

Co-tutelle/Dual degree
UNIVERSITE Lille – Nord de France
Faculté de Médecine
et
Taipei Medical University
Graduate Institute of Medical Sciences

Année 2016

N° _____

THESE

POUR L'OBTENTION DU GRADE DE
DOCTEUR DE L'UNIVERSITE LILLE – NORD DE FRANCE

Discipline: Hématologie et Neurosciences

Présentée par
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**Dedicated, virally-inactivated, platelet lysates and
platelet microparticles in regenerative medicine and
neuroprotective therapies**

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List of Publications

Publications, integrated in this thesis

Major publications

Chou ML, Lin LT, Devos D, Burnouf T. Nanofiltration to remove microparticles and decrease the thrombogenicity of plasma: *in vitro* feasibility assessment. Transfusion 2015;**55**: 2433-44.

Chou ML, Burnouf T, Chang SP, Hung TC, Lin CC, Richardson CD, Lin LT. TnBPTriton X-45 treatment of plasma for transfusion efficiently inactivates hepatitis C virus. PLoS One 2015;**10**: e0117800.

Related publications

Chou ML, Burnouf T, Wang TJ. *Ex vivo* expansion of bovine corneal endothelial cells in xeno-free medium supplemented with platelet releasate. PLoS One 2014;**9**: e99145.

Gouel F, Do Van B*, **Chou ML**,* Jonneaux A, Moreau C, Bordet R, Burnouf T, Devedjian JC, Devos D. The protective effect of human platelet lysate in models of neurodegenerative disease: involvement of the Akt and MEK pathways. Journal of Tissue Engineering and Regenerative Medicine 2016 (accepted). (*co-second author)

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Lin HC, Chang HW, Hsiao SH, **Chou ML**, Seghatchian J, Burnouf T. Platelet-derived microparticles trigger THP-1 monocytic cell aggregation and release of pro-coagulant tissue factor-expressing microparticles *in vitro*. Transfus Apher Sci 2015;**53**: 246-52.

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Patent applications

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- a) Method for removing tangible component-derived fine particle from plasma (patent application #JP2015100410)
- b) Preparation of platelet pellet lysate and its use for treating neurological disorders (European patent application #1.305332.5-1466)

International congresses participation

Oral presentations:

- a) **Chou, M.L.**, Gouel, F., Devedjian, J.C., Devos, D., Burnouf, T. Human platelet lysates exert a neuroprotective effect in a dopaminergic neuron cell model of Parkinson's disease. ISBT, London, UK. 2015 June.
- b) **Chou, M.L.**, Burnouf, T. Removal of microparticles (MPs) from plasma for transfusion by 75 nm nanofiltration. ISBT, Seoul, South Korea. 2014 May.
- c) **Chou, M.L.**, Burnouf, T., Wang, T.J. Isolation and *ex vivo* propagation of corneal endothelial cells in a xenogeneic and recombinant growth factor-free medium supplemented solely with human platelet lysate. ISBT, Kuala Lumpur, Malaysia. 2013 Dec.

Poster presentations:

Chou, M.L., Lin, H.C., Chang, H.W., Hsiao, S.H., Seghatchian, J., Burnouf, T. Platelet-derived microparticles triggers THP-1 monocytic cell aggregation and release of pro-coagulant tissue factor-expressing microparticles *in vitro*. ISBT, Indonesia. 2015 Nov.

Related presentations:

- a) Burnouf T., Sung P.S., Hsiao S.H., **Chou M.L.**, Shieh S.L. 75nm-nanofiltration of plasma removes platelet microparticles that trigger neutrophil extracellular traps (NETs) *in vitro*. ISBT, London, UK. 2015 June.

ISBT: International Society of Blood Transfusion

ACKNOWLEDGEMENTS

This work was accomplished within the scope of a dual PhD diploma agreement between Taipei Medical University (TMU; Graduate Institute of Medical Sciences - GIMS and Graduate Institute of Biomedical Materials & Tissue Engineering -GIBMTE), Taiwan and Lille 2 University (Laboratoire de Pharmacologie Médicale, Unité INSERM U1171), France under the directions of Prof. Thierry BURNOUF, Prof. Liang-Tzung LIN of TMU, and Prof. David DEVOS of Lille 2 University.

I would like to express my sincere gratitude to **Professor Régis BORDET**, Vice-President de la Commission Recherche of Lille 2 University, for hosting me and welcoming me to perform this dual degree in his laboratory. I appreciate his encouragements during my study, and for giving me this precious opportunity.

I equally would like to express my sincere appreciations to **Professor Chao-Ching HUANG**, Dean of the College of Medicine of TMU, for having accepted to be a committee member and for examining my thesis work. I appreciate all his time, his wisdom, and the suggestions he gave me.

I would like to express my sincere gratitude to **Professor Fabrice COGNASSE** who has accepted to be a “rapporteur” for this PhD thesis, and gave me great suggestions during my thesis. I am grateful for his immense knowledge, which leads me towards a better way for my future career. It is highly appreciated.

I would also like to express my sincere appreciations to **Professor Didier LUTOMSKI** who has also accepted to be the “rapporteur” for this PhD thesis. My deep thanks for his advices for my experiments and encouragement for my PhD work. It is my great honor to have you as rapporteur.

I wish to express my sincere gratitude to **Professor Hung-Ming CHANG** for accepting to be in my committee, and also for giving me many valuable advices for my PhD study and for sharing his so precious experience especially for guiding my animal experiments. It is my great honor and it is highly appreciated.

I am especially grateful to **Dr. David BLUM** for accepting to be a member of my PhD thesis committee. My most sincere thanks for sharing treasured experiences in science, providing me many advices for my thesis, and giving me the great opportunity to understand better and integrate his research achievements and goals. I am grateful for all your supports and encouragements, which are truly appreciated.

I express my very sincere gratitude to **Professor Liang-Tzung LIN**, my thesis advisor at TMU-GIMS. I appreciate his constant help during my PhD studies, many advices for my research work, as well as support and encouragement during my thesis. It is highly appreciated.

I would like to express my most sincere gratitude to **Professor David DEVOS**, my thesis advisor at Lille 2 University, for providing me enthusiastic, continuous and immediate support during my PhD study, and giving me many encouragements for the research target of developing a treatment for patients. I appreciate very much his comments, constructive advices and leading my thesis in a good direction. I am quite grateful for opening my vision in the field of neurology.

I would like to express my sincere appreciation to **Professor Thierry BURNOUF**, my thesis advisor in TMU-GIBMTE, who gave me this opportunity to integrate this dual degree for my PhD and enlarge my scope of knowledge in research. I appreciate his patience, constantly listening carefully to understand me. I appreciate his good temper, always being a good mentor to let me know how to be a better researcher. I appreciate his instruction to lead me in a good direction not only during my PhD study but also in my future career. It is my honor to be his student and I thank him for always being at my side.

I certainly want to extend my huge thanks to my colleagues in Taipei. We are like a big family. My sincere thanks to Ivy (Yu-Wen WU) for always supporting me, listening me and encouraging me. She is like my sister and the best team worker. My sincere thanks to Zoe (Joe-Wei WU) for your much help during my studies and making me happy when I am upset. My sincere thanks to Erin (Yu-Chun HUANG), Eric (Ying-Jan WENG), Cindy (Yu-Chun KAO), Vincent (Ming-Sheng CHEN), Candy (Ting-Yi RENN), Ericsson (Chang-chin HUANG), Anita (Chi-Ting CHANG), Gaogao (Shun-Hung HSIAO), Ethan (Yao-Ting Ethan HSIEH), Ouada NEBIE, Mariángela Vargas ARROYO, Rose (Yu-An LIN), Linda (Yuan-Ling SUN), Lucinda (Kuan-Jung LU). Thanks for all the help and company to make my study very colorful.

I would like to give my huge thanks to the team in Lille. My sincere thanks to **Dr. Jean-Christophe DEVEDJIAN** for his support in my thesis work, for his stimulating discussions, and many helps during my stay in Lille. I appreciate all his effort for my studies. My sincere thanks to **Dr. Flore GOUEL** for her kindness, sweetness and nice personality. I feel always happy to stay in the same office as her. My sincere thanks to **Aurelie JONNEAUX, Kelly TIMMERMAN, Dr. Charlotte LALOUX** and **Dr. Bruce DO VAN** for their kindness, beautiful smile and numerous help during my PhD study.

I also wish to share the appreciation I have for my Professors at TMU. My sincere thanks to Pf. Ching-Hua SU who gave me continuous encouragements not only during PhD project but also during my master program. My sincere thanks to Pf. Ching-Li TSENG for her kind advices, frequent helps and beautiful smile. My sincere thanks to Pf. Shing-Chuan SHEN, who is my mentor for these three years, and gave me much substantial support when I faced difficulties. My sincere thanks to Pf. Yen-Chou CHEN for his help and suggestions any time. My sincere thanks to Pf. Jung-Shan CHANG for his tough questions during the seminar or qualifying exams and helped me to become more professional. My sincere thanks to Pf. Yuan-Soon HO, Pf. Wan-Wan LIN, Pf. Shie-Liang HSIEH, Pf. Szu-Ting CHEN, Pf. Chao-Wen CHENG for their advices and experimental supports during my PhD thesis.

I would like to give my huge thanks to Doctors who are like my brothers. My sincere thanks to Dr. Tsong-Yi OU for his warm-hearted support during my master and PhD study. My sincere thanks as well to Dr. Yuan-Hau LIN for his constant and kind support to ensure the cohesion of our PhD group.

I would like to give my huge thanks to my friends and classmates who have encouraged or helped me during my thesis. My sincere thanks to Chien-Pei LU for her nice assistance for all the documents during my master and my PhD, with her constant beautiful smile anytime. My sincere thanks to Austin Changou CHUN, Chin-Ming LEE and the all members in TMU Core Facility Center and Animal Center for their kind aid and helpful suggestions for my experiments. My sincere thanks to my classmates Zhao-Feng CHEN, Shu-Wen LIU, Hung-Sheng HUA, Tzu-Chun KAN, Yi-Hsien SHIN, Wei-Ran WANG, Su-Chuan Lai, Yen-Ju CHAN, Fan-Li LIN and Chih-Hsuan HSIA for all their kindness and many memories shared with them. My sincere thanks to Wei-Ning CHENG and Fang-Mo CHANG for your true cheers during my master and PhD work. My sincere thanks to Chia-Lin LI and Pei-Shan SUNG for their great help in the experiments, as well as to Yen-Ming CHEN for his nice warm coffee. My sincere thanks to Grégory Simon for his patience and his knowledge teaching me French for these two and half years.

I am very grateful to all my friends during my PhD study for contributing to unforgettable memories. Thanks to all of you!

Last but not the least, I would like to express my huge and deep gratitude to my family. I highly appreciate their love, support, encouragement and patience during my engagement in pursuing this scientific target.

Ming-Li CHOU
December 2016

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
A/G	Albumin/gamma
ANSM	Agence Nationale de Sécurité du Médicament
APC	Allophycocyanin
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
aPTT	Activated partial thromboplastin
ATP	Adenosine triphosphate
ARE	Antioxidant response element
BBB	Blood-brain barrier
BC	Buffy coat
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BVDV	Bovine viral diarrhea virus
C18	Octadecyl
CACs	Circulating angiogenic cells
CCL	Chemokine (c-c motif) ligand
CMV	Cytomegalovirus
CMVp	Cytomegalovirus-virus promoter
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CTGF	Connective-tissue growth factor
Ctrl	Control
Cx	Cortex
CXCL	Cxc chemokine ligand
db	Dibutyl
db-cAMP	Dibutyl cyclic adenosine 3',5'-monophosphate
DAB	3,3'-diaminobenzidine
Dkk-1	Dickkopf-1
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	EGF receptor

EP	Electrophoresis
FCM	Flow cytometry
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FVIII	Factor VIII
GDNF	Glial-derived neurotrophic factor
GE	Genome equivalents
GFs	Growth factors
GMP	Good manufacturing practices
GP	Glycoproteins
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV
HIV	Immunodeficiency virus
HGF	Hepatocyte growth factor
hOB	Human osteoblast
Hp	Hippocampus
HPPL	Heat treatment PPL
IEF	Isoelectrofocusing
IGF	Insulin-like growth factors
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IMIG	Intramuscular immunoglobulins
i.n.	Intranasal
INR	International normalized ratio
IP	Isoelectric point
ires	Internal ribosomal entry site
iTregs	Foxp3 ⁺ -induced regulatory T cells
IVIG	Intravenous immunoglobulins
kDa	Kilodaltons
L	Leukoreduced plasma
LPS	Lipopolysaccharide
LTR	Long terminal repeat
LUHMES	Lund Human Mesencephalic neurons
MAO-B	Monoamineoxidase-B
MCP-3	Monocyte chemotactic protein-3

MIP-1 α	Macrophage inflammatory protein-1 alpha
MM	Molecular mass
MMP	Matrix metalloproteinase
MPs	Microparticles
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSC	Mesenchymal stromal cells
N; Nano	Nanofiltered plasma
NAT	Nucleic acid testing
neo	Neomycin resistance gene
NF	Nuclear factor
NLAC	National laboratory animal center
NO	Nitric oxide
NPMP	Natural-PMP
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NS5A	Nonstructural protein 5A
NSCs	Neural stem cells
NTA	Nanoparticle tracking analysis
OA	Osteoarthritic
OB	Olfactory bulb
P	Starting plasma
PAI-1	Plasminogen activator inhibitor-1
PAS	Platelet additive solution
PBS	Phosphate buffer saline
PC	Platelet concentrate
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
pERK	Extracellular signal-regulated kinases
PF4	Platelet factor 4
PFA	Paraformaldehyde
PFP	Platelet free plasma
PGF	Placenta growth factor
PL	Platelet lysates
PLT	Platelet
PMPs	Platelet-derived microparticles
PMP-CACs	PMP-pretreated cacs
PMS	1-methoxy phenazine methosulfate
pNTFs	Platelet neurotrophins

PPL	Platelet pellet lysate
PPL-coagulant assay	Procoagulant phospholipid (PPL)-coagulant assay
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Phosphatidylserine
PT	Prothrombin time
PVDF	Polyvinylidene difluoride
RCTs	Randomized controlled trials
RANTES	Regulated on activation, normal T cell expressed and secreted
RBP4	Retinol-binding protein 4
rNTFs	Recombinant neurotrophins
RLU	Relative light units
SD	Standard deviation
S/D	Solvent/detergent
S/D-C18	S/D treatment followed by C18 filtration
SDF-1 α	Stromal cell-derived factor-1 α
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNARE	Soluble NSF attachment protein receptor
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SOC	Standard of care
ST	Striatum
TC	Total cholesterol
TET	Tetracycline
TEM	Transmission electron microscopy
TF	Tissue factor
TFDA	Taiwan Food and Drug Administration
TFPI	Tissue factor pathway inhibitor
TG	Triglycerides
TGA	Thrombin generation assay
TGF- β	Transforming growth factor-beta
TH	Tyrosine hydroxylase
TIMP	Tissue inhibitor of metalloproteinase
TnBP	Typically tri(n-butyl) phosphate
TP	Total protein
TPMP	Thrombin activated PMP
TRALI	Transfusion-related non-immune acute lung injury

TRAP	Thrombin-receptor-activating peptide
TRIM	Transfusion-related immunomodulation
TRPS	Tunable resistive pulse sensing
TT	Thrombin time
tTA	Tetracycline-controlled transactivator
TZP	Tetragonal zirconia polycrystal
UV	Ultraviolet light
VEGF	Vascular endothelial growth factor
VWF:Rco	Von Willebrand factor ristocetin cofactor
WB	Western blot
WHO	World health organization
ZHO	World health organization
6-OHDA	6-hydrodopamine

ABSTRACT

RESUME FRANCAIS

Garantir la qualité des produits sanguins destinés à la médecine régénérative est crucial. Récemment, les lysats plaquettaires (LP) riches en facteurs de croissance (FC) et en plasma, partiellement substitué ou non par des solutions additives de préservation des plaquettes, s'imposent comme le complément idéal des milieux de culture voués à l'expansion *ex vivo* des cellules souches, et comme thérapie de la réparation tissulaire dans les traumatismes, des pathologies dégénératives, la reconstruction osseuse, certaines maladies métaboliques, et, potentiellement, les maladies neurodégénératives. On voit aussi émerger un intérêt pour les vésicules extracellulaires, ou microparticules (MPs), à titre de système physiologique d'administration de FC. Toutefois, l'expression de phosphatidylsérine à la surface des MPs peut induire des effets thrombotiques et inflammatoires dans le milieu sanguin. Un autre risque transfusionnel est la transmission de virus pathogènes, dont celui de l'hépatite C (VHC), maîtrisable par la mise en place de traitements de réduction virale tels que solvant/détergent (S/D), chauffage, ou nanofiltration.

Notre travail a visé à étudier des technologies d'amélioration de la sécurité des produits sanguins dont les LPs: (a) influence de l'élimination des MPs présents dans le plasma par nanofiltration sur filtres de 75 nm and (b) capacité des traitements S/D, de chauffage à 56°C ou de nanofiltration à inactiver ou éliminer le VHC, respectivement, dans le plasma. Nous avons utilisé les résultats obtenus pour développer un LP de qualité thérapeutique pour des applications ciblant l'usage neurorégénératif. Nous avons ainsi émis l'hypothèse qu'un lysat de culot plaquettaire (LCP) enrichi en facteurs neurotrophiques et dépourvu de plasma est le produit le mieux adapté à la protection des neurones dopaminergiques dans l'option d'une thérapie modificatrice.

Nos résultats montrent que la nanofiltration sur des filtres de 75 nm préserve la composition du plasma et son pouvoir fonctionnel et hémostatique. Des techniques biophysiques spécialisées montrent aussi la capacité de la nanofiltration à décroître

le contenu en MPs et à éviter *in vitro*, dans un milieu plasmatique, la génération de thrombine. De plus, nos résultats révèlent le pouvoir du traitement S/D à 31°C pour 30 minutes à éliminer le pouvoir infectieux du VHC. Pris globalement les traitements de nanofiltration et S/D apparaissent donc comme des méthodes de choix pour l'amélioration de la sécurité du plasma vis à vis de risques thrombogènes et infectieux, respectivement.

Nous avons ensuite utilisé ces résultats pour préparer et caractériser un LCP appauvri en protéines plasmatiques (dont le fibrinogène) et enrichi en un mélange pléiotrope de FC dans le but spécifique d'une administration cérébrale pour traitement des maladies neurodégénératives. Les analyses par ELISA et par approches protéomiques ont montré qu'un chauffage de 56°C pour 30 min réduisait le contenu en protéines et modifiait favorablement la composition relative en facteurs plaquettaires neurotrophiques. Ce chauffage, qui améliore de façon inattendue, l'action neuroprotectrice contribue aussi à la réduction du pouvoir infectieux du VHC, de même que les traitements par S/D et nanofiltration sur filtres de 20nm. Ce LCP exerce une neuroprotection élevée dans des modèles de la maladie de Parkinson (MP) (a) *in vitro* (cellules LUHMES différenciées en neurones dopaminergiques et exposées au MPP⁺) et (b) *in vivo* (souris intoxiquées par MPTP). De plus, nous montrons que l'administration intranasale (i.n.) du LCP chauffé induit une diffusion de FC et protège l'expression de la tyrosine hydroxylase (TH) dans la *substantia nigra pars compacta* et le striatum dans le modèle souris/MPTP. Des événements neuro-inflammatoires pouvant aggraver le développement des maladies neurodégénératives, nous avons vérifié que le LCP n'induit pas l'expression de marqueurs inflammatoires (COX-2, iNOS) par des cellules microgliales BV2, et pouvait même diminuer celle de COX-2 lors d'une exposition à des lipopolysaccharides. De plus, nous avons identifié que le LCP contenait 1×10^{12} MP/mL d'une taille moyenne de 160 nm. Isolées, ces MPs n'ont pas d'effet thrombotique *in vitro* quand elles sont ajoutées au liquide céphalo-rachidien et exercent de plus un rôle neuroprotecteur des cellules LUHMES exposées à l'agent neurotoxique.

En conclusion, nos résultats établissent la faisabilité technique à préparer des lysats plaquettaires viro-inactivés pour des usages en médecine régénérative, et en particulier pour une thérapie modificatrice des maladies neuro-dégénératives du système nerveux central, comme la maladie de Parkinson.

OBJECTIFS

L'objectif principal de cette thèse a été de développer un lysat plaquettaire spécifique (PL) capable d'exercer une activité neuroprotectrice dans des modèles cellulaires et animaux de la maladie de Parkinson (PD). PD a été choisi comme un modèle représentatif des maladies neurodégénératives parce que des modèles expérimentaux fiables de cette maladie ont été mis au point, permettant d'y induire une neurotoxicité et d'étudier le pouvoir de neurorestoration de différentes thérapies expérimentales. De plus, PD est, avec la maladie d'Alzheimer, l'une des principales causes pathologiques de décès chez les personnes âgées à l'échelle mondiale.

Qualité et sécurité sont deux éléments essentiels à prendre en compte de manière prioritaire dans tout projet de recherche translationnelle impliquant l'utilisation de PL en médecine régénérative. Les exigences réglementaires sont particulièrement aigues pour tout produit sanguin thérapeutique préparé à partir d'une source de sang homologue (ou allogénique). Un critère primordial de qualité est la sécurité virale. Une marge de sécurité virale adéquate peut être assurée par une combinaison de procédures de réduction virale visant à compléter les mesures existantes de sélection des donneurs et de dépistage des dons par les établissements du sang (EFS en France). En effet, en dépit des procédures de dépistage mises en œuvre pour une sélection efficace des dons de sang utilisés pour fabriquer les produits thérapeutiques, le risque de contamination par des agents pathogènes par le sang, comme le VIH, le VHC et le VHB, reste élevé dans les zones endémiques. Il l'est aussi dans les pays aux ressources économiques limitées. L'amélioration de la sécurité des produits sanguins passe par l'usage de technologies d'inactivation ou d'élimination des pathogènes dès lors que celles-ci ne portent pas atteinte à l'activité fonctionnelle des cellules et des protéines présente dans le produit sanguin labile. Le traitement solvant détergent (S/D), diverses approches de traitements thermiques et la nanofiltration sont, parmi d'autres, les méthodologies les plus en vue aujourd'hui pour assurer une marge suffisante de sécurité virale aux produits plasmatiques. L'autre contributeur de qualité et de sécurité des produits sanguins identifié

récemment est la présence, qui peut être préjudiciable ou bénéfique, de microparticules d'origine cellulaire. Un effet néfaste potentiel de ces microparticules est l'induction de thromboses dans un milieu plasmatique ou sanguin.

Dans cette thèse, nous avons supposé que la sécurité virale de PL destiné à être utilisé dans la neuroprotection peut être obtenue par inactivation virale (par traitement S/D et/ou traitement thermique), ou par élimination virale (par nanofiltration). En outre, nous nous sommes aussi concentrés sur le rôle éventuel joué par les microparticules sur la sécurité et/ou de l'efficacité du plasma pour transfusion en étudiant l'impact joué par une étape de nanofiltration sur fibres creuses de 75 nm de porosité. Ces développements ont d'abord été réalisés en utilisant le plasma comme modèle de produit sanguin, choisi en raison de sa complexité protéique. Les résultats obtenus ont ensuite été appliqués au développement d'un lysat plaquettaire spécifique pour administration au cerveau par voie intra-cérébro-ventriculaire (ICV) ou par voie intranasale (i.n.), et caractérisé par une faible teneur en protéines proche de celle du liquide céphalo-rachidien (LCR). Ce LP est appauvri en fibrinogène et autres protéines plasmatiques potentiellement toxiques, et contenant un mélange physiologique concentrée de neurotrophines dérivées des plaquettes.

Mes études ont donc été divisées en 3 objectifs principaux:

Objectif 1: Evaluer la capacité d'un traitement S/D à inactiver le VHC, choisi comme modèle de virus présent dans le sang et dans le plasma. Nous avons le virus VHC issu de cultures cellulaires (VHCcc), des isolats cliniques et du virus préparés à partir de cultures d'hépatocytes humains primaires, ceci pour les "spikings" et la détermination du niveau d'infectiosité du VHC.

Objectif 2: Evaluer la capacité de la nanofiltration 75-nm sur fibres creuses à éliminer les MPs du plasma pour transfusion et étudier l'impact de leur élimination sur les propriétés fonctionnelles du plasma.

Objectif 3: Utiliser les informations recueillis à partir des objectifs 1 et 2 pour développer un PL viro-inactivé à une forte activité neuroprotectrice dans un modèle cellulaire dédié utilisant des cellules LUHMES exposées à des neurotoxines, et dans un modèle animal de souris intoxiquée par la neurotoxine MPTP.

RESULTATS ET DISCUSSION

Mise en évidence de l'inactivation du VHC par le traitement S/D

La transfusion sanguine est une procédure médicale de resuscitation essentielle lors du traitement de traumatismes graves, ou pour la thérapie de substitution en cas de déficits congénitaux ou acquis en certaines protéines sanguines. La collecte et le traitement du sang sont étroitement réglementés, et les produits sanguins doivent répondre à des exigences de qualité et de sécurité bien établies.

Un critère essentiel à la qualité des produits sanguins allogéniques est la sécurité vis à vis des agents pathogènes. Un premier objectif de notre travail a donc été d'étudier les méthodes d'inactivation ou d'élimination virale susceptibles d'être mises en oeuvre sur un LP utilisé en médecine régénérative, puisqu'aucune technologie spécifique n'est actuellement disponible. En particulier, le risque de transmission du VHC reste l'une des principales préoccupations de la sécurité transfusionnelle. En dépit des progrès réalisés dans les tests de dépistage viraux, l'OMS a estimé cette année encore la prévalence des infections transmissibles par transfusion de 0,32 à 1,03% pour VHC par don de sang.

Plusieurs méthodes ont été mises au point pour inactiver les virus dans les produits sanguins en utilisant des technologies ayant des effets néfastes limités sur l'efficacité clinique des composants sanguins. Dans les 10 à 15 dernières années, trois méthodes ont été autorisées dans divers pays pour l'inactivation des virus dans les dons individuels de plasma pour transfusion et/ou les concentrés de plaquettes. Elles reposent sur des technologies consistant en l'ajout d'un photo-inactivateur et une l'exposition à la lumière visible ou aux ultra-violets (UV).

Une autre méthode, appliquée à un pool de plasma est le traitement S/D réalisé à l'échelle industrielle, procédé mis au point pour inactiver les virus enveloppés sans altérer la capacité hémostatique du plasma. Un traitement d'inactivation virale S/D, utilisant 1% de TnBP et 1% Triton X-100, a été le premier introduit pour traiter des pools de plasma de 100 à 2500 dons à l'échelle industrielle. L'élimination des agents S/D est obtenue généralement par chromatographie d'interaction hydrophobe à grande échelle sur C18. Un traitement simplifié S/D en poches utilisant 1% de TnBP et 1% de Triton X-45 a été mis en œuvre pour le plasma et cryoprécipité. Le traitement S/D maintient l'activité fonctionnelle des protéines et a été validé pour son efficacité à inactiver les virus enveloppés. Cependant, en raison de l'absence d'une méthode de culture du VHC appropriée *in vitro* et de la difficulté technique et éthique pour utiliser un modèle d'infectiosité sur animaux, tels que le chimpanzé, l'efficacité du traitement S/D à inactiver le VHC dans le plasma n'a pas encore été évaluée.

Dans la dernière décennie, un modèle de culture et d'infectiosité du VHC *in vitro* a été développé sur des cellules du foie. Nous avons utilisé le VHCcc marqué par la luciférase pour démontrer que le traitement S/D (1% -TnBP et 1% de Triton X-45) est capable d'inactiver le VHC dans un mélange complexe de protéines, comme le plasma. Nos données révèlent la capacité du traitement S/D à inactiver le VHC en 30 minutes à 31°C. La méthode est aussi efficace pour l'inactivation du VHC d'isolats primaires de patient. Nous montrons aussi que ce traitement S/D-C18 suivie d'une filtration est capable d'éliminer le VHC sans altérer la fonction hémostatique du plasma. En outre, en conformité avec les recommandations internationales, nous avons vérifié le maintien des propriétés hémostatiques du plasma traité. Nous avons confirmé que la procédure S/D-C18 maintient l'activité coagulante évaluée par tests PT et aPTT et dosages spécifiques des facteurs de la coagulation. Le procédé S/D-C18 est aussi efficace pour inactiver le virus de la dengue aux caractéristiques structurales similaires à celles du VHC. Donc, le traitement S/D suivi d'une filtration C18 présente une grande efficacité pour inactiver le VHC dans le plasma, ce qui confirme qu'il peut être considéré éventuellement pour traiter le LP.

Présence et fonctions des microparticules dans le plasma; l'impact de la nanofiltration

Il est apparent que les vésicules extracellulaires, également appelées microparticules (MP) sont présentes en grande quantité dans les composants cellulaires du sang et plasma pour transfusion. Ces MP sont présentes soit lors de la collecte de sang ou générées au cours des étapes de traitement, telles que la filtration pour déleucocytation, la centrifugation, et le stockage. Des données récentes suggèrent qu'un nombre élevé de MPs peut être associé à certains états pathologiques, tels que le cancer et maladies inflammatoires. Des quantités élevées de MPs dans les composants sanguins (tels que le plasma, les concentrés de plaquettes ou de globules rouges) sont soupçonnés de déclencher des événements thrombotiques, des syndromes inflammatoires et être responsables de réactions potentiellement graves.

Nos études ont montré que le plasma traité par nanofiltration 75 nm (Planova 75N, Asahi Kasei medical, Tokyo, Japon) pour éliminer les MPs conserve un profil protéique et de lipoprotéique, et des teneurs en facteurs de coagulation normales. En outre, la nanofiltration n'a pas modifié la teneur en lipoprotéines tels que le HDL, IDL et VLDL particules qui ont une taille comprise entre 8 et 50 nm.

Il est de plus en plus admis que, en raison des limites technologiques de chacune des méthodes actuellement disponibles, différentes méthodologies biophysiques spécialisées devraient être combinées pour évaluer les MPs. La cytométrie de flux trouve une réduction des MPs marquées par l'annexine V et CD41a de 80%, mais cette méthode ne détecte que des particules de plus de 500 nm, et le bruit de fonds décroît la sensibilité. La méthodologie "DLS" a montré aussi que les MPs de plus grande taille sont éliminées par nanofiltration. Deux populations de MP ont été détectées dans le plasma nanofiltré, avec une taille moyenne d'environ 9 et 50 nm,

Nous avons également utilisé l'analyse par "NTA" puisque cette méthode permet d'analyser des particules isolées plus petites que par DLS, bien que les interférences provenant d'agrégats de protéines sont possibles. La nanofiltration élimine les MPs de taille supérieure à 50-100 nm. En utilisant une membrane ayant une taille de pores appropriée, la "TRPS" permet d'identifier les particules qui traversent un pore de taille précise, ce qui permet de les compter avec précision et d'en estimer la taille. Aucune MP ne fut décelable dans le plasma nanofiltré par analyse TRPS doté d'un pore de 100 nm à même de compter les particules dans une gamme de 50 à 200 nm. En revanche, avec les plasmas déleucocytés, on a observé un blocage de la membrane de 100 nm en raison du grand nombre de MP de grande taille. Lors d'une analyse sur des pores de 400 ou 800 nm de nombreuses MPs furent décelées. Le "spiking" d'un tampon PBS avec une quantité connue de PMP isolées, puis nanofiltrer sur le nanofiltre de 75 nm indique une capacité d'élimination de 8 logs, comparables à celle rapportée pour les virus pour des filtres de conception similaire.

Nous nous sommes penchés également sur l'impact de la nanofiltration sur la génération de thrombine *in vitro* dans le plasma. L'analyse par test de génération de thrombine (TGA) avec le réactif "RC faible" à faible concentration de micelles phospholipidiques montre la génération de thrombine par le plasma de départ et celui leucoréduit mais pas par le plasma nanofiltré. Donc, la nanofiltration 75-nm élimine les MPs responsable de la génération de thrombine *in vitro*. Les analyses par coagulation (test PPL-coagulant) ont confirmé l'élimination des MPs exprimant la phosphatidylsérine. La méthode Zymophen MP-FT (facteur tissulaire) ne révèle pas la présence de MPs exprimant le FT, conformément à la littérature. Par conséquent, nous concluons que 75 nm plasma nanofiltré présente une activité hémostatique normale due à la préservation des facteurs de coagulation, mais l'épuisement en MP par nanofiltration contribue à diminuer son activité thrombogène *in vitro*.

Pris dans leur ensemble, on en conclut que le S/D et le traitement par nanofiltration sont des techniques utiles au développement de produits sanguins pour administration chez l'homme d'une plus grande sécurité virale et thrombotique.

Développement d'un lysat plaquettaire pour des applications thérapeutiques dans les maladies neurodégénératives

Nous avons utilisé les informations précédentes pour développer un lysat plaquettaire thérapeutique pour l'administration chez les patients souffrant de maladies neurodégénératives. Nous avons à l'esprit les objectifs suivants pour définir un produit optimal:

- (A) Sélectionner, en tant que matériel de départ, un type de concentré plaquettaire facilement disponible au monde d'origine autologue ou allogénique,
- (B) éviter une surcharge en protéines, en particulier lorsque l'administration ICV est considérée, afin de ne pas saturer le LCR dont la teneur totale en protéines est d'environ 1 g/L,
- (C) Assurer une teneur élevée en neurotrophines plaquettaire
- (D) Vérifier l'absence d'effets inflammatoires *in vitro* et/ou lors de l'administration dans un modèle animal
- (E) Possibilité de développer une forme d'administration i.n. non invasive utile chez certains patients
- (F) Mettre au point un procédé de préparation à même de permettre l'introduction d'étapes de réduction virale, industrialisable, et adaptés aux dons allogéniques ou autologues, et de faible coût.

Il était également important pour sa mise au point de sélectionner une maladie neuro-dégénérative pour laquelle des modèles d'évaluation fiables *in vitro* et *in vivo* existent. Nous avons utilisé des modèles développés pour la maladie de Parkinson pour évaluer la capacité thérapeutique potentielle d'un lysat plaquettaire dans les maladies neurodégénératives, et plus généralement dans les pathologies neurologique.

Dans notre étude, nous avons utilisé des cellules LUHMES pour les tests cellulaires. Ces cellules ont été obtenues auprès du laboratoire du Dr David Devos à l'Université de Lille, France. Les cellules LUHMES sont des cellules humaines immortalisées, non

cancéreuses, d'origine fœtale, sous-clone des cellules mésencéphaliques MESC2.10 cells. Ces cellules peuvent être différenciés en neurones dopaminergiques et représentent un modèle fiable *in vitro* pour l'étude de la maladie de Parkinson. Ces cellules sont utilisées pour évaluer la capacité neuroprotectrice et neurorégénérative lors d'une intoxication *in vitro* par le MPP⁺ dont le précurseur MPTP provoque chez l'homme le parkinsonisme. Les premières expériences réalisées au laboratoire du Dr Devos, puis confirmées à l'Université Médicale de Taipei, ont montré que le traitement des cellules avec des lysats de plaquettes réduit de manière significative la neurotoxicité induite par le MPP⁺.

Les concentrés plaquettaires (CP) utilisés ont pour la plupart été obtenus par aphérèse, sans déleucocytation et maintenus en présence de plasma, méthode disponible dans le monde entier. Les premières expériences dans le laboratoire du Dr Devos ont suggéré que les protéines plasmatiques peuvent nuire à la viabilité des cellules LUHMES. Par conséquent, il a été décidé d'isoler les plaquettes pour préparer le LP (dénommé PPL). L'élimination du plasma a permis d'éliminer les protéines majeures qui sont présentes dans la plupart des LP utilisés en médecine régénérative, constituant ainsi un premier pas vers notre objectif de développer un produit à faible teneur en protéines pour administration cérébrale. Par ailleurs la remise en suspension du LP dans 1/10ème du volume initial du CP a conduit à un enrichissement de 40 fois des facteurs neurotrophiques. Cette approche a aussi permis la réduction efficace du taux de fibrinogène plasmatique qui est néfaste à la culture (induction d'une coagulation du milieu), et évitant l'ajout d'héparine.

Le LP a été obtenu par 3 cycles de congélation/décongélation éviter le recours à l'ajout d'agents de dégranulation des plaquettes comme le chlorure de calcium. In en résulte une concentration en PDGF-AB, BDNF, TGF- β , b-FGF, et VEGF beaucoup plus élevée que dans les LPs traditionnels. L'analyse par SDS-PAGE et électrophorèse bi-dimensionnelle a confirmé l'élimination de nombreuses protéines plasmatiques, tout en révélant la composition encore complexe du LP.

Nos expériences cellulaires ont d'abord établi la non-toxicité du PPL sur les cellules LUHMES. Un pré-traitement d'une heure des cellules LUHMES par 2-5% PPL a montré un effet de protection marqué contre le MPP⁺. Cet effet protecteur est retrouvé avec des PPL préparés à partir de CP frais (jour de la collecte), ou après 3, 6 ou plus de 6 jours de conservation, même si l'effet neuroprotecteur semble décroître à partir de 6 jours de conservation des CPs.

Nous avons décidé d'évaluer l'impact d'un traitement thermique du PPL à 56°C pendant 30 min (dénommé HPPL). Il induit une diminution significative complémentaire de la teneur totale en protéines, respectant ainsi nos objectifs d'une diminution de la charge en protéines. Il faut noter que ce traitement thermique conduit à renforcer l'activité neuroprotectrice *in vitro* à des concentrations en PPL beaucoup plus faibles, ce qui suggère l'élimination de facteur inhibiteur. Le chauffage modifie la composition en protéines. Ainsi, les isoformes du PDGF, qui favorisent la survie des neurones dopaminergiques, sont enrichies proportionnellement aux autres protéines après traitement thermique. En revanche, EGF, BDNF et bFGF sont retrouvés en proportion moindre après chauffage suggérant une action neuroprotectrice limitée dans ce modèle de maladie de Parkinson.

Nous avons également mis l'accent sur l'étude de l'impact des MPs dans les PPLs. PPL et HPPL contiennent une quantité importante (environ 1×10^{12} /ml) de MPs. Testées dans le modèle LUHMES, ces PMPs exercent un effet neuroprotecteur contre MPP⁺ et peuvent donc jouer un rôle dans l'activité neuroprotectrice du PPL et du HPPL.

Un autre objectif était de contrôler l'absence d'activité pro-inflammatoire du PPL et du HPPL. En effet, l'activation des cellules microgliales peut induire une neuro-inflammation néfaste. Testés sur une lignée de cellules microgliales BV2, le PPL et le HPPL ne déclenchent pas l'expression de protéines inflammatoires comme COX-2 et iNOS, et réduisent celle de COX-2 lors d'un contact avec les lipopolysaccharides.

Récemment, l'administration i.n. a attiré l'intérêt des chercheurs car il s'agit d'un traitement non-invasif. Dans nos expériences, les souris ont été soumises à l'administration i.n. par HPPL. Nous avons observé une accumulation significative de PDGF-AB (27-31 kDa) chez le bulbe olfactif, le striatum et le cortex, ainsi que celle de PF4 (11 kDa) dans le cortex. De plus HPPL protège les neurones dopaminergiques dans le striatum et la SN d'une intoxication de la souris par MPTP. Cette forme d'administration pourrait être une option pour une utilisation chez des patients aux stades précoces de l'apparition des troubles neurologiques, si toutefois cette forme d'administration se révélait possible aussi chez l'homme.

HPPL peut être obtenu en utilisant des CP allogéniques. Des étapes d'inactivation virale doivent être introduites durant la préparation. Le VHC a été utilisé comme modèle de vérification de l'efficacité d'inactivation des traitements car ce virus est pathogène à l'échelle globale et il peut infecter le système nerveux central. Et induire des dysfonctionnements cognitifs. Notre travail révèle la possibilité d'appliquer deux types de traitement de réduction des virus, S/D et nanofiltration, aux LPs destinés à être utilisés en médecine régénérative. Nous avons aussi testé la capacité du traitement thermique à 56 ° C pendant 30 minutes à inactiver le VHC.

S/D traitement:

Notre étude initiale visait en partie à démontrer la possibilité d'utiliser un nouveau modèle VHC pour évaluer l'efficacité d'un traitement S/D à base de 1% de TnBP et 1% de Triton X-45 pour inactiver le VHC dans les produits sanguins. Nos études sur plasma ont révélé la possibilité d'utiliser ce modèle d'infectivité pour évaluer un traitement d'inactivation virale appliqué à un mélange complexe de protéines, comme le plasma et (b) la capacité de cet traitement S/D à inactiver le VHC de manière efficace (> 2,8 logs). Des expériences supplémentaires ont donc été effectuées pour vérifier la capacité d'un traitement S/D à inactiver le VHC dans les PPLs développés pour traiter les troubles neurodégénératifs. Les résultats confirment la capacité du traitement à inactiver efficacement le VHC, et probablement d'autres virus enveloppés, lorsqu'il est appliqué au PPL. Cela

représente la première démonstration de la capacité d'appliquer le traitement S/D à un LP pour des applications en médecine régénérative, et en particulier dans le traitement potentiel des maladies neurodégénératives.

Traitement thermique:

Traitements thermiques traditionnels utilisés dans l'industrie du fractionnement du plasma pour l'inactivation des virus sont généralement effectuées soit sur des solutions de protéines ou des préparations lyophilisées. Les traitements thermiques liquides, tels que ceux appliqués aux facteur de coagulation, sont effectuées à une température de 60°C pendant 10 heures, mais nécessite l'ajout de stabilisants pour protéger les protéines. Des conditions alternatives ont été décrites où une température de 50°C pendant 3 heures est efficace pour l'inactivation d'un plasma stabilisé. Comme l'activité neuroprotectrice est augmentée par le traitement thermique à 56°C pendant 30 min et sans stabilisants, nous avons décidé de vérifier si cette étape pouvait également contribuer à inactiver le VHC. Les données une inactivation complète du VHC ($> 2,5$ logs). Par conséquent, ce traitement thermique peut être considéré comme étant une étape d'inactivation d'au moins certains virus sanguins dont le VHC.

Nanofiltration:

La nanofiltration est un procédé habituel dans l'industrie du fractionnement pour accroître le niveau de sécurité virale des immunoglobulines, facteurs de coagulation, inhibiteurs de protéase thérapeutiques. Cependant, la nanofiltration des fractions traditionnelles de LPs serait probablement difficile, en raison de la teneur élevée en protéines diminuant la filtrabilité. Pourtant, dans notre étude, nous avons cependant pu démontrer pour la première fois qu'il est techniquement réalisable de nanofiltrer notre fraction HPPL sur des filtres de 20 nm, car elle est appauvrie en protéines plasmatiques et les protéines thermosensibles comme le fibrinogène sont éliminées. Par ailleurs nos données indiquent que, comme prévu, 20 nm, la nanofiltration permet d'éliminer $\geq 2,5$ log du VHC. Ces données sont en accord avec l'expérience

acquise avec les méthodes de nanofiltration qui démontre la robustesse de cette procédure pour retenir les virus selon un principe d'exclusion stérique, la taille du VHC étant proche de 40-45nm. L'impact que nanofiltration peut avoir sur l'activité neuroprotectrice du HPPL du fait de l'élimination des PMPs a retenu notre attention car cette étape peut avoir des effets bénéfiques ou néfastes relatif à l'activité thrombogène/inflammatoire, d'un côté, ou rôle neuroprotecteur de l'autre côté. Nous montrons en effet que les PMPs exercent un rôle neuroprotecteur contre les neurotoxines dans le modèle LUHMES.

En résumé, nos études révèlent plus de 2,5 log de réduction de HCVcc par traitement thermique, S/D-C18 et nanofiltration 20 nm. Fait important, l'activité neuroprotectrice du lysat soumis à un traitement thermique, S/D, puis une nanofiltration n'a pas été affectée de manière significative.

Au cours de notre travail, toutes les expériences ont été réalisées à l'aide d'un PPL remis en suspension dans un tampon PBS pour maintenir/normaliser le pH pendant le traitement thermique. Cependant, dans le contexte du développement d'un produit pour application clinique, il est nécessaire de formuler le PPL dans une solution pharmaceutique enregistrée, tel que le sérum physiologique. Nos données montrent que le traitement thermique de PPL formulés dans une solution saline maintient l'activité neuroprotectrice dans le modèle cellulaire sur LUHMES *in vitro*.

En bref, cette étude apporte la démonstration qu'un lysat de plaquettes contenant des facteurs de croissance neurotrophiques en forme concentrée, mais aussi appauvri en protéines plasmatiques peut induire une protection très marquée des neurones dopaminergiques dans un modèle cellulaire de la maladie de Parkinson. Nos données sont en accord avec d'autres résultats récents démontrant que l'administration de lysats plaquettaires par ICV améliore la neurogenèse dans un modèle animal d'accident vasculaire cérébral, et que, par ailleurs l'administration i.n. de lysats plaquettaires dans un modèle de souris de la maladie d'Alzheimer soutient l'expression du marqueur TH, améliore les paramètres neuropathologiques et les fonctions cognitives. À la lumière de ces résultats, d'autres études pourraient être

menées pour comprendre les implications mécanistiques des composants de nos PPL et HPPL. Il serait intéressant de sérier plus spécifiquement dans ces lysats plaquettaires, le rôle joué par les neurotrophines les plus importantes, mais aussi celui d'autres composants, tels que les neurotransmetteurs ou les PMP, sur les voies de signalisation de la neuroprotection. Ceci permettrait de conforter le rôle thérapeutique possible de lysats plaquettaires comme nouvelle "stratégie de modification des maladies neurodégénératives".

PERSPECTIVES

Nos travaux ont montré la possibilité d'utiliser des concentrés de plaquettes, non leucoréduits et maintenus en suspension dans le plasma, comme matériel de départ pour isoler une fraction de lysat plaquettaire appauvrie en protéines plasmatiques et en protéines indésirables, dont plus particulièrement le fibrinogène. Ce lysat possède une faible teneur en protéines, ce qui le rend plus compatible pour une administration intracérébrale, et il possède une teneur élevée en neurotrophines du fait de sa concentration lors de la fabrication. De manière inattendue, l'introduction d'une étape de chauffage à 56°C pendant 30 minutes a contribué à diminuer encore plus la teneur totale en protéines, tout en modifiant la proportion en protéines plaquettaires dont les neurotrophines, et en améliorant les effets neuroprotecteurs *in vitro* dans le modèle de cellules LUHMES exposées à la neurotoxine MPP⁺. Cette préparation est dénuée d'effet inflammatoire propre *in vitro* sur des cellules microgliales BV2, réduit l'expression de COX-2 lors de leur exposition aux LPS, et n'a pas montré exercer une toxicité aiguë lors de l'administration ICV chez la souris. La capacité de procéder à une administration du lysat en mode i.n., illustrée par la démonstration de la migration de PDGF-AB et de PF4 dans différentes zones cérébrales chez la souris, de même qu'une observation de la protection du marqueur TH par les neurones dopaminergiques, sont des données encourageantes pour soutenir les évaluations de ce mode d'administration qui pourrait compléter l'administration ICV au moins pour certains patients à des stades évolutifs moins de la maladie. Compte tenu de la possibilité du développement de lysats

plaquettaires d'origine allogénique, avec préparation sur des pools issus de donneurs multiples, pour le traitement futur des maladies neurologiques, nous avons développé un mode d'obtention de lysats facile à mettre en œuvre industriellement, dans lequel 3 étapes (traitement thermique, traitement S/D, nanofiltration 20 nm) peuvent être introduites pour assurer une marge de sécurité virale élevée contre le risque infectieux. Les données acquises lors de ce travail de thèse à visée translationnelle devraient nous rapprocher de la mise en œuvre d'études pré-cliniques de toxicité et d'efficacité chez des primates, puis chez l'homme. Celle-ci détermineront de l'avenir des lysats plaquettaires de types HPPL dans le traitement non seulement de la maladie de Parkinson, mais aussi des autres maladies neurodénégatives, et, plus globalement, des affections neurologiques.

ENGLISH ABSTRACT

Ensuring optimal quality and safety of blood products for regenerative medicine is mandatory. Recently, platelet lysates (PL) rich in growth factors (GFs) have emerged as a powerful clinical-grade supplement of growth media for *ex vivo* expansion of mesenchymal stromal cells (MSC) and as a therapeutic product to promote healing in trauma, degenerative pathologies, bone reconstruction, some metabolic diseases, and, possibly, neurodegenerative disorders. The interest in platelet extracellular vesicles, also called platelet-derived microparticles (PMPs), as physiological delivery system of GFs, has emerged. However, the presence of phosphatidylserine on their bilayer lipid membrane may cause thrombotic and inflammatory side effects in a blood medium. Another transfusional risk is the transmission of pathogenic viruses, including hepatitis C virus (HCV), requiring the implementation of dedicated viral inactivation or removal methods such as solvent/detergent (S/D) or heat treatments, or nanofiltration.

Our thesis first evaluated technologies to improve the safety of therapeutic blood products including those for regenerative medicine: (a) impact of removal of MPs in plasma by 75nm-nanofiltration and (b) capacity of S/D or 56°C heat treatments, or nanofiltration to inactivate/remove HCV. These results were used for developing and improving a customized clinical-grade platelet pellet lysate (PPL) for specific applications in neuroregeneration. We hypothesized that a tailor-made PPL enriched in multiple neurotrophic growth factors and depleted of plasma proteins could be a potent neuroprotective agent and exert neuroprotection of dopaminergic neurons for brain administration as disease modifying strategy of neurodegenerative disorders.

Our data show that 75 nm-nanofiltration preserved protein and lipoprotein profile, coagulation factor content and global coagulation activity of plasma. Specialized biophysical methods showed the capacity of this nanofilter to remove MPs and to avoid an *in vitro* MP-associated generation of thrombin in plasma. Second, our data

using luciferase-tagged infectious cell culture-derived HCV (HCVcc) particles spiked to human plasma revealed the capacity of the S/D treatment to fully inactivate HCVcc within 30 minutes of treatment at 31°C, as shown by the baseline level of reporter signals, total loss of viral infectivity and absence of viral protein NS5A. Taken together, plasma nanofiltration and S/D treatment are valuable techniques to improve the safety of plasma for transfusion with regards to thrombogenicity and risks of HCV transmission, respectively.

We used these results to prepare and characterize a tailor-made, virally-inactivated, heat-treated platelet pellet lysate (HPPL) depleted of plasma proteins (in particular fibrinogen), and enriched in a physiological pleiotropic mixture of neurotrophins for brain administration. ELISA analysis and proteomics studies revealed that heat-treatment at 56°C for 30 min decreased the total protein content, modified the relative protein content, and influenced favorably the balance of platelet components with neuroprotective activity. This heat-treatment, which was found to unexpectedly improve the neuroprotective activity, also contributes to reduce HCV infectivity, as do the S/D and 20-nm-nanofiltration. PPL exerted strong neuroprotective effects in Parkinson's disease (PD) models (a) *in vitro*, using LUHMES cells exposed to MPP⁺ neurotoxin, and (b) *in vivo*, in mice intoxicated by MPTP neurotoxin. In addition, we show that brain delivery of HPPL by intranasal (i.n.) administration induces a diffusion of growth factors and protect the expression of tyrosine hydroxylase (TH) expression in the *substantia nigra pars compacta* and striatum in the mice/MPTP model. Since neuro-inflammation can be detrimental in neurodegenerative disorders, we verified that the PPL did not stimulate the release of inflammatory markers (e.g. COX-2, iNOS) by BV2 microglial cells in culture, and could restrict COX-2 expression when cells were exposed to LPS. In addition, the PPL was found to contain 1×10^{12} MP/mL with a mean size of 160 nm. Interestingly, these MPs were found not to exert a thrombogenic impact *in vitro* when spiked to cerebro-spinal fluid and, furthermore, induced a neuroprotective activity in LUHMES cells exposed to neurotoxin.

Altogether, our data demonstrate the technical feasibility of developing virally-safe customized platelet lysate preparations for use in regenerative medicine, and most specifically for potential disease-modifying strategy in the treatment of neurodegenerative disorders of the central nervous system, such as PD.

CHINESE ABSTRACT

近年來，富含生長因子(GF)的血小板裂解液(PL)被認為是一有力的臨床級添加物，將其添加至細胞培養基中可使間葉幹細胞(MSC)進行擴增，且有促進癒合的作用，故也可視為一治療產品。然而在再生醫學中，此類血液製品有必要被確保其安全性以及最佳品質，因此需考慮如血小板衍生微粒(PMP)以及病原性病毒的傳染等的影響。PMP 表面雙層脂質膜上的磷脂絲胺酸，被認為會引起血栓和發炎等副作用，需利用奈米過濾去除血液中的微粒；而病原性病毒的傳染，包括 C 型肝炎病毒，會影響血液製品的安全性，需透過化學劑(S/D)、熱處理或奈米過濾等專門消毒方法去除病毒的活性。

論文首先探討是否能透過消毒技術來提高再生醫療中血液製品的安全性：(a) 利用 75 奈米過濾去除 PMP; (b) 透過 S/D 或 56°C 加熱處理滅活 HCV 或透過奈米過濾去除 HCV。其次探討是否能開發和改進臨床級血小板沉澱裂解液(PPL)，使之能應用於神經再生。我們假設客製化的 PPL 富含多種神經滋養生長因子，且透過熱處理使其不含血漿蛋白（特別是纖維蛋白原），如此一來，此客製化的 PPL 能提升多巴胺神經元的神經保護作用，可作為神經退化性疾病的新治療策略。研究結果如下：

第一，透過消毒技術來提高再生醫療中血液製品的安全性：(a)透過特殊生物檢測方法可發現，經由 75 奈米過濾後，PMP 被確切移除，且避免了體外 PMP 相關的凝血酶的產生，但依舊保留血漿蛋白、脂蛋白、凝血因子含量和球蛋白凝血活性。(b) 根據訊號強度分析指出，將具有冷光標記感染性細胞衍生的 HCV (HCVcc)顆粒加入血漿中，使血漿受到感染後，再將該 HCVcc 在 31°C 的作用環境下透過 S/D 處理，30 分鐘內即可發現其病毒感染力完全喪失，且偵測不到病毒蛋白 NS5A。綜合上述結論可知，透過 S/D 處理和奈米過濾技術可有效降低血栓形成機率和 HCV 的傳染風險，進而提高輸血之安全性。

第二，開發和改進臨床級 PPL，使之能應用於神經再生醫學：(a)根據 ELISA 分析和蛋白質組學研究顯示，經由 56°C 熱處理，30 分鐘後的蛋白質組成會被修飾且

更具有神經保護活性。故熱處理能改善神經保護活性，若進一步使用 S/D 和奈米過濾等方法將有助於 HCV 失活。(b)在帕金森氏症模型的離體實驗（以 MPP⁺ 神經毒素刺激 LUHMES 細胞），和動物實驗（給予小鼠 MPTP 神經毒素）中，PPL 皆被證實有顯著的神經保護作用。(c)在黑質緻密物中酪胺酸羥化酶(TH)蛋白表現的研究顯示，透過鼻腔給予 (i.n.) PPL 的方式可作為未來神經退行性疾病的另一種治療策略。(d)根據 LUHMES 研究顯示，訊息轉導途徑的特異性抑制會調控 PPL 的神經保護作用。(e)先前研究指出，神經過度發炎最終亦會導致神經退化性疾病，故我們利用內毒素 LPS 刺激 BV2 細胞誘導其產生發炎介質（例如 iNOS, COX-2），證實 PPL 給予並不會加重其發炎反應，且能抑制 COX-2 蛋白的表現。此外，檢測發現 PPL 含有 1×10^{12} MP/mL，其平均大小為 160 nm。

綜觀上述可知，對於細胞治療和再生醫學，我們的研究提供了製備安全的客製化血小板裂解物技術的可行性，特別是可作為中樞神經系統的神經保護製劑。

INTRODUCTION

1 Therapeutic blood products

1.1 Importance of blood products in human therapy

Blood is composed of blood cells (erythrocytes, leucocytes and platelets) and plasma. Blood transfusion is an essential life-saving medical procedure, illustrated by the fact that several blood products are on the World Health Organization (WHO) Model List of Essential Medicines.¹

Blood drawn from donors can be separated by blood establishments into blood components used for direct transfusion to patients. Plasma can be pooled and fractionated into biological protein therapeutics by plasma fractionation centers.

Blood collected in the presence of an anticoagulant (typically a citrate-based sterile solution) can be processed into red blood cell concentrates, platelet concentrates, plasma for transfusion, and cryoprecipitate. Plasma can be fractionated into coagulation factors, immunoglobulins, albumin, various other products including fibrin-based biomaterials.²

Blood products are used most often to treat patients with trauma leading to blood losses, or with congenital or acquired deficiencies. Table 1 shows the main traditional therapeutic products currently made from human blood, either from individual donations or from the small or large pooling of donations.

Table 1 - Overall range of blood products classified based on the pooling or not of individual donations.

Single-donor blood components	Small-pool blood components	Large-pool, unfractionated virally inactivated plasma product	Large-pool products obtained by fractionation of plasma
• Whole blood • Red cell concentrate • Platelet concentrate (obtained by apheresis) • Leucocyte concentrate • Plasma for transfusion • Cryoprecipitate • Cryo-poor plasma	• Platelet concentrates (obtained from whole blood) • Cryoprecipitate	• Plasma for transfusion, solvent-detergent (S/D) treated	• Albumin • Blood coagulation factors • Protease inhibitors • Anticoagulants • Intramuscular immunoglobulins (IMIG) • Intravenous immunoglobulins (IVIG)

(Adapted from WHO, 2007)³

Recommendations from WHO regarding national blood policy and organization highlight that the safety of collected blood is very instrumental for the quality of blood products and related biological products used to prevent or treat life-threatening injuries or diseases. Therefore, the safety and quality of blood components are tightly regulated in most countries by national regulatory authorities, such as the Taiwan Food and Drug Administration (TFDA) in Taiwan, and the Agence Nationale de Sécurité du Médicament (ANSM) in France. Production of blood products by blood establishments should thereby comply with the requirements of good manufacturing practices (GMPs), implying that they must meet established quality and safety criteria set at national levels, following international guidelines and/or Pharmacopoeia. It is necessary to establish a legal framework and implement safe and adequate blood collection procedures to strengthen every country's national health care policy and infrastructure.⁴

Table 2 - Therapeutic blood products, main indications, and form of administration

Products	Main indications	Form of administration
Albumin		
Human serum albumin	Volume replacement	IV
Blood coagulation factors		
Factor VIII	Haemophilia A	IV
Prothrombin complex	Complex liver diseases; warfarin or coumarin derivatives reversal	IV
Factor IX	Haemophilia B	IV
Factor VII	Factor VII deficiency	IV
Von Willebrand factor	Von Willebrand factor deficiency (type 3 and severe forms of type 2)	IV
Factor XI	Haemophilia C (factor XI deficiency)	IV
Fibrinogen	Fibrinogen deficiency	IV
Factor XIII	Factor XIII deficiency	IV
Activated PCC	Haemophilia with anti- factor VIII (or factor IX) inhibitors	IV
Protease inhibitors		
Antithrombin	Antithrombin deficiency	IV
Alpha 1 antitrypsin	Congenital deficiency of alpha 1 antitrypsin with clinically demonstrable panacinar emphysema	IV Aerosol
C1-inhibitor	Hereditary angioedema	IV
Anticoagulants		
Protein C	Protein C deficiency	IV
Fibrin sealant (fibrin glue)	Haemostatic/healing/sealing agent (surgical adjunct)	Topical

Products	Main indications	Form of administration
Intramuscular immunoglobulins (IMIG)		
Normal (polyvalent)	Prevention of hepatitis A (also rubella, and other specific infections)	IM
Hepatitis B	Prevention of hepatitis B	IM
Tetanus	Treatment or prevention of tetanus infection	IM
Anti-Rho (D)	Prevention of haemolytic disease of the newborn	IM
Rabies	Prevention of rabies infection	IM, topical
Varicella/zoster	Prevention of chickenpox infection	IM
Intravenous immunoglobulins (IVIG)		
Normal (polyvalent)	Replacement therapy in immune deficiency states	IV, CV
Cytomegalovirus (CMV)	Prevention of CMV infection (e.g. after bone marrow transplantation)	IV
Hepatitis B	Prevention of HBV infection (e.g. liver transplant)	IV
Rho (D)	Prevention of haemolytic disease of the newborn	IV

(Adapted from WHO, 2007)³

IV: intravenous; IM: intramuscular; SC: sub-cutaneous

Human blood is a source of important therapeutic products in every country throughout the world, provided that sufficient level of blood collection is in place. As illustrated in Table 2, blood products are used to treat a large range of bleeding, immunological, and enzymatic disorders due to congenital or acquired deficiencies, or trauma. Most products are administered intravenously, but some are also formulated for intramuscular, sub-cutaneous, topical treatments, or for aerosol delivery.³ No product is currently used by intranasal delivery.

Transfusion of therapeutic plasma products is rigorously controlled in high-income countries with an established regulatory system. Plasma for transfusion is a blood fraction that is used in cases of surgical bleeding, liver disease, single coagulation factor replacement therapy, thrombotic thrombocytopenic purpura, etc.⁵ Additionally, the shortage of fractionated plasma products in developing countries leads to situations where plasma may be transfused to treat patients facing other pathologies.^{6,7} Sub-optimal screening of donors and testing of donations, and lack of regular controls and government support, are reasons why plasma for transfusion may be at higher risk of transmitting viral infections.⁸

2 Pathogen safety measures for blood products

Absence of pathogen transmission is one of the major factors contributing to safe blood transfusion. Over the last 30 years, much progress has been made to continuously decrease the potential risk of transmitting infectious agents by transfusion.

2.1 Pathogen safety tripod

The viral safety of blood products is widely recognized to be based on the strict implementation of additive measures, typically known as the “safety tripod” including:

- Careful epidemiological control of the donor population.
- Individual screening of each blood donor candidate to exclude from donating individuals presenting a set of risk factors e.g. due to social behavior, travels, or other exposure risks.
- Serological testing or nucleic acid testing (NAT) of each individual blood donation against the most pathogenic known infectious agents, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV), as well as hepatitis A virus (HAV) and parvovirus B19.

2.2 Blood-borne pathogens

2.2.1 Range of blood-borne pathogens

Blood products can potentially transmit several blood-born infectious agents. Table 3 presents an updated list of such infectious agents potentially transmissible by blood products, in the absence of testing of the starting donations, or additional precautionary measures, in particular viral inactivation/pathogen reduction treatments.

Some blood products, when technologies have been developed and validated by suppliers, and licensed by regulatory authorities, are subjected to “viral inactivation” or “pathogen reduction” treatments intended to reduce the risks of transmitting viruses during the window-phase period (donations that are non-reactive for tested viral markers but actually infective) and non-tested viruses (such as emerging agents like Zika virus, Dengue virus Ebola virus, etc.).

Blood-borne infectious viruses include both enveloped and non-enveloped viruses, an important criteria considered for the development and implementation of virus inactivation treatments.

Historically, the major viral threats of pathogen infections, from the whole range of blood products, have resulted from contaminations by HIV, HBV, and HCV.

Table 3 - List of infectious agents potentially transmissible by blood products; evidence or assumption of risks of transmission

Infectious agents	Family	Enveloped	Size (nm)	Cellular blood components	Plasma (or cryoprecipitate)	Fractionated plasma products*
HIV I&II	Retro	+	80-100	+	+	+
Human T-cell leukemia virus I & II	Retro	+	80-100	+	–	–
Simian foamy virus	Retro	+	80-100	?	?	–
HBV	Hepadna	+	40-48	+	+	+
Hepatitis Delta virus	Delta	+	36	+	+	+
HCV	Flavi	+	40-50	+	+	+
Dengue virus	Flavi	+	40-60	+	?	–
HGV	Flavi	+	40-60	+	+	+
West Nile virus	Flavi	+	40-60	+	?	–
Zika virus	Flavi	+	40-60	+	?	–
Chikungunya	Toga	+	40-60	+	–	–
Cytomegalovirus	Herpes	+	180-200	+	–	–
Epstein Barr virus	Herpes	+	120-220	+	–	–
Human Herpes virus-8	Herpes	+	120-200	?	–	–
TT virus	Circo	+	30-50	+	+	+
SARS coronavirus	Corona	+	80-90	?	?	–
MERS coronavirus	Corona	+	120-160	?	?	–
Ebola virus	Filo	+	100-800	+	?	–
HAV	Picorna	–	27-32	+	+	+
HEV	Calici	–	35-39	+	+	+
Human erythro B19	Parvo	–	18-26	+	+	+

(Adapted from WHO, 2007)³

*: Lack of evidence of transmission is likely due to implementation of virus reduction treatments prior to the emergence of the virus; ?: uncertain

2.2.2 Hepatitis C virus

HCV is classified among the flaviviridae family and is an enveloped, positive single stranded RNA virus. The genome of HCV consists of structural proteins that include core protein, and glycoproteins E1 and E2, and of non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.⁹

The World Health Organization (WHO) estimates that approximately 112.5 million blood donations are collected globally each year. The prevalence of transfusion-transmissible infections is 0.12-1.08% HIV, 0.91-3.7% HBV, and 0.32-1.03% HCV per blood donation in middle or low-income countries.⁴

Manufacturing procedures of therapeutic products made from pooled plasma/blood donations increase the risks of viral transmission if the pool contains a donation from unscreened donor, or a donation collected within the window period of infection (Table 4). Thereby, measures to decrease the infectious risks, most particularly by implementing viral reduction (i.e. inactivation or removal) treatments, are crucial to eliminate the possibility of contamination by viruses.

Table 4 - HCV level of infectivity in plasma and duration of the window period depending upon detection methods of HCV infection

HCV in plasma from unscreened donor blood			Average HCV window period (days)	
Prevalence in donor blood	Viral titer (GE/ml)	Calculated titer in plasma pool (GE/ml)	Without nucleic acid amplification technology	With nucleic acid amplification technology
1/50-1/100	10^4 - 10^6	10^2 - 10^4	82 ^a	9

^a Based solely on anti-HCV detection

2.2.3 Risk of HCV in plasma transfusion

Around 150 to 300 million people suffer from acute or chronic HCV infection globally. In wealthy countries, patients are treated by pegylated interferon-alpha combined with ribavirin, an anti-viral drug. This advanced standard of care (SOC) for HCV, already used for more than a decade, has, however, limited efficacy against the most widely spread HCV genotype 1.¹⁰

By targeting viral NS3/4A protease, NS5A cofactor and NS5B polymerase, some pharmaceutical drugs (Table 5) showed high therapeutic success rate while also inducing less adverse reactions.¹¹

However, the cost of these pharmaceutical drugs makes treatment unaffordable in low-income countries. In addition, there is yet no preventive vaccine of hepatitis C. Approximately 500,000 people die each year of hepatitis C-related diseases¹² and, in some countries, HCV prevalence in the population is over 10%.¹³ HCV infection may progress to chronic stages into liver cirrhosis and cancer, making it a main cause for liver transplantation in some countries.^{14,15}

Table 5 - Licensed HCV antiviral drugs

Inhibitors target	Antiviral drug	Trade names
NS3/4A protease	Boceprevir	Victrelis
	Telaprevir	Incivek, Incivo
	Simeprevir	Olysio, Sovriad, Galexos
NS5A cofactor	Daclatasvir	Daklinza
	Ledipasvir	Combined with sofosbuvir called Harvoni
	Ombitasvir	Viekira Pak and Technivie
NS5B polymerase	Sofosbuvir	Sovaldi
	Mericitabine	-
	Dasabuvir	Viekira Pak and Exviera

HCV transmission is one of the major potential infectious complications of the transfusion of non-virally inactivated plasma and other blood products. One of the most common route of HCV transmission in the world is through the transfusion of unscreened blood and blood products, including plasma.¹⁶ It is estimated that there is a prevalence of HCV in approximately 1/50 to 1/100 in donor blood. The HCV viral titer is approximately 10^4 to 10^5 genome equivalents (GE)/ml and the calculated titer of HCV in plasma pool is 10^2 to 10^4 GE/ml. Moreover, the window period of HCV is from 9 to 82 days, with and without NAT screening, respectively. In some countries, such as Egypt, HCV infection can affect 10% or more of the population.¹⁷ WHO announced this year that approximately 700,000 people die of HCV infection annually and that chronic HCV infection carriers have high risks of developing liver cirrhosis and liver cancer.

Implementing treatments capable to inactivate or remove HCV in all blood-derived therapeutic products is therefore essential.

2.3 Viral reduction treatments

The infectious risks associated to blood products are directly dependent upon the number of donations pooled together. As was shown in Table 6, if red blood cell concentrates and apheresis platelet concentrates for transfusion are single-donor products, by contrast each batch of industrial fractionated plasma products can result from mixing 10,000 to 60,000 plasma donations.

One efficient manner to reduce infectious risks from blood products and safe-guard the blood supply is through implementation of viral reduction treatments capable to inactivate or remove known, emerging, and future viral pathogens.¹⁸

The selected treatments have to be adapted to each blood product in order to maintain its functional activity and therapeutic efficacy. It is technically more challenging to find technologies capable to inactivate viruses in cellular products owe to the fragility of cells and on a context where several blood-borne viruses are intracellular. This explains the current lack of viral inactivation technologies for whole blood and red blood cell concentrates. Table 6 summarizes the viral and pathogen reduction treatment currently applicable to blood products.

The efficacy of these treatments should be validated on a case-by-case basis (for each cell or protein product) to demonstrate absence of protein denaturation and robust capacity to inactivate or remove viruses. International guidelines and regulations are available on this important issue.^{19,20} Large-pool products are typically subjected to at least two dedicated “orthogonal” steps of viral inactivation or removal, using complementary methods. The three most robust viral reduction treatments of protein therapeutics currently in place are S/D, nanofiltration, and heat.

Table 6 - Pathogen reduction treatment of blood products

Blood product	Viral reduction treatment	Comments
Whole blood		Under development
Red blood cell concentrates		Under development
Platelet for transfusion	• Psoralen/UVA²¹ • Riboflavin/UV²²	Recently licensed in Europe and/or USA
Plasma for transfusion	• S/D treatment²³ • Methylene blue/light²⁴ • Psoralen/UVA • Riboflavin/UV	
Cryoprecipitate	• S/D treatment²⁵	
Fractionated plasma products ^{2,26}	• S/D treatment • Pasteurisation • Heat treatment • Caprylic acid treatment • Nanofiltration • Low pH incubation	The type of treatment depends upon the protein products

2.3.1 Solvent/detergent (S/D) treatment

S/D treatment is a well-established viral inactivation treatment of plasma protein products (Table 6). It is performed by incubating a protein solution at 20-37°C, with an organic solvent, typically tri(n-butyl) phosphate (TnBP) used at 0.3-1% (v/v; final concentration), and a non-ionic detergent, typically Tween-80, Triton X-100, or Triton X-45 (< 1%, v/v; final concentration).²⁶ S/D can disrupt the lipid coat of enveloped viruses destroying their infectivity. After treatment, the S/D agents can be removed by various processing approaches encompassing oil extraction, positive protein adsorption chromatography (where the target proteins bind to a resin) or C-18 hydrophobic interaction chromatographic material (on which hydrophobic compounds bind). This process is ineffective against non-enveloped viruses, however one main benefit is the lack of impact of S/D on the functional activity of most blood proteins.

2.3.2 *Nanofiltration*

Nanofiltration is now a well-established removal process of viruses based on filtration of protein solutions onto dedicated nanofiltration cartridges with defined pore-size typically ranging from 15 to 75 nm. The removal of pathogens, viruses and potentially as well as prions,²⁷ is based on size-exclusion.^{28,29} Infectious agents, regardless of their biochemical structure, that are larger than the pore size of the filtration membrane are entrapped (therefore removed), whereas proteins can pass through the filter (and are recovered).

2.3.3 *Heat-treatment*

Three types of heat inactivation methods have been developed to improve the viral safety of therapeutic protein products. Heat treatment can be performed on a protein solution typically at a temperature of 60°C for 10 hours (a process known in the plasma industry as “pasteurization”) usually in the presence of carbohydrates (e.g. sucrose), polyols (e.g. sorbitol), amino acids (e.g. lysine and/or glycine) or fatty acids (e.g. caprylate) to prevent or limit protein heat-denaturation. It has been applied to albumin, as well as some coagulation factors and protease inhibitors preparations.^{30,31} Alternative methods using lower temperature and duration (50°C for 3 hours) have been found effective to inactivate viruses in human plasma.³²

Heat treatments can also be performed on lyophilized proteins exposed to vapor-steam under pressure (“steam-treatment”)³³ or more frequently on the final products already dispensed in the final container (“dry-heat treatment”).³⁴ Dry-heat treatments at 60-68°C for 24-96 hours were shown to ensure the inactivation of HIV in coagulation factor concentrates.³⁵ Treatments at 96 °C for 30 min, or 80 °C for 72 h, can be used to inactivate non-enveloped viruses in coagulation factor preparations also subjected to S/D treatment,³⁶ to benefit from the complementarity of these two methods in the inactivation of viruses.

3 Safety measures for blood products unrelated to infectious risks

Several factors, apart from infectious risks, are important for the transfusion safety of blood products, including immuno-hematological compatibility (to avoid the risks of allo-immunisation in recipients receiving allogeneic blood cell components), bacterial sterility, and apyrogenicity. These risks are generally well controlled in modern transfusion medicine.

However, recently, increased attention has been paid to the possible “pathogenic” role played by blood cell-derived microparticles (MP; microvesicles. Some could be regarded as “nanoparticles” considering their size in the 30-200 nm range.) present in blood products,³⁷⁻³⁹ deserving a particular attention in relation to our work.

3.1 Microparticles (MPs)

There is much recent interest in the study of the characteristics and functional roles of MPs.⁴⁰⁻⁴⁸

MPs encompass a heterogenous group of cell-derived microvesicles that are present in essentially all body fluids. A large number of MPs shed from plasma membranes of virtually all cells types (erythrocytes, white blood cells, platelets, endothelial cells) are circulating in blood. Physically, the generation of MP occurs through membrane budding involving three enzymes (floppase, scramblase, and flippase) and associated with cytoskeletal changes affecting the asymmetry of the cellular lipid bilayered membrane. While phosphatidylcholine and sphingomyelin neutral phospholipids are normally exposed on the outer layer of the membrane of resting cells, the formation of PMP leads to the exposure of the anionic phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine, originally present in the inner layer.^{49,50} Flippase is known to maintain the asymmetry of the phospholipidic layer in resting cells. However, physiological events, such as activation and increase in the intracellular

calcium concentration, lead to flippase inhibition, activation of floppase and scramblase, exposure of cationic phospholipids and shedding of MPs.⁵¹

PS exposed on platelet-derived MPs (PMPs) surface can bind to coagulation factors and lead to thrombin generation. PMPs express surface receptors for FVIII (factor VIII),⁴⁹ FVa to form a prothrombinase complex with FXa,⁵² FIXa,⁵³ and expose glycoproteins (GP), such as GPIIb/IIIa and GPIb/IX.⁵⁴ As such, PMPs membranes have been estimated to generate a thrombogenic activity that is 50-100 times more than that of platelets.⁵⁵ PMPs are anucleated and have a content that reflect their cells of origin (Figure 1). They are surrounded by a phospholipid bilayer, and are heterozygous in size with a range thought to be comprised between 0.05 μm to 1 μm in the circulation.^{37,56}

It has long been considered that platelet-derived MP (PMPs) are the MPs present in the highest proportion in plasma, accounting for 70%-90% of the total,^{37,57} but this belief may have to be revised as new exploration methods of MPs are developed allowing more precise quantitative and qualitative assessment.^{37,58}

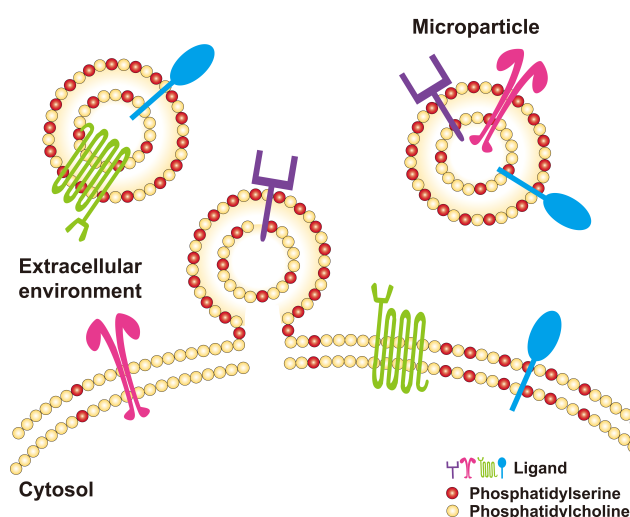


Figure 1 - Microparticles (MPs) budding from plasma membrane

Physiological MPs concentration in plasma can reach $10^{10}/\text{mL}$,^{37,40,59} with a constant slow production associated with various events including cell apoptosis.^{45,60,61,62} prior to clearance from the circulation through phagocytosis by macrophages or fusion with other cells.⁶³

3.2 MPs and pathologies

MPs production in the blood circulation is known to be stimulated by various pathological conditions,^{38,62} such as atherosclerosis, ischemic stroke, deep-vein thrombosis, and cancer.^{37,64,65} A major trigger in the generation of PMPs is an activation state involving thrombin or collagen,⁶⁶⁻⁶⁹ and complement-mediated membrane dysfunction.⁷⁰ Shear stresses in the circulation are also contributing to favoring MP formation, possibly in association with cell aging.³⁸

3.3 MPs in blood products

The collection of blood, and additional processing and storage of blood-derived products, that involve filtration steps, contacts with artificial surface, and storage can lead to the formation of MP from blood cells.³⁸ As such, all transfused cellular blood components and unfractionated plasma protein products, such as plasma for transfusion and cryoprecipitate, contain MPs.³⁸ There is only little information to-date on the characterization and function of PMPs in platelet lysate preparations.³⁷

Whether MPs play a beneficial or detrimental role in therapeutic blood products is still controversial and currently debated. It has been argued that MPs, by their pro-coagulant properties, contribute to the hemostatic activity of plasma and cryoprecipitate. Although coagulation factors in plasma are the main components of the hemostatic effect, MPs are thought to promote the coagulation.^{56,71,56,72,73}

Leucofiltration of whole blood on a negatively charged filter remove MPs and lower plasma procoagulant and hemostatic potential.⁷⁴ Excess of PMPs in plasma for transfusion may however possibly trigger thrombotic events in patients with procoagulant state. Cryoprecipitate contains up to 265-fold more MPs than its corresponding cryosupernatant or original plasma, one therapeutic cryoprecipitate unit potentially containing MPs equivalent to 4×10^9 platelets.^{38,75,76} Cryoprecipitation may lead to physical entrapment of MPs present in plasma, while interactions with cryoprecipitate components such as fibrinogen, fibronectin, and vWF may facilitate co-precipitation.⁷⁵ Functional role of MPs in cryoprecipitate is suggested by its capacity to correct bleeding abnormalities due to platelet dysfunction.⁷⁷ The contribution of MPs to plasma and cryoprecipitate hemostatic activity, but also thrombogenic potential needs clarification.⁷⁶

Indeed, on the other side, MPs have been considered as “submicron clotting bombs”⁷⁸ and responsible for transfusion side-effects.⁷⁹ Clinical data suggest that MPs present in platelet concentrates increase the incidence of venous thrombosis and embolism.

Large retrospective clinical observations have shown that the transfusion of plasma together with packed RBC significantly increases the occurrence of deep vein thrombosis and pulmonary embolism,⁸⁰ and venous and/or arterial thrombotic events increase in pediatric patients with the volume of plasma transfused.⁸¹ In addition, 7% of neonates are exposed to thrombotic events when transfused with plasma.⁸² The role of MPs in such thromboembolic events has not been studied and therefore remains speculative. In addition, data suggest that MPs are efficient cargo of bioactive molecules that can lead to cell activation and inflammatory processes, and may influence inflammatory processed and adaptive immunity.³⁹

MPs in RBC concentrates are suspected to trigger immunomodulatory and proinflammatory conditions.^{38,42,83} and may explain diminished survival seen in transfused cancer patients,⁴⁵ and higher mortality, sepsis or septicemia in patients undergoing cardiac surgery.⁸⁴ MPs derived from RBCs have been linked to the postoperative thrombosis, transfusion-related non-immune acute lung injury (TRALI), and transfusion-related immunomodulation (TRIM).^{42,43,85 86}

In connection with these findings it was identified that PMPs activate neutrophils *in vitro* and increase in CD11b expression,⁸⁷ while, in animal studies, MPs from stored RBC supernatants induce neutrophil priming and activation,⁸⁸ and MPs from platelets and RBCs lead to TRALI.⁸⁹ Soluble CD40L may bind to MPs and accumulate during storage, leading to polymorphonuclear leukocytes activation, endothelial damage and TRALI.⁹⁰ In conclusion, the reduction or control MPs numbers may be compatible with a preservation of the hemostatic activity of plasma, while avoiding undesirable thrombotic effects can have potential application in some patients in need of plasma transfusion.^{80,81,91}

4 Platelet materials in regenerative medicine and cell therapy

4.1 Preparation of platelet concentrates

Platelet concentrate (PC) preparations are obtained following three well-established procedures by blood centers.

Whole blood-derived or pooled platelets from “random donors” are the two types of PC made which from whole blood donations. Whole blood is collected in the presence of a sterile anticoagulant solution, then stored at 22 ± 2 °C for less than 24 hours, and centrifuged at different g forces to isolate either the buffy coat (BC) or the platelet rich plasma (PRP).

- To prepare PC, blood is centrifuged at ca. $3000 \times g$ for 5 min to separate the red blood cells (bottom layer), the BC (intermediate layer) and the plasma (upper layer).^{92,93} The BC layer is transferred into another bag and gently resuspended into 20-30mL of plasma from the same donation (alternatively a platelet additive solution can be used at this stage) before a light spin centrifugation at $1000 \times g$ for 6 min. The supernatant containing the platelets is then transferred to a storage bag. A therapeutic unit is prepared by pooling 4 to 6 BC donations to reach a content of 3 to 4×10^{11} . This BC method is popular in Europe, including France, and can generate a product less contaminated in leukocytes especially when combined with a step of leukoreduction.
- In the PRP method, blood is centrifuged at lower g force ($1000 \times g$) for 10 min to separate red blood cells (bottom layer) from the plasma and platelets (upper layer) that are transferred in another bag. A second centrifugation ($3000 \times g$ for 5 min) pellet the platelets that are resuspended in a small volume (50-70 ml) of plasma.⁹² A therapeutic unit is also obtained by pooling 4 to 6 donations. The PRP method is used in USA and many countries in Asia, including Taiwan.

The third procedure to obtain therapeutic PC from single donor involves an automatic procedure called plateletpheresis.⁹⁴ Anticoagulated blood is separated through an extracorporeal procedure using cell separators that separate and collect platelets by repetitive centrifugation cycles.⁹⁵ The collected volume from each donor is larger (200-300 mL) allowing obtaining a therapeutic dose containing 3 to 6 x 10¹¹ platelets without pooling.

Small volume (around 5 to 10 mL) of platelet materials can also be prepared from autologous patients blood (50 to 100 mL) by medical devices using centrifugation to isolate the PRP.

4.2 Traditional therapeutic use of platelet concentrates

Platelet concentrates intravenous infusion is used for therapeutic transfusion to treat active bleeding or in cases of invasive surgical procedure that could cause bleeding. Prophylactic platelet transfusion can also be performed to prevent spontaneous bleeding in patients with low platelet counts (e.g. due to bone marrow suppression, septicemia, cancer chemotherapy, leukemia). Platelet transfusion is also performed in patients with platelet consumption diseases (e.g. immune thrombocytopenia, disseminated intravascular coagulation) and inherited (Wiskott-Aldrich syndrome, Glanzmann thrombasthenia, and Bernard-Soulier syndrome) or acquired (Uremia, diabetes mellitus, myeloproliferative disorders) platelet function defects. Platelet (and plasma) transfusion may also be decided for patients with massive blood loss receiving red blood cell concentrates, as well as in those undergoing cardiopulmonary bypass experiencing thrombocytopenia and impaired platelet function. The appropriate clinical transfusion of platelet concentrates in various pathologies is determined in specific clinical guidelines.^{96,97}

4.3 Novel therapeutic use of platelet concentrates in regenerative medicine

4.3.1 Clinical use

Platelets are well known to play critical roles in hemostasis and coagulation. However, platelets are increasingly recognized to act as instrumental functional players in inflammation, immunity, or tissue repair.⁹⁸ The functions of platelets are largely thought to be mediated through the release of biomolecules including growth factors (GFs) entrapped in their α -granules,⁹⁹ supporting synergistic therapeutic capacity in regenerative medicine and cell therapy.

Therapeutic platelet materials such as PRP, platelet gel and platelet lysate (PL) are typically obtained from autologous PRP preparation or from allogeneic PC prepared in blood centers. The synergetic role of platelet-derived GFs leads to accelerating the healing of soft, hard tissues and cartilage.

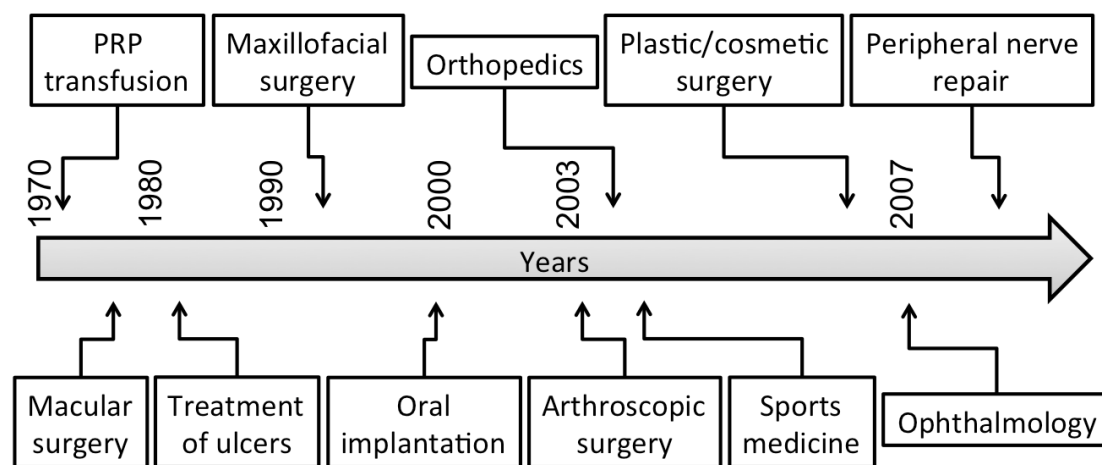


Figure 2 - The time schedule of PRP use in regenerative medicine.

(Adapted from Sánchez *et al.*, 2012)¹⁰⁰

There are three major categories of therapeutic applications for GFs in regenerative medicine (Figure 3). They encompass applications in degenerative, metabolic or autoimmune diseases with different modes of delivery:

- Topical applications for the treatment of degenerative recalcitrant ulcers associated with diabetes, peripheral nerve pathologies, trauma, burns, as well as tendons or ligaments tearing in sport medicine.
- Intra-articular injections to treat knee osteoarthritis pain¹⁰¹ and joint degeneration.¹⁰²
- Ocular applications as eye drops to release symptoms associated to age-related or autoimmune dry eye syndrome.
- In combination with grafts for reconstructive bone surgery in dental, orthopedic, maxilla-facial and plastic surgery.¹⁰³

Under the current status of knowledge, scientific rationale supports that platelet materials can promote tissue repair and regeneration.¹⁰⁴ Meta-analysis and controlled clinical studies concur, for instance to suggest a beneficial clinical effect in the healing of soft tissues and ulcers,¹⁰⁵ osteoarthritis and articular lesions,¹⁰⁶⁻¹¹¹ or dry eye syndrome,¹¹² among others (Figure 3).

However, variability in product characteristics and procedures used for applications suggest that more evaluations should be conducted using standardized products and clinical procedures,^{113,114} and, potentially, specialized preparations targeting specific applications.¹¹³

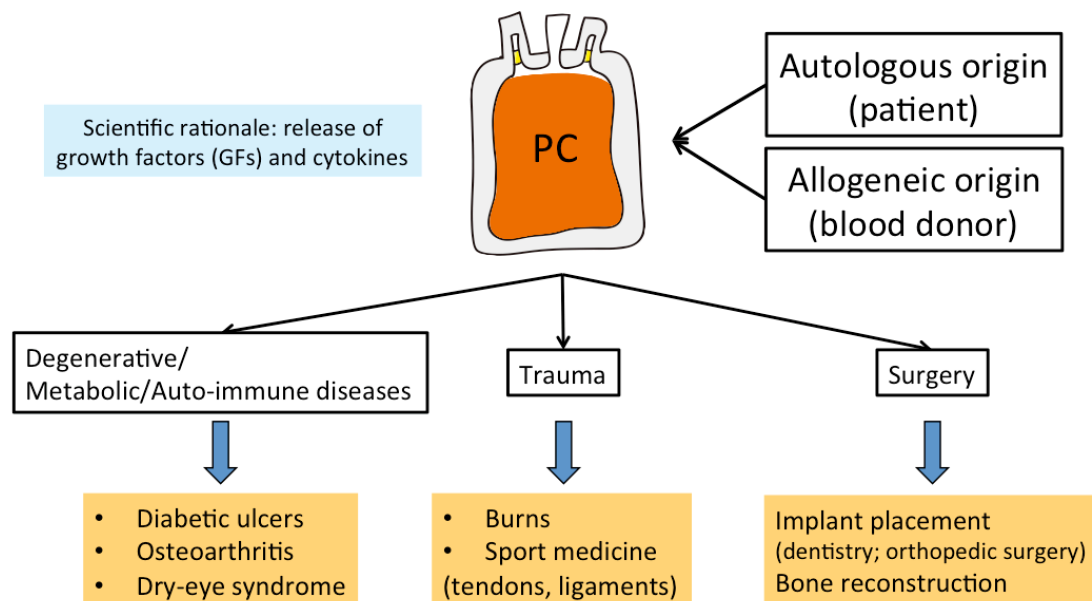


Figure 3 - Allogeneic PC preparation for regenerative medicine and cell therapy.

(Adapt from Chen TM *et al.*, 2008; 2010; 2013)¹¹⁵⁻¹¹⁷

Abbreviations: platelet concentrates (PC); growth factors (GFs)

4.3.2 Cell therapy

The benefit of PL, as a substitute for fetal bovine serum (FBS), for *ex vivo* expansion (Figure 4) of mesenchymal stromal cells (MSC) is clearly proven. PLs provide an abundant source of nutrients and growth factors that can support MSC growth, while meeting the requirements of xenogeneic-free clinical-grade media for cell therapy.^{93,118-121}

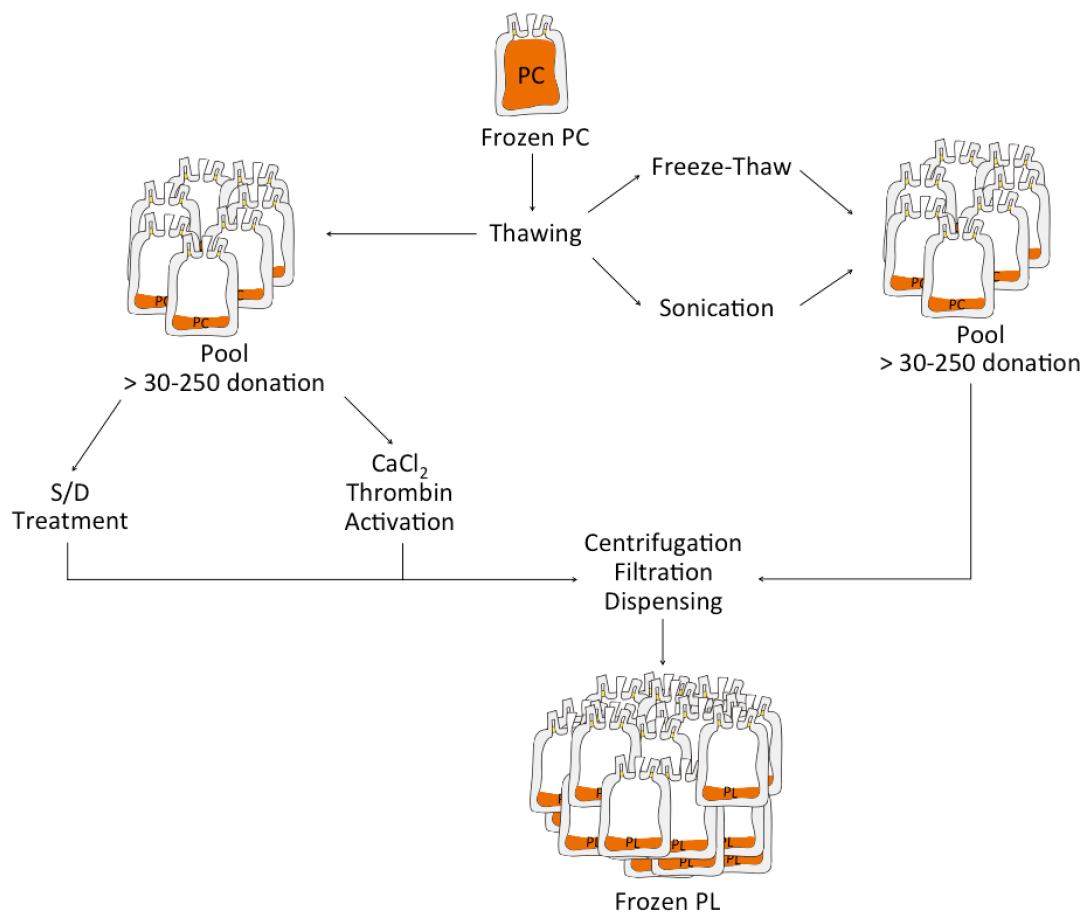


Figure 4 - Processing of allogeneic platelet concentrates into pooled platelet lysates for *ex vivo* cell expansion

4.3.3 *Mode of preparation of platelet lysates (PLs)*

PLs for regenerative medicine are usually produced from fresh platelet concentrates (PC). Interestingly, the pooled PLs employed as supplement for cell expansion are produced from outdated platelet concentrates generated in blood establishments (Figure 5). PC are frozen at -30°C or colder and stored until they are thawed and processed into HPL.¹²² Freeze-thaw induces cell lysis and release of the platelet content. Further lysis of the platelets can be ensured by (a) typically two or three cycles¹²¹ of freezing (-30°C or -80°C) and thawing (+37°C),¹²³ (b) sonication for 30 min at >20 kHz,¹²⁴ or, alternatively, by a chemical treatment by tri-n-butyl phosphate (solvent) and Triton X-45 (detergent) (S/D) that also allows inactivation of lipid-enveloped viruses¹²⁵ and enhanced release of the platelet growth factors.¹²⁶ Such platelet lysates have an essentially intact plasma and platelet proteomes, including for the content in fibrinogen and coagulation factors. Other platelet lysates are prepared by subjecting the platelet concentrates, either frozen or fresh, to a step of activation triggered by addition of calcium chloride and thrombin generation. This leads to platelet degranulation, serum-conversion of the platelet concentrate by conversion of fibrinogen into fibrin, and growth factor release. These lysates are depleted in some proteins including those involved in the coagulation cascade, including plasma fibrinogen.

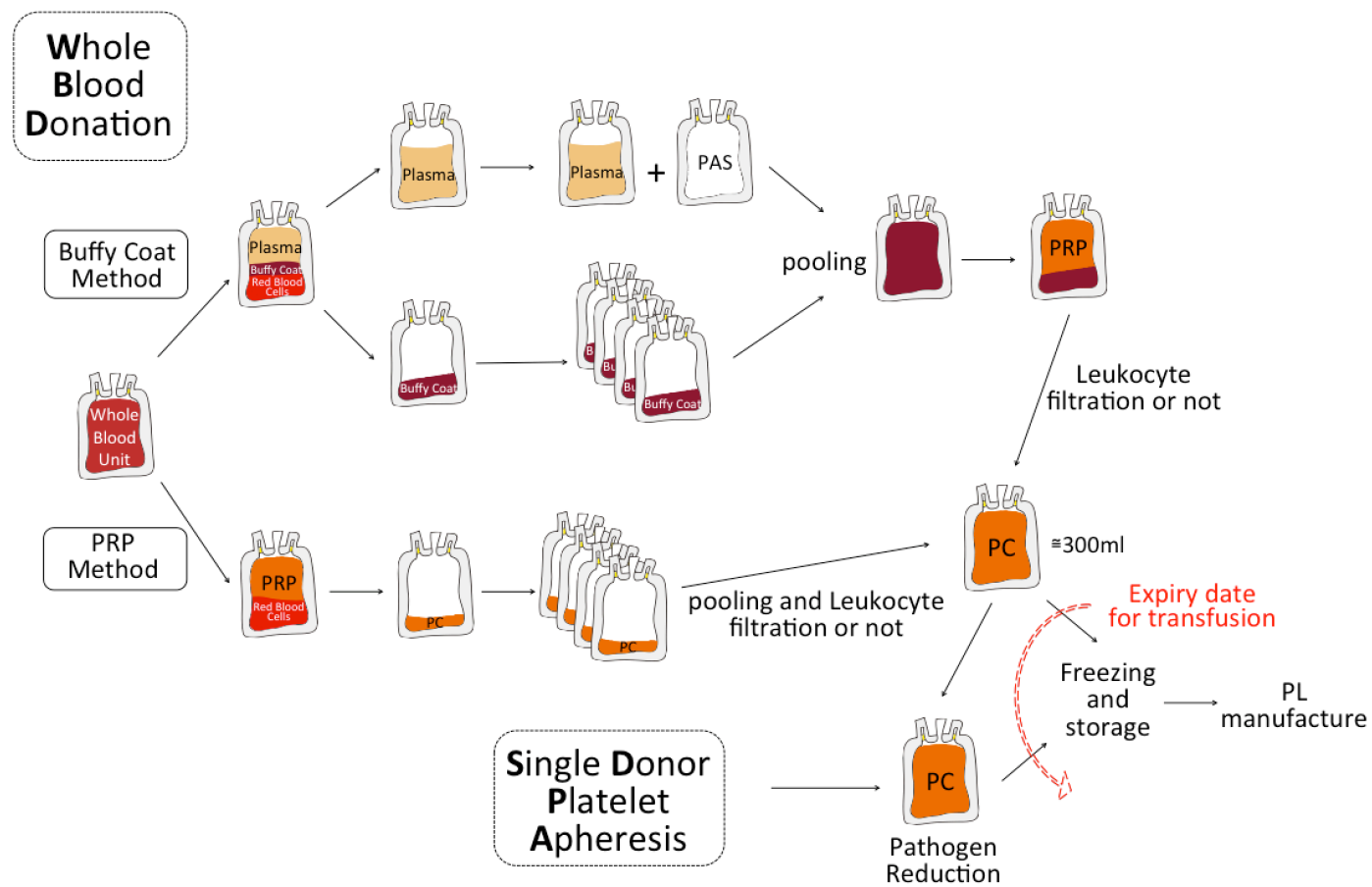


Figure 5 - Mode of preparation of platelet concentrates (PC) used for the production of human platelet lysates (PL).

Abbreviations: platelet concentrates (PC); platelet rich plasma (PRP); platelet additive solution (PAS); platelet lysate (PL).

4.3.4 *Growth factors (GFs) present in platelets*

Nowaday, there are over 300 molecules identified in the platelet secretome.¹²⁷ Based on their granular origin and proposed function, these molecules are classified as below,¹²⁸

- Dense granules consist of some small molecules and ions including ADP, ATP, serotonin, epinephrine, histamine and Ca^{2+} .
- Lysosomes contain α -arabinoside, β -galactosidase, β -glucuronidase, n-acetylglucosaminidase, elastase, collagenase and cathepsin.¹²⁹
- α -granules encompass mixtures of proteins with different functions, including proteins released on the surface of cells called membrane proteins and proteins secreted to microenvironment called soluble proteins. During secretion of these proteins several components are involved, such as soluble NSF attachment protein receptor (SNARE) complex and their regulators, and mediate membrane fusion and granule translocation. The molecular composition of platelets results from synthesis during megakaryocytes or from endocytosis of components from the plasma microenvironment.

Growth factors (GFs), such as platelet-derived growth factor (PDGF)-A, B, C, transforming growth factor (TGF)- β 1, brain-derived neurotrophic factor (BDNF), insulin-like growth factors (IGF)-1, epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and platelet factor 4 (PF4) are shown to have beneficial effects explaining PRP therapeutic effects. However, GFs signaling alone does not explain all biological responses associated with PRP, indicating that some other molecules may also be key to mediate tissue regeneration (Figure 6).¹²⁸

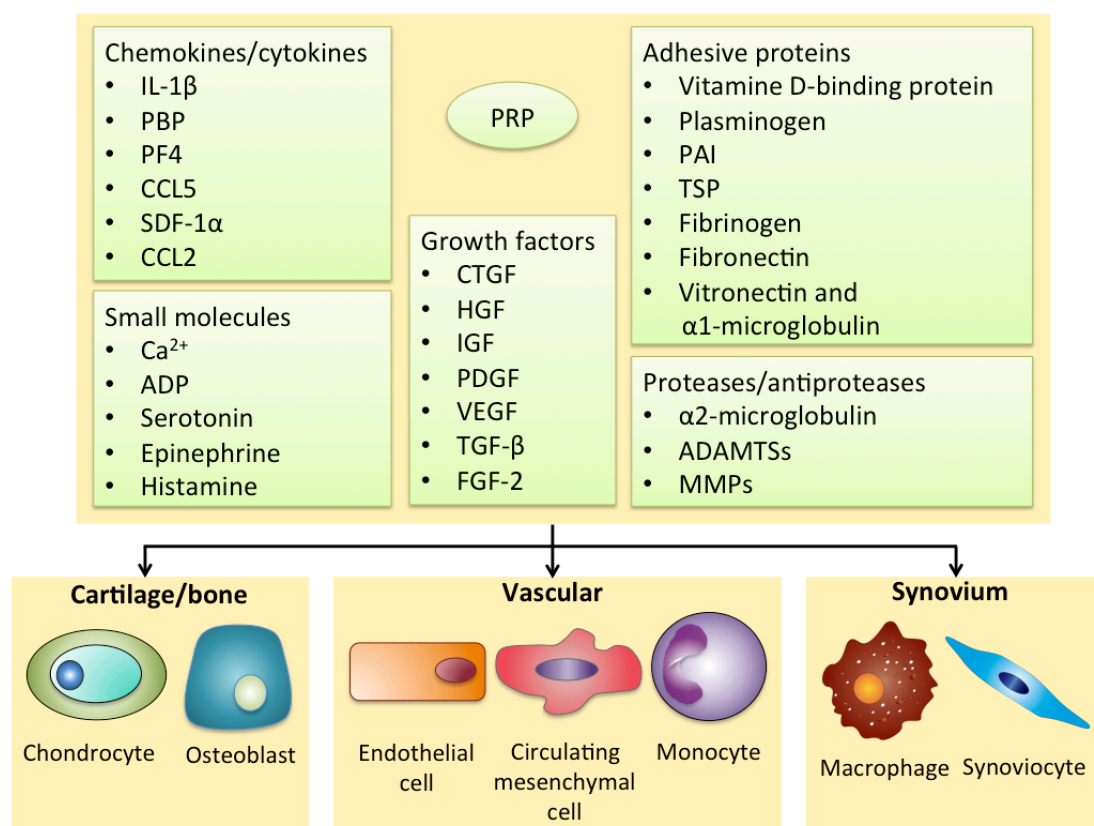


Figure 6 - PRP composition and the target cells

(Adapted from Andia *et al.*, 2013)¹²⁸

Cell proliferation and tissue remodeling triggered by molecules, such as GFs, entrapped in platelets α -granules.^{130,131,132} These GFs mediate the benefit effect in regenerative medicine including PDGF, PF4, VEGF, BDNF, TGF- β , EGF, HGF, IGF, FGF (Figure 7). Some of the growth factors are considered as neurotrophins to promote neurogenesis and survival,¹³³⁻¹³⁵ as described below.

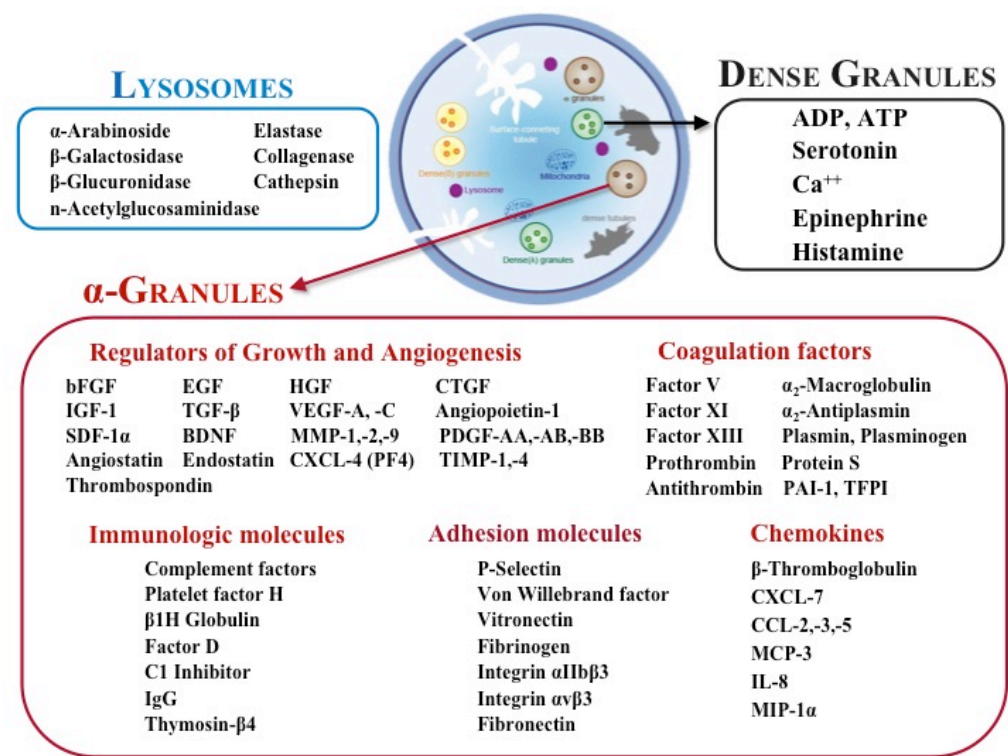


Figure 7 - Platelet components. Platelets contain α -granules, dense granules and lysosomes.

(Figure adapted from Burnouf *et al.*, 2016).¹²¹ Adenosine diphosphate (ADP); adenosine triphosphate (ATP); calcium ions (Ca^{2+}); basic fibroblast growth factor (bFGF); epidermal growth factor (EGF); hepatocyte growth factor (HGF); connective-tissue growth factor (CTGF); insulin-like growth factor 1 (IGF-1); transforming growth factor-beta (TGF- β); vascular endothelial growth factor A (VEGF-A); vascular endothelial growth factor C (VEGF-C); stromal cell-derived factor-1 α (SDF-1 α); brain-derived neurotrophic factor (BDNF); matrix metalloproteinase-1 (MMP-1); matrix metalloproteinase-2 (MMP-2); matrix metalloproteinase 9 (MMP-9); platelet derived growth factor-AA (PDGF-AA); platelet derived growth factor-AB (PDGF-AB); platelet derived growth factor-BB (PDGF-BB); cxc chemokine ligand 4 (CXCL-4); platelet factor 4 (PF4); tissue inhibitor of metalloproteinase-1 (TIMP-1); tissue inhibitor of metalloproteinase-4 (TIMP-4); plasminogen activator inhibitor-1 (PAI-1); tissue factor pathway inhibitor (TFPI); cxc chemokine ligand 7 (CXCL-7); chemokine (c-c motif) ligand 2 (CCL-2); chemokine (c-c motif) ligand 3 (CCL-3); chemokine (c-c motif) ligand 5 (CCL-5); monocyte chemotactic protein-3 (MCP-3); interleukin-8 (IL-8); macrophage inflammatory protein-1 alpha (MIP-1 α); immunoglobulin G (IgG).

The main biochemical features of growth factors as well as their main characteristics and functions are summarized in the Table 7 and Table 8, respectively. Most growth factors have a medium size molecular mass comprised approximately between 6 and 80 kDa and most, apart from EGF, have a basic isoelectric point.

Table 7 - Main biochemical features of platelet growth factors

	Molecular mass (kDa)	Isoelectric point (IP)	Isoforms
PDGF	27–31	9.5–10.4	AA, BB, AB
PF4	11	7.6	
VEGF	19.2	7.77-9.21	A, B, C, D
BDNF	13.6 (or 27.2 dimer)	9.01	-
TGF-β	44-47	8.31-8.83	1, 2, 3
EGF	6.2	5.53	-
HGF	80	8.22	-
IGF	20-21	9.5-9.78	1, 2
FGF	7-38	6.52-11.18	1-21

Table 8 - Summary of the main characteristics and functions of platelet GFs

(Adapted from De Pascale *et al.*, 2015)¹¹⁴

GFs	Action and functions
TGF- β	Acts as a GF in autocrine and paracrine fashions, with long-term healing; inhibits macrophage and lymphocyte proliferation; stimulates mesenchymal stem cells proliferation; regulates endothelial, fibroblastic, and osteoblastic cell mitogenesis as well as collagen synthesis and collagenase secretion.
PDGF	First GF in a wound and is responsible for connective tissue healing; promotes chemotaxis and mitogenesis of mesenchymal stem cells, fibroblastic, and osteoblastic cells; regulates collagen synthesis and collagen secretion.
bFGF	Multifunctional protein with mitogenic effect and regulatory, morphologic, and endocrine role; regulates endothelial, mesenchymal stem cells, osteoblastic cells and fibroblastic cells; promotes mitogenesis of chondrocytes; promotes angiogenesis and formation of new blood vessels from the preexisting vasculature.
EGF	Potent mitogen that increases the expression of several genes leading to DNA synthesis and cell proliferation; regulates mesenchymal stem cells and epithelial cells mitogenesis; promotes chemotaxis of endothelial cells and angiogenesis; regulates collagenase secretion.
VEGF	Stimulates the proliferation and migration of endothelial cells to form immature vasculature; regulates collagenase secretion.
BDNF	Neurotrophin that supports the survival of neuron, and maintains synapses.

4.3.4.1 Platelet factor 4 (PF4)

Platelet factor 4 is known as chemokine (C-X-C motif) ligand 4 (CXCL4) released from α -granules of activated platelets. It is also present in microglia and was shown to have high affinity for heparin.¹³⁶

4.3.4.2 Platelet-derived growth factor (PDGF)

There are three isoforms of PDGF, composed by two dimeric glycoprotein chains of AA, BB or AB. It can be produced or released by various cells, including platelets, activated macrophages, smooth muscle cells and endothelial cells.¹³⁷ In particular in the neurological field, PDGF is expressed widely in the central nervous system (CNS).¹³⁸ It plays essential roles in modulating synaptic transmission,¹³⁹ promoting differentiation of neuron.^{140,141} Also, PDGF also exerts neuroprotective effect,^{138,142} increases the survival rate in dopaminergic neurons cultures to enhance the survival rate.¹³⁷ In 6-hydrodopamine (6-OHDA) rat model, PDGF expression was increased after lesions in *substantia nigra* suggesting its neuroprotective effect.¹⁴³

4.3.4.3 Vascular endothelial growth factor (VEGF)

There are five members in VEGF family such as VEGF-A, -B, -C, -D and placenta growth factor (PGF). VEGF can stimulate angiogenesis, vasculogenesis by induction of hypoxia in brain¹⁴⁴ therefore contributing to neuroprotection, neurogenesis and angiogenesis in focal cerebral ischemia.¹⁴⁵ In a PD rat model, VEGF-B has evidenced neuroprotective action on dopaminergic neurons in the substantia nigra.¹⁴⁶ VEGF plays roles not only in neuroprotection but also in neuronal differentiation, and the regulation of glial and dendritic growth.¹⁴⁷⁻¹⁴⁹ In neurodegenerative diseases, such as ALS, a decrease seen in VEGF leads to selective motor neuron degeneration *via* alteration of neurotrophic and angiogenic mechanisms.¹⁵⁰ In addition, there is a lack of VEGF upregulation in cerebrospinal fluid (CSF) in hypoxemic ALS patients.^{151,152} Finally, intrathecal transplant of neural stem cells (NSCs) overexpressing VEGF can delay disease progression and extend the survival in an ALS mice model.^{153,154}

4.3.4.4 *Transforming growth factor beta (TGF- β)*

TGF- β , a multifunctional cytokines comprised of three isoforms (TGF- β 1, - β 2, - β 3), regulates cell growth, differentiation, and survival and influences the immune system. TGF- β is widely spread in the brain and is increasingly recognized to exert a neural function¹⁵⁵ that protects dopaminergic neurons in the substantia nigra against neurotoxin MPTP in a PD model, and promote survival of spinal cord motoneurons.¹⁵⁶ TGF- β neuroprotective activity may be synergistic of that of glial-derived neurotrophic factor (GDNF).¹⁵⁷

4.3.4.5 *Basic fibroblast growth factor (bFGF)*

bFGF, a heparin binding polypeptide, plays multifunctional roles in developing and adult CNS. Several evidences suggest that bFGF enhances cell survival, neurite outgrowth,¹⁵⁸ promotes axonal branch formation¹⁵⁹ and modulates synaptic transmission.¹⁶⁰ In a preclinical model, bFGF has shown attractive therapeutic effects in neurodegenerative diseases, such as Huntington's disease¹⁶¹ and PD.¹⁶²

4.3.4.6 *Epidermal growth factor (EGF)*

EGF is a low molecular weight polypeptide. By binding to EGFR (EGF receptor), it stimulates cell growth, proliferation and differentiation. EGF can be synthesized in the brain and is found in blood and CSF.¹⁶³ EGF stimulates glial cells and astrocytes proliferation and differentiation.¹⁶³ Also, it can promote dopaminergic neuron survival and stimulate neuritis outgrowth therefore exert its pivotal roles in regulation and protection of the neurons in the brain.^{163,164}

4.3.4.7 *Brain-derived neurotrophic factor (BDNF)*

BDNF is a member of the neurotrophin family, found in the platelets and brain. BDNF is synthesized in CNS and also present in high quantity in its mature form in platelets.^{165,166} BDNF supports neuron survival, growth, differentiation and synapses. Neuroprotective effects of BDNF have been shown in some neurodegenerative diseases, such as AD,¹⁶⁷ PD¹⁶⁸ and Huntington's disease.¹⁶⁹ In a PD rat model, BDNF increases striatum and substantia nigra neurogenesis when administered via an intraventricular system.¹⁴³

4.3.5 Physiological properties of platelet materials

Some studies strongly suggest that PLs exhibit objective beneficial therapeutic effects for some clinical applications (Figure 8). It is important to understand the scientific mechanisms how PLs helps regenerative medicine to make an optimal use of their versatility. Such understanding can help developing specialized PL preparations targeting specific clinical applications.

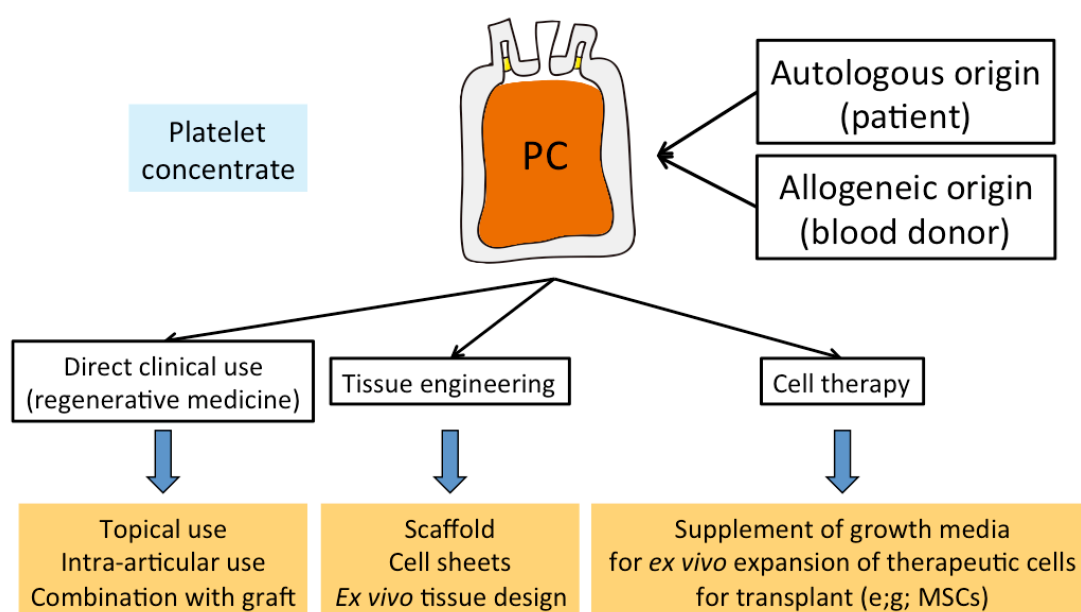


Figure 8 - Range of new therapeutic applications of platelet-derived products

4.3.5.1 Antimicrobial properties

Anecdotal clinical reports have suggested that platelet materials exert antimicrobial activities as evidenced by lowered rate of bacterial infections when using fibrin materials and platelet materials in skin grafts, as well as increased graft take in an infected bed.¹⁷⁰ The mechanisms explaining this effect of platelet materials have remained largely unexplained.¹⁷¹ *In vitro* experiments have shown that the proteins of the plasma compartment, and in particular the complement system, may play an instrumental role in the inactivation of gram-negative *E. coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*.¹⁷²

CaCl₂ activation was reported to decrease the antimicrobial activity against *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*, and a S/D treatment did not affect antimicrobial activity.^{172,173} These data are consistent with observations that cryoprecipitate-derived fibrin sealant, containing active complement, can inhibit the growth *Escherichia coli*, but not a purified, thereby complement component-free pasteurized commercial fibrin sealant.¹⁷⁴ *In vivo*, the degranulation of platelets release cytokines like interleukin (IL)-1 capable of attracting neutrophils to the wound site and inhibit bacteria.¹⁷⁵ Similarly, antimicrobial proteins and peptides of the innate immune defense,¹⁷⁶⁻¹⁷⁹ or platelet α -granules components, such as complement and complement binding proteins may contribute to antimicrobial power.¹⁸⁰

4.3.5.2 *Anti-inflammatory properties*

Platelet materials used in implantology and in the treatment of osteo-arthritis have been reported to exhibit anti-inflammatory and analgesic properties, and reduce post-surgical inflammation.¹⁸¹⁻¹⁸³ These properties have been attributed to platelet components such as RANTES and lipoxin AA4.¹⁸¹

In addition TGF- β is anti-inflammatory in an animal model of asthma where immunosuppressive and anti-inflammatory effects associated with enhancement of Foxp3⁺-induced regulatory T cells (iTregs) were found.¹⁸⁴ HGF, may limit the effect of the inflammatory IL-1 β cytokine in human osteoarthritic (OA) chondrocytes, and inhibit the activation of nuclear factor (NF)- κ B, which is involved in OA pathogenesis;^{183,185} lowered cyclooxygenase (COX)-2 and CXCR4 target genes expression was also observed.¹⁸³

In LPS-activated macrophages, HGF decreases IL-6, a pro-inflammatory cytokine, and increases the anti-inflammatory IL-10.¹⁸⁶ TRAP (thrombin-receptor-activating peptide)-activated platelets inhibit TNF- α expression in monocytes exposed to LPS.¹⁸⁷ In a chondrocyte model, HGF, TGF- β 1, IGF-1, and PDGF-BB decrease the inflammation by inhibiting IL-1 β or suppressing NF- κ B pathway.^{183,185,188,189}

Exposure of tendon fibroblasts to platelet rich plasma *in vitro* was recently observed to induce an inflammatory response through the TNF- α and NF- κ B pathways. The authors conclude that PRP could trigger a transient inflammatory event preceding the tissue regeneration response.¹⁹⁰

Our laboratory has observed that various plasma and platelet lysate fractions suspended in plasma do not stimulate the generation of NO, iNOS, COX-2, and TNF- α *in vitro* markers of inflammatory response in a RAW cell macrophage model. When cells were stimulated by LPS, these fractions were found to contribute to some control of the inflammation, albeit with some variations in the degree of anti-inflammatory capacity. Plasma depleted of platelet/platelet lysate was anti-inflammatory in whereas the S/D-treated platelet lysates, which contained more RANTES, exhibited the strongest anti-inflammatory action under LPS stimulation, with notorious TNF- α and COX-2 inhibition.¹⁹¹ This suggests that components from both the plasma and platelet compartments contribute to the overall anti-inflammatory properties of these preparations. Examples of proteins in the plasma compartment that include anti-inflammatory proteins in plasma include albumin (that can reduce NO and TNF- α levels in patients with cirrhosis and spontaneous bacterial peritonitis)¹⁹² alpha 1-antitrypsin¹⁹³ inter-alpha trypsin inhibitor subunit bikunin,¹⁹⁴ alpha-2-macroglobulin,¹⁹⁵ and activated protein C.^{196 197} IGF-1, a growth factor in plasma, down-regulates NF- κ B.¹⁸⁸

4.3.5.3 REDOX properties

Oxydative stress and damage can play a substantial role in the degeneration and repair of various tissue, such as joints and cartilages,¹²⁸ as well as in bone fractures, or tendon injuries. There is still little information on the capacity of various platelet materials to balance clinically meaningful anti-oxydant/REDOX capacity, regulate detoxifying and antioxidative enzymes, and reduce oxidative stress in clinical use.

VEGF has been shown to activate nuclear factor (erythroid-derived 2)-like2 (Nrf2) pathway *in vitro* and prevent oxidative damage. The platelet proteome enhances the synthesis of endogenous VEGF and increase the antioxidant response element (ARE) through Nrf2 activation in primary human osteoblast (hOB) and in osteoblast-like cell line (SAOS-2), preventing oxidative damage.¹⁹⁸ Similar ARE stimulation by platelet lysate was obtained using tenocyte cultures,¹⁹⁹ but by contrast platelet-rich-plasma increased at least transient expression of ROS and oxidative stress in tendon fibroblasts.¹⁹⁰ Further studies on the benefit that the platelet secretome can play or not in controlling oxidative stress in damaged tissues are needed.

4.4 Physiological contributions of microparticles to tissue regeneration and neuroprotection

The role potentially played by cell-derived MPs, including PMPs, in tissue regeneration and repair is gradually emerging, and certainly as complex as is MP diversity.²⁰⁰ PMPs are decorated physiological microvesicles expressing functional receptors from their cells of origin and thereby can interact with the cellular environment. They play a critical role in cell-cell communication,²⁰¹ and serve as cargo for functional molecules, such as growth factors and miRNA, to target cells and their microenvironment.^{65,202-205}

PMP in the blood circulation are thought to contribute to continuous physiological vascular tissue repair and regeneration,²⁰⁶ maintaining the function of an healthy endothelium.²⁰⁷ Intramyocardial injections of PMPs was observed to induce a marked increase in the amount of new capillaries following ischemia,²⁰⁸ helping to repair myocardial muscle. Also, PMPs have been shown to enhance the re-endothelialization capacity of atherosclerotic patient-derived circulating angiogenic cells (CACs). PMP-pretreated CACs (PMP-CACs) exhibit increased adhesion capacity. Intravenous injection of PMP-CACs to rats with hindlimb ischemia could promote neovascularization of the ischemic limbs, and PMP function was found to be associated to the secretion of RANTES. PMP-CACs could therefore be a therapeutic option to enhance angiogenesis for limb ischemia in atherosclerotic patients.²⁰⁹ Local application of PMPs was propose a therapeutic strategy for targeting angiogenesis-related conditions.²¹⁰

PMPs could improve reperfusion in a rat myocardial infarction model.^{211,212} Interest of PMPs in bone regeneration was revealed through their capacity to increase by 20 to 50-fold the mitogenic response of human trabecular bone-derived cells, accelerating the regeneration of mineralized tissue.²¹³

The possibility that PMPs may be useful in neurosciences and treatment of diseases of the CNS is generating some interest.²¹⁴ PMPs promote neuronal cell proliferation, survival, and differentiation and are considered for treatment of brain injuries,²¹⁵ as they increase the survival, proliferation, and differentiation of neural stem cells.^{211,215,216} Human brain endothelial cells can internalize PMPs *in vitro*,²¹⁷ resulting in changes in phenotype and functions.²¹⁸ PMP functional action involves the pAKT and extracellular signal-regulated kinases (pERK) pathways.²¹⁵ Topical application to injured brain of PMPs entrapped in a biodegradable polymer was effective in an *in vivo* model of cerebral ischemia.²¹⁹ Finally, PMPs transfused to rats undergoing middle cerebral artery occlusion were shown to exert a protective effect on cerebral ischemic damage suggesting new therapeutic approaches for cerebral infarction.²²⁰

5 Can platelet neurotrophins play a role in the treatment of neurodegenerative disorders?

5.1 Neurodegenerative diseases

Neurodegenerative diseases associated with damages of the CNS (encompassing brain or spinal cord) lead to various neurological or psychiatric disorders. In aging societies of Europe, North America and Asia, the prevalence of neurodegenerative diseases is high and is so far still rising. AD and PD are common, chronic, fast-progressing, non-communicable diseases. They affect millions of people and their prevalence is expected to double by 2030 or before. These diseases bear huge economic societal impact (\$100 billion are spent each year to address AD in the USA) and lead to immense emotional burden on patients and their caregivers. Stopping the progression of neurodegenerative diseases through the development of effective, affordable and pragmatic biotherapies amenable for implementation in both developed and developing countries is highly challenging.

PD is the second most frequent neurodegenerative disorder worldwide. It affects approximately 1% of population older than 60 years, and up to 4% in the oldest age groups. The loss of dopamine production in the striatum (as a result of progressive neuronal degeneration in the *substantia nigra pars compacta* (SNpc)) is the primary chemical marker of PD. Clinical diagnosis is based on a combination of bradykinesia, stiffness and rest tremor of the limbs. Axial signs include postural instability, gait disorders, dysarthria and dysphagia. PD is regarded as a neuropsychiatric disease because of the frequent cognitive symptoms (dementia, etc.) and behavioural symptoms (i.e. depression, addiction, etc.) associated with its progression. It is urgent, but quite challenging, to develop pragmatic and affordable effective “disease modifying strategy” providing neuroprotective and/or neuroregenerative benefits.

5.2 Neurotrophins and neuroregenerative diseases

Neurotrophins, as modulators and activators of the neuronal signalling pathways, may exhibit therapeutic benefits that have been evaluated.²²¹ ICV delivery of several recombinant neurotrophins (rNTFs), including BDNF, PDGF-BB, PDGF-CC, TGF- β and VEGF evidenced neuroprotective activities in cell and animal models. Still, in spite of these proof-of-concepts of the neurorestorative activity of NTFs, most randomized controlled trials (RCTs) of high-dose, ICV-administered single recombinant NTFs did not exhibit therapeutic benefits in PD, ALS and AD.²²²⁻²²⁶ In addition to the pharmacodynamic limitations of the continuous administration of single rNTFs by ICV, there are several possible explanations for the repeated failure of single-NTF-based strategies in clinical trials,^{226,227} including:

- 1) The limited diffusion of NTFs within the human brain,
- 2) The inability of a single NTF to orchestrate, alone, the complex interplay of signalling pathways required for an efficient neuroprotection (attraction and proliferation of different cell types at the degenerative site; cell differentiation; and angiogenesis).
- 3) The blunting effect of high-doses of a given rNTF on transduction pathways promoting proliferation and survival. For instance, GDNF failed to protect nigral dopamine neurons against α -synuclein-induced neurodegeneration because of reduced expression of the transcription factor Nurr1 and its downstream target (the GDNF receptor Ret).²²⁸

5.3 Platelet materials have the capacity to exert neuroprotective effect

In platelet α -granules, the combination of physiological bioactive molecules, such as growth factors, serve the tissue healing and repair abilities contributing to the therapeutic benefit.^{113,180,229,230} Platelets are a reservoir of growth factors which originate from megakaryocytes or are captured through endocytosis. Some of them are identified as NTFs (BDNF, EGF, bFGF, PDGF (-AA, BB, AB), TGF- β , and VEGF).

Recent data show the diffusion of cytokines into the striatum after ICV administration of platelet lysate depleted plasma. Local delivery of GFs extracted from platelets to the lateral ventricles could induce angiogenesis, neurogenesis and neuroprotection and reduces behavioural deficits after brain ischemia.²³¹ In addition, some platelet lysates GFs diffuse and exert neuroprotective effects in animal models of neurodegenerative diseases (AD and PD) when administered by the intranasal (i.n.) route.^{232,233} **This led us to hypothesize that (a) the lack of clinical effects of NTFs may rather result from the inability of a single NTF to exert a noticeable neuroprotective action in such complex diseases, and (b) the use of physiological mixtures of platelet NTFs (pNTFs) may be key to promote balanced and effective clinical benefits.**

It is therefore conceivable that **extensive neuroprotection and neurorestoration could be obtained through administration of physiological mixtures of pNTFs.** To confirm our hypothesis our work has consisted in evaluating whether platelet lysates enriched in neurotrophic GFs can exert neuroprotection in well-established *in vitro* and *in vivo* models of PD, using differentiated Lund Human Mesencephalic neurons (LUHMES) intoxicated by 1-methyl-4-phenylpyridinium (MPP⁺) neurotoxin²³⁴ and MPTP intoxicated mice.

6 Models and toxins used in our studies

6.1 The neuronal cellular model system used in this thesis: LUHMES

Our experimental cellular model uses homogeneous cultures of post-mitotic human neurons, the Lund human mesencephalic (LUHMES) cell line. The LUHMES cell line is a subclone generated from MESC2.10 cell line²³⁵ obtained from embryonic ventral mesencephalic tissue with tetracycline (TET)-controlled and immortalized with LINX *v-myc* retroviral vector system.²³⁶ In the absence of tetracycline, tetracycline-controlled transactivator (tTA) is activated to trigger *v-myc* transcription. This phenomenon allows cells proliferation in Advanced DMEM/F12 containing bFGF (Figure 9). To initiate differentiation, the cells are treated with differentiation medium containing Advanced DMEM/F12, non-toxic concentration of TET, dibutyryl cyclic adenosine 3',5'-monophosphate (db-cAMP) and GDNF. The presence of TET abrogates tTA activation to block *v-myc* production, and those cells freely transform to post-mitotic neurons.

LUHMES cells are conditionally immortalized human neuronal precursor cells.²³⁵ We used them in our work to (i) test and screen the effects of PPLs prior to use in animal models, (ii) characterize the molecular mechanisms of the neurodegenerative processes and the protection afforded by pNTFs, and (iii) establish the feasibility of molecular biology experiments. This model using LUHMES is largely thought to be the best available model of dopaminergic cell types for the analysis of PD-related processes. The neuronal status of LUHMES cells and its robustness have been extensively studied.^{234,237-239} LUHMES cells present an extremely high (> 99%) rate of conversion to post-mitotic neurons expressing dopaminergic markers, presenting neurite outgrowth and electrophysiological properties. LUHMES cells respond to MPP⁺ (the most valuable neurotoxin for inducing parkinsonism in animal models) in much the same way as primary cells. The LUHMES cell line is therefore a powerful tool for characterizing neurotoxins' modes of action in cell death and neurite growth inhibition.^{240,241}

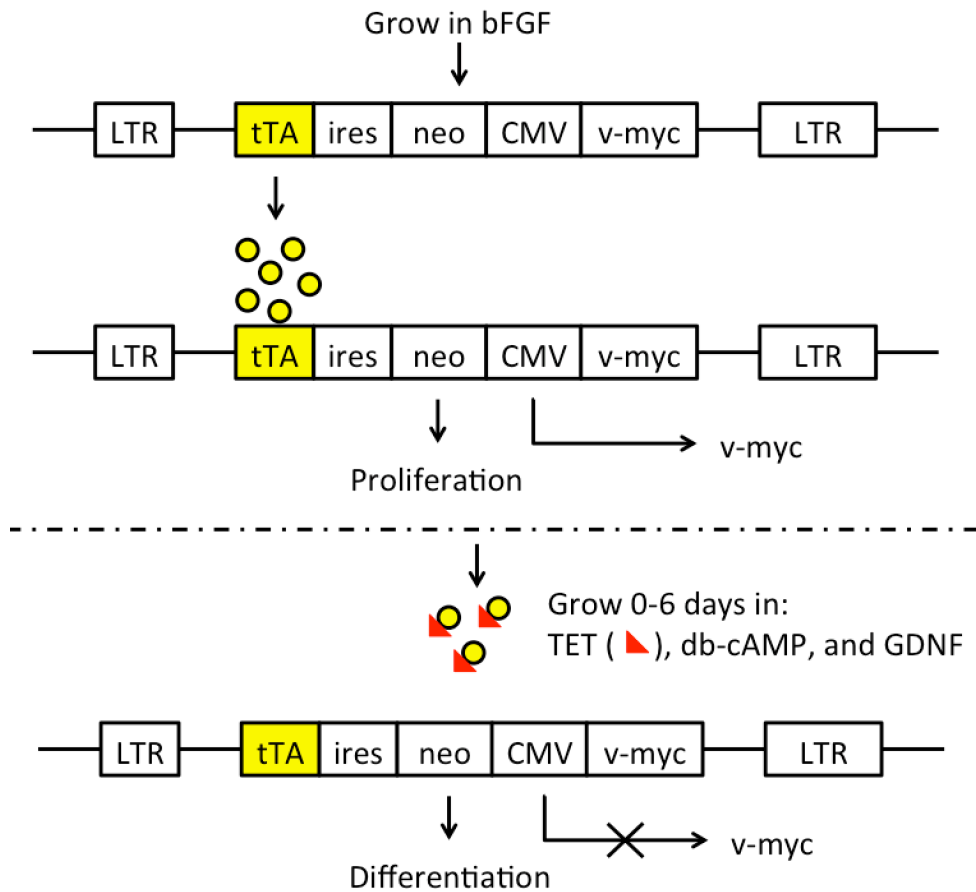


Figure 9 - LUHMES proliferation and differentiation.

(Adapted from Lotharius *et al.*, 2002)²³⁵ Abbreviations: basic fibroblast growth factor (bFGF), long terminal repeat (LTR), yellow circles: tetracycline-controlled transactivator (tTA), internal ribosomal entry site (ires), neomycin resistance gene (neo), cytomegalovirus-virus promoter (CMVp), purple triangles: tetracycline (TET), dibutyryl (db), glial cell line-derived neurotrophic factor (GDNF).

6.2 MPTP and MPP⁺ neurotoxins

Lipophilic MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is precursor of neurotoxin MPP⁺ (cation 1-methyl-4-phenylpyridinium) can cross blood-brain barrier (BBB) and converted to MPP⁺ by monoamineoxidase-B (MAO-B) in glial cells. MPP⁺ can interfere the oxidative phosphorylation in mitochondria by inhibiting the complex I and it causes ATP reduction even cell death. In our study, we used MPTP in mice experiment and MPP⁺ in cell culture.

6.3 MPTP mice model

Lipophilic MPTP is used as a trigger to investigate the mechanisms involved in the damage of dopaminergic neurons in PD, especially in the striatum and substantia nigra. The C57BL/6 mice strain is sensitive to MPTP as this strains has greater activity of MAO-B in the brain than in the liver. The most reproducible results were found in the male mice at least 8 week-old and at least 22 g.²⁴²

AIMS

The main objective of this thesis has been to develop a scalable specific platelet lysate (PL) preparation capable to exert neuroprotective activity in cellular and animal models of PD. PD was selected as a representative neurodegenerative disorder because experimental models to induce neurotoxicity and to study neurorestoration are reasonably well established, and since PD is, together with Alzheimer disease, a leading pathological cause of social impairment and death in aging populations worldwide.

Quality and safety are therefore two critical elements that must be taken into account as initial primary points in a translational research project involving the use of PL in regenerative medicine. They are mandatory regulatory requirements for any therapeutic blood products prepared from allogeneic blood source. One primary factor of the quality should be the viral safety. An adequate margin of viral safety can be ensured by a combination of viral reduction procedures to complement the donors screening and viral testing strategies already in place in blood establishments. Indeed in spite of screening procedures implemented in the selection of blood donations used to make therapeutic products, the risk of contamination by blood-born pathogens, such as HCV, HIV and HBV, remains high in endemic areas or resource-limited countries.²⁴³ To improve blood products safety, viral inactivation or removal procedures are necessary as long as they do not affect the functional activity of blood proteins. Solvent detergent (S/D) treatment, various heat treatments, and nanofiltration²⁴⁴ are among the procedures commonly used to provide a robust margin of viral safety to plasma products. Another possible, more recently identified contributor to the quality and safety of blood products is linked to the presence, either detrimental, or beneficial, of MPs. One potential detrimental effect of MPs is the risks of inducing thrombosis in a plasma protein and blood cell environment.

In this thesis we hypothesized that the viral safety of PL for use in neuroprotection could be achieved by inactivation by S/D treatment and/or heat-treatment, or removal by nanofiltration. Also, we focused on the possible role played by MPs in the safety and/or efficacy of PLs by studying the impact played by a hollow-fiber nanofiltration step using a 75 nm membrane. These developments were first performed using plasma as a “worst-case” model of blood product, owing to its protein complexity. Results were then applied to a customized clinical-grade platelet lysate, tailor-made for ICV and/or i.n. brain administration and characterized by a low protein content close to that of the CSF, depleted of fibrinogen and other, potentially toxic, plasma proteins, and containing a concentrated physiological mixture of platelet-derived neurotrophins.

My studies have therefore been divided into 3 main objectives:

Specific aim 1: To evaluate the capacity of a S/D treatment to inactivate HCV, selected as a model of blood-borne viruses, in plasma. We used culture-derived HCVcc, clinical HCV isolates and HCVcc infected primary human hepatocytes for spiking experiments and/or to assess HCV infectivity.

Specific aim 2: To evaluate the capacity of 75-nm hollow fiber nanofilter to remove plasma-borne MPs and study the impact on plasma functional properties.

Specific aim 3: Use the information from aims 1 and 2 towards developing a tailor-made, virally safe, PL possessing strong neuroprotective activities in a dedicated cellular model using LUHMES cells exposed to neurotoxins, and in an animal model of mice intoxicated by MPTP neurotoxin.

MATERIALS AND METHODS

Experimental procedure

1 Overall experiment design

In this thesis, we first focused on studying steps intended to improve the viral safety of blood products.

- Blood was collected with a citrate-based anticoagulant and separated into plasma and platelet concentrate. Hepatitis C virus (HCV) particles were spiked into plasma samples to test the capacity of a solvent detergent (S/D) incubation to inactivate its infectivity.
- A 75 nm pore size nanofilter, typically used as a pre-filter prior smaller pore-size virus removal nanofiltration in the plasma fractionation industry, was tested to remove platelet-derived microparticles (PMPs) present in plasma. PMPs in plasma samples, before and after nanofiltration, were characterized by dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS) and transmission electron microscopy (TEM). As PMPs exhibit a higher content of phospholipids on their surface, it was hypothesized that they may induce more risk of thrombogenicity during plasma transfusion. Therefore we tested PMP thrombogenicity in plasma and after spiking to cerebrospinal fluid (CSF).

In addition, we prepared a particular platelet pellet (PPL) from platelet concentrate (PC) as a growth factor preparation intended for neuroregeneration. To ensure better quality and viral safety, this PPL preparation was additionally subjected to heat treatment (HPPL), S/D treatment and nanofiltration. Neuroprotection and neuroinflammation of PPL were tested.

Moreover, content of PMPs in PPL was determined and capacity of PMPs alone to exert a neuroprotective effect was determined. The experimental design is shown in Figure 10.

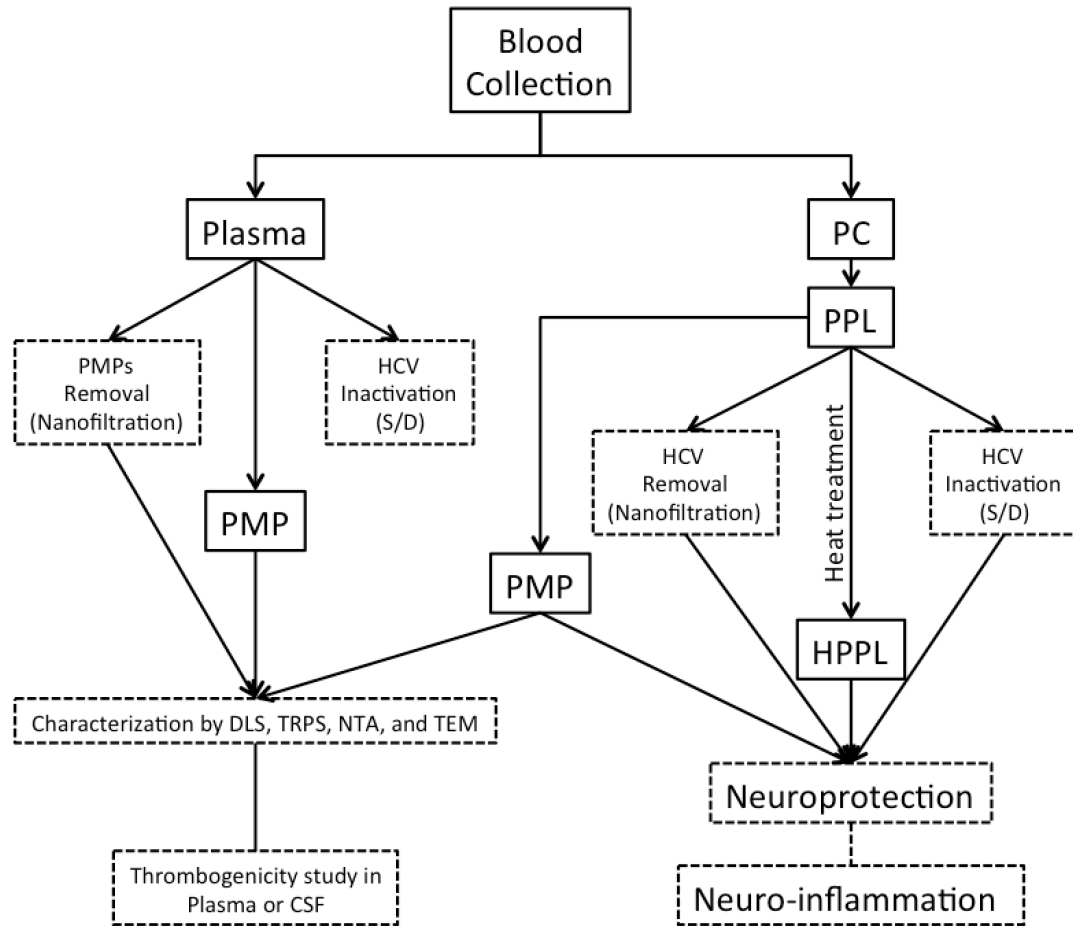


Figure 10 - Overall experimental design. Blood was collected in the presence of anticoagulant then separated into plasma and PC.

PMP present in plasma, nanofiltered plasma, and S/D treated plasma were characterized by DLS, TRPS, NTA and TEM. The thrombogenicity of PMP present in plasma and CSF was analyzed. Also, PPL was prepared from PC then subjected to 3 viral inactivation steps, including heat treatment, nanofiltration and S/D treatment to test their neuroprotective effect and neuroinflammatory effect. *Abbreviations:* platelet derived microparticles (PMPs); hepatitis C virus (HCV); solvent detergent (S/D), cerebrospinal fluid (CSF); platelet concentrate (PC); platelet pellet lysate (PPL); heat-treated PPL (HPPL); intracerebroventricular (ICV); intranasal (IN); dynamic light scattering (DLS); nanoparticle tracking analysis (NTA); tunable resistive pulse sensing (TRPS); transmission electron microscopy (TEM).

2 Blood products preparation

2.1 The collection of plasma and platelet concentrate (PC)

The Institutional Review Board of Taipei Medical University approved the study (no. 201301020). Plasma and non-leukoreduced PCs were provided by Taipei Blood Center (Guandu, Taiwan) and isolated by Haemonetics apheresis machine (Haemonetics Corp., USA) in the presence of a citrate anticoagulant solution from volunteer healthy donors. Blood cells count was determined using ABC Vet blood cell count (ABX Diagnostics, Montpellier, France). Plasma and platelet concentrates were kept at 22 ± 2 °C until being processed typically within 6 hours or, for some experiments, for up to a maximum of 8 days. A total of 30 PCs were used in these studies.

2.2 Preparation of platelet lysate (PL)

PL was prepared by PC suspended in plasma then subjected to three freeze (-80°C) and thaw (30°C) cycles. The material was centrifuged at 6,500 x g for 30 minutes to collect the supernatant called PL for further experiments and stored at -80°C until used (Figure 11).

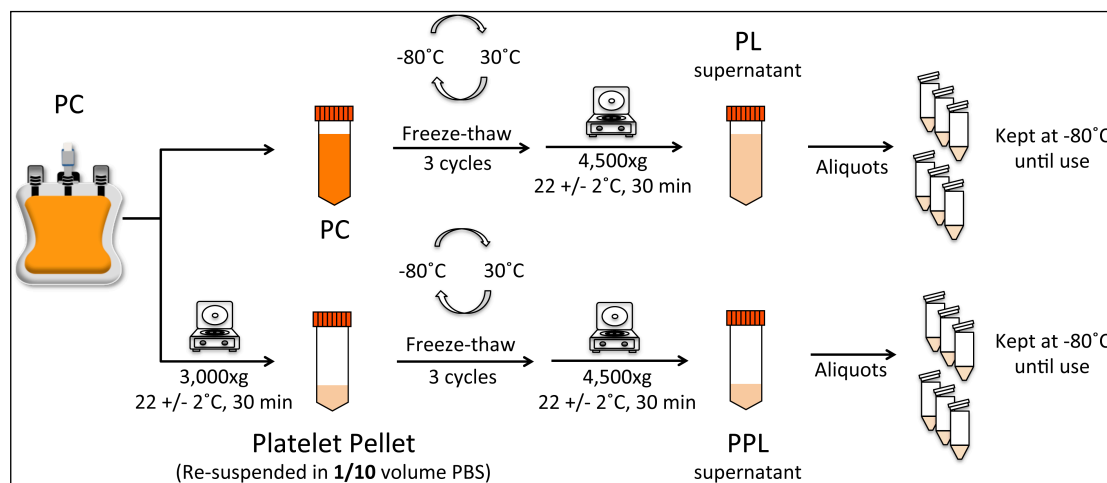


Figure 11 - The preparation of PL and PPL from apheresis PC. PC was subjected to 3 cycles of freeze and thaw steps to prepare PL.

PC was first centrifuged to remove plasma, then subjected to 3 cycles of freeze and thaw steps, and cell debris was removed by centrifugation. All aliquot were stored at -80°C until uses. *Abbreviations:* phosphate buffer saline (PBS); platelet concentrate (PC); platelet lysate (PL); platelet pellet lysate (PPL).

2.3 Preparation of platelet pellet lysate (PPL)

To prepare PPL, PC was centrifuged (3,000 x g, 30 min, 22 ± 2°C) and the platelet pellet collected. The surface of the platelet pellet was gently washed twice by sterile 0.1 µm filtered PBS (GE Healthcare, United Kingdom). PBS (10% of the initial PC volume) was used to resuspend the platelet pellet, then the mixture was subjected to three freeze (-80°C) and thaw (30°C) cycles. Centrifuge (4,500 x g, 30 min, 22 ± 2°C) was then performed for clarification. PPL was kept frozen at -80°C until use. In some experiments, the PPLs were heat-treated in a dry bath (Basic Life, Taiwan) at 37 (control), 45, 56, 65 ± 1°C for 30 min, and cooled down at least 5 minutes on ice then centrifuged (10,000 x g; 15 min; 4 ± 2°C). The supernatant was then stored frozen at -80°C until use.

2.4 Preparation of PMPs

Platelets were isolated from PC then harvested by 3000 g for 15 minutes. The platelet pellet was resuspended in HEPES-Tyrodé's buffer then subjected to 0.1 U/ml thrombin (Sigma-Aldrich) for 60 minutes at 37°C with gentle agitation to activate platelet and release of MPs. The activation of platelets was stopped by final 20 mM EDTA then centrifuge at 3,200 g for 10 minutes. The supernatant was collected and harvested again by high-speed centrifugation at 20,000 x g for 90 minutes at 18°C. Microparticles pellet was be resuspended in Tyrodé's buffer and stored at -80°C until used. This PMP fraction obtained by activation of platelets by thrombin was called TPMP. The preparation is shown in Figure 12.

Moreover, plasma obtained after centrifugation of PC was collected and subjected to 3,000 x g centrifugation for 15 minutes, followed by high-speed centrifugation of the supernatant at 20,000 x g for 90 minutes at 18°C to pellet PMP that were resuspended in Tyrodé's buffer and stored at -80°C until use. This PMP fraction was called NPMP.

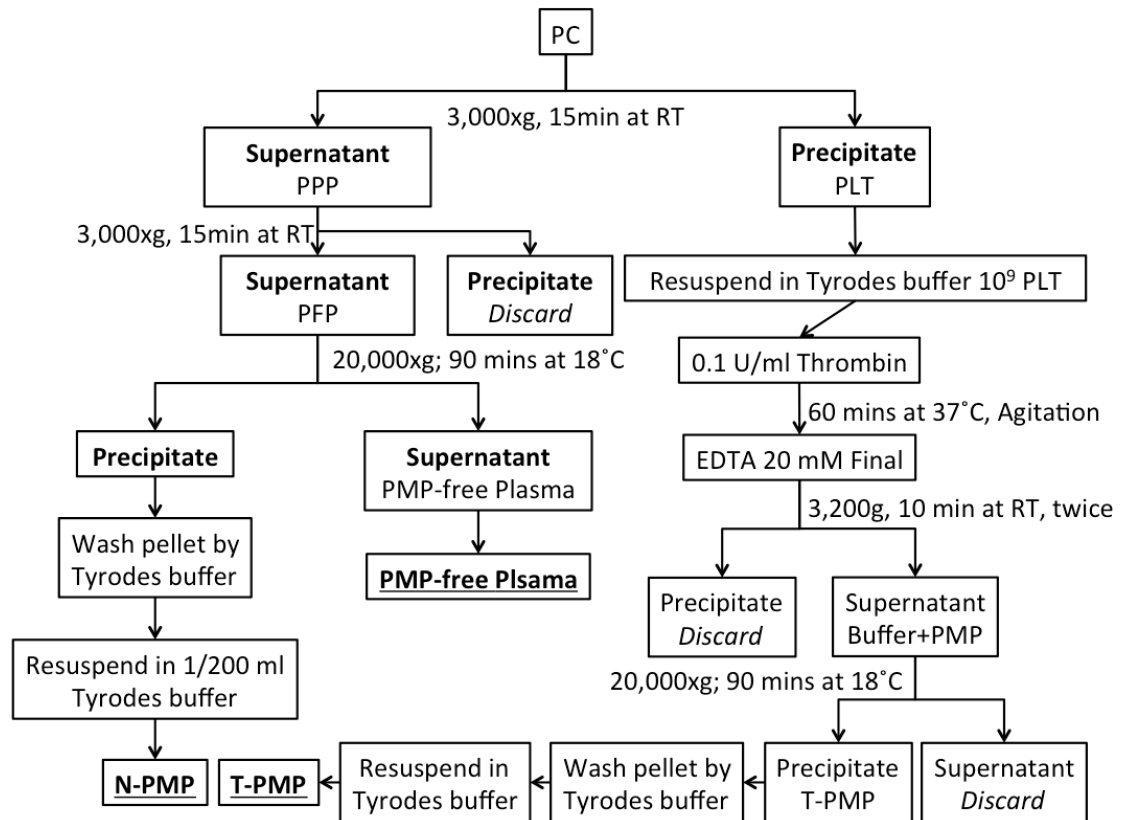


Figure 12 - PMP preparation procedures.

Abbreviations: Platelet concentrate (PC); Platelet (PLT); Platelet poor plasma (PPP); Platelet free plasma (PFP); platelet derived microparticles (PMP); natural-PMP (NPMP); thrombin activated PMP (TPMP); ethylenediaminetetraacetic acid (EDTA).

3 Cell Culture

3.1 Huh-7.5 hepatoma cells culture

Human hepatoma Huh7.5 cell line (obtained from Dr. Charles M. Rice) was chosen to do the HCV infectivity assay. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher, USA) with 10% heat-inactivated FBS (Sigma-Aldrich), 0.5 µg/mL amphotericin B (Sigma-Aldrich) and 50 µg/mL gentamicin (Sigma-Aldrich) in a 5% CO₂ at 37°C humidified atmosphere. After Huh-7.5 cells reached sub-confluence (1×10^4 cells/well in 96-well plate), they were treated with plasma samples. Cell viability was analyzed by XTT assay (Sigma-Aldrich) 72 hours later, as described below.

3.2 LUHMES cells maintenance and differentiation

Undifferentiated LUHMES cells (obtained from Dr. Scholz's laboratory, University of Konstanz, Germany) were seeded at 3×10^6 cells in T75 flask in a 5% CO₂ at 37°C humidified atmosphere and cultured in 50 µg/mL poly-L-ornithine (Sigma-Aldrich) and 1 µg/mL fibronectin (Sigma-Aldrich) pre-coated flask with Advanced DMEM/F12 medium (ThermoFisher) containing N2 supplement (ThermoFisher), 2 mM L-glutamine (ThermoFisher) and 40 ng/mL recombinant bFGF (R&D Systems, USA). To trigger differentiation into mature dopaminergic neuron, 2×10^6 cells were seeded in T75 flask in proliferation medium for 24 hours, then changed to medium containing N2 supplement, 2 mM L-glutamine and 1 mM dibutyryl cAMP (Sigma-Aldrich), 1 µg/mL tetracycline (Sigma-Aldrich) and 2 ng/mL recombinant human GDNF (R&D Systems). LUHMES were cultured in differentiation conditions for 2 days then cultured to 24-well plate for neuroprotection experiment at day five. On day 5 of differentiation, LUHMES cells were subjected to various concentrations or treatments by PPL, and after one hour intoxicated using neurotoxin 30 µM MPP⁺ (Sigma-Aldrich). Cell viability was analyzed by MTT assay (ThermoFisher) 48 hours after MPP⁺ administration, as described below.

3.3 BV2 microglial cell culture

The BV2 cell line was a gift from Dr. Wan-Wan Lin (National Taiwan University, Taipei, Taiwan). It was generated by immortalization using c-myc retroviral vector from primary murine microglial. The cells were cultured at a density of 4×10^5 - 1×10^6 cells/ml in a 5% CO₂ at 37°C humidified atmosphere and maintained in high glucose DMEM medium (ThermoFisher) containing 10% heat-inactivated FBS, 100 U/ml of penicillin (ThermoFisher), 100 µg/ml of streptomycin (ThermoFisher) and 2 mM of L-glutamine. PPL subjected to various treatments was added to BV2 cells (3×10^5 cells/well in 24-well plate) and cells were incubated for one hour then subjected or not to 150 ng/ml of lipopolysaccharide (LPS) (Sigma-Aldrich) for an additional 24 hours. Cell viability was analyzed by MTT assay 24 hours later, as described below.

4 Cell viability

4.1 MTT viability assay

The MTT assay was used to determine cell viability by assessing cell metabolic activity. The assay is based on NAD(P)H-dependent cellular oxidoreductase enzymes in viable cells that are capable of reducing the MTT tetrazolium dye to insoluble purple formazan crystals. Briefly, 0.5 mg/ml of MTT reagent was added to each well and incubated to 2 hours at 37°C. After removing the culture medium from the wells, DMSO (Sigma-Aldrich) was used to solubilize the formazan crystal as described by the supplier. The absorbance was then measured at 570 nm (calibrated by a background value 690 nm) and the results were presented as % viability compared to untreated control condition (100%).

4.2 XTT cytotoxicity assay

In some experiments, cell viability was also measured by XTT assay. XTT is a colorless compound that can be reduced to brightly orange when it is conjugated with mitochondrial intermediate electron acceptor, 1-methoxy phenazine methosulfate (PMS), and then formed water-soluble formazan. It can be quantified at 450 nm.

5 HCV preparation, viral inactivation and viral infectivity assays

5.1 Cell culture-derived HCV particles (HCVcc) preparation

Gaussia luciferase reporter-tagged Jc1FLAG2 (p7-nsGluc2A) construct (HCV genotype 2a) (kindly provided by Dr. Charles M. Rice) was used to generate HCVcc in hepatoma cells by electroporation, as described previously.²⁴⁵ TCID₅₀ method²⁴⁶ was used to determine the HCV viral titer by analyzing NS5A antibodies (1:25,000; gift from Dr. Charles M. Rice) immunofluorescence staining as described. 2% FBS containing medium was used for all virus infectious experiments.

5.2 Viral infectivity assay

Huh7.5 cells were seeded (1×10^4 cells/well in 96-well plate) for 3 hours at 37°C in the presence of plasma samples in serial dilutions in 1) HCVcc-spiked plasma (multiplicity of infection, MOI) = 0.01, 2) HCV plasma subjected to 1% (v/v; final concentration) of Tri-*n*-butyl phosphate (TnBP) (Merck KGaA, Germany) solvent and 1% (v/v; final concentration) Triton X-45 (Sigma-Aldrich) detergent (S/D), and then followed by C18 filtration using Sep-Pak® Plus C18 cartridge 130 mg sorbent per cartridge, 55-105 µm particle size (Waters Corporation, USA). The excess virus was removed by two PBS washes then 2% FBS containing medium was added to the cells that were then incubated for 72 hours. The culture medium was collected to detect the luciferase activity by BioLux™ *Gaussia* Luciferase Assay Kit (New England Biolabs, Canada) and analyzed by a luminometer (Promega, USA). HCV viral inhibition (%) and infectivity were expressed as log₁₀ of relative light units (RLU). All the values were compared to the control.

5.3 Viral inactivation assay using low dose S/D

HCVcc was incubated with low doses (0.01 or 0.05%) of S/D for 30 minutes at 31°C. The virus inoculum was then diluted with medium 50 times to reach a non-cytotoxic S/D concentration ($\leq 0.001\%$) and added to Huh-7.5 cells. After 3 hours of inoculation, cells were washed by PBS then replaced by fresh medium and incubated for another 72 hours incubation, as described before. Infectivity assay was performed by luciferase signal.

5.4 Immunofluorescence staining for viral protein

Immunofluorescence staining of Huh-7.5 cell in the presence of viral particles were fixed by ice-cold methanol then blocked with 3% BSA (Sigma-Aldrich). Cells were treated with 25,000 fold-dilution of mouse monoclonal anti-NS5A 9E10 antibodies for 1 hour at 37°C then washed with PBS three times. 400 fold-dilution of Alexa Fluor 488 goat anti-mouse IgG (H + L) antibody (ThermoFisher) was then added to the cells and the mixture was incubated for 1 hour at 37°C. Three times PBS washes were performed, then the cells were stained with Hoechst dye (Sigma-Aldrich) for nuclei identification. The observation was done using fluorescence microscopy and micrographs were randomly taken from three fields per sample.

5.5 HCV binding analysis using clinical isolates

A surface binding analysis, described previously,²⁴⁷ was used with some modifications to determine whether S/D treatment subjected to C18 filtration has the capacity to inactivate clinical HCV isolates. Briefly, clinical HCV viruses were isolated from hepatitis C carrier patients (viral load > 1 x 10⁶ IU/ml; HAV, HBV, and HIV markers present negative) and were provided by Prof Liang Tzung Lin (Taipei Medical University). Obtaining HCV-positive blood samples from patients at Chi-Mei Medical Center Biobank was approved by the respective Institutional Review Board. Samples were treated by S/D and C18 filtration as described before and incubated with Huh-7.5 monolayer cells. PBS was used to wash out unbound virus particles and viral RNA from cell bound virus was collected. Trizol kit (ThermoFisher) was then used to extract total cellular RNA that was quantified by COBAS AMPLICOR™ HCV MONITOR test (Roche Molecular Diagnostics, USA).

5.6 Primary human hepatocytes infected by HCVcc

The method to assess HCVcc infectivity in primary human hepatocytes (ThermoFisher) was described previously,²⁴⁸ and used with some modifications. Cells were seeded in pre-coated plates with collagen-I and grown in William's medium E (ThermoFisher) with primary hepatocytes supplements (ThermoFisher). After two days, cells were washed by PBS and incubated with plasma samples (MOI = 0.1) for 3 hours at 37°C. Excess virus was washed by PBS twice and replaced by fresh culture medium. After 5 days, the supernatant was collected and analyzed for expression of luciferase activity.

5.7 HCV inactivation steps in PPL samples

Various PPL samples were spiked with luciferase-tagged HCVcc and then subjected or not to the different viral inactivation or removal steps:

- (A) PPL was spiked with HCVcc then heated at 56°C for 30 min,
- (B) HPPL was spiked with HCVcc then inactivated by 1% TnBP and 1% Triton X-100 (S/D) for 1 hour at 37°C, followed by C18 column (S/D-C18) filtration,
- (C) S/D-C18-HPPL was spiked with HCVcc, then filtered through 0.001 m² of Planova 20N nanofilter (20 nm pore size; Asahi Kasei, Japan) at a flow rate of 0.1 ± 0.1 mL/min at 22 ± 0.5 °C and a transmembrane pressure less than 1 bar following the manufacturer's recommendation.

For all experiments, Huh-7 cell monolayers (2 x 10⁴ cells/well in 48-well plate) were infected by the different spiked samples at a final MOI of 0.03 for 2 h. Following washes and incubation for at least 6 days, luciferase signal in the supernatant was analyzed and HCVcc infectivity expressed as log₁₀ RLU.

6 MPs removal procedure

6.1 Preparation of PMPs and spiking

PMPs were spiked to 0.1 μm filtered PBS then 75 nm filter nanofiltration was performed using the same conditions as for plasma samples. Fractions eluted from the nanofilter were collected in 5 fractions of 4 mL each (flow through (FT) 1 to FT5). They were collected and kept for further analysis.

6.2 Plasma Leukoreduction and nanofiltration

150 mL apheresis plasmas were freshly prepared then processed for nanofiltration. 50 mL of plasma was kept as starting plasma sample, and 100 mL of plasma was run through Sepacell RZ-1000N or RLX-5 leukoreduction filters (Asahi Kasei Medical, Tokyo, Japan) by gravity filtration at a flow rate at 7-8 mL/min at 22 ± 2 °C. 50 mL of leukoreduced plasma was then subjected to 0.001 or 0.01 m^2 Planova 75N nanofilter (75 nm pore size; Asahi Kasei, Japan) at a flow rate of 0.5 ± 0.1 mL/min at 35 ± 0.5 °C and a transmembrane pressure less than 1 bar following the manufacturer's recommendation. PMPs-spiked-PBS was also subjected to 0.001 m^2 75 nm nanofiltration at a constant flow rate, as previously described. Pressure was continuously recorded during the duration of the nanofiltration.

7 Assessment of PMPs by biophysical methods

Four biophysical methods were used to assess PMP characteristics as described below.

7.1 Flow Cytometry (FCM)

FCM was performed using FACSCanto II (BD Biosciences) equipped with FACSDiva software (BD Biosciences). 30 µl of plasma thawed at 35°C was stained with 10 µl Annexin V-fluorescein isothiocyanate (FITC) (BD Biosciences, USA) and 10 µl CD41a-allophycocyanin (APC) (BD Biosciences, USA) for 30 minutes at room temperature. FCM analysis was performed immediately after staining. Fluorescent-calibrated beads of 3000, 900, and 500 nm (Megamix beads, Biocytex, France) were used to define the gating areas corresponding to MPs. PMPs were detected based on double-positive events for Annexin V-FITC and CD41a-APC.

The percent reduction in MPs was quantified by calculating the total number of detectable particles and double-positive events (Annexin V and CD41a) in plasma after nanofiltration.

7.2 Dynamic light scattering (DLS)

DLS is a method allowing to determine the differential size distribution profile of MPs populations by temporal fluctuations in scattered light intensity.²⁴⁹

Plasma samples were diluted 100 fold (starting plasma and leukoreduction plasma) using 0.2 µm-filtered sodium citrate (Sigma-Aldrich), or without dilution (nanofiltered plasma). DLS analysis was performed using Zetasizer Nano ZS (Malvern Instruments, UK) at 25°C in triplicates. Particle size (nm) distribution graphs were processed by Cumulant analysis Zetasizer software.

7.3 Nanoparticle tracking analysis (NTA)

NTA is a technique designed to visualize and analyze individual particles size by the rate of Brownian motion.

Plasma samples were diluted 100 fold in 0.1 μm filtered saline solution (Thermo Scientific, USA) and, after mixing, 300 μl of the solution was analyzed by Nanosight NS300 system (Malvern Instruments, UK). The scattered light and Brownian movement was recorded in the field of frame over 60 seconds to determine the particle size distribution and concentration, then analyzed by NTA software. The particle size (nm) was calculated by their movement velocity and applying the Stokes-Einstein equation.

7.4 Tunable resistive pulse sensing (TRPS)

TRPS is a technique to measure single particles passing through a size-tunable nanopore one at a time. qNano (Izon, New Zealand) is a TRPS instrument that determines the number of individual MPs and their diameter while they pass through the nanopore under electric field or pressure.

Samples were diluted 2-fold (plasma and PMP-spiked PBS samples) in 0.1- μm -filtered PBS (Hyclone) or without dilution (nanofiltered plasma and nanofiltered spiked PBS samples) and analyzed by TRPS. The nanopore elastomeric membranes that were used had pore sizes of 100, 400, or 800 nm, depending upon the samples tested to avoid pore clogging.^{250,251} Calibrators were used with standard particles size of 100 nm (CPC100), 400 nm (CPC400) or 800 nm (CPC800) (Izon).

8 Assessment of PMPs by functional methods

PMPs were also assessed using four functional methods, as detailed below. Furthermore, Isolated PMPs were used to do spiking experiments to understand the extent of PMP log removal achieved by 75nm nanofiltration and they added to PPL-deficient plasma to study the impact of the coagulation time.

8.1 Thrombin generation assay (TGA)

TGA is an assay designed to determine thrombin generation over time, as triggered by the presence of phospholipids micelles containing recombinant human tissue factor and CaCl_2 in Tris-Hepes-NaCl buffer. 40 μl of plasma samples were incubated with 10 μl of low (RC-low) or high (RC-high) concentrations of phospholipids (note: the concentration of phospholipid is not disclosed by the manufacturer) and 50 μl of TGA substrate. The mixtures were read immediately and continuously for 60 minutes at 1 minute-intervals, at 37°C. The generation of a fluorescence signal by thrombin cleavage of a fluorogenic substrate over time as the coagulation cascade is activated and measured by Thermo Varioskan Flash (Thermo). Thrombin generation was expressed using three parameters as indicated by the supplier (the peak height, the time to reach the peak, and the lag time) and as processed by the TECHNOTHROMBIN® TGA (Technoclone, Austria) evaluation software. This allowed to express the total quantity of thrombin generated and the time to reach this maximum level.

8.2 Phosphatidylserine (PS) microparticle (MP) procoagulant activity assay

Because MPs express PS on their surface, they possess a procoagulant activity in a plasma medium that can be measured by the prothrombinase Zymuphen MP-activity assay (Hyphen BioMed, France).

Plasma samples (100 μ l) were incubated with calcium, factor Xa and thrombin inhibitors and subjected to the pre-coated Annexin V coupled with streptavidine and biotinylated plate as recommended by the supplier. After 5 times washes, factor Xa-Va, calcium and purified prothrombin were introduced. When phospholipid expressed by MPs is exposed to Annexin V, the generated thrombin reacted with a chromogenic substrate that was measured at 405 nm. The results are presented as nanomolar PS equivalents.

8.3 Tissue factor (TF) microparticle (MP) procoagulant activity assay

TF-based MPs functional activities were analyzed by a Zymuphen MP-TF assay (Hyphen BioMed).

Briefly, plasma samples (20 μ l) were incubated overnight with 200 μ l of MP-TF assay enhancer solution with a pre-coated murine monoclonal antibody specific for human TF overnight at RT. After 5 washing steps, the last wash solution was kept. An additional 25 μ l of coagulation factor VIIa and 25 μ l of factor X was added to generate factor Xa that reacted with a specific substrate producing a yellow color that could be measured through its absorbance at 405 nm.

8.4 STA-procoag-PPL-coagulant assay

The STA-procoag-PPL-coagulant assay was done on a STA Compact automatic coagulometer (Diagnostica Stago, France).

Briefly, 500 µl of plasma samples (starting, leukoreduced and nanofiltered plasma) or PMPs-spiked PBS samples (before or after nanofiltration) added into microparticles-free plasma were analyzed by Stago compact (Stago, France). The measurement was accomplished in the presence of calcium, phospholipids-depleted substrate plasma and factor Xa to analyze the downstream phase of the coagulation cascade. A shortening clotting time indicates an increase in procoagulant phospholipids.

9 Biochemical analysis

9.1 Biochemical analysis and hemostatic function of protein samples

Plasma samples were kept at -20 to -80°C no longer than one month. For analysis, samples were thawed (30°C) and analyzed within two hours.

Total protein (TP), albumin, immunoglobulin G (IgG), IgA, IgM, triglycerides (TG), cholesterol (TC), apolipoprotein A1 (ApoA1), and B (ApoB) were analyzed by ADVIA 1800 chemistry system (Siemens, Germany). Prothrombin time (PT), activated partial thromboplastin (aPTT), international normalized ratio (INR), clottable fibrinogen (von Clauss method), and d-dimers were determined by Sysmex CA-1500 system (Siemens). Thrombin time (TT), FVIII and FIX coagulant activity, and Von Willebrand factor ristocetin cofactor (VWF:Rco) activity by Sysmex CA-7000 (Siemens). The protein zone electrophoresis and lipoprotein analysis were performed using SPIFE 3000 (Helena, USA). All the functional assays were performed at Taipei Medical University Hospital or Union Clinic Laboratory (Taipei, Taiwan) under their routine validated procedures. All the results were compared to the normal range for these plasma proteins.

9.2 Cell lysis and total protein determination

BV2 cells were cultured under different conditions and after 24 hours of intoxication, cells were collected and lysed by 50 µl of RIPA buffer (Sigma-Aldrich) containing protease inhibitors. Samples were homogenized by several strong vortexing steps (15 seconds, 5 minutes intervals for a total of 30 minutes) at 4°C, followed by 12,000 x g centrifugation for 20 minutes at 4°C to collect the protein extract (supernatant). Total protein was determined by Bio-Rad coomassie assay (Pierce, USA), using BSA as a standard.

9.3 Animal tissues homogenization and lysis

Animal brain tissues were isolated and stored in Tris-sucrose buffer (10 mM Tris-HCl, 320 mM Sucrose and pH 7.4) at -80°C. After freezing the tissues in liquid nitrogen for 10 seconds, tetragonal zirconia polycrystal (TZP) beads were added to the tubes, then tissues were homogenized at 1,800 rpm for 10 seconds for three times. Homogenized samples were lysed by RIPA buffer for GF expression by ELISA test or tyrosine hydroxylase (TH) protein expression by Western blot.

9.4 SDS-PAGE

30 µg of protein samples (plasma, PPL, cell lysate, and homogenized animal tissues) were mixed with 4x sample buffer (T-Pro Biotechnology, Taiwan) and heated at 95 °C for 5 min, then immediately put on ice for 5 min. SDS-PAGE was performed under non-reducing and reducing conditions at 70 V for 30 min then 120 V for 1.5 h to separate proteins by their molecular mass.

9.5 Two-dimensional electrophoresis

For two-dimensional gel electrophoresis, PPL samples were first desalted using 2-D Clean-up kit (GE Healthcare). A quantity of 200 µg protein was diluted in 125 µl rehydration buffer (8M Urea, 4% CHAPS and 0.5% IPG buffer) and loaded onto 7 cm strip and rehydrated immobilized dry strip gel at 30 volts for 12 to 14 hours. Ettan IPGphor 3 isoelectrofocusing (IEF) (GE Healthcare) was performed at a pH 3-10 gradient and set for the following IEF programs of 150 Voltage-hours (Vhs) (step and hold mode), 250 Vhs (step and hold mode), 500 Vhr (gradient mode), 10000 Vhs (gradient mode), and 2500 Vhs (step and hold mode). The strips were then equilibrated in the equilibration buffer (6 M Urea, 2% SDS, 1.5 M Tris-HCl pH 8.8, 0.01% Bromophenol blue and 30 % Glycerol) containing 50 mg of DL-dithiothreitol (DTT) for 15 minutes then 125 mg of idoacetamide (IAA) in equilibration buffer for 15 minutes. Afterwards the strip was loaded for SDS-PAGE using 4-12% polyacrylamide gradient gel and run for 3 hours at 20 mA. Strip gel was then subjected to protein detection using Protein Gel Fast Stain Solution staining (Strong Biotech Corporation, Taipei, Taiwan).

9.6 Western blots

Western blot analysis was conducted to detect fibrinogen, COX-2, iNOS and TH protein expression. Briefly, samples were mixed with 4x sample buffer and heated to 95°C for 5 min. Proteins were separated by SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) (Pall, USA) membranes. The membranes were blocked with 5% skim milk and sequentially incubated with fibrinogen antibody (GeneTex, California, USA), iNOS antibody (1:500) (Santa Cruz, Calif, USA), COX-2 antibody (1:500) (Genetex, Irvine, California, USA), or β -actin antibody (1:3,000) (Proteintech Group, Inc, USA). Horseradish peroxidase-conjugated secondary antibody (1:5,000) were used and followed by enhanced chemiluminescence (ECL) detection (GE Healthcare). The immune complexes were visualized with the UVP system (Upland, USA) and bands were quantified by BioSpectrum 610 UVP imaging system (Upland). Data of specific protein levels are presented as relative multiples in relation to the control.

9.7 Growth factor and cytokine content by ELISA and cytokine array

Growth factors were determined in triplicates using Quantikine ELISA kits (R&D Systems) following supplier's instructions, as previously described.^{125,166,252} PPL samples were diluted 500-fold (for both 37°C and 56°C-treated samples) for PDGF-AB (R&D Systems) determination; 500- and 50-fold (37°C and 56°C, respectively) for BDNF (R&D Systems); 10-fold and without dilution (37°C and 56°C, respectively) for bFGF (R&D Systems); 5-fold (both 37°C and 56°C) for VEGF (R&D Systems); 100-fold (both 37°C and 56°C, respectively) for EGF (R&D Systems); without dilution (both 37°C and 56°C) for HGF (R&D Systems); 400-fold (both 37°C and 56°C) for TGF-β (R&D Systems); and 1x10⁶-fold for PF4 (R&D Systems). Dilution factors were 200-fold, 100-fold, 1-fold, 2-fold, 50-fold, 2-fold, 100-fold, and 1x10⁵-fold, respectively, for PL. For TGF-β1 determination, 40 µl samples were acidified at 20 µl 1N HCl for 10 minute then neutralized by 20 µl of 1.2 N NaOH/0.5 M HEPES.²⁵² 30 and 50 µg of brain tissue-lyzed samples were loaded into wells to detect PDGF-AB and PF4 by ELISA, respectively. Human XL cytokine array was used to detect the relative content of 102 cytokines/growth factors in duplicates in 150 µg PPL, heat-treated or not at 56°C or 65°C, following manufacturer's instructions (R&D Systems). Signal intensities were quantified using Imagine J software.

9.8 Measurement of nitric oxide (NO) production

Nitric oxide production was used to determine BV2 cells inflammatory status. After 24 hours of treatment with 2% PPL, with or without 150 ng/mL LPS, the culture medium of BV2 was collected and centrifuged at 300 x g for 5 minutes at 4°C. 100 µl of Griess solution (Sigma-Aldrich) was added to 100 µl of supernatant, and the mixture was incubated for 20 minutes prior to determining the quantity of NO produced. The NO content was determined at 540 nm and compared to that obtained using untreated cells (control).

10 Animal experiment

Male C57BL/6 mice of 6–8 weeks of age were purchased from *National Laboratory Animal Center (NLAC)* (Taiwan), housed in the Center for animal facility (Taipei Medical University, Taipei, Taiwan), and provided food and water ad libitum. All experiments were carried out in accordance with Institutional Animal Care and Use Committee or Panel (IACUC/IACUP) (IACUC Approval No. LAC-2014-0088).

10.1 HPPL Intranasal (i.n.) diffusion in mice model

A total of 6 mice were used and divided into a control group (3) and a HPPL group (3) treatment. The, non-anesthetized, mice received 48 μ L HPPL or PBS intranasal (i.n.) administered (3 μ L per nostril for 8 times, 6-minutes intervals for 60 min) using a micropipette, and holding the mice in an upright position. Mice were perfused intracardially with Ringer's solution (154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl_2 , 2.4 mM NaHCO_3 , pH 7.3) following the last i.n. administration. Whole brains were dissected into the following areas: olfactory bulb (OB), striatum (ST), cortex (Cx), hippocampus (Hp). All samples were stored in a Tris-sucrose Buffer (pH 7.4) containing protease inhibitors at -80°C . Prior to homogenization, the samples were put into liquid nitrogen for 10 seconds and then homogenized with TZP beads at 1,800 rpm for 10 seconds for 3 times, and then RIPA buffer (Sigma-Aldrich) was added and the mixture vortexed 15 seconds every 5 min, 6 times. The suspension was centrifuged at 13,800 $\times g$ for 20 min, the debris were removed, and the supernatant was used (a) to determine the total protein content using the Bio-Rad protein assay and (b) to perform ELISA analyses.

10.2 Parkinson Diseases animal model treatment procedures

In our study, a total of 30 mice (8 week-old male C57BL/6 mice) was used. They were divided into:

- six groups (for Western blot) of 3 mice, each, comprising control and PPL, HPPL, MPTP, MPTP/PPL and MPTP/HPPL treatment groups,
- or four groups (for IHC) of 5 mice, each, comprising control, MPTP, HPPL and MPTP/HPPL treatments.

Mice were intoxicated with 30 mg/kg 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) (Sigma-Aldrich) or received saline by intraperitoneal injection for 5 continuous days.

2 days prior to intoxication, mice started to receive 48 μ l (3 μ l per nostril for 8 times) of PBS or PPL or HPPL by intranasal (i.n) delivery, 3 times per week for 2 weeks. On week 3, mice received PBS or PPL or HPPL again one day before sacrifice (Figure 13).

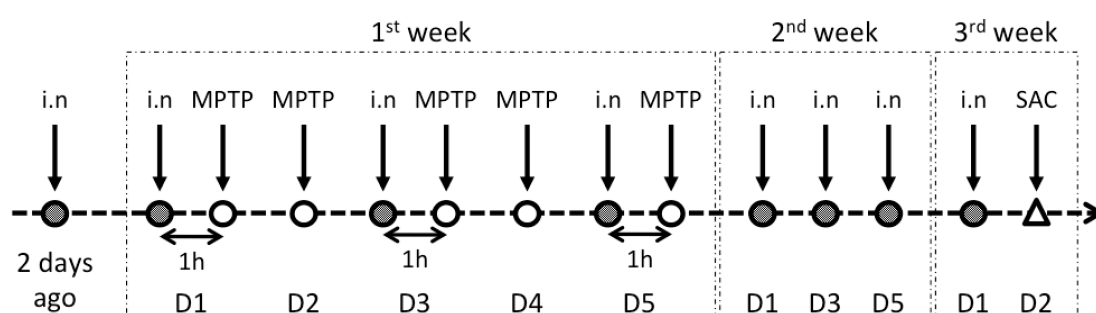


Figure 13 - MPTP mice experimental schedule. C57B6/L mice were intoxicated with MPTP for 5 days.

PPL or HPPL intranasal administration was performed 8 times before, during and after MPTP intoxication. *Abbreviations:* intranasal administration (i.n.); sacrifice (SAC).

10.3 Mice brain tissue preparation for Western blot analysis

Mice were perfused intracardially with Ringer's solution. Brain were dissected immediately then isolated striatum for TH western blot analysis. All samples were stored in a Tris-sucrose Buffer (pH 7.4) containing protease inhibitors at -80°C. Homogenized and lysis procedure were done as described before.

10.4 Mice brain tissue preparation for IHC staining

Mice were perfused intracardially with 4% paraformaldehyde (PFA) in PBS and the whole brain dissected immediately, then stored for additional 3 hours in PFA. Brain tissues were transferred to 30% sucrose overnight, then frozen coronal sections with 20 μ m thickness were cut and stained in buffer. Cryo-sections were incubate with primary tyrosine hydroxylase (TH) (Genetex, USA) antibodies followed by biotinylated secondary antibodies, avidin-biotin-peroxidase complex (Vectastain Elite) and 3,3'-diaminobenzidine (DAB). Multiple slices through the brain were observed by optical microscopy (ZEISS AXioskop2, Germany) coupled to a CCD (Optronics, USA). Images were analyzed by HistoQuest analyzing software (TissueGnostics, Austria)

11 Statistical analysis

Our results are reported as mean $\pm$ standard error (SEM) or $\pm$ standard deviation (SD) from 3 independent experiments unless otherwise indicated. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. A *P* value of < 0.05 was considered as statistically significant.

RESULTS

1 Plasma for transfusion treatment by Solvent/Detergent to inactivate HCV

1.1 Experimental design of HCV viral inactivation studies

HCV virally inactivation studies were performed using either Huh-7.5 hepatoma cell line or primary human hepatocyte cells. HCVcc stocks were spiked into apheresis plasma samples at a 1 to 9 ratio in order not to substantially modified the biochemical composition of the test material, following the international recommendations and regulatory guidelines.²⁵³ HCVcc inactivation steps were then achieved by adding 1% (v/v; final concentration) of TnBP solvent and 1% (v/v; final concentration) Triton X-45 (Sigma-Aldrich) detergent (S/D), followed by strong vortexing for 5 sec and under mild mixing conditions at 30°C ± 2°C for 30 minutes. The S/D reaction was stopped by octadecyl carbon chain (C18) treatment that is known to eliminate the S/D agents.²³ So, 300 µl of S/D treated plasma samples were chromatographed by filtration through a 130 mg C18 cartridge. All the samples from each step were collected and evaluated by viral infectivity assay. The flowchart of the experiment is shown in Figure 14.

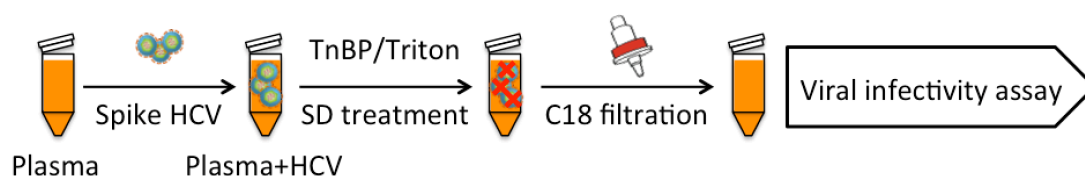


Figure 14 - Viral inactivation experimental design

1.2 Elimination of Huh-7.5 cell cytotoxicity in S/D treated plasma subjected to C18 filtration

We first confirmed that plasma treated by S/D and subjected to C18 filtration did not exert cell toxicity in the infectivity assay. XTT cell viability assay was performed on Huh-7.5 cells incubated with the starting plasma sample alone, S/D in PBS buffer (to test the toxicity induced by S/D) and S/D treated plasma followed by C18 filtration (Figure 15). The results showed that plasma alone or S/D treated plasma followed by C18 filtration did not cause cell toxicity. By contrast, and as expected, S/D in PBS (final concentration of 1% solvent and 1% detergent) was highly cytotoxic. The C18 filtration step, performed to remove the S/D agents, was shown to be highly efficient. Cytotoxicity due to the S/D agents was eliminated and cell viability was similar to that obtained when using mock conditions. The slight reduction in cell viability when growing Huh-7.5 cells in the presence of C18-filtered S/D plasma, compared to the starting plasma, may reflect the removal of some hydrophobic nutrients or plasma derived growth factors during this viral inactivation procedure.

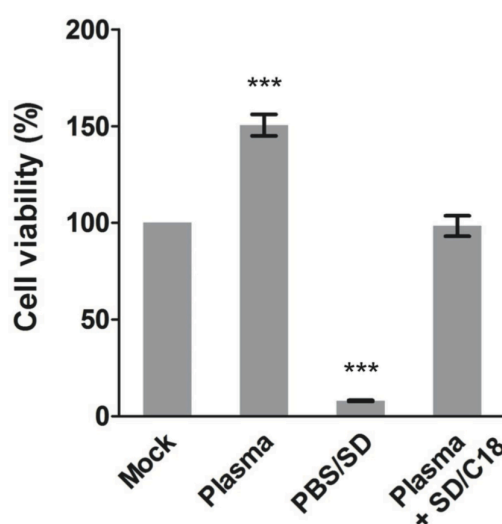


Figure 15 - S/D cytotoxicity on Huh-7.5 cells was eliminated by C18 filtration.

Starting plasma, PBS with S/D and SD/C18 plasma samples were incubated with Huh-7.5 for 72 hours then analyzed by XTT cell viability assay. *** $P < 0.001$ as compared with the Mock.

1.3 A low concentration of S/D is sufficient to inactivate HCVcc infectivity

To further understand the robustness and capacity of the S/D procedure to inactivate HCV in plasma-free condition, HCVcc was treated using much lower concentrations of S/D agents: 0.01% (final concentration of 0.005% TnBP and 0.005% Triton X-45) and 0.05% (final concentration of 0.025% TnBP and 0.025% Triton X-45) under mixing for 30 minutes at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$. HCV infectivity was tested in Huh-7.5 cells incubated with 50-fold dilution by 2% FBS medium of the S/D-HCVcc mixtures (to reach S/D sub cytotoxicity concentration) without C18 treatment. HCVcc infectivity data indicated that using 0.05%, but not 0.01%, of S/D significantly decreased the viral reporter signals (Figure 16). The results indicated that as little as 0.05% of S/D is sufficient to inactivate HCVcc infectivity in plasma-free condition.

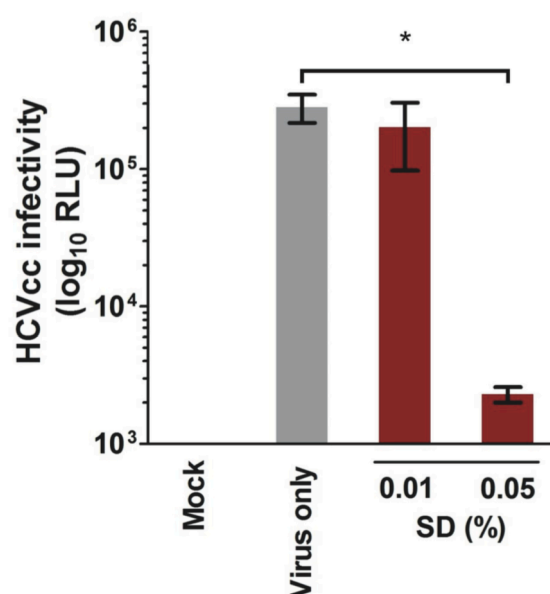


Figure 16 - HCVcc was inactivated by low concentration of S/D in plasma-free condition.

Low dose of S/D (0.01 and 0.05%) were incubated with HCVcc for 3 hours then 50-fold dilutions were performed to reach sub-cytotoxic concentration to treat Huh-7.5 cells. HCVcc infectivity was detected by luciferase reporter activity (RLU). * $P < 0.05$ as compared to the mock data.

1.4 Inactivation of HCVcc infectivity by S/D treatment of plasma sample

To understand the potential to inactivate HCVcc by S/D treatment (1% TnBP and 1% Triton X-45) of plasma, infectivity assays were performed using Huh-7.5 cells challenged by HCVcc spiked plasma that was immediately S/D treated for 30 minutes and subjected to C18 filtration. Nonstructural protein 5A (NS5A), which plays a key role in HCV RNA replication, was used to detect the replication.

The results of NS5A immunostaining showed that the S/D treatment of HCVcc-spiked plasma followed by C18 filtration, eliminated the NS5A signals, which was by contrast strongly detected in the starting HCVcc-spiked plasma only (Figure 17, A). The results confirmed the complete inhibition of HCVcc replication in plasma subjected to S/D treatment. In addition, there were more than 2.5 log HCV removal found in the TCID₅₀ assay (Figure 17, B) and reduced luciferase reporter signals (Figure 17, C) in the HCV-spiked plasma followed by S/D treatment and C18 filtration. Taken together, the data confirmed that the S/D treatment was highly effective at inactivating the infectivity of HCVcc in plasma.

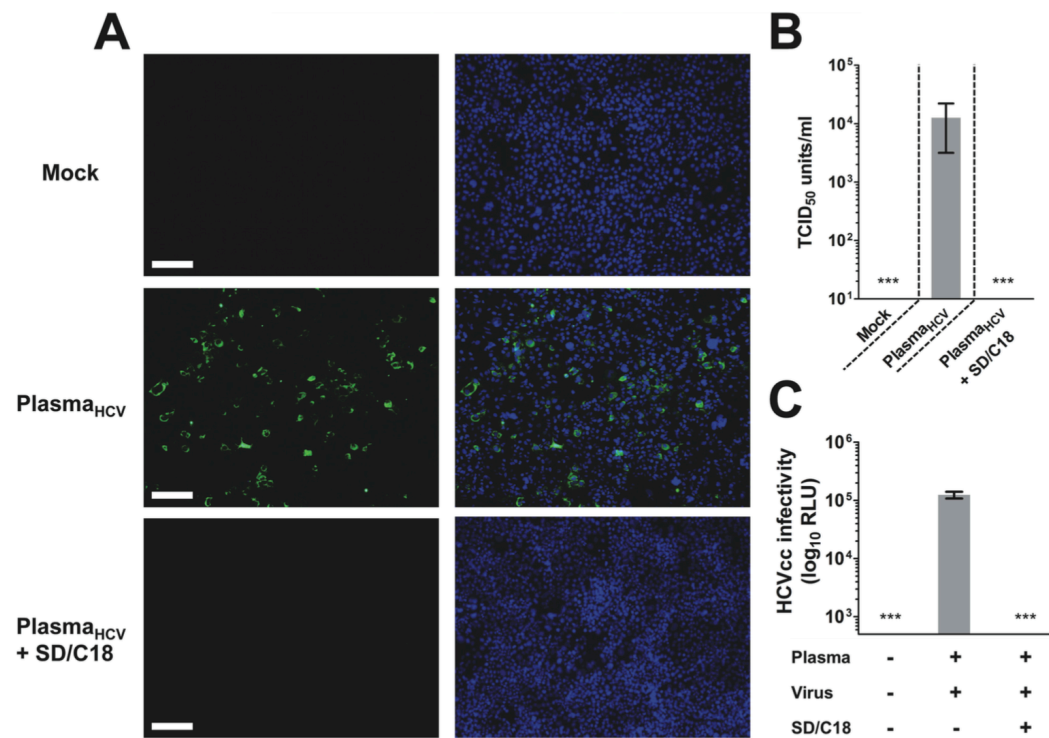


Figure 17 - HCV infectivity was removed of HCV-spiked plasma subjected to S/D treatment and C18 filtration using h-7.5 cells culture model.

Infectivity was determined by (a) viral NS5A immunofluorescence staining (scale bar = 100 μ m) (b) TCID₅₀ (units/mL) analysis and (c) HCV infectivity assay by luciferase reporter measurement *** P <0.001 as compared to the mock data.

1.5 S/D treated plasma followed by C18 filtration does not change the biochemical protein composition and plasma hemostatic ability

To assess whether S/D treatment alters plasma protein composition and functionality,²⁵³ plasma biochemical protein composition and hemostatic functionality were analyzed and compared to the normal physiological range.^{254,255}

The total protein (TP) levels, triglyceride (TG), total cholesterol (TC) and apolipoproteins (Apo) contents remain within the normal range in starting plasma, C18 filtered plasma and S/D-C18 treated plasma (Table 9). S/D-C18 treatment of plasma led to a decrease in lipidic/hydrophobic molecules such as TG, TC and ApoB, without affecting TP content. There is no further change in the S/D-treated plasma composition following C18 filtration. This observation is consistent with the fact that the S/D treatment targets lipids without altering proteins composition.²³ In summary, our results indicate that S/D-C18 treated plasma preserve its overall protein content, especially the TP level, within normal physiological ranges and has a lower content in lipidic components, such as TG, TC and ApoB.

Table 9 - The biochemical composition of plasma, plasma subjected to C18 filtration and S/D treated plasma followed by C18 filtration.

Parameter	Normal Range	Control	C18	SD + C18	P Value
Total Protein (g/dl)	6.0–7.8	6.30 ± 0.11	6.25 ± 0.07	5.80 ± 0.05	NS
Triglyceride (mg/dl)	< 250	96.00 ± 3.78	82.73 ± 11.92	53.33 ± 1.20	NS
Cholesterol (mg/dl)	< 199	139.7 ± 8.7	134.6 ± 15.8	94.3 ± 1.4	NS
ApoA1 (mg/dl)	> 88	104.0 ± 7.9	106.4 ± 7.8	106.7 ± 1.6	NS
ApoB (mg/dl)	< 151	75.00 ± 7.63	78.93 ± 9.17	44.33 ± 0.33	NS

To further ascertain that the plasma hemostatic function is not affected by S/D and C18 treatment, the global coagulation activity including prothrombin time (PT), international normalized ration (INR) and activated partial thromboplastin time (aPTT) were determined. The S/D-C18 treated plasma still exhibited normal *in vitro* hemostatic functions based on normal PT, INR and aPTT parameters (Table 10). Taken together, this scale-down evaluation indicated that S/D-C18 plasma maintain normal biochemical proteins profile and hemostatic function.

Table 10 - Hemostatic function of plasma, plasma subjected to C18 filtration and S/D treated plasma followed by C18 filtration.

Parameter	Normal Range	Control	C18	SD + C18	P Value
PT (sec)	11–13.5	11.37 ± 0.06	11.6 ± 0.20	12.73 ± 0.08	NS
INR	0.8–1.2	1.047 ± 0.006	1.067 ± 0.017	1.170 ± 0.005	NS
APTT (sec)	22–41	33.87 ± 0.68	34.57 ± 1.31	34.93 ± 0.06	NS

Control: starting plasma; C18: plasma subjected to C18 column; SD+C18: plasma treated with S/D then subjected to C18 column. NS = no significant difference ($P > 0.05$)

1.6 Confirmation of the inactivation of clinical HCV isolates by S/D treatment

To further assess the reliability of the S/D viral inactivation process as applied to plasma for transfusion and to other biological samples, experiments were also conducted using various clinical HCV isolates of different genotypes. The virus binding capacity²⁴⁷ was used to test the efficacy of the S/D process for patient-derived HCV isolates. Even though the virus binding capacity assay is not initially designed to examine HCV infectivity, still, it is actually quite valuable to directly determine the capacity of S/D treatment to inactivate clinical HCV isolates as well as to prevent HCV isolates binding onto the Huh-7.5 cells. Clinical HCV particles (genotypes 1b, 2a, and 6) in serum were subjected to 1% TnBP/1% Triton X-45 S/D treatment at 30°C for 30 min, C18 filtration and then subsequent incubation with Huh-7.5 cells. After washing out the unbound viral particles, total RNA of surface-bound HCV was quantified. The results showed that the S/D-C18 process eliminated the signals of clinical HCV RNA completely compared to the controls not subjected to the S/D-C18 procedure (Figure 18).

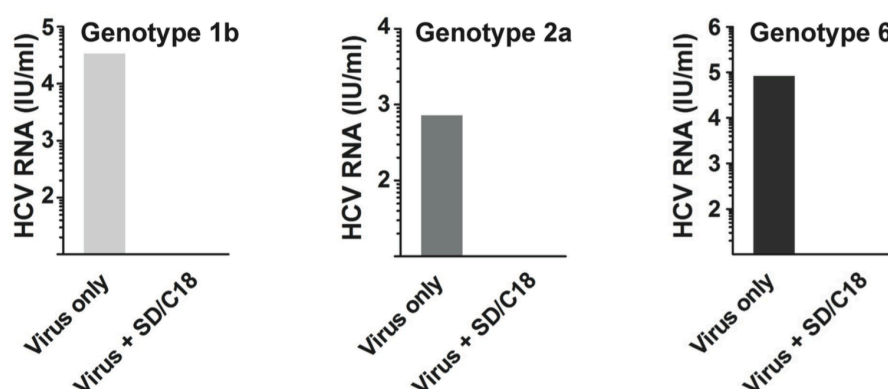


Figure 18 - Inactivation of clinical HCV isolates by S/D treatment and C18 filtration determined by virus binding capacity assay with HCV genotype 1b, 2a and 6 in Huh-7.5 cells.

HCV isolates from patients were treated with SD followed by C18 filtration and incubated with Huh-7.5 cells. Total HCV bound on Huh-7.5 cells were analyzed by COBAS AMPLICOR HCV MONITOR test.

1.7 S/D treatment reduces the HCVcc infectivity in primary human hepatocytes

We next assessed HCVcc infectivity using primary human hepatocytes. Primary hepatocytes were incubated with S/D-C18 treated HCVcc-spiked plasma and the luciferase reporter signals were measured.

As expected, there was no detectable HCVcc infectivity revealed in the primary human hepatoma cells when exposed to HCVcc-spiked plasma subjected to S/D-C18 treatment (Figure 19). These results confirmed the elimination of HCVcc infectivity by S/D-C18 treatment of plasma.

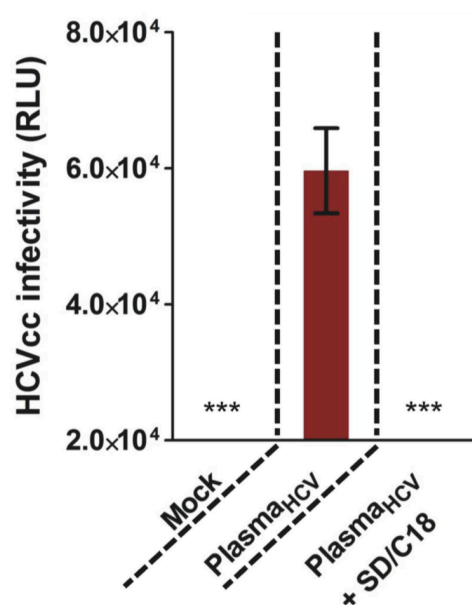


Figure 19 - S/D treatment inactivates HCVcc infection in primary human hepatocytes.

Primary hepatocytes were incubated with HCV-spiked plasma and SD/C18 treated HCV-spiked plasma samples. The supernatants from culture medium were analyzed following 5 days for quantify viral infectivity. (***) $P < 0.001$ as compared with the Mock

In conclusion, these outcomes demonstrate that S/D viral inactivation procedure using 1% TnBP and 1% Triton X-45 at 30°C, followed by C18 filtration, is very efficient to destroy, in a robust and consistent manner, HCV infectivity in plasma for transfusion and other plasma materials such as clinical isolates. These data also suggest that this procedure would likely be efficient in the inactivation of HCV and other lipid-enveloped viruses in other blood-derived protein solutions such as the platelet lysates we developed for regenerative medicine (results are reported in a separate section).

2 Plasma microparticles removal and *in vitro* thrombogenicity decrease by 75-nm nanofiltration

Plasma was used as a model to assess the capacity of nanofiltration to impact content of microparticles and proteins.

2.1 Experimental design of MPs removal studies

The mean blood cells count was first determined in each freshly collected apheresis plasma. RBC, WBC, and platelet number were $< 0.89 \times 10^{12}/L$, $< 0.1 \times 10^9/L$, and $15.7 \times 10^9/L$, respectively.

MPs removal studies were performed using plasma that was kept at $22 \pm 2^\circ C$ for less than 24 hours. Plasma was first subjected to leukoreduction in order to reduce the risk of clogging of the filter by excessive residual cells. We used two types of leukoreduction filters to compare cell removal efficacy and impact on nanofiltration: Sepacell RZ-1000N or the PLX-5 leukoreduction filters. Leukoreduced plasma was then subjected to nanofiltration on Planova 75N hollow fiber device with a nanopore size of 72 ± 4 nm, as illustrated in Figure 20.

It was possible to nanofilter plasma in a consistent way, using a flow-rate of 0.5 mL/min and a temperature of $37^\circ C$ (to decrease plasma viscosity). Nanofilter capacity was over 12 mL and 120 mL using the nanofilters with a surface area of 0.001 m^2 or 0.01 m^2 , respectively. In both cases, the transmembrane pressure remained low (≤ 0.25 bar) far below the maximum limit of 1 bar recommended by the supplier. There were no detectable blood cells after nanofiltration. Plasma samples including starting plasma (P), leukoreduced plasma (L) and nanofiltered plasma (N), were collected at each steps of the procedure to assess plasma global coagulation activity, perform specific protein assays, and determine MP biophysical and functional assays (Figure 20).

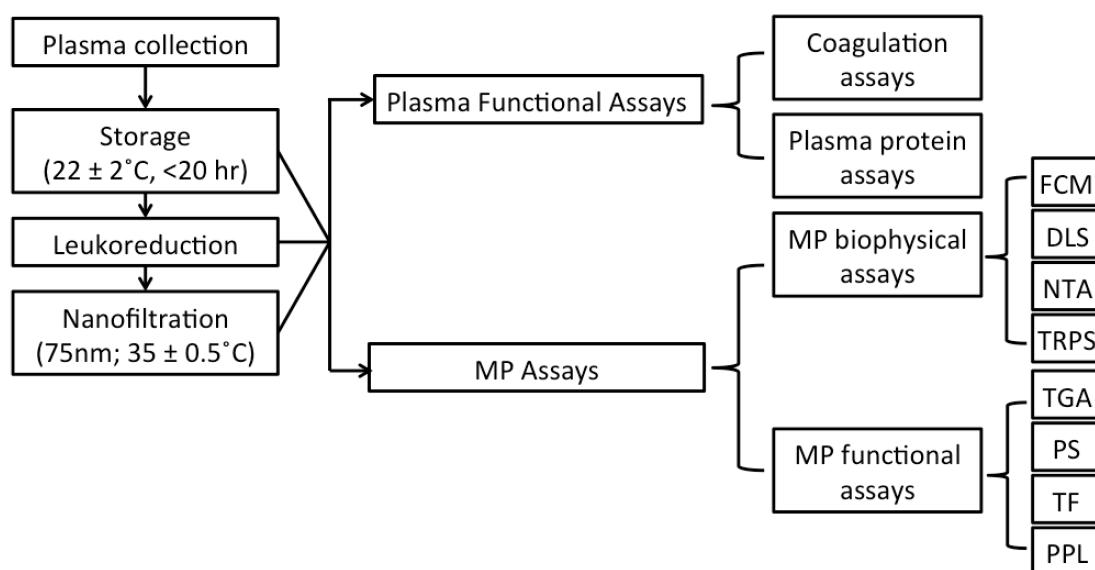


Figure 20 - Experimental design to study MPs removal by nanofiltration.

For detailed explanations, see text. Abbreviations: microparticles (MPs), flow cytometry (FCM), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), thrombin generation assay (TGA), phosphatidylserine (PS), tissue factor (TF), procoagulant phospholipids (PPL).

2.2 There is no significant alteration in abundant protein content, lipoprotein composition and coagulation activity after plasma nanofiltration on Planova 75N

To assess whether nanofiltration affects plasma proteins composition and functionality, plasma biochemical proteins composition and coagulation activity were analyzed.

Zone electrophoresis analysis did not evidence significant changes in albumin (P: 59.6; N: 59.9%), α -1 (P: 2.05; N: 2.3%), α -2 (P: 7.4; N: 7.8%), β (P: 12.4; N: 12.0%), and γ (P: 18.5; N: 18.0%) repartition between starting and nanofiltered plasma. SDS-PAGE patterns (Figure 21) under non-reducing (left) and reducing (right) conditions were equivalent

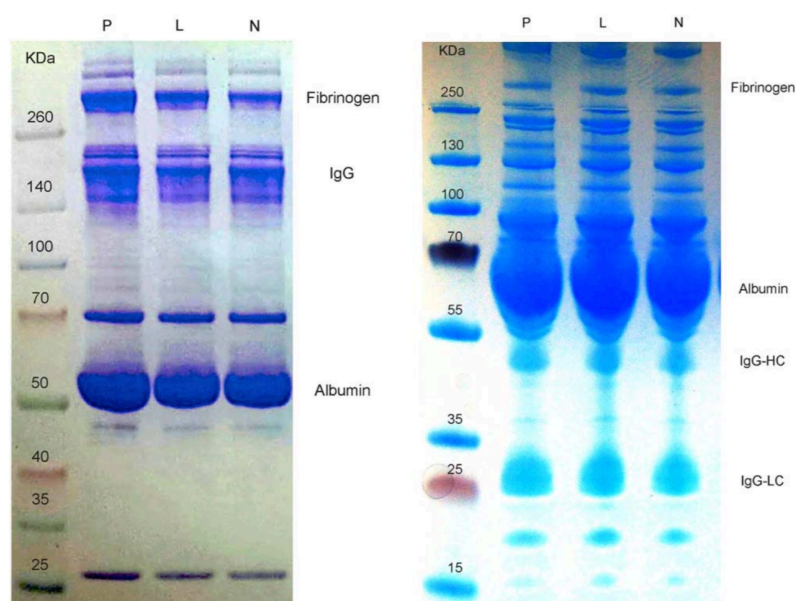


Figure 21 - SDS-PAGE under nonreducing (left) and reducing (right) condition in conditions with starting plasma (P), after leukoreduction (L), and after nanofiltration (N).

Compared with starting plasma, nanofiltered plasma had a recovery of TP, albumin, IgG, IgM and IgA over 90%, and FVIII, FIX, vWF and fibrinogen over 80% recovery; PT, aPTT and TT global coagulation activities did not exhibit significant changes suggesting satisfactory preservation of the hemostatic activity (Table 11). Moreover, there were no detectable d-dimers ($< 0.19 \mu\text{g/mL}$), reflecting a lack of activation of the coagulation cascade.

TG, TC, HDL, LDL, Apo determination and lipoprotein electrophoresis suggest that no significant differences in starting, leukoreduced and nanofiltered plasma (Table 12). In summary, nanofiltered plasma maintains global proteins content and coagulation activity.

Table 11 - Protein and global coagulation assays in starting plasma (P), after leukoreduction (L), and nanofiltration (N)

Step	Protein assays									Global coagulation assays (sec)		
	Total protein (g/L)	Albumin (g/L)	IgG (mg/dL)	IgM (mg/dL)	IgA (mg/dL)	FVIII (IU/mL)	F IX (IU/mL)	VWF:RCO (IU/mL)	Fibrinogen (mg/dL)	PT	aPTT	TT
P	65.0 ± 1.0 (63.0-66.0)	42.3 ± 0.3 (42.0-43.0)	858.7 ± 22.3 (814.0-882.0)	37.3 ± 1.2 (35.0-39.0)	234.3 ± 8.6 (218.0-247.0)	86.3 ± 5.6 (75.2-92.3)	106.0 ± 5.2 (97.1-115.2)	89.7 ± 5.5 (80.0-99.0)	290.2 ± 15.5 (265.8-319.0)	11.6 ± 0.2 (11.3-12.0)	35.4 ± 0.8 (34.5-37.0)	18.7 ± 0.6 (18.0-19.8)
L	64.7 ± 1.3 (62.0-66.0)	42.3 ± 0.7 (41.0-43.0)	850.0 ± 27.8 (795.0-884.0)	37.3 ± 1.2 (35.0-39.0)	231.7 ± 12.4 (207.0-246.0)	84.8 ± 5.2 (74.5-90.4)	108.0 ± 4.0 (100.1-112.4)	93.0 ± 4.4 (86.0-101.0)	281.9 ± 25.7 (242.3-330.0)	11.1 ± 0.1 (10.8-11.3)	33.7 ± 0.1 (33.6-34.0)	18.8 ± 0.5 (17.8-19.6)
N	60.7 ± 1.5 (58.0-63.0)	40.3 ± 1.2 (38.0-42.0)	803.3 ± 17.7 (774.0-835.0)	34.0 ± 1.0 (33.0-36.0)	217.0 ± 6.4 (206.0-228.0)	78.4 ± 4.3 (73.3-87.0)	95.1 ± 1.2 (92.8-96.8)	77.3 ± 0.7 (76.0-78.0)	245.9 ± 37.8 (172.9-299.1)	12.2 ± 0.9 (11.2-14.0)	35.3 ± 1.9 (32.8-39.0)	19.3 ± 0.6 (18.4-20.4)

* Data are expressed as mean ± SEM (range); n = 4.

aPTT = activated partial thromboplastin time; PT = prothrombin time; TT = thrombin time; VWF:RCO = von Willebrand factor ristocetin cofactor.

Table 12 - Content and ratio of triglyceride (TG), total cholesterol (TC) and lipoprotein in conditions with starting plasma (P), after leukoreduction (L), and nanofiltration (N)

Step	Triglyceride (mg/dL)	Cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	CHO/HDL ratio	ApoB/ApoA1 ratio	ApoA1 (mg/dL)	ApoB (mg/dL)	Lipoprotein electrophoresis			
									α-Lipoprotein (%)	Pre-β (%)	β-Lipoprotein (%)	Chylomicron (%)
P	125.0 ± 26.9 (72.0-159.0)	154.3 ± 6.7 (142.0-165.0)	33.7 ± 0.7 (33.0-35.0)	97.7 ± 2.9 (93.0-103.0)	4.6 ± 0.2 (4.3-5.0)	0.77 ± 0.03 (0.70-0.80)	109.0 ± 1.2 (107.0-111.0)	84 ± 1.5 (82.0-87.0)	32.6 ± 3.0 (27.1-37.3)	12.3 ± 0.6 (11.6-13.4)	51.9 ± 3.0 (47.7-57.8)	3.2 ± 1.3 (0.8-5.4)
L	125.3 ± 24.8 (76.0-155.0)	153.7 ± 4.1 (146.0-160.0)	34.7 ± 1.3 (32.0-36.0)	97.7 ± 2.9 (93.0-103.0)	4.5 ± 0.3 (4.1-5.0)	0.77 ± 0.03 (0.70-0.80)	110.3 ± 1.9 (108.0-114.0)	84.7 ± 1.2 (83.0-87.0)	31.0 ± 3.9 (24.0-37.3)	11.9 ± 0.9 (10.5-13.5)	53.7 ± 4.0 (49.0-61.6)	3.3 ± 0.7 (1.9-4.1)
N	86.0 ± 13.9 (63.0-111.0)	132.0 ± 4.7 (123.0-139.0)	31.0 ± 1.0 (30.0-33.0)	82.0 ± 3.5 (75.0-86.0)	4.3 ± 0.2 (4.1-4.6)	0.73 ± 0.03 (0.70-0.80)	102.0 ± 2.3 (98.0-106.0)	75.0 ± 3.1 (69.0-79.0)	33.0 ± 2.7 (29.3-38.2)	12.2 ± 1.1 (9.9-13.4)	52.2 ± 2.5 (47.5-55.9)	2.6 ± 1.3 (1.1-5.2)

* Data are expressed as mean ± SEM (range); n = 4.

CHO = cholesterol.

2.3 MPs removal by 75-nm nanofiltration determined by biophysical analysis

FCM revealed more than 80% reduction of particles after nanofiltration compared to the starting plasma based on the mean number expressed by total particles (P: 622273 to N: 34977), Annexin V staining particles (P: 54350 to N: 2387) and CD41a staining particles (P: 47186 to N: 2037). Since we noticed that these rare events were also found in control PBS and fluorescent antibodies solution, their detection in nanofiltered plasma was attributed to a background signal.

By DLS analysis (Figure 22), the starting (P) and leukoreduced plasma (L) contained a similar distribution of MPs with mean sizes between 9 to 1,000 nm and residual events of around 7,000 nm. On the contrary, the mean sizes of particles in nanofiltered plasma (N) was 9-100 nm.

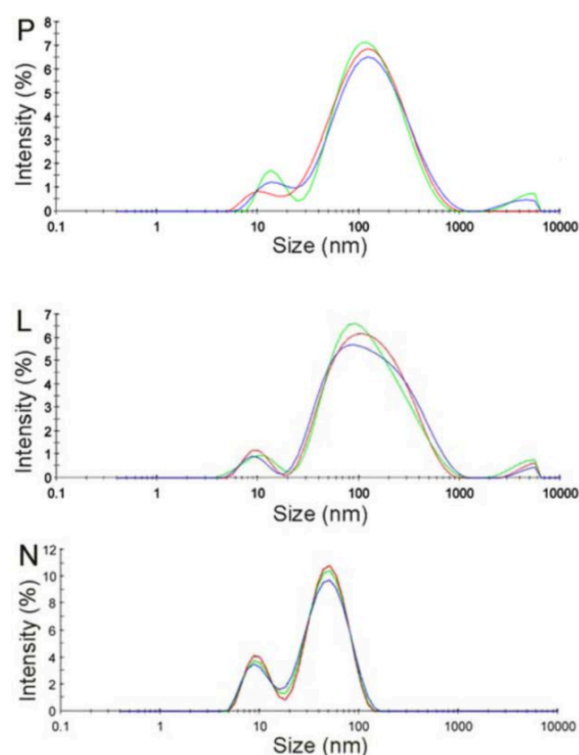


Figure 22 - DLS biophysical assessment of MPs.

The respective percentage of light scattering intensity exhibit MPs population. The results show triplicate determination. Starting plasma (P); leukoreduced plasma (L); nanofiltered plasma (N).

NTA size distribution (Figure 23) showed that 30% of MPs in the starting plasma (P) and leukoreduced plasma (L) were above 100 nm, with a range from 10 to 290 nm. Major MP peaks in the starting plasma were at 36, 58, and 98 nm and at 37, 45 and 100 nm in leukoreduced plasma. By contrast only 10% of MPs in nanofiltered plasma (N) contained MPs above 100 nm, and the major peak was at 29 nm.

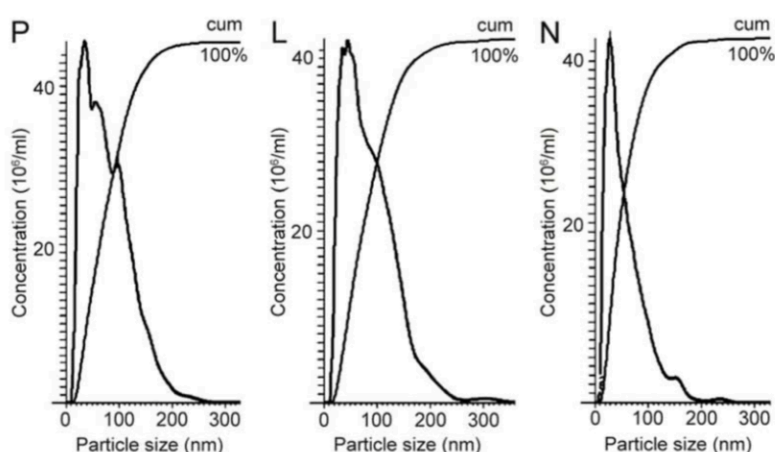


Figure 23 - NTA biophysical assessment of MPs.

MPs were analyzed by the movement velocity. Plasma samples were diluted 100-fold. Starting plasma (P); leukoreduced plasma (L); nanofiltered plasma (N). The cum line indicates the progressive accumulation of detected events.

TRPS data (Figure 24), showed numerous MPs in both starting plasma (P) and leukoreduced plasma (L) when detected by a membrane with a 800 nm nanopore (which can detect particles in the 400-1,600 nm range). In starting and leukoreduced plasma, 800 nm nanopore membrane cannot be used to detect the particles less than 400 nm as their size is too small (as indicated by the supplier). In addition, the 400 nm nanopore membrane could not be used due to clogging by big size MPs present in these two plasma types. In contrast, there were no detectable MPs when 100 nm of nanopore membrane (operating range is 50 to 200 nm) was used to assess nanofiltered plasma (N) suggesting that particles above 50 nm were removed.

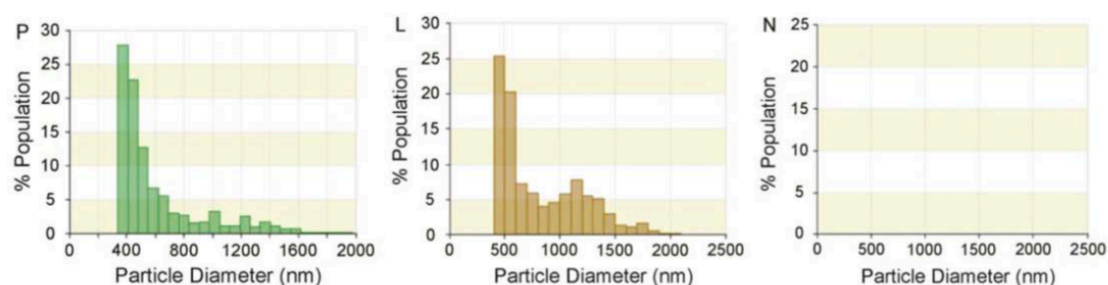


Figure 24 - TRPS biophysical assessment of MPs.

Starting and leukoreduced plasma samples were applied to 800 nm pore size membrane and nanofiltered plasma was subjected to 100 nm pore size membrane. Starting plasma (P); leukoreduced plasma (L); nanofiltered plasma (N).

Moreover, we further performed PMPs spiking experiments to confirm the removal capacity of 75-nm nanofiltration (Figure 25). PMPs prepared from PC were present as monodisperse particles with a mean and median size of 101 nm and 89.6 nm, respectively, and with a diameter range around 27-303 nm when observed by TEM (Figure 26). PMPs- spiked PBS (9.25×10^9 PMPs/mL) subjected to 75 nm nanofiltration showed no detectable particles when evaluated by TPRS using the 100 nm nanopore membrane, indicating a more than 9 log PMP removal capacity by Planova 75N.

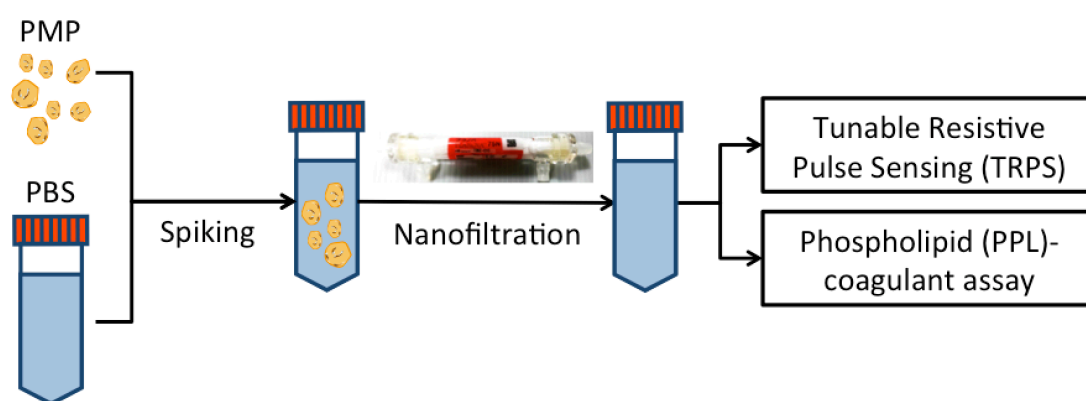


Figure 25 - Experimental design of PMPs spiking for nanofiltration.

PMP were added to PBS then run through 75-nm Planova. The filtrate was then analyzed by TRPS and PPL-coagulant assay.

The TEM observation (Figure 26) showed that the monodisperse vesicles with heterogeneous size in around 27 to 303 nm and presented a mean size at 101 nm and a median size at 89.6 nm.

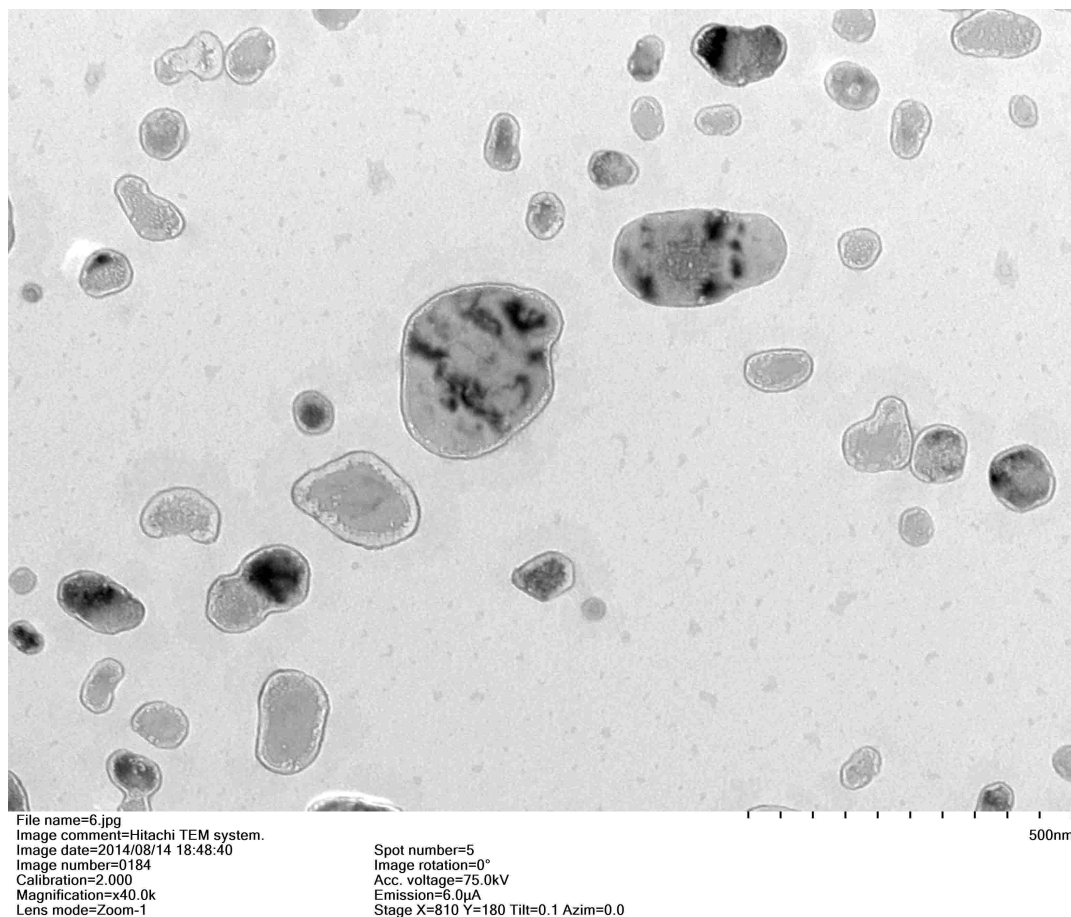


Figure 26 - Isolated PMP observed by TEM.

PMPs have heterogeneous population size from approximately 25 to 300 nm.

2.4 A reduction of thrombogenicity in nanofiltered plasma is evidenced by *in vitro* functional methods

In the TGA assay, reagent “RC-low” contains low concentration of phospholipid micelles (and therefore can be used to assess thrombogenicity due to MPs) whereas reagent “RC-high” contains high concentration. In both the starting plasma (P) and leukoreduction plasma (L), thrombin was generated in the presence of “RC-low” reagent, with a mean lag phase, a mean peak thrombin concentration and a time to the peak at around 22 to 25 minutes, 190 nM and 35 minutes, respectively (Table 13). On the contrary, in nanofiltered plasma (N), there was no thrombin generation observed. Interestingly, when “RC-high” reagent was used, all plasma samples triggered thrombin generation and no significant difference was found between each condition.

Table 13 - Thrombin generation assay with RC low and RC high reagents

RC low (A)					
	Lag phase (min)	Thrombin (nmol/L)	Time to peak (min)	Velocity index	AUC
P	24.5 ± 3.8 (18-34)	183.8 ± 31.3 (142.8-275.7)	34.8 ± 5.6 (25.0-45.0)	21.8 ± 6.6 (8.6-39.4)	2238.3 ± 305.0 (1761.0-3126.2)
L	22.3 ± 3.8 (14-30)	191.0 ± 39.0 (106.0-291.5)	34.5 ± 5.8 (24.0-45.0)	18.9 ± 6.0 (6.2-29.2)	2354.1 ± 448.1 (1205.1-3266.0)
N	0.0† (0-0)	0.0† (0-0)	0.0† (0-0)	‡	0.0† (0-0)
RC high (B)					
	Lag phase (min)	Thrombin (nmol/L)	Time to peak (min)	Velocity index	AUC
P	17.8 ± 3.0 (12.0-23.0)	140.3 ± 33.6 (59.6-208.1)	30.0 ± 4.8 (24.0-44.0)	14.8 ± 4.2 (2.8-22.5)	1538.1 ± 353.8 (574.1-2217.3)
L	20.3 ± 0.8 (19.0-22.0)	101.1 ± 13.2 (83.5-140.2)	31.0 ± 4.4 (25.0-44.0)	12.9 ± 4.0 (3.8-23.4)	1182.0 ± 46.5 (1052.7-1258.7)
N	22.8 ± 2.4 (16.0-27.0)	127.1 ± 29.7 (67.2-198.1)	32.3 ± 4.6 (24.0-45.0)	18.0 ± 6.0 (3.7-30.5)	1245.9 ± 281.1 (616.6-1888.4)

* Data are expressed as mean ± SEM (range); n = 4.

† p < 0.001.

‡ In the absence of thrombin formation, the velocity index is not expressed.

AUC = area under the curve.

In the Zymuphen prothrombinase assay (Table 14), the mean value of phosphatidylserine (PS)-bearing MPs in nanofiltered plasma (-0.01 ± 0.01 nM) was significantly lower than in starting plasma (4.77 nM) and leukoreduced plasma (3.64 nM). Moreover, tissue factor (TF)-bearing MPs presents low activity in starting plasma (0.27 ± 0.02 pg/mL) and there was no detectable activity after leukoreduction and nanofiltration.

The procoagulant phospholipid (PPL)-dependent clotting time assay (STA) showed a significant prolongation in coagulation time of nanofiltered plasma (119.7 seconds compared to 27.5 seconds for starting plasma, and 46.6 seconds for leukoreduced plasma) also suggesting substantial removal of MPs containing procoagulant PPL. To further confirm the coagulation activity associated to PPL-expressing PMPs, PMP-spiked PBS was added to PPL-deficient plasma. The results showed that a dramatic prolongation of clotting time of PMP-spiked PBS after nanofiltration (188.4 ± 3.0 ; 182.4-191.5 range) with a value similar as PBS control, but a decrease when PMP-spiked PBS was added (69.1 ± 0.3 ; 68.5-69.4 range). Thus pro-coagulant PMP spiked to PBS can be removed by Planova 75 nm filtration.

Table 14 - PS and TF base MP expression assay and PPL coagulant activity

Sample	(A) PS (nmol/L)	(B) TF (pg/mL)	(C) PPL coagulant assay (sec)
P	4.77 ± 0.27 (4.5 to 5.0)	0.27 ± 0.02 (0.25 to 0.28)	27.5 ± 1.3 (25.9 to 30.0)
L	3.64 ± 0.1 (3.5 to 3.8)	≤ 0.05	46.6 ± 15.9 (28.8 to 78.2)
N	$-0.01 \pm 0.01^*$ (-0.03 to 0.02)	≤ 0.05	$119.7 \pm 2.6^*$ (114.6 to 122.9)

Data are expressed as mean $\pm$ SEM (range); n = 4.

* $p < 0.001$.

Altogether, these data, using a wide range of biophysical and functional methods concur to confirm our initial hypothesis that nanofiltration on Planova 75 nm filter is an efficient approach to remove MPs from plasma, buffer, and probably other fluids, and can be used to decrease the risks of side-effects such as the occurrence of thrombotic events in some biological fluids like blood.

In order to examine the possible functional relevance of PMPs in neurological applications, further experiments were carried out where PMPs were spiked to cerebrospinal fluid (CSF) and analyses were done to observe a possible triggering of thrombin generation. Our results showed that there was no evidence of thrombin generation in the TGA assay.

3 Neuroprotective effect of platelet-derived material – platelet pellet lysates (PPL) against Parkinson's diseases (PD) inducing neurotoxin

3.1 Which questions did we ask ourselves when conducting this study?

To address whether PPL can be applied in neurodegenerative diseases to protect dopaminergic neurons from neurotoxins, we first asked ourselves 5 questions (Figure 27).

- 1) *How PPL differ from PL?* PL, a combination of plasma and releasates from platelets is the type of materials typically used in regenerative medicine. However, we considered that its high protein content could affect the brain physiology especially if used by ICV. In addition to limit infusion volume, a concentrated form would be preferable. We therefore prepared one platelet-derived material depleted of plasma proteins and enriched in growth factors (GFs), which we called “PPL”. A further heat treatment was implemented that resulted in a higher purity product called “HPPL”.
- 2) *Is PPL neuroprotective in vitro and in vivo?* We then tested neuroprotective effect of PPL in LUHMES dopaminergic cell model and MPTP mice model of PD selecting a mode of delivery by i.n. administration.
- 3) *Does PPL induce neuroinflammation?* We tested our PPL in BV2 microglia cell line to know if the inflammatory effect is influenced by PPL.
- 4) *Is PMP neuroprotective in vitro?* We test PMP neuroprotective effect *in vitro* due to the presence of numerous PMPs in both PPL and HPPL.
- 5) *Make PPL virally safe.* In order to apply the PPL preparation process to allogeneic platelet concentrate sources (not only autologous sources) and perform clinical evaluations using PC from volunteer donors, PPL should be subjected to viral inactivation or removal steps. Impact of these treatments on neuroprotective activity should however be confirmed initially *in vitro*.

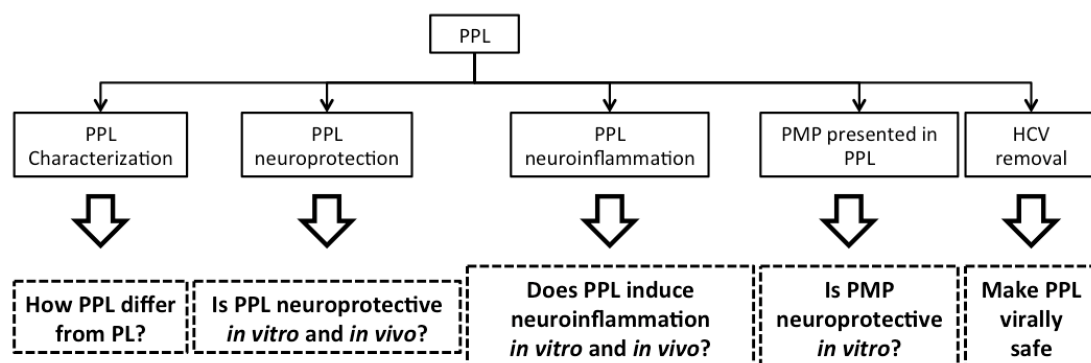


Figure 27 - Questions addressed for the neuroprotective study of PPL?

Abbreviations: platelet pellet lysate (PPL); hepatitis C virus (HCV); platelet lysate (PL); platelet-derived microparticles (PMP).

3.2 Overall experimental design for PPL used in PD model

PPL and heat-treated HPPL were characterized by biochemical analysis to determine the protein composition and examine their potential content in PMP. PPL and HPPL samples were then tested for their neuroprotective effects both *in vitro* and *in vivo* as well as for their possible neuroinflammatory effect.

Viral inactivation steps were introduced, in the hypothesis of using PPL from allogeneic sources for clinical use, to understand their capacity to inactivate or remove HCV as well as the impact on neuroprotection. Moreover, understanding the possible contribution of PMP in PPL and HPPL neuroprotective efficacy can be achieved through removal by nanofiltration experiments as well as by testing the neuroprotective effect of isolated PMPs *in vitro* (Figure 28).

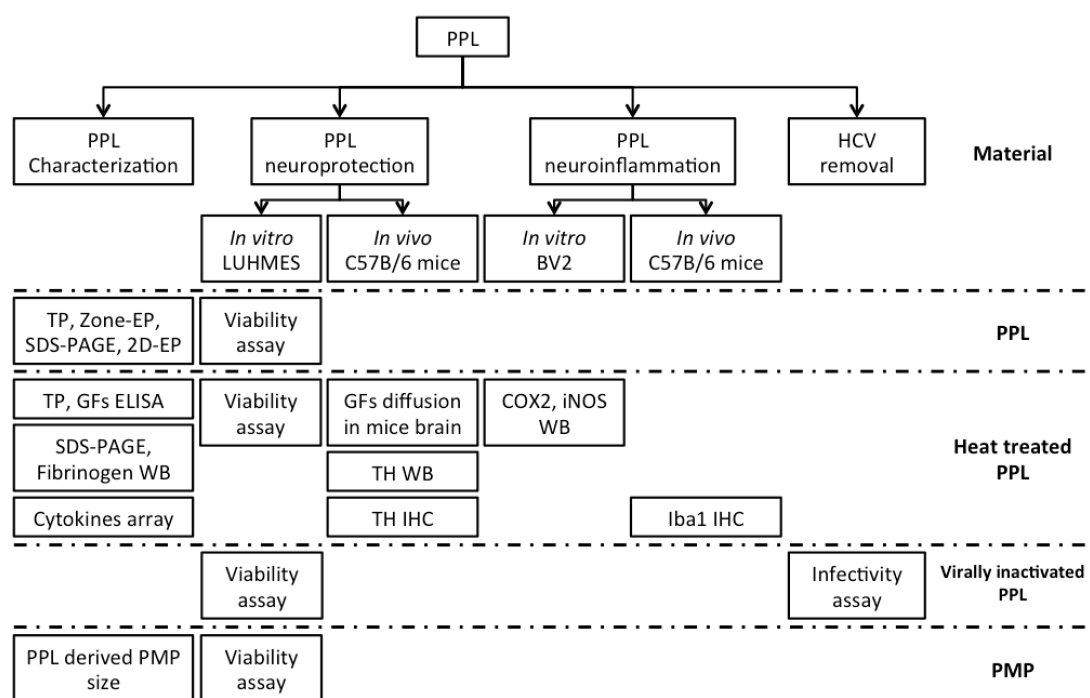


Figure 28 - Experimental design to assess PPL, virally inactivated PPL, and PMP neuroprotective effect.

PPL samples were characterized by biochemical analysis, by testing their neuroprotective effect *in vitro* and *in vivo*, by evaluating the neuroinflammatory impact *in vitro* and *in vivo*, and by testing HCV inactivation/removal capacity of three virus reduction procedures. *Abbreviations*: platelet pellet lysate (PPL); hepatitis C virus (HCV); LUHMES cell line (LUHMES); BV2 cell line (BV2); total protein (TP); electrophoresis (EP); growth factor (GF); western blot (WB); platelet derived microparticles (PMP); tyrosine hydroxylase (TH); immunohistochemistry stain (IHC).

3.3 Blood cell count of starting platelet concentrates (PC) and characterization of platelet pellet lysates (PPL)

Mean blood cell count in the PC used to prepare PL and PPL were $1240 \pm 252 \times 10^9/\text{L}$ of platelets, $0.08 \pm 0.05 \times 10^{12}/\text{L}$ of RBC, and $0.5 \pm 0.2 \times 10^9/\text{L}$ of WBC. Total protein content of PPL (ca. 11 mg/ml) was significant lower than that of PL (ca. 65 mg/ml) (Figure 29, A). In zone electrophoresis, the albumin/gamma (A/G) ratio (0.5 to 1.6) was lower in PL compared to PPL (Figure 29, B), with proteins migrating in the albumin (32.3 to 60.9) and gamma (11.5 to 19.3) regions being less and in alpha 1 (14.1 to 1.1), alpha 2 (18.4 to 6.5) and beta (23.7 to 12.2) regions being higher (Figure 29, C).

The SDS-PAGE pattern of PPL showed major bands corresponding to proteins with a molecular mass of approximately 60, 48 and 15 kDa under non-reducing and 68, 48 and 15 kDa under reducing conditions, with a distribution of proteins based on their molecular mass (Figure 29, D, left part). In addition, two-dimensional (2D) electrophoresis of PPL using isoelectrofocusing (IEF) separation from pH 3 to 10 followed by SDS-PAGE separation evidences the complexity of the platelet proteome (Figure 29, D, right part). Overall, PPL showed unique proteins patterns differing from those of PL.

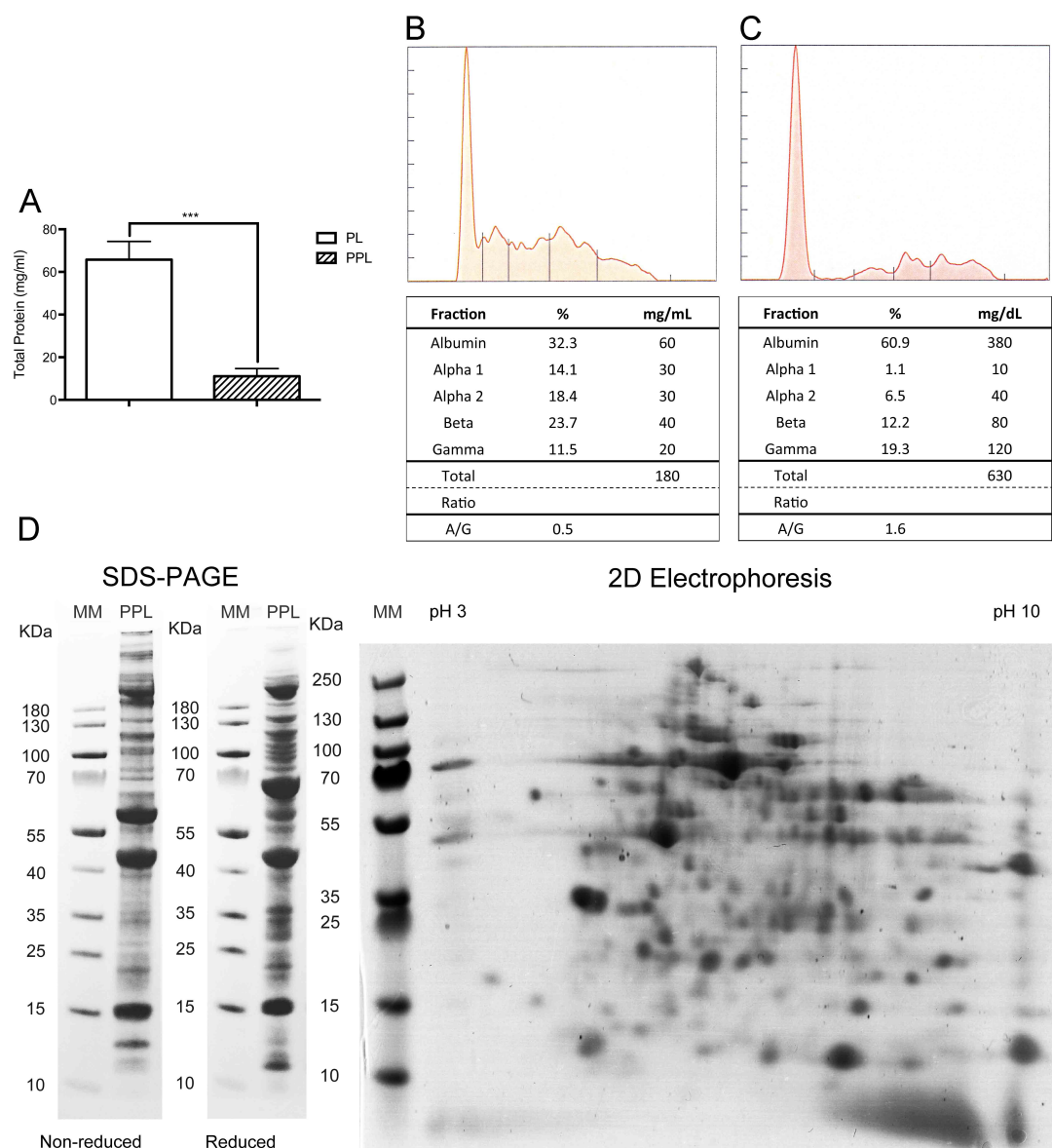


Figure 29 - PL and PPL protein characterization.

A: Total protein content (mg/ml) of PL and PPL. B, C: PL (L) and PPL (R) pattern by zone electrophoresis. D: PPL SDS-PAGE pattern under non-reducing and reduced conditions. E: PPL two-dimensional electrophoresis pattern. *Abbreviations*: platelet concentrate (PC); platelet lysate (PL); platelet pellet lysate (PPL); kiloDaltons (kDa); molecular mass (MM); sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). ** $P < 0.01$ as compared to PL.

3.4 PPL exerts protection in LUHMES cells against neurotoxin MPP⁺ when prior added

We first verified whether PPL may exert potential toxicity on LUHMES cells. 0.1% to 5% PPL, added to the cell cultures, did not induce any significant toxicity (Figure 30, A). By contrast MPP⁺ neurotoxin had a significant toxic effect on LUHMES. When the cells were then treated with MPP⁺ neurotoxin 1 hr after PPL pre-treatment, a significant protection was observed by 2% of PPLs prepared from PC which were stored under mild mixing for several days (one (0), 3, 6 and 7-10 (> 6) days) at controlled RT (20-24°C) after collection (Figure 30, B). Additionally, a dose-response effect was shown on cells treated with different PPL dosage (0.025 to 15%) (Figure 30, C). Maximum protection of PPL against the neurotoxin effect was found at a concentration of 2%. Altogether, PPL was found to exert a significant neuroprotective effect in the LUHMES cell model against the MPP⁺ neurotoxin.

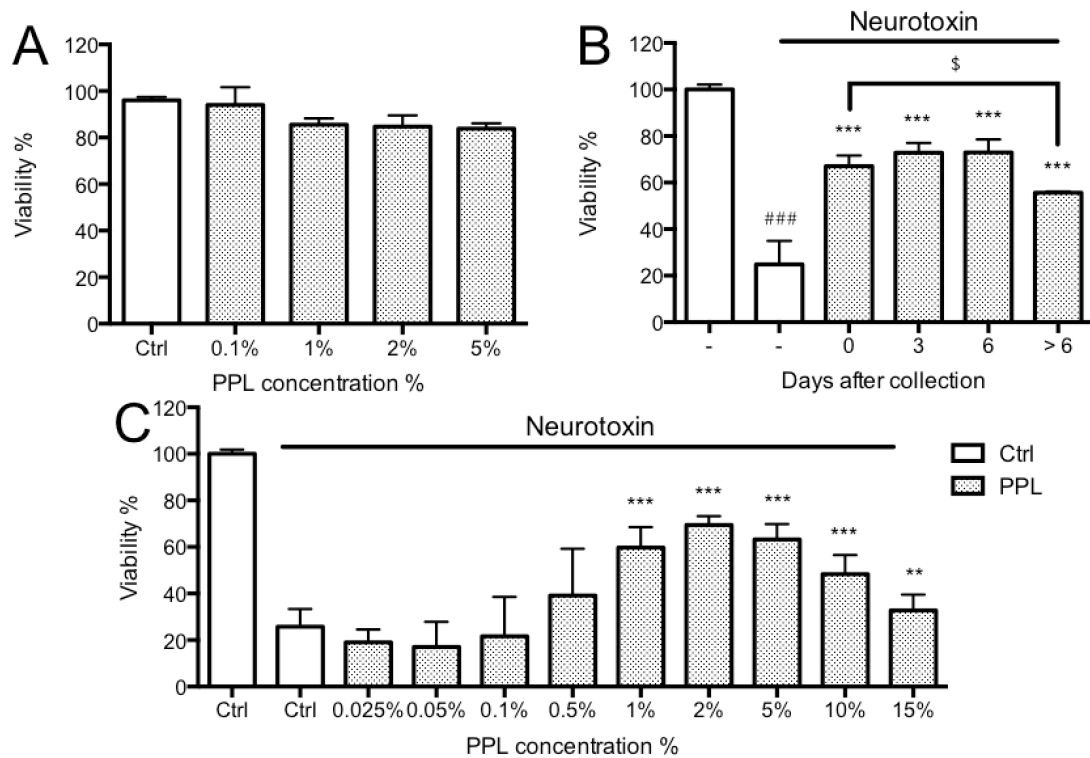


Figure 30 - PPL was not toxic and exerted protective effects against the MPP⁺ neurotoxin in a LUHMES cell model.

Data show the viability of LUHMES cells under different conditions A: non-heat-treated PPL (PPL treated at 37°C) at different concentration (0.1 to 5%) without MPP⁺. B: 2% PPL prepared from PC stored for 0, 3, 6, and >6 days, and subjected to MPP⁺. C: non heat-treated PPL (PPL-37°C) at different concentrations (0.025-15%) and subjected to MPP⁺. The LUHMES viability in all conditions was expressed as percent of the control group not exposed to MPP⁺ (100%). *Abbreviations:* control (Ctrl); platelet pellet lysate (PPL). ###*P*<0.001 as compared with the control. ***P*<0.01, ****P*<0.001 as compared to the control (w MPP⁺), \$*P*<0.05 as compared to the PPL prepared from PC at 0 days after collection.

3.5 Heat treatment of PPL improves the neuroprotective

We then compared the neuroprotective activity of 0.5-15% of not-heat treated PPL (Figure 31, A), and of PPL heat-treated at 45°C (Figure 31, B), 56°C (Figure 31, C) and 65°C (Figure 31, D). A highly significant protective activity was found even in the low concentration range of 0.5% of all heat-treated (45°C, 56°C and 65°C) PPLs. The comparative protective effects using various doses of non heat-treated or heat treated PPL is shown in Figure 31, E.

This indicated that the neuroprotective efficacy remained similar when subjecting PPL to heat-treatment at the highest temperatures (56°C and 65°C). On the contrary, non-heated PPL and PPL only heated at 45°C exhibited a dose response effect with no protection at the lower dosage (0.5%), and inhibition or toxicity at the higher dosages (10-15%), potentially due to protein overload.

PPL treated at 56°C for 30 min (HPPL) was selected for most of further experiments of neuroprotection.

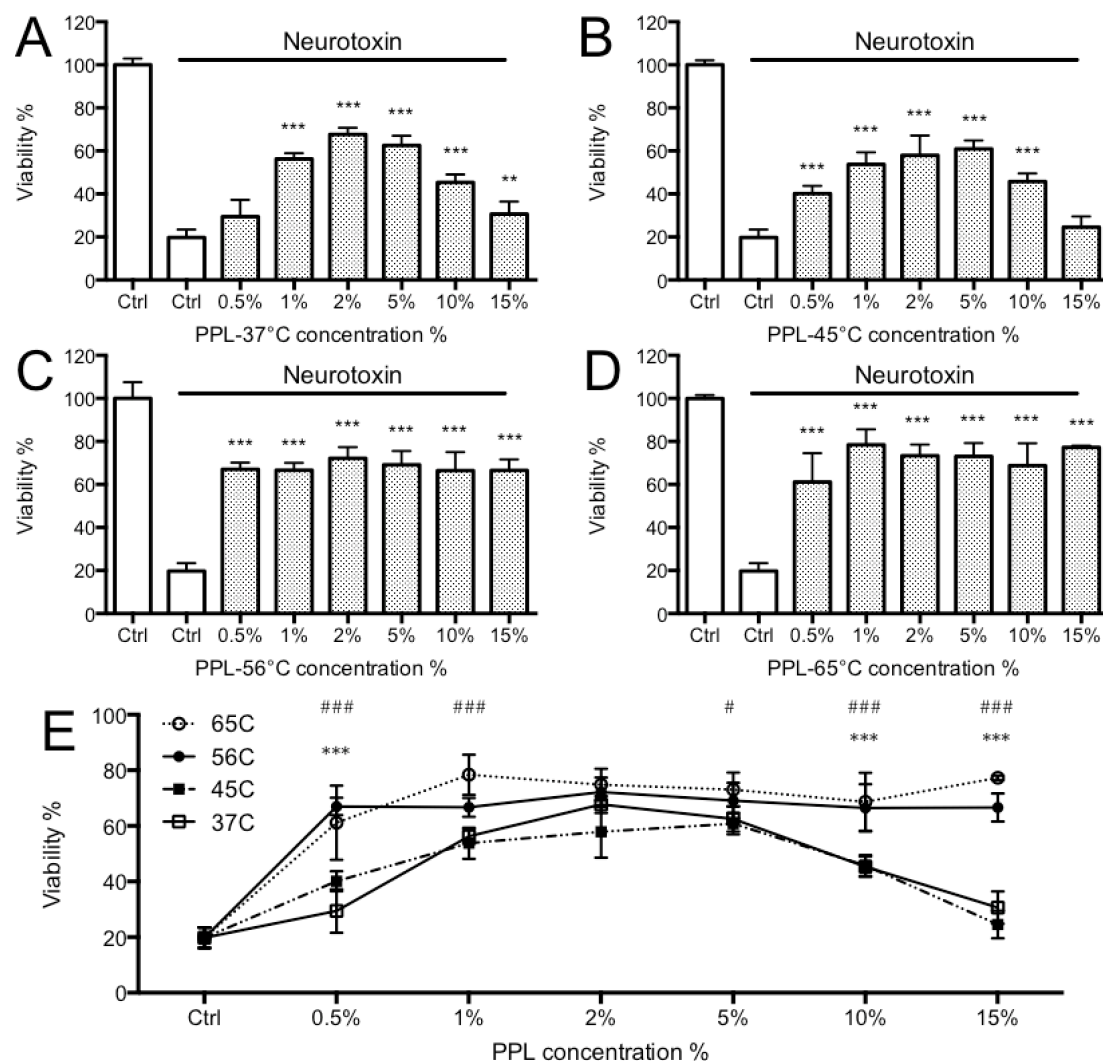
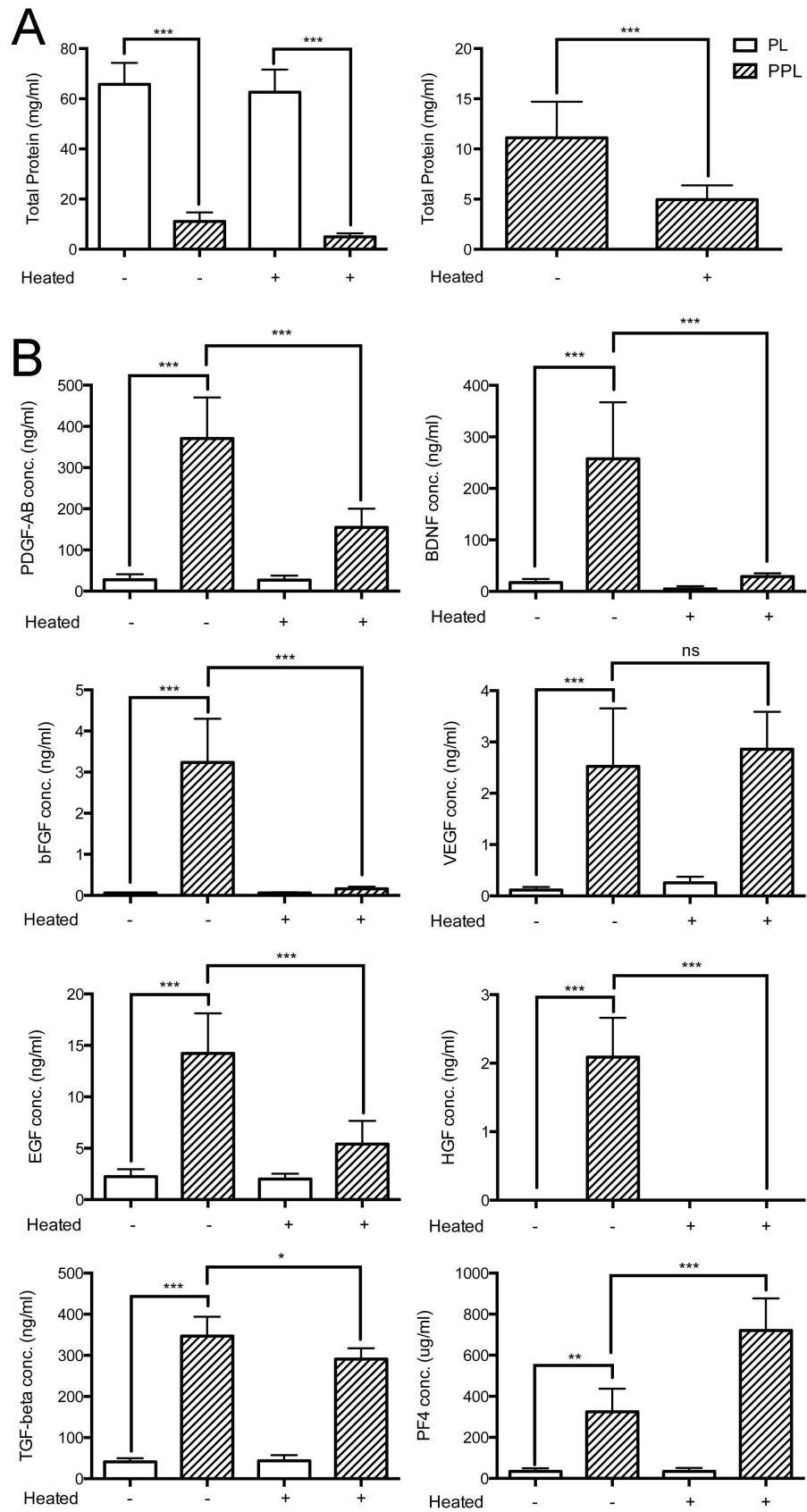


Figure 31 - Neuroprotective effect of the treatment of LUHMES cells by 0.5-15% PPL, heat-treated or not, prior to MPP⁺ exposure.

A: PPL not heat-treated, or heat-treated at B: 45°C, C: 56°C, or D: 65°C. E: Extent of neuroprotection provided by treatment of LUHMES cells with increasing doses of non heat-treated PPL (37°C) or heat-treated at 45, 56, or 65°C. Data are expressed as % of the viability of LUHMES cells grown in standard medium and not exposed to MPP⁺ (100%). *Abbreviations:* control (Ctrl); 1-methyl-4-phenylpyridinium (MPP⁺); platelet pellet lysate (PPL). (A, B, C, D) ***P*<0.01, ****P*<0.001 as compared to the control (w MPP⁺); (E) * 56°C vs 37°C PPL, # 65°C vs 37°C PPL, ***/###*P*<0.001; */#*P*<0.05.

3.6 Protein and GFs content were modified by heat treatment of PPL

To know whether heat-treatment can influence neurotrophic growth factors, PPL was subjected to 56°C for 30 min. As heat-treatment induced some protein precipitation of heat-sensitive proteins, the total protein content of the supernatant was significantly lower in HPPL than in the corresponding non-heated PPL and the PL control (Figure 32, A). The concentration of GFs was measured by ELISA (Figure 32, B) and expressed as a ratio of the total protein content (Figure 32, C). Some GFs such as PDGF-AB, BDNF, bFGF, EGF, HGF and TGF- β exhibited a lower concentration in heated PPL whereas VEGF and CXCL4/PF4 content remained essentially unchanged. The relative GFs content, expressed per mg of total proteins, showed a higher proportional decrease in BDNF, bFGF and HGF whereas PDGF-AB and EGF remained at a constant ratio, while that of VEGF, TGF- β and PF4 significantly increased, reflecting a differential heat sensitivity of these proteins.



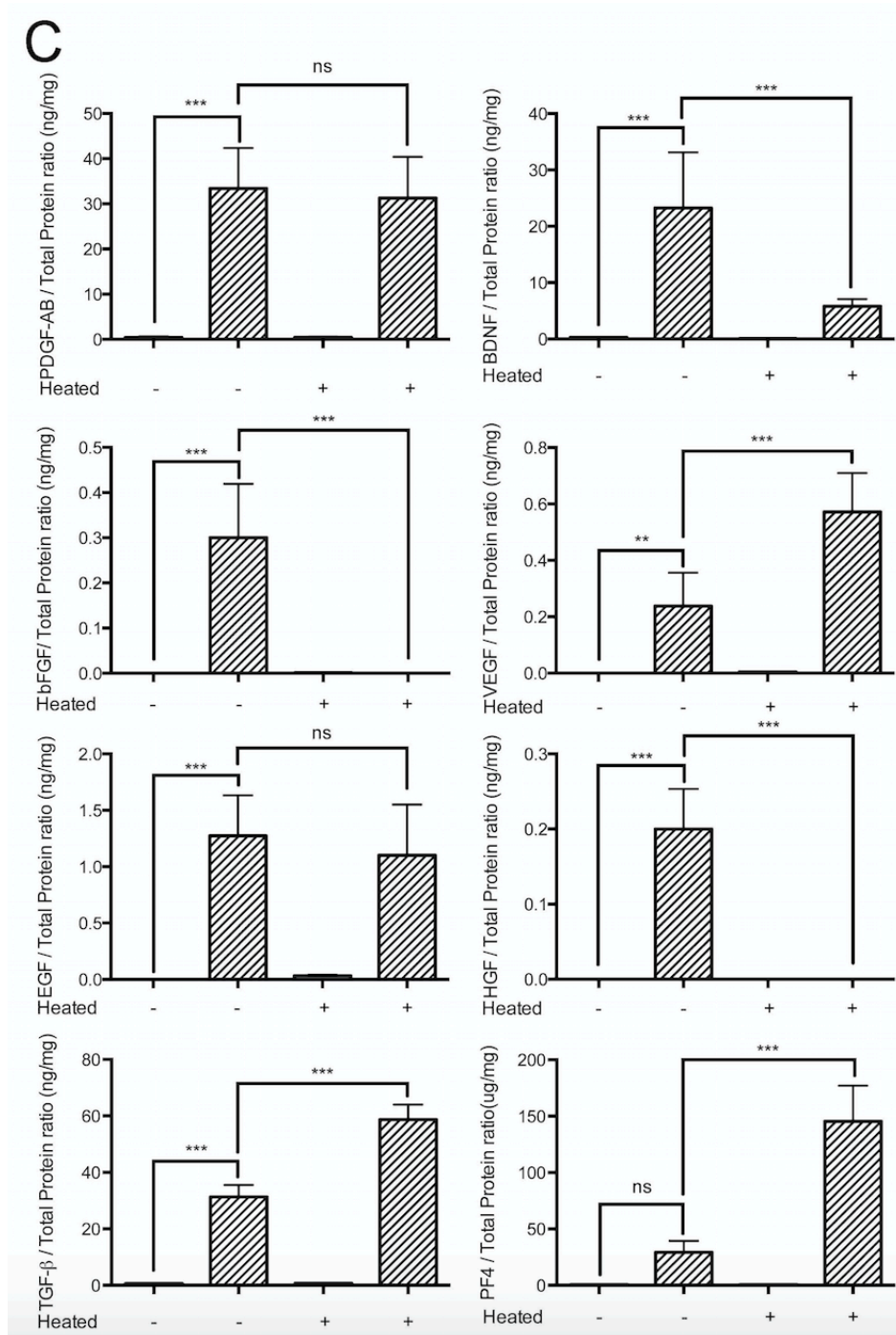


Figure 32 - Protein characterization of PL and PPL subjected to heat-treatment (56°C) or not.

A: Total protein content (mg/ml); B: content expressed in ng/ml and; C: in ng/mg proteins in PDGF-AB, BDNF, FGF, VEGF, EGF, HGF, THG-β, and PF4. *Abbreviations:* brain derived neurotrophic factor (BDNF); epithelium growth factor (EGF); basic fibroblast growth factor (bFGF); hepatocyte growth factor (HGF); platelet-derived growth factor-AB (PDGF-AB); platelet factor 4 (PF4); transforming growth factor-β (TGF-β); vascular endothelium growth factor (VEGF); platelet lysate (PL); platelet pellet lysate (PPL). ns= non-significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to PL, heated PL, or PPL.

Further explorations of the impact of heat-treatments were performed to check the protein profile of PPL, subjected to different temperatures, by SDS-PAGE under non-reducing or reducing conditions (Figure 33, A). The major impact of the heat-treatments was a gradual removal of proteins from various molecular mass, most obviously at 56 and 65°C.

Furthermore, Western blot analysis showed that the heat treatments at 56°C and 65°C removed platelet-borne fibrinogen (MM around 270 kDa) (Figure 33, B).

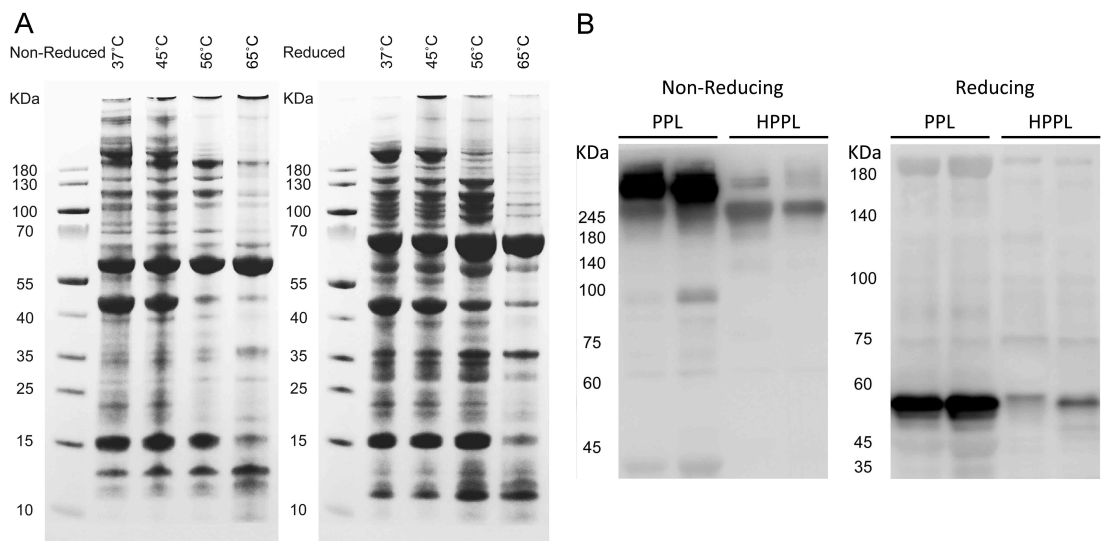


Figure 33 - Comparison of the protein profiles of PPL heat-treated at different temperatures.

A: heat-treated PPL analyzed by SDS-PAGE under non-reducing (L) and reducing conditions (R) B: fibrinogen Western blot analysis under non-reducing (L) and reducing conditions

The comparison of 102 cytokines by cytokine array showed a relative enrichment in some cytokines in heat-treated PPL at 56 or 65°C, compared to the untreated PPL (Figure 34); those included adiponectin, lipocaline-2, PDGF-AA, -AB/BB showed a relative impoverishment whereas angiogenin, angiopoietin-1, BDNF, CD14, complement factor D, dkk-1, EGF, ENA-78, endoglin, RANTES, RBP4, thrombospondin-1, Vitamin D BP showed a relative decrease.

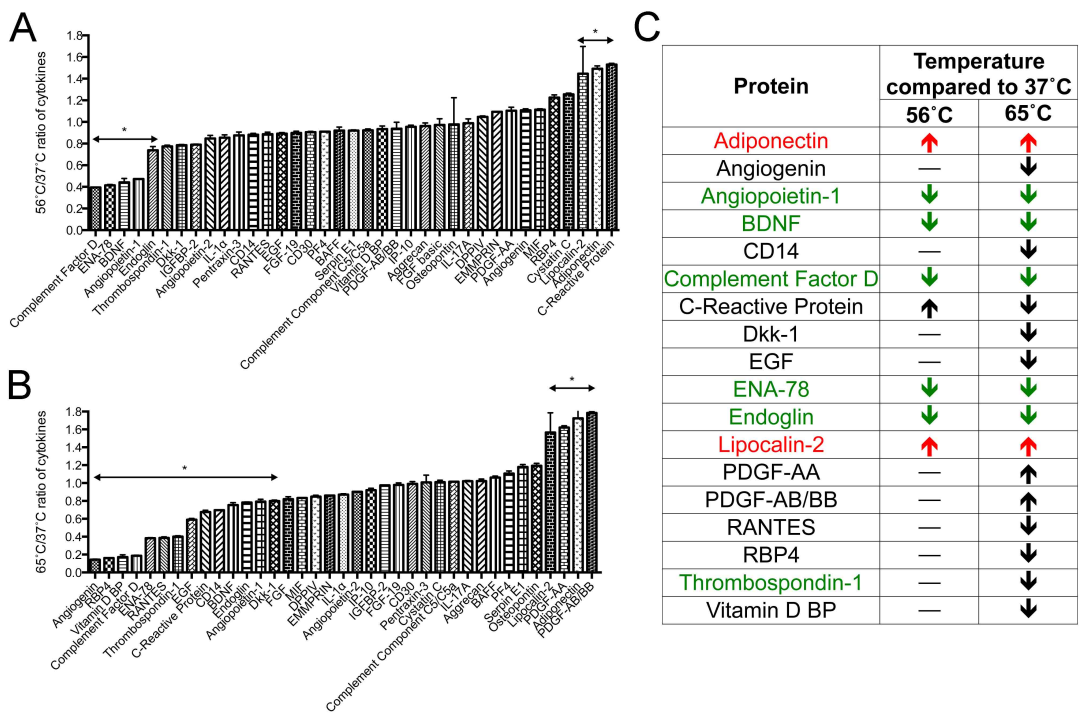


Figure 34 - Cytokine array showing the relative variations of cytokines in heat-treated (at 56°C or 65°C) PPL compared to the non-heated PPL (37°C).

A: Relative cytokine reactivity ratio in 56°C and 37°C PPL. B: Relative cytokine reactivity ratio in 65°C and 37°C PPL. C: Table showing cytokines found to increase or decrease in relative value in 56°C or 65°C PPL compared to 37°C PPL. *Abbreviations:* brain derived neurotrophic factor (BDNF); dickkopf-1 (Dkk-1); epithelium growth factor (EGF); CXCL5 (ENA-78); kiloDaltons (kDa); platelet-derived growth factor (PDGF); retinol-binding protein 4 (RBP4); regulated on activation, normal T cell expressed and secreted (RANTES) * $P < 0.05$, compared to the constance relative ratio of 1.0 at both temperatures.

3.7 Neuroprotective or neurotoxic PMP?

Our previous data showed that PMP exhibits thrombin generation in a plasma medium. Removal of PMP in plasma may be important for the safety of blood derived products administered intravenously. However, it is still unknown whether PMP can contribute to any neuroprotective or neurotoxic effect for dopaminergic cells subjected to neurotoxin stress.

NTA analysis revealed that PMPs are present in high quantity in the PPL materials. They were found at a mean dose of 1.06×10^{12} particles/mL in PPL and 6.17×10^{11} particles/mL in HPPL.

Therefore we designed specific experiments to understand whether the PMP present in PPL can play a role in neuroprotective activity, and if such activity can depend upon the type of PMP. To test this question, we incubated differentiated LUHMES with NPMP and TPMP with or without MPP⁺. Under these conditions, it was observed that both NPMP and TPMP were not toxic and exerted a neuroprotective effect two day after incubation (Figure 35).

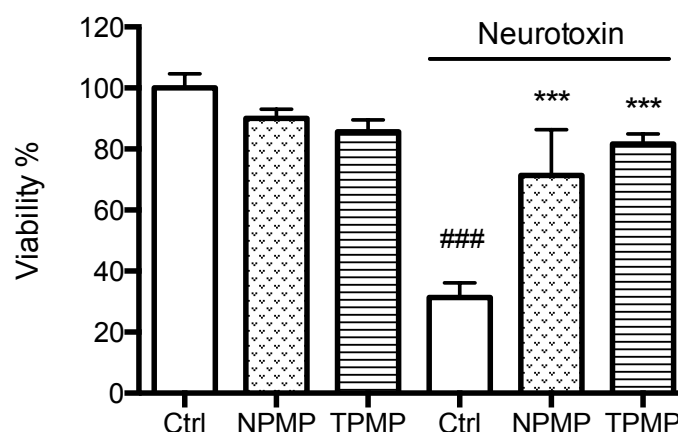


Figure 35 - PMP exert a neuroprotective effect.

NPMP and TPMP culture with LUHMES cells with or without MPP⁺. *Abbreviations:* Control (Ctrl); naturally presented in plasma PMP (NPMP); thrombin activated derived PMP (TPMP). ###*P*<0.001 as compared with the control (w/o MPP⁺). ****P*<0.001 as compared to the control (w MPP⁺).

3.8 PPL and HPPL do not increase microglia derived COX2 and iNOS expression induced by LPS

Microglia cells are the resident macrophages in the brain and play an important role to maintain CNS physiological function. BV2 cells line is an *in vitro* microglia cell model that we used to evaluate the viability when incubated with PPL and HPPL, with or without the presence of LPS.

Our results indicated that PPL and HPPL do not decrease the BV2 cells viability with or without LPS treatment (Figure 36, A). Under LPS exposure, PPL and HPPL diminished inflammatory cytokines COX2 expression (Figure 36, B and C), but did not exert significant effect on iNOS expression compared to the LPS group (Figure 36, B and D).

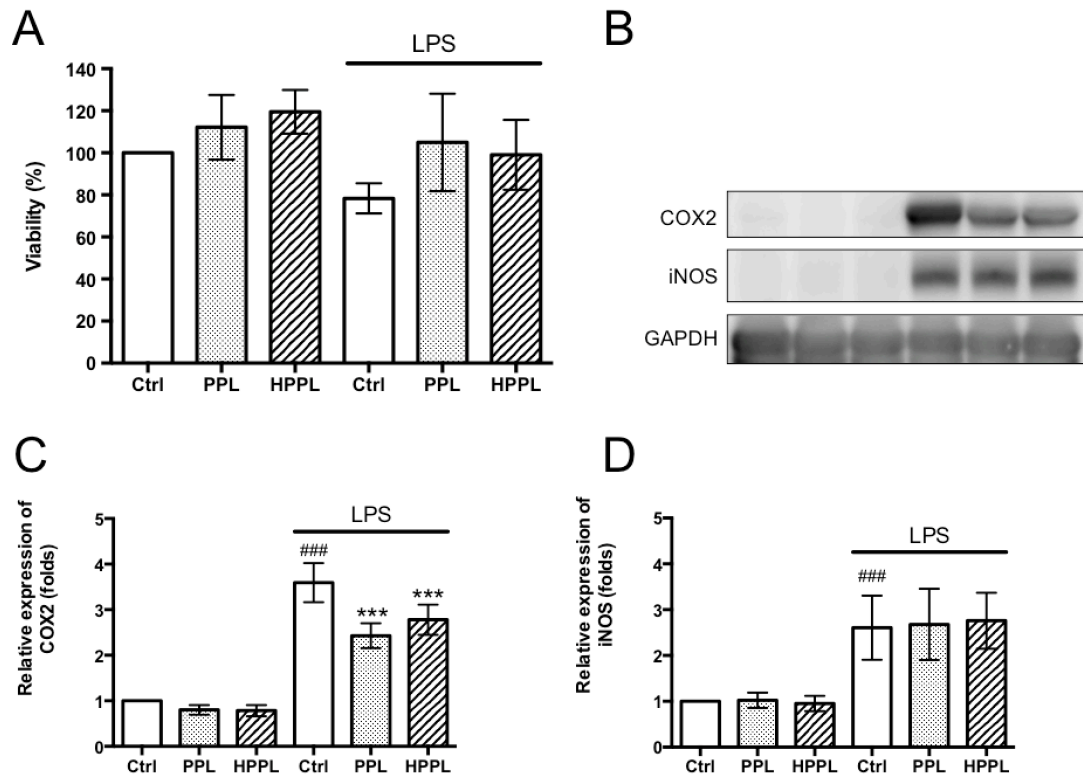


Figure 36 - 2% PPL did not exert cytotoxicity and inhibited LPS-induced COX2 protein expression in the BV2 cell model.

A: Data show the viability of BV2 cells exposed to PPL (non-heat-treated PPL, 37°C) and HPPL (heat-treated PPL, 56°C/30min) with or without LPS. Data were expressed as percent of the control group not exposed to LPS (100%). B, C, D: Cells were pre-treated by 2% PPL for 1 h and subsequently submitted to LPS exposure for 24 h; cell lysates were obtained and analyzed for COX2 (C) and iNOS (D) protein expression. Abbreviations: control (Ctrl); Lipopolysaccharides (LPS); platelet pellet lysate (PPL). $^{\$}P<0.05$, $^{\#}P<0.05$, $^{###}P<0.001$ as compared with the Ctrl (w/o LPS). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as compared with the Ctrl (w LPS).

3.9 Growth factors (GF) derived from HPPL diffused in mice brain when administered by intranasal route

We performed i.n. administration of HPPL to test whether HPPL-GF can diffuse to the different parts of the mice brain. We applied HPPL intranasally with interval of 6 minutes within one hour. After the last HPPL administration, we sacrificed mice by cardiac perfusion using Ringer's buffer.

Our results indicated that human PDGF-AB (used as a marker for HPPL GF due to its relative abundance in HPPL and its known role in neuroprotection), can diffuse in olfactory bulb, striatum and cortex (Figure 37, A), but there was no significant detectable increase in the hippocampus. PF4, also tested as a model for higher molecular mass platelet component, was found to be significantly elevated in the cortex (Figure 37, B).

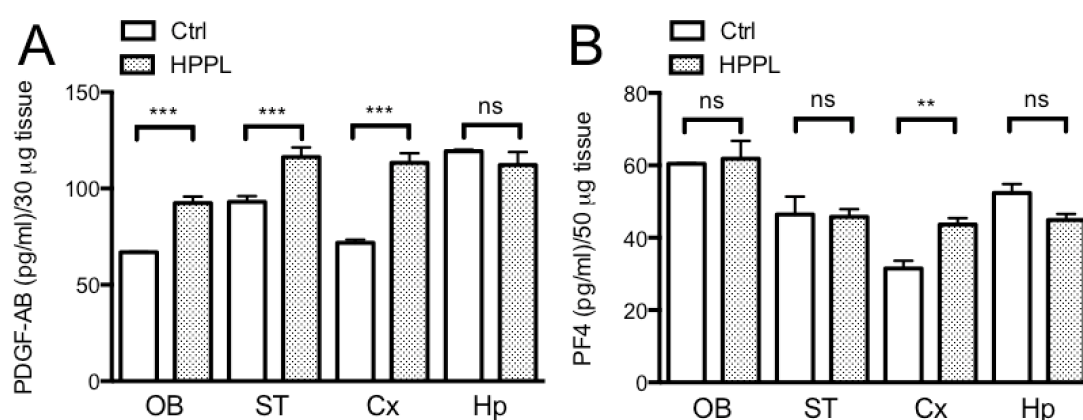


Figure 37 - Analysis of the diffusion of human PDGF-AB and PF4 to different brain areas in mice receiving i.n. delivery of HPPL.

A: human PDGF-AB B: human PF4 in olfactory bulb, striatum, cortex and hippocampus in mice brain. ns = no significant, ** $P < 0.01$, *** $P < 0.001$ as compared to the control (without HPPL i.n. administration).

3.10 HPPL protected dopaminergic neuron labeled by tyrosine hydroxylase (TH) marker from MPTP neurotoxin in the striatum

Male C57BL/6 mice received i.n. administration of PPL or HPPL by intranasal administration once before the first MPTP intoxication. After subsequent two weeks of PPL i.n. administration, mice brains were isolated and analyzed by Western blot to quantify TH expression by dopaminergic neurons. A significant increase ($P < 0.05$) of TH expression in the striatum was found in the MPTP-mice group receiving HPPL i.n. administration suggesting a neuroprotective effect (Figure 38). An increase was also observed when using PPL but it did not reach significance.

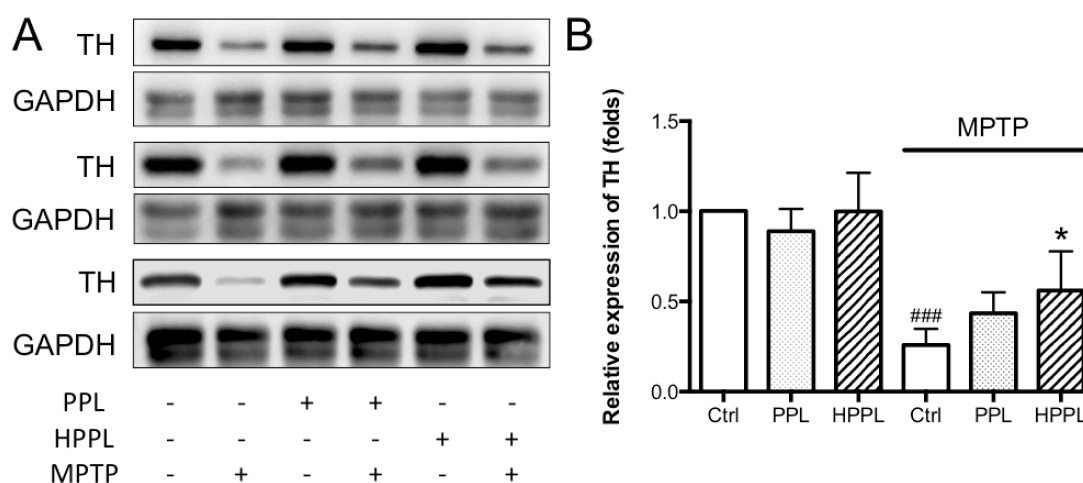


Figure 38 - PPL and HPPL administered i.n. exhibit a neuroprotective effect in a MPTP mice model.

Mice received PPL (non-heat-treated PPL, 37°C) and HPPL (heat-treated PPL, 56°C) through i.n. administration, with or without MPTP. The striatum brain tissues were recovered and analyzed for TH protein expression. A: TH and GAPDH expression in striatum was analyzed by Western blot. B: There was a relatively higher quantitative expression of TH of neurons in MPTP intoxicated mice receiving HPPL. *Abbreviations:* control (Ctrl); platelet pellet lysate (PPL). ### $P < 0.001$ as compared with the control (w/o MPTP). * $P < 0.05$ as compared to the control (w MPTP).

3.11 HPPL exerts neuroprotection in MPTP mice *substantial nigra* and striatum without triggering microglia accumulation

We further explored the distribution of dopaminergic neurons or terminals in the *substantial nigra* (SN) or striatum by tyrosin hydroxylase (TH) marker. Our results indicated that the dopaminergic neurons and terminals were decreased by MPTP neurotoxin, as expected, showing the efficacy of our experimental model to affect neurons in the SN and striatum. Interestingly, HPPL i.n. treatment could strongly rescue and/or protect dopaminergic neurons and terminals of both the SN and striatum from the lethal effects of MPTP (Figure 39 and Figure 40). Inhibition of microglia activation has shown to be associated with an improved neuroprotective effect in MPTP mice.²⁵⁶ We therefore analyzed microglia expression in the striatum using Iba1 marker. Our results suggested that microglia accumulation was induced by MPTP intoxication whereas this induction was avoided by i.n. HPPL administration (Figure 41).

A

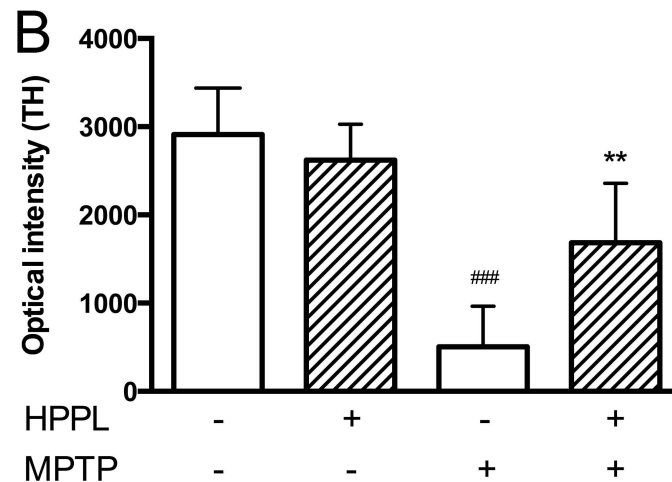
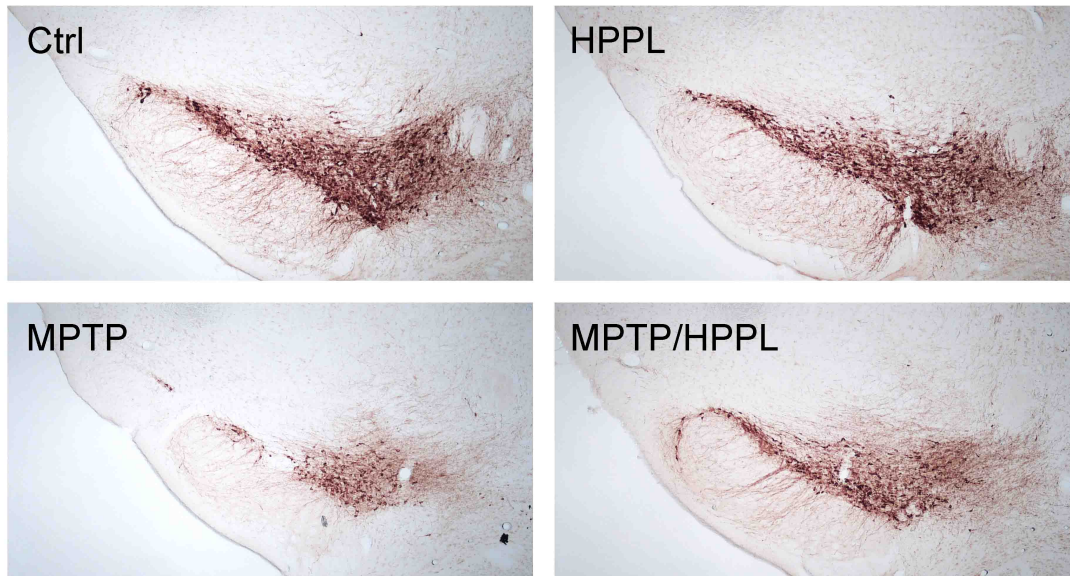


Figure 39 - Expression of TH marker by dopaminergic neurons in mice substantia nigra.

Brain cryo sections show the distribution of dopaminergic cells in substantia nigra in control group (Ctrl), and in groups treated with HPPL only (HPPL), MPTP only (MPTP) and MPTP and HPPL (MPTP/HPPL). A: HPPL was found to protect dopaminergic neurons against MPTP intoxication in substantia nigra. B: There was a relatively higher quantitative expression of dopaminergic neurons in MPTP intoxicated mice receiving HPPL. ### $P < 0.001$ as compared with the control without neurotoxin. ** $P < 0.01$ as compared to the control with neurotoxin.

A

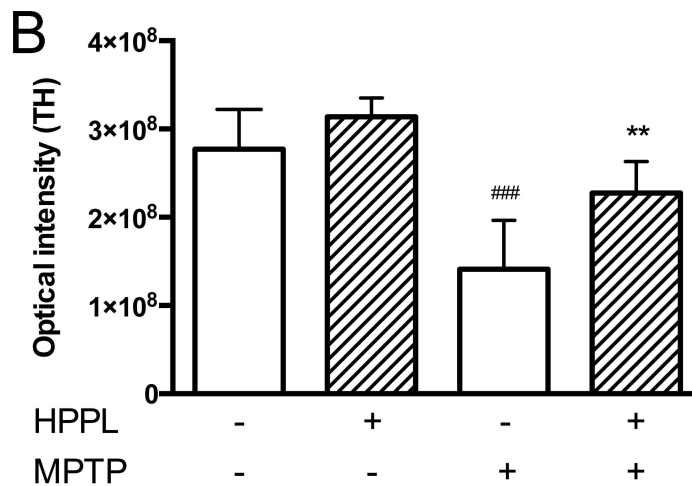
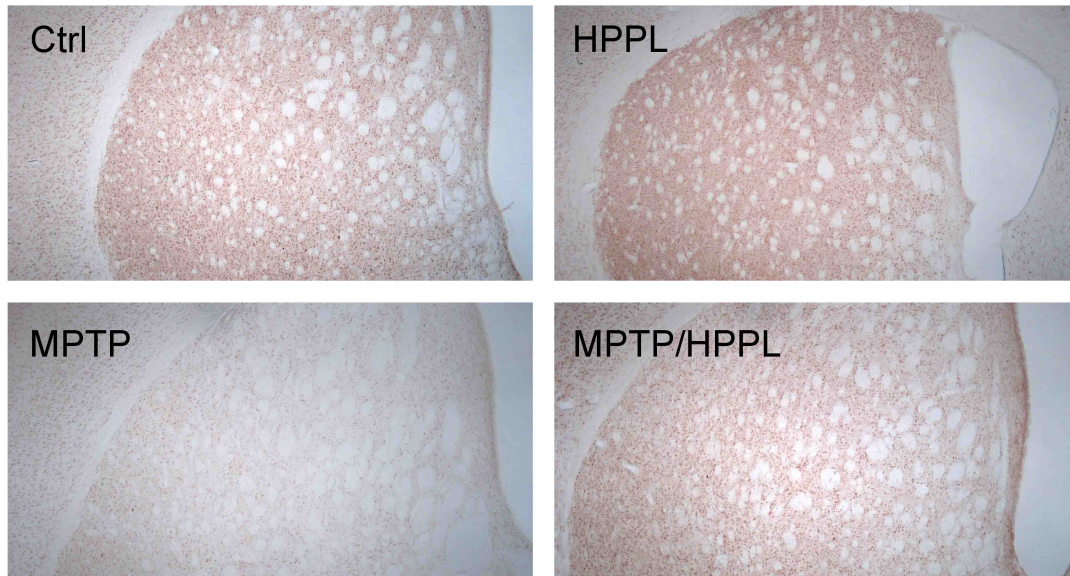


Figure 40 - Expression of TH marker by dopaminergic terminals in mice striatum.

Brain cryo sections show the distribution of dopaminergic terminals in striatum in control group (Ctrl), and in groups treated with HPPL only (HPPL), MPTP only (MPTP) and MPTP and HPPL (MPTP/HPPL). HPPL was found to protect dopaminergic terminals against MPTP intoxication in striatum. B: There was a relatively higher quantitative expression of dopaminergic terminals in MPTP intoxicated mice receiving HPPL. ### $P < 0.001$ as compared with the control without neurotoxin. ** $P < 0.01$ as compared to the control with neurotoxin.

A

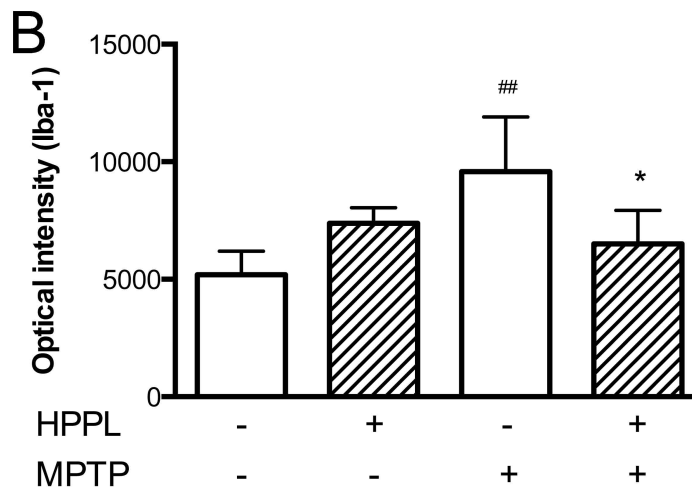
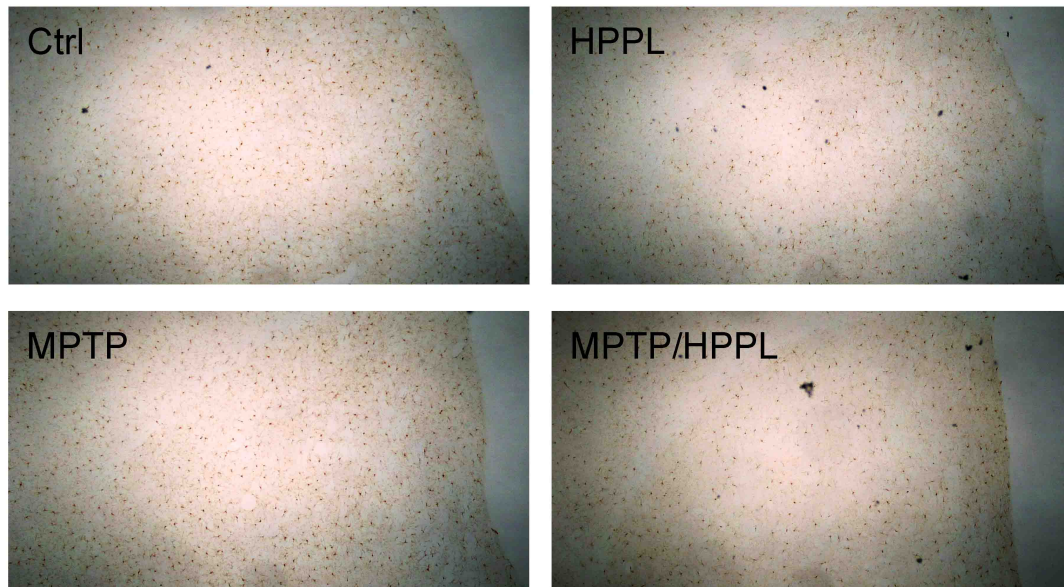


Figure 41 - Expression of Iba1 marker by microglia in mice striatum.

Brain cryo sections show the distribution of microglia in the striatum in control group (Ctrl), and in groups treated with HPPL only (HPPL), MPTP only (MPTP) and MPTP and HPPL (MPTP/HPPL). HPPL was found to diminish MPTP induced microglia activation in striatum. B: There was a relative decrease in quantitative expression of Iba-1⁺ by microglia in MPTP intoxicated mice receiving HPPL. ^{##}*P*<0.01 as compared to the control without neurotoxin. **P*<0.05 as compared to the control with neurotoxin.

3.12 Exploration of the impact of three viral inactivation/removal steps during PPL preparation

Future clinical applications possibly using allogeneic single-donor, small pool, or large-pool PPL would require the implementation of viral inactivation or removal treatments.

We therefore evaluated three different viral reduction steps applied to PPL.

PPL samples were spiked with HCVcc just prior to heat inactivation (56°C for 30 minutes), S/D treatment followed by C18 filtration (SD/C18), and nanofiltration using a 20 nm pore size filter (Planova 20N). PPL samples were taken prior to and after the respective viral reduction treatments and evaluated by cell culture infectivity assay in Huh-7.5 cells to determine the infectious dose.

Data showed that ≥ 2.5 logs of HCVcc were inactivated by each of these three treatment steps (Figure 42), indicating that the combination of these three “orthogonal” treatments that use different principles of virus inactivation or removal would be over 7.5 logs. It is indeed accepted by regulatory authorities to add log of inactivation or removal when viral reduction treatments use different principles of virus reduction as is the case here when using heat, S/D and nanofiltration treatments.

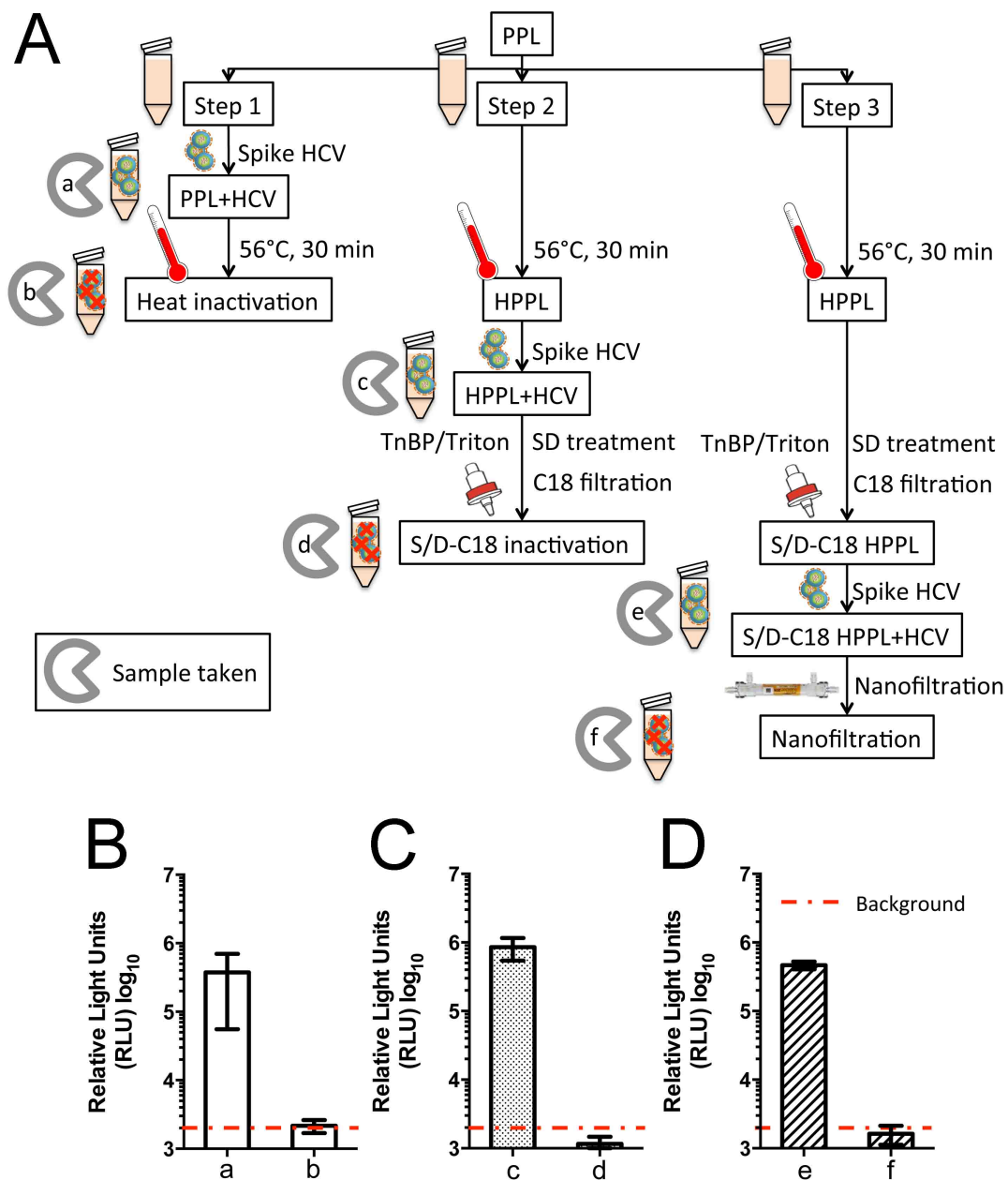


Figure 42 - 56°C/30 min heat treatment, S/D-C18, and Planova 20N could each inactivate or remove ≥ 2.5 logs of HCVcc infectivity signal.

A: The viral inactivation procedures. Samples were collected at a, b, c, d, e, and f steps then performed HCVcc infectivity assay. B: The reduction of HCVcc infectivity after heat treatment; C: The reduction of HCVcc infectivity after S/D-C18 treatment; D: The reduction of HCVcc infectivity after 20 nm nanofiltration.

3.13 Microparticles size distribution profile altered by three viral inactivation/removal steps

NTA analysis shows that PMPs are present in high quantity in the PPL/HPPL materials. After defining the impact of the three viral inactivation or removal treatments, we decided to further explore whether PMPs distributions could be affected. We then determined the samples subjected to viral inactivation steps in DLS method.

PPL samples were subjected to heat treatment to obtain HPPL. Then both PPL and HPPL were subjected to either S/D and C18 filtration (S/D-C18) or 20 nm nanofiltration as described before. Our results indicated that the mean size was decreased in HPPL (mean size at 112 nm) compared to PPL (mean size at 189 nm). Moreover, in PPL and HPPL subjected to 20 nm nanofiltration, there is a shift in the mean size of particles distribution in nanofiltered PPL (mean size at 30 nm) and HPPL (mean size at 12 nm) (Figure 43).

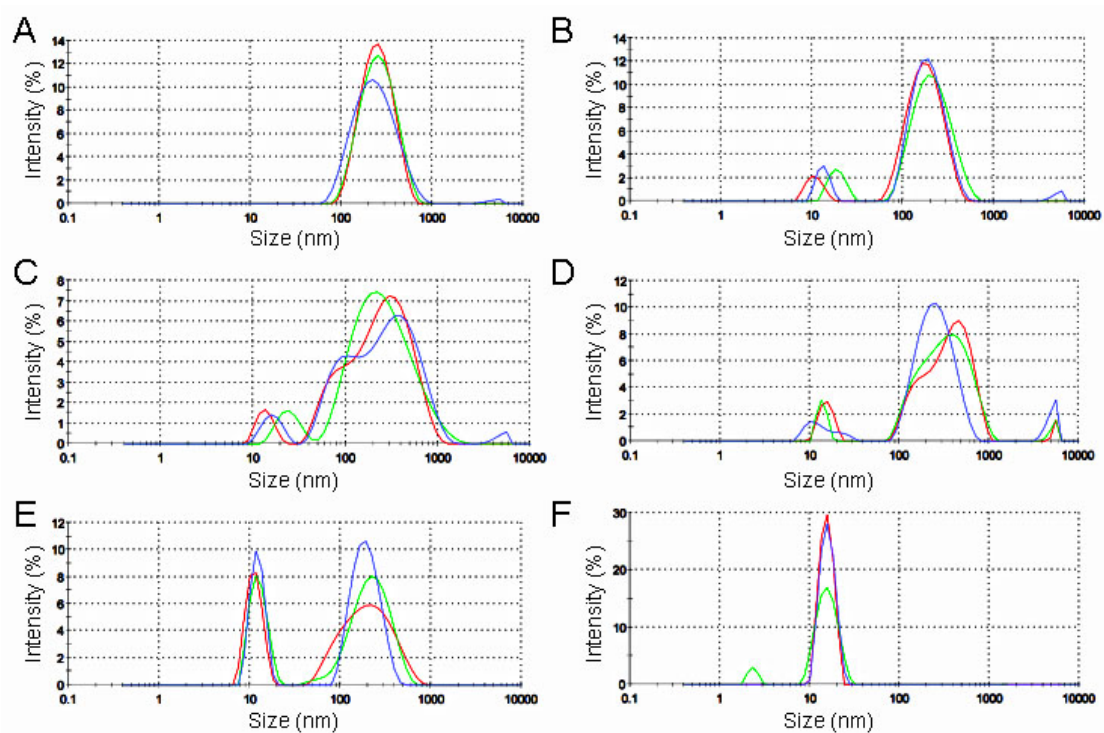


Figure 43 - PMPs distribution in PPL altered by viral inactivation steps by DLS analysis.

In DLS method, the microparticles distribution in A: PPL; B: HPPL; C: S/D-C18 PPL; D: S/D-C18 HPPL; E: 20-nm nanofiltered PPL; F: 20 nm nanofiltered HPPL samples.

3.14 Neuroprotection effect of PPL subjected to viral inactivation steps

Once we determined the capacity of these three treatments to inactivate or remove HCV, we decided to understand whether they could, when implemented individually or in combination, affect the neuroprotective activity of PPL.

PPL samples were therefore subjected to heat treatment to obtain HPPL. Then, both PPL and HPPL were subjected to either S/D and C18 filtration (S/D-C18) or 20 nm nanofiltration as described before. The LUHMES cell model was used to assess neuroprotection. Our results indicated that both S/D-C18 treatment of PPL and nanofiltration of PPL and HPPL did not affect neuroprotection. By contrast, treatment of HPPL by S/D-C18 treatment was observed to decrease the neuroprotective activity, possibly due to protein adsorption on the C18 column (Figure 44).

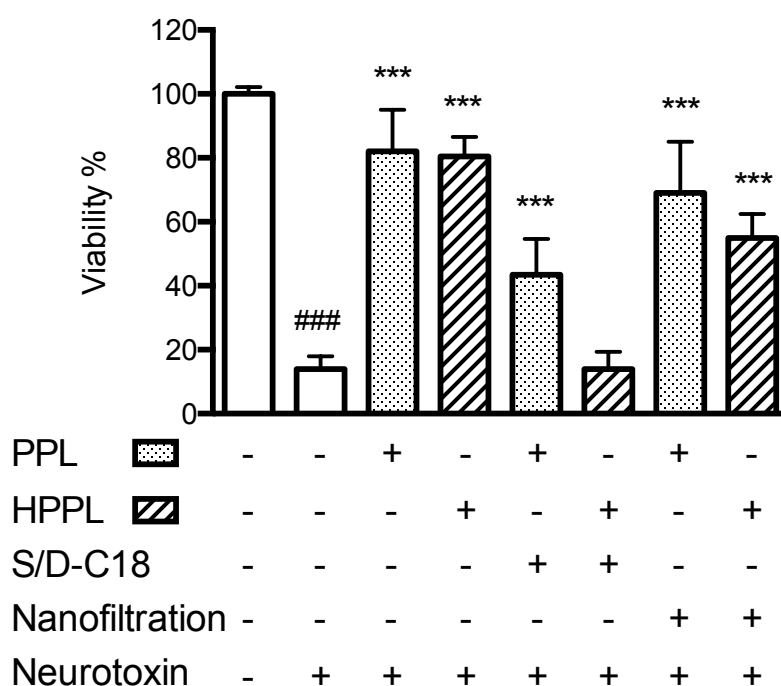


Figure 44 - Viral inactivation step by S/D treatment, and viral removal step by nanofiltration preserves the neuroprotective effect of PPL and HPPL.

$P < 0.001$ as compared with the control without neurotoxin. *** $P < 0.001$ as compared to the control with neurotoxin.

3.15 PPL suspended in PBS and saline are preserved their neuroprotective effect

in vitro

We then considered that clinical applications of PPL may preferentially be done using PPLs suspended in physiological saline solution rather than in a PBS buffer system (PBS buffer was preferred for our experimental studies for standardization purposes to ensure stable pH during *in vitro* cell model explorations).

A specific preparation of PPL was made where after centrifugation of PC, the platelet pellet was frozen/thawed three times and resuspended in either 10 fold smaller volume of PBS or of Saline. The respective PPLs were heat-treated at 56°C for 30 min.

Both heat-treated PPLs were found to exert a similar neuroprotective activity in the LUHMES cell model against neurotoxin exposure (Figure 45)

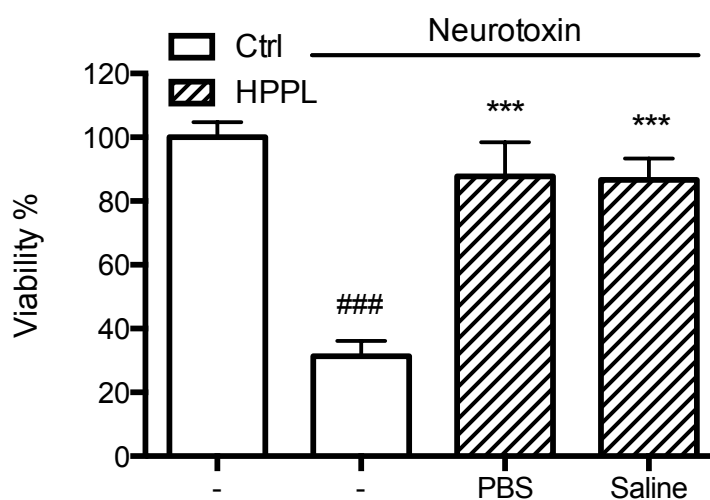


Figure 45 - HPPLs prepared by PC resuspended in PBS or saline solution both showed significant neuroprotective effects in LUHMES exposed to MPP⁺ neurotoxin.

$P < 0.001$ as compared with the control without neurotoxin. *** $P < 0.001$ as compared to the control with neurotoxin.

Our results, both *in vitro* and *in vivo*, concur to confirm that a physiological platelet lysate preparation depleted of plasma proteins, concentrated 10-fold, and subjected to a step of heat-treatment at 56°C for 30 min, exerts a strong neuroprotective activity in cellular (LUHMES cells) and animal (MPTP mice) models of PD. Interestingly PDGF-AB was found to migrate into mice brain after i.n. delivery and to protect TH expression in the *substantia nigra* and striatum of MPTP intoxicated mice.

Further, our data provide evidence that PPL preparations do not induce acute toxicity *in vitro* to various neuronal cells and *in vivo* after i.n. administration in mice models. The PPL was found in an *in vitro* model using BV2 microglial cells not to induce inflammatory reactions based on the expression of inflammatory markers, and to reduce the expression of COX-2 inflammatory marker during BV2 exposure to LPS.

As a means to evaluate the capacity to use allogeneic PPL in clinics we also verified that various viral inactivation or removal treatments, that are well documented to provide a high margin of viral safety to fractionated plasma products could reasonably be considered for allogeneic PPL without detrimental impact on the neuroprotective activity.

DISCUSSION

Demonstration of HCV inactivation by S/D treatment

Blood transfusion is an essential life-saving medical procedure used for the treatment of trauma or for replacement therapy in congenital or acquired deficiencies in blood components. The collection and processing of blood is tightly regulated, and blood products should meet established quality and safety requirements.

One key criteria in the quality of allogeneic blood products is pathogen safety. A first objective of our work has therefore been to study methods of viral inactivation or removal that could eventually be applied on PL used for regenerative medicine since essentially no specific technology is currently used routinely. In particular the risk of transmission of HCV remains a major concerns. Despite of the progress achieved in donors' screening and donation testing, WHO still estimated this year a prevalence of transfusion-transmissible infections of 0.32-1.03% HCV per blood donation in middle or low-income countries.⁴

Several methods have been developed to inactivate viruses in blood products using technologies having minimal impacts on the clinical efficacy of blood components. In the last 10 to 15 years up to three methods have been licensed in various countries to inactivate viruses in single donations of plasma for transfusion and/or platelet concentrates. They use technologies relying on the addition of a photo-inactivator and exposure to light or ultra-violet (UV).¹⁸

Another method, applied to pooled plasma for transfusion is S/D treatment at industrial scale, a method developed to inactivate lipid-enveloped viruses without altering its hemostatic properties.²⁵³ The S/D viral inactivation treatment, by using 1% TnBP and 1% Triton X-100, was the first introduced to treat pools of plasma of 100 to 2500 donations at industrial scale. The elimination of the S/D agents is achieved typically by large-scale hydrophobic interaction chromatography using a C18 packing material. A simplified S/D treatment using 1% TnBP and 1% Triton X-45 has been implemented to virally-inactivate plasma and cryoprecipitate in a bag system.^{257,258} S/D treatments preserve the protein functional activity²⁵⁹ and were validated to inactivate lipid-enveloped viruses efficiently.²⁶⁰ However, due to the absence of an appropriate HCV *in vitro* infectivity model, and to the difficulty and ethical barrier in using an animal infectivity model, such as chimpanzee, the extent of inactivation of HCV by S/D treatment of plasma has not been evaluated yet.

In the past decade, HCV *in vitro* models were based on hepatoma cells using recombinant full-length genomes.²⁶¹⁻²⁶³ We used HCVcc with luciferase-tagged system to demonstrate that the S/D treatment (1% TnBP and 1% Triton X-45) is able to inactivate HCV in a complex protein mixture like plasma. Our data using luciferase-tagged infectious cell culture-derived HCV (HCVcc) particles spiked to human plasma revealed the capacity of the S/D treatment to fully inactivate HCVcc within 30 minutes of treatment at 31°C as shown by the baseline level of reporter signals, total loss of viral infectivity, and absence of viral protein NS5A. This S/D inactivation step not only inactivates patient-derived HCV isolates but also abrogates HCVcc infection to primary human hepatocytes. Interestingly, another similar HCV model was used recently to study the capacity of other pathogen reduction procedures to inactivate HCV in plasma and PC.²⁶⁴

We show that S/D treatment followed by C18 filtration is able to eliminate HCV infectivity from plasma spiked with high doses of the virus, without altering plasma hemostatic function. The Sep-Pak® Plus C18 cartridge we used was sufficient to remove S/D-related toxicity. Moreover, based on the recommendations from international guidelines,²⁵³ we studied the plasma hemostatic properties in a scale-down process mimicking the HCV inactivation process. We confirmed that this S/D-C18 procedure maintains the PT and aPTT within the normal range, with data that are close to or slightly better than those previously published,^{257,258} suggesting a good preservation of coagulation factors such as fibrinogen, FVIII and FIX. This can be explained by the incubation time of the S/D treatment in our study that is much shorter than that of other studies (120 minutes) evaluating the influence of S/D treatment on plasma proteins function and hemostatic activity.^{257,258} The S/D-C18 process is also sufficient to inactivate high load of enveloped Dengue virus, a virus with similar structural features as HCV, in plasma.²⁶⁵ Taken together, the minipool S/D treatment followed by C18 filtration exhibits a high efficiency to inactivate HCV infectivity in plasma, confirming that it can be applied as an efficient method for the reduction of transfusion-associated HCV infectious in plasma or platelet products.

Presence and physiological roles of microparticles in plasma; impact of nanofiltration

It is becoming apparent that extracellular vesicles, also called microparticles (MPs) are present in high amounts in cellular blood components and plasma for transfusion. These MPs are either derived from the blood circulation of the donor where they are present under normal physiological conditions, or generated during blood processing steps, such as leukoreduction filtration, centrifugation, and storage. Recent evidence suggests that high number of MPs can be associated with some pathological conditions, such as cancer and inflammatory diseases.²⁶⁶ High amounts of MPs in blood components (such as plasma, platelet concentrates, or red blood cell concentrates) are suspected to trigger thrombotic and inflammatory disorders and be responsible for potentially severe post-transfusion reactions.³⁷

Our studies showed that nanofiltered plasma processed through a 75-nm nanofilter (Planova 75N, Asahi Kasei Medical, Tokyo, Japan) to remove parts of MPs still preserved its protein and lipoprotein profiles, coagulation factors content, and global coagulation activities. Although we did not specifically measure anti-coagulant proteins here, a previous study demonstrated that a nanofiltration process on a 35 nm filter, with a pore size that is smaller than the 75 nm nanofilter used in our study, was able to maintain anticoagulant proteins including protein C, protein S and antithrombin.²⁶⁷ Additionally, 75 nm nanofiltration did not alter the content in lipoproteins such as HDL, IDL and VLDL particles which have a size range between 8 to 50 nm.²⁶⁸

It is increasingly admitted that, due to technological limitations in each currently available methodologies, different specialized biophysical methodologies should be combined to assess MPs. This is what we did to demonstrate the removal capacity of MPs by nanofiltration. In the FCM technique, there was an 80% reduction of double positive MPs (AnnV and CD41a). However, it is known that the particle detection threshold of standard FMC is above approximately 500 nm, and the background signal may cause some inaccuracy in MPs counting, thereby making it mandatory to

use other methods. The DLS methodology showed that larger-size MPs were removed by nanofiltration. However, the scatter light from the bigger particles is more intense than from the smaller one. Moreover, the presence of proteins or lipoproteins can also interfere with MP detection in this method. Two populations of MPs were detected in nanofiltered plasma with a mean size of approximately 9 and 50 nm, which is consistent the previous observation that in a 0.2 μm -filtered plasma two populations at 10 and 100 nm have been detected by others.²⁴⁹ These results suggest those these two populations may correspond to proteins, lipoproteins or micro-aggregates rather than only MPs. We also used the NTA instrumentation since this method can analyze isolated particles with sizes smaller than those detected by DLS, although interferences from protein aggregates still need to be considered.²⁶⁹ A progressive shift towards a smaller particles presence was observed in nanofiltered plasma indicated that the nanofiltration is able to remove MPs with a size exceeding 50-100 nm. By using an appropriate pore size membrane, TRPS allows to identify particles going through the pore, thereby allowing to count MPs number precisely and to estimate the size range.²⁴⁹ There were no detectable MPs in nanofiltered plasma by TRPS analysis, using a 100 nm pore size membrane (which can count particles with a range at 50 to 200 nm). By contrast, starting or leukoreduced plasmas could easily block this 100 nm pore size membrane due to the presence of a large number of large size MPs. When these samples were evaluated using 400 or 800 nm pore size membranes, many particles could be detected by TRPS, confirming the presence of many larger size MPs are presented in starting and leukoreduced plasma. We were interested to determine the MP log removal capacity of the 75-nm nanofilter. For this, we needed to use a “clean” buffer solution to avoid interferences from biological materials. PBS was spiked with a known quantity of isolated PMP and run through the 75-nm nanofilter. Data suggested that more than 9 log of PMPs could be removed by nanofiltration, consistent with the capacity of over 8 log reported for hollow-fiber nanofilters of similar design used for virus removal.²⁹

In our study, we next checked whether nanofiltration had the capacity to avoid *in vitro* thrombin generation, an event that can be triggered by the phospholipids present in MPs. The TGA assay has recently become a well established technique to detect the risks of thromboembolism in plasma derived immunoglobulins, therefore we first applied it in our study.²⁷⁰ “RC low” reagent, used in this assay, contains low concentration of phospholipid micelles and is used to directly monitor the thrombogenicity originating from MPs. When using the “RC low” reagent, a thrombin generation was observed in the starting and leukoreduced plasmas but not in nanofiltered plasma. Thus, 75-nm nanofiltration removed MPs that are capable to generate thrombin *in vitro*. Interestingly, when using the “RC high” reagent, which contains high concentration of phospholipid micelles, nanofiltered plasma was still capable to generate thrombin to an extent similar to that of the starting and leukoreduced plasmas. This phenomenon results, in our opinion, from the fact that under these conditions of an higher concentration of phospholipid micelles, nanofiltered plasma, with a coagulation factor content within physiological range, recovers its normal hemostatic potential associated with a physiological content of phospholipids. Interestingly, similar TGA observations, namely thrombin generation only when using the RC-high reagent and not the RC-low reagent, have been reported in the literature for the industrial S/D-treated plasma,²⁷¹ a product which, in clinical use, exhibits good clinical hemostatic efficacy.²⁷² Again, in prothrombinase and PPL-coagulant assay, we confirmed the fact that nanofiltration was capable to remove procoagulant-active PS bearing MPs. In PMPs-spiked experiment, a similar evidence was obtained, with a significant prolongation of the clotting time after nanofiltration. Analyzed by Zymophen MP-TF method, we did not find evidence of presence of detectable coagulant TF, a finding consistent with the belief that TF-bearing MPs are absent in plasma under normal physiological conditions.^{273,274} Therefore we conclude that 75-nm nanofiltered plasma exhibits the normal hemostatic activity associated with the preservation of the coagulation factors, but the depletion in MPs by 75-nm nanofiltration contributes to decreasing its *in vitro* thrombogenic activity. These results should be considered in line with other observations in our laboratory showing that some types of PMPs (released by thrombin activation of platelets) are capable to trigger monocytes (THP-1 cells)

aggregation and release of monocyte-derived TF-bearing MPs,²⁷⁵ as well as aggregation and release of neutrophils extracellular traps (NETs) by neutrophils.²⁷⁶ Therefore PMPs may trigger thrombotic events in blood through both direct thrombin activation due to exposure of PS, but also activation of monocytes or neutrophils.

Taken together, S/D treatment and plasma nanofiltration are valuable techniques to consider in the development of blood protein products for administration in humans. They can be instrumental to improve safety with regards to the risks of transmission of HCV and other lipid-enveloped viruses, and potential thrombogenic risks in some patients with hyper-coagulable state.

Development of a platelet lysate for therapeutic applications in neurodegenerative diseases

We tried to use the information gathered on S/D treatment and nanofiltration to develop a therapeutic platelet lysate for safe administration in patients suffering from neurodegenerative diseases. We had in mind the following targets to define an optimal product:

- (a) Select, as a source material, a type of platelet concentrate that is readily available worldwide, either from autologous or allogeneic sources
- (b) Avoid a protein overload, especially when ICV administration is considered, in order not to saturate the CSF (which has a total protein content of approximately only 1 g/L)
- (c) Ensure a high content in a range of platelet-derived neurotrophins
- (d) Verify the absence of inflammatory effects *in vitro* and/or upon administration in an animal model
- (e) Possibility for effective non-invasive i.n. administration in some patients
- (f) Develop a process:
 - a. In which viral reduction steps can be introduced
 - b. That can be scalable in case of industrial future production from allogeneic sources
 - c. That is amenable to preparation from both allogenic or autologous sources and can be cost-effective

It was also important in order to develop such a product to select a neurodegenerative disease for which reliable *in vitro* and *in vivo* models. This is the reason why we used models of PD to assess the platelet lysate intended for application in neurodegenerative diseases, and more generally neurology.

In our study, we used LUHMES cells for the cellular experiments. These cells were available from Dr. David Devos's laboratory at the University of Lille, France. The LUHMES cells are conditionally-immortalized, non-transformed human foetal cells that are a subclone of MES2.10 mesencephalic cells.²³⁵ They can be differentiated

into the dopaminergic neuron-like phenotype and represent an established *in vitro* model for PD,^{234,237} thanks to their physiological relevance and consistency.²⁷⁷ Sensitive to various inducers of oxidative insults, LHUMES cells are used to evaluate the neuroprotective and neurorestorative capacity of drug candidates.^{234,238,277} MPP⁺ precursor, MPTP, causes parkinsonism in man and is a neurotrophin typically used in animal models of PD,²⁷⁸ making MPP⁺ (a mitochondrial complex I inhibitor) a valuable dopaminergic neuron specific toxin in pre-clinical cellular models.²⁷⁷ MPP⁺ neuronal toxicity involves oxidative phosphorylation in mitochondria, reduction in dopamine levels, and gradual cell death.²⁷⁷ Early experiments performed at Dr. Devos's laboratory and then confirmed at Taipei Medical University showed that the treatment of cells with platelet lysates reduced significantly the neurotoxicity induced by MPP⁺. Therefore this cell model could be used to develop further a platelet lysate meeting the above-mentioned targets.

The PCs used a starting material were mostly obtained by apheresis, were not subjected to leukofiltration and were maintained in 100% plasma. This preparation method of PC is used worldwide (together with the preparation from collected whole blood) and the lack of leukofiltration and maintenance in plasma instead of replacement with PAS can contribute to limiting the cost. We confirmed that the blood cell count of these starting PC was within the expected range of this standardized collection procedure. Early experiments in Dr. Devos's laboratory suggested that the plasma proteins may impair LUHMES cells viability. Therefore, it was decided to isolate the platelets to prepare the lysates. Removing plasma also allowed to remove the bulk proteins found in most platelet lysates used for regenerative medicine, avoid the risks from inflammatory biological modifiers release by platelets into plasma,²⁷⁹ and was a first step towards our objectives to develop a product with low protein content for brain administration. In addition, the isolation of platelets by centrifugation was of interest to avoid possible experimental interferences from any neuroprotective (such as IGF-1) or anti-inflammatory (such as protease inhibitors) components that can also be present in the plasma compartment,^{191,280} (and could also be valorized in the future as complementary neuroprotective purified preparations). Removing plasma and resuspending the

platelets in 1/10th of the initial volume, indeed dramatically decreased the protein load of the platelet lysate, and resulted in ca. 40-fold enrichment in neurotrophic factors. In addition, on a practical experimental basis, plasma-born fibrinogen was removed efficiently to prevent fibrin generation and growth medium clotting during cell culture tests, thereby avoiding the addition of heparin anti-coagulant, as typically done when non-fibrinogen depleted platelet lysates are used for *ex vivo* expansion of cells.⁹³ Other possible drawbacks of heparin include (a) complexation of neurotrophic growth factors,²⁸¹ impairment of cell behavior,^{282,283} and we observed that it damages the fibronectin coating used in the LUHMES cell cultures.

Platelet lysis was obtained by freeze/thaw cycles to avoid addition of degranulation agents like calcium chloride that could affect the capacity to conduct further animal experiments.^{93,121} The 10-fold platelet pellet concentration, resulted in PDGF-AB, BDNF, TGF- β , b-FGF, and VEGF concentration much higher than that reported in standard PLs.^{93,94,113,125,166,284} IGF-1 was not detected in PPL, consistent with its presence in the plasma compartment.¹²⁶ We noticed a high concentration of PF4 (CXCL4) consistent with its high content in platelet α -granules.²⁸⁵ The SDS-PAGE and 2D-electrophoresis patterns confirmed the depletion in many plasma proteins, but still revealed the complex composition of the PPL proteome.²⁸⁶

Several recombinant single GFs have been demonstrated to possess neuroprotective activities. PDGF is widely expressed in the CNS where it exerts functions in modulating synaptic transmissions and promoting neuronal differentiation. The neuroprotective effect of recombinant PDGF-BB infused by ICV was observed in 6-OHDA rats PD model.¹⁴³ VEGF could enhance Schwann cells survival,²⁸⁷ whereas impairment of VEGF induction caused motor neuron degeneration.²⁸⁸ Recombinant VEGF-B₁₈₆ administered by intra-striatal infusion leads to the protection of dopaminergic neuron in the striatum and the SN in 6-OHDA rats.¹⁴⁶ BDNF,¹⁴³ bFGF,¹⁶² TGF- β ¹⁵⁶ present in brain exert neuroprotective functions in dopaminergic neurons in SN against MPTP intoxication. Also, TGF- β exhibits a synergistic neuroprotective effect combined with GDNF to in MPTP mice model.¹⁵⁷ However, all single recombinant neurotrophins evaluated so far have failed in clinical trials.^{226,227} This

led us to hypothesize that the pleiotropic combination of neurotrophins/growth factors in platelet lysates may represent an alternative way for effective neuroprotection.

Our cell experiments first established that PPL was not toxic to LUHMES cells at the doses tested. MPP⁺ neurotoxicity on LUHMES cells was consistent with previous studies.²³⁴ MPP⁺ neurotoxicity was strongly prevented by 1 hr-pre-treatment of the LUHMES cells by 2-5% PPL. PPL prepared from fresh PC (0 day after blood collection), 3, 6 or more than 6 days exhibited neuroprotective effect, even though there was a reduction when using the PPL prepared from 6-days old PC.

We decided to evaluate the impact of a heat treatment of PPL as our previous experiments had shown its benefits (as applied to a non-concentrated platelet lysate suspended in plasma) for the *ex vivo* expansion of corneal endothelial cells.²⁸⁹ Heat-treatment at 56°C for 30 min, in particular, was associated to a significant decrease in the total protein content, thereby complying with our objectives to decrease the protein load. Interestingly, heat-treatment led to a neuroprotective activity similar to PPL but at much lower concentrations, suggesting the removal of inhibitory factor leading to enhancement of neuroprotective activity. Heat-treatment at 56°C for 1 hour in plasma-derived eye drops rich in GFs has been shown to dramatically decreased the IgE content as well as the complement activity, while retaining also, the biological activity in the protection of the ocular surface cells.²⁹⁰ Moreover, our platelet lysate heat-treated at 56°C for 30 minutes was capable to maintain the typical hexagonal morphology of corneal endothelial cells with good adhesion properties, and to maintain the expression of important membrane markers such as Na-K ATPase, zona occludens-1, phospho-connexin 43 and N-cadherin in corneal endothelial cells.²⁸⁹ Substantial differences in protein composition were indeed found when comparing heat-treated and non-heat-treated PPL by electrophoreses and cytokine array. In particular, we noticed that PDGF isoforms, which promote survival of dopaminergic neurons,²⁹¹ were enriched in relative terms compared to the total protein content, after heat-treatment. By contrast, angiogenin, which is neuroprotective in a mutant superoxide dismutase 1 mice model of ALS,²⁹² and on

neuroblastoma cells²⁹³, decreased to approximately 10% of initial levels after 65°C treatment. EGF, BDNF, and bFGF were decreased by heat-treatments, possibly suggesting a limited neuroprotective action in this model of PD disease. However, it is conceivable that these growth factors play a critical role in models convenient for other neurodegenerative diseases.

We also focused on the presence of PMPs in PPL as they have been recently shown to induce angiogenesis and neurogenesis after stroke, and also to promote proliferation and differentiation of neural stem cells.^{215,219} PMPs present the capability to achieve cell-to-cell communications and deliver platelet-borne bioactive molecules including GFs, cytokines, micro RNA, etc.³⁷ Interestingly, PPL and HPPL were found to contain a high amount (approximately 1×10^{12} /mL) of PMPs as detected by NTA. We therefore tested whether PMPs exhibit neuroprotective effect in the LUHMES model against MPP⁺. In our studies, both NPMP and TPMP protect dopaminergic cells against the neurotoxin, therefore possibly playing a (partial) role in the neuroprotective activity of PPL and HPPL. I.n. administration of stem cell have been suggested to provide an alternative strategy for the delivery of therapeutic molecules to the CNS,²⁹⁴ but accumulating evidence suggest that the regenerative effect is due to the paracrine actions of mesenchymal stem cells associated to their content in cell-derived microparticles/microvesicles.²⁹⁵ This indicates that microvesicles secreted by stem cells can be a landmark in the treatment of neurodegenerative diseases, as they exhibit substantial advantages over cell-based therapeutics. The neuroprotective effect specifically associated with the PMPs present in PPL and HPPL should therefore be further evaluated.

Another target was to control the lack of pro-inflammatory activity of PPL and HPPL. Indeed, neuroinflammation, such as microglia activation, plays an important role in neurodegenerative disorder.²⁹⁶ Therefore we tested PPL and HPPL impact in a BV2 microglia cell line model. Our results suggested that both platelet lysates did not trigger microglia inflammatory proteins, including COX-2 and iNOS, expression under LPS pressure compare to LPS stimulation alone. In addition, an anti-inflammatory action against COX-2 was found under LPS stress. It should be noted that no acute

toxic impact was observed by Dr. Devos's group when they delivered PPL by ICV to mice.

Recently, i.n. delivery is attracting attention as it is a non-invasive treatment. Evaluating this mode of delivery was important as it is generally recognized that GFs present in platelet lysates can possibly reach the brain along the olfactory nerve or trigeminal nerve to olfactory bulb (OB) or brainstem across the epithelial barriers and nasal mucosa through:

- (a) intracellular pathways to olfactory sensory neuron or trigeminal ganglion cells, or
- (b) by paracellular and transcellular pathways
 - (i) entry into the blood vessels to reach circulation, or
 - (ii) entry into the lymphatic vessels to reach cervical lymph nodes, or
 - (iii) direct entry into the cranial compartment to reach cerebrospinal fluid (CSF) and distribute in brain different areas.²⁹⁷

One aspect to consider is that i.n. delivery is thought to be less effective in humans than in rodents. Nevertheless, there are several clinical trials on going in humans using i.n. administration to deliver stem cells, insulin and glutathione in order to cure neurodegenerative diseases. Other studies are on-going in AD pathologies where large proteins, such as anti glutamate immunoglobulins (160 kDa), are administered by i.n and restores the learning ability in rats intoxicated by β -amyloid protein A β ₂₅₋₃₅.^{297,298} A recent study in rodent shows that GFs from a platelet lysate suspended in plasma could reach brain after i.n. delivery.²³³ In our experiments, the mice were subjected to HPPL i.n. administration. We observed a significant accumulation of PDGF-AB (27-31 kDa) in mice olfactory bulb, striatum and cortex as well as that of PF4 (11 kDa) in mice cortex. Inducing neurotoxicity in animals using MPTP is thought to represent a satisfactory model of PD animal, explaining why numerous studies have used this model to test various neuroprotective strategies.²⁹⁹ Therefore, in our study, using the MPTP intoxicated mice model is helpful to reveal processes or mechanisms involved in the pathogenesis of PD, as well as potential therapeutic strategies. HPPL i.n. delivery was found to protect dopaminergic neurons in both SN

and striatum against MPTP, as evaluated by Western blot and IHC analysis. I.n. delivery of autologous HPPL (or PPL) could be a practical option for use by patients at early stages of neurological disorders. Moreover, microglial activation, a neuroinflammation phenomenon, has been described in the brain of PD patients³⁰⁰ and in the MPTP model.²⁵⁶ Decreasing microglia activation is considered to favour neuroprotective effects in MPTP mice.²⁵⁶ Our studies have shown that i.n. administration of HPPL to mice intoxicated with MPTP can decrease the up-regulation effect of Iba-1, a marker of microglia activation, that was seen following MPTP administration.

The other possible way to prepare HPPL is by using allogeneic PC donations as source material. Therefore viral inactivation steps have to be introduced during preparation. HCV was used as a model as this virus is considered as being pathogenic worldwide. In addition, HCV is a relevant virus since its RNA can be associated with CNS tissue in infected individual and HCV is known to induce central nervous system abnormalities including cognitive dysfunction. Therefore it is important to make sure that patients with neurodegenerative disorders would not be exposed to the risk of HCV through ICV or i.n. delivery of PPL. The development of highly standardized platelet lysates for regenerative medicine will almost inevitably go through the preparation of products from pools of platelet concentrates to limit individual donors variations. To date, pooled hPL for use as supplement for *ex vivo* expansion of stem cells and other cells are produced from mixing 40 to 50 therapeutic PC, corresponding to donations from up to close to 250 donors.¹²¹ An inherent risk associated with larger pooling of blood donations leads to higher probabilities of contamination by blood-born pathogens,²⁶ and therefore potential infections of recipients.

Safety nets that are currently in place to improve the margin of safety of single-donor or small-pool blood products include epidemiological control of donor population, screening of potential donors at individual level and testing of donations for known serological viral markers (anti-HIV, anti-HCV, HBsAg, and others based on national legislations). Nucleic acid testing (applied on individual donations or “mini-pools”) is performed in some countries in order to decrease the risk of false-negative

“window-phase” donations: targeted viruses include HIV, HBV, HCV, HAV, parvovirus B19, and more recently, in reaction to emerging infectious, WNV, Dengue virus, or Zika virus. However, it is widely admitted that the most reliable means to ensure virus safety reside in the implementation of dedicated virus/pathogen reduction treatments. Viral safety of allogeneic platelet lysates for regenerative medicine can be addressed at two distinct, and potentially complementary, levels:

- Pathogen inactivation treatments of the starting platelet concentrates are now available and started to be licensed in several European countries, and in the last two years in USA.¹⁸ Methods in place generally rely on UV-photoinactivation with or without addition of a photosensitizer, such as sporalen or riboflavin.^{301,302} A short wave UVC method is under development in France.³⁰³ Wider implementation of these technologies for transfusion purposes will generate more pathogen-reduced source platelet concentrates for use in regenerative medicine or cell therapy. Whether these materials could also be used in all or some fields of regenerative medicine is largely unknown, but their capacity to be used expanding BM-MSC,^{304,305} islet cells for transplant³⁰⁶ and human haematopoietic and epithelial cell lines³⁰⁷ has been found. Similarly, HPL made from short-wave UV-treated PC could expand BM-MSC.³⁰⁸
- Viral reduction treatment during the processing of platelet lysates following the model used in the industrial plasma fractionation industry. Our laboratory has already demonstrated that it is possible to submit PC to a S/D treatment in order to provide more efficient extraction of growth factors in the lysate through lipid membrane disruption,¹²⁶ as well as viral inactivation. The S/D-platelet lysate could successfully be used to isolate and expand AT-MSCs.³⁰⁹ However, capacity of the treatment to inactivate HCV or other lipid-enveloped viruses has not been shown. No other large-scale viral inactivation treatment has been studied yet.

In our work we have evaluated for the first time the capacity of applying two types of virus reduction treatment, S/D and nanofiltration, to platelet lysates intended for use in regenerative medicine. We have tested the possibility of the heat-treatment of PPL at 56°C for 30 min to inactivate HCV used as a model pathogenic virus.

S/D treatment:

Our initial study was intended in part to demonstrate the possibility to use a new HCV infectivity model³¹⁰ to assess the efficacy of a S/D treatment using a combination of 1% TnBP and 1% Triton X-45 to inactivate HCV in blood products. So far it has been very difficult to establish an *in vitro* infectivity assay for HCV. Plasma was used as a test material as it is known that S/D treatment of plasma is technically achievable.²³ Results demonstrated (a) the feasibility of using this infectivity model to assess a viral inactivation treatment applied to a complex protein mixture like plasma and (b) the capacity of this S/D treatment to inactivate HCV efficiently (> 2.8 logs).³¹¹ These findings confirmed our previous studies that S/D treatment could also inactivate another flavivirus, Dengue virus.²⁶⁵

Based on this data, additional experiments were done to verify the capacity of the same form of S/D treatment to inactivate HCV in the dedicated PPL preparation we developed to treat neurodegenerative disorders. Data confirmed the ability of the S/D treatment to inactivate HCV, and presumably other lipid-enveloped viruses, when applied to treat PPL. This represents the first demonstration of the capacity to apply S/D treatment to a platelet lysates for regenerative medicine applications, in particular in the potential treatment of neurodegenerative diseases.

Heat-treatment:

Traditional heat-treatments used in the plasma fractionation industry for inactivation of viruses are typically performed either on protein solutions or on freeze-dried preparations.

Most liquid heat-treatments, such as those carried out on coagulation factor or protease inhibitors concentrates, are performed at a temperature of 60°C for 10 hours but required the addition of stabilizers to protect proteins from denaturation.^{26,30} However, alternative conditions have been described where a temperature of 50°C for 3 hours has been used to experimentally inactivate various viruses in whole plasma efficiently.³² As the neuroprotective activity of PPL was increased by heat-treatment at 56°C for 30 min without stabilizers, we decided to verify whether this step could also contribute to inactivate HCV. The data revealed that, rather unexpectedly, a significant and complete inactivation of HCV was achieved. More than 2.5 logs of HCV were inactivated over the duration of the heat-treatment of PPL. Therefore this 56°C heat treatment of PPL without stabilizers can be considered as being a dedicated step of inactivation of at least some plasma-borne viruses such as HCV. Further studies would nevertheless be needed to verify whether these conditions could be sufficient to inactivate or at least decrease the infectivity of non-enveloped viruses, as well as resistant lipid enveloped viruses. Prior studies showed that treatment of plasma at 50°C for 3 hours in the presence of a complex mixture of stabilizers can inactivate over 6.6 log of HIV, and over 4 logs of model viruses of HCV and herpesvirus. In addition, the inactivation was > 5 logs for poliovirus (model for HAV), 4.6 log for reovirus type 3, and 3.2 log for bovine parvovirus, a model for human parvovirus B19. Therefore at least a partial inactivation of similar viruses could be expected when the heat-treatment is conducted at higher temperature and without stabilizers as applied to PPL.

Nanofiltration:

Nanofiltration is now a routine procedure used in the plasma fractionation industry to ensure a higher level of viral safety to immunoglobulins, coagulation factor, and protease inhibitor concentrates²⁹ However, nanofiltration of complex platelet lysate fractions on small pore size membranes of 15-35 nm to trap viruses^{29,267} would likely be challenging because high protein content and viscosity may impair filterability. Still, in our study, we however could demonstrate for the first time that it is technically achievable to nanofilter a heat-treated PPL fraction, depleted of plasma

proteins and heat-unstable proteins such as fibrinogen on a 20-nm membrane. It is very likely that the low protein content of HPPL together with the depletion in fibrinogen and other molecular mass proteins contribute to facilitate unexpectedly the nanofiltration process. Furthermore our data show that, as expected, 20-nm nanofiltration allows the removal of ≥ 2.5 log of HCV. This data are consistent with the experience developed with nanofiltration methods that demonstrates the robustness of this procedure to remove viruses by a size-exclusion mechanism.^{28,29} Capacity to implement nanofiltration of HPPL on 20-nm filters is an important step in viral safety in the hypothesis of producing this platelet fraction from large pools of HPPL. The impact that nanofiltration can have on neuroprotective activity and safety of HPPL deserves attention as this step also removes PMP that can have beneficial or detrimental impacts with regards to thrombogenic/inflammatory activity, on one side, and regenerative/neuroprotective roles on the other side, as we have found.

Other methods of virus reduction are unlikely to be amenable to processing of HPPL. Pasteurization (defined as a heat treatment in the liquid state at 60°C for 10 hours, typically in the presence of stabilizers) may be feasible but usually needs to be carried out in the presence of high amount of stabilizers. Low pH treatment or caprylic acid incubation, that are other known viral inactivation methods, are likely non-applicable to PPL due to risks of protein denaturation or precipitation of many proteins. Recently, our laboratory has shown that pure platelet lysates (suspended in plasma depleted of fibrinogen by CaCl_2 activation) cannot be filtered on a new hollow fiber anion exchange membrane (QyuSpeed D; QSD) thought to have the capacity to remove pathogens by partitioning. However, growth media supplemented with 10% platelet lysate removed $\geq 5.58 \log_{10}$ and $\geq 3.72 \log_{10}$ of porcine parvovirus and the 263K prion strain of hamster-adapted scrapie, respectively without affecting expansion capacity of various cells.³¹² Whether HPPL could be run through this QSD device has not been tested but remains conceivable considering the lower protein content than standard platelet lysates.

Therefore our studies revealed more than 2.5 log reduction of HCVcc by heat treatment, S/D-C18 and 20 nm nanofiltration. Importantly, the neuroprotective

activity of the lysate subjected to heat treatment, S/D (in PPL), then nanofiltration was not significantly affected.

During our work, all experiments have been achieved using a PPL resuspended in PBS buffer to maintain/normalize the pH during heat treatment. However, within the context of developing a product for clinical application, it is required to suspend the PPL in an already approved pharmaceutical fluids such as physiological saline. Our data show that heat treatment of PPL formulated in saline maintains the neuroprotective activity in the LUHMES *in vitro* model.

Altogether our study proves evidence that a platelet lysate containing concentrated neurotrophic growth factors and depleted of plasma proteins can induce strong protection of dopaminergic neurons in a cellular model of PD. Our data are in agreement with recent findings showing that brain delivery of platelet lysates enhances neurogenesis in an animal model of stroke,²³¹ and that intranasal administration of platelet lysates in an AD-disease mouse model improves neuropathological parameters and cognitive functions.²³² In light of these findings, further studies could be conducted to understand the mechanistic role of platelet lysate components, most likely neurotrophins, but also other components, such as neurotransmitters or PMP, on the neuroprotective signaling cascades.³¹³⁻³¹⁵ Such data would support a possible therapeutic role of platelet lysates as novel “disease modifying strategy” of neurodegeneration.

PERSPECTIVES

Our work has shown the possibility to use platelet concentrates, non-leukoreduced, and suspended in plasma to isolate a platelet lysate fraction depleted of plasma proteins and fibrinogen, with a low total protein content, a high content of neurotrophins and PMPs, that can be compatible with ICV administration. The introduction of a heat-treatment at 56°C for 30 min contributed to further decrease the total protein content, without decreasing the PMP content, while improving the neuroprotective effects *in vitro*. This preparation was devoid of inflammatory effect *in vitro* using microglial cells and did not exert acute toxicity during ICV administration in mice. The capacity to deliver the HPPL i.n. was shown with a demonstration of the migration of PDGF-AB and PF4 to various brain areas, and with a protection of the TH markers on dopaminergic neurons. In view of the hoped development of allogeneic, large-pool HPPL in the future for the treatment of neurological diseases, we have developed an easily scalable process, in which 3 steps (heat-treatment, S/D treatment, 20-nm nanofiltration) can be introduced to ensure a high margin of safety against blood-borne viruses.

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