primates, which showed that the activity and the pharmacokinetic properties of filgrastim were equivalent to those of nartograstim (Tanaka et al. 1997).

Therefore, despite the relatively low recovery, which is comparable to the scarce data found in the literature, the secondary structure of nartograstim, when purified from cells produced in auto-induction or chemically defined medium, displayed expected conformation and biological activity. Moreover, nartograstim was produced in an antibiotic-free

medium in bioreactor, which could decrease both its production costs and its environmental footprint.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-020-11014-y.

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Authors' contributions F.A.P.E. conducted experiments and wrote the manuscript. D.E.M., G.R.R., E.M., and C.L. conducted experiments. E.C. conceived the research and contributed with analytical tools. P.B. contributed with reagents and analytical tools and supervised animal experiments. P.L.H. supplied the plasmid and revised the manuscript. G.C.B. and V.M.G. conceived and designed the research, supervised the experiments, and wrote the manuscript. F.A.P.E., E.C., P.B., G.C.B., and V.M.G. analyzed the data. All authors read, revised, and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The protocol for animal experiments was approved by the *Instituto Butantan* Animal Care and Use Committee (protocol n° 853/11) and is in accordance with Brazilian law regarding the scientific use of animals and with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA).

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