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RESEARCH

Physicochemical and biological characterization of Transfer Factor and consistency of its production process

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ABSTRACT

Transfer Factor (TF) is a complex drug applied in cellular immunodeficiencies, based on peptides isolated from human lymphocytes. The TF production process combines its extraction from lysed lymphocytes, followed by tangential flow filtration, viral inactivation by pasteurization and sterile filtration. In this work, TF was characterized by physicochemical and biological procedures, while the process consistence was also analyzed. As results; the average peptide concentration was 2.45 ± 0.28 mg/mL, DTH was 1.69 ± 0.02 mm (control 1.55 ± 0.05 mm, p = 0.3928), the indirect pyrogen measurement by rabbit body temperature was 36.5 ± 0.2 °C, endotoxin content was below 0.625 UE/mL. Furthermore, the SDS-PAGE profile showed a main band around 17 kDa, obtained with a purity higher than 95%, and 2.23 ± 0.26 ng/dose DNA. The size-exclusion-HPLC was able to separate up to nine fractions, while the dynamic light scattering profiles revealed only the presence of two peptide fractions (100-1000 nm, 1000-10 000 nm). The process was reproducible among batches, according to retention times results, as detected by size exclusion-HPLC. Peptide concentration showed very small variation among the different steps of the purification process: starting material (5.62 ± 1.21 mg/mL), diafiltered (2.75 ± 0.13 mg/mL) and final product (2.54 ± 0.18 mg/mL). Altogether, the Hebertrans® TF batches were shown to fulfill all the approved quality attributes and the purification process showed a high degree of reproducibility and consistency.

Keywords: Transfer Factor, Hebertrans®, immunodeficiency, biopharmaceutical products

RESUMEN

Caracterización biológica y físico-química del Factor de Transferencia y consistencia de su proceso de producción. El factor de transferencia es un fármaco complejo aplicado para el tratamiento de inmunodeficiencias celulares, a base de péptidos aislados de linfocitos humanos. Se obtiene al combinar la extracción de linfocitos lisados, la filtración de flujo tangencial, la inactivación de virus por pasteurización y la filtración estéril. En este trabajo se caracterizó fisicoquímica y biológicamente al factor de transferencia y la consistencia de su proceso productivo. Los análisis mostraron los siguientes resultados: concentración promedio de péptidos de 2,45 ± 0,28 mg/mL; DTH de 1,69 ± 0,02 mm (control 1,55 ± 0,05 mm, p = 0,3928); medición indirecta de pirógenos con una temperatura corporal en conejos de 36,5 ± 0,2 °C; contenido de endotoxinas menor de 0,625 UE /mL. En la caracterización molecular, el perfil de SDS-PAGE reveló la presencia de una banda principal entre 15 y 20 kDa (pureza > 95 %), y un contenido de ADN de 2,23 ± 0,26 ng/dosis. Se observaron hasta ocho fracciones mediante cromatografía de exclusión molecular-HPLC, mientras que el perfil de dispersión dinámica de la luz mostró dos fracciones peptídicas (100-1000 nm, 1000-10 000 nm). Hubo reproducibilidad entre los lotes con respecto a los tiempos de retención detectados mediante HPLC de exclusión molecular. La concentración peptídica mostró una variación muy pequeña entre etapas del proceso de purificación: material de partida (5,62 ± 1,21 mg/mL), diafiltrado (2,75 ± 0,13 mg/mL) y producto final (2,54 ± 0,18 mg/mL). En resumen, los lotes de TF Hebertrans® cumplieron con todos los requisitos de calidad aprobados y el proceso de purificación mostró un alto grado de reproducibilidad y consistencia.

Palabras clave: Factor de transferencia, Hebertrans®, inmunodeficiencia, productos biofarmaceutical

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Introduction

Transfer factor (TF) is a mixture of approximately 200 substances isolated from human lymphocytes, including peptides with a molecular weight ranged 3500-6000 kDa and oligoribonucleotides attached to peptides [1]. This pharmaceutical product is able to transfer antigen -specific signals for cell-mediated immunity (CMI), its transference of immunity being assumed as resulting from the action of small molecules [2].

More precisely, TF is produced by CD4+Th1-lymphocytes during the immune response to a target antigen. TF biological activity depends on antigen-specific inducing, suppressing and non-specific (adjuvant-like) components [3, 4]. The inducing fractions enhance the antigenic stimulus, leading to the production of gamma-Interferon (IFN-γ), Interleukin-2 (IL-2) and Tumour Necrosis Factor-α (TNF- α) by CD4+Th1-

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lymphocytes. Besides, the CMI developed against the target antigen also induces the expression of Interleukin 6 (IL-6) and Interleukin 8 (IL-8) by activated monocytes. The regulation of IL-6 and IL-8 and TNF-α production is associated with Toll-like receptor (TLR2 and TLR4) expression, nuclear factor kappa-light-chain-enhancer of activated B-lymphocytes (NF-kB) and cyclic adenosine monophosphate activities [5-7].

Therefore, the clinical use of TF can be associated with the function of several immune components and with the regulation of cytokine synthesis [7]. An important advantage of using TF as therapeutic agents is that it may induce a rapid immune response against pathogens (within 24 h), and, thereby, reducing the time for the patient's immune response of 9-13 days in one day, against the common 10 to 14-day period. Another significant aspect of TF application in major diseases (such as cancer, rheumatoid arthritis, hepatitis, heart diseases, Alzheimer, and others), is that the formation of TF by CD4+Th1-lymphocytes has been observed [5].

During its production process, TF can be isolated from different biological sources by dialysis or ultrafiltration of disrupted human lymphocytes [8, 9]. TF can also be obtained from ultrafiltrated cryolysed lymphocytes or lymphoid organs (lymph nodes and spleen) from different animal species [8-13], bird eggs [14], and bovine colostrum [15]. Furthermore, raw TF preparations can also been purified using different chromatography types [16], with heterogeniety of the biological starting material having as its main limitation. This derives from the 20-40 % proportion that lymphocytes subsets represent among white blood cells, and the differences cuased by ethnicity, gender and lifestyle habits, with the health adult lymphocyte levels in the range 0.80-4.00 × 10^9/L [17].

In the case of Hebertrans®, it is a TF product manufactured from human blood by the Center for Genetic Engeneering and Biotechnology of Havana, Cuba [18]. However, data about the consistency of its manufacturing process as to deliver a high quality product able for human use, while guarateed, has not been previously reported. Therefore, in this work, the consistency and reproducibility of the Hebertrans® production process were assessed, including its physical, chemical and biological properties.

Materials and methods

Buffy-coat isolation from human lymphocytes

Certified human whole blood extracted from healthy donors with less than 48 h of extraction was employed to isolate buffy-coats. Whole blood was centrifugated at 2548 × *g* during 7 min at 2-8 °C, and stored at 4 ºC for 24 h. Buffy-coats were separated in 50 mL-sterile bags with a cell viability above 95 %, an erithrocyte fraction volumen lower than 0.5 mL, leucocyte amount 1.5 × 10^9 cells/donor, and it was stored at 2-8 °C. Then, buffy-coats were subjected to hemolysis by adding sterile and cold NH_4Cl solution (83 %). Cellular suspensions were continuously centrifuged at 2548 × *g* (52 L/h flow-rate) at 4 ºC. The cell pellets were resuspended in phosphate buffered saline (100 mM PBS, pH 7.3), previouly cooled at 2-8 °C. A second hemolysis step was carried out, as previously described, for 10 min and final cellular suspentions were storaged at –20 °C.

Transfer factor preparation

Cells were subjected to several alternate freeze-thaw cycles for cell lysis. In every cycle, the cell suspension previously frozen at –20 °C was incubated at 37 °C for 2 h, and then frozen again at –20 °C for 6 h. Disrupted cells were diluted in 100 mM PBS, pH 7.3 and centrifugated at 2548 × *g* (model DL6R, Shanghai, China) at 2-8 °C for 3 h. Supernatants were collected and diafiltered through a 10 kDa ultrafiltration cassete (Sartocon Slice-200, Germany) installed to a Crossflow system (Sartoflow Advanced System, Germany). The permeate mixture was subjected to a pasteurization step at 60 ± 1 °C for 10 h, using a glass reactor (ACE Glass, US), and the pasteurized material then filtered through a 0.2 capsule (Sartorius, Germany) for bioburden reduction.

Total white blood cell counts by flow cytometry

The specific white blood cell populations were identified by labelling with anti-CD3 and anti-CD19 monoclonal antibodies conjugated with fluorochromes as biomarkers. A Flow Cytometer CyFlow Space (Sysmex-Partec, Germany) equipment was employed [19].

Peptide quantification

Peptide concentration was quantified according to the procedure described by Lowry *et al*. [20], with a standard curve of bovine serum albumin from 10 to 100 µg/mL. Absorbance was measured at 730 nm on an Ultrospec UV/Visible 2000 Spectrophotometer (Pharmacia Biotech. Cambridge, England).

Determination of TF biological activity by delayed-type hypersensitivity

The biological activity was determined by the delayed-type hypersensitivity (DTH) assay, with some modifications [21]. The test was performed in isogenic BALB/c mice, supplied by the National Animal Breeding Center (CENPALAB, Bejucal, Cuba). Mice received recombinant Streptokinase (rSK) as a positive control of the experiments, by intradermal injection into the footpad of the right hind limb. Skin reaction was measured 48 h after antigen injection. TF samples were considered that passed the test when statistically significant differences were found (Student's t test, $p < 0.05$) between the increase (greater than 4 mm) in the induration reaction of the inoculated mice in relation to the mice inoculated with placebo (100 mM PBS, pH 7.3).

Determination of pyrogen content in TF samples

It was carried out according to the USP rabbit test [22]. The increase in body temperature after the intravenous injection of the DLE samples in rabbits was measured using an Automatic Pyrogen Test System (Ellap APT 75, Denmark). A constant TF dose of 0.14 U/kg of rabbit body weight was inoculated. The test criteria were: negative test (rabbit with not temperature difference ≥ 0.5 ºC), valid test (one or more rabbits with a temperature difference ≥ 0.5 ºC, the assay continues), and positive (the sum of temperature differences > 3.3 ºC; samples were declared with no compliance to the pyrogen test).

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Endotoxin quantification in TF samples

Endotoxins were determined using the LAL Kit (Bio-Whittaker, Germany), based on the activation of the Limulus Amebocyte Lysate (LAL) proenzyme by endotoxins from Gram-negative bacteria. The p-nitroaniline (pNA) released by the activated enzyme from the Ac-Ile-Glu-Ala-Arg-pNA substrate was measured by spectrophotometry at 405 nm, its absorbance directly proportional to the endotoxins' concentration in the samples. A calibration curve (0.10 to 1 EU/mL) was used, prepared with an Endotoxin Stock Solution. The concentration of the samples was calculated with a conventional regression program, by interpolating the samples' absorbance values into the standard curve.

Determination of TF profile and purity by SDS-PAGE

Samples (20 µg) were analyzed by electrophoresis in 15 % gels to evaluate the electrophoretic profile and purity under reducing conditions [23]. The separated peptides were stained with silver nitrate (Bio-Rad Laboratories, Richmond, USA) and peptides molecular sizes were estimated using an adequate molecular weight marker (Bio-Rad, USA).

DNA quantification in TF samples

The 260/280 nm absorbance ratio was measured to every TF batch in 20-µL samples, with the aid of a NanoDrop Photometer NP 80 equipment (NanoDrop, US). DNA was also analyzed with the adequate fractionation of DNA molecules by 1 % agarose gel electrophoresis. Electrophoresis runs were done in TBE buffer (216 g Tris, 110 g boric acid and 18.6 g EDTA) at 120 V, and bands were stained at 10 µg/mL ethidium bromide.

Dynamic light scattering of TF samples

For dynamic light scattering (DLS) measurements, TF samples were assessed using a Delsa TM Nano (Beckman Coulter, US) at 25 °C, 633 nm, refractive index 1.3328, scattering insensitive 3101 cps and using water as diluent. Two measurements were taken on each sample to check the repeatability of results. The intensity size distributions were obtained by analyzing the correlation functions, using the Multiple Narrow Modes algorithm of the equipment's software.

Determination TF profile and retention time (RT) by size exclusion chromatography (SE-HPLC)

Chromatographic separation was performed using 500 µL of each TF sample applied on a Superdex™ 75, 10 × 300 mm analytical HPLC column (GE Healthcare, US). Elution was carried out isocratically with 100 mM PBS, pH 7.3. The absorbance of eluted fractions was measured at 280 nm. Cytochrome C (100 µg) was used as molecular weight standard, which was subjected to the same operating conditions.

Statistical analysis

Experimental results were compared through a single tail-ANOVA, at a confidence interval of $\alpha = 0.05$. Analyses were made with the aid of Statgraphics Plus software (Version 5.0; StatPoint Technologies, Warrenton, Virginia, US). Mathematical analyses were performed using Microsoft Excel 2016 software (Microsoft, Redmond, Washington, US).

*R*esults and discussion

TF discovery and description of its immunological mechanism date back to 1954 [4]. Since then, manufacturers have addressed TF production as an alternative to treat several disorders, including cellular immunodeficiencies, cancer, infectious parasitic and allergic diseases, among others [5]. Despite, a complete biological and physico-chemical characterization, together with consistency testing of a TF production process operated under GMP compliance, were not published yet. This is a key issue, since TF is commonly isolated from a variety of biological sources, with high batch-to-batch variability. Therefore, in this study, multiple TF batches were analyzed according to several quality parameters, such as peptide concentration, DTH, pyrogen and endotoxin contents, SDS-PAGE purity, DNA content, and DLS and SE-HPLC profiles.

It is known that the TF action mechanism involves activation of T-lymphocytes, to release chemokines that attract macrophages and cytotoxic CD8+ T-lymphocytes to the application site [6, 7]. Hence, it was necessary to identify the lymphocyte population present in the analyzed samples of the cell biomass used as starting material. This identification was done by flow cytometry, with a monoclonal anti-CD3 antibody linked to a fluorescent surface marker (Figure 1). In this assay, the forward scatter (FSC) axis stands for cell size and the side scatter (SSC) for complexity or cytoplasmic granularity, according to the size and shape of the nucleus. These parameters support the identification of lymphocyte regions (R2) after the hemolysis of pooled, concentrated and isolated lymphocytes. The percentage of CD3+ cells was 40.58 % in respect to total cell population of every analyzed sample, while the cell concentration was 157 537 ± 32 150 cells/mL [19].

The average ratio of the purification process was 2.26 ± 0.36 µg/buffy-coat ($p = 0.7348$) and 1.14 ± 0.13 µg/g biomass ($p = 0.5487$), with small standard deviations, but statistically non-significant differences, and showing process consistency (Table 1). These results are in contradiction with those reported by Medina-Rivero *et al*. (0.43 µg/buffy-coat) [1]. The referred differences can be explained in that the Transferon® production process started from 1000 healthy donors and involved five freeze-thaw cycles for cellular lysis. Meanwhile, TF (Hebertrans®) production involved 1404 ± 610 donors and six freeze-thaw cycles.

Regarding the estimation of TF doses in the final product (Hebertrans®), it is essential to determine the peptide concentration used as active pharmaceutical ingredient, considering that one unit of TF is equivalent to 1 mg/mL of peptide. In this sense, the average peptide concentration was 2.51 ± 0.17 mg/mL (quality specification: > 0.5 mg/mL), for a number of doses per batch (13.7 L) of 34 387.

In general, biological assays are carried out *in vitro* by measuring the biological response in cells, once the target therapeutic protein is added to the specific assay. In the case of TF, the biological potency was measured by a DTH-skin test, which is the classical method for the *in vivo* evaluation of cellular mediated immunity [21]. It is mediated by the interaction between antigen presenting cell with CD4+ T helper and CD8+ cytotoxic T cells, leading to the local inflammation in the following 48-72 h, due to the mobilization

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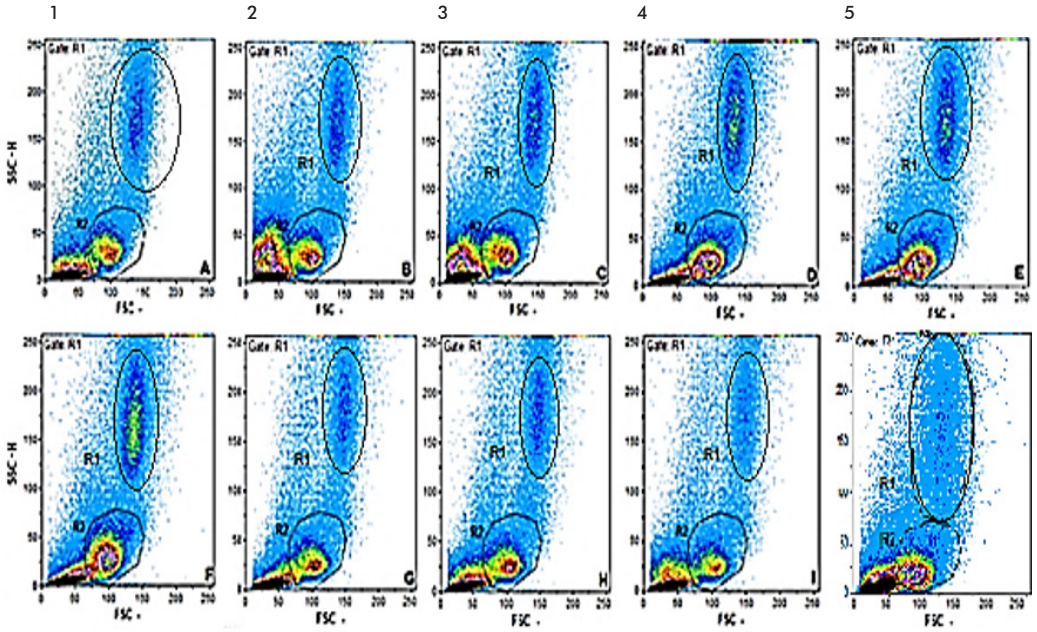

TF samples	Cell number		
	Counts	Cells/mL	%
1	46 020	133 555	34.46
2	53 697	132 115	40.64
3	59 348	132 725	44.72
4	82 744	195 015	42.43
5	81 331	191 640	42.44
6	87 478	199 770	43.79
7	47 200	134 630	35.06
8	52 411	131 640	39.81
9	38 304	131 510	29.13
10	90 755	192 770	47.08
Average	63 929	157 537	40.58
SD	19 569	32150	–

Figure 1. Graphs of polymorphonuclear and lymphocyte populations in Hebertrans® transfer factor (TF) batches (1-10). Data were obtained by flow cytometry analysis with anti-CD3 and anti-CD19 monoclonal antibodies conjugated with fluorochromes as biomarkers. Relevant cell populations (R2) were directly stained for each of the differential population markers, following the hemolysis of pooled, concentrated and isolated lymphocytes. Cell number parameters for each sample are tabulated in the inset.

Table 1. Peptide concentration, recovery and biological activity of ten samples of Hebertrans® transfer factor production batches

TF samples	Buffy coat (U)	Biomass (g)	Cell suspention (mL)	Peptide concentration (mg/mL)	Ratio		Biological activity	
					μg TF/Buffy-coat	μg TF/g Biomass	TF Samples/Placebo (μm)	TF Sample/Control (μm)
1	1105	2264	2205	2.53	2.29	1.12	123×10^{-2}	$2.67 \times 10^{+2}$
2	1305	2231	2205	2.62	2.01	1.21	4.99×10^{-3}	$3.57 \times 10^{+2}$
3	1230	2217	2430	2.33	1.89	1.05	9.63×10^{-6}	$3.20 \times 10^{+2}$
4	1004	2173	2173	2.23	2.22	1.08	1.53×10^{-4}	$3.87 \times 10^{+2}$
5	1083	2168	2405	2.63	2.43	1.18	$8.26 \times 10^{+2}$	$1.23 \times 10^{+2}$
6	1020	2071	2215	2.40	2.35	1.10	5.36×10^{-5}	$4.33 \times 10^{+2}$
7	1162	2067	2260	2.36	1.54	0.87	7.30×10^{-6}	$1.11 \times 10^{+2}$
8	999	2064	2060	2.57	2.57	1.24	2.34×10^{-1}	$1.05 \times 10^{+2}$
9	1010	2059	2090	2.59	2.56	1.25	4.98×10^{-4}	$1.48 \times 10^{+2}$
10	1020	2264	2180	2.80	2.75	1.31	4.84×10^{-6}	$1.15 \times 10^{+2}$
Average	1094	2146	2222	2.51	2.26	1.14	–	–
SD	107	77	119	0.17	0.36	0.13	–	–
p	0.0556	0.1800	0.2493	0.8170	0.7348	0.5487	$p < 0.05$	$p > 0.05$

of lymphocytes and monocytes to the site of intradermal inoculation of the antigen. Therefore, in this study, the inflammatory edema provoked at the administration site was used as an indirect measurement of TF biological activity. After the application of 0.5 mL of TF sample per animal, statistical differences ($p < 0.05$) were estimated, between the inflammatory edema of placebo group and those produced by analyzed samples, showing no statistically significant differences among TF samples ($p > 0.050$) (Table 1).

Moreover, one of the most important contaminants of biopharmaceutical products are pyrogens, because they induce fever, shock, and changes in physiological functions, even at very low concentration (pg/mL) [24]. Pyrogens can be derived from bacteria (mainly endotoxins shed from gram-negative bacteria), viruses or fungi. To demonstrate the level of pyrogens in the TF samples, a rabbit pyrogens test was applied [22]. Rabbits were injected intravenously with TF samples and, then, temperature measured intrarectally (Table 2). The average of rabbit temperature before assays ($t = 0$) was 39.08 ± 0.46 °C. Once TF was applied, rabbit temperature increased up to 39.9 ± 0.47 °C, evidencing no statistically significant differences ($p = 0.8840$), which demonstrated that the TF manufacturing process rendered a pyrogen-free product.

The biopharmaceutical manufacturing process has also to guarantee impurity clearance, such as bacterial endotoxins, which are well known for their immunogenic, pro-inflammatory and pyrogenic effects. When the body is exposed to endotoxins, a systemic inflammatory reaction can occur, leading to multiple pathophysiological effects such as endotoxin shock, tissue injury and death [25, 26]. These are directly related to the endotoxin amount present in the product dose administered to the patient. Because doses vary from

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Table 2. Rabbit temperature before and after the intravenous administration of Hebertrans® transfer factor and endotoxin concentration

TF samples	Rabbit initial temperature (°C)	Temperature difference (°C)			Sum of differences (°C)	Rabbit final temperature (°C)	Endotoxin concentration (UE/mL)
		Rabbit 1	Rabbit 2	Rabbit 3			
1	39.51	0.14	0.13	0.10	0.40	39.90	6.25
2	38.94	0.15	0.08	0.13	0.40	39.34	7.78
3	39.23	0.05	0.08	0.03	0.35	39.58	6.25
4	38.54	0.21	0.02	0.11	0.35	38.89	6.25
5	38.87	0.08	0	0.12	0.20	39.07	6.25
6	39.62	0.32	0.14	0.23	0.40	40.02	6.95
7	39.44	0.21	0.19	0.05	0.50	39.94	7.17
8	38.35	0.13	0.19	0.15	0.50	38.85	6.25
9	38.43	0.12	0.16	0.13	0.40	38.83	7.78
10	39.22	0.01	0.13	0.12	0.25	39.47	5.86
Average	39.08	0.14	0.11	0.11	0.38	39.39	6.68
SD	0.46	0.09	0.07	0.05	0.10	0.470	0.69
p	–		0.6065		–	0.8840	–

product to product, the endotoxin limit is expressed as K/M, where K corresponds to 5.0 EU/kg, while M represents the maximum human dose/kg that can be administered in a single one-hour period [22, 25, 26].

Regarding the endotoxin content measured in TF samples, the analysis of this production process evidenced a high capacity to deliver a final product with endotoxin concentration as low as to 6.68 ± 0.69 EU/mL, equivalent to 0.14 mg/kg. This value was 36-fold lower than those approved for a human dose. These results can be explained considering the endotoxin properties, which can form high molecular weight aggregates (up to 106 Da) in water and tend to be adsorbed to different surfaces, as amphipathic molecules [26]. Thus, these contaminants can be removed by the TFF step (10 000 Da cut off) during the TF purification process.

Residual DNA is another significant contaminant for biopharmaceutical products produced in mammalian cell lines, due to the risk of oncogene-bearing DNA fragments when found in the final product. Those molecules bear tumor-inducing potential, and could carry defective genes critical to subvert the normal physiological functions [27]. This is a key issue of the TF production process, since large pools of T-lymphocytes are used as starting material and the purification process is devoid of robust DNA removal steps despite showing certain capacity for its fractionation. Nevertheless, the production process was able to reduce the residual DNA amount measured after the cell lysis step (124.14 ± 13.04 mg/batch), to 42.90 ± 7.64 mg after the TFF step, and 76.96 ± 10.20 mg after pasteurization and sterile filtration, showing 1.61-fold of DNA removal factor (Table 3). This represents a residual amount of 2.23 ± 0.26 ng DNA/dose. The residual DNA limits approved by regulations for animal cell culture as substrate should not exceed 10 ng per dose [27]. Thus, this value is within the levels of residual DNA approved for pharmaceutical products produced from this type of biological substrates. Furthermore, the residual DNA was not either visualized (detection limit: < 10 µg/µL) by DNA electrophoresis (Figure 2).

Regarding the molecular weight and migration pattern of TF among batches, SDS-PAGE was performed under reducing conditions. A consistent electrophoretic pattern was shown for all TF samples (Figure 3, lanes 1-9) with a major protein band of a high purity degree

Table 3. DNA quantification of samples belong to different stages of Hebertrans® transfer factor

TF samples	Protein concentration (mg/mL)			DNA concentration (ng/µL)			DNA content (mg)			DNA ratio (ng/dose)		Equivalent doses	
	SN	DF	FP	SN	DF	FP	SN	DF	FP	DF	FP	DF	FP
1	6.25	2.74	2.57	7.75	2.40	4.60	116.25	32.88	60.26	0.88	1.79	37538	33667
3	5.26	0.15	0.15	7.85	3.90	5.90	117.75	53.82	78.47	1.51	2.39	35742	32851
5	4.06	0.05	0.05	9.60	3.00	6.45	144.00	41.10	85.14	1.04	2.42	39593	35244
7	6.60	0.21	0.21	8.70	3.00	6.45	130.50	41.10	85.14	1.08	2.34	38223	36432
9	5.43	0.08	0.08	7.48	3.33	5.74	112.20	45.62	75.77	1.24	2.22	36716	34188
Average	5.52	0.32	0.32	8.28	3.13	5.83	124.14	42.90	76.96	1.15	2.23	37562	34476
SD	0.99	0.21	0.21	0.87	0.55	0.76	13.04	7.64	10.20	0.24	0.26	1465	1396
p value	0.0048*			0.0018*			0.0018*			0.0001***		0.0092**	

TF: Transfer factor. SN: Supernatant. DF: Diafiltered fraction. FP: Final product. Protein concentration in the DF and FP fractions had an specification of > 50 mg/mL; DNA ratio in ng/dose of < 10 ng/dose.

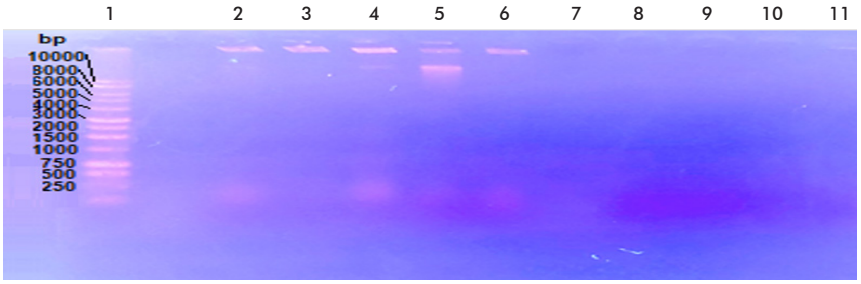

Figure 2. DNA electrophoresis of Hebertrans® transfer factor (TF). Lanes: 1, 1 kb DNA molecular weight marker (catalog number G5711; Promega, USA); lanes 2-6, supernatants of TF production batches 1, 3, 5, 7 and 9, respectively; Lanes 7-11, fiafiltered samples of TF batches 1, 3, 5, 7 and 9, respectively. Samples were run in a 1 % agarose gel.

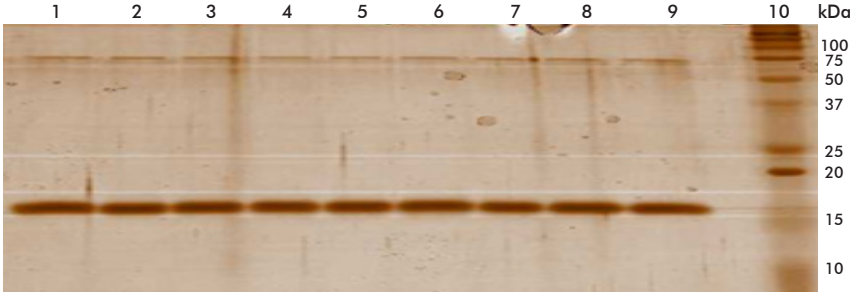

Figure 3. SDS-PAGE performed under reducing conditions of transfer factor (TF) Hebertrans® samples (20 µg). Lanes: 1 to 9, samples of TF batches 1 to 9, respectively; 10: Protein molecular weight marker PPM-SERVA6 (10-250 kDa).

(> 98%), nearly 17 kDa in weight, and another slightly visible band around 75 kDa (< 2 %). These results (two bands) are characteristic of TF, coincident with those reported by other researchers [1]. Interestingly, a group of nine peaks was detected in the size-exclusion-HPLC (Figure 4). The first peak showed a molecular weight of 17 kDa (RT 42 min), similar to the 17-kDa band identified by SDS-PAGE, the second peak being estimated at 14 kDa (RT 45 min), and the other peaks below 10 kDa (RT 48-84 min). This pattern did not interfere with consistency, a high consistency profile being corroborated for samples in all the TF batches tested.

In this sense, the determination of molecular homogeneity by size-exclusion-HPLC is also important, because it can detect aggregation level that could impacts on TF immunogenic capacity. The analysis of ten consecutive TF batches revealed slight aggregation (> 10 kDa) of TF peptides (Figure 4), which is consistent with results reported by Avila *et al*. [1]. Besides, to study aggregation level of peptides, a DLS analysis was also performed. Results evidenced only two populations of molecules with a diameter ranged from 100 to 10 000 nm (Figure 5), which corresponded with 0.4-40 kDa [28]. It can be hypothesized that this apparent contradiction in molecular weight with the cut-off of the ultrafiltration membrane step (< 10 kDa) can be explained by aggregation of peptides and/or with residual DNA, which can be produced during pasteurization step. High temperatures can have different effects on proteins, leading to an accelerated aggregate formation, which can include thermodynamic instability and hydrophobic interactions. For instance, aggregation of human serum albumin has been detected at 40 °C. Fedurkina *et al*. [29] found that creatine kinase aggregation did not depend on protein concentration at 51 °C, but did at 60 °C. Therefore, further experiments have to be done to identify the TF peptide aggregation status after the pasteurization step.

In summary, the reported TF production process provides a biopharmaceutical product of high batch-to-batch reproducibility and process consistency. This was demonstrated by the results of peptide concentration, biological activity, pyrogen and endotoxin content, DNA content, molecular weight, retention time and profile in SE-HPLC determinations. Hence, the Hebertrans® biopharmaceutical product batches comply with National Regulatory Agency regulations for blood products of clinical use, supporting its application to treat human diseases. Furthermore, blood products are still highly demanded, because their homologous recombinant versions, when available, neither display their specific biological properties nor are obtained in the amounts required for specific treatments. Despite, they also should comply with strict regulatory policies regarding safety of the product itself and its raw materials. In this sense, the impact of the aggregation status of the peptide mixture and the viral clearance capacity of the TF production process are susceptible of further research for improvement.

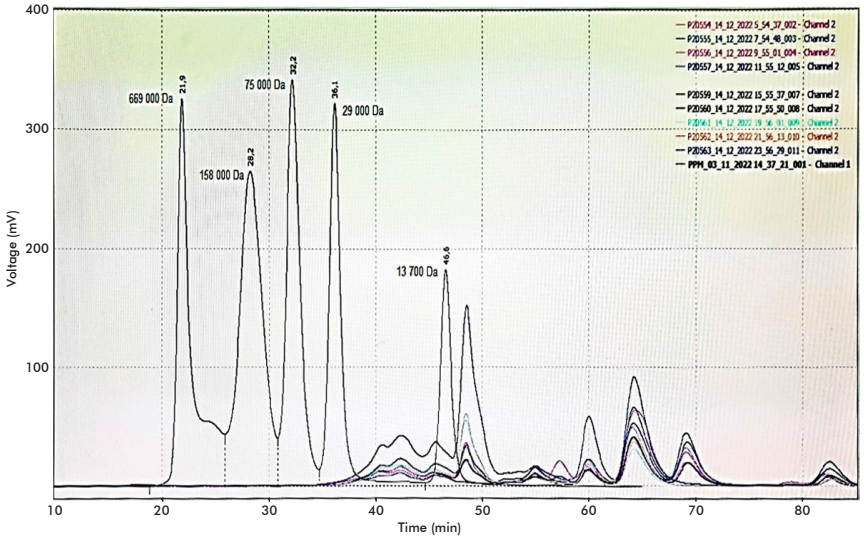

Figure 4. SE-HPLC profiles of TF batch samples analyzed using the AZURA analytical systems to obtain area percentage and retention time (RT) for peaks. Molecular weight marker: Thyroglobuline, Mr 669 kDa, RT 21.9 min; Aldose, Mr 158 kDa, RT 28.2 min; Conalbumin, Mr 75 kDa, RT 32.2 min; Carbonic anhydrase, Mr 28 kDa, RT 36.1 min; Ribonuclease A, Mr 13.7 kDa, RT: 46.6 min).

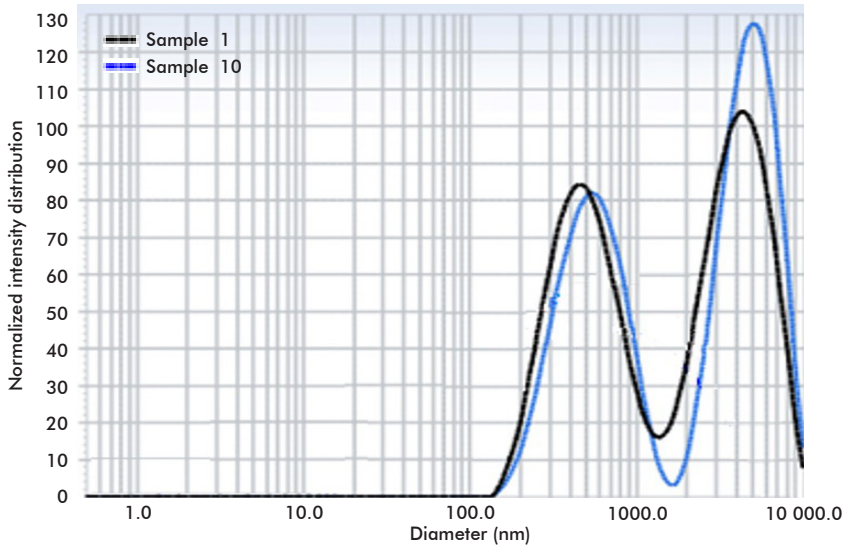

Figure 5. Dynamic light scattering profiles of two transfer factor (TF) Hebertrans® batches.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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