Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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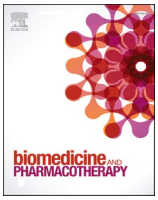

Advanced cell therapy with low tissue factor loaded product NestaCell® does not confer thrombogenic risk for critically ill COVID-19 heparin-treated patients

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ABSTRACT

Since the COVID-19 pandemic started, mesenchymal stromal cells (MSC) appeared as a therapeutic option to reduce the over-activated inflammatory response and promote recovery of lung damage. Most clinical studies use intravenous injection for MSC delivery, raising several concerns of thrombogenic risk due to MSC procoagulant activity (PCA) linked to the expression of tissue factor (TF/CD142). This is the first study that demonstrated procoagulant activity of TF+ human immature dental pulp stromal cells (hIDPSC, NestaCell® product) with the percentage of TF+ cells varied from 0.2% to 63.9% in plasma of healthy donors and COVID-19 heparin-treated patients. Thrombogenic risk of TF+ hIDPSCs was evaluated by rotational thromboelastometry (*in vitro*) and in critically ill COVID-19 patients (clinical trial). We showed that the thromboelastography is not enough to predict the risk of TF+ MSC therapies. Using TF-negative HUVEC cells, we demonstrated that TF is not a unique factor responsible for the cell's procoagulant activity. However, heparin treatment minimizes MSC procoagulant (*in vitro*). We also showed that the intravenous infusion of hIDPSCs with prophylactic enoxaparin administration in moderate to critically ill COVID-19 patients did not change the values of D-dimer, neither in the PT and PTT times. Our COVID-19 clinical study measured and selected the therapeutic cells with low TF (less than 25% of TF+ hIDPSCs). Our data indicate that the concomitant administration of enoxaparin and low TF-loaded is safe even for critically ill COVID-19 patients.

1. Introduction

Coronavirus disease 2019 (COVID-19) is an emerging disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) [1]. The first disease cases were reported in December 2019 in Wuhan, Hubei province (China), and spread faster for more than 220 countries, including Brazil [2]. Since SARS-CoV2 had already infected more than 424 million people, more than 5.89 million have died due to the disease [2]. Despite the efforts to develop therapies to scuffle COVID-19, to date, no approved effective treatment is available that can halt the progression of the disease and can address the critical cases with a high fatality, driving public fear in the "Corona crisis" [3,4]. Thus, any treatment that could reduce case fatality by alleviating severe COVID-19 and speed up the recovery of critically ill patients is in great demand [4].

In this context, intravenous infusion of mesenchymal stromal cells (MSCs) emerge as a promising therapeutic approach for COVID-19 patients [5,6]. This is because the MSCs exhibit immunomodulatory/anti-inflammatory and regenerative properties that, combined, can halt the cytokines storm in COVID-19 and repair injured tissues [5,7]. Moreover, on the one hand, the pulmonary

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first-pass effect has been considered an obstacle for intravenous stromal cell delivery for many diseases [8], on the other hand, this effect is particularly interesting for COVID-19-related pneumonia, since 50–80% of the infused MSCs tend to trapped within the lungs [9]. For these reasons, more than 390 clinical trials evaluating the efficacy of MSCs for treating COVID-19-related respiratory diseases were registered on ClinicalTrial.gov.

Although the MSCs can be easily isolated from different tissues (adipose, bone marrow, menstrual blood, umbilical cord, and dental pulp), the therapeutic potential of these cells can change according to their origin [10]. This occurs because each MSC population shares a transcriptomic signature in common with its tissue origin [11]. In this sense, studies showed that human dental pulp stromal cells (hDPSCs) exhibit an anti-inflammatory potential three times higher than any MSC [12,13], making these cells potential candidates for the COVID-19 treatment.

Based on this, we developed a novel and scaling-up methodology to isolate human immature dental pulp stromal cells (hIDPSCs) from the dental pulp of deciduous teeth [14,15]. The hIDPSCs comprise a population of therapeutic cells with a unique molecular profiler, which despite its ectomesenchymal origin (neural crest), exhibit the criteria for defining multipotent MSCs proposed by the International Society for Cell Therapy (ISCT) [16], but express high levels of the neurological marker nestin [17]. Currently, these cells have been produced under a good manufacturing process (GMP) by the Brazilian company Cellavita Scientific Researches (Valinhos-SP), and correspond to the active component of the NestaCell® product.

Although the safety and efficacy of the intravenous infusion of the hIDPSCs (at a dose of 1×10^6 and 2×10^6 cells/weight kilogram) were previously demonstrated in two clinical trials for Huntington's disease (Phase I and II, ClinicalTrials.gov Identifier NCT02728115 and NCT03252535, respectively) [18], *in vitro* studies, based on thromboelastography (TEG) and calibrated thrombogram (CAT), have shown that, for expressing the tissue factor (TF)/CD142, MSC can trigger the instant blood mediated inflammatory reaction (IBMIR), increasing the risk for thromboembolic events [5,19–24]. This data is particularly concerned for patients with COVID-19 exhibiting a hypercoagulable state due to the cytokine storm [25]. For this reason, in a new updated guideline, the TF/CD142 assessment was included as part of the minimal criteria to maximize patient safety [26].

Tissue factor (TF), also known as CD142, thromboplastin, or factor III, is an integral membrane glycoprotein (46 kDa, with 263 amino acids), encoded by the gene *F3* (*locus* 11p.12.), which is found is expressed in high levels in vascularized organs (brain, placenta, lungs, heart, kidney, intestine, testis, and uterus) and blood cells (monocytes, macrophages, granulocytes, and platelets) [27]. However, the TF can also be found expressed on the surface of MSC from different sources [28–30], reducing the clotting time (CT) in a dose-dependent manner, as described by studies based on TEG and CAT [4,20,24,31,32]. For this reason, some authors have emphasized the need to evaluate the portion of TF+ MSC and correlate this data with CT using *in vitro* techniques to mitigate the thrombogenic risk of MSC-based products, especially for critically ill COVID-19 patients [4,31].

Despite this concern, most *in vitro* studies describing the procoagulant potential of TF+ MSCs were performed using platelet-poor plasma (PPP) obtained from healthy donors [20,32]. Moreover, these studies employed cell doses 12–200-fold higher (12×10^6 to 200×10^6 cell/kg) than the typically used dose for the therapeutic purpose, (1×10^6 cell/kg) [31,33–35]. Thus, novel studies evaluating the procoagulant potential of TF+ MSC in PPP from severe COVID-19 patients are mandatory to predict the eventual thrombogenic risk of cellular products dedicated to the advanced cell therapy for COVID-19.

Thus, this study evaluated for the first time the procoagulant potential of different lots and sub-lots of hIDPSCs (NestaCell® product), which varied in TF+ cells percentages using the rotational thromboelastometry (ROTEM). However, unlike previous studies, we performed the assays using the PPP from healthy donors and critically ill COVID-19 patients. In addition, we assessed the procoagulant potential of intravenous infusion of hIDPSCs (NestaCell® product) in moderate to severe COVID-19 heparin-treated patients (ClinicalTrial Identifier NCT04315987).

Although our *in vitro* results confirmed that TF decreases the CT in a dose-dependent manner in PPPs from healthy donors and critically ill COVID-19 patients, we demonstrated that the heparinization reduces thrombogenic risk. Confirming these results, we provided evidence that the advanced cell therapy with the NestaCell® low TF-loaded product does not increase the thrombogenic risk for COVID-19 heparin-treated patients.

2. Material and methods

2.1. Ethical aspects

The clinical protocol and Informed Consent Form (FICF) and all other applicable documents were submitted to the Research Ethics Committees of the qualified centers that participated in the study, which reviewed and issued them approval opinions following current legislation. Thus, this study was approved by the National Research Ethics Commission (CEP/CONEP) (process number 4044840) and, conducted in Good Clinical Practice compliance (ClinicalTrials.gov identifier NCT02728115).

2.2. Isolation, expansion and characterization of hIDPSCs (NestaCell® product)

The human immature dental pulp stromal cells (hIDPSCs) used in this study were isolated from deciduous teeth from four healthy children aged between 6 and 12 years (all procedures were performed after Informed Consent Form signature), as previously described by Kerkis et al. [14] and patented by the Brazilian facility Cellavita Pesquisas Científicas Ltda. (US patent number US20160184366A1). The hIDPSCs were cultivated until the fifth passage (which corresponds to the active component of NestaCell® product) in basal medium (Dulbecco's modified Eagle's medium (DMEM)/Ham's F12, supplemented with 15% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 2 nM nonessential amino acids, all from Gibco, Carlsbad, USA). Cells isolated from each dental pulp donor resulted in four different lots of NestaCell® product, which were identified as hIDPSC-1, −2, −3, and 4. The sub-lots resulting from the expansion of each lot of NestaCell® product were identified as hIDPSC-1.1, −1.2, 1.3, *etc.* The MSC phenotype of these cells was confirmed using the criteria defined by the ISCT [16,36]. The hIDPSCs employed in this study are CD105-, CD73- and CD90-positive, and CD45-, CD34-, CD11b-, and HLA-DR-negative, as previously described by Kerkis et al. [14]. The cell manufacturing process was performed according to the good manufacturing practices (GMP) required by the Brazilian Health Regulatory Agency (ANVISA, RDC 508/21) for advanced therapy products. NestaCell®'s manufacturing process is protected by the US patent number US20160184366A1.

2.3. Flow cytometry

To evaluate the percentage of TF/CD142-positive, all sub-lots of hIDPSC (NestaCell® product) or HUVEC cells (used as a control for not expressing TF), at a concentration of 1.0×10^6 cells per 100 µL, were incubated overnight at 4°C with the mouse monoclonal anti-human TF/CD142 (Thermo Fischer Scientific, Carlsbad, USA, catalog number MA1–43029), at a dilution of 1:100. Next, cells were washed twice in PBS and incubated for two hours at 4°C with the secondary anti-mouse IgG conjugated with FITC at a dilution of 1:4000. Cells were washed twice, resuspended in 100 µL of PBS, and analyzed in the flow cytometer.

For the detection of phosphatidylserine (PS), 1.0×10^6 cells before and immediately after the analysis in rotate thromboelastometry (ROTEM) were incubated for 20 min with 5 μL of Annexin V-FITC (Invitrogen, Carlsbad, USA, catalog number A13199). Next, cells were washed and resuspended in 200 μL of PBS and immediately analyzed in the flow cytometer. All acquisitions were performed using the C6 Accuri Flow Cytometer (BD Bioscience, USA). A total of 10.000 events were acquired (in triplicated). Results were analyzed using the FlowJo software v.10.0.7r2 (Tree Star Incorporation, USA).

2.4. Platelet-poor plasma (PPP) obtaining from health donors and COVID-19 patients

Whole blood was collected from four healthy donors (identified as A to D) and five patients with COVID-19 (identified as E to I). The blood from healthy donors was collected in the Vital Brazil Hospital (Instituto Butantan. São Paulo, Brazil) and, the blood from patients with COVID-9 (aged 65–80), was collected in the Intensive Care Unit (ICU) from Hospital das Clínicas (São Paulo Medical School, University of São Paulo). All procedures were approved by the Ethical Committee (Plataforma Brasil, process number 1806596). We used as eligibility criteria for healthy donors the age (25–35 years), the absence of any known disease, and donors with did not make use of any drug, neither had consumed alcohol 48 before the blood collection. The plasma from COVID-19 was obtained from patients exhibiting the criteria for critically ill COVID-19 proposed in the Guideline for Care of Critically Ill Adult Patients with COVID-19 in the Americas, published by the World Health Organization (available in https://iris.paho.org/bitstream/handle/10665.2/53895/PAHOIMSEIHCOVID-1921010_eng.pdf?sequence=6&isAllowed=y). These criteria included: $FiO_2/PaO_2 \ll 250$, or 2; chest x-ray with bilateral patchy infiltrate; respiration rate $\ll 30$, or oxygen saturation $\ll 90\%$ and presence of ARDS. All these data were included in the section. Blood was collected into 4.5-mL BD Vacutainer tubes containing 3.2% sodium citrate and inverted to assure proper anticoagulation. Platelet-poor plasma (PPP) was obtained by centrifuging the whole blood at $2000 \times g$ (RCF) for 20 min at room temperature. PPP was obtained from four healthy donors, without any reported disease or coagulopathy, and not infected by SARS-CoV2. Additionally, PPP was aliquoted and stored at − 80 °C until use. PPPs were isolated in a BSL-1 laboratory. All procedures were performed in a biological safety cabinet (BSC) as recommended by the Centers for Disease Control and Prevention (CDC, available in https://www.cdc.gov/sars/guidance/f-lab/app5.html).

2.5. Cell dose range for coagulation studies

Cell preparation for testing occurred immediately before the clotting assay. For the experiments, cells were thawed, counted using a hemocytometer, and resuspended in saline (0.9% NaCl) to a working concentration of (i) 1.0×10^6 cell/mL or (ii) 2.0×10^7 cells/mL, as proposed by George et al. [37]. These cell doses are within the range of $1–3 \times 10^6$ cells/Kg, which was previously demonstrated as clinical safety [35]. Clotting assays were performed in a final concentration of (i) 15,000 hIDPSC/mL (NestaCell® product), which corresponds to $1–2 \times 10^6$ cells per adult human weighing around 60–80 Kg, as proposed in the literature [24,26,32,37], or 0.2×10^5 hIDPSCs, which is equivalent to the therapeutic dose employed in the combined Phase I and II clinical trials of hIDPSC (NestaCell® product) for severe COVID-19 pneumonia (2.0×10^7 cells/patient), as described in *clinicaltrial.gov* (NCT04315987, available in https://clinicaltrials.gov/ct2/show/NCT04315987).

2.6. Rotation thromboelastometry (ROTEM)

Clotting time (CT) was analyzed by the rotation thromboelastometry (ROTEM), using the ROTEM four-channel system (Pentapharm GmbH, Munich, Germany), a methodology based on thromboelastography that provides global information on the dynamics of clotting kinetics in real-time. For this, 36 μL of hIDPSC suspension (at 1.0×10^6 cell/mL or 0.2×10^5 cells/mL) were added to a cuvette (cup) containing 20 μL of 0.2 M CaCl2. Next, it was added 304 μL of PPP from healthy donors or patients with COVID-19, totaling a final assay volume of 360 μL in the cup. Considering that cell death can lead to false-positive results due to the exposure of phosphatidylserine residues, the cell viability of each sample was analyzed before being subjected to ROTEM analysis using the trypan blue assay. All analyzed samples showed a percentage of viability higher than 95%.

As negative controls, an identical volume of cell vehicle (36 μL of saline) was added to the PPPs. To confirm that proteins involved in coagulation cascade are preserved in the PPPs from both healthy donors and patients with COVID-19, two positive controls were employed: (i) phospholipid and ellagic acid solution (provided by the TTPA Clot kit, BIOS Diagnóstica, Sorocaba, Brazil), and (ii) an external source of tissue factor from rabbit brain (provide by TP Clot kit, BIOS Diagnóstica, Sorocaba, Brazil). These kits allow to confirm that both intrinsic and extrinsic coagulation pathways, respectively, are preserved, avoiding false negative results, as preconized by the Brazilian Pharmacopeia (ANVISA, 2019). Each sample was run in duplicate, and values of CT were averaged.

2.7. Reversal of procoagulant activity with enoxaparin sodium

Increasing concentrations of enoxaparin sodium (final concentrations of 0.01, 0.1, 0.5, and 1 U/mL) were added to cells suspension (1.0×10^5 and 0.2×10^5 cells/mL) before adding to the PPPs from healthy donors and critically ill COVID-19 patients and, they tested suing ROTEM. The concentrations of enoxaparin tested were based on the study published by Moll et al. [38]. Control samples were prepared in similar ratios with saline only, as proposed by George et al. [37].

2.8. hIDPSCs intravenous infusion in COVID-19 patients

A total of 90 patients with moderate to critically ill COVID-19 were enrolled in the randomized Phase I/II clinical trial in order to evaluate the safety and efficacy of hIDPSCs (NestaCell® product) for the severe COVID-19 pneumonia treatment (ClinicalTrial.gov ID NCT04315987, available in https://clinicaltrials.gov/ct2/show/NCT04315987). The patients included in this study showed the following criteria: aged $\gg 18$ years, confirmed presence of COVID-19, pulmonary impairment greater than or equal to 50%, oxygen saturation $< 95\%$. Patients with autoimmune disease, with diabetes, malignant neoplasm, hematopathy, active hemorrhage, severe malnutrition, known or self-reported HIV or syphilis, pregnant or lactating woman, or those that make continuous use of immunosuppressive agents in the past six months were excluded from the clinical trial. The patients were divided into two groups of 45 participants each: (i) control/placebo, which receives an intravenous administration of cell vehicle (saline) and, (ii) treated, which were subjected to four intravenous infusions of 2×10^7 hIDPSCs/patient on days 1, 3, 5 and 7. A day before (day 0) and one day after each intravenous infusion of NestaCell® product (days 2, 4, 6, and 8), a blood sample was collected and subjected to the D-dimer, prothrombin time (PT), and partial thromboplastin time (PTT) tests. These tests were performed by the laboratory of Vera Cruz Hospital (Campinas, Brazil), in which the patients were hospitalized. For the clinical trial, it was used lots/sub-lots of NestaCell® product containing less than 25% of TF+ cells. Thus, the patients were treated with the lots/sub-lots 1.1, 2.1, 3.1, 3.2, and 4.1 (Table 1). The description of cohort, including the clinical parameters before (day 0) and after the last cell infusion (day 8) is shown in Supplementary Table 1.

Table 1
Percent of TF-positive cells identified in different lots and sub-lots of hIPDSCs (NestaCell® product), obtained from four different donors.

Donor	Lots/sub-lots	TF-positive cells (%)
hIPDSC-1	1.1	10.63
	1.2	29.41
	1.3	37.95
	1.4	56.67
	1.5	63.93
hIPDSC-2	2.1	21.14
	2.2	29.28
	2.3	41.66
hIPDSC-3	3.1	0.21
	3.2	0.26
hIPDSC-4	4.1	0.26
	4.2	33.07

2.9. Data analysis

Statistical analyses were performed through simple linear regression (to evaluate the correlation between the percentage of TF-positive cells and the clotting time) and analysis of variance (ANOVA) one-way and two-way, followed by the Tukey *post hoc* test, both with a significance level of 5%. The analyses were performed using GraphPad Prism v.5.04 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Percentage of TF+ cells varies between the lots of different donors and sub-lots obtained from the same donor

Table 1 shows the percentage of TF+ cells derived from four

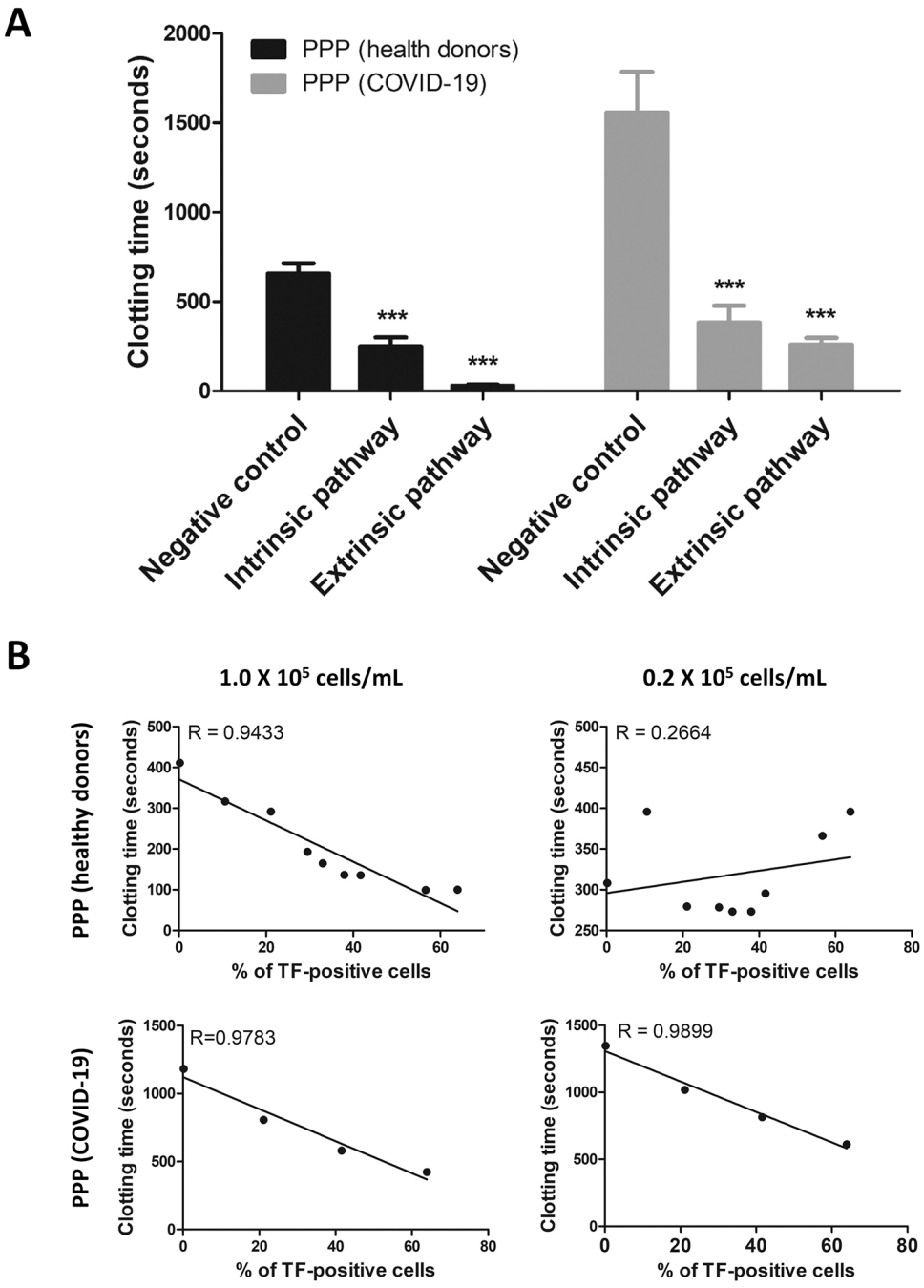

Fig. 1. A) The clotting time of PPP from healthy donors (black) and critically ill COVID-19 patients (gray) after the addition of saline (negative control), ellagic acid in phospholipid solution (positive control to activate the intrinsic coagulation pathway), an external source of tissue factor (positive control to active the extrinsic coagulation pathway). Results show the addition of ellagic acid in phospholipid solution or tissue factor reduce the CT in relation to the negative control, demonstrating the label factors of both intrinsic and extrinsic coagulation pathways are preserved in PPPs from health donors and critically ill COVID-19 patients, respectively. Results also show an increase in CT of negative control of PPPs from critically ill COVID-19 patients as an expected consequence of the heparinization used to reduce the thrombogenic risk in these patients. B) Simple linear regression showing the mean of clotting time (CT) in function of the percentage of TF-positive hIDPSCs (analysis performed in triplicate). Results show that, for the dose of 1.0×10^5 cells/mL, the higher the percentage of TF+ cells, the shorter the CT. However, the CT values remains into the normal range (480–900 s, or 8–15 min). Similar result was observed in PPPs from critically ill COVID-19 patients using the cell dose of 0.2×10^5 cell/mL. However, this effect was not verified in PPPs from health donor for this same cell dose. Analysis performed in triplicate, using all lots/sublotes of NestaCell® product described in Table. Bars show the standard deviation.

independent donors, each presenting one lot. The lots differ in the rate of TF+ cells ranging from 0.2% to 63.9%. One sublot of the hIDPSC-3 lot showed a lower rate of CD142+ cells. All other lots (hIDPSC-1, −2 and −4) vary significantly in CD142+ cells percentage. Note that all hIDPSCs (NestaCell® product) had been isolated and cultivated under the same conditions, according to the GMPs required by the Brazilian Health Regulatory Agency (ANVISA) for advanced therapy products and adopted by the Cellavita Pesquisas Científicas. All cells used in the present study passed through rigorous quality control.

3.2. CT in PPP from healthy and COVID-19 donors is correlated with the percentage of TF+ in high doses of hIDPSCs

We investigate the procoagulant potential of two doses of hIDPSCs (NestaCell® product). The dose 1.0×10^5 cells/mL corresponds to the usually tested dose of 1.0×10^6 cells/kg employed in a clinical trial involving MSC from different sources [32]. The dose 0.2×10^5 cells/mL corresponds to that (20×10^6 per patient and injection) used in the combined Phase I and II clinical trials of NestaCell® product for severe COVID-19 pneumonia.

First, we evaluated procoagulant activity in PPP from healthy and critically ill COVID-19 patients according to TF+ cells percentage. We showed that the clotting time (CT) decreased significantly in both PPPs after adding the activators, demonstrating the label factors of both intrinsic and extrinsic coagulation pathways are preserved in the studied samples (Fig. 1A). However, we also showed that PPP from critically ill COVID-19 patients has significantly prolonged CT compared to PPP from healthy donors. The difference observed in CT between healthy and COVID-19 patients can be justified by the administration of 1 mg/Kg heparin, as proposed by Moll et al. [38], which, by inactivating thrombin, prevents fibrin formation and inhibits thrombin-induced activation of platelets and of factors V and VIII [39], reducing the thrombogenic risk of critically ill COVID-19 patients. Therefore, we confirmed that heparinization of critically ill COVID-19 patients increases CT, reducing the risk for thromboembolism (Fig. 1A).

Next, we showed a correlation between the percentage of TF+ cells and the CT for the high cell dose of 1×10^5 cells/mL and low cell dose 0.2×10^5 cells/mL in PPP from both healthy donors and COVID-19 patients. We demonstrated that the CT decreases when the TF+ cells number in the hIDPSC population increases (Fig. 1B, Table 2). This is also true for low cell doses for critically ill Covid-19 patients (Fig. 1B). Importantly, for the low cell dose for healthy patients, we did not observe such a correlation (Fig. 1B).

Table 2
Results of ROTEM analysis of platelet-poor plasmas (PPPs) from four health donors (A–D) and five patients with critically ill COVID-19 (E–I) before (negative control) and after activation with ellagic acid and phospholipids (activators of intrinsic coagulation pathway - PTT) and external source of TF/CD142 (extrinsic coagulation pathway - PT), showing a significant clotting time (CT) reduction after activation, demonstrating that both intrinsic and extrinsic coagulation pathways are preserved in all analyzed PPPs. Analysis performed in triplicate.

			Coagulation pathway	
PPP	Sample	Negative control $\bar{x} \pm SD$	Intrinsic (PTT) $\bar{x} \pm SD$	Extrinsic (PT) $\bar{x} \pm SD$
Health	A	725.0 ± 16.9	292.0 ± 1.4	22.5 ± 2.1
	B	653.0 ± 14.1	206.5 ± 3.5	34.0 ± 1.4
	C	583.0 ± 1.4	204.5 ± 0.7	31.0 ± 2.8
	D	663.0 ± 7.0	293.5 ± 3.5	32.5 ± 2.1
	$\bar{x} \pm DP$	**656.0 ± 58.1**	**249.1 ± 50.3**	**30.0 ± 5.4**
COVID-19	E	1726.0 ± 19.7	305.0 ± 5.6	191.0 ± 9.8
	F	1306.0 ± 11.3	303.5 ± 3.5	268.5 ± 3.5
	G	1726.0 ± 19.7	353.0 ± 5.6	224.0 ± 2.8
	H	1306.0 ± 11.3	426.0 ± 1.4	288.5 ± 4.9
	I	1719.0 ± 11.3	526.0 ± 2.8	274.0 ± 7.0
	$\bar{x} \pm DP$	**1556.5 ± 228.7**	**382.7 ± 94.3**	**258.1 ± 38.3**

PPP – platelet-derived plasma; results show the mean ($\bar{x}$) followed by the standard deviation (SD) of the clotting time observed for each duplicate. PTT – partial thromboplastin time. PT – prothrombin time.

3.3. CT in PPP for hIDPSC differs between healthy donors and critically ill COVID-19 patients

To investigate whether COVID-19-derived PPP could affect the procoagulant activity of the hIDPSCs, the lots and sub-lots of NestaCell® product were grouped according the percentage of TF-positive cells in: 0.2% (hIDPSC-3.1, −3.2 and 4.1), 10–25% (hIDPSC-1.1 and −2.1), 26–45% (hIDPSC-1.2, −1.3, −2.2, −2.3 and −4.2) and 46–65% of TF-positive cells (hIDPSC-1.4 and −1.5).

Next, we compared the average CT registered for each group with the CT of the respective negative controls. Interestingly, this statistical approach demonstrated significant differences between the procoagulant activity of hIDPSCs tested in PPP from healthy donors and critically ill COVID-19 patients (Fig. 2A, B).

For healthy donors, results showed that, for the cell dose of 1.0×10^5 cell/mL, the sub-lots of hIDPSCs with 24–65% of TF+ cells exhibit low CT than those with 0.2–25% of TF+ cells (Fig. 2A). However, we did not observe the difference in CT between sublots with various TF+ cell percentages in an assay that used 0.2×10^5 cell/mL (Fig. 2B) Altogether, these data confirm previous observations that the procoagulant activity of MSCs depends on the dose of cell used. In addition, we demonstrate that the procoagulant activity of MSCs is in correlation with TF+ cells percentage when high cell doses are used.

Furthermore, we observed a statistically significant difference in CT between the healthy patients in control and for high cell dose for all tested hIDPSC populations with 0.2, 10–25%, 26–45%, and 46–65% of TF+ cells. However, we did not observe a statistically significant difference in CT for the high cell dose between the hIDPSC population, which contain 0.2% and 10–25%, as well as 26–45% and 46–65% (Fig. 2A). For low cell doses, there was no statistical difference in the CT between the same populations (0.2–25% and 26 – 65%) of hIDPSC with TF+ cells (Fig. 2B). Therefore, the MSCs even with a very low percentage of TF+ cells present significant procoagulant activity.

In addition, the results showed that both tested doses of hIDPSC (1.0×10^5 and 2.0×10^5 cell/mL) in PPP from critically ill COVID-19 patients also decreased CT (Fig. 2). However, in PPP from COVID-19 patients, the lots/sub-lots of hIDPSCs with 26–65% of TF+ cells showed the lowest CT, followed by the lots/sub-lots with 10–25% of TF-positive cells (Fig. 2A, B).

Although the lots/sub-lots with more than 25% of TF-positive cells can significantly reduce the CT of heparinized plasma of COVID-19 patients (Fig. 2A, B), results showed that CT registered for these lots are higher than the CT for negative control rate obtained from healthy donors (Fig. 2A,B). These results suggest that heparin treatment of critically ill COVID-19 patients decreases the hypercoagulative state of plasma, reducing the risk for thromboembolism even when MSC are used at a high dose and high TF+ cell percentage. Furthermore, the prophylactic administration of anticoagulants could reduce the eventual thrombogenic risk related to the intravenous administration of MSC.

3.4. TF- cells reduce TC similar to TF+ cells

Since we observed that hIDPSC with only 0.2% of TF+ cells showed CT similar to hIDPSC with 10–25% of TF+ cells in PPP of healthy donors (Fig. 2), we evaluated CT of HUVEC cells, which does not express TF as confirmed by flow cytometry (Fig. 3A). We registered that the addition of the HUVEC cell line (at the doses of 1.0×10^5 and 0.2×10^5 cells/mL) was able to reduce the CT of PPP from both healthy donors and critically ill COVID-19 patients (Fig. 3B). However, we did not observe a dose-dependent response between HUVEC cells doses used in the study (Fig. 3B). Thus, these data suggest that other factors besides the TF can

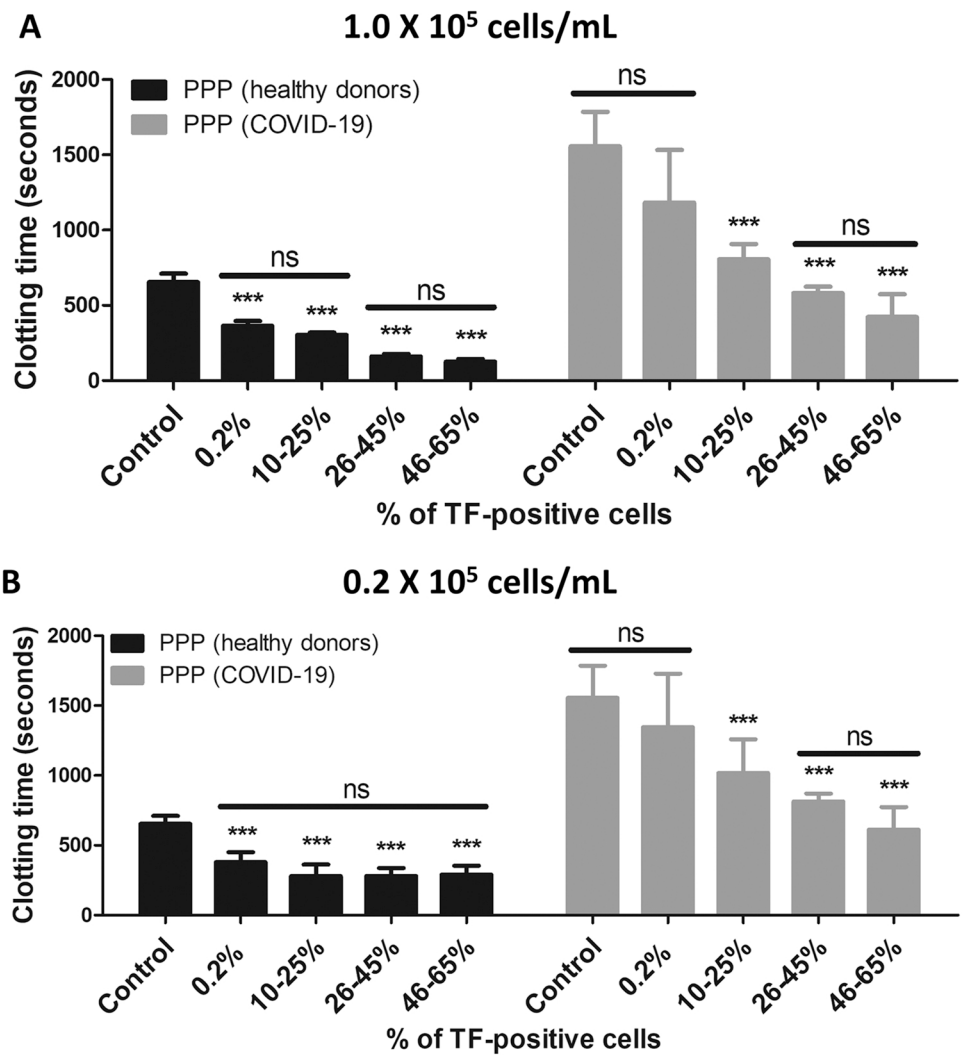

Fig. 2. Results of ROTEM analysis using platelet-poor plasmas (PPPs) from four health donors and five patients with critically ill COVID-19 before (control) and after the addition of 1.0×10^5 and 0.2×10^5 cell/mL (hIDPSC). Results confirm that the PPP from critically ill COVID-19 patients has significantly prolonged CT when compared to PPP from healthy donors, which can be justified by the heparinization of these patients. Results show a statistically significant difference for healthy patients in the control CT and CT for high cell dose for all tested hIDPSC populations with 0.2–25%, 26–65% of TF+ cells. However, we did not observe a statistically significant difference in CT for the low cell dose between the hIDPSC population, which contain 0.2–65%. Notably, the high cell dose and high TF+ cell percentage showed the CT in critically ill heparinized COVID-19 patients is similar to that observed in negative control with high cell dose in PPP of healthy patients. Bars represent the standard deviation. Analysis performed in triplicate, using all lots/sublotes of NestaCell® product described in Table 1.

reduce the CT observed in the ROTEM.

3.5. ROTEM can increase the phosphatidylserine exposure in cell membrane

Although the exposure of phosphatidylserine (PS) on the cell surface is recognized as a marker of apoptosis, Wei et al. [40] showed that the exposure of PS on the bone marrow-derived MSC is crucial for the uptake of microvesicles. However, the exposure of negatively charged PS at the outer leaflet plasma membrane in cells can activate the intrinsic coagulation pathway and convert the encrypted form of TF to decrypt [41]. For these reasons, the exposure levels PS on the outer leaflet plasma membrane of HUVEC cells (TF-negative) and hIDPSC from two sub-lots with 0.2% and 63.9% of TF-positive cells were analyzed using annexin V-FITC.

We perform these analyses before and immediately after to subject the cells to ROTEM analysis to evaluate whether the piston rotation movement could promote changes in the plasma membrane. Results showed that 50% of all analyzed cells were PS-positive (Fig. 3C–E) before the ROTEM analysis. However, after the ROTEM analysis, 100% of cells were PS-positive (Fig. 3C–E), suggesting that the piston rotational movement of the ROTEM can induce changes in the plasma membrane, increasing the PS exposure.

3.6. Prophylactic administration of enoxaparin can avoid the procoagulant activity of hIDPSCs

We also showed that the addition of enoxaparin sodium (0.01, 0.1, and 1.0 U/mL) in lots/sub-lots of hIDPSC with 0.2%, 21.1%, 41.6%, and 63.9% of TF+ cells altogether avoided the clot formation, making the cells uncoagulable even after 30 min of analyzes (Table 3). This data suggest that the prophylactic administration of enoxaparin can mitigate the eventual thrombogenic risk of MSCs.

3.7. hIDPSCs do not confer thrombogenic risk for critically ill COVID-19 heparin treated patients

Clinical research followed our *in vitro* study included 90 patients (45 treatment and 45 placeboes) with critically ill COVID-19. The patients were randomized to receive four doses of 2×10^6 cells (20 million of NestaCell®) intravenously on days 1, 3, 5, and 7 (total dose 80×10^6 cells per subject). A matching placebo was administered IV on the same days as the cells in all patients. NestaCell® product used in the present study was positive for TF, and only cells with CD142 being less than 25%: from 0.26% to 23.28% were administered IV in COVID-19 patients. Commonly used clinical laboratory coagulation indexes, such as D-dimer (DD), prothrombin time (PT), and partial thromboplastin time (PTT), were measured on days 0, 2, 4, 6, and 8. All COVI-19 patients received enoxaparin as concomitant treatment.

We observe no difference in DD, PT, and PTT values before (day 0)

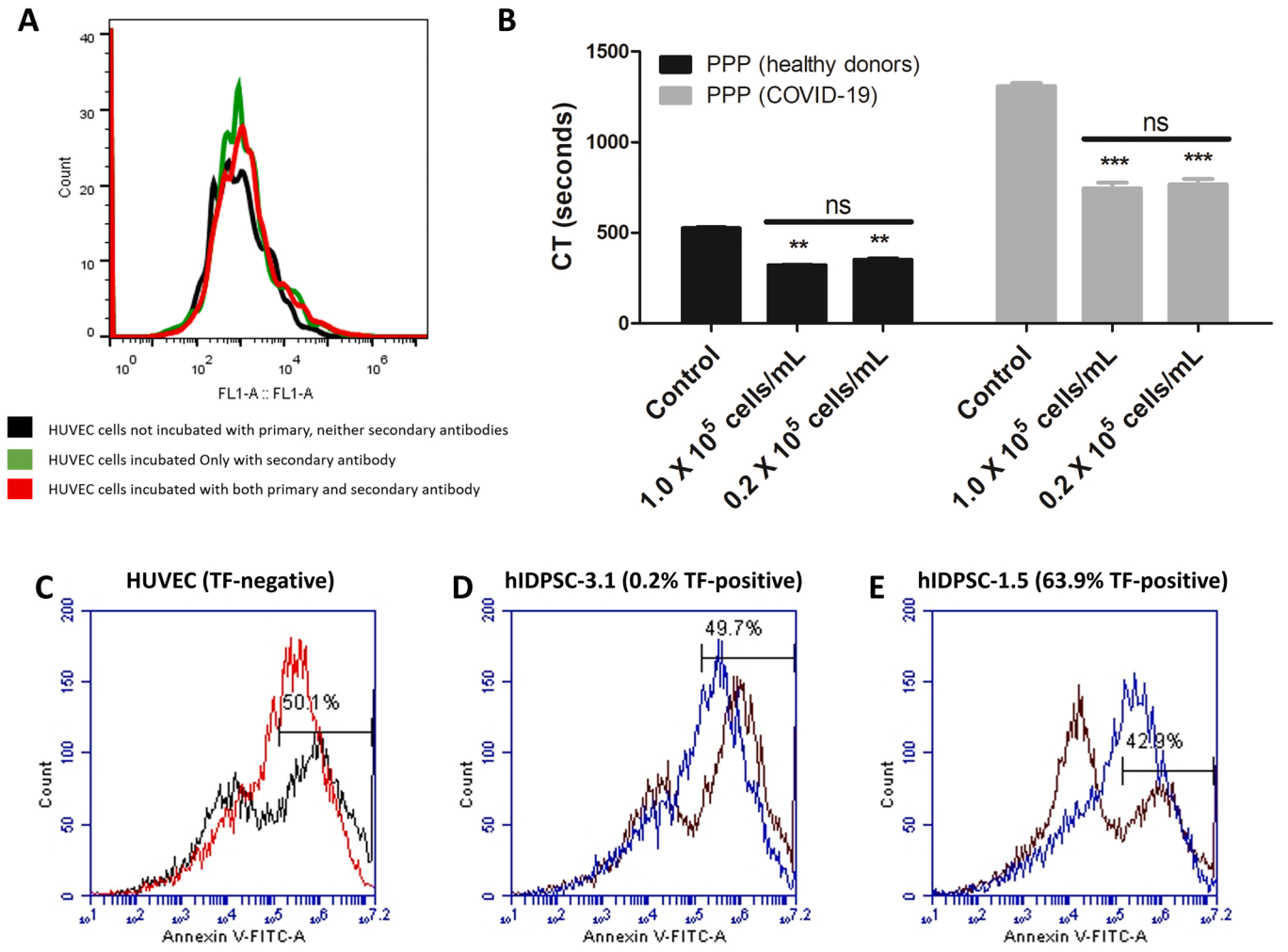

Fig. 3. A) Results of flow cytometry, confirming the absence of tissue factor expression in HUVEC cells. Total of 10,000 events analyzed in triplicate. B) Results of rotation thromboelastometry (ROTEM) showing that the addition of the two doses of HUVEC cells (1.0 × 105 and 0.2 × 105 cells/mL) in PPP from both healthy donors or critically ill COVID-19 patients statistically reduces the clotting time (in seconds). Detection of phosphatidylserine (PS) exposure on the outer plasma membrane before and immediately after the ROTEM analysis. Results show that, before the ROTEM analysis, about 50% of both TF-negative (HUVEC cells, C) and TF-positive (lot 3.1 of hIDPSCs with 02% of TF+ cells, D and lot 1.5 with 63.9% of TF+ cells, E) are PS-positive (line in black). However, after the ROTEM, 100% of all cells, independently of the TF-positive percentage are PS-positive (lines in red – for HUVEC cells, and blue – for hIDPSCs, C-E). Total of 10,000 events analyzes. Analyses performed in triplicate.

Table 3
Results of rotation thromboelastometry (ROTEM) showing the mean of clotting time ($\bar{x}$), in seconds, followed by the standard deviation (SD) showing that the addition of enoxaparin sodium (at 0.01, 0.1 and 1 μg/mL) completely avoids the clot formation, making the samples uncoagulable (in 30 min of analyzes).

				PPP (healthy donors)		PPP (COVID-19)	
	hIDPSC identification			Control	Enoxaparin	Control	Enoxaparin
Cells/mL	Donor	Batch	% of TF	$\bar{x} \pm SD$	0.01/0.1/0.5/1.0 U/mL	$\bar{x} \pm SD$	0.01/0.1/0.5/1.0 U/mL
1.0×10^5	hIPDSC-3	3.1	0.21	391.7 ± 73.4	Uncoagulable	1182.4 ± 121.2	Uncoagulable
	hIPDSC-2	2.1	21.14	291.7 ± 90.9	Uncoagulable	806.4 ± 98.5	Uncoagulable
	hIPDSC-2	2.3	41.66	135.5 ± 61.8	Uncoagulable	580.2 ± 145.8	Uncoagulable
	hIPDSC-1	1.5	63.93	100.3 ± 6.2	Uncoagulable	423.0 ±141.1	Uncoagulable
	HUVEC	–	0.0	322.0 ± 2.8	Uncoagulable	978.5 ± 12.2	Uncoagulable
0.2×10^5	hIPDSC-3	3.1	0.21	235.8 ± 155.8	Uncoagulable	1346,5 ± 381,5	Uncoagulable
	hIPDSC-2	2.1	21.14	279.5 ± 82.2	Uncoagulable	1018,0 ± 240,4	Uncoagulable
	hIPDSC-2	2.3	41.66	295.5 ± 85.6	Uncoagulable	814,0 ± 57,6	Uncoagulable
	hIPDSC-1	1.5	63.93	284.6 ± 61.9	Uncoagulable	612,2 ± 161,5	Uncoagulable
	HUVEC	–	0.0	252.1 ± 5.65	Uncoagulable	1258.5 ± 21.5	Uncoagulable

and after NestaCell® administration (days 2, 4, 6, and 8) in placebo and NestaCell® treated patients (Fig. 4). Statistically, we did not observe a significant difference between placebo and NestaCell® treated patients on days when DD, PT, and PTT were measured (Fig. 4). These data support our *in vitro* findings and demonstrate that the intravenous infusion of NestaCell® product is safe when used in the dose above, multiple administrations, even for moderate to severe COVID-19 patients, which can exhibit an increased prothrombogenic risk.

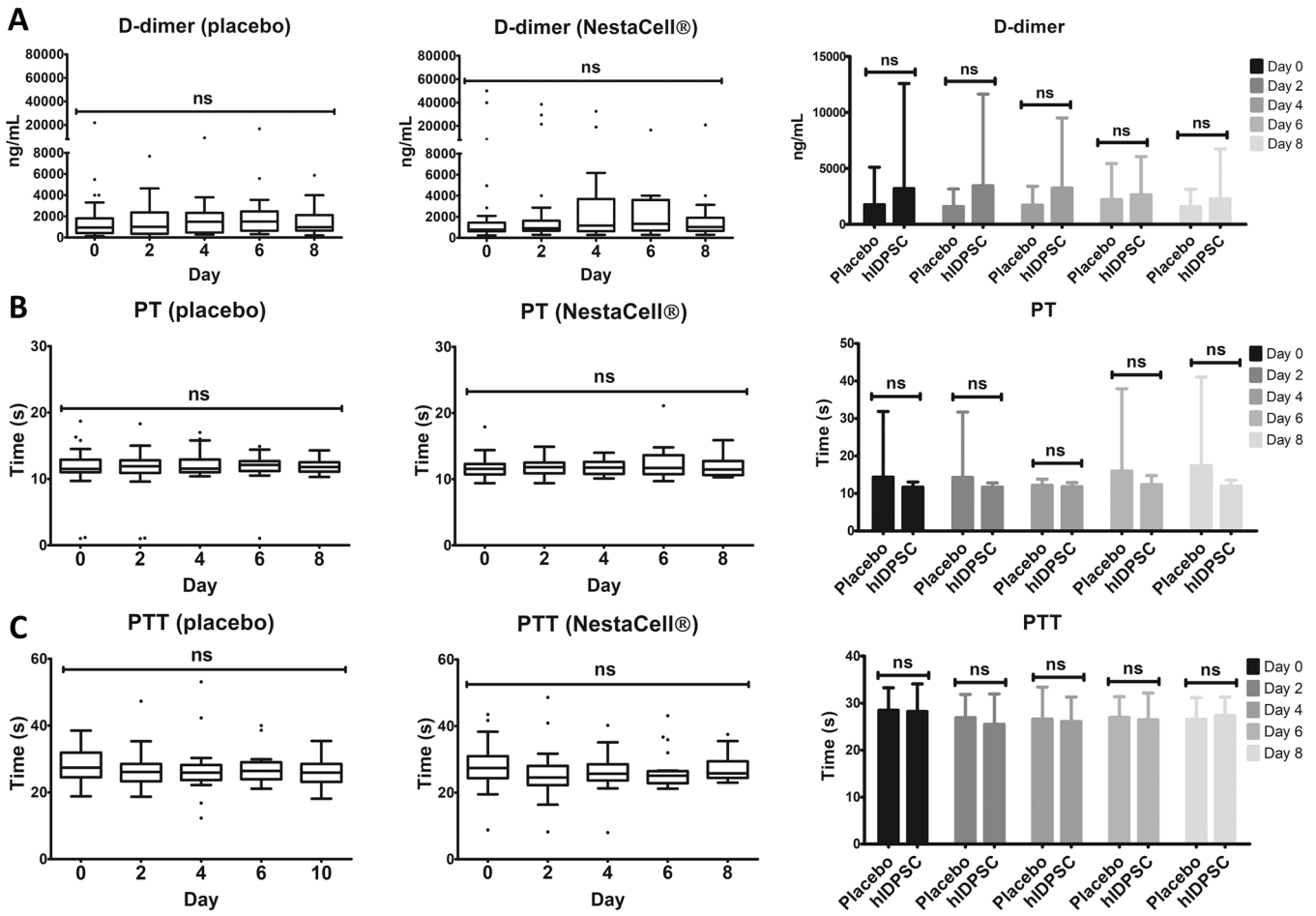

Fig. 4. Results of: A) D-dimer (A), B) prothrombin time (PT) and, C) partial thromboplastin time (PTT) after de hIDPSC intravascular infusion (days 2, 4, 6 and 8). Results show that the cell infusion does not promote changes in the values of D-dimer (A), neither in the PT (B) and PTT (C), indicating that the NestaCell® product is safety even by moderate to critically ill COVID-19 patients.

4. Discussion

Due to their anti-inflammatory and regenerative properties, MSCs have been widely explored for therapeutic purposes for different diseases, including the emerging COVID-19. This is because, the SARS-CoV2 causes an aggressive inflammatory response, which not promotes local damages (in the lungs), but also leads to systemic damages, increasing the risk for thrombogenic events [25].

However, for expressing TF/CD142, a glycoprotein of cell surface enables to trigger the IBMIR and, therefore, increases the risk for thromboembolic events [5,20–23,31], the safety of MSC-based therapies, especially for patients with COVID-19 has been questioned. This is because, previous studies, based on *in vitro* techniques demonstrated that TF-loaded MSCs reduce the clotting time in a dose-dependent manner [5,20–23,31]. Despite this, these studies were performed in platelet-poor plasmas (PPPs) from health donors [20,32], and using cell doses 12–200-fold higher (12×10^6 to 200×10^6 cell/kg) than the typically used dose for the therapeutic purpose, (1×10^6 cell/kg) [31,33,34]. Moreover, these studies suggest that differences in the number of TF+ cells are related to the origin of these cells (tissue and donors), or to their production method [31,33,34].

For these reasons, we analyzed, for the first time, the procoagulant potential of hIDPSCs from different donors (lots) produced in GMP using a well stabilized and patented method (which correspond to the NestaCell® product), avoiding any interference of the manufacturing process. In addition, we analyzed the expression levels of TF/CD142 in sublots derived from the same donor (lot). These sub-lots are obtained by expansion of cryopreserved hIDPSCs in passage two (P2) obtained from the dental pulp cell isolation using a scaling-up methodology developed by us [14,15].

Interestingly, we demonstrated that the percentage of TF+ cells varied significantly between the sublots of the same lot of hIDPSC fabricated under GMP conditions and rigorous control quality. This result indicates that variation in the percentage of TF+ cells occurs independently of the donor or the manufacturing process, being regulated by other biological processes still unknown. This data is particularly important for the industrial production of MSCs, since to date, there is no manufacturing process that can be adopted in order to guarantee a stable percentage of TF+ cells in lots or sublots of MSC products.

Based on these data, we also analyzed, for the first time, the procoagulant potential of hIDPSCs in PPPs from health and critically ill heparinized COVID-19 patients (using ROTEM) in order to predict the thrombogenic risk of NestaCell® product for patients with increased thrombogenic risk. Our results confirmed a correlation between the percentage of TF+ cells and CT for the high cell dose for healthy and COVID-19 patients. We also demonstrated that heparinization of critically ill COVID-19 patients increases CT, reducing the risk for thromboembolism even when MSC are used at a high dose and with a high TF+ cell percentage, as previously demonstrated by *in vitro* studies using PPPs from health donors [19,37,42–44]. In this sense, it was currently demonstrated that the use of heparin anticoagulant does not influence the performance of human MSCs [45]. Confirming these results, we verified that the high cell dose and high TF+ cell percentage present the

CT rate in COVID-19 heparin treated patients was similar or higher to that observed in negative control with high cell dose in PPP of healthy patients.

We also showed that the hIDPSC population with 0.2% of TF+ cells already present procoagulant activity, decreasing CT independently from cell dose. Reinforcing this result, we observed that TF- HUVEC cells were also able to reduce the CT similar to TF+ hIDPSCs, demonstrating that other mechanisms besides the TF could trigger the CT observed in PPP from healthy donors in the ROTEM. Our data suggest that the piston rotational movement of the ROTEM can induce changes in the plasma membrane, increasing the PS exposure, thus reflecting on CT. Confirming this hypothesis, we demonstrated an increase in PS exposure on the cell surface of TF- cells (HUVEC cells) and TF+ cells (hIDPSCs). PS is the major component of cell-based coagulation, enhancing coagulation through the charge-based binding of coagulation factor zymogens and cofactors to increase the formation of the tenase and prothrombinase complexes [46]. For this reason, the use of MSC populations with low PS exposure has been preferentially recommended in order to mitigate the thrombogenic risk [46].

Under physiological conditions, TF is found encrypted in cholesterol- and neutral phospholipid-rich (sphingomyelin and phosphatidylcholine) microdomain of the plasma membrane [12,34]. However, changes on the membrane phospholipid asymmetry occurred since those induced by the binding of interleukin (IL-6) to its receptor (IL6R) can lead to exposure of encrypted to decrypted TF [35,36]. MSC, including hIDPSCs, constitutively express IL-6 once this pleiotropic cytokine plays a crucial role in MSC proliferation, responsible for these cells' immunomodulatory and neuroprotective potential [37,38]. However, once decrypted, the TF elicits the extrinsic coagulation pathway. It proceeds as Ca2+-dependent extracellular signaling to sequentially activate zymogens (factors VII (FVII), X (FX), and prothrombin (FII)) for the generation of coagulant mediators: factor VIIa (FVIIa), Xa (FXa), and thrombin (FIIa), respectively [17]. As a result, FIIa cleaves fibrinogen (FBG) into fibrin monomers that cross-link to produce insoluble blood clots [17]. For this reason, studies have been correlating the expression levels of TF with the MSC procoagulant potential by *in vitro* techniques [9–15]. Thus, our data indicate that the increase in PS exposure on cell surface activates the intrinsic coagulation pathway, and leads to decryption of TF/CD142, activating the extrinsic coagulation pathway.

However, we demonstrated that the *in vitro* enoxaparin addition to PPP from healthy donors with 0.2–63.9% of TF+ cells avoided clotting formation. This result was confirmed by our clinical results, which did not show statistical differences in D-dimer, PT, and PTT values between the patients treated with NestaCell® and treated with placebo (saline). Supporting that MSC-based therapies are safety for COVID-19 patients, independent clinical trials based on the intravenous infusion of three doses of 4×10^7 umbilical cord-derived MSC [47], 150×10^6 Whatson's jelly-derived MSC [48] or even 1×10^6 adipose-tissue-derived MSC [49] did not show any thrombogenic risk. Although these studies did not evaluate the percentage of TF+ cells, they use a prophylactic administration of low-molecular wight heparin.

5. Conclusion

Our study confirmed that the TF/CD142 can reduce the clotting time in a dose-dependent manner. However, as already reported by the literature, we demonstrated that the adding enoxaparin in the cells, it is possible to avoid the clotting formation, mitigating the thrombogenic risk of hIDPSC-based therapy. Supporting this finding, we observed that the intravenous infusion of hIDPSCs (NestaCell® product) with prophylactic enoxaparin administration in moderate to critically ill COVID-19 patients did not change the values of D-dimer, neither the PT and PTT times. Our data also provides evidence that the ROTEM is not enough to predict the risk of TF+ MSC, since this method does not allow to distinguish whether the procoagulant potential evidenced by this technique is related to the TF levels, or the PS exposure. In this sense, we also provide evidence that the ROTEM can promote changes in membrane asymmetry, increase PS exposure, lead to TF decryption, and illicit the intrinsic coagulation pathway. In summary, our data suggest that the intravenous infusion of hIDPSCs (NestaCell® product) is safe even for patients with increased risk for thromboembolism, since administrate with anticoagulants.

Authors contribution

Conceptualization R.P.A. and I.K; methodology R.P.A., I.K., B.C.P., V.G., B.P., T.B.M., F.D., H,V., M.V., C.W.V., E.P.; formal analysis R.P.A., I.K.; writing R.P.A. and I.K.; supervision I.K.; funding acquisition I.K.

CRediT authorship contribution statement

We informed that the **Rodrigo P. Araldi** and **Irina Kerkis** were responsible for the article Conceptualization, Formal analysis and Writing. **Rodrigo P. Araldi**, **Irina Kerkis**, **Bruna C. Policíquio**, **Vivian Gonzaga**, **Thais B. Mendes**, **Fernanda D'Amélio**, **Hugo Vigerelli**, **Cristiane W. Valverde**, **Eduardo Pagani** were responsible for the Methodology development; and **Irina Kerkis** was responsible for the Supervision.

Conflict of interest statement

The authors declare no conflict of interest.

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112920.

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