Original article

Impact of blood source and component manufacturing on neurotrophin content and *in vitro* cell wound healing

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Background - We evaluated neurotrophin (NF) levels and their impact on in vitro cell wound healing in eye drops from differently prepared blood sources (cord blood [CB], and peripheral blood [PB]) in the same donor, to avoid intrasubject biological variability.

Materials and methods - Twenty healthy adult donor PB samples, and twenty CB samples acquired at the time of delivery were processed to obtain serum (S), platelet-rich plasma (PRP), platelet-poor plasma (PPP), and S retrieved from PRP after activation with Ca-gluconate (PRP-R). The levels of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) were assessed with a Luminex xMAP (Luminex Corporation), and by using multikine kits from R&D system, and were statistically analysed in the eight different preparations. The impact of S, PRP, PPP, PRP-R from both sources on a cell line responding to NF supplementation (MIO-M1, UCL Institute of Ophthalmology, London, UK) was tested with a scratch wound assay, and analysed by IncuCyte S3 equipment.

Results - All the preparations from CB showed higher NF levels, except for BDNF where no difference was found as compared to PB. PRP showed higher NF levels with respect to S, PPP and PRP-R in this decreasing order. Younger donors in PB contributed with higher NF levels. The scratch assay showed different cell migration results, with a complete wound closure only recorded with the supplementation of CB-S, and a progressive reduction by using PRP, PRP-R, and PPP from both sources.

Discussion - Protocols of preparation and choice of blood source determine different NF levels in the final products. The therapeutic use of a natural neurotrophin pool from blood sources might have a clinical impact in several different settings. Efforts are needed to standardise the manufacturing and the product content in order to establish and modulate the posology of the final supplementation.

Keywords: *BDNF, NGF, cord blood, peripheral blood, blood manufacturing.*

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INTRODUCTION

Several diseases of the ocular surface, including dry eye disease (DED), corneal ulcer, persistent epithelial defect, neurotrophic keratitis (NK), ocular surface burn, recurrent

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corneal erosion, ocular graft-*versus*-host disease, and limbal stem-cell deficiency have been successfully treated with the topical application of blood-based eye drops[1,2].

Corneal wound healing is a complex process[3] which is mediated by multiple epitheliotropic factors such as epithelial growth factor (EGF), platelet-derived (PDGF) and transforming growth factors (TGF) α and β. In eyes with ocular surface diseases, deficiency or imbalance of tear film growth factors (GFs) leading to ocular surface damage has been shown[4]. The rationale for the use of blood-based products is based on the promotion of cell proliferation and migration through the supply of metabolically active substances, and in particular GFs, cytokines and vitamins, thus replenishing the composition of natural tears.

A role of neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-line derived neurotrophic factor (GDNF) has also been suggested in ocular surface homeostasis by stimulating innervation[5,6]. Neurotrophins (NFs) are critical modulators for the peripheral nervous systems, and their supply may be beneficial for the restoration of sub-basal corneal nerves that have been impaired in DED[7].

An emerging field of interest is the application of blood-based products as neuroprotecting agents in neurodegenerative diseases[8,9].

Either autologous (from patients themselves) or allogeneic (from peripheral blood [PB] of adult donors or from cord blood [CB] sampled at birth) sources have been used in ophthalmic practice to prepare different products mainly under the forms of serum (S), platelet-rich plasma (PRP), platelet-poor plasma (PPP), and S retrieved from PRP after activation with Ca-gluconate (PRP-R). Despite the extensive literature published so far, several issues are still under discussion with respect to standardisation of product processing and storage, and characterisation of active substances contained in the final product. Evidence of the differential levels of GFs in different eye drops has been reported[10-13] with the aim of correlating the clinical outcomes with GFs supplied by the blood-based product[14-16]. The clinical efficacy of these biological eye drops might vary due to problems in reproducibility of the methods and the different GF concentrations among preparations, and there is a need for product standardisation in order to allow comparison of clinical outcomes across different studies[17].

The aim of this paper was to estimate the levels of GFs, with a focus on NFs contained in blood components derived from PB and CB, prepared under different protocols, starting from same donor samples to exclude intraindividual variability. The comparison was performed among the forms of S, PRP, PPP and PRP-R. The biological effect on cell migration exerted by these differently obtained products was then evaluated by *in vitro* wound healing assay.

MATERIALS AND METHODS

This is a prospective study on blood samples obtained from the Emilia Romagna Cord Blood Bank and from the Transfusional Service of the IRCCS Azienda Ospedaliero-Universitaria di Bologna, Italy. The study was performed in the pre-pandemic era (January-September 2019) in respect of the principles of the Declaration of Helsinki, and approved by the Ethical Committee of the Policlinico S. Orsola Malpighi (100/2016/O/Sper). Donors were provided with full information concerning the scientific rationale of the study and were then asked to sign an informed consent. Blood samples were collected from twenty donors by venipuncture at the time of donation and twenty CB samples at the time of delivery.

Each sample was divided into four parts, and each one was processed to obtain S, PRP, PPP, and PRP-R, as described below.

Cord blood

- *Obstetric data* - Data were retrieved anonymously from clinical records with respect to gestational age of the mother, sex and birth weight of the new-born, duration of labour, and mode of delivery.
- *Collection method* - Blood samples from twenty CB units were collected from twenty healthy new-borns with Apgar scores 9 or 10. All steps from the recruitment to the processing and registration of the CB were performed according to guidelines provided by the Foundation for the Accreditation of Cellular Therapy (FACT) and the Italian regulations in force. CB collection for transplantation purposes was performed when the placenta was still *in utero* by puncturing the umbilical vein with a sterile system (cord blood collection set, JMS, Singapore) in a bag containing 20 mL citrate-phosphate-dextrose (CPD) and from *ex utero* placenta vessels in 9 mL Vacuumtube (Biomed Device, Modena, Italy) without any anticoagulant. This study

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used CB units not suitable for haematopoietic stem cell transplantation. The CB units were then sent to the processing facility laboratory in the CB Bank, for further preparation procedures.

- *Assessment of CB unit* - Cord blood was collected from spontaneous term births free of complications and from Caesarean births (≥37th week of pregnancy), selected by trained and qualified health personnel. The units underwent a series of checks and tests to establish the blood characteristics and their suitability for preservation as blood component for therapeutic use; for clinical validation, maternal infectious disease markers (HIV, HCV, HBV *Treponema pallidum*, CMV, Toxoplasmosis and HTLVI/II) evaluations were performed. The number of white blood cells (WBCs), total nucleated cells (TNCs), and platelets (PLTs) were counted with an auto analyzer (XN-1000, Sysmex, Kobe, Japan) and expressed as number $\times 10^6$ mL. The ABO blood group was also determined (Neo, Immucor Gamma, Dreieich, Germany).

Peripheral adult blood

- *Haematological characteristics* - Collected adult PB was tested for infectious disease markers of HIV, HCV, HBV and *Treponema Pallidum* according to the Italian regulations in force. The number of WBCs, TNCs, and PLTs were counted with an auto analyzer and expressed as number $\times 10^6$ mL. The ABO blood group was also determined.
- *Collection method* - The PB was sampled during normal donation procedures from twenty volunteer adult donors and collected in a 9 mL Vacuumtube without any anticoagulant and in a 9 mL Vacuumtube with sodium EDTA. PB samples were processed following Standard Operating Procedures[18].
- *Assessment of PB samples* - The WBCs, TNCs, and PLTs were counted and expressed as number $\times 10^6$ mL. The ABO blood group was also determined as above.

Preparation protocol of blood components from a single donor

The blood components produced for this study were: S, PRP, PPP, and PRP-R, prepared as follows.

- *S preparation*: whole PB collected without anticoagulant was allowed to clot for at least 60 minutes (min) at room temperature. CB collected without anticoagulant was allowed to clot from 24-36 hours (h) at 4°C, due to the temporary storage before transportation from the delivery room to the processing facility (PF), often located in another city. Serum was obtained by centrifugation of blood at 2,500 × *g* for 10 min to remove clot, supernatant was carefully removed and aliquoted.
- *PRP preparation*: whole PB and CB were centrifuged at low-speed centrifugation 200 × *g* for 10 min to obtain a PRP. An aliquot of PRP was stored without manipulation until use. Samples underwent three cycles of freezing at −80°C and slow thawing at room temperature before use either for neurotrophin determination or *in vitro* experiments. The remaining PRP was divided into 2 Falcon tubes.
- *PPP preparation:* one Falcon tube was centrifuged at 2,000 × *g* for 15 min, and supernatant was carefully removed and aliquoted.
- *PRP-R preparation:* 10% v/v of Calcium Gluconate (S.A.L.F., Merck, Milano, Italy) was added to the other Falcon tube. Clotting was induced by allowing the sample to incubate for 10 min at 37°C; the sample was then centrifuged at 2,500 × *g* for 10 min. Supernatant was carefully removed and aliquoted.

Aliquoting in sterile tubes was performed under a laminar flow hood. The tubes were finally stored at −80°C until growth factor determination and *in vitro* use.

Growth factor dosage

Samples from the four preparations obtained from the two sources were tested in Luminex Assay for the presence of selected growth factors, specifically NFs, and in particular BDNF, NGF, GDNF, FGF and EGF. Samples were thawed before each assay, and the amount necessary for each test was removed. Samples were quantified using multiplex magnetic bead-based sandwich immunoassay following manufacturer's instructions (R&D System, Minneapolis, MN) and as previously described[12]. Values with a coefficient of variation above 10% were discarded before the final data analysis. Data were analysed by the xPONENT® Software for Luminex MAGPIX instrument (Luminex Corp., Northbrook, IL, USA).

Cell culture model

The biological activity of the preparations obtained in the present study was evaluated in *in vitro* experiments using a cell line known to respond to neurotrophin administration[19]. Spontaneously immortalised human Muller cell line MIO-M1 (Moorfields/Institute of

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Ophthalmology-Muller 1) was obtained from the UCL Institute of Ophthalmology, London, UK[20]. The MIO-M1 cells were cultured and maintained in DMEM L-Glutamax (Gibco, Thermofisher Scientific, Waltham, MA, USA), 5% Fetal Bovine Serum (FBS), in the incubator at 37°C and 5% CO_2, according to the manufacturers' instructions.

Scratch wound assay

To investigate the wound healing effect of the different blood components on MIO-M1 cells, the IncuCyte S3 instrument and technology was used (Essen BioScience, Ann Arbor, MI, USA). The cells were seeded in a 96-well Essen ImageLock plate (Essen Bioscience) at a density of 10^4 cells/well and cultured in DMEM L-Glutamax culture medium plus 10% FBS until confluence in the incubator at 37°C and 5% CO_2.

Once the cells had formed a confluent monolayer, they were scratched by using the WoundMaker (Essen BioScience) device to obtain homogeneous wounds (700-800 μm width scratches devoid of cells), and rinsed in PBS to remove detached cells and debris. The cells were then treated in DMEM L-Glutamax medium with the addition, respectively, of 5% S, PRP, PPP and PRP-R derived from PB or CB. The culture plate was inserted in an IncuCyte S3 instrument located inside the incubator at 37°C and 5% CO_2. Each wound image was automatically recorded by IncuCyte S3/SX1 optical module phase contrast with a 10× objective, every hour for 48 h. Digital images were analysed using IncuCyte 2019B software to quantify over time the Wound Confluence (%) as the wound area occupied by cells and the Wound Width (μm) as the distance between wound boundaries.

Statistical analysis

Statistical analysis was performed with Python computer software. Descriptive statistics for tests and variables analysed in subjects were reported as geometric mean and 95% confidence interval[21]. The difference between the two blood types (CB and PB) was estimated using a robust linear model including the effect of the source and the preparations (on the logarithmic transformed data), and p-values were adjusted for multiple testing using Benjamini-Hochberg, using 0.05 as threshold of significance. The full table with all the co-efficients can be found in *Online Supplementary* **Table SI**.

We compared the concentrations in PB and CB samples using the geometric mean and 95% confidence interval of each preparation (S, PRP, PPP, PRP-R) in the five NFs tested (BDNF, EGF, NGF, GDNF, and FGF). We analysed the distributions of PB and CB samples, dividing the four preparations (S, PRP, PPP, PRP-R) and the five NFs tested (BDNF, EGF, NGF, GDNF, and FGF). For each distribution, we reported the minimum, maximum, 1st and 3rd quartiles, and median using a box plot representation. In both blood sources, we estimated the Spearman's rank correlation coefficient (r) between the five NFs (BDNF, EGF, NGF, GDNF, and FGF) among the four preparations (S, PRP, PPP, PRP-R). Strength of correlation ranged from −1 to +1 and was categorised considering the absolute values as: "very weak" (0-0.19); "weak" (0.20-0.39); "moderate" (0.40-0.59); "strong" (0.60-0.79); "very strong" (0.80-1.0)[22]. Significance was estimated as two-tailed as

Table I - *Characteristics of mothers and babies*

Donors of cord blood (CB) samples	
Characteristics	**Value**
Number	20
Mother's age (years)*	33 (21-39)
Gestational age (weeks)*	40 (39-42)
ABO blood group	
A	5
B	3
0	12
Parity	
P=1	7
P>1	13
Babies' sex	
Male	8
Female	12
Weight at birth (g)*	3,337 (2,490-4,210)
Donors of peripheral blood (PB) samples	
Characteristics	**Value**
Number	20
Age (years)*	40 (25-57)
Sex	
Male	7
Female	13
Blood group	
A	6
B	1
0	13

*Data expressed as median (95% confidence interval).

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there was no prior assumption as to the possible effects of the protocol on the correlations.

RESULTS

The characteristics of mothers and babies, donors of CB samples, and those of adult donors of PB samples are reported in **Table I**.

The NF levels in the four different preparations from CB and PB are reported in **Table II**.

Statistically significant higher levels of EGF, FGF, and GDNF were shown in CB samples as compared to PB samples. An higher contribution of NGF was detected in the S from CB as compared to PB source, whereas no statistical significance between the two sources was found in NGF levels in the other preparations. There was no difference in BDNF levels in S from either CB or PB, whereas the other preparations from PB evidenced a higher level of BDNF as compared to those from CB.

The comparison among the different preparations with respect to the levels of NFs is shown in **Figure 1**, with the PRP preparations showing the highest contributions in both sources, except for NGF levels where the contribution from S appeared highest in both sources.

The correlations among the number of WBCs, TNCs, PLTs, ABO group, donor age and sex, and NF levels were also analysed. Strong inverse correlations between donor age and BDNF and FGF were found in samples from PB, and in NGF in CB samples (Spearman's rho, range −0.62 to −0.75; $p<0.01$). A moderate direct correlation was found only between weight at birth and BDNF and FGF levels (rho 0.45 and 0.66, respectively; $p<0.01$). A low or weak direct correlation was found between PLTs and NF levels in both sources, except for EGF in CB source where a moderate strength was found (see *Online Supplementary* **Figures S1 a,b** and **S2 a,b** for more details).

The MIO-M1 cell line reached confluence after 48 h from

Table II - *Summary of the statistics extracted from the dataset*

GFs	Preparations	PB (95% CI)	CB (95% CI)	q-value
BDNF pg/mL	**PRP**	12,920.4 (5,902.0-28,284.9)	20,583.5 (11,291.0-37,525.3)	(0.0002)***
	PPP	5,712.8 (1,739.0-18,765.5)	2,578.8 (999.0-6,659.2)	($6E^{-05}$)***
	S	15,556.9 (6,994.0-34,605.7)	13,908.6 (8,326.0-23,235.4)	0.31
	PRP-R	11,376.2 (5,476.0-2,3635.5)	4,905.3 (1,682.0-14,307.6)	($3E^{-06}$)***
EGF pg/mL	**PRP**	472.5 (347.0-642.6)	871.2 (547.0-1,388.0)	($2.9E^{-11}$)***
	PPP	22.9 (11.0-49.2)	46.4 (16.0-135.7)	($4.5E^{-05}$)***
	S	353.0 (259.0-481.3)	604.1 (340.0-1,072.3)	($3.1E^{-08}$)***
	PRP-R	64.7 (40.0-103.4)	319.4 (185.0-553.1)	($2.6E^{-20}$)***
NGF pg/mL	**PRP**	3.9 (2.0-7.9)	4.6 (2.0-14.09)	0.3
	PPP	3.6 (2.0-7.8)	4.0 (1.0-12.6)	0.48
	S	4.4 (1.0-14.6)	6.5 (4.0-11.8)	(0.019)*
	PRP-R	4.5 (2.0-10.8)	3.2 (1.0-10.0)	0.056
GDNF pg/mL	**PRP**	1.0 (1.0-1.7)	1.5 (1.0-3.2)	(0.0002)***
	PPP	0.4 (0.0-0.8)	1.0 (0.0-2.8)	($5.3E^{-07}$)***
	S	0.5 (0.0-1.4)	1.2 (0.0-2.8)	($1.4E^{-05}$)***
	PRP-R	0.4 (0.0-0.8)	0.7 (0.0-1.9)	($4.5E^{-05}$)***
FGF pg/mL	**PRP**	19.8 (4.0-109.9)	160.8 (81.0-316.7)	($1.6E^{-11}$)***
	PPP	9.0 (1.0-56.2)	34.4 (9.0-138.6)	($1.5E^{-05}$)***
	S	8.9 (2.0-35.8)	93.4 (27.0-321.8)	($1E^{-12}$)***
	PRP-R	8.7 (2.0-34.6)	51.7 (2.0-1,504.4)	(0.00014)***

Geometric mean and 95% confidence intervals as the exponential transformation of the confidence intervals of the arithmetic mean of the logarithic transformed data of each concentration for each preparation from both sources: peripheral blood (PB) and cord blood (CB). PB and CB groups were compared using a one-way ANOVA test computing the p-value associated with the corresponding F statistic of the test (performed on the logarithmic transformed values). p-values were adjusted (q-values) using Benjamini-Hochberg correction for multiple tests. Statistical significance is shown: p-values *0.001, **0.01, ***0.05.

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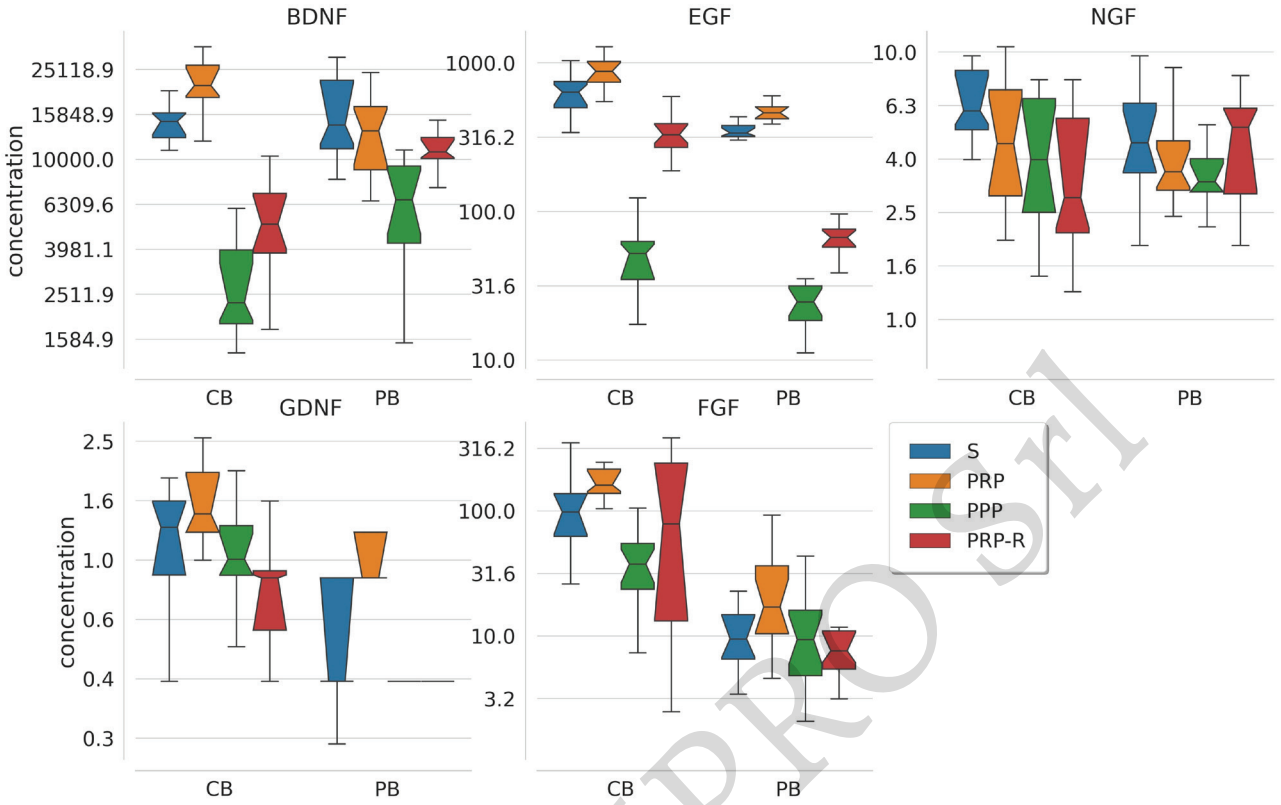


Figure 1 - Distributions of the values (with logarithmic axes scale in base 10) of the four preparations (S, PRP, PPP, PRP-R) in the five source types (BDNF, EGF, NGF, GDNF, and FGF)
CB and PB distributions are represented separately for comparison. Each distribution is represented independently: minimum and maximum distribution shown by lines and median using the internal notch (representing the uncertainty of the median). Box spread represents the 1st and 3rd quartiles of distribution.

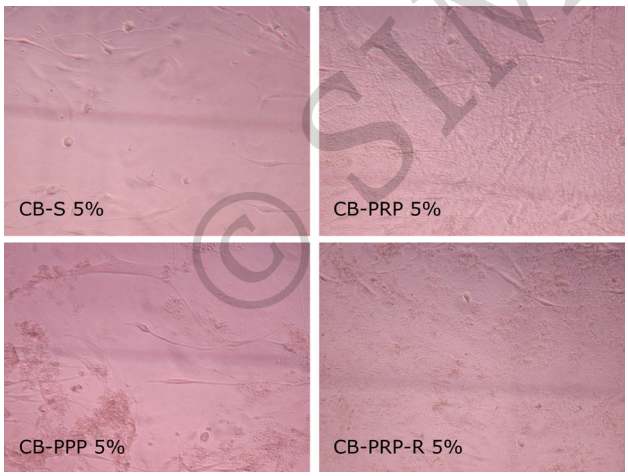


Figure 2 - MIO-M1 cells cultured in DMEM L-Glutamax culture medium plus blood components derived from cord blood (CB): 5% of Serum (S), PRP (Plasma-Rich Platelet), PPP (Plasma-Poor Platelet), or PRP-R (S retrieved from PRP after activation with Ca-gluconate

seeding. When cultured in presence of different blood components added to culture media, the MIO-M1 cells showed a bipolar morphology with irregular membrane and formation of cytoplasmic projections.

No morphologically evident differences were noted with respect to the product or the source used. Representative images from MIO-M1 cells grown in the presence of the four different products derived from CB as a source are reported in **Figure 2**.

The effect of different blood components on the wound healing of MIO-M1 is summarised in **Figure 3** for CB as a source, and in **Figure 4** for PB as a source. In these series of experiments, defined lots of eight blood components were employed, and the NF content mirrored the inter-relationships among NFs inside each preparation form, as already shown in **Figure 1**.

In the experiments using preparations from CB as a source (**Figure 3**), a complete closure of the initial wound at

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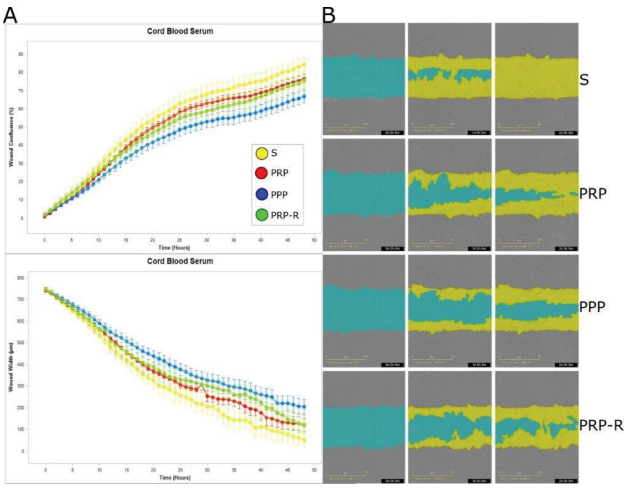


Figure 3 - (A) Representative graphs of Wound Width and Wound Confluence of MIO-M1 cells treated with 5% of S, PRP, PPP and PRP-R produced from cord blood (CB); three replicates for each experimental condition. Data are reported as mean ± SEM. (B) Representative images of MIO-M1 cells treated with 5% of S, PRP, PPP, PRP-R derived from CB taken from an IncuCyte S3 instrument with 10x objective at 0 (at the scratch, left column), 24 (middle column), and 48 (right column) hours after adding the different blood components. Blue: initial scratch wound area; yellow: MIO-M1 cells migrated in the wound area
S: Serum; PRP: Platelet-Rich Plasma; PPP: Platelet-Poor Plasma; PRP-R: S retrieved from PRP after activation with Ca-gluconate.

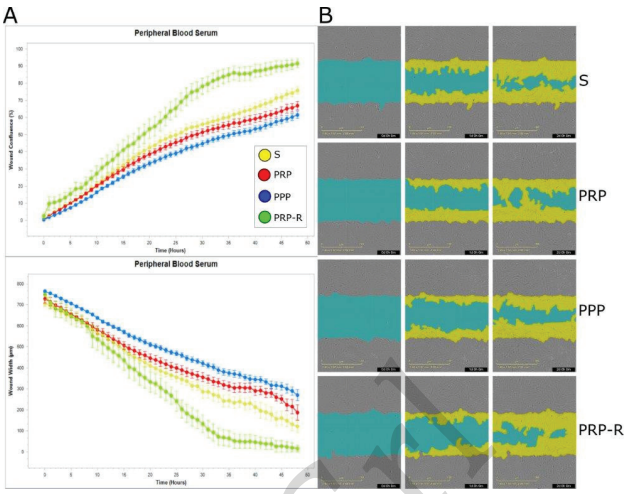


Figure 4 - (A) Representative graphs of Wound Width and Wound Confluence of MIO-M1 cells treated with 5% of S, PRP, PPP and PRP-R produced by peripheral blood (PB); three replicates for each experimental condition. Data are reported as mean ± SEM. (B) Representative images of MIO-M1 cells treated with 5% of S, PRP, PPP, PRP-R derived from PB taken from an IncuCyte S3 instrument with 10x objective at 0 (at the scratch, left column), 24 (middle column), and 48 (right column) hours after adding the different blood components. Blue: initial scratch wound area; yellow: MIO-M1 cells migrated in the wound area
S: Serum; PRP: Platelet-Rich Plasma; PPP: Platelet-Poor Plasma; PRP-R: S retrieved from PRP after activation with Ca-gluconate.

48 h was observed only in the experiments performed with the S form. The Wound Width appeared progressively reduced by using PRP, PRP-R, and PPP, in this order.

In the experiments using preparations from PB as a source (**Figure 4**), a complete closure of the initial wound at 48 h was never observed. The Wound Width appeared progressively reduced by using S, PRP, PRP-R, and PPP, in this order.

DISCUSSION

Data show that different blood sources and different manufacturing protocols impact on NF levels and the biological activity of the final blood components. In particular, they confirmed the higher content of growth factors in all the preparations from CB, except for BDNF level where no difference was found as compared to PB. The preparation in the form of PRP showed higher NF content with respect to S, PPP and PRP-R, in this decreasing order. Younger donors in PB, and high weight at birth in CB appear to contribute with higher NF levels. Biological activity evaluated with the scratch assay showed different effects on cell migration, with a complete wound closure only recorded at 48 h with CB-S.

Methodology of blood collection and subsequent processing steps, including clotting time, anticoagulant type, centrifugation strategy, and storage length, are known to be critical issues, and these might impact on the content of a final product. Reviewing the literature on manufacturing parameters is beyond the scope of the present work; for each blood component, we applied the protocols of preparations in use in our lab. The focus of this work was not to develop the "best" preparation but to compare different preparations starting from the same donor sample, to exclude the impact of intraindividual variability on NF content. To the best of our knowledge, this approach had not been applied previously.

The use of blood-derived topical products has become increasingly popular over recent years as an option in severe ocular surface disorders. However, in spite of this, local legislation varies worldwide and harmonised

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guidelines are still needed to improve and standardise the quality of the final products[17]. A rationale based on a "5 Ws and 2 Hs" protocol is proposed as a useful approach, with the attempt to clarify Who, Why, When, Where, What, and How (for both standardisation of the product and for treatment delivery) to use these treatment options[17].

Autologous blood-derived eye drops have been extensively used in the past and can still be considered the most popular approach, mainly prepared in the form of S or PRP. However, recent reviews[17,23-25] and a Cochrane meta-analysis[26] concluded that while autologous S eye drops provide some benefits in reducing subjective symptoms, the lack of controlled trials, the heterogeneous disease severity and type, and poor product characterisation lead to inconclusive results on resolution of clinical signs.

Blood-based platelet and plasma eyedrops have been introduced more recently and include PPP, PRP with its variant PRGF (Plasma Rich in Growth Factors) to treat ocular surface diseases. These preparations are now being introduced as potentially successful options for retinal disorders[27], although some papers have provided data advising them to be used with caution[28,29].

The GF effect on cell migration, proliferation and survival is a complex paradigm, mediated by a balance in GF levels, and this is an even more urgent issue in biological products.

The different effect of some blood products has been explored on corneal cells in *in vitro* and *in vivo* models[11,30,31]. Lower concentrations of blood components supplemented to culture media (range 3-5%) seem to promote corneal epithelial cell migration, whereas higher concentrations (10% or above) may worsen it, as previously suggested by others[32,33]. Data from the present work seem to support this hypothesis, as the complete wound closure was obtained by adding S and not PRP, suggesting that the delivery of more growth factors is not always the best solution.

A few papers reported the levels of supplementation of growth factors, the form of product, and their potential correlation with clinical outcomes[34,35]. In a recent study, we demonstrated that reduction in corneal epithelial damage is positively associated with EGF, TGFα, and PDGF content in the CB-S used, whereas levels of IL-13 are positively associated with a decrease in symptoms[36].

The issue of NF content in blood-derived products is of scientific interest and could have a clinical impact. The importance of the neural pathway in the ocular surface pathophysiology has been highlighted, and it has now been recognised that neurosensory abnormalities play an etiological role in DED onset[37]. Furthermore, recent evidence shows that topical administration of NFs exerts a beneficial role on corneal nerves[7], and neuroprotection role in both animal models[38] and in pilot studies in humans[8,39], and that these factors can be delivered by blood-based products[8,40,41].

In agreement with previous papers, our data show that young donors contribute with higher GFs levels in PB[42,13].

A limitation of this study is that we characterised only five NFs out of the many growth factors potentially involved, and the outcome on cell migration could have been related to other unknown factors.

CONCLUSIONS

The blood processing procedures, the choice of the source of the blood sample, and the selection of the form of preparation should be selected carefully since the application of different methodological approaches may introduce bias and cause a wide difference in clinical trial outcomes, leading to high interstudy variability. According to the most recent legislation, standard operating procedures on blood sampling and preparation now include the use of certified and validated devices for specific use, blood processing in a sterile environment under a laminar flow hood, and microbiological controls for the detection of aerobic and anaerobic bacteria and fungi. In addition, and with respect to PRP preparations, the control of platelet number is also required.

Assessment of the optimal dosage underpins novel therapeutic candidates. Furthermore, establishing an evidence-based posology is required to compare clinical outcomes. Data from this study would suggest characterising the final blood component and associating a "standard of product". This would promote the introduction of a biomarker of delivery content, for instance, indicating at least one GF level expressed per unit among those most targeting the disease to be treated. Further studies are needed to better understand blood-based derivatives and to establish a standard product.

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AUTHORSHIP CONTRIBUTIONS

Marina Buzzi and Piera Versura share seniorship.
Conception and design: MB, PV, SV; acquisition of data: SV, CD; analysis of data: NC, EG, CD; interpretation of data SV, MB, PV; drafting of manuscript: all Authors; final approval of the version to be published: all Authors.

DISCLOSURE OF CONFLICTS OF INTEREST

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
A patent covering the topic of this manuscript has been filed (WO2018229718) on December 20, 2018, and is owned by the University of Bologna, IRCCS Azienda Ospedaliero-Universitaria di Bologna, and the University of L'Aquila. As a hypothetical conflict, PV and MB, declare that they are inventors of the same patent application, but they guarantee the validity of scientific results as this manuscript is a natural step of the translational research. The other Authors declare no conflicts of interest.

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