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Plasma Medicine

Concepts and Clinical Applications

Edited by Yusuf Tutar and Lutfi Tutar

PLASMA MEDICINE - CONCEPTS AND CLINICAL APPLICATIONS

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http://dx.doi.org/10.5772/intechopen.68652
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Contributors

Mikel Sánchez, Nicolás Fiz, Juan Azofra, Jaime Oraa, Ane Garate, Pello Sánchez, Diego Delgado, Sabino Padilla, Ane Miren Bilbao, Jorge Guadilla, Beatriz Aizpurua, Ramune Sepetiene, Raminta Sidlauskiene, Vaiva Patamsyte, Zilan Xiong, Kazuo Shimizu, Jaroslav Kristof, Jean Filipov, Emil Dimitrov, Borelli Zlatkov, Jesus Alcaraz, Marina Viegas Moura Rezende Ribeiro, Êurica Adélia Nogueira Ribeiro, Luiz Eduardo Feliciano Ribeiro

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First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Plasma Medicine - Concepts and Clinical Applications
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p. cm.

Print ISBN 978-1-78923-112-0

Online ISBN 978-1-78923-113-7

eBook (PDF) ISBN 978-1-83881-338-3

Meet the editors

Prof. Dr. Yusuf Tutar is currently working at the University of Health Sciences, Mekteb-i Tıbbiye-i Şahane, Istanbul, Turkey. He obtained his MSc and PhD degrees from the Oregon State University and Texas Tech University, respectively. He pursued his postdoctoral studies from the Rutgers University Medical School and National Institutes of Health (NIH/NIDDK), USA. His research mainly focuses on biochemistry, biophysics, genetics, and molecular biology with a specialization in the fields of prion, drug design, cancer, protein structure and function, protein folding, microRNAs, pseudogenes, molecular cancer, proteomics, genomics, and protein expression and characterization by the spectroscopic and calorimetric methods.

Dr. Lütfi Tutar is currently an assistant professor at the Department of Molecular Biology and Genetics, Faculty of Art and Sciences, Ahi Evran University, Kırşehir, Turkey. His interdisciplinary research focuses on bioinformatics analysis of high-throughput data, microRNAs, small RNAs, and heat-shock proteins (HSPs) in human diseases and other multicellular organisms.

Contents

Preface

Plasma can be defined as the extracellular matrix of blood cells. Plasma components, their role in human health risk evaluation, and their functional and clinical analyses are covered in this comprehensive book.

The first section explains the handling and processing of samples along with the meaning of the test results. The second section in clinical practice covers the different applications of plasma. The use of platelet-rich plasma in dry eye disease and in orthopedics and sports medicine for therapeutic strategies is reviewed. The other two chapters cover plasma exchange and growth factors in clinical practice.

The third section deals with the physical plasma-ionized gas, which is one of the four fundamental states of matter. This homonym has begun to emerge because it can interact with living systems. The physical plasma biomedical applications are reviewed in drug delivery and wound healing medical applications. This approach revolutionizes the therapeutic approaches in medicine and may open up new concepts and clinical applications. The book is an essential source for researchers in the field and provides a platform for different professions.

Yusuf Tutar
University of Health Sciences
Istanbul, Turkey

Lutfi Tutar
Ahi Evran University
Kırşehir, Turkey

Section 1

Plasma for Diagnostics

Chapter 1

Plasma for Laboratory Diagnostics

Ramune Sepetiene, Raminta Sidlauskiene and Vaiva Patamsyte

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76092

Abstract

Main clinical and scientific aspects of methodology how to choose the right sample for testing to be involved in *Plasma for Laboratory Diagnostics* chapter as plasma is in use sufficiently widely. Different approach by clinical and research laboratories formed the necessity to discuss the basic laboratory terms and conditions to obtain correct results. Evaluation of preanalytical variables with impact on handling, processing and storage of samples with subsequent meaning for laboratory test results should be known by physicians, biomedical scientists, laboratory technicians and so on. The impact of chemical additives for diagnostics tools, the differences between plasma and serum samples for different laboratory tests and the subsequent analysis, management and traceability of specimens with standardisation value should be taken into account.

Keywords: plasma, laboratory, preanalytics, diagnostics

1. Introduction

Summarised results for *Plasma application for laboratory diagnostics* in one chapter for qualitative testing to be called "*Handbook.*" The understanding of laboratory phases, mainly preanalytics, is necessary both for clinical and research laboratories to obtain, compare and repeat or reproduce analysis. Main standardisation requirements are taken from clinical laboratories, and their approach should be useful for researchers to plan, organise the scientific work fulfilling their research interest. This is the first attempt to improve research by the requirements for clinical laboratories.

2. Challenges and limitation for plasma use in laboratory testing

A literature *review*, *metaanalysis and grounded theory* of known methods and obtained results in laboratory practice worldwide with value of quality assurance are taken. The suppositional contents are as follows.

2.1. Preanalysis. Factors before testing

Human blood with additives for plasma preparation is a subject for variables, affecting preanalytical process, which takes app 50% of total laboratory turnaround (see **Figure 1**).

The preanalytical phase is divided into *outside laboratory phase* and *inside laboratory phase* for samples processing. There are no standards defining the quality of preanalytical phase. Known and following criteria should be taken as quality criteria in each individual laboratory. See **Table 1**.

Quality of the sample must be desired through all laboratory phases, starting from the preanalytical, which covers more than 50% of the total laboratory investigation circle [1].

2.2. Influence of unavoidable factors

Unavoidable factors such as age, race, gender and pregnancy are important considering clinical chemistry and haematology subjects for testing and interpreting results. Biochemistry variables for serum iron [2], CK and creatinine [3] are always taken into account by gender performance; other various substrates and enzyme activity or concentration (uric acid, bilirubin, alkaline phosphatase, etc.) are taken into account depending on age. Race differences should

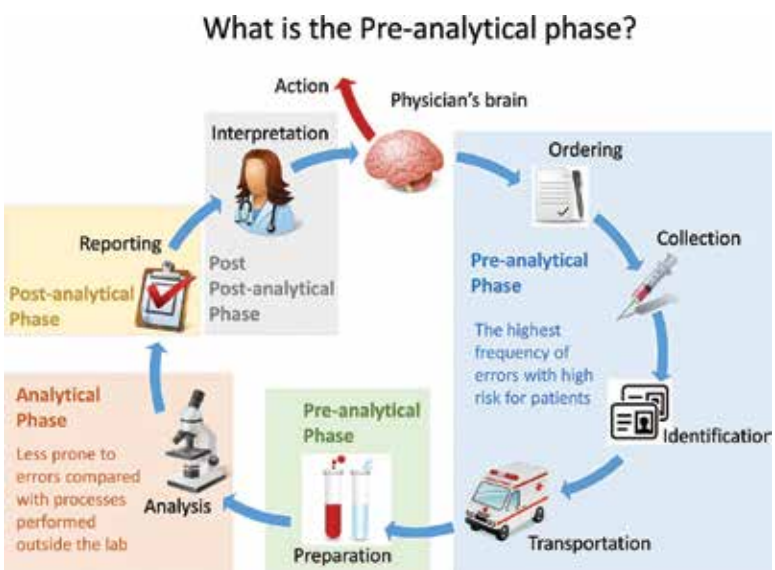

Figure 1. Preanalytical phase.

Process	Importance	Remarks for materials
Outside laboratory phase		
Unavoidable factors	Age, race, gender, pregnancy	Fill request form properly
Avoidable, variable factors	Caffeine, smoking, alcohol, drugs	Ask patient and mark
Patient preparation	Diet, starvation, exercise, altitude	Ask patient and mark
Preparation of sampling	Define and enter request into system, proper tube labelling	Request form and information system are mandatory. Patient and sample identification procedures
Sampling process	Patient's ID, timing, use of tourniquet, site of sampling selection, position of needle, run order of tubes	Use of tubes, needles, disinfection materials strongly according procedures.
Transportation	Difference of collecting and transporting tubes	Transporting containers, cooling systems according procedures
Laboratory phase		
Sample treatment	Registration, identification, centrifugation, distribution, extraction	Identification and registration procedures, authorised and secured laboratory information system
Sample/specimen storage	Selection of site and temperature, timing, utilisation	Storage and freezing devices with temperature control according procedures

Table 1. Preanalytical phase and its importance for quality testing.

be evaluated for blood count in haematology and enzymology within clinical chemistry, while the mean variable for plasma volume changes is pregnancy. During healthy pregnancy plasma volume increases by an average of about 1250 ml, with progressive increase up to 35th week of gestation, after which little or no further increase occurs. The frequently observed fall in plasma volume in the last 6 weeks of pregnancy is a false of measurement due to poor mixing of tracer when the woman lies supine, and this posture obstructs the circulation to the lower limbs [4]. The differential changes are biologically plausible. Erythrocytes mass rises proportionately to the need to carry the extra oxygen necessary in pregnancy [5]. The greater plasma volume is needed to cope with the very large increases in blood flow to organs which require different amount of extra oxygen. Increased plasma volume is greater than the increase in red blood cell mass, thus there is a fall in haemoglobin concentration, haematocrit and red blood cell count. It is worth to mention that despite this haemodilution, there is usually no change in mean corpuscular volume (MCV) or mean corpuscular haemoglobin concentration (MCHC) [6].

2.3. Meaning of variable subjects for plasma specimens

The variable diet, starvation, exercise are more important for serum analytes in clinical chemistry. Triglycerides, aspartate aminotransferase, bilirubin, glucose are very sensitive to diet and drinking habits. Starvation reduces blood cholesterol, triglycerides and urea concentrations. In contrast, creatinine and uric acid are elevated after long time (4 weeks) starvation period. Changes may occur to an increase in reabsorption of the measured analyte or metabolism changes [1].

Enzymes, such as pyruvate kinase, creatine kinase are raised from 2.5 to more than 4 times after a marathon rise. Sodium and potassium measurable in plasma after the same marathon rise elevate only by one fold. To reduce possible misinterpretation of laboratory findings, sampling after 12 h fasting and normal activity is highly recommended to ensure preanalytical procedures [1].

Significant changes may occur in blood at high altitude. β_2-globulin and C reactive protein (CRP) may rise 43–65%. Ht and Hb are less sensitive—they increase only by 8%. The opposite phenomenon with decreasing values is demonstrated at plasma osmolality, plasma renin analytes [7].

Studies concluded that caffeine, smoking, alcohol or drugs intake may either decrease or increase different analytes [8]. Thus, it is highly recommended for blood sampling—the early morning before coffee, cigarette or other intakes were eliminated.

2.4. Interference factors. Lipaemia, haemolysis

Haemolysis is defined as the release of blood cells content into plasma or serum. The colour intensity depends form haemoglobin released from erythrocytes. Sometimes this can occur due to platelet and or granulocyte lysis. An effect of haemolysis is classified according to the different mechanisms: increase of intracellular contents extracellularly; optical interference due to the haemoglobin colour; interference by intracellular contents with the different mechanisms of reaction. Laboratory should document haemolysed samples procedures as the responsibility of the laboratory diagnostics results belongs from the relevant interpretation of events [1].

After haemolysis, lipaemia is the most frequent interference factor that can influence results of clinical laboratory methods. Plasma or serum should be always considered due to varying degrees of an increased lipoprotein content. Turbidity is caused by an increased triglyceride concentration, which may variate from slight to milky, and these samples are called lipaemic. The most common preanalytical cause of lipaemia is short time from meal taken or parenteral administration of lipid emulsions to blood sampling. The best way to detect the degree of lipaemia is measuring lipaemic index on analytical platforms. Laboratory staff should keep preanalytical procedures under control. Unlike for other interferences, lipaemia can be removed, and measurement can be done in a clear sample, using a protocol for removing lipids. On the other hand, sample has to be chosen carefully, since its dependency on the analytes that have to be tested [9].

2.5. Plasma or serum samples *in vitro* diagnostics. Advantages and disadvantages for use

Scientists and clinicians are still on debates—what type of sample a laboratory should use. Serum is still considered to be the gold standard remaining the required sample matrix for some biochemistry, immunology assays.

Both plasma and serum are liquid parts of the blood. The main difference between them performs a clotting process. Plasma specimen is prevented from clotting and is more reflective of the systemic blood circulation in the body.

There are diagnostically relevant differences between the results obtained from serum or plasma in laboratory. This happens due to several physiological and technical reasons:

- clotting factors: fibrinogen, platelets, glucose affecting analyte;
- analytes (potassium, lactate dehydrogenase, phosphate, ammonia, lactate, neurone-specific enolase, dopamine and serotonin) may be released from the cells during clotting process;
- the anticoagulant may interfere with the assay or contaminate with its cations: Lithium (heparinate) with flame photometry, when calibrated with lithium [1, 10].

There are different tubes for plasma or serum collection (see **Figure 2**) and sometimes different applications due to the variable pathophysiological effects of platelets and clotting process to be involved for laboratory findings [11] (**Table 2**).

It is necessary to perform a rapid centrifugation to obtain more stable plasma. The extended contact with blood cells is complexable event including particles of the cells and metabolites after DNA, proteins degradation [12]. Thus, an immediate plasma centrifugation and extraction is desirable ASAP after blood collection.

There are proposed various methods and solutions for miniaturised blood plasma extraction. Macro-scale depending on the desired sampling volume: blood transfusion volume (500 ml), analytic venous sample (1–50 ml), or blood droplet from a finger-prick (up to 200 μl). Whatever the blood volume processed, the two conventional mechanisms—centrifugation or filtration—exploited for plasma separation at the macroscale remain as necessity. Different solutions are

Figure 2. Vacuum tubes for plasma collection. Images reproduced by kind permission of Becton, Dickinson and Company, All rights reserved. Unless otherwise noted, BD, the BD Logo and all other trademarks are property of Becton, Dickinson and Company.

Functions	Plasma	Serum
Time saving	✓	
Higher yield/specimen volume	✓	
Prevention of clotting effect	✓	
Prevention of changes induced with coagulation process	✓	
Contamination with NH_4^+, Li^+, Na^+, K^+		✓
Inhibition of metabolic reactions by heparin		✓
Interference of ions distributed intra- and extra-cellular space		✓
Inhibition of enzymes by metal binding to EDTA and citrate		✓
Binding of ionised calcium to heparin		✓

Table 2. Advantages (marked) and disadvantages of plasma and serum (according Guder et al., 2016) [26].

available for miniaturised blood plasma extraction within three main formats: the microfluidic chip format; the CD format and the paper format, based on different resources [13].

Remarks for testing:

Time saving. Serum sample must be allowed to clot, this time is variable from 10 min to 30 min and even longer.
Higher specimen volume or yield. Up to 20% more plasma than serum can be yielded after centrifugation.
In general, serum is used widely for the serological diagnosis of infectious diseases. There are some tests, that is, complement fixation or bacterial agglutination tests, where serum must be used only.

2.6. Types of plasma samples. Additives for tubes

The colour codes of anticoagulants are described in ISO/CD 6710: EDTA = lavender/red; citrate 9:1 = light blue/green; citrate 4:1 = black/mauve; heparinate = green/orange; no additives (for serum) = red/white (ISO 6710). To obtain cell-free plasma for laboratory use, the anticoagulated blood should be centrifuged for at least 15 min at 2000–3000 × *g*, temperature should be set from 15 to 24°C [1].

2.6.1. EDTA

A salt of ethylene tetraacetic acid. Dipotassium (K2), tripotassium (K3) and disodium (Na2) are used. EDTAK2 or K3 blood is used in haematology and considerations which one additive to use are still under debates. The cell volume measured after centrifugation, decreases when concentration of this anticoagulant is higher and this is mainly seen with the tripotassium (K3). This fact has been reported by different authors due to erythrocytes dehydration in hypertonic medium. Despite automated analysers work differently, but their MCV is not affected by K3-EDTA concentrations up to 10 times normal, while high concentrations of K2-EDTA, result a slight MCV increase [14, 15].

2.6.2. Citrate

Trisodium citrate with 0.100–0.136 mol/L citric acid. Buffered citrate with 5.5–5.6:84 mmol/L trisodium citrate with 21 mmol/L citric acid. The International Society for Thrombosis and Haemostasis (ISH) recommended to use the Hepes-buffered citrate for all investigations of haemostasis. A mixture of one citrate part with nine parts of blood is recommended for coagulation tests (ISO 6710), see: plasma for coagulation testing. A mixture of one citrate part with four parts of blood for ESR testing.

2.6.3. Heparin

12–30 IU/mL of sodium, lithium or ammonium salt of heparin, with 3–30 kDa of molecular mass. Calcium-treated heparin with concentration of 40–60 IU/mL of blood (dry heparinisation) and 8–12 IU/mL of blood (liquid heparinisation) are in use for ionised calcium determination.

2.6.4. Specificity for glucose samples (grey cap) for blood transporting stability only

Additives: potassium oxalate and sodium fluoride or sodium fluoride/Na2 EDTA or sodium fluoride (no anticoagulant, will result in serum sample).

It is worth to follow CLSI recommended Order of Draw [16].

Anticoagulants are useful to inhibit blood or plasma from clotting ensuring the quantity of additive has no impact for following analysis. Anticoagulation effect is achieved by binding calcium ions or by inhibiting thrombin activity. The very important step is mixing ensuring effective distribution of solid or liquid anticoagulant within the whole blood.

2.7. Plasma for coagulation tests

Coagulation tests (routine and special) in modern laboratory require a lot of knowledge, continuous education, sensitive automatic analytic system, broad spectrum reagents with different purpose, quality manual and high-level communication with clinicians, phlebotomists and nurses with preanalytics algorithms detailed description or specimen collection and handling instructions (**Table 3**).

The sample types for coagulation tests to use in laboratory medicine are: platelet rich plasma (PRP), platelet poor plasma (PPP) and whole blood (**Figure 3**).

PRP is a component of blood (plasma) with concentrations of platelets above normal values. PRP typically contains 3–8 times more platelets concentration than normal plasma. PRP is used for platelet function assays (diagnostics constitutional and acquired thrombopathy, follow-up on anti-platelet treatment) and in a variety of clinical applications, based on the premise that higher content of platelet-derived growth factors should promote better healing. Platelet derivatives represent a promising therapeutic modality, offering opportunities for treatment of wounds, ulcers, soft-tissue injuries, and various other applications in cell therapy [17].

Specimen	Tests	Advantages	Limitations	Centrifugation
PPP	PT, aPTT, fibrinogen, single coagulation factors assays	Representative of circulating blood	Common preanalytics	One step: 1500–2000 × *g* 10–15 min
PRP	Platelet function assays	Two aims possible: (1) Diagnostics (2) Therapy	Sensitive for handling	Two steps: 1st: slow centrifugation: 170 × *g* calculated at the interface blood-plasma for 10 min to eliminate a red blood cells contamination. 2th: fast centrifugation: 2200–2400 × *g* for 20 min. Centrifugation at 4000 rpm
Whole blood	Platelet reactivity tests	Centrifugation is not required, the tests may be performed quickly. This sample type is used for POC analyser often.	Red and white cells can impact on the test results	Without special preparation

POC, point of care.

Table 3. Samples for coagulation.

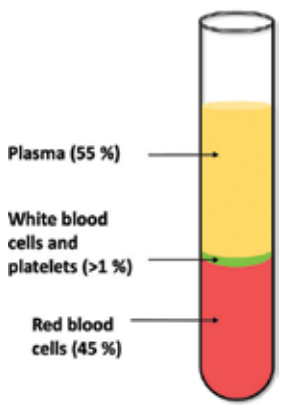

Figure 3. Plasma for coagulation tests.

Trueness of results in modern haemostaziology within modern laboratories depends on three major issues:

- Management preanalytics in the pre-laboratory phase and laboratory phase
- Sensitivity and possibilities of analytical system
- Interpretation of tests together with clinical data (clinical condition, anticoagulants, anti-platelet treatment)

http://dx.doi.org/10.5772/intechopen.76092

2.7.1. Some preanalytics aspects

Though one mistake in preanalytics may distort the result and clinical interpretation may be wrong.

2.7.1.1. Clot in tube for coagulation tests?

The clot can be formatted *in vitro* in sample via this reasons:

- Slow blood flow into the tube
- Long pressure of tourniquet
- Significant (not acceptable for good practice) manipulative procedures with needle in vein
- Not enough mixing specimen with anticoagulant right away after blood drawing.

2.7.1.2. Serum or clotted sample?

If primary tube is taken inside laboratory, it is not difficult to determine whether blood was taken with proper anticoagulant. But errors may occur when samples get into with secondary tubes. It is not possible to state if proper anticoagulant or serum was taken instead of plasma. A serum does not has fibrinogen or other coagulation factors (FII, FV or FVIII) and high molecular weight of VWF as well. Serum analysis can give high levels of some factors (FVII) due to their activation and coagulation times testing for PT, aPTT and TT are not measured. False diagnosis can be established if plasma factor evaluation is wrong, as it often happens in the case of a certain type of Von Willebrand disease. Problems may occur with Lupus anticoagulant determination.

Modern laboratories try to provide equipment which allows to detect the clots in specimen after the detection of significantly longer clotting times, but a visual check for a clot in a sample should be recommended or use of two wooden applicators/sticks for whole blood. If a clot is found, it is necessary to reject a sample from further investigation [18].

2.7.2. Haemolysis impact for coagulation

Reasons of *In vitro* haemolysis may be due to incorrect blood drawing or inadequate handling of blood after collection. Haemolysis can occur *in vivo* when cell lysis happens inside of vessels by autoimmune haemolysis, severe infections, DIC or post-transfusion reactions.

Analytical systems with mechanical clot detection principle are not affected by the interference factors due to haemolysis, but results of tests may be not accurate because of products of cell lysis, including tissue factor which can activate coagulation. It is called a biological impact of haemolysis. Total effect of haemolysis may decrease the level of fibrinogen, but increase a level of D dimers. Prothrombin time may drop down because of lower concentration of fibrinogen; aPTT may lengthen or shorten depending which process prevails: decrease

of fibrinogen or activation of coagulation. Haemolysis may distort other coagulation tests results, for instance decrease of antithrombin level [19].

If possible, the sample with grossly haemolysis must be rejected. If tests must be performed (e.g., *in vivo* haemolysis, it is not possible to take a new sample), for this case, it is recommended to use mechanical detection systems, not excluding, that potential activation is possible as well.

Samples visually seem haemolysed via haemoglobin substitution, should not be rejected and must be analysed with mechanical or electromechanical clot detection method [20].

CLSI H21-A5:2008 Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Haemostasis Assays; Approved Guideline—Fifth Edition standard says:

> *When evident, the presence of visible hemolysis, as evidenced by a pink to red tinge to the plasma, should be noted. Lysis of red blood cells and resultant release of intracellular or membrane components may cause clotting factor activation. This activation of coagulation factors may impact clotting time results, whether using an optical or mechanical end-point detection system, although there is discrepancy in the literature about the impact of hemolysis on clot-based assays.[67,68] Until additional studies are published, due to the potential with result interference, grossly hemolyzed samples should not be used. Pink or red-tinged plasma specimens may further impair end-point detection when using an optical system, due to its interference with light transmittance. Plasma may be tinged when there is red cell lysis or when the patient has been administered a hemoglobin substitute. Samples that appear hemolyzed due to the presence of hemoglobin substitutes are not a cause of specimen rejection, and these samples should ideally be tested with an instrument that uses mechanical end-point detection.*

The best way to choose correct method when existing studies which were done with analytical systems and reagents combination and studied how haemolysis impact on routine (PT, APTT, fibrinogen) tests [21].

2.7.3. Specimen handling, storage, transportation

Transportation is a challenge of samples when specimens or samples are taken out to the external laboratories. Tubes should not be subjected to vibration, shaking, vortexing, continuous mixing or agitation. Transport and sorting as bulk goods is not compatible with reliable coagulation diagnostics. Generally, blood samples for coagulation analyses should not be shaken, and dropped samples should be discarded [1].

The transportation often causes a delay of sample analysis, so customer must be sure that specimens will be analysed in time. Blood samples for coagulation diagnostics should be stored at room temperature (20–25°C) until analysis. Storage at lower temperature, or on ice, may strongly influence some of the coagulation assays [1].

A storage of uncentrifuged samples at room temperature up to 6 h may yield acceptable results, although a shorter delay is desirable. Whole blood assays should be performed within 4 h after blood sampling. Data about handling and storage (time and temperature) of samples before transportation are different. Thus a laboratory must take decision how long the analysis may delay [16].

2.7.4. Continuous improvement of preanalytics in coagulation testing

Coagulation laboratory must regular review how to improve preanalytics in medical institution. Bostic et al. [19] studied, how possible to reduce quantity-not-sufficient specimens (QNS) via several methods and to measure effect of expired collection tubes on the amount of blood that can be drawn. During study period the rate of QNS specimens dropped from 0.7 to 0.3%. It was a significant difference in the amount of blood drawn into nonexpired vs. expired (**Image 1**).

The authors of study published a laboratory bulletin about Proper Blood Collection for Coagulation Studies [19].

2.8. Plasma for molecular-miRNR testing

Extracellular RNAs (exRNAs) found in biological samples have a potential to be used as clinical biomarkers for various diseases as well as treatment monitoring. Differences in miRNA expression profiles are associated with tumorigenesis and can be used to classify cancer type, identify the developmental origin, select and monitor treatment [22]. Due to relatively easy acquisition and handling blood plasma is an attractive specimen to be used for miRNA extraction compared to tissue samples. Even though miRNAs have been a major focus for

Image 1. Photograph of sodium citrate tubes containing blood from a single draw performed in a study period. The first three tubes from the left were expired and showed decreasing blood retrieval ability with older expiration dates (from right to left). The black arrow points to the minimum recommended fill level (etched line).

over a decade now, the majority of methodologies used for miRNA extraction and profiling come from a scientific research and are not approved for clinical use yet.

The major points affecting the miRNA extraction and application are choice of coagulant, plasma preparation and biological condition. Among the three most widely used anticoagulants (EDTA, citrate, and heparin), EDTA is shown to be the least interfering chemical in the subsequent miRNA profiling, while heparin and citrate interfere with enzymes used for various PCR [23]. Circulating miRNAs are either encapsulated in vesicles or found in complexes with proteins and lipoproteins, therefore are considered to be relatively stable. However, it is recommended that plasma preparation is done within 2 h of phlebotomy since blood cells start to release miRNAs into the collected sample causing changes in miRNA profile. Even mild haemolysis is also considered as an interfering factor associated with miRNA contamination from blood cells. Visual identification of minor haemolysis is difficult, therefore a simple measurement of absorbance can be an accurate and time saving solution. Prepared plasma samples could be quickly tested for haemolysis by measuring the absorbance peak of free haemoglobin at 414 nm [24]. Plasma samples with $A_{414} > 0.18$ show signs of miRNA released from erythrocytes which might interfere with the overall profile of circulating miRNAs [24]. It is worth noting that lipaemia affects the A_{414} measurement; therefore, it is recommended to perform a second measurement at A_{385} to detect the presence of lipaemia and use it to calculate a lipaemia-independent haemolysis score which could be adopted as a pre-analytical quality control [25]. Circulating miRNAs are often found in association with lipoproteins, therefore biological conditions, such as fasting, might have an impact on miRNA profiling [23]. The use of miRNA biomarkers in clinical setting is only starting therefore to standardise the pre-analytical procedure of sampling and sample preparation it is worth to have as little environmental variables as possible.

2.9. Quality manual for laboratory testing

A medical laboratory or clinical laboratory are laboratories for the examination of materials derived from the human body with a purpose of providing necessary information for diagnosis, prevention and treatment of disease as well as for follow up evaluation of health of a patient during the treatment. The 'International Organization for Standardization' (ISO) is an 'International Authority' for setting up standard guidelines for various organisations and laboratories. Each quality manual is based on internationally accepted standards and provides guidance for public health and clinical laboratories on writing policies and procedures that support a quality management system. It comprises a main document providing information and written procedures for laboratory quality (standard operating procedures, forms, and processes). It is worth to remember always to address quality manual before starting testing, sampling or consulting patients [24].

Acknowledgements

The chapter was supported by project "Age-related remodelling of aorta and dilatative pathology of ascending aorta: search for epigenetic biomarkers", project number: SEN-05/2016.

Conflict of interest

There is no conflict of interests.

Author details

Ramune Sepetiene[1]*, Raminta Sidlauskiene[2] and Vaiva Patamsyte[3]

*Address all correspondence to: sepetiene@yahoo.co.uk

1 Laboratory of Molecular Cardiology, Lithuanian University of Health Sciences and Clinical Diagnostics Laboratory of Kaunas City Outpatients Diagnostics Centre, Kaunas, Lithuania

2 Paliesiaus Clinic, Vilnius, Lithuania

3 Lithuanian University of Health Sciences, Kaunas, Lithuania

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Section 2

Plasma in Clinical Practice

Chapter 2

The Use of Platelet-Rich Plasma in Dry Eye Disease

Marina Viegas Moura Rezende Ribeiro,
Eurica Adélia Nogueira Ribeiro and
Luiz Feliciano Ribeiro

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76090

Abstract

Dry eye affects 35% of population, and it is a cause of chronic pain and discomfort. The conventional treatment with lubricants is often not sufficient in moderate to severe cases, which can lead to complications such as keratopathies, corneal opacities, ocular perforations, and visual loss. Platelet-rich plasma (PRP) eyedrops have already been used in ocular surface diseases due to their role in epithelialization and the presence of growth factors and vitamins that are similar to human tears. We intend to make a literature review of the use of platelet-rich plasma in dry eye disease, and present the results of a 13 case series, of diabetic severe dry eye patients that used this alternative treatment.

Keywords: dry eye, platelet-rich plasma, wound healing

1. Introduction

In this chapter we will cover a brief literature review on the use of platelet concentrate in dry eye, especially in moderate and severe cases that are generally refractory to conventional treatment, and we will cite some results of its use in other areas of medicine by several authors.

2. Dry eye: definition and classification

The tear has several important functions to the ocular surface, such as lubrification, transport of oxygen, carbon dioxide and other metabolites, immunological actions, and maintenance a stable corneal surface, among others. It is rich in immunoglobulins, growth factors, and vitamin A. It

is separated in three layers: the lipid layer, which protects the tear against evaporation and is secreted by Moll, Meibomian, and Zeiss glands [1]; the mucin layer produced by Manz glands, Henle crypts, and corneal and conjunctival cells—this one stays between the hydrophobic ocular surface and the hydrophilic tear film [2]; and the last and the most prevalent layer, the aqueous layer, produced by Wolfring and Krause glands [3].

The most recent definition of dry eye is that it is a multifactorial disease of the tears and ocular surface, which is accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles [4]. The inflammation of the ocular surface can be both the cause and the consequence of dry eye: dysfunction of the lacrimal glands alters the tear composition, leading to hyperosmolarity that stimulates more inflammation [5], and another factor recognized in dry eye pathogenesis is oxidative stress [6].

There are several risk factors for dry eye, all of them are controversial. These are female sex, menopause therapy, omega-3 deficiency, refractive surgeries; use of some medications as antihistaminic drugs, antihypertensive drugs, diuretics, antidepressants, and others; hepatitis C; radiation therapy; Asian race; HIV and HTLV infection; chemotherapy; isotretinoic acid use; large facetectomy incisions; low humidity environments; ovarian dysfunction; and sarcoidosis [7].

This disease is actually classified into two primary categories: these are tear-deficient and evaporative categories. The tear-deficient dry eye group generally can be due to Sjogren's syndrome or non-Sjogren's syndrome. Sjogren's syndrome is an exocrinopathy in which lacrimal secretion deficiency occurs due to an autoimmune process that affects the lacrimal glands, salivary glands, and other organs of the body [8]. Non-Sjogren's syndrome is caused by lacrimal diseases or lacrimal obstruction and by reflex alterations, without autoimmune factor role. Some causes are age-related dry eye, congenital alacrima, familial dysautonomia, sarcoidosis, lymphoma, AIDS (acquired immunodeficiency disease syndrome), gland denervation, lacrimal obstruction as in pemphigus, trigeminal injury, diabetes, neurotrophic keratopathy, use of contact lenses, and motor reflex block due to VII pair injury [9, 10]. The evaporative causes of dry eye disease are due to oil deficit, lid changes, use of contact lenses, or ocular surface diseases, as allergic conjunctivitis, and some of the iatrogenic dry eye that occurs after the use of systemic or topical medications or after surgeries or nonsurgical procedures [8, 11].

Dry eye can be also classified according to severity. One of the schemes is proposed by Delphi Panel [8, 12]. This classification in grades 1–4 (mild to severe) is based on the frequency or intensity of the dry eye symptoms and discomfort, the blurred vision and visual symptoms, conjunctival injection or redness, conjunctival staining, corneal staining, changes in the cornea and tear as in ocular surface, alterations in the glands and lids, tear film break-up time (TFBUT), and Schirmer's test values.

3. Epidemiology

Dry eye disease affects from 5 to 50% of population; this discrepancy is probably observed due to the absence of a consensus on the diagnosis of dry eye and the lack of standardization

in its classification [13]. It is more frequently in women [14], probably because of the hormone effects in the ocular surface and eye glands [4].

It is more common in Asia and Europe, with only one study showing the prevalence in South of Equator [4], and it seems to be also more frequent in older people, possibly because of the aging alterations in the lids, glands, ocular surface, and adnexal tissue [15].

4. Diagnosis and treatment

Diagnosis is controversial, and literature shows the lack of correlation between some objective tests and symptoms; this is probably due to the difficulty in understanding dry eye pathophysiology [16].

There are some guidelines in this theme, and one of them is the American Academy of Ophthalmology that relates that dry eye diagnosis is obtained after a clinical approach that include asking the patient about exposition to dry eye risk factors and the most common symptoms and signs like redness, itching, photophobia, dryness, foreign body sensation, or pain. It is also important to ask the patient about the exposition to some kind of pollutant, if he is a smoker, if he has any systemic disease (like dermatological, allergic, or rheumatic diseases), checking the hygiene of eyelashes and eyelids, use of medications, eyedrop use, previous ocular surgeries [17], and use of a screening questionnaire as the OSDI (Ocular Surface Disease Index) [18]. The clinical history is followed by a complete ophthalmological exam, including evaluating the eyelids, skin, nerves, visual acuity, and biomicroscopy, and some specific tests like tear breakup time, tear film osmolarity determination, and ocular surface staining with fluorescein lissamine green [4]. The assessment of the tear meniscus (less than 0.35 mm is abnormal) can be performed, verifying if the blink rate is decreased and evaluate the quality of the tear (if there are mucus and debris) and corneal topography [3].

Management of dry eye disease will depend on the cause of this condition and its severity. There is currently no cure for dry eye, and any causal factors that are amenable to treatment should be treated. It includes modifications of the environment; suspension of topical or systemic medications, associated with worsening when possible; artificial tear lubrification; and eyelid hygiene [19].

Generally, the conventional treatment is the lubricants, but it does not resolve all the cases. There are a wide variety of artificial tears, like 1% sodium hyaluronate, hypotonic solutions, those that contains lipids or substances with bioadhesive properties and formulas that have substances that protects the cell's stress; but none of them has the natural tear properties [20, 21].

Other therapies are topical cyclosporine and corticosteroids [22], but these have some disadvantages such as eye irritation and ocular pressure elevation and cataract, respectively [23, 24]. In moderate cases we can also use systemic supplements with omega-3 and linoleic acids and the increased consumption of water, lacrimal occlusion, and glasses use. In severe dry eye, in addition to all these treatments, we can take another measure like systemic cholinergic agonists, systemic anti-inflammatory agents, mucolytic agents, contact lenses, correction of

palpebral alterations, permanent lacrimal punctal occlusion, tarsorrhaphy, and, finally, the autologous serum tears [17].

The Ebers Papyrus 1534 BC is the first reference in the history of a blood derivative in the eye. In 1975, Ralph et al. used it in dry eye [25]. Fox et al. and later Tsubota related the use of autologous serum as an alternative treatment of severe dry eye cases [26, 27]. During many years of study, fetal bovine serum, allogenic serum, and umbilical cord serum have been used; however, they are heterologous, the risks of infections or allergic reactions are increased, and their use is not possible in any center [28]. It does not have preservative, and it is rich in vitamins, fibronectin, growth factors, and cytokines and has biomechanical properties similar to natural tear film, and they do not have preservative which is common in artificial tears [29]. Epidermal growth factors and vitamin A are important for proliferation, differentiation, and maturation of the ocular surface epithelium [30]. Fibroblast growth factor-beta is also involved in epithelial healing, fibronectin promotes cell migration, albumin has antiapoptopic activity, alpha-2-macroglobulin has anti-collagenase action, platelet-derived growth factor promotes hepatocytes growth factor, and substance P and insulin-like growth factor aid in the migration and adhesion of epithelium to the stroma [31]. The serum also maintains intracellular ATP at acceptable levels and cell membrane integrity [32–35].

Some authors related the use of autologous serum drops in dry eye with some promisor results [35, 36].

Kojima et al. in 2005 described increased TFBUT in treated group comparing to control group (using artificial tears) [37]. Lee and Chen studied the use of autologous serum in 23 patients by 18 months and found improvements in symptoms and corneal staining pattern in approximately 75% of patients [38].

Other indications for the use of autologous serum have been the epithelial defects [39], graft-versus-host disease [40], neurotrophic keratopathy [41], trabeculectomy ampoules [42], after refractive surgery [43, 44], Mooren's ulcer [45], and other keratitis [46].

Tananuvat et al., in a randomized prospective study, found that control eyes had improvement in symptoms, signs, and rose bengal staining compared with the baseline. However, some advantages neither Schirmer's test results nor tear breakup time improved in treated group [34].

Urzua et al. described in a double-blind crossover clinical trial 12 severe dry eye syndrome patients in which autologous serum treatment showed a statistically significant higher OSDI decrease (50%) versus conventional treatment (22%). There were no significant changes in objective parameters (Oxford corneal staining and TBUT) [47]. A review to evaluate autologous serum efficacy compared to lubricants, in dry eye disease, concluded that there is a great heterogeneity considering the preparation and storage of the eyedrops, as well as the adequate use of this therapy, and that new studies that standardize these items would be necessary [19].

Another alternative, platelet-rich plasma (PRP), can be used in severe dry eye. It is prepared in double centrifugation of total plasma. Some of the important growth factors this hemoderivate has are platelet-derived angiogenesis factor, platelet-derived epithelial growth factor, and platelet factor 4 [48].

The epidermal growth factor accelerates the healing process and epithelial migration in the cornea, besides stimulating the DNA synthesis of the epithelial cells, and is also associated

with the production of mucin 1 by some conjunctival cells. Transforming growth factor b1 (TGF-b1) has an increased levels in the epithelium during corneal stromal repair processes; it stimulates the production of collagen, fibronectin, and proteoglycans and together with the platelet-derived growth factor (PDGF) has an important anti-inflammatory action [49]. Vitamin A is one of the major epitheliotropic factors in autologous serum; it is 100 times more concentrated than in natural tear and prevents squamous epithelial metaplasia [50].

Fibronectin is an important protein to corneal reepithelization, promoting healing and phagocytosis [51]. Annexin A5 has been investigated as an alternative to fibronectin eyedrops. It interacts with some integrins and stimulates the secretion of plasminogen activator-type urokinase, whose expression is increased in epithelial defects. Albumin is one of the most important proteins in the blood. It reduces the natural degradation of cytokines and growth factors in areas of tissue injury and shows antiapoptopic activity [39, 52, 53]. Alpha-2-macroglobulin neutralizes proteolytic enzymes. It is useful in ocular burns and marginal ulcers [32, 39]. Fibroblast growth factor-beta is a factor that promotes corneal healing, increasing cell proliferation and motility [54].

The insulin-like growth factor helps epithelial cell migration [55]. Neural growth factor (NGF) is the most well-known neurotrophin. It restores the function of injured neurons and can be effective in trophic ulcers [56].

One of the PRP advantages is that it has not cytokines derived from leucocytes and monocytes, which are present in autologous serum and can be deleterious in patients with immune diseases [57]. PRP also regulates expression of several genes in the cellular differentiation improving biological activity of the corneal epithelial cells when compared with autologous serum [58].

The PRP protects the ocular surface from scar formation more than autologous serum, because of reduction of myofibroblasts. It has been observed in PRK where patients who had done this surgery has a decreased incidence of haze [59]. PRP causes reduction in inflammation by indirect action, through the reduction of osmolarity, thus, diluting the pro-inflammatory factors existing in the ocular surface. This also occurs because of the presence of the growth factor-rich plasma interleukin-1 receptor antagonist as well as the presence of metalloproteinase [60].

Platelets have a lot of important functions that are repairing tissue damage; coagulation prevents blood loss, secreting proteins, cytokines, and other mediators; inducing tissue regeneration by cell migration, proliferation, and angiogenesis, and preventing infections because of its antibiotic action. They also have an anti-inflammatory and analgesic action [61].

5. The use of PRP in medicine and odontology

Platelet-rich plasma has been used in several medical and dental areas. The platelet-rich plasma has approximately 1 million/ml platelets [62], and it has been used to the revitalization of necrotic pulp teeth [63], in periodontitis [64], in revascularization of young teeth [65], and in other oral conditions [66].

Platelet-rich plasma has been used in orthopedic therapies like cartilage repair [67], also in bone regeneration, and in tendons, ligaments, and articular lesions [68].

In dermatology, PRP has been used for wound healing of acne scars [69], vitiligo [70], venous ulcers [71], alopecia [72], skin rejuvenation [73], and some cases of lichen sclerosus [74].

In gynecology, there are studies where PRP had been used in cases of infertility [75], in uterine prolapse [76], in inducing endometrium proliferation [75], and in scars of cesarean sections [77].

It has also been used in otolaryngology [78], in urinary diseases [79], and in other medical specialties.

6. The use of PRP in ophthalmology

PRP has been reported in corneal ulcers [80–82], in chemical burns [83, 84], in restoration of lacrimal function [85], in blepharoplasties surgeries [86], in ocular surface syndrome after refractive surgeries [44], and in graft-versus-host disease [87].

In dry eye, there are few studies reporting the use of PRP. Alio et al., in 2007, evaluated 18 symptomatic patients that used this treatment for 1 month and observed that symptoms of dry eye improved in 89% of patients, that conjunctival injection was present in 38.9% of patient, that 86% of the symptoms decreased, that lachrymal meniscus improved in 56% of the cases, and that corneal staining also decreased. In impression cytology increase in conjunctival goblet cells was observed [88].

Ribeiro et al., had studied about diabetic dry eye patient. They evaluated 12 patients with grades 2–4 of severity [8, 12]; 41.67% had a reduction in Schirmer's gradation, 58.33% had an increase in TFBUT (tear film breakup time), visual acuity improved in 41.66% of patients, and OSDI questionnaire score significantly improved in 100% of patient. The authors also found that before treatment, 91.67% had severe dry eye and after treatment, 50% were classified as normal, 25% as mild dry eye, 16.66% as moderate dry eye, and just one patient had instead a severe dry eye (**Figures 1–5**) [89].

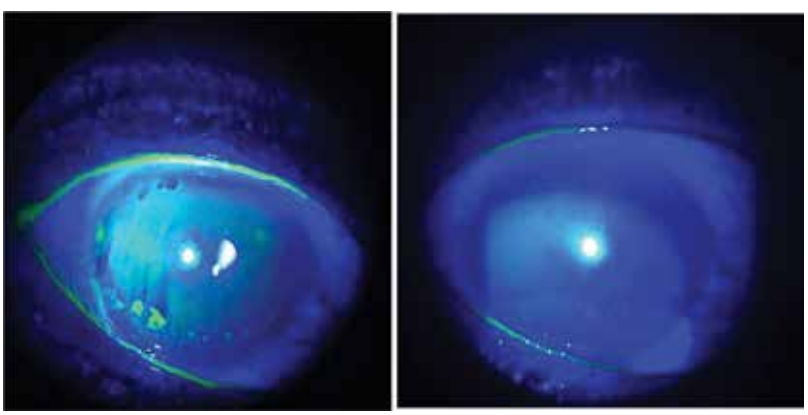

Figure 1. Before and after treatment with PRP in patient with diabetic dry eye.

http://dx.doi.org/10.5772/intechopen.76090

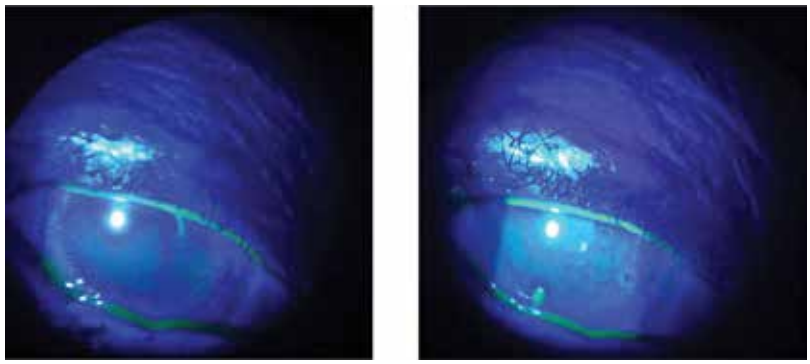

Figure 2. Before and after treatment with PRP in patient with diabetic dry eye.

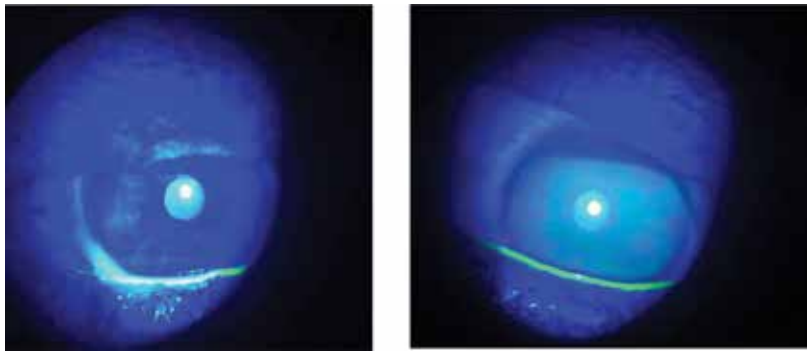

Figure 3. Before and after treatment with PRP in patient with diabetic dry eye.

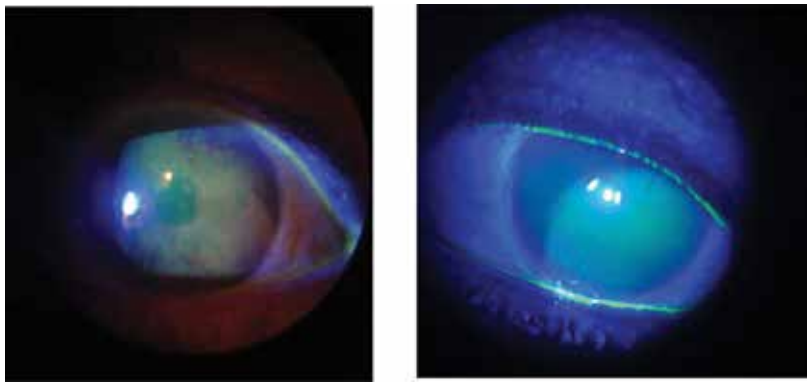

Figure 4. Before and after treatment with PRP in patient with diabetic dry eye.

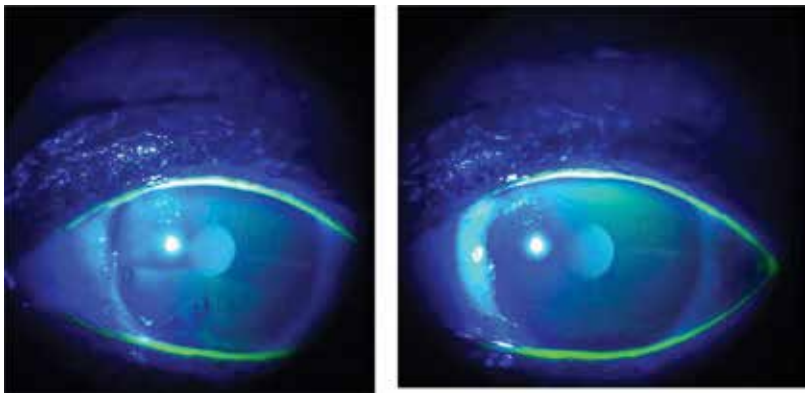

Figure 5. Before and after treatment with PRP in patient with diabetic dry eye.

Recently, in 2017, Alio reported a 368-patient prospective, interventional nonrandomized study, where moderate to severe dry eye was included. The results were as follows: Schirmer's test value had a significant improvement, subjective symptoms had an improvement in 87.5% of patients, OSDI scores were statistically significant, 28.8% experienced an increase of one or more lines of vision, decrease in corneal fluorescein staining was observed in 76.1% of patients, and only one patient reported intolerance to the use of PRP due to discomfort at the time of instillation [90].

Another alternative that has been reported in dry eye treatment is the plasma rich in growth factors (PRGF). Lopez-Plandolit et al. observed that PRGF treatment was associated with improvement in score dry eye questionnaire values and as results from impression cytology. In 75% of patients, no further medication was required. But squamous metaplasia did not reduce significantly [49]. It is very similar to PRP; however, there are no researches in human use compared to all the hemoderivates in dry eye.

7. The methods of obtaining platelet-rich plasma

The two main methods of obtaining platelets are by autologous donation and by the technique of platelet apheresis. The advantage of the apheresis technique is that the platelet concentration is higher in the final concentrate and should contain at least 5.5 × 10 10 platelets, while its disadvantage is the cost that is extremely superior to the autologous total donation technique [48].

In the apheresis technique for platelet collection, Rezende et al. used the Haemonetics MCS + 9000 automatic cell separator and the 995-E apheresis-specific kit (Haemonetics Corp.). In that system, by means of a venipuncture, the blood of the patient himself is drained to a separation device. An optical refraction analyzer separates the platelet layer, and the remaining blood is completely returned to the patient, determining the end of a cycle. Sodium citrate can be used as an anticoagulant in the ratio of 1 to every 9 ml of whole blood processed. In 2 cycles, 72 ml of platelet concentrate was collected; the hematimetric indices of the patient and also the platelet concentrate indices are evaluated before and after the procedure (Coulter AcT Diff) [81].

Platelet growth factors were generally obtained in a ranked room; hence, the handling of platelet concentrate was performed within a Category II-Type A biological safety cabinet. Then, 2800 microliters of 10% calcium chloride was added, and the final product was maintained at +37°C for approximately 30 min. Subsequently, the unit was subjected to centrifugation (900G), and the supernatant serum, which contained the platelet growth factors, was transferred to four 50 ml falcon-type tubes (Becton-Dickinson) and maintained at −80°C (Revco). Research of bacteria, aerobic and anaerobic, and fungal agents was performed in a systematic way.

The release of serum with platelet growth factors in the study by Rezende et al. was done as follows: weekly, thawed Falcon tube containing approximately 10 ml of autologous serum with platelet growth factors that was transferred to flasks, and the patient is advised to keep the biological medicine at a temperature below −10°C (freezer) and to unfreeze it naturally immediately before each use [81].

Alio et al. used the autologous donation technique to obtain platelets, when patients were submitted to venipuncture and 80–100 ml of blood were collected in sterile 10 ml tubes containing 1 ml of sodium citrate to avoid coagulation. These tubes were left at room temperature for 10 min, and only the supernatant (upper tube fraction) was collected as the final product. The platelet concentrate was then prepared under sterile conditions in a laminar flow room. Two to three milliliters of this concentrate was then placed in sterile eyedrops. The eyedrops were kept at −20°C, and only when the patient was going to use an eyedrop bottle, thaw it and then keep it at +4°C, discarding that bottle at the end of a week, when a new one is thawed. The patients were advised to use these eyedrops four times a day for 1 month [88].

Both methods require the patient to perform an autologous blood donation. Autologous blood does not transmit disease; there is no occurrence of hemolytic (alloimmunization), allergic, immunological (immunomodulation), or acute lung injury by transfusion and common complications in heterologous donations [91].

The contraindications for autologous donations are anemia or other types of pathological hemodilution; conditions that lead to oxygen saturation and hemoglobin saturation (less than 11 mg/dl); hepatopathies; nephropathies; coagulopathies; hemoglobinopathies; decompensated heart diseases; and presence of infectious diseases such as Chagas, syphilis, HIV, HTLV, hepatitis B and C, and others transmissible by blood considered as "relative" contraindications, since the patient will receive the same, but there may be contamination of the health team in the handling of that blood. In HIV, there may be reactivation of the virus when reinfused [91, 92].

Complications of autologous donation are those inherent to a donor, such as hypotension, anemia, angina, and contamination of the material of the blood bags [91].

8. Conclusions

It can be concluded from these studies that the therapeutic response with PRP was actually satisfactory in severe or moderate dry eye cases which do not respond to conventional

therapy. However, randomized clinical trials are needed so that standardized protocols for the production, storage, and use of this therapy should be created. Prospective studies should also be conducted to evaluate these long-term outcomes.

Conflict of interest

The authors declare no conflict of interest in this subject.

Author details

Marina Viegas Moura Rezende Ribeiro[1]*, Eurica Adélia Nogueira Ribeiro[2] and Luiz Feliciano Ribeiro[3]

*Address all correspondence to: dra.marinaribeiro@gmail.com

1 Centro Universitário Tiradentes de Alagoas, Universidade Federal de Alagoas, Maceió, Brazil

2 Universidade Federal de Alagoas, Maceió, Brazil

3 Instituto de Olhos de Maceió, Maceió, Brazil

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Chapter 3

PRP Injections in Orthopaedic Surgery: Why, When and How to Use PRP Dynamic Liquid Scaffold Injections in Orthopaedic Surgery

Mikel Sánchez, Diego Delgado, Ane Garate, Pello Sánchez, Jaime Oraa, Ane Miren Bilbao, Jorge Guadilla, Beatriz Aizpurua, Nicolás Fiz, Juan Azofra and Sabino Padilla

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76091

Abstract

Platelet-rich plasma (PRP) products can be described as any autologous blood platelet concentrate within a plasma suspension. PRP products include plasma and twofold or greater increases in platelet concentrations above baseline levels. The injection of activated PRP in its liquid formulation delivers growth factors locally and simultaneously mimics and amplifies the spontaneous healing response in injured areas and in special cell niches, which would otherwise be inaccessible. This in situ generated transient three-dimensional scaffold will gradually release growth factors and maintain their concentration at the site of the scaffold formation. The combination of liquid PRP with surgical techniques in orthopaedic surgery allows a wide range of therapeutic strategies in the management of injuries in the field of orthopaedics and sports medicine. The use of different therapeutic elements, including PRP as biological stimuli and rehabilitation and physiotherapy treatments as mechanical stimuli, provides extremely favourable synergies that will help fulfil the physician's objective, to stop the progression of disease and to improve function in the shortest period of time

Keywords: platelet-rich plasma, orthopaedic surgery, injections

1. Introduction

Virtually, all the cells of the musculoskeletal tissues are mechano-sensive and experience mechanical stress through the distortion of the extracellular matrix (ECM) complex. The exposure of musculoskeletal cells to nonphysiological stimuli, either mechanical or biochemical, leads to swings of the tissue pendulum towards profound alterations of components of the ECM both cellular and acellular as well as the physical and chemical properties of the ECM. Such stimuli lead to cell microenvironment damage, non-resolving inflammation and disease. In addition to specific features of each tissue (vascularization, innervation and type of cells), abnormal biomechanical loading as obesity, a sedentary lifestyle leading to metabolic disorders, joint injury or high intensity and prolonged sports activities make musculoskeletal tissues vulnerable to injury. Through such overuse or disuse, these nonphysiological stimuli may well produce a consequential disruption in tissue homeostasis (**Figure 1**).

A new innovative approach to the treatment of acute and chronic sports injuries uses engineering biology assisted by the application of platelet-rich plasma (PRP) in its different formulations. Generally, PRP products can be described as any autologous blood platelet concentrate within a plasma suspension. PRP products include plasma and twofold or greater increases in platelet concentrations above baseline levels: not insignificantly, their concentration of leukocytes and erythrocytes varies widely [1] from a complete absence of these cells to a high concentration of them. In this chapter, the described PRP has a platelet concentration between

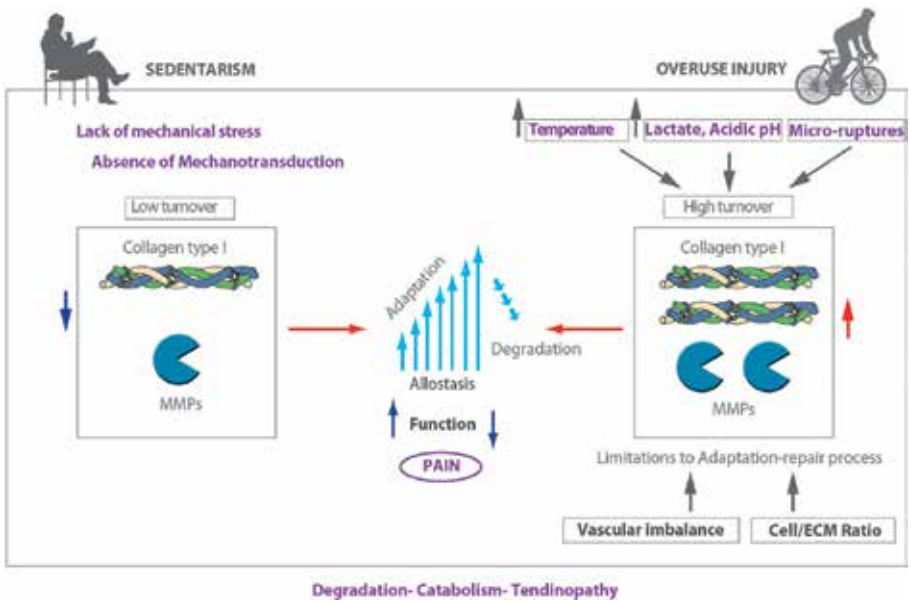

Figure 1. Both an excess and insufficiency of physical activity associated with factors such as vascular imbalance, intrinsic hereditary risk factors and a novel environment may disrupt the fragile homeostasis maintained by the tenocyte, stromal fibroblast and tissue-resident macrophages. A localised, predominantly catabolic context associated with a high temperature, ECM fragments and acidosis with building up of lactic acid may be the root cause of inflammation-degeneration of the ECM.

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2- and 2.5-fold higher than blood and no leukocytes (PRGF®-Endoret®, BTI-Biotechnology Institute, Vitoria-Gasteiz) [2]. PRP can be activated with $CaCl_2$ offering a variety of autologous formulations whose versatility endows this technology with a myriad of applications in orthopaedics [3–5].

This chapter addresses the following questions: Why would surgeons want to harness the biological features of PRP in the operating theatre? When are PRP injections indicated as an adjuvant to? How should the injections be introduced to obtain beneficial outcomes in surgery?

2. Reasons to use PRPs as repair process enhancers in orthopaedic surgery: the scientific rationale behind it

The physicochemical features of PRP liquid formulation, once activated, make it appropriate to reach wide areas of soft and hard tissues such as the tendons, muscles, ligaments, menisci, cartilage and bone. Platelet growth factors and fibrin, together with plasmatic growth factors (HGF, IGF-1) present within PRP, stimulate in a pleiotropic manner cell proliferation and migration, angiogenesis, synthesis and deposition of ECM components and tissue remodelling in the musculoskeletal tissues [6–8]. The surgical site is opened in the normal manner, and in the following 2: 4 minutes, the liquid-activated formulation must be injected as a solution into soft tissues. Because of its local and gradual activation and homogeneous distribution and interaction with the ECM of different tissues, it is converted into a matrix-like malleable transient structure [2]. There is a direct interplay between components of a tissue's ECM (collagens, glycosaminoglycans and adhesive proteins) and the adhesive proteins and growth factors released gradually from the degrading fibrin clot which will influence cellular growth, differentiation and morphogenesis [9]. Therefore, the injection of PRP in its liquid formulation delivers growth factors locally and simultaneously mimics and amplifies the spontaneous healing response in injured areas and in special cell niches, which would otherwise be inaccessible. This in situ generated and moulded plastic nano-scaffold of fibrin interacts with ECM proteins and cells, binding to fibronectin [10], generating a transient three-dimensional scaffold, which will gradually release growth factors and maintain their concentration at the site of the scaffold formation (**Figure 2**)

The fibrin molecules, together with growth factors, influence and govern the repair mechanisms to reconstruct structures and restore function, both by harnessing local or resident cells and by stimulating cell migration and proliferation, thereby regulating angiogenesis, modulating inflammation, chemoattracting circulating progenitor cells and guiding tissue remodelling [11, 12]. PRP in situ generated nano-scaffold of fibrin offers a biologically active cell-matrix landscape where adhesive proteins, namely, fibrinogen, fibronectin, vitronectin and thrombospondin (TSP-1), facilitate cell adhesion, migration, proliferation and differentiation. Furthermore, by the release of stromal cell-derived factor 1 (SDF-1) which has been entrapped in the fibrin network, the nano-scaffold mediates the chemotaxis of CD34 progenitor cells and mesenchymal stem cells (MSCs) [7, 13, 14]. Once recruited, MSCs or pericytes [15] adhere to a fibrin network and may exert several functions such as tissue organisation, regulating the fate of other circulating and resident progenitor cells [16] and serving as

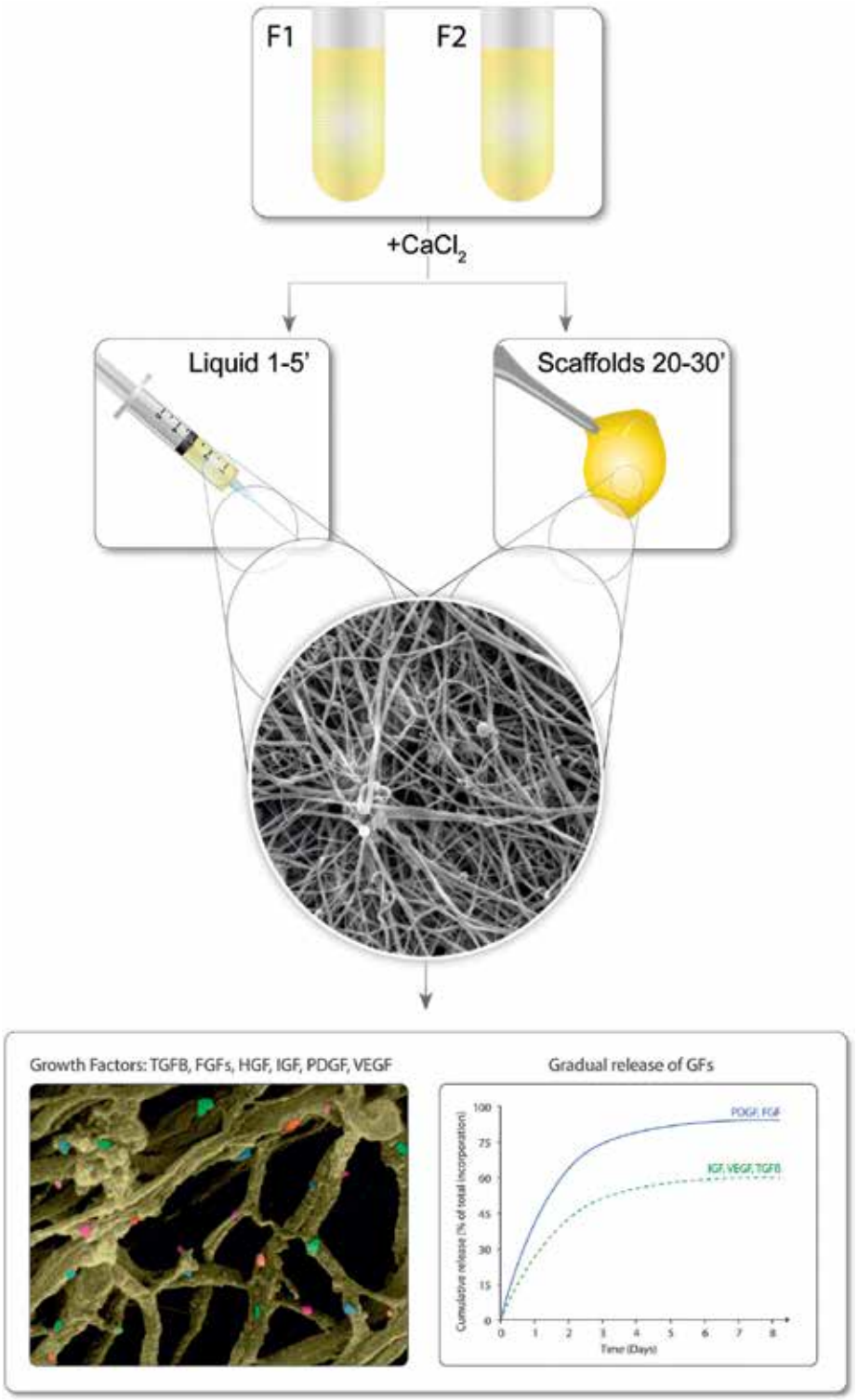

Figure 2. PRP niche therapy approach: injectable dynamic scaffold for molecular intervention.

progenitor cells that replace the damaged tissue, prevent scar-forming cells from entering the damaged area and exerting immunomodulation activities [7, 14].

3. The use of PRP infiltrations in orthopaedics: surgical applications

Although it is not within the scope of this chapter to address the wide range of therapeutic strategies in the management of injuries in the field of orthopaedics and sports medicine, only a holistic approach will fulfil the objective of surgeons, namely, to stop the progression of disease and to improve function in the shortest period of time. In this respect, and as a clinical application of cell mechanotransduction, a rehabilitation programme, which included the employment of PRP in a synergistic manner would play a crucial role in both promoting the repair or remodelling of injured tissue and avoiding the degradation and atrophy of structures such as the bone, periarticular muscles, tendons and ligaments with the goal of full recovery of function [17].

3.1. PRP infiltrations in tendon surgery

There is increasing evidence showing that tendon and ligament adaptation, injury and repair processes share several intracellular pathways, and although it is difficult to draw the line between the cellular and molecular responses that lead to either tissue adaptation or tissue damage, inflammatory processes appear to be at the interface of tendon adaptation and damage [18–20]. Repetitive mechanical loading, as is the case in early stages of tendinopathy, and tendon overuse induce the activation of NF-κB in stromal fibroblasts and thereby the synthesis of matrix metalloproteinases (MMPs), two isoforms of cyclooxygenase (COX)-1 and COX-2 and PGE2 by inflammatory tenocytes and stromal fibroblasts, mast cells and other immunocompetent cells [18, 21–23]. PGE2 is a major systemic and local inflammatory mediator that decreases the production of collagen and causes aberrant differentiation of TDSCs into adipogenic and osteogenic lineages [23], which might partially account for the presence of fibrocartilage, calcifications and adipose tissue in injured and chronic degenerative tendons [18, 23, 24].

An excellent series of in vitro and in vivo studies demonstrated that blood-derived BDDT induced tenocyte proliferation, stimulated the synthesis of type I collagen and neovascularization [9] and promoted differentiation of TDSCs into active tenocytes, but, significantly, the addition of leukocytes into the releseate increased the synthesis of PGE2 and the gene expression of MMP-1, MMP-13 and IL-1β and decreased the expression of alfa-SMA as a marker of active tenocytes. Among the myriad mediators conveyed by blood-derived BDDT, HGF and lipoxin A4 (LX4) have been shown to exert an anti-inflammatory and pro-resolution of inflammation effect on injured tendons [21–23].

3.1.1. Surgical treatment of acute ruptures of tendons

In the case of tendons such as the Achilles, patellar or quadricipital, the volume of blood extracted is approximately 60–70 mL (six to nine tubes). Blood is taken a few minutes prior to surgery, before any fluid or drugs are administered to the patient, in the operating theatre

itself. PRP should be prepared, while the patient is being prepared in the operating theatre and applied by injection immediately after activation ex vivo (**Figure 2**).

The injury site is accessed via a medial approach [25], the hematoma is evacuated and the necrotic tissue is debrided. Then, the tendon is repaired using non-reabsorbable material previously soaked in liquid-activated PRP. The PRP liquid freshly activated is infiltrated into the healthy tendon and the tendon/bone repair zone. Both the repair zone and the proximal and distal end stumps are injected (**Figure 3**). The use of small syringes means that large pressures are exerted on the ECM of the tissue during infiltration. We therefore recommend the use of 10 mL Luer lock-type syringes with 21G needles. Upon infiltration, the needle should be oriented as closely as possible parallel to, and longitudinal with, the tendon as possible for an optimal diffusion of PRP (**Figure 3**). Repair concludes with closure of the peritenon. The peritendinous regions are also infiltrated in order to recruit mesenchymal stem cells, pericytes and endothelial cells [15]. Approximately 12 mL of PRP is used during this phase. Before closing the overlying skin, the affected area is covered with a fibrin scaffold. Once closed, the subcutaneous tissue is irrigated with freshly activated PRP. An ultrasound examination of the Achilles tendon is performed in week 3, and, if healing problems, especially intratendon cyst formation, are detected, liquid-activated PRP is infiltrated under ultrasound guidance in the outpatient manner.

It is mandatory to coordinate and integrate functional recovery in light of the changes to biological mechanisms. Achieving a shorter immobilisation time allows physiotherapy to be speeded up because of the formation of a more efficient repair tissue [4, 25, 26].

The application to ruptures of major tendons at other sites follows the same methodology. The sequence in which the PRP liquid is applied is the same, with the volumes being varied in each individual case (**Figure 3**). Two maxims must always be followed: the use of suture systems and repair techniques that respect the tendon's native biology as far as possible and the achievement of mechanically stable configurations that allow early rehabilitation.

3.1.2. Surgical treatment of chronic tendinopathy

In patients with a tendinopathy in whom conservative management, including percutaneous infiltration of PRP, has failed, surgery may be indicated [5]. This procedure, which is based on longitudinal tenotomy with removal of the area of failed healing response together with the application of PRP, is intended to remove the degenerative tissue, induce neovascularization and provide the tendon with a physical support and three-dimensional structure where local and neighbouring cells (e.g., from the paratendon) can proliferate and synthesise both blood vessels and ECM. We have summarised the process in the following steps (**Figure 4**):

1. The tendon injury sites are located and excised. Longitudinal tenotomies should be performed throughout the whole thickness of the tendon in the same direction as the fibres. The aim of this process is twofold: to access the whole tendon in order to remove all the degenerative tissue and to generate a repair stimulus in the injured tendon.

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2. Liquid-activated PRP (8–10 mL) is then injected into the tendon fibres at both the excision site and the proximal and distal ends of the injury site following the procedure depicted previously for tendon ruptures.
3. Once the subcutaneous tissue has been closed, it is infiltrated with freshly liquid-activated PRP.

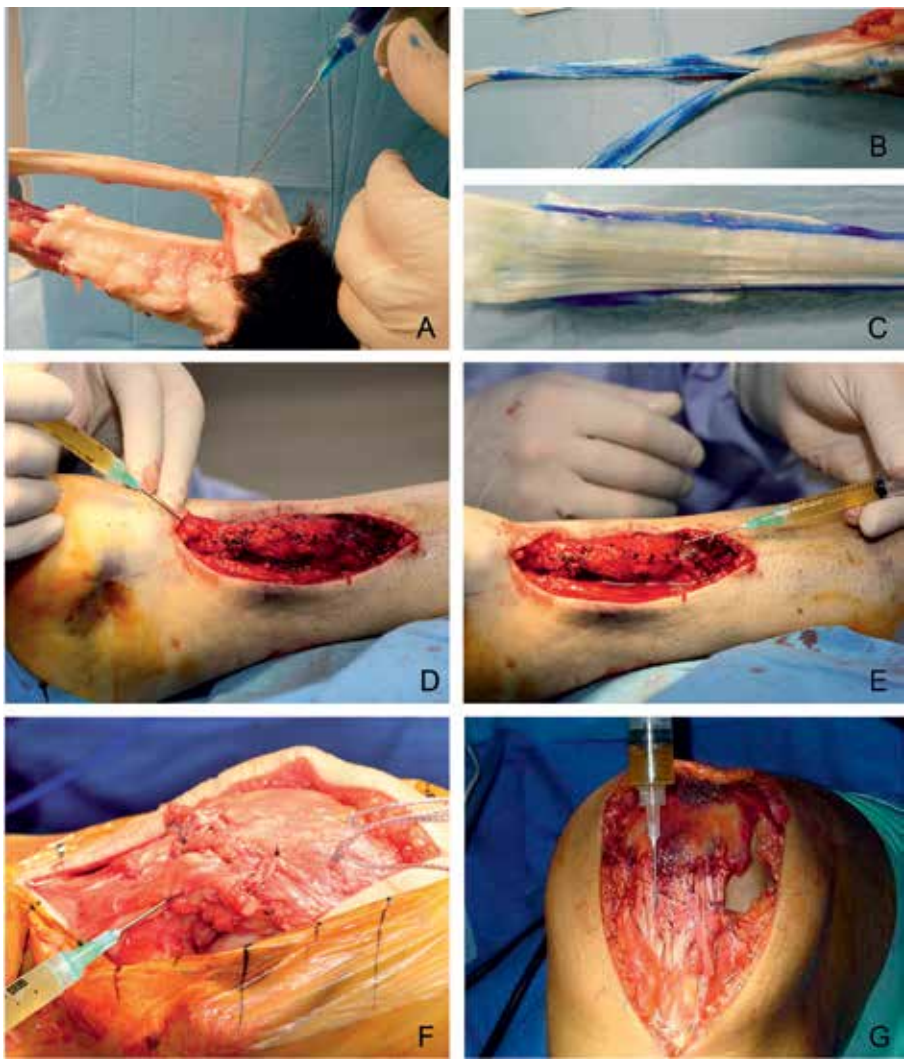

Figure 3. Injection at the site distal and proximal to the rupture in the direction of the tendon fibres (A, B). PRP is injected into the healthy tendon as well. To determine the effect of infiltration angle, and the size of syringe and diameter of the needle on the diffusion, PRP stained with methylene blue was injected in Achilles tendon of the sheep (C, D, E). The optimal diffusion of the PRP was obtained when the needle was oriented as closely as possible parallel to, and longitudinal with, the tendon. Complete rupture of the quadricipital tendon (F, G).

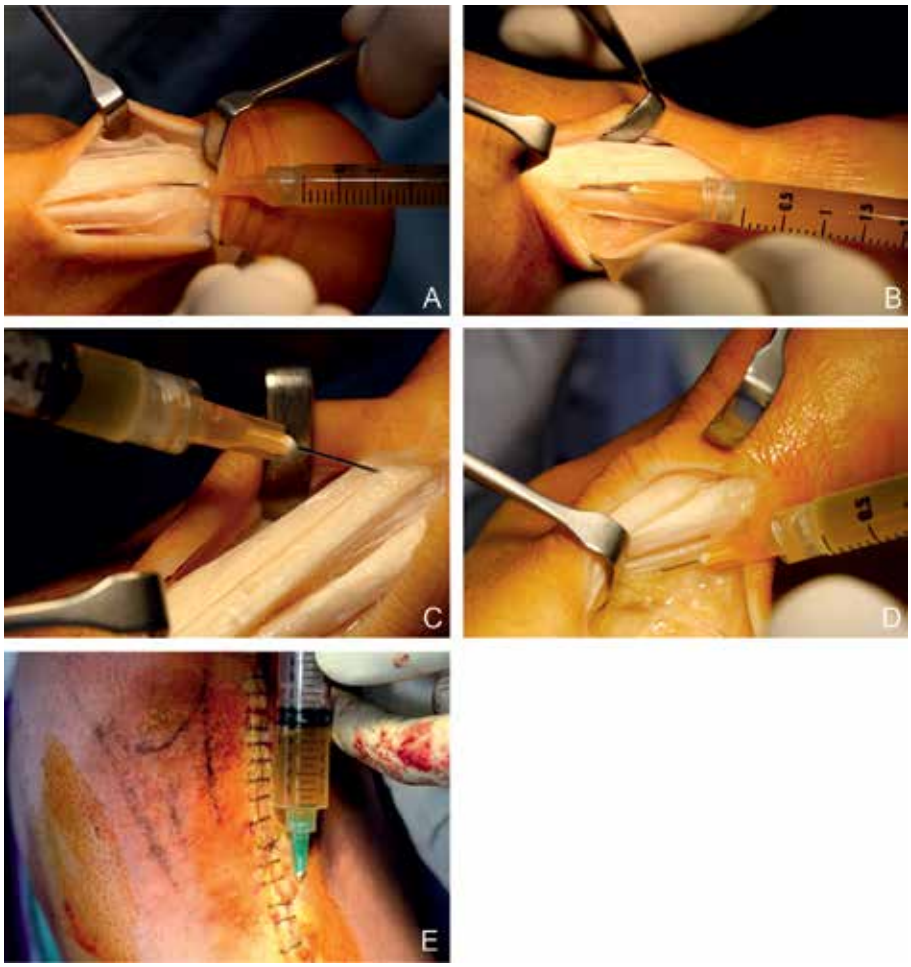

Figure 4. After having performed longitudinal scarifications in the tendon, liquid-activated PRP (8–10 mL) is injected into the tendon fibres at both the excision site and the proximal and distal ends of the damaged area (A–D). Infiltration of the subcutaneous tissue with freshly liquid-activated PRP (E).

3.1.3. Management of postsurgical Achilles tendon complications with PRP

PRP application in combination with surgery meets the criteria for treatment of major complications from Achilles tendon rupture and repair, namely, versatility, biocompatibility, biosafety and efficacy. After having carefully cleaned the necrotic area, we proceeded to apply PRP. We injected 3 mL of the activated liquid both in the distal and proximal tendon stumps as well as in healthy areas of the tendon as described previously. In addition, the paratendon construct was richly injected with PRP. In one case, an autologous semitendinosus tendon was used to fill the Achilles tendon gap. Before the graft was anchored, we injected PRP liquid into the newly formed tissue (during week 3 after the first operation), among the tendon fibres of the graft and into the reconstructed tendon [27].

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3.1.4. Surgical treatment of rotator cuff tears

The three factors that cause tissue damage of connective tissue in the musculoskeletal system coincide in the aetiology of rotator cuff injuries: (1) mechanical factors, (2) overuse-related micro−/macrotraumas and (3) the vascular decompensation inherent to this structure. Indeed, biopsies have shown a structure with a disproportionately low degree of vascularization and cellularity for its high level of functional demands. This tissue undergoes constant demands where the cell phenotypes cannot adapt themselves to the high level of motor demands and where stroll fibroblasts are chronically activated. The commitment and fragility become even more evident in gliding tendons in which the part of the tendon that is in contact with the bone develops an avascular fibrocartilaginous tissue in response to the compression forces. During the surgery, we infiltrate approximately 8–10 mL of liquid-activated PRP, distributing it as follows [28]:

1. Into the body of the damaged and sutured tendon in order to promote a chemotactic and angiogenic effect in it.
2. Into the myotendinous junction, where the majority of healthy cells are present, and the subacromiodeltoid bursa, a likely source of multipotent cells.
3. Into the tendon/bone region and into the cancellous bone of the humerus in order to stimulate mesenchymal stem cells in the cancellous bone.
4. Finally, we inject a further 8–10 mL of the remaining PRP into the subacromial space in order to bathe the entire sutured region.

Rotator cuff injuries tend to have a poor prognosis as more than 50% of sutured tendons may not heal. This fact highlights the importance of strictly observing the PRP protocol. An ultrasound examination is performed at week 3 and week 6, and the tendon suture and subacromial space are infiltrated again (8–10 mL of liquid-activated PRP).

3.2. PRP injections in cartilage diseases

In spite of advances in pharmacological and surgical techniques, the treatment of cartilage injuries is still a challenge. Articular cartilage is a tissue that is remarkably resilient to compressive and shearing forces. Yet, it is highly fragile to alterations of the synovial membrane and subchondral bone, two well-vascularized tissues from where systemic and local inflammation insults arise. These aggressions are mediated by pro-inflammatory cytokines and inflammatory macrophages and synoviocytes, which damage articular cartilage as in the case of rheumatoid arthritis or osteoarthritis [29]. However, synovial membrane and subchondral bone are also the egress point and source of nutrients and MSCs for mounting a chondrogenic reparative response, which is driven by the recruitment and chemotactic homing of synovium and bone marrow-derived stem cells mediated by SDF-1, TGF-β and fibronectin. This is the case in microfracture techniques and in the combinatorial strategy using intraarticular (IA) and intraosseous (IO) infiltrations of blood-derived BDDT such as PRP [30]. In doing so, PRP tackles the four synovial joint tissues and acts as a dynamic autologous liquid scaffold that, in a sustained and gradual manner, conveys chemotactic endogenous MSC homing and chondrogenic factors

such as SDF-1, TGF-β and fibronectin [31, 32]. In addition, PRP dampens inflammatory stress at the level of joint tissues, by both inhibiting the NF-κB on chondrocytes and macrophages [33] and upregulating the antioxidant response element NF-E2-related factor 2 (NrF2-ARE) pathway in osteoblasts [34]. Improvements of clinical outcomes of patients with knee and hip OA were reported applying this strategy [35, 36] which might primarily be mediated by HGF, CTGF, IGF-1 and PDGF, among others [33, 34, 37], thereby paving the way to cartilage regeneration; however, elusively, it remains.

3.2.1. PRP and chondral surgery

In joint diseases, the whole joint is affected: cartilage, subchondral bone, synovium, ligaments, neural tissue, etc. Thus, all components of the joint are essential to maintain homeostasis, and both genetic and acquired or environmental factors can break this balance, causing degeneration of cartilage, subchondral bone and other joint components and becoming a clinical problem [36]. The use of PRP as treatment in joint pathology is based on its capacity to restore homeostasis joint, to have inductive and protector effects on chondrocytes and to act on the synovial membrane, stimulating the production of hyaluronic acid and other molecules. All these properties contribute to the promotion of a biological environment that is conducive to slowing the joint cartilage degeneration and relieving clinical symptoms [37].

3.2.2. Fracture/avulsion and osteochondritis dissecans

The first step is to debride the wound bed and to separate the fragment carefully. The bony surface of the said fragment is refreshed to achieve an appropriate appearance. When a bleeding bed is obtained by spongialization, an intraosseous infiltration of 3 mL of liquid-activated PRP is conducted. Next, the osteochondral fragment is fixed into its original niche and its stability is endured. Finally, 2 mL of PRP is infiltrated into space between the wound bed and the fragment using a fine needle. When the fragment is reinserted, the region around all edges is filled and sealed.

3.2.3. Osteochondral injuries with an inviable fragment

In this case, the subchondral bone is debrided removing all damaged tissue as in the osteochondritis dissecans. Therefore, a spongialization is conducted in order to achieve a bleeding bed. The Pridie procedure or microfractures are performed to drill the bone, and a trocar is introduced in order to infiltrate liquid-activated PRP. Consequently, MSCs are stimulated, generating cell and molecular signals that promote the repair processes of joint cartilage. Moreover, a three-dimensional fibrin matrix is formed from PRP, which traps the cells that have come to the lesion area. As a result, the synthesis of the new tissue is promoted, performing a similar mechanical function as the original.

3.2.4. Extensive osteochondral injuries and necrosis

First, the injured tissue has to be debrided in order to achieve a bleeding spongy bone. Next microfractures are conducted and serum is aspirated by intraarticular wash. Finally, liquid-activated PRP is administrated by an intraosseous (3–5 mL) and an intraarticular (8 mL) injections.

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The use of autologous osteochondral grafts are recommended when subchondral bone is affected by osteonecrosis of the medial condyle of the knee. Infiltrations of liquid-activated PRP help to integrate such graft. These infiltrations are conducted into the bed and bone osteochondral graft and in the interface where the allograft is implanted. At the end of surgery, serum is aspirated, intraarticular space washed and PRP infiltrated in an intraarticular manner. During the post-operative period, three intraarticular injections of PRP are performed on a weekly basis. Initially, the patient has to walk assisted by crutches with minimal load.

3.2.4.1. Avascular osteonecrosis of the hip

This condition is the final point of several factors. Below, we describe the steps and times for this surgery and the use of PRP [37]. This arthroscopic protocol describes the "light bulb" technique and the biological support to achieve satisfactory results during a mean follow-up of 14 months (**Figure 5**).

1. Both diagnosis and treatment of associated intra-articular damages are addressed by arthroscopy.
2. During stages I and IIA, arthroscopic vision allows to perform several image-guided perforations in order to decompress the necrotic cephalic region. With a trocar, liquid-activated PRP is administrated into this region and into the surrounding healthy bone with it. When the femoral head is deformed, an osteoplasty is conducted and PRP infiltrated.

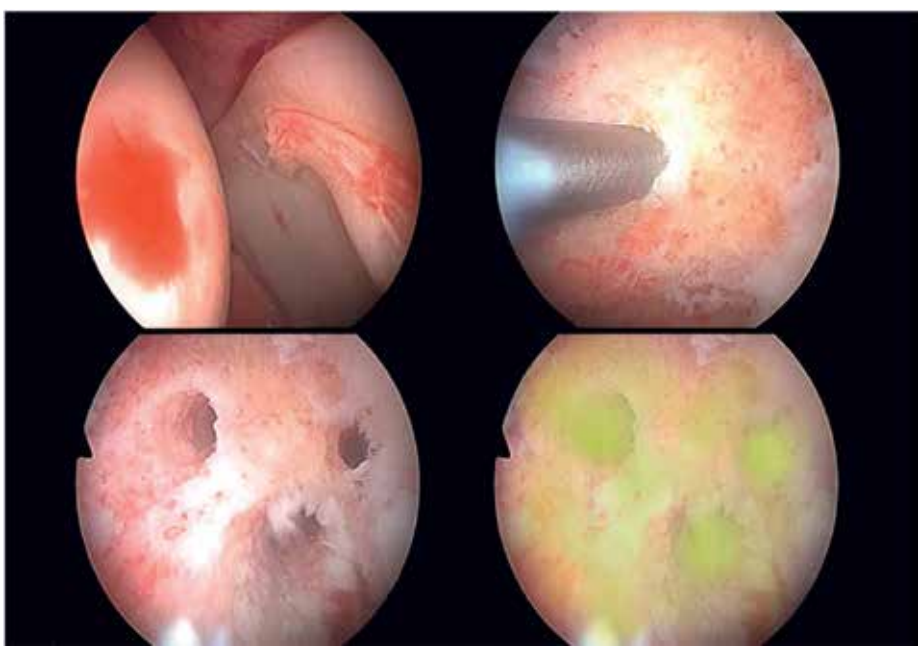

Figure 5. Arthroscopic diagnosis of associated damage. Creation of perforations down to the necrosis bed. Intraosseous infiltration with PRP.

3. In stages IIA and IIB, where the condition curses with cystic and sclerotic changes, a debridement and removal of necrotic tissue are performed with trephines, curettes and a synoviotome. When the healthy bone is available, autologous bone graft is impacted into the femoral in order to adapt it properly. The preparation of graft is carried out by using the ipsilateral iliac crest bone graft and liquid-activated PRP. When the size of the injury allows it, a demineralized bone matrix/PRP mixture can be applied.
4. Finally, 8 mL of liquid-activated PRP is injected into the joint. In the next weeks, infiltrations are repeated (three or four times) under ultrasound guide.

3.3. PRP infiltrations in bone damage

When a fracture occurs, the first tissue-based phenomena to be manifested are tissue destruction, vessel rupture and cell necrosis. This results in bleeding that stimulates and activates defence systems to prevent excess bleeding and contamination of the injury site (both of which may be life-threatening). Although the fracture site tends to be hypoxic, with an altered pH and mechanical instability (the local reaction attempts to isolate the fracture site to prevent infection), cells such as platelets, endothelial cells and macrophages are responsible for orchestrating a cell-based response by releasing growth factors such as PDGF, TGF-β, IGF I and IGF II, FGFs, VEGF, BMPs, IL-1, IL-6, TNF-α and PGE2 [38]. This group of bioactive molecules promotes the attraction/migration of osteogenic and MSCs from the periosteum and bone marrow, as well as fibroblasts from the surrounding soft tissue to the fracture site, where they form an extensive network based on fibrin and other plasma proteins.

These growth factors play a key role in the initial phases of recruitment/migration, MSC mitogenesis and angiogenesis, which are essential. Simultaneously, the development of new blood vessels is stimulated and under the influence of angiogenic factors such as angiopoietin 1 and VEGF. MSCs and osteoprogenitor cells continue to express BMPs, which induce chondro−/osteogenesis and ECM synthesis. These MSCs initially form aggregates that express transcription factors sox9 and col2 (to express cartilaginous proteins) and then differentiate into chondroblasts (by the third or fourth day). The TGF-β expressed by both platelets and endothelial cells during the initial stages of callus formation, and subsequently by chondrocytes and osteoblasts, appears to be key to both MSC chemotaxis and proliferation and the chondrogenesis and formation of endochondral bone [30, 39].

3.3.1. Treatment of fractures assisted with PRP

We have developed a set of basic guidelines for the application of PRP during the minimally invasive treatment of bone fractures (**Figure 6**) [5].

1. Once the whole context of the fracture has been assessed to determine the most appropriate treatment, 36 mL of peripheral venous blood is withdrawn. Occasionally, due to the type of bone and fracture, it may be necessary to extract further amounts of blood.
2. Reduction and percutaneous osteosynthesis of the fracture under radiographic guidance. If the fracture does not require osteosynthesis, the process can be performed under radiographic control ensuring optimal sterility.

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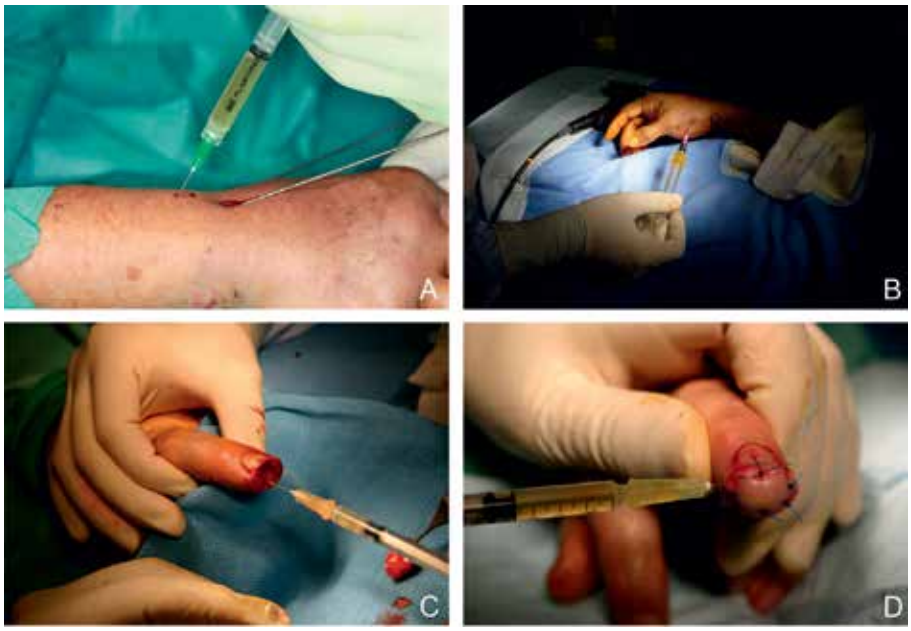

Figure 6. Infiltration of liquid PRP at the fracture site, after stabilisation, under radioscopic control: Colle's fracture (A), Bennett's fracture (B) and a distal finger/hand phalanx fracture (C, D).

3. Liquid PRP is then activated for injection at the previously reduced and stabilised fracture site, under radiological control, to form the fibrin clot that is responsible for sustained release of the cell signals that induce the biological repair programme.
4. The volume of PRP infiltrated depends on the size of the fracture, although it is normally around 8 mL.
5. Healing of the fracture is then monitored clinically and radiographically. If signs of consolidation delay are detected, liquid PRP is injected a second time between weeks 4 and 6 in the same manner as the first infiltration (radiographic guidance and between 6 and 8 mL).

3.3.2. Surgical fracture treatment

Generally, irrespective of the type of osteosynthesis material expected to be used (always on the basis of the most appropriate surgical indication), this biological bone regeneration therapy is used with PRP in either its liquid form or as a fibrin membrane or clot during surgical fracture repair. Our group has developed a set of basic guidelines for the application of PRP during the surgical treatment of fractures [5].

The fracture to be treated is assessed to determine the amount of PRP required and therefore the volume of blood to be extracted. If surgical treatment of the fracture site does not require the use of allo- or autografts, the fracture is reduced/stabilised. Sound stabilisation of the fracture site is a key factor in the subsequent repair process. Liquid-activated PRP is infiltrated at the fracture site and at its bony ends, using a Luer Lock syringe fitted with a needle of the appropriate gauge (**Figure 7**).

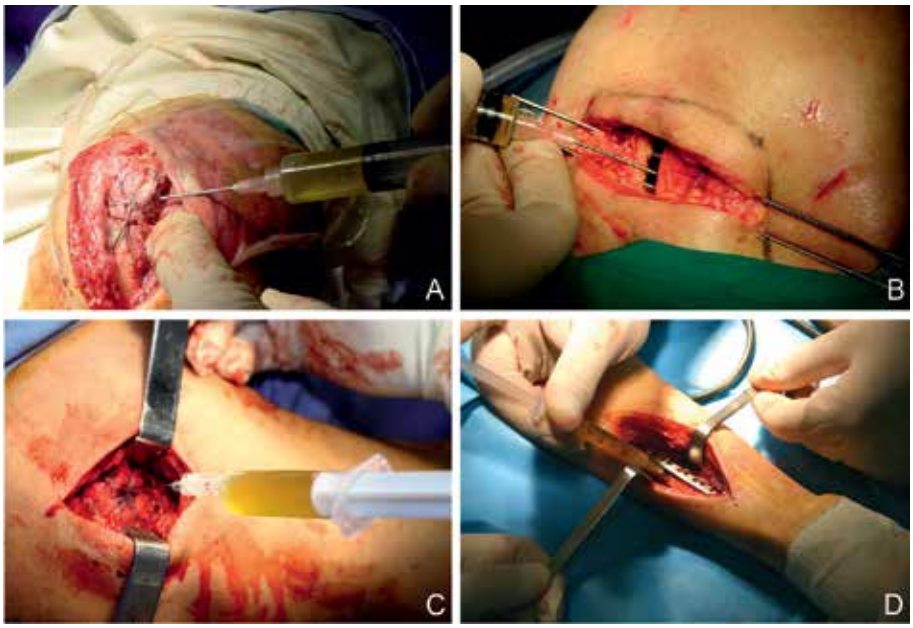

Figure 7. Infiltration of liquid PRP at the fracture site, after stabilisation and osteosynthesis, into an olecranon fracture (A), into the os acromiale (B), into a fracture of the humeral head (C) and (D) at the radial fracture.

It is particularly important to stress that in the case of fractures of the fibula, calcaneus and other sites where the skin tends to heal poorly, we apply PRP on the skin margins of the surgical wound to enhance spontaneous epithelisation and to induce a bacteriostatic and anti-inflammatory effect. If fracture repair presents signs of delayed consolidation, a further percutaneous infiltration is performed at the fracture site following the same basic steps as described previously.

3.3.3. Treatment of nonunions

When the nonunions present a stable fracture area and appropriate osteosynthesis, the procedure consists in a percutaneous infiltration under anaesthesia. It is important to locate and infiltrate the edges of the bones accurately by using an image amplifier. In these cases, a trocar is used to perform the injection to allow several controlled perforations in the injured site. The fracture region and contiguous bone areas are infiltrated with 6–8 mL of liquid-activated PRP. This treatment is repeated in a weekly basis up to a total of three infiltrations (**Figure 8**).

When an adequate fixation is not reached, debriding and bleeding of the bony edge fragments are conducted in the nonunion area. Next, the region is stabilised using appropriate osteosynthesis material. Liquid-activated PRP is infiltrated into the edges of the bone fragments as in the previous case. When the nonunion presents a bone defect, a bone graft (auto- or allograft) is used together with liquid-activated PRP.

If the criteria described before are followed, treated patients should evolve favourably, presenting clinical and radiographic results that show full resolution in between 2 to 6 months [40]. Similar outcomes have been achieved by other authors such as Seijas et al. [41].

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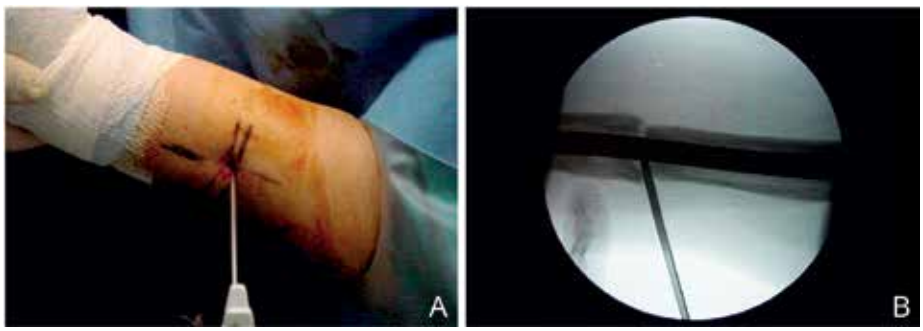

Figure 8. Percutaneous infiltration of liquid-activated PRP at the area of a humeral nonunion (A) under radiological control (B).

3.4. PRP and meniscal surgery

The abundant ECM (between 60 and 70% of tissue weight) presented in meniscus determines the reparation of this tissue. Cells such as fibrochondrocytes and fibroblast are dispersed throughout the ECM. The peripheral area or meniscal wall presents the tissue vascularity (limited to 10: 30%), the largest number of cells, and it receives nerve endings [42]. Due to these characteristics, the recovery capacity of meniscus is highly influenced by this outer portion, since that is where the repair stimuli are generated [43]. Meniscus participates in the stability to the knee and in the support of compressive, traction and shearing forces. In addition, it absorbs part of the mechanical stress received by the knee and takes part in the lubrication of the knee. Thus, injuries in this structure compromise joint function, and it is recommendable to enhance its limited regenerative capacity to achieve an optimal repair. Because promising results are showed by PRP on meniscal cells in laboratory experiments [44], it has been emerged as a novel technique for treating meniscal tears [45].

3.4.1. Meniscectomy

Bearing in mind the special conditions of the meniscal wall, liquid-activated PRP needs to be infiltrated into this structure during a partial meniscectomy. The injection is conducted in an extra-articular way (from outside to inside). However, when the posterior horn of the external meniscus is infiltrated, the injection is performed from inside to avoid vascular or nerve damage. A 21G needle and a 3 mL syringe are used in order to spread the PRP into the meniscus, since a high pressure is required because of the high density of this tissue compared with other structures. Finally, an intraarticular infiltration is performed with 8 mL of liquid-activated PRP is infiltrated in an intraarticular manner.

The maintenance of the meniscal wall is a key element to achieve a partial repair and healing process of the meniscus, and it should be maintained whenever possible. This region presents the cellularity and vascularization needed to generate the biological stimuli for repair and regeneration.

3.4.2. Meniscal sutures

The meniscal sutures are a suitable technique to preserve the structure of the knee and consequently reach greater stability and protection of cartilage. The infiltration protocol is similar to that described in the meniscectomies, but in this case, PRP infiltration is applied not only into the meniscal wall but also into the suture region. An intraarticular infiltration of PRP is carried out when the whole process is finished. After 14 days other intraarticular injections of PRP could be conducted to improve the repair process, depending on the evolution of patient.

3.5. PRP in the management of neuropathies

PRP products hold an important therapeutic potential as a neuroprotective, neurogenic and neuroinflammatory therapeutic modulator system [46–50] and as enhancer of sensory and motor functional nerve-muscle unit recovery [51–53]. They are applied either as a filler of nerve conduits or vein-muscle grafts across nerve gaps post-trauma by ultrasound-guided perineural and intraneural infiltrations or as scaffolds to bridge or wrap the injured nerve stumps [54–56]. Moreover, there are non-traumatic peripheral injuries such as compression, adhesion and fibrosis [46], where this novel approach may diminish undesirable consequences such as fibrotic scars and denervated organ atrophy, since this adjuvant therapy can speed up the functional recovery of the nerve-muscle unit [55–58]. The therapeutic potential of PRP for nerve repair lies in the prolonged and gradual delivery system of biomolecules and in its function as a transient guidance scaffold for axonal sprouting [51, 57]. Considering Schwann cells (SC) as key in the nerve repair process, they are an idoneal target for the synergic action of neurotrophic and neurotropic factors of PRP. Thus, the release of biomolecules from the fibrin matrix at the beginning of regeneration process would induce several biological effects of SC aimed to repair [58–60].

In surgical repair by PRP as in the case of end-to-end neurorrhaphy, nerve compression and nerve entrapment, we recommend combining intraneural and perineural infiltrations of liquid PRP with the application of a PRP membrane as scaffold, which wraps the injured tissue.

4. Guidelines for the appropriate use of PRP infiltrations

Good treatment commences with a correct overall diagnosis that entails the highest number of factors implicated in the disease and considers all the best options.

1. It should be remembered that inactivated PRP can be stored for 3–4 hours without losing its efficacy. However, once activated, it must be used immediately, in the ensuing 2–3 minutes after activation. This aspect provides us with room for manoeuvre when scheduling its use in the theatre room.
2. The volume of the infiltration syringe and the diameter of the needle used will affect the diffusion of PRP within the tissues. The use of small syringes means that large pressures are exerted on the ECM structures during infiltration, thereby accounting for local disruption of the components.

3. Upon infiltration, the needle should be oriented as closely as possible, parallel to and longitudinal with the tendon. This results in the optimal diffusion of PRP while allowing the position of the needle to be controlled as closely as possible with ultrasound guidance.
4. The application of PRP should not alter the surgical technique commonly used for their repair. The main result of combining PRP with surgical treatment is to shorten and lessen the intensity of the initial defence phase and to accelerate the proliferative and trophic phases during the tissue repair process.
5. It is fundamental to combine the application of PRP with other rehabilitation treatments and physiotherapy as mechanical stimuli. Indeed, the combination of different therapeutic elements provides extremely favourable synergies.
6. Given the heterogeneous composition and products of PRPs, it is difficult to ascertain general guidelines in order to optimise them. Rehabilitation and other systemic factors such as nutritional imbalance, overuse or disuse of tissues and life style may account for the majority of degenerative processes.

Conflicts of interest

SP is a researcher of BTI (Biotechnology Institute), a dental implant company that investigates in the fields of oral implantology of PRGF-Endoret technology.

Author details

Mikel Sánchez[1]*, Diego Delgado[2], Ane Garate[2], Pello Sánchez[2], Jaime Oraa[1], Ane Miren Bilbao[1], Jorge Guadilla[1], Beatriz Aizpurua[1], Nicolás Fiz[1], Juan Azofra[1] and Sabino Padilla[3]

*Address all correspondence to: mikel.sanchez@ucatrauma.com

1 Arthroscopic Surgery Unit, Hospital Vithas San José, Vitoria-Gasteiz, Spain

2 Advanced Biological Therapy Unit, Hospital Vithas San José, Vitoria-Gasteiz, Spain

3 University Institute for Regenerative Medicine and Oral Implantology – UIRMI (UPV/EHU-Fundacion Eduardo Anitua), Vitoria–Gasteiz, Álava, Spain

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Chapter 4

Plasma Exchange in Clinical Practice

Jean J. Filipov, Borelli K. Zlatkov and Emil P. Dimitrov

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76094

Abstract

Plasma exchange (PEX) is a treatment method with increasing range of indications. However, due to the small number of randomized trials, its effectiveness is still under debate in certain conditions. The aim of our chapter is to present the major principles of PEX, discuss safety issues and reveal current data for treatment effectiveness of the method. Novel indications for PEX will also be discussed.

Keywords: plasma exchange, indications, contraindications, safety

1. Introduction

Plasma exchange (PEX) is an invasive therapeutic method, separating plasma from blood cells. Thus, pathogenic antibodies or other large molecules are removed and plasma is replaced by human albumin and/or fresh frozen plasma (FFP). The method was first developed in the first half of the twentieth century. Over the years a significant improvement in the PEX technique, patient safety and broadening of indications were observed. Selective techniques were also introduced into practice, leading to selective removal of proteins and reduction of protein loss during the standard procedure, especially fibrinogen. Thus, improved effectiveness and patient safety was achieved.

2. Plasma exchange: basic principles

Generally, in PEX, blood is pumped out of the patient's circulation and is transferred to the filter, separating plasma from blood cells. Afterwards, blood cells are pumped into the patient's vein. Patient's plasma is substituted by human albumin and/or FFP.

2.1. Vascular access

In most of the cases, central venous catheters are used in PEX, especially in acute conditions. They can be placed in internal jugular, femoral and subclavian veins. However, if life-long treatment is needed (e.g., LDL apheresis), arteriovenous fistula creation may be required. In addition, as the blood flow is low (90–150 ml/min), large peripheral veins can be used (cubital veins). Single-vein access is also possible but in cases where centrifugal separation of plasma is used.

2.2. Separation techniques

Plasma is separated from blood cells via two major methods—centrifugal and hollow-fiber membrane separator. In addition, more selective methods were developed.

2.2.1. Centrifugal separation

The separator is a disposable rotating centrifugal bowl. Blood runs into the bowl and centrifugal force separates blood cells from plasma. Blood cells are pumped back into patient's circulation, whereas plasma is separated in sterile bags. The process can occur simultaneously or intermittently. There is no upper limit for the size of the molecules removed by centrifugal PEX. Usually the blood flow ranges between 90 and 150 ml/min. A major disadvantage of centrifugal PEX is platelet count reduction, which may reach up to 50% [1].

2.2.2. Membrane PEX

In this type of PEX, highly permeable hollow fiber membrane filters are used. The fibers have pores with diameter ranging from 0.2 to 0.5 μm. As blood runs through the fibers plasma is separated from the blood cells, which are returned in patient's circulation. All immunoglobulins are effectively cleared by this method. However, its effectiveness is poorer in immune complexes and cryoglobulins. The risk for platelet count reduction is small. Yet, there is a risk for hemolysis, especially if faster blood flow is used (normal values for the method are 90–200 ml/min). Synthetic membranes are used; plasma filters should not be reused [1].

2.2.3. Selective separation techniques

The abovementioned plasma separation techniques remove plasma from whole blood, thus causing loss of normal proteins, especially coagulation factors and albumin. In order to reduce protein loss, selective PEX techniques were introduced into practice.

2.2.3.1. Double cascade PEX

Cascade filtration is a semi-selective separation technique, in which after initial separation of plasma from blood cells, additional filtration of plasma is performed with different diameters of fiber pores, so that target protein fractions are filtered and the rest are pumped back in circulation. This technique showed up to 70% reduction in albumin loss after the procedure [2].

2.2.3.2. Cryofiltration

The method is used to remove cryoglobulins in several immune diseases. After plasma is initially filtrated, it is cooled to 4°C. This causes precipitation of cryoglobulins and they do not pass the second membrane. Afterwards, the cooled plasma is warmed to body temperature again and is returned to the patient.

2.2.3.3. Thermofiltration

Similar to cryofiltration, plasma is firstly separated from whole blood. Before the selective filtration, the filtrate is warmed up to 40°C, causing aggregation of VLDL and LDL molecules. Then second filtration is performed and the filtrate is introduced back into patient's blood. The method is not widely used due to the fact that little is known about the changes in large molecules after being exposed to higher temperatures [2].

2.2.3.4. Unselective adsorption

Unselective adsorption uses charcoal or ion exchange raisins to remove exogenous or endogenic toxins from blood (hemoperfusion) or from filtered plasma (plasmaperfusion). These methods are most commonly indicated in exogenous intoxications. There are reports that hemoperfusion was effective in sepsis, septic shock and disseminated intravascular coagulopathy [3]. Currently, plasmaperfusion is gaining ground over hemoperfusion due to its improved effectiveness and improved safety profile.

2.2.3.5. Selective adsorption

In selective adsorption the initial filtrate runs through prearranged immunosorbents. Thus, specific antibodies can be selectively removed, whereas albumin and clotting factors are returned to the patient. There are two types of selective adsorption—*immunoadsorbtion (IA)* and *selective plasma adsorption*. In IA, either the plasma runs through column bearing antigens directed against certain antibodies or antibodies against certain plasma constituents. In selective plasma adsorption, plasma components are removed by binding to ligands other than antibodies and antigens (e.g. heparin and dextransulfate in LDL adsorption).

Different immunoadsorbtion (IA) techniques exist, but protein A-based IA is the most commonly used one. Protein A is a *Staphylococcus aureus*-derived molecule that binds to the Fc-region of immunoglobulin G (IgG). The principle of the procedure is similar to the previous selective methods—plasma is firstly separated from blood and then the filtrate runs through protein A—containing filters. Thus, immunoglobulins (IgG) and immune complexes are removed and the filtrate is pumped back into circulation. The method is well tolerated and is used in the following situations: acute antibody-mediated rejection, presensitized kidney transplant (KT) candidates, systemic lupus erythematosus (SLE), Guillain-Barrè syndrome, Goodpasture syndrome, myasthenia gravis, hemolytic uremic syndrome (HUS) and so on.

Other adsorbents can carry antibodies against proteins to be removed too. Polyvinyl-alcohol gel, bound to tryptophan, is used for removal of anti-acetylcholine-receptor antibodies in myasthenia gravis; phenylalanine-bound polyvinyl-alcohol gel for selective removal of anti-DNA antibodies; and cardiolipin antibodies in SLE [2].

2.3. Anticoagulation

Practically in all PEX procedures, anticoagulation is needed. In centrifugal PEX, usually citrate anticoagulation is used, whereas in membrane PEX, heparin is the anticoagulant of choice [1]. Citrate has advantages in patients with high bleeding risk, as it has no influence on systemic coagulation, but it is associated with increased incidence of hypocalcaemia. The usual dose of heparin is bolus dose 2000–5000 IU, followed by infusion of 500–2000 IU per hour. Anticoagulants are administered pre-filter. Low-molecular weight heparins (LMWH) can be used too. They are associated with lower incidence of side effects and more selective prevention of clotting. In our institution low-molecular weight heparins are used in doses, 0.01 ml/kg body weight or generally 0.8–1.0 ml LMWH per procedure.

2.4. Substitution fluids

PEX requires large volumes of replacement fluids. A single procedure was found to reduce plasma macromolecule levels by 60% [4]. The use of crystalloids is ineffective, as they are not capable of preserving the intravascular oncotic pressure. Gelatin-based plasma expanders have limited practical importance, as they have shorter half life compared to albumin-based fluids. Therefore, the most widely used substitution fluid is human albumin. Replacement volume reaches 50 ml/kg, 4–5% human albumin per procedure. The major disadvantage of albumin replacement is the lack of coagulation factors. Therefore fresh frozen plasma (FFP) can be applied after PEX. In certain diseases, the replacement fluid should consist of FFP only— for example, HUS, thrombotic thrombocytopenia purpura (TTP) and so on. Other indications for FFP use are reduction of plasma fibrinogen level below 1.25 g/l, increase of prothrombin time more than 2 s above normal values and increased risk of bleeding (pulmonary hemorrhage, 48 h after biopsy/surgery) [1]. FFP should be used with caution, as its application is associated with hypotension, citrate-associated paraesthesia, urticaria, anaphylaxis and blood-borne infections.

2.5. Treatment volume: frequency of PEX

2.5.1. Treatment volume

A formula for determining the needed volume of single PEX was suggested by A.A. Kaplan [5]:

$$Volume\ PEX = \left[0.065 * body\ weight(kg)\right] * (1 - Hct) \quad (1)$$

where *kg: kilograms and Hct: hematocrit*.

An easier way to assess the needed PEX volume is 30–50 ml/kg body weight.

2.5.2. Frequency of PEX

PEX is usually performed daily or every other day. The duration of treatment is 10–14 procedures, but it can be guided by clinical outcomes and laboratory results (auto-antibody titers, platelet count, etc).

2.6. PEX: mechanism of action

PEX and IA have beneficial effects on different diseases due to the following mechanisms [2]:

- Elimination of pathological constituents—alloantibodies/autoantibodies, paraproteins, circulating immune complexes, toxins and so on.
- Substitution of plasma proteins: clotting factors, hormone carrier proteins, immunoglobulins and so on.
- Modifying immune cells' functions: deblocking of reticuloendothelial system and modifying lymphocyte response.

2.6.1. Immunosuppressive treatment in PEX

Despite the mentioned beneficial effects, PEX is not effective in immune disease when used alone, as it influences pre-existing pathological molecules and has no influence on their formation. It was established that the procedure causes rapid decrease in antibody titers, which is followed by increased antibody production and B-cell proliferation [6]. In addition, the combination PEX and immunosuppressive therapy has better results compared to using plasma exchange and immunosuppression alone.

Several combinations have been suggested. Initially, steroids of 1–2 mg/kg for 2–3 weeks or cyclophosphamide of 2–3 mg/kg for 2–3 weeks, followed by azathioprine of 1–2 mg/kg for several months after cyclophosphamide treatment, were suggested. Later, cyclophosphamide pulses and PEX were found superior to oral intake and PEX [7]. Over the last years new immunosuppressive agents have been introduced as concomitant therapy in PEX—cyclosporine A, tacrolimus and mycophenolic acid [8]. In addition, biological agents are more widely used—monoclonal antibodies (e.g., rituximab) and intravenous immunoglobulin (IVIG). Rituximab is usually applied, 375 mg/m^2/weekly, for 2–4 weeks. IVIG is applied, 100 mg/kg, after each PEX [9, 10].

2.6.2. Additional medications

Calcium gluconate and potassium chloride can be infused to counterbalance PEX-associated hypocalcaemia and hypokalemia.

3. Contraindications to plasma exchange: complications

3.1. Contraindications to PEX

The major contraindications to PEX are hemodynamically unstable patients, sepsis, history for allergy to human albumin or FFP.

3.2. Complications

Generally, the procedure is safe, and though the incidence of all complications peaks to 40%, the risk for life-threatening adverse events (defined as death, hypotension-requiring vasopressor agent, arrhythmias, medical intervention and hemolysis) is low, ranging between 0.025 and 4.75% [11, 12]. There are three groups of complications in PEX, which are summarized in **Table 1**.

As the most serious complication, death has incidence of up to 0.05%, though most of the patients were with severe pre-existing conditions [2]. However, the complication rate varies across registries, as mortality was 0% in the Swedish apheresis data base, encompassing more than 20,000 procedures. Yet the same trend was observed—overall complications' rate reached 4.3%, of which just 0.9% were serious adverse events [13].The highest risk for complications was detected in unstable patients, hypotension, active bleeding, bronchial

Vascular access-associated	Hematoma
	Infection/sepsis
	Pneumothorax
Substitution therapy-associated	Anaphylaxis to FFP
	Death, due to anaphylaxis
	Coagulopathy
	Blood-borne infections
	Hypocalcaemia
	Hypokalemia
Other	Hypotension
	Dyspnea
	Low platelet count
	Hemolysis
	Drug and vitamin removal
	Death, due to cardiac arrest, pulmonary edema and pulmonary embolism
	Anaphylactoid reactions, hypotension, flushing due to ACE inhibitors and the use of dextran sulfate systems for LDL apheresis

FFP: fresh frozen plasma, ACE inhibitors: angiotensin-converting enzyme inhibitors, LDL: low-density lipoprotein.

Table 1. Complications in plasma exchange.

obstruction and anemia [14]. In addition, the complications are significantly more in PEX with FFP substitution, compared to human albumin only.

Similar results were observed in our institution. In 51 PEX procedures no life-threatening complications were detected. Two episodes of hypotension were established, not requiring vasopressor agents. Two patients developed paraesthesia. Laboratory results prior and after PEX remained stable (hemoglobin level, white blood cell count, platelet count and potassium and calcium levels). An expected drop in fibrinogen, immunoglobulin A and G levels, was detected, without bleeding or infection episodes, associated with the procedure [15].

In conclusion, though the procedure is relatively safe, due to the risk for serious complications, it should be performed by experienced personnel.

3.3. Indicators to monitor during PEX treatment

3.3.1. Clinical indicators

The basic clinical parameters should be monitored prior to and after the procedure—blood pressure, heart rate and body temperature. Clinical assessment can be performed at shorter intervals of time during the procedure in unstable patients.

3.3.2. Laboratory indicators

Full blood count, plasma calcium, plasma potassium, fibrinogen levels and prothrombin time should be evaluated after each procedure. Other laboratory tests can be performed prior to and after PEX treatment, including antibody titers.

4. Plasma exchange: clinical indications

4.1. Clinical indications: classification

PEX was prescribed with different volume, duration, frequency, number of performed procedures and different concomitant (immunosuppressive) therapy over the years. This is the reason for the relatively small number of randomized controlled trials (RCTs) concerning plasma exchange.

In order to evaluate the present data for the effectiveness of PEX in the treatment of different diseases, the American Society for Apheresis (ASFA) has classified the indications into four categories, according to the possible beneficial effect of PEX [16, 17]:

- *ASFA category 1: Disorders for which apheresis is accepted as first-line therapy, either as a primary stand-alone treatment or in conjunction with other modes of treatment.*
- *ASFA category 2: Disorders for which apheresis is accepted as second-line therapy, either as a stand-alone treatment or in conjunction with other modes of treatment.*

- *ASFA category 3: Optimum role of apheresis therapy is not established. Decision-making should be individualized.*
- *ASFA category 4: Disorders in which published evidence demonstrates or suggests apheresis to be ineffective or harmful.*

4.2. Clinical indications: renal diseases

4.2.1. Rapidly progressive glomerulonephritis

Rapidly progressive glomerulonephritis (RPGN) is a glomerular disease, associated with crescent formation in over 50% of the glomeruli, necrotic changes and rapid deterioration of kidney function. Kidney findings can be coupled with pulmonary hemorrhage, thus defining the Goodpasture's syndrome. RPGN can be detected also in systemic lupus erythematosus (SLE), IgA nephropathy, post-infectious glomerular disease and systemic vasculitis [anti-neutrophil cytoplasmic antibodies (ANCA)-associated RPGN].

4.2.1.1. Anti-glomerular basement membrane disease

Anti-glomerular basement membrane (anti-GBM) disease or Goodpasture's syndrome encompasses diseases with antibodies against the glomerular basement membrane. A term within this definition is Goodpasture's disease, indicating disease associated with antibodies against the α3 chain of collagen type 4, present in alveoli and glomeruli. Goodpasture's syndrome can present with renal and pulmonary involvement—rapidly progressing renal failure, hemoptysis and pulmonary failure. PEX and immunosuppression (steroids, cyclophosphamide) are the cornerstones of anti-GBM disease. Alveolar involvement is associated with high mortality; therefore, the presence of pulmonary hemorrhage is absolute indication for PEX (ASFA category 1) [16, 17]. Dialysis-independent patients with anti-GBM disease also fall in this category. In dialysis-dependent cases without alveolar hemorrhage, the effectiveness of the method is reduced and is classified as ASFA category 3. Treatment should be performed daily/every other day for at least 14 days. Though ant-GBM antibody titers can be evaluated, the best way to assess effectiveness of the treatment is clinical outcomes [16].

4.2.1.2. ANCA-associated glomerular disease

The major representatives of this group are Wegener's granulomatosis with polyangiitis, microscopic polyangiitis and Churg-Strauss syndrome. They are characterized with RPGN, systemic involvement, minimal or no immune deposits in the vascular wall and usually have elevated ANCA titers (ANCA/+/positive), though 10% of the cases are ANCA /−/negative. Treatment of ANCA/+/and ANCA/−/is similar and is based on immunosuppression and PEX. Immunosuppressive treatment includes high-dose steroids and cyclophosphamide. Rituximab can substitute cyclophosphamide too [18]. PEX is performed daily/every other day for 6–9 procedures. In cases of rapidly progressing kidney failure daily procedures are recommended. PEX is indicated in patients with pulmonary hemorrhage and on dialysis (ASFA category 1), whereas for dialysis-independent patients the effectiveness is significantly lower, therefore, categorized by ASFA as category 3 [16, 17].

4.2.2. Infection-associated glomerular disease

Infection-associated glomerular disease consists of the following major subgroups of diseases:

- Bacterial infection-related diseases: Post-streptococcal glomerulonephritis (PSGN), infective endocarditis-related glomerulonephritis and shunt-associated glomerulonephritis
- Hepatitis C virus (HCV)-associated glomerular disease
- Hepatitis B virus (HBV)-related glomerular disease
- Human immunodeficiency virus (HIV)-related glomerular disease
- Protozoal infection-related glomerular disease

The cornerstone of infection-related glomerular disease is treatment of the underlying infection. However, in cases of histologically detected crescents, rapidly progressive GN, complicating PSGN or schistosomiasis-related glomerulonephritis immunosuppressive treatment can be considered. In HCV – related glomerulonephritis, associated with presenting with mixed cryoglobulinemia (IgG/IgM), presenting with nephrotic proteinuria/acute flare of cryoglobulinemia/rapid deterioration of kidney function, immunosuppressive treatment can be coupled with PEX [18]. PEX is effective in cryoglobulinemia and is categorized as ASFA category 1 and is superior to IA. Generally, 3–8 procedures are required. Immunosuppression with rituximab was superior to other immunosuppressive agents [16].

4.2.3. Membranoproliferative glomerulonephritis

In cases of membranoproliferative glomerulonephritis (MPGN), an underlying disease (SLE, HCV or HBV infection, monoclonal gammopathies and rheumatologic disorders) should be ruled out. Idiopathic MPGN is treated with immunosuppressive agents. PEX is rarely indicated, except for HCV-associated MPGN, complicated with cryoglobulinemia (Section 4.2.2). In all other cases of MPGN the use of PEX is under debate due to the small number of studies and the controversial results [19].

4.2.4. Minimal change disease and focal segmental glomerular sclerosis

Minimal change disease (MCD) and focal segmental glomerular sclerosis (FSGS) are disorders presenting with nephrotic syndrome. Several pathogenic mechanisms for increased protein loss in urine have been suggested, including the presence of permeability factors, increasing the permeability of glomerular basement membrane (GBM) for proteins. Several molecules were considered for permeability factors (e.g., cardiotrophin-like cytokine 1), but currently no definite molecules proved to increase GBM permeability. Due to the similar pathogenesis and podocyte involvement, MCD and FSGS are considered by most of the researchers as different steps in the progression of a single glomerular disease. Both MCD and FSGS have primary and secondary forms. The treatment of the secondary forms is based on the treatment of the underlying disease, whereas primary forms are treated with steroids or cyclophosphamide, mycophenolate mofetil and calcineurin inhibitors [18]. Plasma exchange is not effective in FSGS and MCD in native kidneys.

LDL apheresis was found to have beneficial effects in steroid-resistant FSGS cases [20]. Long-term efficacy of low-density lipoprotein apheresis for focal and segmental glomerulosclerosis is present. However, the data for this selective method in FSGS are insufficient and this modality is not widely available. PEX is indicated in recurrent FSGS after kidney transplantation (ASFA category 1), despite the inconsistent data for the efficacy in preserving graft function. At least nine procedures should be performed in recurrent FSGS, though the PEX can be prolonged for several months after improvement in proteinuria, by performing weekly or monthly procedures. In addition, pre-transplant PEX was found to reduce the incidence of recurrent FSGS [16, 17].

4.2.5. Membranous nephropathy

Membranous nephropathy (MN) is a glomerular disease, presenting with nephrotic syndrome. It has idiopathic and secondary forms (neoplasia, SLE, viral diseases). In primary MN auto-antibodies against the M-type phospholipase A2 receptor were detected, possibly involved in the pathogenesis of the disease [21]. Treatment of MN is based on angiotensin-convertase enzyme (ACE) inhibitors, steroids, combined with cyclophosphamide or calcineurin inhibitors. Current guidelines do not suggest the use of PEX, though currently there are reports for successful treatment of MN with PEX and rituximab/IVIG [9].

4.2.6. IgA nephropathy: Henoch-Schönlein purpura

IgA nephropathy (IgAN) is characterized by mesangial proliferation and deposits of immunoglobulin A in the mesangium. Henoch-Schönlein purpura (HSP) is a small vessel vasculitis, involving intestines, skin, joints and the kidney. Histologically the renal findings in HSP are similar to IgAN. A rare presentation of the disease is acute kidney injury due to crescentic glomerular involvement. Small studies indicate beneficial effects of PEX in crescentic IgAN [22]. However, guidelines do not support plasma exchange in IgAN or HSP (ASFA category 3), even in the presence of crescents or severe extra-renal manifestations of HSP, due to the scarce data supporting plasmapheresis in these cases [16, 18].

4.2.7. Lupus nephropathy

PEX was found to have no significant effect on patients with lupus nephropathy (LN) in the randomized controlled trial [23] (AFSA category 4). A recent meta-analysis also established no significant effect of PEX in the treatment of proliferative LN [24]. In addition, IA was not superior to PEX in LN [16, 17]. PEX and IA are used in other presentations of SLE (the issue will be discussed in a different section).

4.2.8. Systemic amyloidosis

Plasma exchange is ineffective in systemic amyloidosis and falls into ASFA category 4, both for AA and for AL sub-forms [16]. However, β2 microglobulin adsorption was partially effective in β2 amyloidosis [17].

4.2.9. Kidney transplantation

PEX is used in three major directions—pre-transplantation treatment of sensitized/AB0 incompatible patients, antibody removal in rejection and recurrent disease after KT.

4.2.9.1. HLA-sensitized patients and AB0 incompatible KT

Desensitization protocols are used in candidates for KT in order to increase the donor pool in organ transplantation. Treatment of patients with HLA antibodies and positive cross-match reaction proved to be effective with excellent results for the 1-year graft survival. Different protocols exist, yet PEX or IA are the cornerstones of HLA desensitization protocols, accompanied by immunosuppressive treatment with IVIG or rituximab or both [25, 26]. Currently, bortezomib is being introduced in the immunosuppressive regimen. However, HLA desensitization was not effective in deceased donors [16]. The treatment should aim for negative cross-match prior to KT. Despite the good short-term results, in the long term, there is increased incidence of rejection and poorer graft survival.

In AB0-incompatible KT, again protocols using PEX/IA, combined with IVIG/or/and rituximab, are used (ASFA category 1) [16, 17]. The procedures are performed prior to and after KT. The treatment aim is reduction of anti-AB0 antibody titers from 1:4 to 1:32. Currently, short- and long-term graft survival is similar to AB0-compatible transplantation [27].

4.2.9.2. Antibody-mediated rejection

Antibody-mediated rejection (AbMR) is associated with histologically detected graft injury, positive C4d staining and the presence of circulating donor-specific antibodies. Both IA and PEX are used in the treatment of acute AbMR, in association with IVIG, 100–200 mg/kg, after each procedure [28]. Treatment should be accompanied with anti-T cell treatment with thymoglobulin. Rituximab can be added to the immunosuppressive protocol [29]. Usually 5–6 procedures are performed.

Unfortunately, PEX in chronic AbMR is not as effective as in acute AbMR due to the irreversible changes in the graft [29].

4.2.9.3. Posttransplant recurrent glomerulonephritis

PEX/IA is the part of the first-line treatment in recurrent FSGS (Section 4.2.4). Recurrent anti-GBM disease is also an indication for aggressive plasma exchange. In recurrent ANCA-associated glomerulonephritis, similar treatment to native kidneys should be initiated [30]. In MPGN, PEX can also be considered, though the data are scarce. Currently, there are no data to support the use of PEX in recurrent IgAN and membranous nephropathy. PEX also had controversial results in recurrent LN after KT [30].

4.3. Clinical indications: hematology

4.3.1. Thrombotic microangiopathies

Thrombotic microangiopathies (TMAs) are acute syndromes, characterized by hemolytic anemia, thrombocytopenia and organ involvement due to microvascular thrombosis. It consists of two clinical aspects, having similar pathogenesis— hemolytic-uremic syndrome (HUS), presenting in children with predominant renal involvement, and thrombotic thrombocytopenic purpura (TTP), mainly in adults with severe neurologic presentation. The etiology encompasses the presence of autoantibodies, drugs, systemic diseases and pregnancy. The diagnosis should be made as early as possible, so that adequate treatment can be initiated.

4.3.1.1. PEX treatment in HUS

Plasma exchange was found effective mainly in atypical HUS, especially in the presence of complement factor gene mutations (ASFA category II) and the presence of factor H autoantibody (ASFA category I). Treatment should be started as early as possible, with treatment volumes of 50 ml/kg, daily procedures for at least 5 days and with subsequent reduction of the PEX procedures per week. Substitution should be performed with FFP only. The decision to stop treatment should be taken based on the patient's response and condition. In addition to PEX, treatment with rituximab and eculizumab can be added to the therapy [16].

4.3.1.2. PEX treatment in TTP

TTP is a potentially fatal disease and PEX has significantly improved survival in these patients. Therefore it is the first-line treatment in TTP. PEX is initiated at similar doses and daily procedures should be performed until platelet count rises above 150×10^9/l for three consecutive days. Supplementation should be made with FFP/cryoprecipitate poor plasma. Afterwards, procedures can be performed less frequently, though no data exist. Additionally, steroids and rituximab can be used in the treatment.

4.3.1.3. PEX in drug-related TMA

TMA is associated with the use of several drug classes—calcineurin inhibitors, medications reducing platelet aggregation (ticlopidine, clopidogrel) and so on. Of these, PEX proved to be an effective option in ticlopidine-associated TMA. In all other medications, PEX was not associated with clear improvement in patient outcomes [16, 17].

4.3.2. Multiple myeloma

Multiple myeloma has a wide spectrum of renal involvement, spanning from myeloma cast nephropathy, AL amyloidosis to cryoglobulinemia and membranoproliferative glomerulonephritis. Chemotherapy is the crucial part of the treatment. AL amyloidosis is not significantly influenced by PEX [16]. The effect of PEX in myeloma cast nephropathy was also evaluated in the past. However, the results so far are conflicting. Therefore, the use of PEX in everyday practice is not recommended [31].

4.3.3. Waldenström macroglobulinemia

Increased serum levels of plasma proteins increase serum viscosity, leading to small vessel damage, especially small veins. Clinically, hyperviscosity presents with retinopathy and neurological symptoms (headache, somnolence, coma and seizures). Hyperviscosity syndrome is usually detected in Waldenström macroglobulinemia and multiple myeloma. Generally, the treatment of the diseases is chemotherapy. PEX is applied in cases of symptoms associated with hyperviscosity. Generally, when substitution volume is 50 ml/kg, human albumin is used. Symptoms are relieved after 1–3 procedures; after that PEX can be discontinued or prophylactic procedures monthly can be performed [16, 17].

4.3.4. Autoimmune hemolytic anemia

Autoimmune hemolytic anemia (AIHA) is a disorder in which autoantibodies cause either intravascular or extravascular destruction of red blood cells (RBCs). AIHA is categorized in two major groups—warm AIHA (antibodies reacting at body temperature) and cold agglutinin disease (CAD, hemolysis occurring at temperatures between 0 and 5°C). The first-line treatment for warm AIHA is prednisolone; rituximab is used as the second-line agent. In CAD the primary goal is avoidance of exposure to the cold; in severe cases rituximab is the drug of choice. The results for PEX treatment in warm AIHA are conflicting; therefore, its use is limited to severe cases of fulminant AIHA. In CAD, PEX has shown no effect in terms of improvement of long-term outcomes. Due to the risk of agglutination at room temperature for CAD, the procedure should be performed at higher temperatures for both extracorporeal circuit and room temperature.

4.3.5. Aplastic anemia

Aplastic anemia (AA) and pure red cell aplasia (PRCA) are rare hematopoietic stem cell disorders. In AA there is pluripotent progenitor cell involvement, causing pancytopenia and hypocellular bone marrow. In PRCA only erythroid progenitors are affected, leading to normochromic, normocytic anemia, reticulocytopenia, severe reduction in marrow erythroid precursors and normal myelo- and lymphopoiesis, as well as platelet production. The diseases can be idiopathic, as well as secondary, due to infection, neoplasia, chemicals and drugs. As the pathogenesis of the conditions is mostly immunological (the presence of autoantibodies was established), immunosuppressive agents are usually used as first-line treatment. PEX can also be considered in immunosuppression-resistant cases. PEX is performed until hematopoiesis/erythropoiesis recovers [16].

4.3.6. Hematopoietic stem cell transplantation

PEX is used in the case of AB0-incompatible hematopoietic stem cell transplantation (HSCT) and in HLA desensitization protocols. There are two types of AB0-incompatible HSCT—major and minor ones. In major AB0-incompatible HSCT, natural isoagglutinins in the recipient against the donor's A and/or B blood group antigens are present. They cause acute hemolysis of the RBCs present in infused hematopoietic progenitor cell (HPC) products. In minor AB0-incompatible HSCT, isoagglutinins cause hemolysis only if the antibodies are in high titers (above 1:128). In major AB0-incompatible HSCT, PEX and IA are used to reduce the titers of natural isoagglutinins. Procedures are performed daily, substitution volume is usually 50 ml/kg, and substitution fluid includes albumin and donor- and recipient-compatible FFP. The reduction of isoagglutinin titers below 1:16 is aimed prior to HSCT.

In HLA-sensitized patients there is reduced graft survival after HSCT. Reports indicate that successful procedure after desensitization is performed. PEX is used to remove donor-specific antibodies and is coupled with immunosuppression (IVIG, rituximab, bortezomib). However, the data considering the use of PEX in desensitization protocols are scarce. PEX usually is used every other day, aiming at negative cross-match test prior transplantation.

PEX is not recommended in graft versus host disease (GVHD). In these cases extracorporeal photopheresis (ECP) has a beneficial effect [17].

4.4. Clinical indications: neurology

4.4.1. Neurological diseases from ASFA category 1

In the following neurological conditions, PEX has beneficial effects and is considered as first-line treatment: Guillain-Barre Syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, myasthenia gravis, Sydenham's chorea, N-methyl D-aspartate receptor antibody encephalitis and progressive multifocal leukoencephalopathy associated with natalizumab [16, 17]. The diseases are autoantibody mediated; therefore, PEX has a crucial role in removing the main pathogenic factor. In addition, immunosuppression is performed (steroids, calcineurin inhibitors, rituximab and IVIG). Generally, plasma exchange is performed 5–6 times for 10–14 days, or 2–3 procedures per week, where substitution volume is 50 ml/kg and albumin solutions are preferred. The procedure is performed until symptoms resolve, though maintenance PEX can also be considered.

4.4.2. Neurological diseases from ASFA category 2

The following diseases belong to this category: acute disseminated encephalomyelitis, acute neuromyelitis optica, Lambert-Eaton myasthenic syndrome, acute demyelinating multiple sclerosis and voltage gated potassium channel antibodies. The conditions are also autoimmune mediated, but the first-line treatments are immunosuppressive agents (steroids, IVIG and rituximab). PEX comes as a second-line treatment. Technically, the procedure is performed as the recommendations in Chapter 4.4.1.

4.4.3. Neurological diseases from ASFA category 3

Several neurological conditions fall into this category: chronic focal encephalitis, post-IVIG Guillain-Barre Syndrome, chronic progressive multiple sclerosis and paraneoplastic neurological syndromes. In these diseases, the most important part of the treatment is immunosuppression/anticancer treatment. There are conflicting results for the use of PEX/IA, in most of the cases aiming at slowing down the progression of the disease. Usually 3–6 PEX procedures every other day are performed. If IA is considered then 2–3 procedures/week must be conducted. Maintenance protocols have also been suggested [16].

4.4.4. Neurological diseases from ASFA category 4

In amyotrophic lateral sclerosis, dermatomyositis/polymyositis and inclusion body myositis, the use of PEX was not superior to conservative treatment alone; therefore, its use in clinical practice is currently not recommended.

4.5. Clinical indications: rheumatology

4.5.1. Systemic lupus erythematosus

SLE is an autoimmune disease, with involvement of several organs. It is an incurable, chronic, remitting and relapsing disease. Immunosuppressive agents are first-line treatments for SLE

(steroids, cyclophosphamide, azathioprine, biological agents, etc.). PEX was regarded as a treatment option due to the presence of pathogenic autoantibodies. However, the trials so far failed to establish improvement of the prognosis in mild SLE. In severe SLE (presence of TTP, cerebritis, alveolar hemorrhage and cryoglobulinemia), PEX coupled with immunosuppression demonstrated improvement in clinical outcomes. Therefore, severe SLE is classified as ASFA category 2. Lupus nephritis is not significantly influenced by PEX (ASFA category 4), except for the cases with LN and TTP [18]. Plasma exchange is performed daily/every other day; usually 3–6 procedures are sufficient in lupus cerebritis and alveolar hemorrhage.

4.5.2. Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is a hypercoagulable state characterized by episodes of vascular thrombosis and the presence of antiphospholipid antibodies. It can be associated with SLE, though non-SLE APS is also present. Catastrophic APS is a rare disease, characterized by APS and multi-organ failure. The mainstay of the treatment is treatment of etiological factors and anticoagulation. The role of PEX is not clearly defined; it is suggested that it is involved in antibody and cytokine removal. In order to optimize the effect, substitution fluids should contain FFP as a source for proteins C and S; therefore, substitution with FFP and albumin is performed. The procedures are performed daily, there is no clear guideline in terms of duration and clinical response remains the most important indicator.

4.5.3. Scleroderma

Scleroderma is a progressive disease of unknown origin, with skin and visceral organ involvement. Currently, the cornerstone of the treatment is D-penicillamine. Immunosuppressive agents are used too. So far two therapeutic options have been evaluated—PEX and extracorporeal photopheresis (ECP). Despite several reports indicating beneficial effects of the procedures, the data are conflicting; therefore, the disease is categorized as ASFA category 3.

4.5.4. Polyarteritis nodosa

Polyarteritis nodosa (PAN) is a vasculitis, involving medium-sized arteries, presenting with multi-organ involvement. The disease is idiopathic, or secondary, associated with infection [hepatitis B (HBV)]. In HBV-associated PAN the current treatment strategy includes steroids and antiviral agents. PEX is used as second ß line agent, and its beneficial effect is explained with removal of immune complexes. Idiopathic PAN is treated with steroids and cyclophosphamide. PEX in these cases is not recommended [17]. The usual substitution volume and albumin solutions are used. In HBV-associated PAN, 2–3 procedures per week are performed.

4.6. Clinical indications: endocrinology and metabolic disease

4.6.1. Thyroid storm

Thyroid storm is an extreme manifestation of thyrotoxicosis. Conservative treatment is the first-line therapy and encompasses medications which stop the synthesis, release and

peripheral effects of the thyroid hormones. Once these first- and second-line choices fail to have effects, third-line treatment, such as PEX, is considered. PEX reduces serum levels of the hormones, as well as provides thyroglobulin, thus binding free thyroid hormones. Therefore, FFP and albumin should be considered for PEX in the thyroid storm. The procedures are performed daily until clinical improvement is established.

4.6.2. Diabetes mellitus type 1

Autoimmune destruction of the β cells of the pancreas is a key factor for the development of diabetes mellitus (DM) type 1. Therefore, PEX was evaluated as a possible treatment of DM type 1. Though several reports demonstrated improvement in clinical outcomes, the overall results are conflicting and PEX is not recommended in the treatment of the disease.

4.6.3. Familial hypercholesterolemia

Familial hypercholesterolemia (FH) is an autosomal dominant disorder, associated with mutations of hepatocyte apolipoprotein-B (apo-B) receptors, resulting in decreased hepatic LDL removal. It is characterized with elevated total cholesterol and LDL levels, early atherosclerosis and death from cardiovascular events (especially in homozygotes). Conservative treatment reduces LDL from 10 to 49%. In progressive diseases, interventional techniques including PEX and LDL apheresis are considered. Generally, the indications for LDL apheresis are failure of the conservative treatment (LDL reduction <50%) and progressive coronary artery disease. The results for LDL apheresis in homozygotes are excellent, and FH in these cases is classified in ASFA category 1. Apart from PEX, there are several selective LDL removal techniques:

- immunoadsorption
- electrostatic removal of apo-B lipoproteins by dextran sulfate columns
- heparin extracorporeal LDL precipitation (HELP) by precipitation of apo-B in the presence of heparin and low pH
- hemoperfusion-based direct adsorption of lipoprotein
- membrane differential filtration, filtering LDL from plasma.

Volumes in LDL apheresis vary; for PEX, standard volume of 50 ml/kg is suggested. The procedure is performed once per 1–2 weeks. The patients may require arteriovenous fistula for the treatment.

4.6.4. Fulminant Wilson disease

Wilson disease is an autosomal recessive genetic disorder, characterized by impaired biliary copper excretion and copper accumulation in the liver, brain, cornea and kidneys. Fulminant forms are associated with severe liver failure and multi-organ failure. The ultimate treatment is liver transplantation (LT), but PEX can be used as bridging therapy, due to the reduced donor pool. PEX is beneficial due to rapidly reducing copper levels, as well as providing coagulation factors via plasma infusion. The reports however are scarce. Due to the wider availability, PEX is usually preferred to molecular adsorbents recirculating system (MARS).

Substitution fluid should consist of FFP/FFP and albumin. The frequency is daily/every other day, until clinical and laboratory improvement is detected.

4.7. Clinical indications: cardiology and pulmonology

4.7.1. Lung allograft rejection

Different therapeutic apheresis modalities were evaluated after lung transplantation in the following conditions—bronchiolitis obliterans syndrome (BOS) and antibody-mediated rejection (AbMR). BOS is an increasing airflow obstruction, due to chronic rejection. AbMR after lung transplantation is an important cause for graft loss and diagnostic criteria are currently assessed. The first-line treatment for the two conditions is immunosuppression. In BOS ECP can be considered as second-line treatment. ECP probably decreases levels of effector T cells while at the same time expanding regulatory T cells, thus influencing the immune response. ECP had beneficial effects in several studies, in patients unresponsive to immunosuppression. In addition, it did not increase the risk for infection complications. Unfortunately, the data so far are scarce. Different approaches have been suggested, for example, 24 procedures for 6 months [16]. PEX is recommended as treatment of choice in resistant AbMR, though the results are inconclusive.

4.7.2. Cardiac allograft transplantation

There are two apheresis modalities used in cardiac transplantation. PEX is used in sensitized patients and in the treatment of antibody-mediated acute rejection, together with immunosuppressive agents [32]. PEX is crucial in desensitization protocols, whereas in AbMR, the results are still controversial. ECP is an option in cellular rejection and rejection prophylaxis [17]. The procedures are performed until improvement in laboratory, clinical and histological findings is achieved.

4.7.3. Idiopathic dilated cardiomyopathy

Idiopathic dilated cardiomyopathy (IDC) is characterized by cardiac enlargement and deteriorating heart function of unknown origin. External factors were detected, as well as autoantibodies against the myocardium. Current treatment encompasses conservative treatment (ACE inhibitors, diuretics, etc.). However, PEX and IA also were evaluated. IA showed improvement in clinical outcomes in adult patients. Small studies also demonstrated beneficial effects from PEX in ICD in adults and children [17, 33]. IA usually is performed daily/every other day for a total number of five procedures. Similar treatment protocol for PEX is suggested.

4.8. Clinical indications: dermatology

4.8.1. Pemphigus vulgaris

Pemphigus vulgaris (PV) is an autoimmune, potentially fatal disease, with mucocutaneous involvement. The cornerstone of the treatment is immunosuppressive agents and steroids. PEX and IA have been tested, aiming at reduction of the antibody titers. Clinical results,

however, are conflicting. Currently PEX/IA can be considered in severe cases of PV (ASFA category 3). PEX is performed daily/every other day, in cases of IA it is done three times per week, and then gradually tapered. Procedures are performed until clinical improvement is noted and a significant drop in autoantibody titer is achieved.

4.8.2. Toxic epidermal necrolysis

Toxic epidermal necrolysis (TEN) is a life-threatening skin disorder, characterized by widespread erythema, necrosis, epidermal detachment, erosion of mucous membranes and systemic clinical symptoms (fever, sepsis, multi-organ failure). The etiology of the condition encompasses medications, infections, solid organ transplantation and bone marrow transplantation. The major aspects of current treatment are etiologic treatment, supportive care, fluid resuscitation and treatment of infectious complications. Due to the marked heterogeneity of the reports considering PEX in TEN, currently it is considered as part of the treatment only in refractory cases. The procedure is performed daily/every other day, and up to five procedures are usually performed.

4.8.3. Psoriasis vulgaris

Psoriasis is a skin disease that is accompanied by systemic inflammation and is characterized by epidermal hyperproliferation and dermal inflammation. Different treatment modalities are used—from topical medications, ultraviolet light to systemic agents (immunosuppressive agents and biological formulations). PEX is not indicated in psoriasis (ASFA category 4). However, ECP was found to have beneficial effects in disseminated forms.

4.9. Clinical indications: gastroenterology

4.9.1. Acute liver failure (ALF)

Acute liver failure (ALF) can develop in the setting of healthy liver (fulminant hepatic failure) or on top of chronic liver disease. The condition is associated with high mortality; prognosis depends on etiology. Generally, conservative treatment is the cornerstone of treatment. In patients with ALF and poorer prognosis for improvement, liver support systems are used for bridging therapy to liver transplantation. Liver support systems generally are of two types—cell-based (currently experimental) and non-cell-based support systems— and include PEX, albumin dialysis, MARS and selective plasma exchange. Apheresis probably improves outcomes in ALF due to removal of toxins and inflammatory cytokines. Use of PEX had better clinical outcomes compared to patients not treated with PEX. PEX combined with MARS improved bilirubin clearance versus PEX only, though clinical outcomes were similar in both groups. A recent study demonstrated that high-volume PEX (treated volume reaching 15% of body weight) effectively improves survival, compared to standard medical care. Unfortunately, the technique is not available worldwide. The procedures (PEX or high-volume PEX) should be performed daily, until clinical and laboratory improvement is noted or liver transplantation is performed. Substitution fluid should include FFP and albumin.

4.9.2. Liver transplantation

PEX is increasingly being used in AB0-incompatible liver transplantation (LT). The most beneficial effect was detected in living donation; the procedure is coupled with immunosuppression (IVIG, rituximab). In AB0-incompatible LT in living donation, PEX is a first-line treatment prior to the operation. In deceased donation, PEX can also be applied in AB0 incompatibility. The reports are limited in number. In addition, the effectivity of PEX is reduced in the setting of cadaver transplantation and urgent LT. In these cases crossover LT can be considered. PEX had a beneficial effect in antibody-mediated rejection after LT. However, the reports are predominantly retrospective ones, and further evaluation of PEX effectivity in humoral rejection after LT is needed. Procedures are performed daily until negative cross-match test is achieved. No titers for natural agglutinins were suggested. In humoral rejection, clinical and laboratory parameters should be evaluated.

4.9.3. Inflammatory bowel disease

The cornerstone of inflammatory bowel disease (IBD) treatment is conservative treatment (immunosuppressive agents and biological agents). PEX is not indicated in IBD; however, cytapheresis techniques were evaluated. The results are still insufficient to incorporate these invasive methods in everyday practice.

4.10. Clinical indications: sepsis and poisoning

4.10.1. Sepsis

Sepsis, especially associated with multi-organ failure, is a condition with mortality peaking up to 70%. The mainstay of treatment is antibiotics, fluid resuscitation and so on. The possible beneficial effect of PEX is removal of inflammatory molecules and replenishing anticoagulant proteins. Selective techniques have been evaluated too. Despite the promising results from retrospective studies, prospective trials showed conflicting results of PEX use in improving clinical outcomes. The substitution volume is 50 ml/kg, and substitution fluid should be FFP [17]. The procedures should be performed in intensive care unit and are performed daily.

4.10.2. Exogenous intoxications

This category encompasses three conditions—drug overdose/poisoning, envenomation and mushroom poisoning. The mechanism of action of each agent is different; therefore, different treatment options are used. The basic treatment options currently are stabilization of airways, breathing, circulation, gastric lavage, oral charcoal administration and forced diuresis. More aggressive approaches include hemodialysis and hemoperfusion. PEX was evaluated in mushroom poisoning and demonstrated improvement in survival, especially if early initiation is performed. The reports concerning PEX in envenomation are anecdotal. Data for PEX in drug poisoning are insufficient too. Generally, PEX can be used in drug poisoning with molecules having high-protein binding. The usual substitution volume is recommended, substitution fluid is albumin, but FFP can also be considered, especially if coagulopathy is present. PEX is performed daily until clinical symptoms resolve.

4.11. Clinical indications: oncology

4.11.1. Hematological malignancies

The use of PEX in multiple myeloma and Waldenström disease has already been discussed. Generally, PEX is not used in other hematological malignancies. However, other techniques (e.g., ECP) are recommended in cutaneous T-cell lymphoma [17]. In addition, leucapheresis and plateletpheresis can be performed in life-threatening leukemia/myeloproliferative disorders [2].

4.11.2. Solid tumors

Several reports indicated improvement in clinical outcomes in solid tumors and metastatic cancer [2]. A possible explanation is that via PEX, inhibitory molecules are removed from plasma, thus improving immune response. Due to the heterogeneity of the studies and the conflicting results, no clear indications for PEX in these cases are defined. Further studies in this field are needed.

5. Conclusion

There are three major obstacles for the adequate evaluation of PEX effectiveness—the small number of patients enrolled, small number of randomized controlled trials and high cost of the procedure. However, with the advance of the technique and adequate collection of data on PEX use, these obstacles can gradually be overcome in the future. A more interesting perspective is the development of more selective techniques, as well as the use of magnetic separation and cell filtration. Thus, we can expect wider use of apheresis in medical practice in the future.

Author details

Jean J. Filipov[1,2]*, Borelli K. Zlatkov[1,2] and Emil P. Dimitrov[1,2]

*Address all correspondence to: jeanphillipov@yahoo.com

1 Department of Nephrology and Transplantation, University Hospital “Alexandrovska”, Sofia, Bulgaria

2 Medical University—Sofia, Sofia, Bulgaria

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Chapter 5

Clinical Applications of Plasma Growth Factors

Jesús Alcaraz Rubio and Juana María Sánchez López

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76089

Abstract

The use of plasma rich in growth factors has become a technique increasingly used in various fields of medicine. Since its inception in use in sports medicine and dental implants in the mid-80s, gradually it has expanded its field of use in clinical specialties. The power cell tropism for certain tissues, attributed to growth factors, has currently talked of a new medical discipline as Regenerative Medicine. Not only has experienced an ever increasing boom in various medical specialties, but simultaneously has increased exponentially types and methodology for obtaining application forms even for the same pathology. So much so that now its use has exceeded the capacity to produce scientific evidence for successful clinical application.

Keywords: plasma growth factors, medicine regenerative, platelet rich plasma

1. Introduction

The use of plasma rich in platelet growth factors has become a technique increasingly used in various fields of medicine. Since its inception in use in sports medicine and dental implants in the mid-80s, gradually it has expanded its field of use in clinical specialties as diverse as otolaryngology, plastic surgery and dermatology, general surgery, ophthalmology, obstetrics and gynecology or neurosurgery between other. The power cell tropism for certain tissues, attributed to growth factors, has currently talked of a new medical discipline as regenerative medicine. Not only has experienced an ever increasing boom in various medical specialties, but simultaneously has increased exponentially types and methodology for obtaining application forms even for the same pathology. So much so that now its use has exceeded the capacity to produce scientific evidence for successful clinical application. The objective of this

work is to review the clinical applications where there has been more scientific evidence and those where although still lack solid scientific basis are interesting from the point of view of clinical and preclinical.

2. Fields of application of platelet RICH plasma

They are numerous and ever growing fields where PRP is being implemented and its various fractions. Let us review for specialty applications where there seems to be more consensus, emphasizing those where there is scientific evidence of greater support; furthermore task not without difficulty given the controversy in the medical community even for the same clinical application and the absence of scientific studies weight as properly designed clinical trials for this purpose; although as will most indications are based on case series and even isolated clinical cases difficult to reproduce by the authors as to design more scientific studies force.

2.1. Rheumatology, traumatology or sports medicine

This is certainly the field of greatest projection in the use of PRP, far surpassing even the existing clinical evidence. In vitro PRP shown to regulate cytokine processes involved in neovascularization, proliferation of tenocytes, fibroblasts, myocytes and chondrocytes, and the recruitment of inflammatory cells with inhibitory effect of proinflammatory cytokines (IL-1) with anti-inflammatory and regenerative activity (**Tables 1** and **2**).

2.1.1. Epicondylitis

Epicondylitis is a tendinopathy limiting, with a clear tendency to become chronic and random partial response to conventional therapy with steroid injections and rehabilitation. Studies using PRP in these patients as single infiltration resulted in significant functional improvement in analgesic and 85% of them without imparting any adverse effect. Further deepening is necessary anyway, since existing studies contained small numbers of patients [1, 2].

2.1.2. Plantar fasciitis

As in the rest of tendinopathy with a tendency to become chronic, has reviewed a study of a case series of patients with plantar fasciitis refractory to treatment with NSAIDs, immobilization, physical therapy and corticosteroid infiltration, who are PRP infiltrated with a significant functional improvement and pain in 90% of them. Also in this case more studies are needed to objectify the benefit in this pathology [3, 18].

2.1.3. Knee osteoarthritis

A relatively new application that is currently bringing together most of the current clinical research. Case series studies of patients compared with single infiltration of hyaluronic acid,

Area	Clinical applications	Scientific evidence
• Skeletal muscle pathologies	• Tendinopathies • Meniscopathy • Ligament injuries • Bone fractures • Fasciitis • Muscle tears • Osteoarthritis	• Preclinical and clinical Studies (cases-control)
• Surgical wounds	• Gynecological surgery (abdominal) • Cardiovascular surgery (sternal and vascular access) • Plastic surgery (skin flap)	• Clinical studies (case series).
• Burns	• Skin and corneal	• Clinical studies (clinical isolates).
• Chronic ulcers	• Diabetic • Vascular • For pressure	• Clinical Studies (Case-control)
• Ophthalmology	• Corneal ulcers • Dry eye	• Preclinical and clinical studies (case series)
• Otolaryngology	• Tympanoplasty	• Preclinical and clinical studies (case series)
• Cosmetic surgery and dermatology	• Facial expression lines • Hair Implants	• Preclinical and clinical studies (case series).
• Neurology and neurosurgery	• Suture of peripheral nerves and neurorehabilitation	• Preclinical and clinical studies (clinical isolates)

Table 1. Applications of PRP in different fields of medicine and scientific evidence.

document up to 35% of respondents to the PRP infiltrate, compared with 10% hyaluronic acid. While these studies collect causística few patients, the importance is that for the first time the concentrations of growth factors contained in PRP infiltrated specified, stressing the importance of leukocyte fraction not potentiate the proinflammatory effect of the final product obtained. In recent years most extensive series of clinical cases have reported their success in implementing PRP injections; perhaps the largest series the Spanish belonging to a group that included the treatment of 261 patients with 3 injections of PRP 15 days set apart from each other, with a follow up of 1 year, with a functional improvement in 67% of them, on especially those younger patients and those with a more incipient development of the disease [4–6, 11, 15, 16].

	Study	Results	Authors
• Tendinopathy chronicle	• Injury chronic more than 6 weeks. • Refractory epicondilitis • Chronic pain lateral epicondyle. • Achilles tendinopathy. • Progress of a patient with Achilles rupture. • PRP vs. saline infiltration. • Chronic refractory tendinitis: PRP vs. saline infiltration.	• 97% good results • 8% poor results. • 79% success rate. • 60% improvement at 8 weeks, 81% at 6 months and 93% at 12 months. • PRP did not generate damage and faster recovery. • Regeneration and faster return to activity. • Same results though less inconvenience. • No significant differences.	• Gandia and col. • Edwards y Calandruccio. • Mishard and Pavelko. • Sanchez and col. • Filardo and col. • De Vos and col. • De Jonge and col.
• Patellar tendinitis	• Animal study. • Plasma in rat patellar tendon. • 20 athletes with chronic patellar tendinitis (three injections of PRP). • Patellar tendon in 31 patients during 6 months. • PRP vs. Others treatments.	• Greater immunogenic response without abnormal marcadors. • Increased levels of collagen I and II. • 70% complete recovery at 6 months, the rest 80% with decreased pain. • Better results and better quality. • PRP more effective in degenerative conditions	• Taylor and col. • Kajikawa and col. • Kon and col. • Filardo and col. • Van Ark and col.
• Elbow tendinitis	• Corticosteroid injections vs. PRP. • Using PRP in epicondylitis. • PRP Injections vs. corticosteroids infiltrations.	• 79% good results with PRP vs. 51% with corticosteroids. • Increased angiogenesis, wound healing and accelerates higher histological grade. • Long-term benefits.	• Gosens and Perbons. • Lyras and col. • Coombes and col.
• Plantar fasciitis	• Cronica. • PRP vs. another treatments. • Chronic plantar fasciitis.	• 6/9 symptomatic relief als 8 weeks and 77.9% complete resolution of symptoms a year. • Best treatment to avoid relapses. • Ecure method and reduces pain.	• Barret and Erredge. • Glazer and col. • Martinelli y col.
• Ligament acute injury	• ACL reconstruction. • ACL reconstruction.	• Shortening the return to activity by 27%. • There is no consensus, faster transformation of LCA with PRP.	• Samspson and col. • Ventura and col.

	Study	Results	Authors
• Muscle injuries	• 11 elite athletes with muscle tears. • 20 professional athletes. • 8 football players and 6 of basketball. • PRP in animals.	• Return to the fastest competition and 30% shortens recovery • Good results. • Faster return to competition. • It shortens the recovery period of the muscle.	• Wright-Carpenter. • Sanchez and col. • Cugat and col. • Hammond and col.

Table 2. Applications PRP in trauma and orthopaedic surgery.

2.1.4. Other applications

It has been seen that the PRP is useful in chronic Achilles tendinopathy especially when infiltrations Ozone [8, 9, 10], patellar tendinopathy [5, 6, 11], previously used in repairing cuff rotators [12, 13, 14], repair the anterior cruciate ligament [11] or with plasty erector or tibial bone-tendon graft-bone, meniscal repair the knee joint [5, 6], in the reconstruction of the glenoid labrum or hip and ultimately partial and total muscle injury made here open repair and strengthening PRP later [7, 19].

2.2. Chiropody

In most cases, the goal of medical research is not only to extend the patient's life, but to improve the quality of the patient. Growth factor rich plasma is a novel and relatively recent technique applicable to tissue repair. It consists of a simple system for obtaining platelet and autologous plasma proteins from a patient's blood sample. In the field of podiatry the application of PRGF allows to improve the evolution of patients with regenerative needs in lower limbs such as: accelerate ossification postoperatively, shorten the resolution time in diabetic ulcers or improve scarring among many others. Several studies on the application of growth factors have shown excellent results in different medical specialties among which podiatry is found; therefore it is considered a technique of high effectiveness and clinical interest for its contribution to the scientific community [17].

2.3. Dentistry and maxillofacial surgery

Maybe another field where the PRP has seen a more visible development. However there is a strong controversy and debate as to the usefulness of the PRP in the recovery of dental alveolar bed with lyophilized bone plasty, objectifying alveolar increased, improving the healing of the soft tissues and facilitating greater cohesiveness particulate graft, which would useful in dental implantology. Others are more pessimistic when it comes to reproduce these results, due to the large differences in growth factors present in the PRP, according to the method of obtaining the final product applied [20–23]. This led to think that the higher the concentration of these factors would be more effective regeneration, promoting the use of systems that got a

higher concentration of growth factors, systems that were approved and used without thinking of the concentration obtained product end. Far from achieving the desired effect, in vitro otherwise completely it observed when the concentration factor exceeds a certain level. Hence the strong controversy arose in its use in many cases fueled by the lack of systematic obtaining PRP that may be incorrect.

2.3.1. Aggressive periodontitis

Platelet-rich plasma has emerged as an alternative in periodontal therapy. PRP appears to increase the speed of the healing process since it is biologically possible that a higher concentration of platelets can assist in wound healing due to the higher concentration of platelets and initiate a faster cellular response than the normal blood clot [6, 7]. Today we have an evidence-based learning curve that shows us a first stage where it was used as a cementing biomaterial and as a stimulant for the regeneration of bone tissue. In a second stage, it is applied for the healing of soft tissue wounds based on biological evidence, which has generated great expectations in several medical specialties, among which is dentistry. The clinician today increasingly understands the need to make decisions based on scientific evidence. Until now, we know that biologically it is possible that a higher concentration of platelets can aid in healing.

2.3.2. Dental implants

In the field of Implantology, we report the use of PRP in the preparation of maxillary bone for placement of implants; thus it is described that in the alveoli to which PRP are placed they show a greater buccolingual/palatal bone width, accompanied by a higher bone density and a faster tissue coverage compared to patients in whom this compound was not used [33]. Probably, the benefits of PRP on implants may be related to the type of bone on which they act, since most of the studies that show better clinical indices correspond to sites without grafts or autologous grafts where factors such as vascularization may play an important role. In a radiographic clinical study of 11 patients who were implanted in the posterior mandibular area without grafting, no implant failure was observed, and it was shown that the use of PRP may lead to early bone apposition around the implant, and that improves soft tissue healing. Previous in vitro studies have demonstrated a PRP stimulatory effect on osteoblast proliferation that appears to begin in vivo at the second week, increase from the third week, and is maintained during the fourth week, so the local application of PRP would increase the amount of newly formed bone around the implant and bone density. In studies on canines, it has been observed that the application of PRP significantly increases the contact between bone and implant ($P = 0.028$). B15. In the dental area, most studies have focused on bone regeneration. With respect to the use of plasma in sinus elevation and increased alveolar ridge published a study that determined the advantages of its use in conjunction with lyophilized bone, but also indicate that it is necessary to have more studies that support this method. With regard to repair of bone defects and use of PRP in a study with 10 patients with diagnosis of periodontitis we used bone graft associated with PRP in the cases group, and bone graft and serum in the control group. Comparatively a greater reduction of the sacks and better quality of bone was obtained in the patients of

the case group with respect to the control group. Evaluated the use of PRP with tricalcium phosphate compared with the use of alloplastic material only in intraosseous defects. They conclude that the associated use of the materials presents better clinical and radiographic results. Conducted a study in which they evaluated the ability to reduce bone resorption in fractured alveoli, in a case group of 14 people who used PRP compared to 6 people in whom it was not used. It was concluded that the use of PRP decreases bone resorption.

Several clinical procedures have used the PRP observing their qualities in the dental area. Presented platelet gel for use as an adhesive in bone grafts and one that aided the consolidation. The platelet gel is obtained and processed immediately in the operating room. Marx et al. [23] observed that a platelet concentrate obtained by blood centrifugation caused a high concentration of platelets in the graft and with them, the presence of growth factors and that the cells of the spongy bone also possess receptors for these.

It was also described that the use of PRP and PRF offer a new and useful therapeutic tool in the acceleration of healing and bone maturation in maxillofacial and reconstructive surgery. In this regard, Marx et al. and Fennis et al. [29], demonstrated that PRP improves bone regeneration and that platelets can act as local regulators of the healing process; in turn, the application of the PRP and the CFs it contains, increase the microcirculation of the gingival mucosa surrounding the wound. Other studies have shown that with a single 20 pM application of a recombinant factor PDGF-BB type, a significant effect can be achieved in increasing capillary density. A similar effect could be achieved in patients treated with PRP [30]. The characteristics of the PRP suggest that it could be of great use in implant procedures, and generally in procedures involving the preservation of bone and soft tissue. The use of PRP has several advantages such as a safe autogenous preparation, free of worries about communicable diseases such as HIV, hepatitis or Creutzfeldt-Jakob disease; and is convenient for the patient, since the blood is collected in the immediate preoperative. However, although PRP therapy has been used for decades, there is no agreement in the literature as to whether this procedure influences the success of bone integration of an implant. Our observations have shown lower rates of failure with the use of PRP compared to the conventional technique without PRP, although this difference is not statistically significant and, although the calculated risk and treatment measures show a beneficial effect of PRP, it is reduced.

2.3.3. Periodontics

In the field of periodontics, the use of PRP has been described as adjuvant of regenerative therapy. Some authors found that there was a significantly greater increase in the periodontal ligament when the injured sites were treated with PRP [31, 32]. Since the beginning of the research with the PRP, studies have been published that showed optimal results in bone regeneration with its application alone or combined with grafting. In general, most of the studies where PRP is applied agree that there is a visible improvement of soft tissue healing and a greater cohesiveness of the particulate grafts, since it facilitates their manipulation and transport to the surgical bed; however, it is important to emphasize that the real role of growth factors is in relation to differentiated cells (preosteoblasts or osteoblasts), promoting their proliferation and differentiation and not on the stem cells of the tissue (able to differentiate

into cells of the bone tissue), which would explain some controversies regarding the main role of these factors in the formation of bone tissue. However, the application of PRP in the specialty of periodontics continues to be of great utility since it behaves as a matrix for particulate grafts in the regeneration of bone defects (GTR) left by periodontal disease and in the area of periodontal cosmetic surgery (for root coverage), where it has been reported that it may be a good alternative to connective tissue grafts in root coverage surgery to promote the formation of lost soft tissues and decrease postoperative inflammatory response, as reported in the studies reviewed.

2.4. Gynecology

Gels have been used for handling PRP surgical wounds in various major surgeries, with positive effects on aspects like reduction in postoperative pain with less conventional analgesic requirement. In a case-control study, application of recombinant PDGF gels in dehiscence abdominal surgery, showed a significantly shorter closure, compared to controls, no significant side effects [24, 25].

Furthermore the PRP has been used in vitro as "plug" in the management of premature rupture of fetal membranes, watertight sealing of defects in biological membranes [26].

However it has not been informed of the effect of PRP in areas such as infection or trans and postoperative bleeding.

2.5. Cardiovascular surgery

While it has been used to promote healing of cardiovascular surgical wounds, especially at the sternal level, but also in wounds caused by peripheral vascular access, several studies have shown that the topical application of PRP decreases the frequency of chest infection, improves hemostasis, postoperative pain, the amount of wound drainage and even decreases the days of postoperative hospital stay. Although studies have not shown a significant effect on the management of these [21, 27].

2.6. Plastic surgery

In a case series, we observed that the application of a skin flap on a surgical bed which was previously applied PRP, qualitatively reduced the volume of capillary bleeding from the surgical bed, decreased need for drainage or compression bandages, as well as a reduction in postoperative pain [28].

On the other hand there are few studies strongly demonstrating the usefulness of PRP in the management of burns. Experimental studies have shown that applying a gel burns PRP stimulates an intense inflammatory response, with a significant increase of extracellular matrix proteins, fibroblast proliferation, collagen and granulation tissue. However it has not documented a real acceleration epithelialization of wounds. On the other hand, a study that applied the subconjunctival injection of PRP in 10 patients with ocular burns showed a significantly

faster epithelialization of the cornea and the conjunctiva [26]. Another study that addressed the use of PRP gel for wound management including friction burns demonstrated a significant improvement in its use [27]. Until now, there are no other studies that support the utility of PRP in burns. However, due to the good experimental results, there is a theoretical possibility that due to the reported increase in inflammatory action this could stimulate the formation of hypertrophic scarring in superficial burns and in deeper burns [27].

Therefore there is still no strong scientific evidence to recommend the PRP in the management of burns. There are variables such as type of scarring, burn extent, thickness thereof, time of application or rate of infection that still require more in-depth study [37].

2.7. General surgery

It has been postulated that the use of PRP as rich fibrin glue for placing meshes in correcting inguinal hernias material improves tolerance, postoperative pain, decreasing the amount of suture material for fixing the same, however even better designed studies are needed to refine this clinical application [31].

On the other hand it seems to be well-founded scientifically use in both diabetic ulcers, how in pressure ulcers, significantly speeding up the closure of the same, decreasing the pain without significant side effects. Several studies, including a meta-analysis, have shown that the application of PRP in chronic diabetic ulcers significantly accelerates their closure, decreases pain and even works in the most severe wounds without significant collateral events reported [28]. A study that analyzed the cost of this therapy over conventional treatment over 5 years showed that the management of PRP improves the quality of life of these patients significantly and significantly reduces the costs of their care [29]. Evidence for the efficacy of platelet-rich plasma in these chronic lesions does not appear to be contradictory, so that PRP treatment in diabetic ulcers is well founded, however it remains unknown whether this overall effect of PRP on chronic wounds, if its beneficial effects are maintained in the long term, decreasing the percentages of amputation or if the outcome may be influenced by other factors of the wound, concomitant treatment or the particular patient. In this sense, a recent study that included 49 patients with chronic wounds of various etiologies (pressure ulcers, venous ulcers, diabetic ulcers, etc.) also demonstrated the degree of improvement (area reduction, wound closure) in 97% of cases regardless of wound origin [29, 30].

2.8. Ophthalmology

Experimental studies in vitro demonstrated that PRP increases the migration of fibroblasts and conjunctival keratinocytes. Similarly, some clinical studies have objectified positive effects of PRP in corneal ulcers and keratoconjunctivitis sicca Sjogren's syndrome, for which there is currently no satisfactory treatment [33, 35].

In the case of eye burns subconjunctival injection of PRP in a number of patients showed significantly more rapid epithelialization of the cornea and conjunctiva, however this has not been demonstrated later [32].

2.9. Otolaryngology

On preclinical and clinical use in type 1 patients with perforated eardrum Tympanoplasty studies could be useful in defect closure, however still required study more scientific evidence to corroborate this fact, not having such studies with controls [34, 36].

2.10. Dermatology

Experimental studies have shown that dermal papilla cells exposed to significantly increase their proliferation PRP, which was associated with increased Akt and ERK signaling and upregulation of fibroblast growth factor 7 and the B-catenin, which are recognized hair growth factors. Even though scientific studies are needed more power, PRP opens a range of possibilities in the treatment of psoriasis, vitiligo, alopecia, lichen planus and other cosmetic applications [28, 39].

2.11. Neurology and neurosurgery

Preclinical experimental studies have shown healing capacity and neuroregeneration with functional recovery in applying jointly PRP and suturing the edges of the injured nerve, due to a significant increase of axons in the distal segment, however these studies are still experimental, although it has documented some isolated case report with positive results. Similarly this opens a door to the possibility of nerve stimulation in patients with neurological hypoxic ischemic tare origin especially following active neurorehabilitation programs. Still needed are appropriately supported in this respect clinical trials to assess the potential clinical benefit that PRP can contribute in this field [38, 40].

2.12. Anesthesiology

Nerve growth factor (NGF) is the founding member of the neurotrophin protein family. It was discovered over half a century ago through its ability to promote sympathetic and sensory neuronal survival and axonal growth during the development of the peripheral nervous system, and are the paradigmatic neurotrophic factor-derived targets underlying the neurotrophic hypothesis. Since that time, NGF has also been shown to play a key role in the generation of acute and chronic pain and in hyperalgesia in various pain states. NGF is expressed at high levels in damaged or inflamed tissues and facilitates the transmission of pain by nociceptive neurons through a variety of mechanisms. Genetic mutations in NGF or its receptor TrkA tyrosine kinase, lead to a lack of congenital sensitivity or a decrease in the ability of humans to perceive pain. B16. In humans, NGF levels are elevated in a variety of acute and chronic pain states including rheumatoid and spondyloarthritis in neurogenic overactive bladder and interstitial cystitis induced cancer pain prostatitis and in patients with degenerative intervertebral disc disease (Lee et al. [38]). The functional link between these increased levels of NGF and pain was determined through a variety of animal and human studies that modulate NGF levels and observe the resulting effects on the level of pain experienced. In humans, intramuscular injections of NGF in one trial resulted in an increase in pain scores and increased pressure pain sensitivity in NGF injected muscle compared to baseline; these effects were resistant

to local muscle anesthesia. NGF also induced localized and long-lasting non-inflammatory mechanical and thermal hypersensitivity in human skin after local injection. Similarly, local injection of NGF into the masseter muscle induced mechanical allodynia and hyperalgesia that persisted for at least 7 days after administration of NGF B16. The results of clinical trials of tanezumab (a monoclonal antibody that sequesters NGF and does not let it act) that are currently underway, particularly those related to the progression of arthritis or osteonecrosis, are the next determinant of if and when realized that potential. In the event that tanezumab is shown to have an acceptable long-term safety profile. The key role of tanezumab in the management of pain in patients with chronic diseases may depend on a greater understanding of their different effects on symptom control (i.e., analgesia) vs. disease modification (chronic pain or persistence) B16.

2.13. Other applications

From the standpoint of experimentation, the scientific evidence regarding the role of PRP in stimulating proliferation of various cell lines both epidermal and mesenchymal, have been used as a support for growing and clonal expansion in vitro of same laboratory.

3. Conclusion

Surely we are facing a new era of treatment in the new field of what is called regenerative medicine with an extraordinary range of possibilities for clinical applications increased, but that requires a process of scientific and medical systematization which allows channel it safely and effectively in applications where there is scientific evidence really enough weight so apply. To do this it is necessary to two things: first the consensus of the authors engaged in the production and application of this therapy in order to standardize procedures for obtaining those more effective and allow adequate traceability and monitoring of the end product, depending clinical application given their intended and secondly the design of clinical trials which management and establish appropriate guidelines to that effect.

Today we are still far from achieve, given that almost all existing clinical applications, the scientific evidence is weak, based on case series or case-control studies in the most positive assumptions. The growing presence of various protocols to obtain, low control over the final product component and variety of clinical applications, difficult first reach some sort of consensus on the process or procedures more reliable and adequate collection and secondly development of appropriate clinical trials to test them in different pathologies susceptible to it.

Revised everything published about the conclusion you reach is that the PRP is well tolerated technique, considered from 2 years as a medicine ago, restricted its use to prescribing physicians, dentists and podiatrists, lacking tab Currently technique, and cannot be considered standard treatment for any medical condition where intended to be used, if it is accepted that it can be used as adjunctive therapy along with conventional therapies to implement clinical and functional improvement of the patient.

They are necessary in the future of basic research and translational medicine to better understand the pathophysiological mechanisms underlying its regenerative effects.

Similarly sheet to establish a sound scientific studies are necessary in the form of clinical trials to standardize the techniques for obtaining both depending on the cellular composition of the final product as a protein obtained and is reproducible by all authors and specific management guidelines for each clinical application where feasible use in regenerative medicine.

Author details

Jesús Alcaraz Rubio* and Juana María Sánchez López

*Address all correspondence to: jesusalcaraz@telefonica.net

Unión Murciana de Hospitales, Murcia, Spain

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Section 3

Plasma Homonym in Medicine

Chapter 6

Microplasma Drug Delivery

Kazuo Shimizu and Jaroslav Krištof

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73498

Abstract

There are several techniques to perform drug delivery. One of the newest methods of drug delivery through the skin is the application of plasma. Reactive species generated by plasma can change the chemical composition of the skin, change the structure and extract lipids of the lipid barrier, create pores in or etch the surface of the skin. These changes have an influence on the barrier function of the skin which can be decreased. The main barrier of the skin is called stratum corneum. The structure and composition of the stratum corneum and function of the components is described. Possible interaction of plasma particles with skin is presented and compared with interaction of plasma species with carbon or hydrocarbon surfaces. Active species which can effectively interact with lipid molecules is introduced. Hydrophilic drugs and drugs with high molecular weight can penetrate very difficult through the skin or cannot penetrate the skin at all. As a model, a drug, Cyclosporine A, was studied. Cyclosporine A is a lipophilic drug with a molecular weight of 1203 Da which is used during and after organ transplantation to prevent rejection. The Hairless Yucatan micropig was used to simulate human skin. A film electrode was used to generate plasma that was used for skin treatment. An AC voltage ($V_{0\text{-}p}$ = 0.6–1.5 kV, 25 kHz) was applied with flowing gas (5 L/min). The barrier function of the skin was evaluated by a Franz cell experiment and high performance liquid chromatography (HPLC) for a particular drug. An effective amount of drug in human body was determined by pharmacokinetic model.

Keywords: microplasma, plasma drug delivery, stratum corneum, transdermal drug delivery

1. Introduction of atmospheric plasma for medical application

Plasma applications are used in many applications from etching of surfaces [1], deposition of superconducting materials [2], and improvement of adhesion properties of polymers [3] to sterilization [4] and various medical treatments. Plasma in biomedical field is intensively

studied these days. Research is mainly oriented to wound healing [5], cancer treatment [6], dermatology [7] and drug delivery to the cells [8]. Drug delivery through the skin is also studied very intensively and for a very long time [9, 10] but using plasma is a relatively new approach and only some studies exist [11–15]. For successful penetration of the drug through the skin, it is necessary to know the composition and structure of the stratum corneum and its lipid barrier. The stratum corneum is the main barrier of the skin, so knowledge of the function(s) of the molecules which participate in this barrier is crucial. Plasma sources can produce different kinds of particles with lifetimes from several nanoseconds to several seconds [16–18]. The configuration of using a specific gas or mixture of gases can prefer certain type of particles. Each particle can have different effects on the skin such as penetration depth, reactivity, and the ability to convert to more reactive particles. The effects of the particles to carbon and hydrocarbon surfaces will be described. The success of using of plasma for transdermal delivery of certain molecules and also feasibility of using model drug, Cyclosporine A, will be presented.

2. Plasma discharges

These days many types of plasma discharges exist. If we want to use plasma in medical applications (plasma drug delivery in our case), mostly atmospheric discharges are required. Atmospheric discharges can be generated from DC, through low frequency AC, radiofrequency to microwave frequencies. Generally, we can divide plasma sources into three categories:

1. Discharges where plasma is directly in contact with the sample (skin). In the case of direct plasma treatment of the tissues or skin, the human body serves as one of the electrodes and partial current flows through the tissue. The plasma has a low temperature (up to 45°C). The sample is several millimeters from plasma source and active species directly treat sample [19].
2. Plasma is blown out by gas flow to the sample (skin) from the place where the plasma was created. The distance between the plasma nozzle and the sample can be set from several millimeters to centimeters [20]. The diameter of the plasma plume can reach the diameter of the needle [21].
3. Plasma is not in contact with the sample (skin). The sample is treated by long living particles such as certain radicals, ions, metastable species or particles with very long lifetimes (for example, ozone). Plasma usually has a very high temperature up to several thousand degrees or very small dimensions. A typical example of high temperature plasma source for medical treatment is "PLASON," which is used for the production of NO radicals [22], or microwave plasma torches [23]. On the other hand, a high temperature is sometimes necessary for the production of certain type of species. Sources with low dimensions are surface plasma discharges. Microplasma DBD (**Figure 1**) is a plasma source used in Shimizu et al. [11], where electrodes had a thickness in micrometer dimensions that allowed a decrease of ignition voltage to hundreds of Volts. Advantages of surface DBD sources is that they can be very large; and the electrode can be very thin and placed on polymer which can copy the surface of treated sample [24]. A disadvantage is that the distance between the electrode and the sample must be very short, approximately 1–2 mm.

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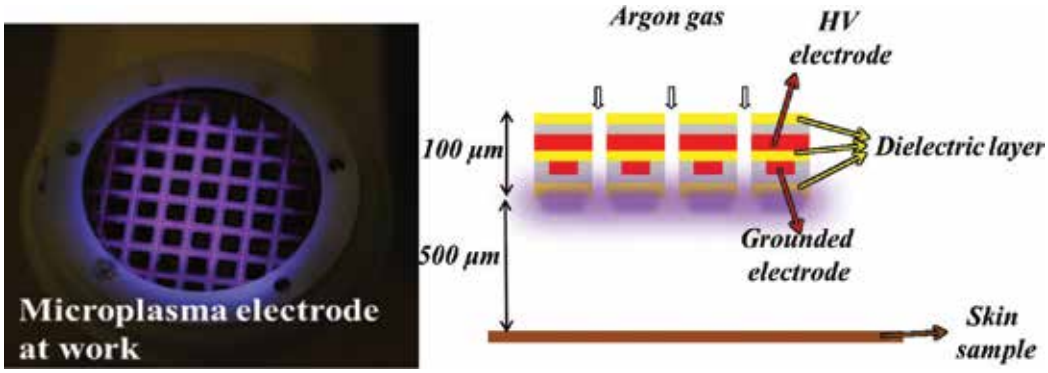

Figure 1. Microplasma discharge at work (left). Schematic of electrode and skin treatment (right) (reproduced from Ref. [13], with the permission of the American Vacuum Society).

3. Stratum corneum structure

The stratum corneum is an upper layer of the skin. This layer is the main barrier of the skin which protects the body against loss of water and ensures that molecules from outside will not enter the body. The stratum corneum is composed of corneocyte cells placed in a lipid-rich matrix. Human stratum corneum (thickness of 10–20 μm) contains from 10 to 25 layers of corneocytes. Corneocytes are composed of fibrillary keratin and water inside a cornified envelope. The cornified envelope is composed of crosslinked fillagrin, loricrin and involucrin [25]. Lipids in the lipid matrix are organized in lamellar layers following a tri-layer broad-narrow-broad arrangement [26]. The width of the tri-layer structures can be 6 or 13 nm. These lamellae can be packed in dense orthorhombic, less dense hexagonal or disordered liquid phase [25]. The lipid matrix consists of ceramides (41%), cholesterol (27%), cholesterol esters (10%), fatty acids (9%) with a small fraction of cholesterol sulfate (2%) [27] and glucosylceramides. Lipid chains are usually in a solid crystalline or gel state. At higher temperatures, lipids change their state to a liquid crystalline and they are more permeable (**Figure 2**).

After disruption of the stratum corneum, cholesterol synthesis leads to repairing of the barrier and it starts 90 min after barrier disruption [28]. The renewal of the stratum corneum occurs every 14 days [29]. Lipid-rich matrix is used for the transdermal delivery (TDD)—intercellular pathway, and it is composed of hydrophilic domain—head of ceramides and lipophilic domain—tail of cermaides. The stratum corneum can be divided into three main layers with different compositions and barrier functions [30]. The layers can be characterized by the concentration of K, Na, ceramides and fillagrin (**Figure 3**).

The concentration of Na is high in the whole stratum corneum and the concentration of K is high in the upper layer but low in the rest of the stratum corneum. The concentration of ceramides decreases from the upper layer to the lower layer. The concentration of arginine, which is the product of fillagrin, is the highest in the middle layer and lowest in the rest of the stratum corneum. The upper layer of the stratum corneum with dimension of 2/5 of the whole thickness (~ 8 μm) appears as a layer which allows passive flow of ions inside and

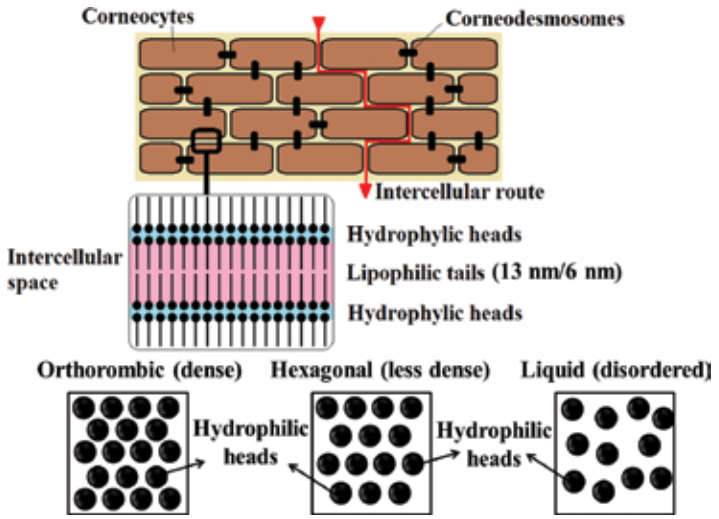

Figure 2. Model of the stratum corneum and with lateral and lamellar organization of lipids in the lipid matrix [26].

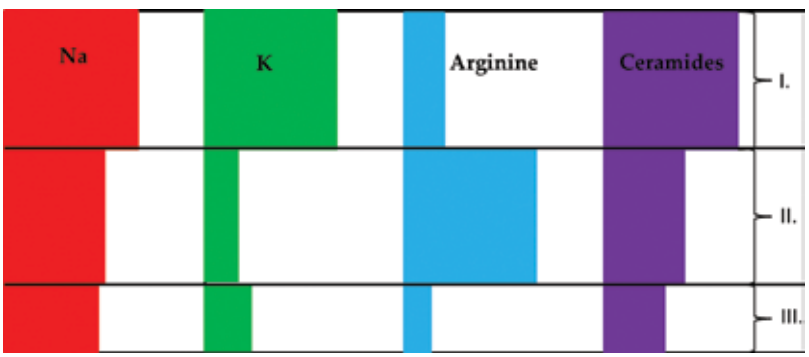

Figure 3. Three layers of the stratum corneum characterized by normalized amount of Na, K, arginine and ceramides [30]. The thickness of red, green blue and violet rectangular indicates relative concentration. I. Denotes the upper layer, II. Denotes the middle layer, and III. Denotes the lower layer.

outside. The amount of Na and K can be changed by the influence of the outer environment. Liquids and ions can easily penetrate the corneocytes in this layer and intracellular Na can be flushed out when corneocytes absorb water or other liquids. The middle layer of the stratum corneum appears as a first real barrier. The thickness is approximately 2/5 of the whole stratum cornea as in the previous case. Ions such as K^+ and Cr^{6+} cannot penetrate into this layer. Hydration of the skin is a function of the second layer because of the high concentration of arginine. It was observed that Cr^{3+} was able to penetrate into the middle layer of the stratum corneum, but not into the third layer, which is the reason why the third layer can be called a second barrier. The third layer has a thickness of 1/5 of the stratum corneum (~ 4 μm) and the barrier function of the stratum corneum increases toward the viable epidermis.

3.1. Corneocytes

Corneocytes contain a lipid envelope bounded to the exterior protein envelope. Corneocytes of the lipid envelope function as a semipermeable membrane that allows water molecules to penetrate but not the larger hygroscopic molecules [31]. Intercellular lipid lamellae and lipids of the lipid envelope of corneocytes are connected through an ester bond (R—CO—O—R1) [32].

3.2. Role of ceramides

The percentages of some ceramides in the lipid matrix are 29.9% of ceramide 6, 21.7% of ceramide 2, 14.8% of ceramide 4, 13.9% of ceramide 5, 11.9% of ceramide 3 and 7.8% of ceramide 1 [33] (**Figure 4**).

The permeability of this membrane can be increased by decreasing the concentration of cholesterol and increasing the amount of short-tailed fatty acids and unsaturated fatty acids. A decrease of ceramides also decreases the barrier function of the skin. Ceramides form a multilayer lamellar structure with other lipids. Ceramides also act as a water modulator and their decrease also decreases the water-holding capacity [34]. The majority of ceramides of the stratum corneum has an even number of carbons and the most abundant has 44 and 46 carbons. Ceramides with number of carbons higher than 60 were also observed in a non-negligible concentration [35]. The permeability of the synthetic lipid membrane was not changed with the length of the ceramides. The changes in head groups of ceramides also did not change the permeability [36]. Other research showed that the chain length of ceramides has a huge influence on the barrier permeability. It was also observed that a synthetic barrier composed of a limited amount of ceramides (3 in this case) has some effect on barrier properties. A pig skin containing lamellae with a wider distribution of ceramides has higher permeability than synthetic lamellae with three ceramides. This can be explained by mismatches between various lengths in the lipid lamellae. The wider distribution of ceramide chains affects lamellar

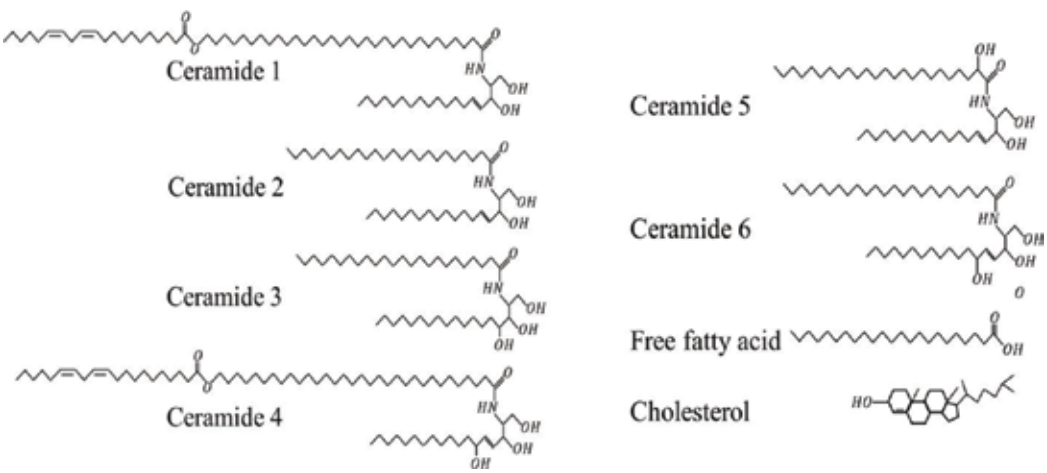

Figure 4. Structure of some ceramides fatty acid and cholesterol in the lipid matrix [33].

and also lateral organization. The wider distribution leads to a hexagonal organization, and synthetic ceramides with a low ceramide chain distribution leads to an orthorhombic organization [37]. A long-periodic phase is formed in the presence of a certain level of unsaturated ceramides, and a long-periodic phase is associated with fluid domain inside. A saturated ceramide EOS-S is important for the stabilization of the orthorhombic phase [38].

3.3. Role of fatty acids

The reduction of free fatty acid (FFA) chains was observed in skin diseases which are known by impaired skin barrier function. The reduction of the fatty acid chain can be caused by an increase in the amount of shorter FFA chains such as with 16 or 18 carbons. A shorter chain allows an increase in vibrations followed by conformational disordering. An increase in the number of unsaturated FFA reduces the packing density of the lipid organization [36]. An analysis of the composition of free fatty acids of human stratum corneum showed a dominant presence of saturated free fatty acids with carbon chain lengths from 16 to 30. The most abundant were FFAs with 24 and 26 carbons (50% of all FFAs). The content of unsaturated FFAs appeared to be around 2% of all FFAs; mostly with 18 carbons and with traces of FFAs with 16, 17 and 20 carbons; 1% of di-unsaturated FFAs (chain length of 18 carbons) [35].

3.4. Role of cholesterol

Cholesterol is situated near the ester bond of ceramides and this position allows the formation of a hydrogen bond between the cholesterol ─OH group and the carbonyl group C═O. The presence of cholesterol has an influence on the formation of the long periodicity phase of ceramides (13 nm). There is a minimal amount of required cholesterol for the formation of the long periodicity phase of ceramides (without cholesterol, long periodicity phase is not formed). Cholesterol does not influence only the presence of long periodicity phase but also the packing density in the long periodicity phase [39]. Cholesterol is very important for correct skin barrier function [40].

4. Plasma treatment

4.1. Sputtering/etching

Sputtering is very often the result of an interaction of the target surface or molecule with ion fluency. When the energy of an ion exceeds a certain value during collision, an atom absorbing this energy can leave its position in the molecule and a vacancy is created (free bond). If the atom that has left still has enough energy, other atoms can be released by following collisions and other vacancies can be created. Some of the atoms can be released from the target material which leads to a process called "physical sputtering."

4.1.1. Physical sputtering

The key parameters of physical sputtering are the binding energy in the target molecule and also the energy and mass of the impinging ion. The dependency on temperature is weak. The

threshold energy is relatively high (in range 10–100 eV) depending on the target/impinging ion combination. The sputtered yield changes with the angle of incident, the substrate material or the roughness of surface. Eroded species consist of atoms or small clusters of target material. Investigation of metallic targets with impinging metallic ions showed that the mechanism of sputtering depends on the ratio of impinging ion mass (M1) and the mass of the atom in the sample (M2) [41]. In the case of M1 < M2, the threshold energy does not depend on the mass M1 or M2. As the mass M2 increases, the probability of reflection of ion increases. The mechanism of sputtering follows the next steps:

1. Ion penetrates through the surface layer.
2. Ion is implanted in sublayer and deform structure of material.
3. Atom of the first layer is sputtered by impinging ion.

If M2 ≥ M1, the threshold energy for sputtering is higher than in the first case. The threshold energy also does not depend on the mass of M1 and M2, and sputtering follows the mechanism:

1. Ion penetrates through the surface layer and creates vacancy, releasing atom.
2. Impinging ion and also released atom deform structure of material.
3. Atom of the first layer is sputtered by released atom.

The threshold is higher in the second case because atoms of the target are sputtered by secondary atoms which were released by ion bombardment. Pure physical sputtering can be achieved mostly in discharges with inert gases. Bombardment of lipids inside the stratum corneum will follow mostly the second mechanism when argon is used and the first one in the case of helium. Physical sputtering is also present in nitrogen, oxygen or air plasma, but chemical sputtering can be much more dominant.

4.1.1.1. Argon bombardment/argon plasma

Argon is one of the simplest media for treatment of biological material. Excited states of Ar (Ar*), metastable states (Ar_m) and Ar ions (Ar^+, Ar_2^+) [42] can be produced in any electrical discharges. Ionization energy of Ar^+ and Ar_2^+ is 15.8 and 15.5 eV, respectively. Argon metastable states have a relatively long lifetime equal to 38 s [43] in vacuum. However, this time is reduced in atmospheric pressure. The energy difference between the ground state and the metastable state of Ar is 11.55 eV, which can be released by collision. A molecular dynamics simulation demonstrated the sputtering of a lipid-like material by argon ions (**Figure 5A** and **B**). The sputtering threshold energy was between 10 and 20 eV. The yield of sputtered particles increases to four carbon atoms per ion impacting the surface at an energy of 50 eV and increases more slowly up to 10 at 100 eV [44]. If we suppose a similar behavior of lipids and polymers during bombardment, argon ion bombardment of the polymers can lead to decreasing of side chains of the polymer, volatile CH_4, CO, CO_2, $HCOOCH_3$ and H_2, and disordered polymer structure [45]. The sputtering of polymer surface by Ar ions leads to graphite-like

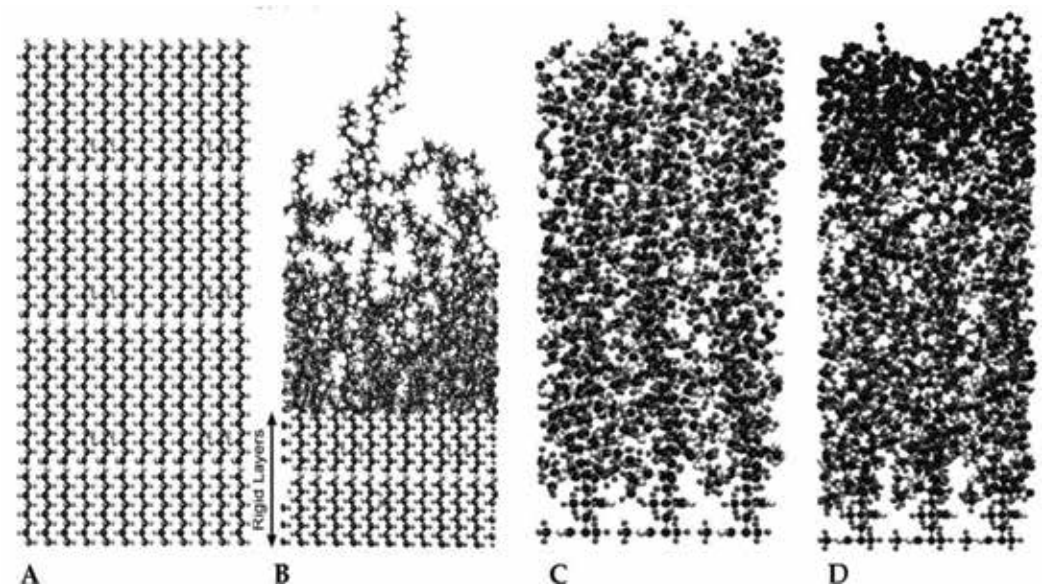

Figure 5. Molecular dynamic simulation. A. Lipid-like surface (black—C, gray—H, white—O). B. Lipid-like surface after bombardment by argon ions. [44] C. Virgin polymethylmethacrylate (composed of C—black, H—gray and O—White) D. Polymethylmethacrylate after bombardment by argon ions (damaged, carbon rich layer is formed on surface) [48].

structure, decreasing the rate of sputtering [46]. If the surface consists of too much of O and H, a thin C-rich layer is not created because released O and H atoms can cooperate in surface sputtering and react with free carbon bonds [44]. Comparison of the lipid-like surface and polymethylmethacrylate in **Figure 5A** and **B** shows forming of carbon-like surface in the case of polymer, but this surface is not formed in the case of the lipid-like surface. Etching of skin surface was confirmed by measuring the thickness of the stratum corneum in the skin cross section (**Figure 6**) [11]. The first layer of the stratum corneum was etched after 5 min of irradiation. If argon plasma treatment is realized in atmospheric air, air particles can participate in treatment with processes other than physical sputtering. Chemical sputtering or surface functionalizing can start to be effective. In this case, plasma treatment causes wettability and hydrophilicity of surfaces by increasing the number of functional groups such as oxygen or nitrogen. Argon plasma treatment is able to increase oxygen functional groups on polymer surface if argon plasma discharge is working on atmospheric air [47]. Molecular dynamics (MD) simulation of bombardment of polymers showed that the main products of etching are CO or H_2, polymer units and C_xH_y fragments during transient sputtering. After the formation of a damaged layer, the products of sputtering are H, H_2, CO, and C_n. Oxygen leaves polymers more difficult than hydrogen because oxygen is larger and less mobile, and that is the reason why oxygen is concentrated under a damaged layer with strong C—O—C bonds and the surface of polymer is created in the layer of amorphous carbon. This process is not valid for all polymers. If polymers compose of many H or O atoms, amorphous layer of carbon is not formed because released H and O atom can etch carbon atoms [48].

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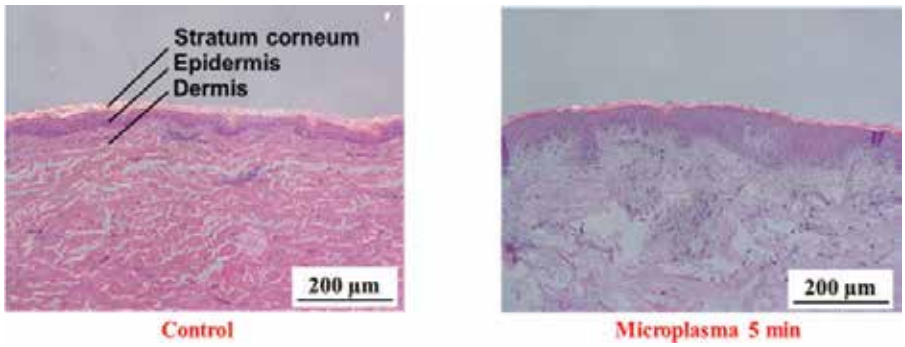

Figure 6. Cross section of pig skin before atmospheric plasma irradiation, the stratum corneum thickness: 18.09 ± 1.64 µm (left—control), and after atmospheric plasma irradiation (right), the stratum corneum thickness: 13.40 ± 1.46 [11, 49].

4.1.1.2. Helium bombardment/helium plasma

Helium is an inert gas with light atomic mass, which means that the sputtering of any surface is lower in comparison with argon. All atoms in the lipid matrix of the stratum corneum are heavier than helium, so atoms are sputtered mostly by the first mechanism described in Section 4.1.1. Investigation of sputtering of polymers by high energy (1 keV) He and Ar ions demonstrated that light atoms such as hydrogen are sputtered at first, followed by heavier carbon and oxygen [50]. On the other hand, the penetration depth increases with decreasing atomic mass, which means that an atomic mass of helium allows penetration deeper inside the surface than argon [51]. However, helium plasma treatment of crystalline carbon with low energy ions (ion temperature ~0.1 eV) demonstrated mass loss and disorder in carbon structure [52]. Helium plasma-treated polycarbonate containing C, O, H atoms did not change the number of C and O atoms, but a change of structure by breaking carbonate groups was observed [53]. Helium is effective in increasing oxygen and also nitrogen functional groups coming from surrounding atmospheric air [47].

4.1.2. Chemical sputtering

Unlike physical sputtering, chemical sputtering (sometimes known as reactive etching) has no or a very low threshold. Chemical sputtering varies strongly with surface temperature and it is highly selective depending on the target/impinging particle combination. The impinging particle also involves neutral reactive species which help to increase the number of sputtered atoms. Eroded species consist of molecules involving impinging atoms and target. The number of sputtered atoms increases by decreasing the energy of bonds and also by decreasing the molecular weights of the atoms in the molecule. The rate of reactions on the surface depends on its structure. If the surface is damaged by sputtering, the reaction rate can increase by orders of magnitude. Atoms react with free bonds after ion bombardment. Chemical sputtering is much more effective, sometimes 1 or 2 orders.

4.1.2.1. Reactive etching/plasma treatment by gases containing oxygen

The dominant mechanism of sputtering by oxygen plasma discharge is the bombardment of the sample by ions creating vacancies. Oxygen molecules, ozone or radicals react with these defects and form H_2O, CO or CO_2. When an unsaturated bond is created in argon plasma, this bond cannot react with any chemically active species to form a volatile by-product. The only way to form those carbon-carbons unsaturated bonds is to react with others carbons and form a highly crosslinked material. Physical sputtering of hydrocarbon film by argon ions can be increased by a beam of molecular oxygen, which leads to chemical sputtering. Molecular oxygen alone cannot sputter the surface but can be adsorbed. Bombardment induces reactions between adsorbed molecular oxygen and bonds created by Ar^+ bombardment on hydrocarbon film. The sputtered amount of atoms and molecules is proportional to the flux of molecular oxygen and the ratio of O_2/Ar^+. An increase of molecular flux increases the number of adsorbed molecules and the number of chemical reactions on the surface [54]. A similar effect can be achieved in Ar/O_2 plasma discharge where except molecular oxygen, other active species such as O_3, radicals O and metastable states $O_2(a)$ and Ar_m are also present. Unsaturated bonds created by Ar/O_2 plasma treatment lead to crosslinking and oxidation [55]. Pure oxygen post-discharge treatment of hexatriacontane resulted in a mass decrease, which corresponds to etching, but no chemical modifications were observed [56]. In this case, only long living particles can interact with hexatriacontane. Wertheimer et al. [57] showed that oxygen radicals $O(^3P)$ and metastable $O(^1D)$ alone are not very effective in interacting with a polymer surface with saturated aliphatic carbons. When hexatriacontane was treated in N_2-O_2 discharge, grafting with etching compete to each other. The mass of hexatriacontane and the amount of oxygen increases followed by etching, and decreases the amount of oxygen [56]. Joubert et al. showed that molecular oxygen does not participate in plasma surface reaction when they compared discharges of N_2O plasma and N_2-O_2 plasma with the same concentration of atomic oxygen. They concluded that O_2 only provides oxygen radicals [58].

4.1.2.2. Reactive etching/plasma treatment by gases containing nitrogen

Nitrogen is a very stable molecule and its concentration of atomic radicals is low in comparison with oxygen. On the other hand, nitrogen creates a lot of metastable states. It was observed that even so nitrogen states coming from nitrogen discharge can cause chemical sputtering [59]. Bombardment of carbon film by N_2^+ ions with an energy under threshold of physical sputtering demonstrated the formation of CN radicals. If these radicals are formed on the surface, it creates HCN and OHCN after reaction with hydrogen and water. If the radical is formed in sublayers, C_2N_2 molecule is created [60]. In the case of nitrogen plasma of hydrocarbon film, we can also observe erosion of the surface and formation CN radicals. However, an admixture of methane can cause deposition of C_xH_y groups. Erosion will compete with deposition and CN, CNH and C_2N_2 groups can be incorporated into the film [61, 62].

4.1.2.3. Reactive etching/plasma treatment by gases containing hydrogen

Hydrogen is the lightest atom, so similar to helium, the sputtering effect of hydrogen ions H^+, H_2^+ and H_3^+ is much lower than by heavy ions. Hydrogen atomic radicals can be formed in discharge which leads to more effective reactive etching. Etching by hydrogen plasma causes incorporation of H and reduction of dangling bonds, unlike oxygen plasma which increases the number of dangling bonds [63]. It was observed that sputtering of the surface with a combination of hydrogen atoms and argon ion enhances sputtering more than their sum. A hydrogen-rich surface is easier to etch than a surface with a deficiency of hydrogen or a more crosslinked carbon network, as the hydrogen atoms can react with free carbon bonds after bombardment by Ar^+ ions. The ratio of the flux of hydrogen atoms and Ar^+ ions is crucial for effective etching. It is the most effective at a ratio 400:1. It means that the atomic flux should be several times higher than the ion flux. If the flux ratio is too low, most of the ion-induced defects recombine before they can be passivated by incident H [64, 65]. Another way to increase sputtering is using an N_2-H_2 mixture. Ions N^+, NH_2^+, NH_3^+, NH_4^+, N_2H^+ and H_3^+ increase the etching rate to a higher value than N_2 or H_2 plasma alone [66].

4.2. Lipid peroxidation

Lipids in the lipid membrane of the stratum corneum do not have to be etched by ions but radicals created in plasma or secondary created in the stratum corneum can degrade lipids by the process called lipid peroxidation. Lipid peroxidation is an oxidative degradation of lipids. The reaction occurs on unsaturated fatty acids. As molecular oxygen is not reactive enough to start oxidation, it must be changed to a more reactive state such as hydroxyl radical (OH), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroperoxyl radical (HO_2•), lipid peroxyl radical (LOO•), alkoxyl radical (LO•), metastable singlet oxygen (O_2(a)), or iron-oxygen complexes (ferryl-, perferryl radical) [67]. Reactive oxygen species abstract hydrogen atoms from the methylene group and create a lipid radical. The rate of lipid peroxidation exponentially increases with the number of bis-allylic carbons, because this bond is the weakest in the molecule with no relation to chain length. After abstraction of hydrogen, the lipid radical can react with molecular atmospheric oxygen and form a lipid peroxyl radical. At a low concentration of atmospheric oxygen, lipids can react with each other in the lipid membrane [67]. The lipid peroxyl radical can abstract hydrogen from another lipid to form lipid radical (L•) which can react with atmospheric oxygen and again to form lipid peroxyl radical (LOO•). Oxidized lipids form more rigid domains. LOOH is more polar than fatty acids; it can disrupt the structure of the lipid membrane.

Superoxide anion radical (O_2−•) reactivity is low in aqueous environments, but it increases in hydrophobic environment.

Hydroperoxyl radical (HO_2•) is much more reactive than superoxide anion radicals. Deprotonation can lead to formation of O_2−• at a pH of 4.8. Weak reactivity of O_2−• allows penetrating deeper in the lipid bilayer than HO_2•. The poor reactivity and relatively long

half-life of O_2−• allows it to diffuse more effectively from its generation site to targets such as membrane lipid bilayers than HO_2• or other reactive species.

Hydrogen peroxide (H_2O_2) has limited reactivity and can diffuse to target longer time and has potential to create other reactive short-living species near the lipid membrane. Creation of lipid radical can be realized in the presence of iron as a catalyst.

Hydroxyl radical (OH•) reactivity is very high and its lifetime is very short; it can react with any molecule in tissue. It can be created from H_2O_2 by catalysis of iron or ultrasound in water [67]:

$$\text{Fe(II)} - \text{complex} + H_2O_2 \rightarrow \text{Fe(III)} - \text{complex} + OH^- + OH \quad (1)$$

5. Application of plasma discharges in transdermal drug delivery

DBD plasma discharge in direct contact with skin was used for transdermal delivery in the air [15]. The time of plasma application was set up to 2 min in pulsed mode 1–10 μs and frequency ranged from 50 Hz to 3.5 kHz. The delivery of large molecules such as dextran molecules with a molecular weight 10 kDa can penetrate to a depth of 600 μm within 1 h and larger molecules such as albumin (66 kDa), IgG human immunoglobulin (115 kDa) and SiO_2 nanoparticles with a diameter of 50 nm can penetrate to a depth of 200 μm within 1 h. Even liposomes with a diameter of 100 nm were delivered to a depth of 100 μm after 1 h. Remote microplasma or plasma jet in argon gas were investigated for transdermal delivery of galantamine hydrobromide (368 Da) [14] and Cyclosporine A (1200 Da) [13]. After 3 min of microplasma treatment, the permeability of galantamine hydrobromide increased by a factor 2 after 24 h in comparison with non-treated skin. The permeability of Cyclosporine A was not able to penetrate through the stratum corneum without plasma treatment. Argon plasma and microplasma showed

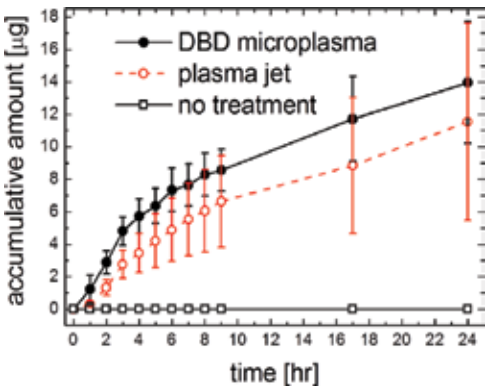

Figure 7. Accumulative amount of penetrated Cyclosporine A through the epidermal layer of the pig skin (four samples were used for microplasma dielectric barrier discharge treatment and five samples for plasma jet treatment) [13] (reproduced from Ref. [13], with the permission of the American Vacuum Society).

similar results. About 9 and 8 μg/cm² of Cyclosporine A penetrated through the epidermal layer using microplasma or plasma jet, respectively (**Figure 7**). The maximum delivery rate was reached after 3 h of delivery and decreased to its minimal value after 9 h (**Figure 8**)

Although, it was shown that microplasma can enhance the permeability of Cyclosporine A, it is only half of the problem which must be solved before clinical use. The second important question is, whether this penetrated amount is sufficient. This problem can be solved with pharmacokinetics, and it was shown that the treated area of the skin must be equal to 225 cm² and the concentration of Cyclosporine A should be 400 mg/ml in propylene glycol. If this condition is fulfilled, a therapeutic concentration in blood can be achieved (**Figure 9**). However, the therapeutic concentration is achieved only for up to 10 h and then another treatment of the skin is needed.

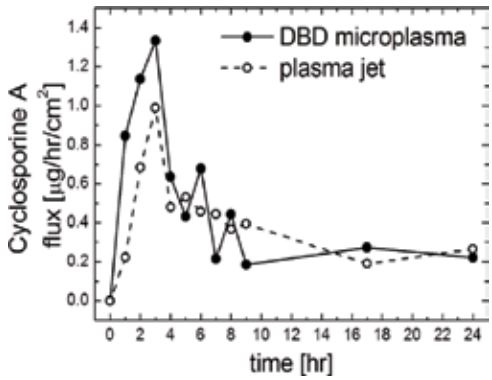

Figure 8. Evolution of the rate of drug delivery through the epidermal layer of the pig skin (four samples were used for microplasma dielectric barrier discharge treatment and five samples for plasma jet treatment) [13] (reproduced from Ref. [13], with the permission of the American Vacuum Society).

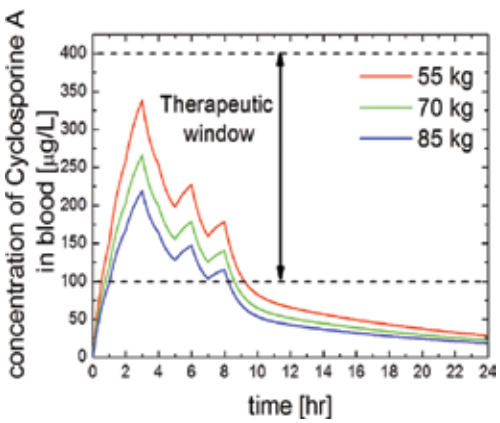

Figure 9. Concentration of Cyclosporine A in blood of adults 55, 70 and 85 kg in weight (400 mg/ml of Cycloporine A in propylene glycol). The two peaks at 6 and 8 h come from experimental data of drug flux containing some errors [13] (reproduced from Ref. [13], with the permission of the American Vacuum Society).

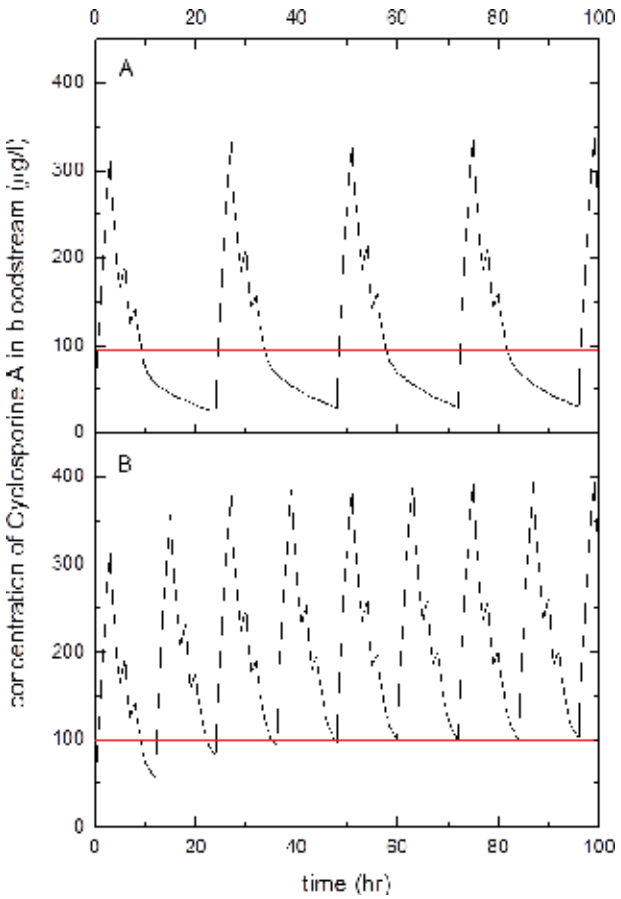

Figure 10. (A) Transdermal drug delivery of Cyclosporine A every 24 h. (B) Transdermal drug delivery of Cyclosporine A every 12 h. The weight of the patient was 55 kg in the model. The red line indicates the lowest therapeutic concentration [68].

Repeated treatment by microplasma and application of Cyclosporine A every 12 h/24 h, to a human of 55 kg is shown in **Figure 10**. As it is seen, treatment every 24 h is not sufficient, and it must be realized at least every 12 h [68]

6. Conclusion

The stratum corneum is the main barrier of the skin. The strength of this barrier is not homogeneous through the whole thickness, but it is stronger in the direction of the living tissues. The lipid matrix is mainly used for drug delivery through the skin. This matrix is composed of lipid molecules which are crucial for barrier properties. If these molecules are peroxided or shortened by breaking bonds, the permeability of the skin can be changed. Lipid molecules are hydrocarbons composed mainly of carbon, hydrogen, oxygen and nitrogen. If plasma is applied on the skin, we suppose that this surface treatment can be compared to

treatment of the other materials, such as polymers. The thickness of the stratum corneum will be decreased. How effective the interaction of plasma can be, depends on the use of a gas or gas mixture. The presence of ions can be very effective to start the interaction with the skin. Usually, oxygen containing plasma can strongly etch the surface with the help of reactivity of active species with free bonds created by ions. If the result is a fluidic or heavy crosslinked structure, the structure depends on the amount of species which is able to react with the free bonds. On the other hand, helium plasma is not so strong of an etching medium and the result of the interaction can be less destructive. However, plasma treatment can also bring some reactive species close to the lipid membrane which can react with lipids without the help of ions and cause peroxidation of lipids and decrease their barrier function. It was shown that plasma sources can influence the skin and help the penetration of various kinds of molecules. Microplasma treatment of the skin and delivery of the Cyclosporine A showed that delivery of a therapeutic amount of drug is possible.

Acknowledgements

We would like to thank to Professor Damon M. Chandler, Mr. Hideto Miyamoto and Dr. Marius G. Blajan of Shizuoka University, for fruitful discussion. This work was supported by JSPS KAKENHI Grant Number JP16H04085.

Author details

Kazuo Shimizu* and Jaroslav Krištof

*Address all correspondence to: shimizu@cjr.shizuoka.ac.jp

Shizuoka University, Hamamatsu, Japan

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Chapter 7

Cold Atmospheric Pressure Plasmas (CAPs) for Skin Wound Healing

Zilan Xiong

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76093

Abstract

In the past 20 years, cold atmospheric pressure plasmas (CAPs) have become a new promising way for many biomedical applications, such as disinfection, cancer treatment, root canal treatment, wound healing, and other medical applications. Among these applications, investigations of plasma for skin wound healing has gained huge success both in vitro and in vivo experiments, and also the mechanism behind it has been studied by many groups. In this chapter, we summarize the state-of-the-art progress in wound healing by CAPs. The plasma devices developed for wound healing, the interactions between plasmas and microorganisms/cells/tissues, the in vitro and in vivo treatments, the clinical trials, and biosafety issues are all included.

Keywords: atmospheric pressure plasma, plasma devices, wound healing, disinfection, cell proliferation, clinical trials

1. Introduction

Wound healing is a complex process involved with infection, cell proliferation/migration, and skin remodeling, see **Figure 1** [1]. For normal wounds, generally the first-stage inflammation occurs in 24–48 h after tissue damage. Bacteria, neutrophils, and platelets are abundant with normal skin appendages present outside the wound. The second stage lasts from after 48 h to around 10 days, during which scab would form on the skin and cell migration and proliferation vigorously occurs. New blood vessels populate the wound area. Skin remodeling starts in the following stage and usually lasts a year or even longer. A scar is usually left and the healed area does not contain normal skin appendages. Wounds can typically be categorized as acute and chronic wounds. Acute wounds contain abrasions, scalds, burns, or post-operative incisions; however, chronic wound does not heal in an orderly set of

stages and often remain in the inflammatory stage for too long and associated with systemic illnesses, age, and repeated trauma such as diabetic ulcers, venous ulcers, arterials ulcers, and pressure sores. The number of patients undergoes chronic wounds increases constantly. It has been reported that around 4.5–5 million people in Germany are concerned with chronic non-healing wounds [2]. Traditional treatments of chronic wounds are expensive and time-consuming; patients usually undergo long-term hospitalization with a poor quality of life.

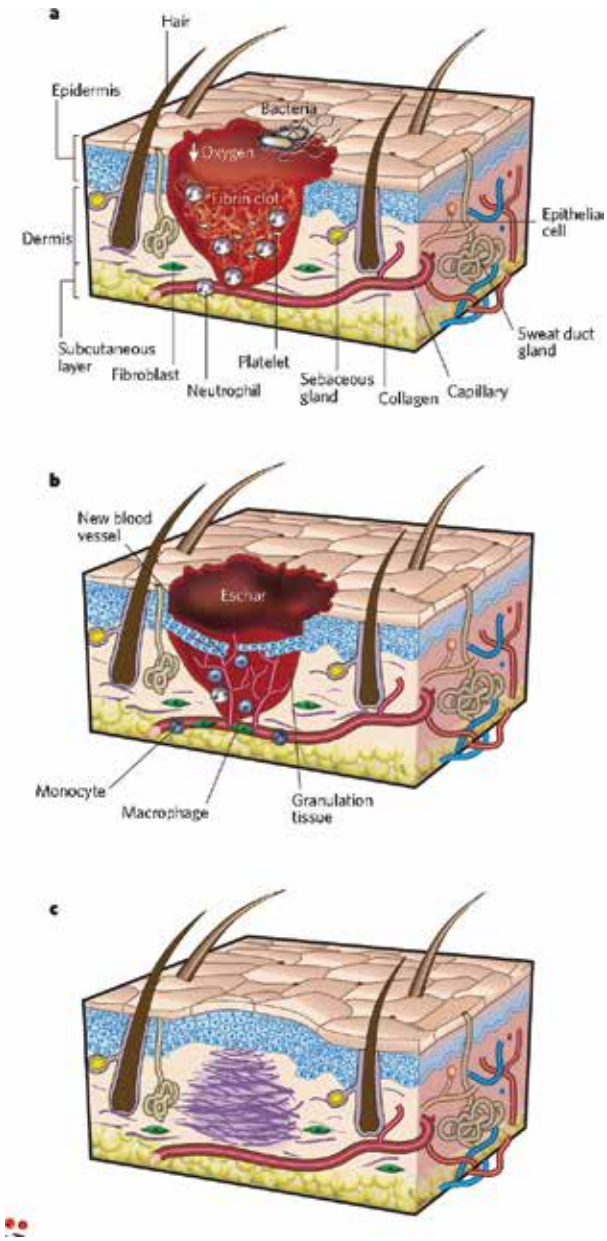

Figure 1. Classic stages of wound repair: (a) inflammation (b) new tissue formation; and (c) remodeling [1].

The first step of chronic wound treatment begins with bacterial disinfection. However, with involvement of multidrug-resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA), treatment of chronic wound encounters more challenges, because the effect of traditional medical antiseptics is largely restrained. Therefore, there is a huge demand for new methods and strategies for skin disinfection and improving wound healing process. One of the potential candidates is cold atmospheric pressure plasmas (CAPs) [3–6].

Plasma, known as the fourth state of matter, other than plasma from blood, has been studied for centuries. Nevertheless, cold atmospheric pressure plasmas (CAPs) also called nonthermal plasmas have attracted huge attention during last two decades for its unique advantage in a new special field—plasma medicine. In 1996, the first paper of plasma medicine came out, which developed a promising way to solve the problems with traditional medical therapies [7]. Since then, related research groups in the entire world started to follow this new field; they extended the research field from initial decontamination to mechanism study, plasma cell interaction, cancer treatment, skin disinfection, blood coagulation, chronic wound healing, and so on, and since then thousands of peer-reviewed research and review papers have been published. There also have been various CAP devices developed [8–13], based on which, in recent years several commercial plasma devices have started to be used in hospital, for example, RF Argon plasma jet-based products by Leibniz Institute for Plasma Science and Technology (INP Greifswald)—kINPen MED has gained Conformité Européenne (CE) marketing certification in 2013 [14].

We report the latest progress of atmospheric pressure plasma for skin wound healing in this chapter and the sections have been arranged as follows: in Section 2, basic plasma components and typical plasma sources for skin treatment are summarized; in Sections 3 and 4, fundamental studies of plasmas interaction with microorganism and cell/tissue are included; in Sections 5 and 6, in vitro and in vivo studies of CAP treatment of skin wounds on animal model are presented; and in Section 6, clinical trials and a very important issue—plasma biosafety—are presented.

2. Plasma generation and designed devices for skin disinfection

CAPs could be generated in lab using plasma source and driven by power supplies with working gas. Working gases such as noble gases (He, Ar), N_2, O_2, air, or their mixtures could be used to ignite the plasma. Typical constituents of plasma include electrons, ions, neutral particles (background gas molecules), UV radiation (UBA, UVB, UVC), heat, reactive oxygen, nitrogen species (RONS), and so on (see **Figure 2**). All these components within plasma make CAPs highly reactive. The constituents and concentration of species in plasma depends on the plasma source, the power input as well as the working gas. When contacting with microorganisms or cell/tissue, components of plasma will play different roles in the treating processes; however, the mechanism has not been fully understood yet. It is well known that UVB (280–320 nm) and UVC (100–280 nm) could be able to cross the epidermis, and UVA (320–400 nm) can even reach the dermis. UVB and UVA can also trigger skin cancer. However, a lot of reports claimed that UV radiation in plasma is low and does not play a significant role in anti-microorganism process except that from microwave-driven discharge [15–18].

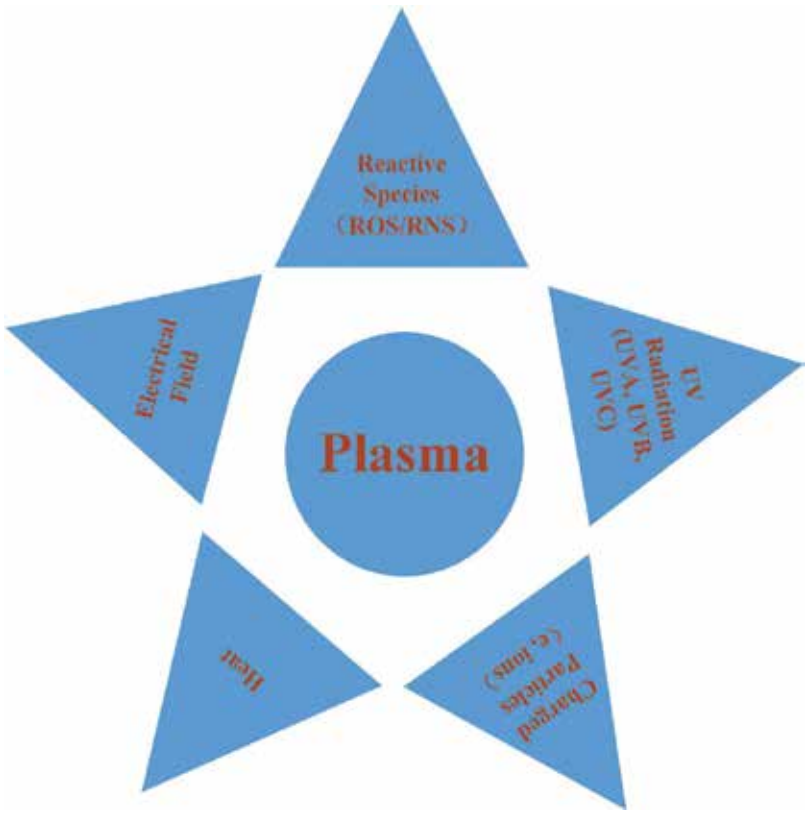

Figure 2. Plasma components.

Charged particles in plasma were concluded to play an essential role in bacterial inactivation by rupturing the outer layer of cell membrane [19, 20]. Mendis et al. reported the electrostatic force caused by charge accumulation on the cell membrane could overcome the tensile strength which leads to rupture [15]. However, other researchers claimed different viewpoints that the anti-bacterial effect of the charged particles is due to the chemical modification of the membrane surface [21]. CAPs are usually designed to operate in near room temperature (less than 40°C); therefore, there would not be substantial thermal effects on microbial cells [22]. The effect of heat can be ignored. The microorganism is under indirect plasma treatment which means that plasma does not directly contact with the target; the electric field was too weak to contribute to the inactivation process. In some cases, when plasma is in direct contact with the samples, the electric field could become high enough to take effect [23]. However, in most of the cases, there is a rare possibility to put the samples within 300 μm away from the electrode to cause damage by the electric field [24]. If the plasma device is using air or O_2-containing mixture or operating in the open air, RONS such as O, O_3, NO, NO_2, OH, and H_2O_2 would present. These highly active species are believed to play an important role in all the plasma treatment processes and have already been reported by many researchers [18, 26–28]. The detailed discussion of the roles of reactive species can be found in [29].

Figure 3 summarizes several typical plasma sources used in different research groups. (a) is called plasma pencil designed by Laroussi group [30]. This device driven by sub-microsecond high-voltage pulse uses He gas flowing through a modified DBD device (with holes in the center of dielectric plates), therefore the plasma created between electrode panels comes out and forms a plasma plume up to 5 cm. Another famous plasma source construct is a floating-electrode DBD device (FE-DBD) which uses the treating object as the second electrode and could generate plasma between the electrode surface and the substrate [31]. FE-DBD could directly use the air inside the short gap as working gas. Safety and stability of FE-DBD largely depends on the power supply, the gas gap, and the electrode shape. Kolb et al. introduced a DC-driven micro-hollow cathode discharge to generate plasma plume outside the tiny hole (c) [32]. It could use air or other gas as working gas. A 51 KΩ ballast resistor is connected to the circuit to restrain the current to 20 mA. The dimension of the plume changes with the gas flow rate as well as the gas temperature. Lu et al. reported a single-electrode plasma jet powered by nanosecond pulse DC [33]. Helium is used as working gas and the length of plasma plume could reach up to 4 cm. The gas temperature is about 300 K and species like O, OH, and N_2^+ are all detected by optical emission spectra (OES) (d). Another structure called plasma needle usually uses bare metal needle as electrode, see (e) and (f) [9, 34]. (g) is a portable DC-driven plasma needle array device called plasma flashlight [35]. This device can directly use a DC battery to power up and create plasma in the open air. (h) is a plasma brush of relatively large area [36]. (i) is also a classical design

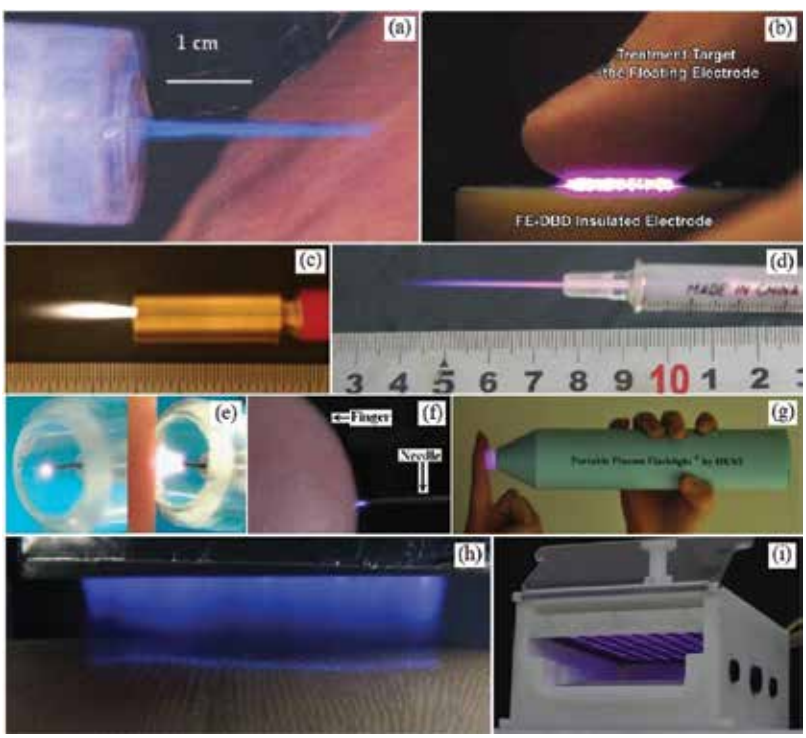

Figure 3. Typical CAP sources.

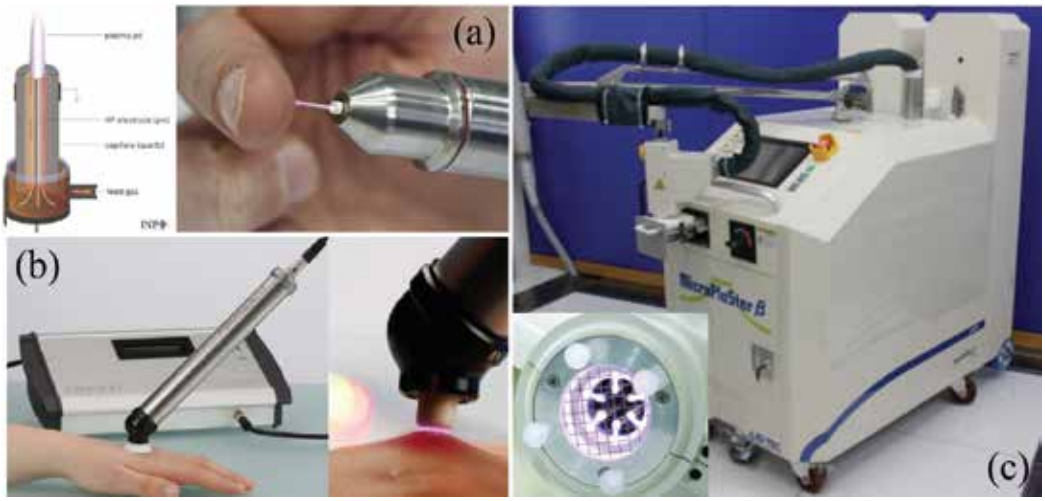

Figure 4. Three commercialized CAPs medical devices.

(surface micro-discharge, SMD) representing 'indirect plasma' [37]. This type of plasma generates on the surface of the electrode, and the plasma does not directly contact with the treatment target. When using air as working gas, SMD could operate in three modes: call ozone mode, transition mode, and NOx mode, respectively. The transition between these modes depends on the input power. More detailed information of the different plasma sources can be found in [38, 39].

With the development of the lab-made plasma sources and fundamental studies, many commercial plasma device products have been delivered into the market. **Figure 4** shows three famous products for skin wound healing based on plasma jet device, FE-DBD and SMD, respectively. **Figure 4(a)** is the world famous kINPen plasma device, which is based on a RF argon plasma jet source and gained CE marketing certification in 2013. (b) is called PlasmaDerm based on FE-DBD. And (c) is MicroPlasma β, which origins from SMD device. The wound healing effect of these three products will be presented in the following sections.

3. Plasma interaction with microorganisms

Plasma sterilization is the first research field of plasma medicine. Since Professor Laroussi published the first paper of plasma sterilization in 1996 on *IEEE Transactions on Plasma Science* [7], there came out thousands of studies on inactivation of bacteria, fungi, and virus using different plasma sources. At the same time, mechanism of plasma interaction with microorganism was studied by using physical, chemical, and biomedical diagnostic methods, such as optical emission spectra (OES), laser-induced fluorescence (LIF), Fourier transform infrared spectroscopy (FTIR), flow cytometry, electrophoresis, ELISA, chemiluminescence assay, and so on. However, the exact mechanism of plasma inactivation microorganisms still

remains unclear. Possible mechanisms proposed by researchers are: (1) electroporation- and oxidation-induced cell wall/membrane dysfunction, which leads to leakage of cellular components; (2) intracellular oxidation and nitrification causing protein damage and gene expression disorder; and (3) direct DNA damage such as causing double-strand breaking.

Bacterial killing effect by CAPs has been investigated for more than 20 years. It is found that CAPs could effectively inactivate different type of bacteria, including gram-positive and gram-negative, anaerobic, aerobic, or facultative anaerobic bacteria [40]. The response of bacteria to CAPs is species-dependent and the Gram-positive bacteria is usually more susceptible to CAPs treatment because of the difference of cell-wall components, which indicates that the CAP-induced damage to the cell membrane and cell wall may be a key factor of antibacterial effect. The most common bacteria found in skin and wound infection are *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, and so on, which have been proved to be effectively inactivated by CAPs [41, 42]. Unlike drugs, another advantage of CAPs is that plasma does not show any resistance after multi-treatment against bacteria. Maisch et al. reported significant decolonization of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* without cell damage of a pig skin sample [43]. Alkawareek et al. also found complete inactivation of MRSA [44].

Many fungi are common constituents of skin flora, and under certain conditions, they would cause diseases. The effects of traditional tools such as chemicals, UV radiation, or heat are often unsatisfactory and sometimes accompanied by undesirable side effects. Unlike bacteria, fungus is more resistance to plasma treatment because of the much more complex cell biology. In 2008, Akishev et al. published the first paper of CAP decontamination of *Aspergillus niger* and *Candida lipolytica* on agar surface using $N_2 + O_2$ plasma jet. After 30–60 treatments, inhibition zone of 30–40 mm was observed [45]. Xiong et al. also inactivated *Candida albicans* on agar surface using a $He + O_2$ plasma jet [46]. They compared the antifungal effect of with/without a cap on the petri dish and found that restraining active species inside the chamber largely improved the antifungal effect. Daeschlein et al. used a low-temperature atmospheric pressure plasma jet to treat clinical isolates of *Trichophyton interdigitale*, *Trichophyton rubrum*, *Microsporum canis*, and *Candida albicans*. They found that plasma irradiation could eradicate fungal growth and no isolate exhibited resistance to plasma treatment [47]. In a new research area of plasma treatment of onychomycosis, Xiong et al. used three kinds of CAPs to treat *E. coli* and *Trichophyton rubrum* living on the back side of a nail model and found that bacteria is easier to inactivate than fungus and the inactivation effect also related to the structure of plasma sources [48].

Researchers also use CAPs successfully inactivate various virus and mechanism has been investigated as well [49–51].

It is known that more than 60% of all infections are caused by bacteria in the form of bacteria which could become resistant to treatment and often develop into a chronic state. A biofilm is often formed by a cluster of cells encapsulated by a 3D extracellular matrix (ECM), [52] which forms a good protection barrier for antibiotics and plasma agents. Therefore, cells inside this community have been demonstrated to exhibit higher antibiotic resistance than planktonic cells. However, CAPs have also shown great decontamination effect against biofilms with longer time than treating planktonic cells under same condition [53, 54]. Koban et al. compared the anti-biofilm (*Candida albicans*) effect by dielectric barrier discharge and plasma jet,

and used 0.1% chlorhexidine digluconate (CHX) and 0.6% sodium hypochlorite (NaOCl) as positive control. They found plasma treatment reduced the colony-forming units CFU significantly compared to chemical disinfectants [55]. In a later research, they investigated the synergistic effect of nonthermal plasma and disinfecting agents against single and multispecies dental biofilms. They found that the combination of plasma and agents increases the antimicrobial efficacy of all tested compounds [56]. Xiong et al. firstly used Laser Confocal Scanning Microscopy (LCSM) technology to obtain the depth of biofilm that plasma could penetrate through [57]. A He-O_2 mixture nanosecond pulse DC-driven plasma jet was used to treat a 10-day growth of *Porphyromonas gingivalis* biofilm, and found that 5-min plasma treatment could at least inactivate the bacteria under 15 μm. In their following work, they successfully inactivated a 25.5 μm biofilm using a plasma flashlight [35]. Puligundla and Mok reviewed the potential application of nonthermal plasmas against biofilm-associated microorganism in 2017. For more details about plasma interaction with biofilms, refer [58].

4. Plasma interaction with skin-related cells/tissues in vitro

The interaction between plasma and human cells largely depends on the plasma source, plasma doses as well as cell type. Researches on plasma cells interaction have been studied by several groups [59–61]. For a unique mixed state with electrical field, charged particles, and controllable reactive species, the response of eukaryotic cell to plasma treatment is very different. It is generally accepted that low dose of plasma treatment could stimulate cell viability and enhance proliferation, differentiation, and migration, while high dose induces cell apoptosis/necrosis [62–65]. It has been found that the resistance against plasma treatment is different between cancer cells and normal cells, which makes plasma selectively killing cancer cells while bring less damage to normal cells and become a potential and powerful tool against cancers [66–68].

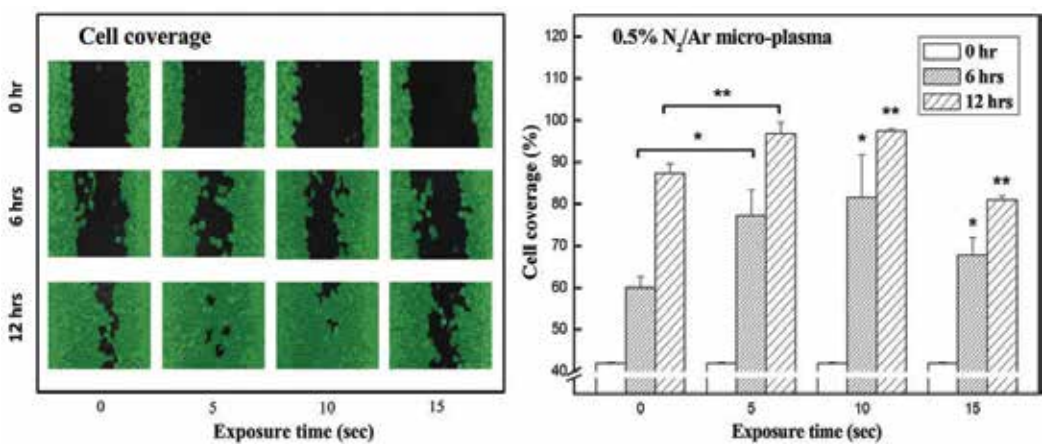

Figure 5. The progression and statistical analyses of cell migration or coverage, 6 and 12 h after plasma exposure times of 5, 10, and 15 s [71].

As mentioned earlier, the second stage of wound repair is related to cell proliferation and migration as well as angiogenesis. Cell types involved in wound healing are mainly fibroblasts and keratinocytes, among which keratinocytes contribute to the major healing processes and fibroblast cells play a guiding role [69, 70]. It has already been reported that CAPs could increases fibroblast cell proliferation and migration by using N_2/Ar microplasma through simulated release of fibroblast growth factor-7 [71], as seen in **Figure 5**. Researchers from INP

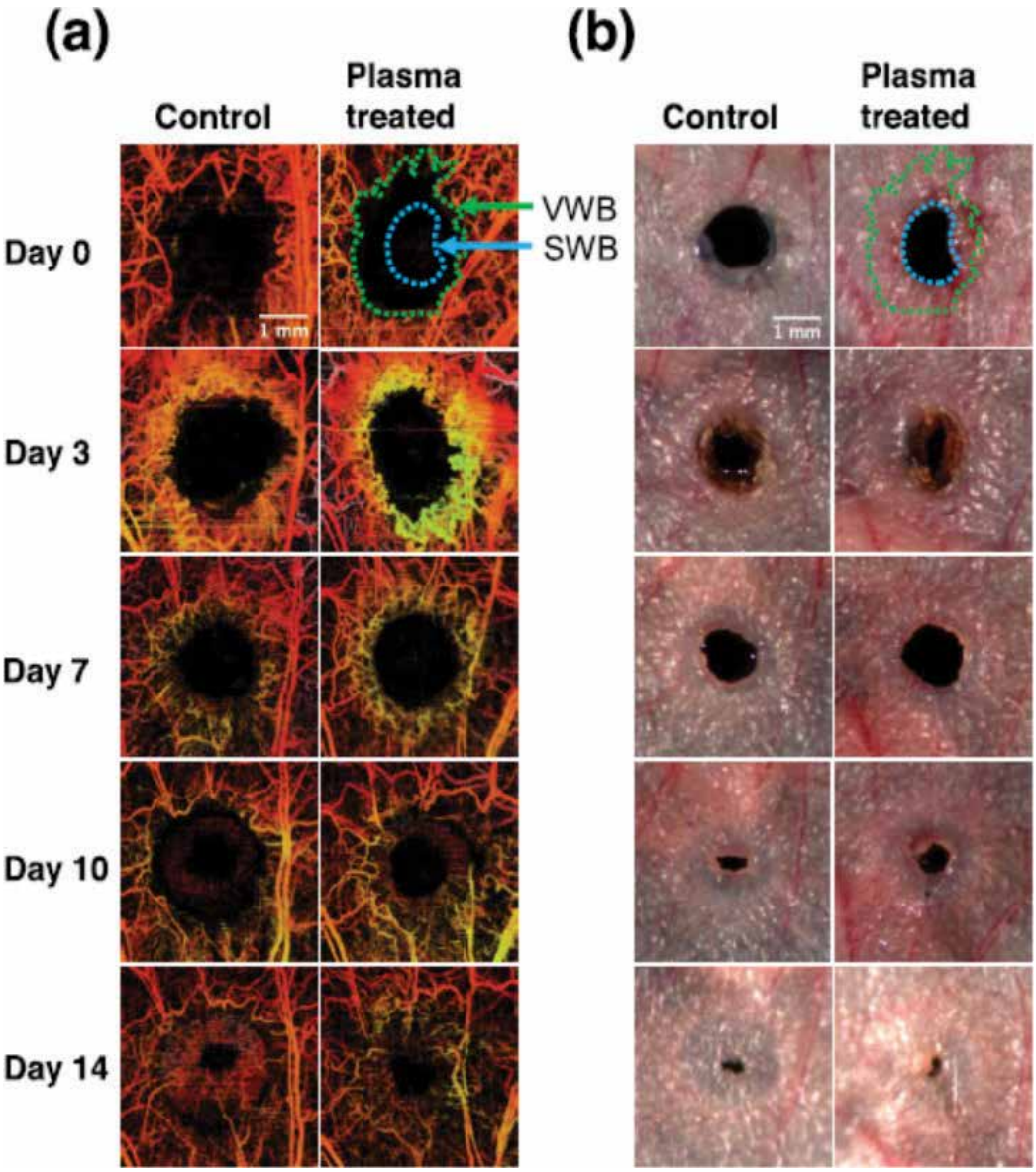

Figure 6. (a) Non-invasive angiographic OCT images and (b) stereoscopic images longitudinally acquired over 14 days after wound generation; relative depth in angiographic OCT images is color-coded as from yellow (superficial) to red (deep) [82].

have done series of investigations of plasma interaction of keratinocyte. They used different plasma sources including plasma jet (kINPen), SMD and DBD, and different keratinocyte cell models/tissues to study the response after treatment. They found increased b1-integrin expression and reduced E-cadherin and EGFR expression of HaCaT-keratinocytes after 30 s treatment [72]. Intracellular level of ROS increased after SMD treatment without dependence on the treatment time or different treatment regimens [73] and DBD and kINPen 09 plasma treatments could also induce oxidative stress in human keratinocytes [71, 72]. Plasma treatment could not only induce cell reactions of stress-sensing but also of proliferative nature, and they propose that stimulating doses of plasma treatment may protect epithelial skin cells in wound healing by promoting proliferation and differentiation through triggering hormesis-like processes [74–78]. Other groups also found the evidence that short-term plasma exposure could enhance keratinocyte proliferation [79].

Angiogenesis is a very important process in the second stage of wound healing involving with growth factors, cytokines, ROS, and NO which could be provided by CAPs. Studies of plasma-inducing angiogenesis have been reported by many groups. Arjunan et al. found that FE-DBD treatment could induce angiogenesis by FGF-2 release regulated by plasma-produced ROS [80]. Hirata et al. used a mouse burn model to investigate the healing process by plasma irradiation, and they found that healing process was improved and the quantity of neovascular vessels was increased after plasma treatment [81]. Kim et al. used angiographic optical coherence tomography (OCT), which successfully captured the plasma-induced angiogenesis process. **Figure 6** shows the en face vascular projections acquired from the angiographic OCT and matched stereoscopic images of the plasma and control wounds over 14 days. They found that the vascular wound area decease of plasma treated wound was more significant [82]. Up to date, very little is known about plasma-induced angiogenesis formation and a lot of work needs to be done in the future to understand the mechanism of plasma effect on angiogenesis.

5. CAPs treatment of in vivo animal m`odels

Based on previous fundamental research of plasma treatment on would healing, treatments on animal models with various wounds and clinical trials are also conducted. Ermolaeva et al. used an argon plasma, which tested the antibacterial effect on both vitro and on the animal model of infected wounds. They found that the 10-min treatment significantly reduced bacterial loads on wound surface and 5-day daily plasma treatment could eliminate bacteria from the infected surface 2 days earlier than the control. Wound closure was accelerated in the plasma-treated animals [41]. Nastuta et al. established a burned wounds model on Wistar rat's skin and used a helium plasma jet to stimulate the wound healing process. They found that both polyurethane wound dressing and plasma-assisted epithelization are positive for the recovery process of burned wounds [83]. Alcantara et al. and Hung et al. also found accelerated wound healing after argon and helium plasma needle and plasma jet treatment [84, 85]. Anke Schmidt used kINPen argon plasma jet device to investigate the wound healing activity on a murine model of full-thickness ear wound; a significant acceleration of

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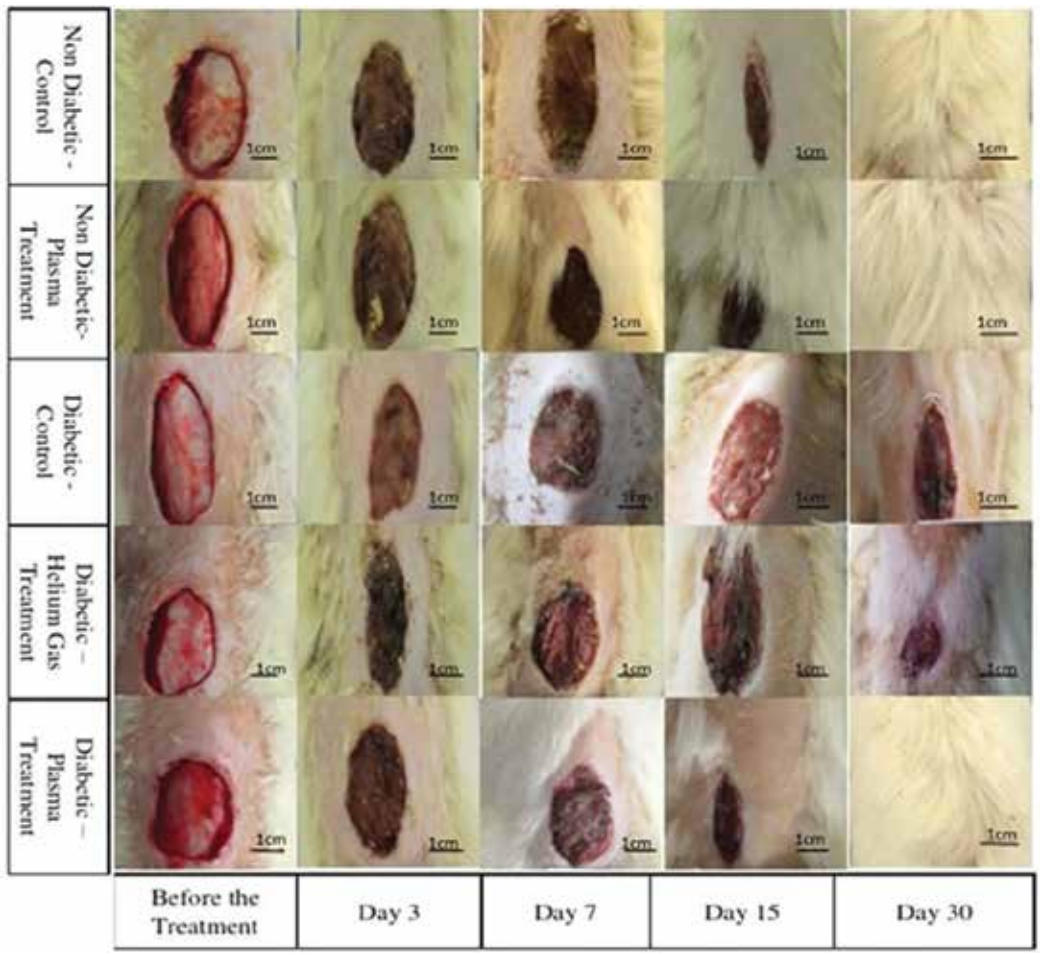

Figure 7. Wound observation for days 3, 7, 15 and 30 after the treatment [89].

wound re-epithelization was observed in days 3–9 [86]. Same results were found by Kubinova et al. without noticeable effects and concomitant activation of pro-inflammatory signaling [87]. Shao et al. investigated the efficacy of a nonthermal N_2/Ar treatment of a laser-induced partial thickness skin wound on a mouse model. Wound-closure kinetics, optical coherence tomography (OCT) and laser Doppler scanning methods were used to measure the healing efficiency and results also show the promotion of wound healing by micro-plasma treatment [88]. Wound healing process in diabetic patients is relatively slow and current therapeutic methods are not completely successful. Fathollah et al. studied the wound healing process by plasma in diabetic rats and found enhanced wound healing rate in the nondiabetic rats and significant wound contraction in diabetic rats after plasma treatment, as seen in **Figure 7**. And also histological analyses show the formation of epidermis layer, neovascularization, and cell proliferation [89].

6. Clinic trials and biosafety concern

Beside animal studies, clinical trials have also been done on patients, especially by using several commercialized products. The world's first plasma source used for clinical trials was the microwave plasma torch MicroPlaSter. Using the first-generation product named MicroPlaSter α, they treated 38 chronic infected wounds on 36 patients with 291 5-min daily treatments and standard wound care, and obtained a significant reduction (34%) of bacterial load without any side effects [90]. In the following study, they compared plasma treatment on various etiologies (Group A), all chronic ulcers (Group B) and Group C for 5-min plasma treatment of chronic venous ulcers. They found a greater reduction in width and length in Group A than control. In Groups B and C, significant reduction in width was found with plasma treatment but not in length [91]. **Figure 8** shows a modified version of MicroPlaSter β and results of treating inflamed ulcer [92]. Isbary et al. reported a successful treatment of

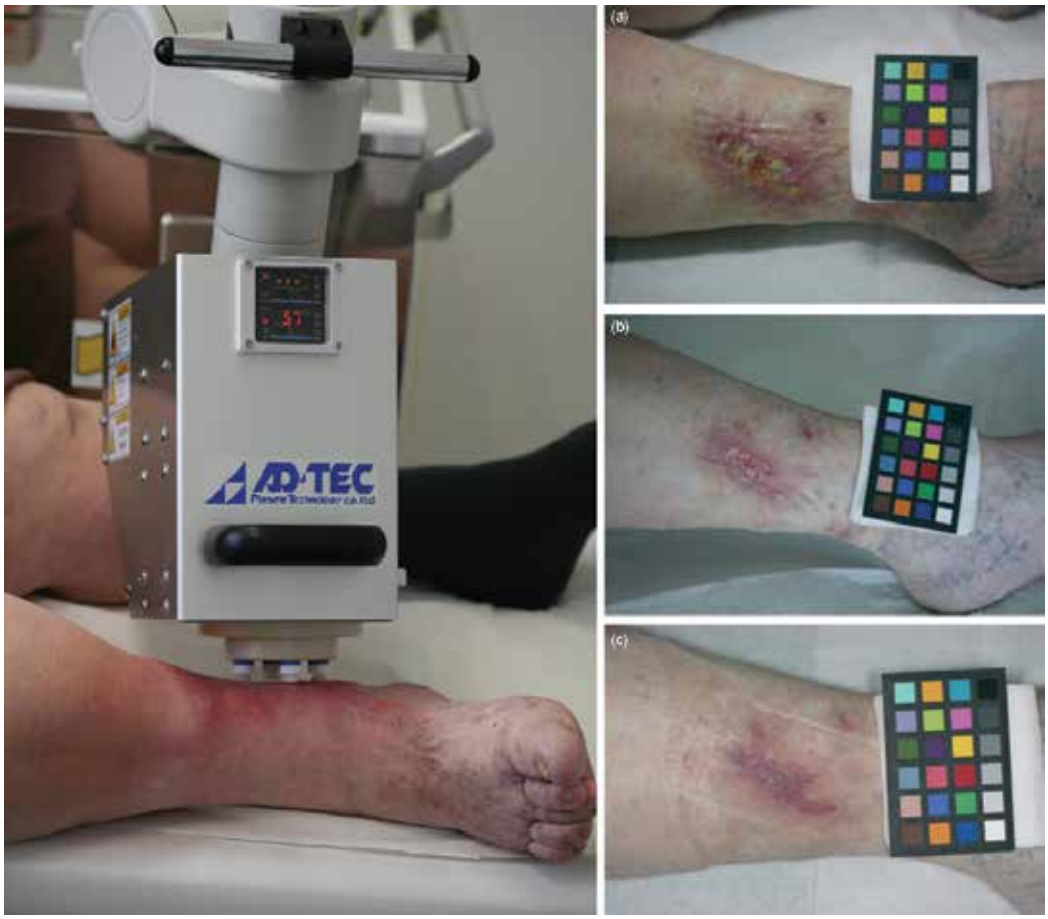

Figure 8. Inflamed ulcer of the right lower leg treatment with cold atmospheric argon plasma generated by MicroPlaSter β [92].

Hailey-Hailey disease by a daily 5-min cold plasma treatment and significant improvement was found after 11 treatments [93]. The argon plasma jet device kINPen MED and DBD plasma source PlasmaDerm successively got the CE marketing for medical devices in 2013. Based on the previous fundamental studies in vitro and on animals, they both reported series clinical trials on human beings focusing on wound healing treatment, especially for the treatment of chronic/infected wounds and microorganism-caused skin diseases. For example, PlasmaDerm was reported to reduced more than tenfold in bacterial colonization on an adult patient with atopic eczema by 30-day treatment of 1 min/day [94]. kINPen MED was reported to significantly reduce the wound volume compared to octenisept in 16 patients with ulcer [95].

Plasma biosafety is definitely a big issue in clinical application. Both of these commercialized products showed tolerable properties (temperature, UV radiation, reactive species, electrical currents, mutagenicity, penetration depth, subjective sensations, cytotoxicity, and histocompatibility) on human skin under controlled conditions and exhibited accelerated wound healing rate [95–98]. Systematic review work of these two devices could be found in [14, 92].

7. Conclusion

Atmospheric pressure cold plasmas could affect different stages of wound healing by helping to activate microorganisms in the first stage and stimulate skin-related cell proliferation and migration in the following period. CAPs have demonstrated high wound healing abilities and may become a promising therapy to replace or assist traditional methods in clinics for wound healing process, especially in chronic wounds. With the certification of several CAP products, more standards and procedures for clinical treatments should be cleared in the future to guide the plasma treatment under an effective and safe way.

Acknowledgements

The author would like to thank for the support from Huazhong scholar program.

Author details

Zilan Xiong

Address all correspondence to: xiongzilan@hotmail.com

State Key Laboratory of Advanced Electromagnetic Engineering and Technology, School of Electrical and Electronic Engineering, Huazhong University of Science and Technology, Wuhan, China

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Edited by Yusuf Tutar and Lutfi Tutar

Plasma can be defined as the extracellular matrix of blood cells. Plasma components, their role in human health risk evaluation, and their functional and clinical analyses are covered in this book. Furthermore, physical plasma-ionized gas is one of the four fundamental states of matter. This homonym has begun to emerge because it can interact with living systems. The physical plasma biomedical applications are reviewed in drug delivery and wound healing medical applications. This approach revolutionizes the therapeutic approaches in medicine and may open up new concepts and clinical applications. The book is an essential source for researchers in the field and provides a platform for different professions.

Published in London, UK

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ISBN 978-1-83881-338-3