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Periodontology, Oral Surgery, Esthetic & Implant Dentistry Open

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Aims and Scope of the POSEIDO Journal

The POSEIDO journal focuses on all aspects of the interconnected clinical and research fields of periodontal sciences, oral and cranio-maxillofacial surgery and medicine, esthetic and restorative dentistry, with a particular interest in implant dentistry, and related research.

Most publications are connected to the dental and maxillofacial field, but some are also from orthopedics, material sciences or other scientific disciplines interconnected with the POSEID research topics (e.g. bone implantable materials, bone regenerative medicine strategies), in order to promote transversal translational research.

POSEIDO is organized as an info journal (international forum), and is therefore publishing a significant quantity of editorial material, as a basis of information, debate and discussion for our community. This editorial material takes particularly the form of **clinical case letters** and **research letters**.

The objective of this strong editorial section is to create links between international research teams, to organize our international research community and to develop a neutral international platform for the publication of debates and consensus conferences in the fast-growing and evolving fields of the POSEID disciplines.

The journal is also publishing a classical content with full-length articles (**original articles and reviews**), following a strict double peer-review process. The journal is particularly interested in original research articles and clinical studies about new techniques, biomaterials and biotechnologies with direct clinical applications in the interconnected fields of periodontology, oral surgery, esthetic and implant dentistry. Review articles are also welcome if they make the clear synthesis of debated topics.

Detailed guidelines for authors can be found on http://www.poseido.info

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Special Review: Editorial of the POSEIDO PACT

The PACT (Platelet & Advanced Cell Therapies) Forum: fostering translational research, transdisciplinarity and international collaboration in tissue engineering and regenerative medicine

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Abstract

The PACT (Platelet & Advanced Cell therapies) Forum Civitatis of the POSEIDO was created to offer a multidisciplinary platform of research, publication, debates and eventually consensus for researchers in the fields of Tissue Engineering and Regenerative Medicine (TERM). In this review, the issues, endeavors and perspectives of this considerable research field are discussed and illustrated, particularly (but not only) through the example of the history, failures and success of probably the oldest method developed in regenerative medicine, the topical use of autologous platelet concentrates (commonly known as Platelet-Rich Plasma – PRP or Platelet-Rich Fibrin – PRF). The History of this domain illustrates very well that the greatest enemy of knowledge is not ignorance; it is the illusion of knowledge. Fighting against illusions in Sciences is a very complex and tricky task, requiring continuing efforts and time. This PACT for a transdisciplinary, translational and international approach in regenerative medicine is an important step in this endeavor.

Keywords. Blood platelet, fibrin, growth factors, regenerative medicine, tissue engineering.

1. A multidisciplinary PACT, this is the right TERM

Tissue Engineering and Regenerative Medicine (TERM) is a very active field of research for many medical disciplines **[1]**. The general concept of this domain is to combine cells, scaffold, biological mediators (the 3 main components of a tissue) or any other

materials and biotechnologies to replace, reconstruct or regenerate a living tissue or organ and to restore a normal function. Dental and orofacial applications are numerous **[2,3]**, starting from dental pulp regeneration, preimplant bone regeneration to extended maxillofacial reconstruction. However the TERM concerns all medical specialties and is a major basic science domain also. As a domain of research in Life sciences, it is one of the best illustrations of the need for medical transdisciplinarity and translational research.

Medical transdisciplinarity is in the essence of the TERM, because the development and results obtained in one medical field (e.g. orthopedic surgery) have often a direct impact in many others (e.g. oral and maxillofacial surgery)**[4]**: for example bone materials, titanium screws and regenerative strategies developed for lower limb reconstruction can often be applied to oral smaller-sized regeneration (the contrary is maybe even more true). In the field of platelet concentrates for surgical use (commonly known as Platelet-Rich Plasma – PRP or Platelet-Rich Fibrin – PRF), the same (or similar) preparations can be used in general surgery **[5]**, ophthalmological surgery **[6]**, plastic surgery **[7]**, orthopedics **[8]**, sports medicine **[9]** or in oral and maxillofacial surgery **[10,11]**. The concepts of regenerative medicine have to be tailored and adjusted to each specialty, but any good publication in one medical domain concerns also the applications in the others.

Translational research is also the founding stone of the TERM, because applied tissue engineering implies the cooperation between basic researchers (material engineers, biologists, etc) and clinicians from all domains. For example, the development of new surfaces for implantable materials **[12]** requires engineers (very specialized such as surface specialists, metallurgists, etc), cell biologists for in vitro testing, sometimes veterinarians for animal investigations of applications, and finally a crowd of clinicians in Humans. When this translational cooperation is not respected, serious confusions can appear. For example, as it was advocated by several authors **[12,13]**, a large part of the literature testing various implant surfaces is significantly biased, as the tested surfaces were simply not characterized properly (or not characterized at all): if a tested product is not clearly define, the results are logically difficult to interpret. If we consider the expertise of surface engineers and the powerful instruments of evaluation available since years, it is surprising to observe such situation. In a series of 5 articles published recently **[13-17]**, it was shown the detailed surface characteristics of 62 implant surfaces available on the market, and all of them presented very different chemistry and topography, while it was the first time - for most products - that these data were so clearly shown and spelled. This example in a very research-intensive field illustrates very well that the notion of translational approach and communication between basic scientists and applied scientists is very needed but still far from being optimal.

In fact, the integration of transdisciplinary (transversal) and translational (vertical) research is the founding need of the TERM. Shall we define this domain as a holistic discipline covering a large range of scientific domains (requiring therefore polyvalent researchers), or as a hub discipline connecting many specialists? Whatever the philosophical approach, this field is extremely multidisciplinary in its essence, and it requires for the team leaders to be able to navigate between the disciplines, vertically and transversally, to change permanently the standpoint to find new solutions to new problems.

This is on this conceptual basis that the PACT (Platelet & Advanced Cell Therapies) Forum Civitatis **(Figure)** of the POSEIDO Academic network **[18]** was designed, to support a multidisciplinary platform of research, publication, debates and eventually consensus for researchers from all disciplines working in this the field of tissue engineering and regeneration. The first objective of this multidisciplinary PACT, it is to support a more

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holistic insight and original standpoints to get the right approach of the TERM. In this first PACT issue of the POSEIDO journal, a series of articles will illustrate very well this need.

Figure. The logo of the PACT (Platelet & Advanced Cell Therapies) Forum, representing the 3 components of a tissue in 3 colors (cells, matrix, mediators) and the lightning of Life, organized like a big P sealing this PACT.

2. A PACT for translational research and transdisciplinarity: the PRP case

Despite the strong interest and fashion for the TERM, the significant investments of the industry and public funding bodies since many years and the considerable literature, the direct clinical applications and results in this domain are still relatively limited and their impact quite modest. Many materials are working quite well, but it is finally more an Evolution than a Revolution. If we follow the developments in the last 10 years, it is an emerging field, and there is a risk that it may remain it for many more years.

The biggest issue in the development of this domain, it is frequently the lack of real conceptual and practical transdisciplinary and translational approach in the research groups. Research groups are often too specialized (in dentistry or other disciplines), and they are lacking the capacity or even the wish to integrate themselves in a more global multidisciplinary ensemble, even for treating complex topics that they do not have the full competence to even consider. In this sense, the literature about platelet concentrates for surgical use (PRP/PRF)**[19]** is a perfect example, as it is also probably the oldest method of regenerative medicine ever developed.

Platelet concentrates are autologous blood extracts prepared through centrifugation of a blood sample of the patient **[19]**. Whatever the method used, the objective of this technology is the same: to gather and collect the platelets (particularly rich in growth factors), the fibrinogen (later activated into a protective fibrin matrix supporting the healing process) and in some cases the cell content (particularly some populations of leukocytes), and to inject or place this preparation into a wounded or surgical site to improve healing and promote tissue regeneration. Historically, the use of this family of blood extracts started in

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the 60-70's with the publications of Matras about fibrin glues **[20]**; it was tested in that time to cover and promote the healing of skin wounds and ulcers. This first approach of tissue regeneration is probably one of the oldest and founding methods of regenerative medicine. It was then mostly based on the concept of regeneration through the use of an autologous scaffold, the fibrin matrix being also the first matrix appearing in a wound after coagulation during a natural healing process **[21,22]**. Fibrin glues are still important surgical adjuvants nowadays.

In the following years, the combination of fibrin with platelets became more frequent, as a logical evolution of this technology, to reinforce the fibrin scaffold but also to use the expected healing properties of the platelets. Therefore, the history of this family of products continues with many tested applications of fibrin-platelet mixtures, tested with some success in neurosurgery **[23]**, ophthalmology **[24]**, general surgery **[25]** and plastic surgery **[26]**, even if these technologies were not widely spread. These products illustrated one of the first forms of regenerative medicine strategies applied to many different medical fields, and they highlighted – more than 40 years ago – the obvious need for medical transdisciplinarity.

The real craze for these technologies developed brutally in the last 15 years, when the concept of "growth factors" was spelled and promoted **[27]**: the early concepts of regenerative medicine (through a coherent fibrin scaffold reinforced with platelet aggregates) were substituted – in the heads of too many researchers – by a pharmacological concept, where a few selected growth factors were expected to regenerate tissues **[28]**. The Industry started to offer and promote many different and often expensive kits for the production of platelet concentrates. Many different techniques were regrouped by mistake under the general acronym PRP (Platelet-Rich Plasma)**[19]**. These technologies were tested in all medical fields even if the most frequent applications can be found in the literature in ophthalmological surgery **[6]**, plastic surgery **[7]**, orthopedics **[8]**, sports medicine **[9]** and particularly in oral and maxillofacial surgery **[10,11]**. As an innovative approach of regenerative medicine, all disciplines were again concerned by these technologies **[4]**. Expectations were very high, as much as the disappointment was a few years later. With a majority of the tested applications of PRP, results were mixed and controversial, and cost-effectiveness was weak. The literature remains very chaotic and difficult to sort and interpret **[28]**. The most logical explanation for this situation is that, despite the number of disciplines interested by the PRP technologies, very little transdisciplinarity could be really observed.

In many aspects, the domain of platelet concentrates is the nightmare of specialist researchers. To be able to apprehend correctly the complexity of blood extracts, serious competences in hematology, immunology, cell biology and endocrinology are needed (at least)**[29]**, as much as the medical and research knowledge of the clinical domains where these products are combined with specialized treatments into a regenerative medicine strategy. Unfortunately, most articles published by dental or orthopedic groups do not integrate a real multidisciplinary team, and the basic knowledge associated with the production of platelet concentrates was often neglected **[19]**. The most famous example was the confusion in the terminology, where all products were gathered under the acronym "PRP", while each protocol in fact leads to a very different combination of cells, matrix and factors **[19]**.

It required 10 years and many scientific debates before a more scientific vision of these materials was reformulated by a transdisciplinary and translational research team **[19,28,30]**: blood is a very complex circulating tissue, PRP and PRF are blood extracts, PRP and PRF are therefore living tissue grafts and not a pharmacological preparation. This aphorism is true in most cases, but it was particularly demonstrated with the L-PRF

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(Leukocyte- and Platelet-Rich Fibrin) clots and membranes, as this material presents a very specific tissue architecture combining fibrin, platelets, circulating cells and all blood components **[31]**. It contains all the complexity of the blood tissue itself **[32]** and was often described as an "optimized blood clot".

To improve the terminology, platelet concentrates were regrouped in 4 big families based on their cell content and fibrin architecture **[19]**: Pure Platelet-Rich Plasma (P-PRP), Leukocyte- and Platelet-Rich Plasma (L-PRP), Pure Platelet-Rich Fibrin (P-PRF), Leukocyte- and Platelet-Rich Fibrin (L-PRF). This major classification is widely cited and serves already as guidelines of the POSEIDO community in this field **[33]**. Thanks to this transdisciplinary and translational approach, the perception of the topic by the scientific community is slowly evolving, even if confusions are still very frequent: there is no Magic of "growth factors", platelet concentrates are in fact very complex autologous tissue grafts and must be very well characterized before they are tested.

Unfortunately, the absence of holistic approach of the topic marked the literature on platelet concentrates with major confusion and illusion of knowledge, the most terrible legacy in Sciences. During many years – and still now – the exact cell content and fibrin architecture of tested platelet concentrates were not clearly characterized, and the most basic information about tested PRP/PRF was missing in most articles; as a consequence, a significant part of the literature on the topic is very difficult to interpret and was called sometimes a "blind library of knowledge" **[19,33]**.

This need for a deep transdisciplinarity and translational approach in the field of platelet concentrates is still extremely strong, as it is very clearly demonstrated by the articles composing this first PACT issue of the POSEIDO Journal. In fact, in many aspects, it is still just starting and this issue is bringing some major breakthrough in the understanding of the field.

3. A PACT against the Merchants to better serve the People and the Industry

Another main issue impacting negatively the development of the field, it may be the huge expectations from funding bodies and the industry in this domain. As tissue engineering and regenerative medicine became much more than just a domain of research – it is almost a Craze – huge resources were invested in a quite short period, stimulating a large literature in all aspects and a need to bring some outcomes from it. This pressure – academic, industrial, mediatic – is a supplementary burden, and it is often pushing scientists to stay even more strictly in one path, and blocking them mentally and financially to take more innovative and risky paths or to go for real debates on the issues encountered in the field.

The field of platelet concentrates is again an excellent illustration of how the commercial and the peer pressure can destroy an outstanding innovative domain. While various platelet gels were tested quietly and with success since many years – a long history started with the work of Matras more than 45 years ago **[20]** – the "Craze for Growth Factors" launched in 1998 **[27]** triggered a massive investment from companies and funding bodies into the field of the PRP-type products. In a few years, many expensive kits and devices were marketed and promoted, and the number of publications on this matter exploded. But with this sudden commercial pressure, scientific communities did not get the possibility to develop the proper concepts and understanding of these techniques. As previously explained, a large part of the literature is incomplete and almost unusable in this field **[19]**.

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The worst consequence was probably the impact of this commercial pressure on the community of users. The PRP fashion was initially very strong (commercially and scientifically) in the domain of oral and maxillofacial surgery, and PRP products were very strongly advertised in all dental meetings. These techniques were in most cases expensive, cumbersome and time-consuming, and the commercial pressure promoting these technologies was in general much higher than the real clinical benefits the practitioners could observe in their patients in their daily practice. The benefit/cost ratio appeared very weak for most users, and the decline of these technologies was very quick. When walking through a dental meeting nowadays, it is easy to observe that PRP technology has almost completely disappeared from the domain. In many countries and communities, platelet concentrate technologies became a source of jokes about the craze for growth factors, or at least a topic considered as not significant. The chaotic literature and the commercial pressure for these products almost discredited these technologies in oral and maxillofacial surgery **[34]**.

In oral surgery, the main platelet concentrate still used, and strongly developing since a few years, is the L-PRF family (Leukocyte- and Platelet-Rich Fibrin)**[19]**. This method appeared very simple, inexpensive and user-friendly, while promoting obvious clinical results justifying very clearly its use **[10]**. The irony of the situation is that this method was developed by a group of clinicians – not by Academic teams nor by the Industry – and was designed as an open-access method, even if some CE/FDA cleared and optimized materials are now available (Intra-Spin L-PRF, Intra-Lock, Boca-Raton, FL, USA)**[33]**. It is expected that it will be the main method of platelet concentrates that will survive in the oral and maxillofacial field, and most probably become also a common gold standard to use in many oral surgical procedures.

However, the relative failure of the early PRP technologies in the oral and maxillofacial field had and still has a very negative impact in the development of other more efficient techniques such as L-PRF. Researchers on L-PRF spent a considerable time in the last 14 years just to repeat and clarify that PRF and PRP are 2 different families of products **[19]**, even if it is obvious when simply observing the products: PRP are liquid solutions that can be softly gelified like fibrin glues, while PRF families only exist as strong fibrin/platelets clots and membranes. It affected also the possibility of publications about these techniques, as the confusion between PRP and PRF was and still remains strong for reviewers and specialists. After the craze for growth factors and the commercial pressure, the suspicion and disinterest towards these technologies was so strong, that it affected even open-access cost-effective technologies that were providing obvious very efficient results. When the discredit touches a field, it concerns the whole field and for a long period. The short-term commercial vision promoted by “Merchants” finally provoked its contrary effects on the long-term **[34]**. Clarity, non-commercial works and cooperation are vital to serve adequately the patients, the research community and the Industry.

A second good example is implant surface design. In theory, the development of new nanomodified surfaces – with specific nanodesign, chemical modification or simply with new micropatterns - was claimed by all specialists to be the future of the field **[35,36]**, to improve even more the quality of osseointegration of the bone implantable materials. Practically speaking, despite the huge literature and investments of the funding bodies and companies, most companies are still using some very basic and quite old surface treatments, as it was clearly shown recently in the ISIS (Implant Surface Identification Standard) project of the POSEIDO network **[13]**: a majority of variations of the SLA-type surfaces (Sand-blasted, Large-grit, Acid-etched)**[15]** or RBM-type surfaces (Resorbable Blasting Media)**[16]**, and a few other minor types (anodized, titanium-plasma sprayed and various forms of coatings

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with calcium phosphates)**[14,17]**. Most companies refuse to use experimental approaches promoted in the literature **[37]**, as the implementation of a new scientific fashion in this domain lead historically several times into significant industrial disappointments **[38]**. In a field where more than 95% of success can be reached by trained surgeons, companies have understood that it is more worthy – and less risky – to use efficient classical surface treatments and to invest in clinical education and surgeon training, than to believe in the miracles of tissue engineering. Some real innovations and improvements are still possible and some families of products such as the SIMN (Subtractive Impregnated Micro/Nanotextured)**[16]** surfaces are opening huge opportunities in surface-led tissue engineering **[39]**; however the commercial and academic pressure in this field in the last 15 years has already damaged significantly this topic. The early craze and excess in this field will impact negatively future major developments during several years: it is clearly negative for patients, scientists and also for the Industry, all stakeholders missing real existing opportunities **[39]** because of short-term illusions.

What happened to the fields of PRPs or implant surfaces may happen to many other branches of tissue engineering and regenerative medicine. The threat is always that results do not meet the expectations quickly enough; in the absence of concrete major results despite the quantity of money invested in the domain by various funding bodies (particularly companies) and sometimes the users, a domain loses its interest before arriving to maturation. As a paradoxical counterproductive effect, an excessive commercial, mediatic or academic pressure to obtain short-term benefits is often the source of commercial confusions and damaging the long-term potential of a whole domain, finally impacting negatively patients, users, scientists and the Industry itself. These examples shall always be kept in mind by researchers and industrials in the field. Like a financial speculative bubble, when a bubble of investment explodes, confusions and failures have global and lasting consequences for all stakeholders.

Platelet concentrates are still very useful technologies if they are selected and used adequately **[7]**; L-PRF has now a considerable impact worldwide in oral surgery **[10,11]**, and this inexpensive user-friendly method is clearly serving the patient and clinician interests. The potential of these blood extracts is still considerable for the industry **[4]**, as it concerns all medical domains, particularly orthopedics and sports medicine **[40]**. The concept of the PACT is not to keep the Industry out of the Community. However a serious Industry needs long-term vision to get long-term stability and benefits - and not short-term minor commercial gains - and to be really efficient and productive for the Communities of Scientists and Patients. This need for clear, honest, transparent, open-minded, non commercial approach **[34]** is the heart of the PACT Forum, and is serving the long-term interests of all stakeholders. In a fashionable research domain such as tissue engineering and regenerative medicine, care must be taken permanently to keep Merchant behaviors out of the scientific Temple. It is our PACT.

4. A PACT against the illusion of knowledge

The interferences of the funding bodies and the Merchant behaviors are often hiding a last threat, much deeper and more insidious: our certitudes, our beliefs. And platelet concentrates and regenerative medicine are very good examples of domains with people "believing in it or not" – a kind of faith, far from what the scientific open, curious and permanently inquiring mind should be.

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Daniel J. Boorstin, a modern American historian who served also as Librarian of U.S. Congress, wrote that the history of Western science confirms the aphorism that the great menace to progress is not ignorance but the illusion of knowledge. The "aphorism" in question was formulated frequently in philosophical history, starting by Socrates in the Antique times. Boorstin spelled this idea repetitively and in many different forms in his books, but we voluntarily selected this version, referring explicitly to the Western science.

Indeed, even if the need for transdisciplinarity and translational approach appears obvious for most scholars, there is another need, which is very often neglected, or misunderstood: this is the need for international – we could even write intercultural – cooperation in this domain. The fact that some countries may appear more developed in sciences - in theory - is often for them the main blockage to consider another standpoint. In Science, certitudes are the worst enemies of Progress, and the Philosophers of Science always insist that the only absolute Truth, it is that there is no absolute Truth. In Science, the perception, understanding and interpretation of a scientific domain (and of the related industry for example) are strongly impacted by cultural parameters (including the perception of social needs and the relationships with the industry). This is often a limit in cooperation. But it can also be a chance, as it opens the possibility to see a problem from different standpoints, and find surprising solutions. More generally, the aphorism repeated so frequently by Boorstin recalls us that a real Scientist shall avoid to be stuck on certitudes and trapped by protective self-fulfilling illusions.

In this first PACT issue of the POSEIDO journal, a series of articles were selected. These articles have one particularity: they demonstrate that the majority of the literature in the field of platelet concentrates for surgical use presents major flaws. In daily practice, investigators observed considerable clinical differences between various platelet concentrate procedures, but the real biological mechanisms remained largely unclear up to now **[41]**; these articles provide a quite unique insight of the complexity of the problem. These articles are both translational and transdisciplinary, as they required the cooperation between engineers, biologists, hematologists, dental and orthopedic specialists. Even if these articles are not directly about clinical results, they are directly tied with the observations of clinicians using these materials.

The first article highlights for the first time the huge diversity of cells present in a small volume of Leukocyte- and Platelet-Rich Plasma (L-PRP) solution. It confirms the major flaw in all articles that did not consider the impact of the cell content on the biology of the platelet concentrates (as the majority of PRPs are in fact L-PRP)**[42]**; it confirms even more clearly that PRPs are not pharmaceutical preparations, but a real autologous tissue graft.

The following series of 3 articles highlights for the first time the impact of the centrifuge characteristics on the cell content and organization of a PRF clot, using the well-identified standard L-PRF protocol as the reference point **[31]**. This study demonstrates that the centrifuge type and vibrations have a considerable impact on the production of PRP/PRF. It reveals this major missing parameter and potential flaw in most publications on platelet concentrates, as many authors are using many different centrifuges without investigating the effect of their devices on their preparations. Finally, the evaluation of the biological signature (in terms of growth factors)**[41]** of different PRF clots obtained with slightly different protocols reveals the considerable impact of tiny protocol changes on the biological patterns of these materials. This issue is both a destructive stone for the literature in the field and a founding stone for a better research in this topic.

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5. Perspectives

The PACT Forum logo **(Figure)** is made of 3 circles representing the traditional 3 components of a tissue: cells, matrix and mediators. The same concept is the basis of the tissue engineering perspective, where researchers always try to combine cells, scaffolds and bioactive molecules. The lightning symbol represents the combination of these 3 components placed into action. It can be seen as the blood support and the integration into the living tissues in vivo, or as the artificial methods to give life to the engineered tissues in vitro. Behind this general symbolism, the notion of scientific PACT itself is very important, when considering the general situation of this field. In this sense, the PACT represents also the need to seal this agreement within our community. It is a PACT to debate and to cooperate freely with an open mind and a long-term vision, a PACT to promote new approach and innovative standpoints, and a PACT to invent new perspectives and solutions to the problems the domain is facing, through trans-disciplinarity, translational research and international cooperation. It is also a PACT of transparency and clarity for the future of the field, far from commercial pressures and keeping Merchant behaviors out of our Temple. This is the PACT offered to the POSEIDO community, and on this PACT we hope to build our Future. There is no better symbol for all these expectations than the Lightning, the power of the innovative idea of the imaginative mind, which suddenly changes the Darkness into Light.

Disclosure of interests

The authors have no conflict of interest to report.

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Research article

Analysis of the Leukocytes in peripheral blood and Leukocyte- and Platelet-Rich Plasma (L-PRP) in rats: A flow cytometry study

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Abstract

Background and objectives. Platelet concentrates for surgical use were often tested as surgical adjuvants in the literature, as a source of platelet growth factors to stimulate healing. Many products are often regrouped under the generic and inaccurate term of Platelet-Rich Plasma (PRP). However, what is tested in many studies is usually a combination of platelets and leukocytes (accurately termed Leukocyte- and Platelet-Rich Plasma – L-PRP). The quantity and impact of leukocytes in these preparations were not yet accurately investigated. In this article, the characteristics of white blood cells in a L-PRP obtained from rats were investigated, in order to point out the main actors and some of the mechanisms that may influence the properties of the platelet concentrates.

Materials and Methods. Blood and platelet concentrate samples were obtained from 64 healthy Wistar rats and leukocyte phenotypes were identified using flow cytometry after labeling leukocytes for CD3, CD4, CD8, CD11bc, CD18, CD25, CD27, CD28, CD45R, CD45RA, CD80, CD90, CD106 (VCAM-1), CD161a and TCRab, TCRgd, RT1B with fluorochrome-conjugated antibodies.

Results. The results have shown that the tested L-PRP contained substantial amounts of leukocytes of many different kinds, particularly T lymphocytes, B lymphocytes, NK cells, monocytes, granulocytes and eosinophils.

Discussion and Conclusion. To highlight the various ways in which these cells can influence their environment will help to better understand the complex interactions of the PRPs with the tissues. This identification of the exact cell content and the understanding of this complex cell equation are important steps towards using these blood concentrates in the best possible way, as a reliable therapeutic option to promote better healing, particularly in infected surgical or wound sites.

Keywords. Blood platelets, infection, leukocytes, platelet-rich plasma, wound healing.

1. Introduction

Platelet concentrates for surgical use represent a relatively novel inductive therapy that could be valuable to accelerate and improve healing processes **[1-3]**. The general concept of these technologies is to concentrate the platelets and their many growth factors, to inject them to stimulate healing and hopefully to promote tissue regeneration. All these techniques are using centrifugation of whole blood, in order to reach high concentrations of platelets and growth factors **[2,4]**. Many products are often regrouped under the generic and inaccurate term of Platelet-Rich Plasma (PRP)**[5]**. Another family is termed PRF (Platelet-Rich Fibrin), when the platelet concentrate was designed and only exists under a strongly polymerized fibrin gel form **[6,7]**.

However, in the vast literature on these technologies, it is often neglected that many platelet concentrate technologies collect also a significant amount of leukocytes **[8,9]**. These cells have a strong direct impact on healing and also produce many molecules including large amounts of growth factors **[10]**. For this reason, a more accurate terminology was proposed and 4 families of products were suggested **[5,6,11,12]**. Two of these 4 main families of platelet concentrates contain higher concentration of leukocytes compared to the amounts of these cells found in peripheral blood: these are termed Leukocyte and Platelet-Rich Plasma (L-PRP) and Leukocyte and Platelet-Rich Fibrin (L-PRF) **[6]**. In fact, these two families with leukocytes are the most frequently used platelet concentrates in many fields of medicine **[3,6,13-15]**. There is still a limited number of studies concerning the detailed composition of leukocytes and their role in these products, and these investigations were mostly done on L-PRF **[7,16]**. Despite a wide spectrum of available diagnostic techniques, we have not found animal and clinical studies identifying extensively the characteristics of white blood cells (WBC) in the various types of L-PRP. Therefore, the objective of this study was to investigate the detailed characteristics of white blood cells in a L-PRP obtained from rats, in order to point out the main actors and some of the mechanisms that may influence the properties of the platelet concentrates.

2. Materials and methods

2.1. Preparation of L-PRP

The study group consisted of 64 healthy male Wistar rats. The Silesian Medical University Bioethics Committee approval was obtained. The rats were anaesthetized with Ketamin (10 mg/kg) after Diazepam (0.1 mg/kg) premedication. For the study, 3.5±0.1 ml of whole blood were collected directly from the heart into a syringe containing 0.7 ml of sodium citrate 105 mmol/l. 4.0±0.1 ml were drawn into a sterile tube and centrifuged for 10 minutes at 1000 RPM (Janetzki K23, Berlin, Germany). This resulted in blood separation into its three basic components: red blood cells, L-PRP sometimes referred to as “buffy coat”, and leukocyte- platelet-poor plasma (L-PPP). Subsequently, L-PPP and L-PRP were removed into 5 ml syringes and centrifuged for 10 minutes at 3000 RPM. After centrifugation, supernatant was removed and 600±50µl L-PRP was obtained. Next, samples with whole blood and L-PRP were examined.

2.2. Cell preparation and flow-cytometric analysis

Blood and L-PRP samples were processed under standardized and optimized conditions within less than 4 hours after collection. The antibody set was designed to identify all major leukocyte populations as well as different lymphocyte subpopulations. For this

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purpose, sample aliquots were stained with 3-to-5 fluorochrome-conjugated mouse anti-rat monoclonal antibodies (Becton Dickinson, San Diego, CA, USA; Invitrogen, Carlsbad, CA, USA; Serotec, Raleigh, NC, USA), as presented in the **Table** (CD3, CD4, CD8, CD11bc, CD18, CD25, CD27, CD28, CD45R, CD45RA, CD80, CD90, CD106, CD161a and TCRab, TCRgd, RT1B). After the staining step, erythrocyte lysis and fixation was performed with FACSLyse Solution (Becton Dickinson). Subsequently, the sample was washed with CellWash solution (Becton Dickinson) and finally resuspended in FACSFlow solution (Becton Dickinson). Acquisition of data **(Figures 1 to 3)** was performed with the use of FACSCanto II flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The data were acquired and analyzed with Diva software (Becton Dickinson).

3. Results

The **Table** shows the results of platelet and leukocyte measurements in blood and L-PRP. In the whole blood, the mean platelet number was 456 ± 78 SD × 10^9/l and the mean leukocyte number 8.501 ± 2.412 SD × 10^9/l. In L-PRP, platelet counts were increased by a mean of 335% (310% to 380%) and leukocyte counts by 350% (320% to 380%) in comparison to the whole blood reference values, showing a strong concentration of both platelets and leukocyte populations in L-PRP. The Mean Fluorescence Intensity (MFI) showed that vascular cell adhesion molecule 1 (VCAM-1/CD106) decreased from 222 to 84 after centrifugation. In the L-PRP tested in this study and in this animal model, the concentrations of most other leukocytes populations were increasing in similar proportions, and the proportions of the various populations (percentages of final leukocyte formula) were relatively similar to the whole blood references. However, granulocytes and monocytes proportions had a tendency to increase during the L-PRP processing, while lymphocytes (T and B) proportions had a tendency to decrease, even if the differences did not appear clearly significant.

4. Discussion

In the literature, most authors focus on the impact of platelet concentration and platelet growth factors amounts **[17-19]**. The presence of the latter in PRPs is believed to accelerate the wound healing process **[14]** and therefore the role of platelet derived growth factors was investigated and discussed in priority in many publications **[2]**. However, most authors were in fact using platelet separation systems collecting also leukocytes **[10,13,20,21]**. The mean leukocyte concentration increase observed in L-PRP was 5 to 8-fold compared to baseline level. In our study, a lower concentration of WBC (3.5-fold on average) was observed, because of the use of a 2-steps harvesting procedure.

Despite the strong potential of leukocytes in the anti-infectious defense and healing process in an injury site (particularly through their antimicrobial molecules and growth factors), some authors still wish to exclude leukocytes from blood concentrate to obtain pure platelet-rich plasma (P-PRP) without leukocytes **[6,22]**. The exact influence of the leukocytes on the mechanism of L-PRP action is obviously very complex and remains to be investigated extensively; its supporters claim that their influence on the inflammatory state is beneficial, while opponents – on the other hand – claim their negative effect in the form of solid enzymes release **[2,12]**. At the time being it is a known fact that leukocytes, and especially neutrophils, are a rich source of not only the natural antibacterial proteins **[23]**, but especially of the growth factors **[10]**.

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		Blood	L-PRP
Platelets (G/L)		**456**	**1530**
WBC (G/L)		**8.5**	**29.75**
Lymphocytes T (CD3+) [%]		**37.81**	**32.62**
[%]	Lymphocytes T CD8+	26.49	24.26
	Lymphocytes T CD4+	70.56	72.81
	Lymphocytes T CD4+CD8+	1.37	1.92
	Lymphocytes T CD4-CD8-	1.58	1.01
	Lymphocytes T TCRab+	96.76	96.6
	Lymphocytes T TCRgd+	1.71	1.63
	Lymphocytes T CD27+	94.65	95.16
	Lymphocytes T CD28+	97.93	98.64
	Lymphocytes T CD8+CD27+	25.05	17.98
	Lymphocytes T CD8+CD28+	24.72	17.88
	Activated lymphocytes T RT1B+	5.05	5
	Activated lymphocytes T CD25+	3.73	3.35
Lymphocytes B (CD45RA+) [%]		**11.6**	**8.78**
[%]	Lymphocytes B CD90+CD45R-	14.61	19.98
	Lymphocytes B CD90+CD45R+	20.42	20.96
	Lymphocytes B CD90-CD45R-	5.57	9.13
	Lymphocytes B CD90-CD45R+	59.4	49.94
Lymphocytes NK (CD161a+) [%]		**1.71**	**1.81**
Monocytes (gran-/CD18+) [%]		**1.3**	**3.33**
[%]	Monocytes CD11bc+	100	100
Granulocytes (gran+/CD18+) [%]		**34.48**	**37.64**
[%]	Granulocytes CD11bc+	100	100
Eosinophils [%]		**2.27**	**1.96**
CD106 median		**222**	**84**

Table. Mean values of selected blood/L-PRP cells.

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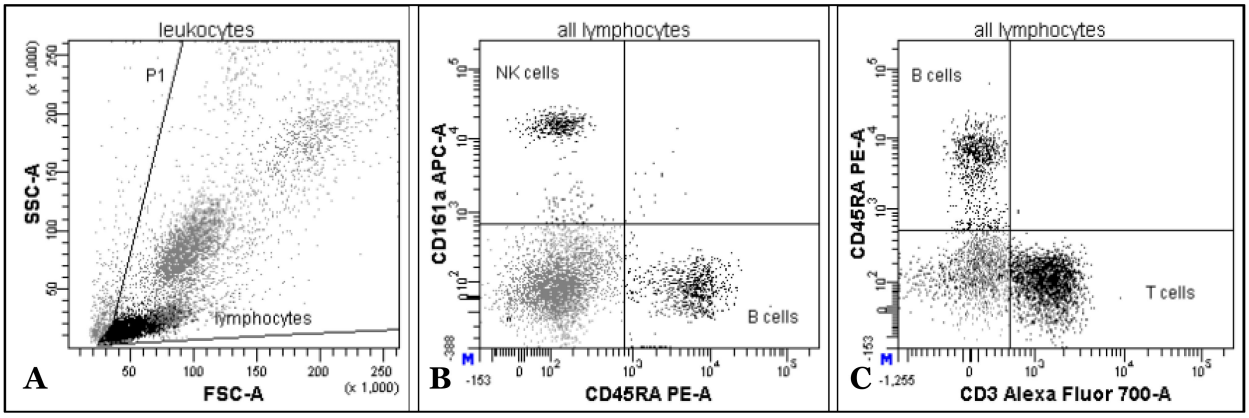

Figure 1. A-C. Rat leukocytes in forward- (FSC) and side scatter (SSC) representation. Main populations of lymphocytes are gated based on their lineage-specific markers: T-cells (CD3), B-cells (CD45RA), and NK cells (CD161a).

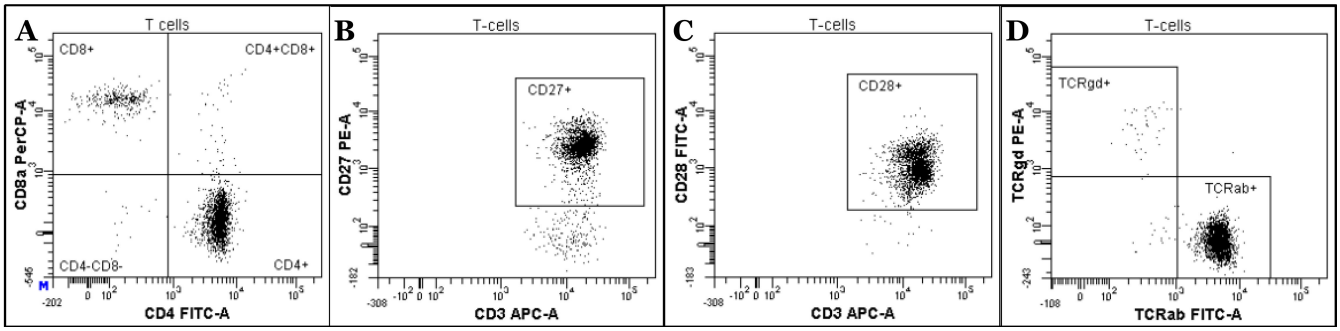

Figure 2. A-D. Main subpopulations of T-cells. Majority of rat T-cells are CD27 and CD28-positive and T-cell surface receptor (TCR) is mostly formed of alpha and beta subunits.

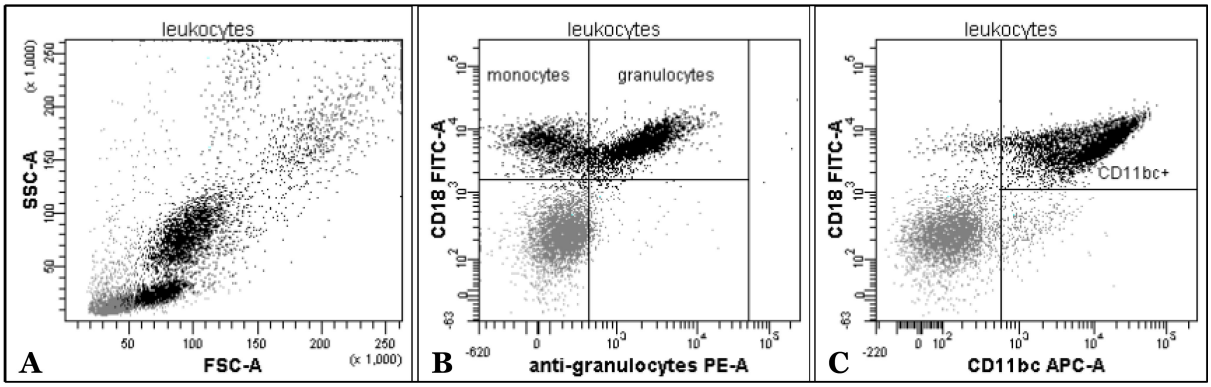

Figure 3. A-C. Monocytes and granulocytes, separated on the FSC-SSC plot and with the use of anti-granulocyte antibody cocktail. Both monocytes and granulocytes are CD11bc and CD18 positive, which form the surface integrin complex.

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To reach plasma with a minimal leukocyte count, the g-force and time of centrifugation need to be decreased. As a consequence, the platelet concentration is lower in comparison to baseline levels and so are, in consequence, the growth factors levels **[24]**. Only in the studies by Aspenberg and Virchenko, where L-PRP was irradiated with 25 Gy to inactivate the WBCs, truly pure platelet concentrate could be obtained **[25]**. However such experimental method is clearly not practical in daily use. Consequently, most PRPs used in the literature have in fact a significant quantity of leukocytes. However, most authors do not mention leukocyte concentration even though they use standard separation systems, and they focus mostly on platelets and growth factors **[6]**. This situation is a major source of confusion and bias in the PRP literature and may explain the many controversies and mixed clinical results obtained with these products in the literature **[5,14,15]**. To clarify this confusion, a terminology was proposed in 2009 **[6]**, and then reinforced in following consensus articles **[5,11,12]**, in order to separate the many different products following at least 2 parameters, the fibrin density and the leukocyte content. For the PRPs, 2 main families were defined: the L-PRPs have a significant quantity of leukocytes, while the Pure PRP (P-PRP) have no or only traces of leukocytes. This first classification was designed to highlight the issue, and it is expected to be extended in the future, when the exact function and impact of the various possible concentrations and proportions of leukocytes will be clarified **[12]**.

Leukocytes are the cells of the immune system defending the body against both infectious diseases and foreign materials **[10]**. According to their appearance under light microscope, there are two broad categories of lymphocytes, namely the large granular lymphocytes and the small lymphocytes. Functionally distinct subsets of lymphocytes correlate with their appearance. Most, but not all large granular lymphocytes are more commonly known as the Natural Killer cells (NK cells). The small lymphocytes are the T-cells and B-cells. Lymphocytes play an important and integral role in the body's defenses. T-cells and B-cells are the major cellular components of the adaptive immune response. T-cells are involved in cell-mediated immunity whereas B-cells are primarily responsible for humoral immunity (relating to antibodies). The function of T-cells and B-cells is to recognize specific “non-self” antigens, during a process known as antigen presentation. Once they have identified an invader, the cells generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen infected cells. B-cells respond to pathogens by producing large quantities of antibodies which then neutralize foreign objects like bacteria and viruses. In response to pathogens, some T-cells, called helper T-cells produce cytokines that direct the immune response whilst other T-cells, called cytotoxic T-cells, produce toxic granules that induce the death of pathogen infected cells. Following activation, B-cells and T-cells leave a lasting legacy of the antigens they have encountered, in the form of memory cells **[10,23,26]**. Finally, B and T lymphocytes have very strong regulatory functions during the inflammatory process, and investigating their role in these complex mechanisms is a very intensive path of research **[27-30]**.

NK cells are a part of innate immune system and play a major role in defending the host from both tumors and virally infected cells. NK cells distinguish infected cells and tumors from normal and uninfected cells by recognizing alterations in levels of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferons. Activated NK cells release cytotoxic (cell-killing) granules, which then destroy the altered cells **[10,23]**. They were named "natural killers" because of the initial notion that they do not require prior activation in order to kill cells which are missing MHC class I.

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Antibacterial properties of L-PRP were investigated in the literature **[31,32]**. However, until now no one can explain the exact mechanism that inhibits bacteria growth, despite several authors have tried. Cieslik-Bielecka et al. described various mechanisms, which can cause microbicidal effects **[23]**. They focused on antibacterial peptides (HDP – Host Defense Peptides), which are produced by the macrophages, epithelial cells, as well as neutrophils and thrombocytes, and are one of the important elements shaping the human natural immunity. Neutrophils are the most common cells with strong phagocytic properties. They constitute the first line of antibacterial defense. The cytoplasm of the neutrophil granulocytes contains numerous granules. The most important ones are primary (azurophil) granules connected to the process of intracellular bacteria destruction and containing numerous bactericidal factors, including defensins, cathelicidins, serprocidins, Bactericidal/permeability-increasing protein (BPI) of gram-negative bacteria, myeloperoxidase and cytoplasmic calprotectin. The secondary (specific) granules are rich in antibacterial proteins such as lysozyme, collagenase, gelatinase, lactoferrin, phospholipase A2, transcobalamin-1 and membrane proteins **[26]**. Neutrophils release enzymes which destroy some specific components of the damaged tissues (to allow cell migration, tissue cleaning and finally tissue reconstruction), and first of all damage microbes, especially bacteria. Neutrophils circulating in the blood become activated by chemotactic factors and this constitutes a signal for the merging of the secretory granules with the superficial membrane. The process of phagocytosis starts by surrounding the organism by pseudopodia, and then closing it inside a phagosome, which undergoes a merge with granules, mainly the primary. The granules release their content exposing the microbe to the activity of a strong mixture of antibacterial proteins. The destruction of the phagocytosed bacteria takes place with the participation of oxygen species, or without oxygen with the use of lactoferrin or lysozyme **[23]**.

Moojen et al. confirmed L-PRP antimicrobial activity against Staphylococcus Aureus **[32]**. Bielecki et al. also used L-PRP in infected bone non unions with good outcomes **[31,33]**. It was justifiable to conclude that L-PRP gel is an inductive biomaterial, which might possess local antimicrobial activity. Some authors have also reported decrease of infections after L-PRP usage in orthopaedic and cardiac surgery **[34]**. Yuan et al. reported a case of infection after intramedullary nailing, which has been a serious problem in orthopaedic surgery **[35]**. In this case, many kinds of treatments had been previously applied, but were not effective. During the operation, they observed a great deal of canal or sinus in most of the callus, and dead bone could be seen from the radiographs. However, it was difficult to remove all of these dead tissues, because if these were removed, the femur would fracture again. Furthermore, in the latter period, the patient refused any open operation under anesthesia. That is why they attempted to use L-PRP to treat the patient, as there were no alternatives to be chosen. To their surprise, the wound healed after L-PRP application.

In Khalafi's study, the L-PRP group had one incidence of sternal infection (0.18%) compared to 11 cases (1.98%) in the control group **[34]**. There were 3 cases (0.53%) of notable drainage from the sternum in the L-PRP compared to 30 cases (5.39%) in the control group. For the leg vein harvest site, the L-PRP group had no reported infections and 61 (10.89%) incidences of excessive drainage, compared to 3 (0.66%) surgical site infections and 212 (48.4%) cases of excessive leg drainage in the control group. Following propensity scoring, they concluded that L-PRP application reduced the odds of chest wound infection by 93%, chest drainage by 96%, and leg wound drainage by 88%.

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VCAM-1 with others adhesion molecules have been found to be significantly increased during viral and bacterial infection **[36]**. However, in our study there was a slight decrease in percentage of CD106-positive leukocytes in L-PRP samples as compared to peripheral blood samples. This may suggest that these rare cells are getting lost during additional centrifugation stages. For other cells, it is interesting to point out that the proportions of the various leukocyte types remain quite stable between blood and final concentrate in the specific L-PRP tested in this study. In another family of platelet concentrates termed L-PRF (Leukocyte- and Platelet-Rich Fibrin), which only exists under a strongly polymerized activated form **[37-39]**, the leukocyte formula of the L-PRF clot is very different from the normal blood composition, with a higher proportion of lymphocytes and a lower proportion of monocytes and granulocytes **[7]**. This composition was advocated to explain the very strong effects in vitro of L-PRF on the bone cell proliferation and differentiation **[40,41]**, and this parameter needs to be evaluated carefully in all PRP and PRF available on the market.

The clinical studies with L-PRP mentioned above illustrate that the associated antibacterial effects of L-PRP (and of L-PRF) play an important role in positive clinical outcomes in many clinical applications. What is also shown is that the characteristics of different L-PRPs are not identical, because their leukocyte content and formula can vary. This aspect is rarely discussed in the literature and more studies are needed in this area. The presence of leukocytes in L-PRP may also influence growth factors levels, as it was already well shown with L-PRF **[4,16,42,43]**. The relative influence of platelets and leukocytes on growth factor levels in PRPs requires further investigation. As it was proven with L-PRF, leukocytes also influence the proliferation and differentiation pathways of many cell types in culture, not only with mediators but also directly **[10,40,41]**. The role of WBC as regulation turntables is essential to fully understand the complex biology of the L-PRP/L-PRF.

5. Conclusion

Until now, the detailed characteristics of white blood cells in L-PRP in rats have never been published in the international literature, and very little is known about the leukocytes populations in most L-PRP available on the market and tested in the literature. Considering the many possible ways in which various leukocyte populations can influence the properties of L-PRP, this study confirms the need to analyze the pattern of white blood cells in blood and L-PRP, before starting to investigate the effects of a platelet concentrate.

Leukocytes have obviously a major impact on the properties and biological activity of platelet concentrates. The ways in which these cells influence the intrinsic biology of L-PRP/L-PRF include their immune and antimicrobial potential as well as their key-role in wound healing processes. That is why the presence of leukocytes in PRPs cannot be neglected and requires further investigation, as this field of research opens new perspectives and possibilities in many clinical situations.

Disclosure of interests

The authors have no conflict of interest to report.

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Author Contributions

All authors participated to the technical design and organization of the study, the treatment of data and to the elaboration of the manuscript. ACB, PP, LS and AS were in charge of the collection of the samples and raw data.

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Research article

The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors and fibrin architecture of a Leukocyte- and Platelet-Rich Fibrin (L-PRF) clot and membrane. Part 1: evaluation of the vibration shocks of 4 models of table centrifuges for L-PRF

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Abstract

Background and Objectives. Platelet concentrates for surgical use (Platelet-Rich Plasma PRP or Platelet-rich fibrin PRF) are surgical adjuvants to improve healing and promote tissue regeneration. L-PRF (Leukocyte- and Platelet-Rich Fibrin) is one of the 4 families of platelet concentrates for surgical use and is widely used in oral and maxillofacial regenerative therapies. The objective of this first article was to evaluate the mechanical vibrations appearing during centrifugation in 4 models of commercially available table centrifuges frequently used to produce L-PRF.

Materials and Methods. The 4 different tested centrifuges were the original L-PRF centrifuge (Intra-Spin, Intra-Lock, the only CE and FDA cleared system for the preparation of L-PRF) and 3 other laboratory centrifuges (not CE nor FDA cleared for L-PRF): A-PRF 12 (Advanced PRF, Process), LW - UPD8 (LW Scientific) and Salvin 1310 (Salvin Dental). Each centrifuge was opened for inspection, two accelerometers were installed (one radial, one vertical), and data were collected with a spectrum analyzer. Each centrifuge was tested in 2 configurations (full-load or half load with 9ml blood collection tubes filled with water) and at the following rotational speeds: 1500, 1800, 2100, 2400, 2700, 3000 and 3300 rpm. Extra rotational speeds were used on some centrifuges. One centrifuge (Salvin) had only one available rotational speed (3400 rpm). For each test, the software documented both radial and vertical vibration.

Results. Very significant differences in the level of vibrations at each rotational speed were observed between the 4 tested machines. The original L-PRF centrifuge (Intra-Spin) was by far the most stable machine in all configurations. At the classical speed of production of L-PRF, the level of undesirable vibration on this centrifuge is between 4.5 and 6 times lower

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than with other centrifuges. Moreover, Intra-Spin always remains under the threshold of resonance, unlike the 3 other tested machines.

Discussion and Conclusion. Each centrifuge has its clear own profile of vibrations depending on the rotational speed, and this may impact significantly the characteristics of the PRP or PRF produced with these devices. This result may reveal a considerable flaw in all the PRP/PRF literature, as this parameter was never considered. It is now necessary to evaluate the impact of the vibration parameter on the architecture and cell content of the L-PRF clots produced with these 4 different machines.

Keywords. Blood platelets, growth factors, leukocytes, platelet-rich plasma, regenerative medicine, wound healing.

1. Introduction

Platelet concentrates for surgical use (Platelet-Rich Plasma - PRP and Platelet-Rich Fibrin - PRF) are blood extracts frequently used nowadays in many medical fields **[1]**, particularly in oral and maxillofacial surgery **[2,3]**, plastic surgery **[4]** and sports medicine **[5,6]**. The objective of all these technologies is to extract (through centrifugation and various handling methods) from a blood sample all the elements that could be use to improve healing and promote tissue regeneration **[7]**, particularly: the platelets (rich in growth factors)**[8]**, the fibrin (serving as a supporting matrix)**[9]** and in some cases the cell content (mostly the various populations of leukocytes)**[9]**. The literature on these products is quite confusing and controversial due to the lack of proper characterization of these many different products **[10,11]**. Recently, they were classified in 4 main families, based on their fibrin and cell content **[11-13]**: the 2 types of Platelet-Rich Plasma (PRP) are platelet suspensions that can jellify into a light fibrin gel after activation (Pure Platelet-Rich Plasma P-PRP, or Leukocyte- and Platelet-Rich Plasma L-PRP, respectively without or with leukocytes). On the contrary, the 2 types of Platelet-Rich Fibrin (PRF) only exist in a strongly polymerized fibrin gel form (Pure Platelet-Rich Fibrin P-PRF, or Leukocyte- and Platelet-Rich Fibrin L-PRF, respectively without or with leukocytes).

In oral and maxillofacial surgery, the use of PRPs is nowadays very scarce, as these various technologies are quite complicated, heavy to use in daily practice, expensive and offering quite mixed clinical results **[2,3]**. On the contrary, the L-PRF family developed very quickly these last years, as this technique is very simple and useful in daily practice, user-friendly and inexpensive **[11]**. Interesting results in vitro **[14,15]** and in some clinical oral applications were already published **[16-20]**, particularly during sinus-lift implant surgery **[21-23]** and gingival healing **[24-26]**. In this open-access method, a 9mL blood sample is taken without anticoagulant and centrifuged immediately during 12 minutes. At the end of the process, a L-PRF clot can be collected in the middle of tube **[9]**. This clot gathers almost all the platelets and half of the leukocytes of the initial blood harvest (with a majority of lymphocytes)**[9]**, and is an active source of growth factors release **[8,27]**. This clot can then be used directly as filling material or mixed with bone grating material **[2]**, or it can compressed into a strong fibrin L-PRF membrane, using an adequate surgical box designed to prepare it without damage (nowadays marketed with CE/FDA clearance as Xpression kit, Intra-Lock, Boca-Raton, FL, USA)**[28]**.

Even if the method is open-access, the early developers carefully optimized this technique, in order to get the best possible and most reproducible clots, membranes and finally clinical results **[28]**. Based on a slow learning through the feedback of the field experience and research, the protocol was tailored by using a high-quality table centrifuge,

specific glass-coated plastic tubes and a specific protocol (12 minutes, 2700 RPM). The relevant literature on the L-PRF was produced using this adequate material since more than 10 years **[2]**. The original open-access method and associated devices used since the early phases of the development of this technique are nowadays marketed with CE/FDA clearance as the Intra-Spin L-PRF system and kit (Intra-Lock, Boca-Raton, FL, USA). This inexpensive system is actually the only L-PRF system available on the market with all certifications and using the original protocol and devices.

Unfortunately, other authors without this experience decided to extrapolate the original L-PRF protocol with cheaper low-quality devices or by modifying some parameters of the initial protocol to adapt to the various centrifuges they decided to use **[29-31]**. The L-PRF clots and membranes produced with these modified materials and methods appeared obviously different (weight and volume of the clots for example) from the original L-PRF method, but this simple observation was often neglected. This chaotic situation of random modification of the protocol was already widely observed in the fields of PRP **[12]**, and it was often advocated that the mixed and controversial results and the absence of consensus in the use of PRP (particularly in sports medicine) was the consequence of this absence of control, characterization and evaluation of the methods and types of PRP in the literature. Some debates appeared in the L-PRF literature **[29-31]**, in order to highlight this absolute need to respect the original protocol and material, or at least to define clearly any variations of the L-PRF protocol/material as a different protocol **[28]**. It was advocated that changes in materials and/or protocols may affect considerably the L-PRF clot content and architecture **[25]**, and must be therefore characterize separately as a specific PRF-like product **[28-31]** and not as the original L-PRF described in the literature. This debate is very important, in order to avoid to create confusing data in the literature that may affect the credibility of the L-PRF technique because of inaccurate articles produced with inadequate methods or materials.

The exact differences between the various materials and methods to produce L-PRF and the characteristics of the different L-PRF products were not clearly demonstrated and published scientifically up to now. The characteristics of the centrifuges are important parameters to start with. All platelet concentrates are produced through the use of a centrifuge. As mechanical instruments, all centrifuges have specific mechanical characteristics that differ significantly among the many possible available models. However, these different characteristics were never evaluated before in the production of PRP/PRF. In the case of small table centrifuges used for L-PRF production, the most relevant parameters to evaluate appeared logically to be the vibrations of the centrifuges during the centrifugation process, the vibration shocks during the acceleration phases and an eventual resonance of the vibrations. All these mechanical characteristics may interfere with the quality and biological signature of the final L-PRF product.

The objective of this series of 3 articles was to point out the impact of the centrifuge characteristics and centrifugation protocol on the cell, growth factors and fibrin architecture of a L-PRF clot and membrane. In this first article, the mechanical vibrations (both radial and vertical) appearing during centrifugation were evaluated in 4 models of commercially available table centrifuges frequently used to produce L-PRF.

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2. Materials and methods

2.1. Description of the tested centrifuges

In this study, 4 different centrifuges, found on the market and used to produce L-PRF, were tested **(Figure 1)**. The country of manufacture being used by some companies as a claim for quality, the country of manufacture of each centrifuge and its main components was checked. The 4 selected centrifuges were purchased from their manufacturers (or distributors).

The first centrifuge was the original centrifuge used during the early development of the L-PRF open-access method and is nowadays marketed under the name Intra-Spin L-PRF centrifuge (Intra-Lock International, Boca-Raton, FL, USA; Made in Germany). It is actually the only CE marked and FDA cleared system for the preparation of L-PRF clots.

The 3 other centrifuges are not CE/FDA cleared for L-PRF, but they can be found relatively frequently available on the market for this use (mostly because they are much cheaper): centrifuge A-PRF 12 (Advanced PRF, Process for PRF, Nice, France; Country of manufacture not indicated on the label, components inside show “Made in China”), centrifuge LW - UPD8 (LW Scientific, Lawrenceville, GA, USA; Components made in China, assembled in the USA) and centrifuge Salvin 1310 (Salvin Dental Specialties, Charlotte, NC, USA; Made in China).

Figure 1. The 4 centrifuges used to produce L-PRF clots and tested in this study. From left to right: original L-PRF centrifuge (Intra-Spin, Intra-Lock), A-PRF 12 (Advanced PRF, Process), Salvin 1310 (Salvin Dental) and LW - UPD8 (LW Scientific).

2.2. Protocol of analysis of the vibrations

Each centrifuge was loaded with 8 blood collection tubes (Serum Vacuette 9 ml, Greiner Bio-One GmbH, Kremsmünster, Austria) filled with water to the manufacturer’s recommended level (approximately 9ml). The tube weights were measured on a high precision balance device (Sartorius M-Prove high precision balance, Model AY123, Sartorius AG, Goettingen, Germany) to ensure that each tube had a substantially equivalent load of water (full tube weights were measured between 18.41 and 18.43 grams).

Each centrifuge was opened for inspection and the placement of two accelerometers (Wilcoxon: Model 780A-IS, 100mV/g, Meggitt, Germantown, MD, USA). One accelerometer was used to access radial vibration on the centrifuges when under load and under

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acceleration. This radial accelerometer was positioned directly on the motor frame of each centrifuge, as close as possible to the bottom of the rotating tube holder. The other accelerometer was used to determine vertical vibration when under load and under acceleration. This vertical accelerometer was positioned on the centrifuge base, as close as possible to the lower edge of the rotating tube holder. The data were collected with a spectrum analyzer-FFT (Fast Fourier Transform) capable and its data processing software (Commtest Model VB7 and software Ascent 2013 Level 2, R3, v13.5.5; Commtest, GE Energy, Christchurch, New Zealand).

Each centrifuge was tested with two configurations: half tube load (3 or 4 tubes depending on capacity) and full tube load (6 or 8 tubes depending on capacity). For each configuration (half tube load and full tube load), tests were run at the following rotational speeds: 1500, 1800, 2100, 2400, 2700, 3000 and 3300 rpm. Extra rotational speeds were used on some centrifuges. One centrifuge (Salvin 1310) had only one available rotational speed (3400 rpm). For each test, the software documented both radial and vertical vibration. Plotted curves showing vibration (m/s^2) vs. frequency (Hz) were obtained from this documentation and recorded.

3. Results

All plotted curves for the tested centrifuges demonstrated a high level of acceleration in a very narrow range of frequencies. These were centered on the excitation frequency (rotational speed). Almost no vibration at other frequencies was noted. Since the software has the capability of combining several curves on a single chart, we were able to obtain for each machine and for a given configuration (half-full or full) a set of curves from which we were able to derive an envelope curve showing the level of vibration vs. the rotational speed. Therefore, all envelope curves could be combined on a single and final chart: one chart for the half-full configuration and one chart for the full configuration. These 2 final charts allow us to compare easily all machines tested.

This experiment highlighted 2 clear results **(Figures 2 and 3)**. First, all centrifuges experienced an increase in the level of vibrations when the rotational speed was increasing. Second, very significant differences in the level of vibrations at each rotational speed were observed between the 4 tested machines. Each machine had its clear own profile of vibrations depending on the rotational speed. The test curves of the 4 machines never crossed. These results were observed for both experimental configurations (half or full tube load).

The original L-PRF machine (Intra-Spin) presented the lower level of vibrations at all speeds in both experimental configurations, and the increase of the vibrations remained very limited when the speed was increasing. This was clearly the most stable machine on this aspect. As this machine served for the development of the L-PRF protocol and significant literature, these values can serve a standard of comparison with other machines.

The LW centrifuge presented a very strong increase of vibrations when the rotational speed was increasing. The vibrations of this centrifuge are 4.5 times higher than the vibrations of the Intra-Spin centrifuge for the production of L-PRF (2700 rpm) in full load configuration, and the difference was even stronger in half load configuration (5.2 times higher).

The Salvin centrifuge offered only one speed of centrifugation (3400 rpm), what was therefore the speed used to produce L-PRF with it. The vibrations of this centrifuge were 6 times higher than the vibrations of the Intra-Spin centrifuge for the production of L-PRF in

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full load configuration, and the difference was a bit stronger in half load configuration (6.3 times higher).

The A-PRF centrifuge presented the strongest increase of vibrations when the rotational speed was increasing. The vibrations of this centrifuge were 6 times higher than the vibrations of the Intra-Spin centrifuge for the production of L-PRF (2700 rpm) in full load configuration, and the difference was even stronger in half load configuration (6.8 times higher).

The results of this study were very clear, and highlighted that each centrifuge had its own vibration profile, and that devices can have considerable differences in terms of intensity of the vibrations.

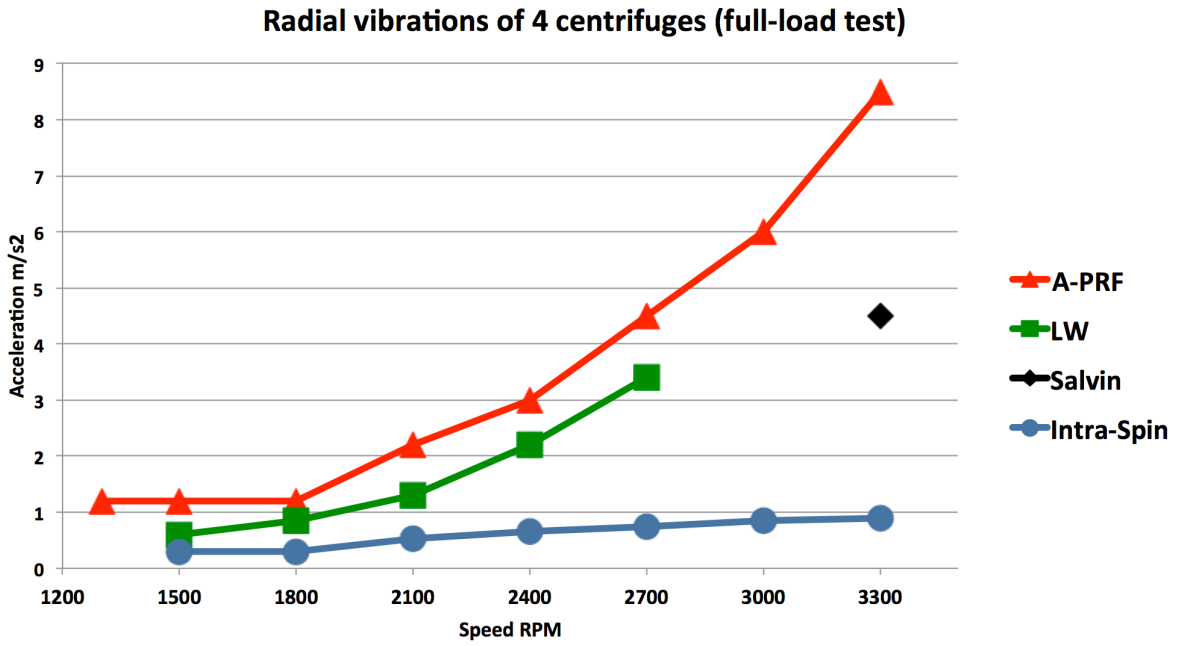

Figure 2. Radial vibrations of the 4 centrifuges during the full-load test. The curves showed the intensity of vibrations at each rotational speed (in RPM, Revolutions Per Minute). The lowest intensity of vibrations was observed always with the original L-PRF Intra-Spin centrifuge. At the classical speed of production of L-PRF, the level of undesirable vibration on the Intra-Spin centrifuge was between 4.5 and 6 times lower than with other centrifuges. Moreover, Intra-Spin always remained under the threshold of resonance, unlike the 3 other tested machines.

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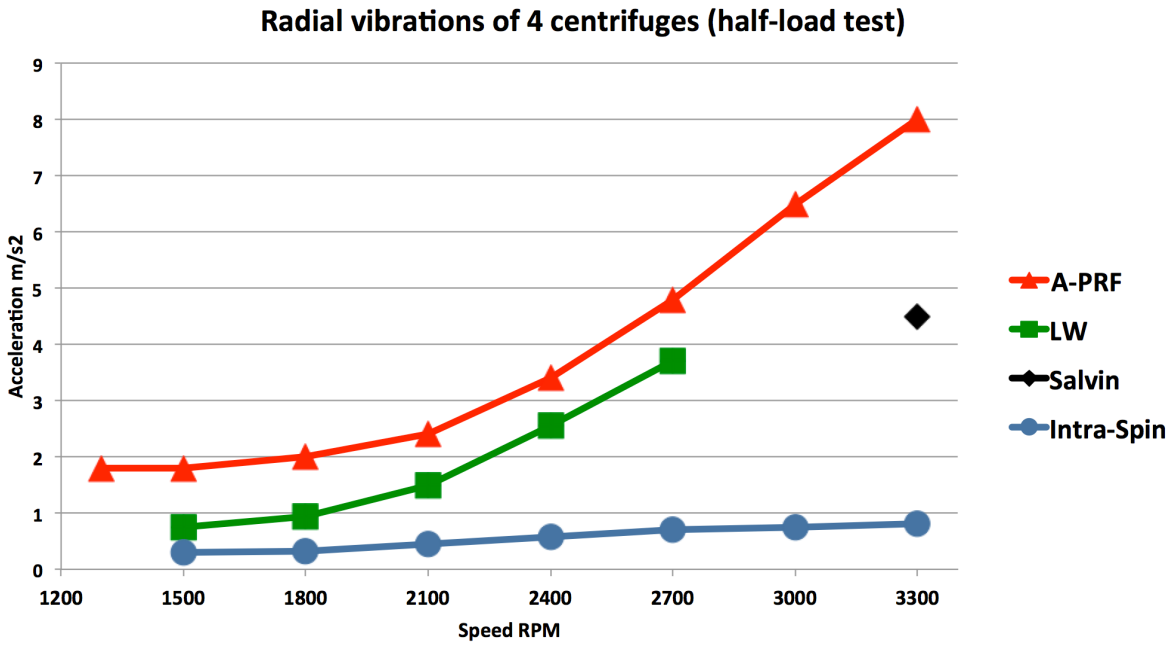

Figure 3. Radial vibrations of the 4 centrifuges during the half-load test. Half-load test was closer from the clinical reality, as clinicians often only use 3-4 tubes of L-PRF for a daily life small surgery. The curves showed the intensity of vibrations at each rotational speed (in RPM, Revolutions Per Minute). The lowest intensity of vibrations was observed always with the original L-PRF Intra-Spin centrifuge. At the classical speed of production of L-PRF, the level of undesirable vibration on the Intra-Spin centrifuge was between 5.2 and 6.8 times lower than with other centrifuges. Moreover, Intra-Spin always remained under the threshold of resonance, unlike the 3 other tested machines. The results were very similar to the full-load test, but the differences between machines were even more marked in this half-load configuration.

4. Discussion

Since the early phases of development of the L-PRF technology, scientists observed easily that the choice of the centrifuge and the protocol of centrifugation was affecting the final aspect (weight and size) of the L-PRF clot **[9,27]**. Good sense observations could reveal that the machines have different levels of vibrations, as it can be perceived easily by simply hearing them and placing a hand on the centrifuge during the centrifugation process. Despite this obvious scientific observation, no one investigated or even considered the quality of the centrifuge as an important parameter in the production of PRP and PRF. PRP are often produced with larger and heavier centrifuges than L-PRF **[27]**, and this may explain why this parameter was not investigated before. However, in the case of L-PRF, this parameter is very perceptible as the L-PRF technique was designed to be used easily in daily clinical practice and therefore with a small and light table centrifuge - therefore with a highest risk of vibrations and resonance during the centrifugation.

This study is the first research evaluating scientifically the intrinsic characteristics of the table centrifuges used to produce platelet concentrates for surgical use. It proves that the devices found on the market have very significant difference in terms of vibrations, and that all tested devices have much higher intensity of vibrations than the original L-PRF centrifuge (Intra-Spin). Moreover, when radial vibrations rise above 1, there is a serious risk that resonance occurs in the centrifuged tubes, what can provoke significant damage to the blood

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cell content of the tubes. At the speed commonly used to produce L-PRF (2700 rpm, or 3400 rpm in the case of Salvin), all tested centrifuges (except Intra-Spin) are largely above this threshold of 1 for resonance, and it is now necessary to evaluate in details the effects of these vibrations on the cell content and fibrin architecture of each L-PRF clot produced with these machines **[9]**.

The A-PRF centrifuge (Advanced Platelet-Rich Fibrin) is an interesting case, as it was suggested to be used with a very low speed (1300 rpm) in order to produce a PRF-like clot called A-PRF. This is actually a quite rare approach, as a too low speed does not allow a good separation of the blood components and the activation of leukocytes. Moreover, the vibrations of this machine at this low speed are already above the threshold of 1 marking the theoretical limit of resonance. In theory, the best configuration for L-PRF would be to have a reasonable speed (around 2700 rpm) for adequate blood separation, and no vibration or resonance to protect the cell content **[9]**, as the adequate collection of the leukocyte appeared as an importance parameter for the clinical effect of these technologies **[32,33]**. Therefore, A-PRF could serve as an interesting example to compare with the original L-PRF, and to illustrate the impact of speed and vibrations on the final aspect and content of a PRF clot.

Finally, this study raises very serious concerns about the whole PRP literature. PRP centrifuges are in general a bit larger and heavier than PRF centrifuges, and should therefore in theory present a lower risk of vibrations (they seem often to vibrate less when a hand is placed on it). However, the centrifuges tested in this study are also used to produce PRP through another protocol, and the inadequate conception of larger centrifuges can lead to the same risk of vibrations integrated to the level of resonance, whatever their size and weight. Moreover, many PRP methods are also using much higher centrifugation speeds and sometimes g forces **[2,3]** than the L-PRF method (often considered to be a very soft method), as they are supposed to make a very sharp separation of the blood components. For all these reasons, it would be interesting to evaluate more seriously the vibrations of all centrifuges available for PRP on the market, in order to evaluate if this has an impact on the final cell content of the PRP and its biological effect.

5. Conclusion

This article is the first study analyzing the intrinsic differences between 4 PRP/PRF centrifuges available on the market. Using the centrifuges in the same conditions and at the same rotational speed, large discrepancy in vibration levels appeared from one machine to another, and 3 of them reach quickly a threshold of resonance. We can therefore extrapolate that if the protocols were identical, the only variable was the vibration characteristics of the different centrifuges. By far, the most stable machine tested is the original machine (now termed Intra-Spin) used since the early development of the L-PRF technology. At the classical speed of production of L-PRF, the level of undesirable vibration on this centrifuge is between 4.5 and 6 times lower than with other centrifuges. Moreover, Intra-Spin always remains under the threshold of resonance, unlike the 3 other tested machines. It is now necessary to evaluate the impact of the vibration parameter on the architecture and cell content of the L-PRF clots produced with these 4 different machines.

Disclosure of interests

The authors have no conflict of interest to report.

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Author Contributions

All authors participated to the technical design and organization of the study, the treatment of data and to the elaboration of the manuscript. DDE, NP, BSK and MDC were in charge of the collection of the materials and raw data.

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Research article

The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors and fibrin architecture of a Leukocyte- and Platelet-Rich Fibrin (L-PRF) clot and membrane. Part 2: macroscopic, photonic microscopy and Scanning Electron Microscopy analysis of 4 kinds of L-PRF clots and membranes

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Abstract

Background and Objectives. Platelet concentrates for surgical use (Platelet-Rich Plasma PRP or Platelet-rich fibrin PRF) are surgical adjuvants to improve healing and promote tissue regeneration. L-PRF (Leukocyte- and Platelet-Rich Fibrin) is one of the 4 families of platelet concentrates for surgical use and is widely used in oral and maxillofacial regenerative therapies. The objective of this second article was to evaluate the impact of the centrifuge characteristics (vibration intensity) on the cell and fibrin architecture of a L-PRF clot and membrane.

Materials and Methods. Four different commercially available centrifuges were used to produce L-PRF, following the original L-PRF production method widely described in the literature (glass-coated plastic tubes, 400g force, 12 minutes). The tested systems were the original L-PRF centrifuge (Intra-Spin, Intra-Lock, the only CE and FDA cleared system for the preparation of L-PRF) and 3 other laboratory centrifuges (not CE/FDA cleared for L-PRF): A-PRF 12 (Advanced PRF, Process), LW - UPD8 (LW Scientific) and Salvin 1310 (Salvin Dental). All clots and membranes were collected into a sterile adequate surgical box (Xpression kit). The exact macroscopic (weights, sizes) and microscopic (photonic and scanning electron microscopy SEM) characteristics and the cell composition of the L-PRF clots and membranes produced with these 4 different machines with 4 different vibration intensity levels were evaluated.

Results. Intra-Spin showed the lowest temperature of the tubes. A-PRF and Salvin were both associated with a significant increase of temperature in the tube. Intra-Spin produced

by far the heaviest clot and quantity of exudate among the 4 techniques. For clot and membrane length and width, Intra-Spin and Salvin presented similar sizes. A-PRF and LW produced much lighter, shorter and narrower clots and membranes than the 2 other centrifuges. Light microscopy analysis showed relatively similar features for all L-PRF types (concentration of cell bodies in the first half of the fibrin mesh). However, SEM illustrated considerable differences between samples. The original Intra-Spin L-PRF showed a strongly polymerized thick fibrin matrix and all cells appeared alive with a normal shape, including the textured surface aspect of activated lymphocytes. The A-PRF, Salvin and LW PRF-like membranes presented a lightly polymerized slim fibrin gel and all the visible cell bodies appeared destroyed (squashed or shrunk).

Discussion and Conclusion. This study illustrated that the centrifuge characteristics (particularly the vibrations) are directly impacting the architecture and cell content of a L-PRF clot. The original L-PRF clot (Intra-Spin) used and validated since years presented very specific characteristics, which appeared distorted when using centrifuges with a higher vibration level. A-PRF, LW and Salvin centrifuges produced PRF-like materials with a damaged and almost destroyed cell population through the standard 400g protocol developed initially for the L-PRF, and it is therefore impossible to classify these products in the L-PRF family. A-PRF, LW and Salvin centrifuges are not suitable for the production of original L-PRF clots and membranes at 400g. Further research would be interesting to evaluate how modifications of the protocol alone (for example reduction of the g forces) may influence the biological signature of the L-PRF clots and membranes, independently from the characteristics of the centrifuge.

Keywords. Blood platelets, growth factors, leukocytes, platelet-rich plasma, regenerative medicine, wound healing.

1. Introduction

Leukocyte- and Platelet-Rich Fibrin (L-PRF) is one of the 4 main families of platelet concentrates for surgical use **[1-3]**. L-PRF is frequently used in oral and maxillofacial surgery as a surgical adjuvant to improve healing and promote tissue regeneration **[4-13]**. The L-PRF technology is very simple and inexpensive (particularly in comparison to the many kinds of Platelet-Rich Plasma PRP available on the market) and the method was developed as an open-access system **[14]**: blood sample is taken in 9ml tubes without anticoagulant and immediately centrifuged at 2700 rpm during 12 minutes. At the end of the process, a large L-PRF clot can be collected in the middle of each tube. This clot can be used directly to fill a cavity **[15,16]** or mixed with a bone material **[4]**, or compressed into a membrane **[10]** or a fibrin cylinder **[17]** using the adequate surgical box designed to prepare it without damage (marketed with CE/FDA clearance as Xpression kit, Intra-Lock, Boca-Raton, FL, USA)**[18]**.

The L-PRF clot or membrane contains most of the platelets and half of the leukocytes present in the initial blood harvest **[19]**. Platelets are mostly activated and serve as a cement to reinforce the strongly polymerized fibrin matrix **[19]**. Leukocytes (a majority of lymphocytes) are trapped within this fibrin network, but are still alive and ready to move in culture **[20]**. The platelet growth factors are trapped within the fibrin network **[21]**. With this architecture, L-PRF is the source of a strong and slow release of growth factors during more than 7 days in vitro **[22,23]**, through the release of the platelet growth factors trapped within the fibrin gel or through the production of new molecules by the leukocytes of the clot **[21]**. In vitro, the L-PRF membranes have strong effects on the stimulation of the proliferation of most cell types (fibroblasts, keratinocytes, pre-adipocytes, osteoblasts, bone

mesenchymal stem cells)**[20,24]** and on the differentiation of the bone cells **[20]**. This result was explained by the growth factors and cell content of the L-PRF **[20]**. Finally, through its specific natural architecture combining a wide cell population (mostly leukocytes), large quantities of mediators (particularly platelet growth factors) into a strong natural fibrin matrix, L-PRF was considered as a tissue and was often described as an optimized natural blood clot **[19]**. This specific architecture in itself may explain most of the positive characteristics of this material **[25-27]**.

The original L-PRF was developed as an open-access protocol, but the material and method were tailored with a lot of care in order to reach the best possible clot and result **[19]**. The protocol was tailored by using a high-quality table centrifuge, specific glass-coated plastic tubes and a specific protocol (12 minutes, 2700 RPM). The relevant literature on the L-PRF was produced using this adequate material since more than 10 years. The original open-access method and associated devices used since the early phases of the development of this technique are nowadays marketed with CE/FDA clearance as the Intra-Spin L-PRF system and kit (Intra-Lock, Boca-Raton, FL, USA). This inexpensive system is actually the only L-PRF system available on the market with all adequate certifications and using the original protocol and devices.

With the development worldwide of this open-access method, many variations of the original method appeared, using different centrifuges (often cheaper models) and/or different protocols. The situation is starting to be confusing as all variations of the materials and methods clearly do not offer the same material than the original L-PRF **[18,28-30]**. Differences between the original L-PRF and various PRF-like materials are obvious and easily observable (for example the size and weight of the clots and membranes), but this simple truth is often not understood because of ignorance and the confusions created by commercial statements and marketing claims **[31]**. The specific fibrin architecture and cell and growth factors contents of the L-PRF are key characteristics of an original L-PRF clot/membrane as characterized in the literature **[19]**, and any modification of the material and protocol can lead to a different biological signature and clinical result **[18]**.

The objective of this series of 3 articles was to point out the impact of the centrifuge characteristics and centrifugation protocol on the cell, growth factors and fibrin architecture of a L-PRF clot and membrane. In the first article, the mechanical vibrations (both radial and vertical) appearing during centrifugation were evaluated in 4 models of commercially available table centrifuges frequently used to produce L-PRF. It was proven that the original L-PRF centrifuge (Intra-Spin) was by far the most stable machine. At the classical speed of production of L-PRF, the level of undesirable vibration on this centrifuge is between 4.5 and 6 times lower than with other centrifuges. Moreover, Intra-Spin always remains under the threshold of resonance, unlike the 3 other tested machines. In this second article, the exact macroscopic and microscopic (photonic and scanning electron microscopy) characteristics and the cell composition of the L-PRF clots and membranes produced with these 4 different machines were evaluated. As a secondary objective, the impact of the vibration parameter on the architecture and cell content of the L-PRF clots was discussed.

2. Materials and methods

The study was conducted in accordance with the Helsinki Declaration (2000) and approved by the Medical Ethics Committee of the University of the Andes (UANDES). All volunteers provided signed informed consent.

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2.1. Description of the tested centrifuges

In this study, 4 different centrifuges, found on the market and used to produce L-PRF, were tested. The country of manufacture being used by some companies as a claim for quality, the country of manufacture of each centrifuge and its main components was checked. The 4 selected centrifuges were purchased from their manufacturers (or distributors).

The first centrifuge was the original centrifuge used during the early development of the L-PRF open-access method and is nowadays marketed under the name Intra-Spin L-PRF centrifuge (Intra-Lock International, Boca-Raton, FL, USA; Made in Germany). It is actually the only CE marked and FDA cleared system for the preparation of L-PRF clots.

The 3 other centrifuges are not CE/FDA cleared for L-PRF, but they can be found relatively frequently available on the market for this use (mostly because they are much cheaper): centrifuge A-PRF 12 (Advanced PRF, Process for PRF, Nice, France; Country of manufacture not indicated on the label, components inside show “Made in China”), centrifuge LW - UPD8 (LW Scientific, Lawrenceville, GA, USA; Components made in China, assembled in the USA) and centrifuge Salvin 1310 (Salvin Dental Specialties, Charlotte, NC, USA; Made in China).

2.2. Preparation of L-PRF

Blood samples were collected at the San Bernardo University of the Andes Health Center from 8 healthy volunteers (age range 25-35 years, ASA 1), with no history of recent aspirin intake or any medication neither disease correlated with the coagulation process. For each volunteer, nine tubes of blood were obtained from the antecubital vein. One tube with 2,5ml of anticoagulant was used for whole blood analysis as a control for normal blood parameters. Eight plastic glass-coated tubes were taken without anticoagulant (with BD Vacutainer Serum 10.0ml tubes, Becton Dickinson, Franklin Lakes, NJ, USA) for the production of L-PRF clots and membranes.

The blood was collected quickly (22 seconds mean value, less 25 seconds per tube) and immediately (before 1 minute) centrifuged at 400g during 12 minutes in the four different centrifuges (two tubes were distributed per centrifuge in a randomized way) at room temperature. To standardize exactly the protocol and isolate only the centrifuge vibration parameter, the 400g centrifugation force used in the original L-PRF method (corresponding to 2700 rpm in the original Intra-Spin centrifuge) was used with all centrifuges, and rpm were adjusted accordingly for each centrifuge, i.e. 2400 rpm for the A-PRF machine and 2300 rpm for the LW centrifuge. Salvin centrifuge has only one preset possible speed (3400 rpm), which lead to a centrifugation force higher than 400g. The temperatures of the surface at the center of the tubes were registered before and after centrifugation with an infrared thermometer (HVACPro, Fluke, Everett, WA, USA).

A total of 64 L-PRF clots/membranes were obtained: 32 membranes were prepared for Scanning Electron Microscopy (SEM) analysis and 32 membranes were prepared for light/photonic microscopy.

2.3. Macroscopic analysis

After centrifugation the L-PRF clot was removed from the tube using sterile tweezers and a smooth spatula to gently release the red blood cells clot inside the tube (**Figure 1A**). The L-PRF fibrin clot obtained was placed on a sterile microscope slide (**Figures 1B, 1C**) placed in an individual tray for weight and size measurements (**Figure 2**). The supernatant

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and red blood cells clot remaining in the tube were also weighted to get the L-PRF fibrin clot / whole blood ratio per tube. Each sterile microscope slide had in every corner a 1mm rubber stop (**Figure 1C)** to allow the compression of the clot with another microscope slide using 100 grams constant pressure for two minutes. This standardized method allowed to obtain from each clot 1mm-thick L-PRF membranes, which were weighted and measured individually (**Figure 3**).

From each volunteer, two membranes were obtained per each centrifuge and after macro analysis (weight, size measurements) were prepared for histologic procedures. One membrane was prepared for SEM evaluation and the second one for light-microscopy analysis. The membranes were kept between the microscope slides during fixation to avoid distortions.

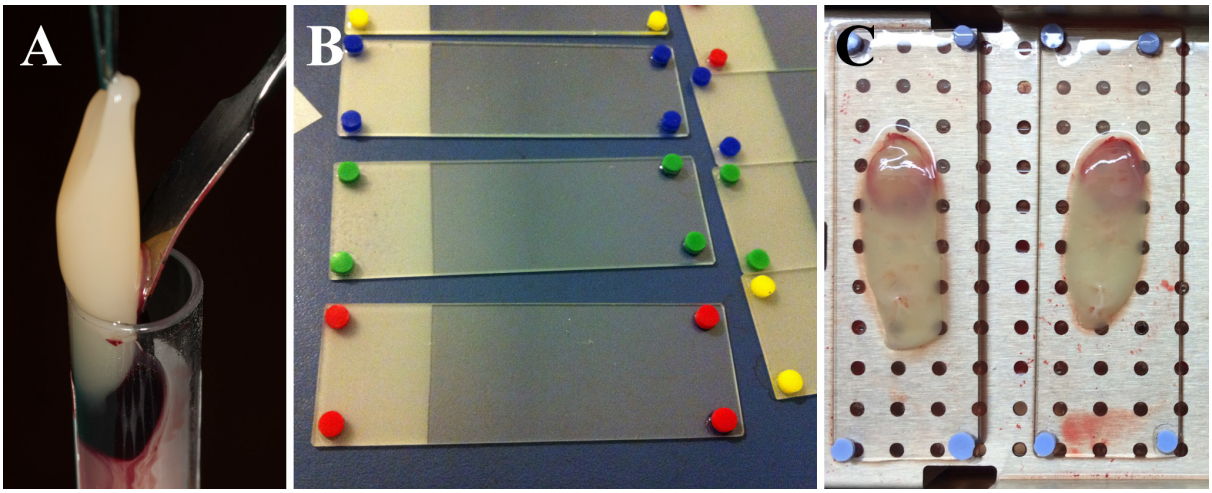

Figure 1. Material for PRF clot handling. L-PRF clots were collected in each tube, and the red blood cell part was gently removed with a smooth instrument and a light lateral pressure **(A)**. Standard glass histological slides were use to support the clots during the macroscopic evaluation **(B)**. Rubber stops were placed on each corner of the slides, in order to perform a standardized compression of all the samples into membranes between 2 glass slides **(C)**. The same procedure was applied for all clots produced during this study, even if the handling was often more difficult with the A-PRF, LW and Salvin products.

2.4. Light microscopy procedure

The membranes were fixed in 10% neutrally buffered formalin for 24 hours at room temperature for paraffin inclusion. Successive sections of 4 microns were performed along the center of the long axis of the membranes and were stained with hematoxylin-eosin. Each section was divided in three areas of equal size: Proximal (Head & Face), Center (Body), Distal (Tail). Each area of these sections was observed through light microscopy and analyzed by counting the visible cell bodies (marked in dark purple, mostly leukocytes) in the center of each area observed with a 40X magnification. The total numbers of counted cell bodies were used to correlate their distribution among the three areas of the membrane (head & face, body and tail). Most of the cells were concentrated in the proximal area (head & face).

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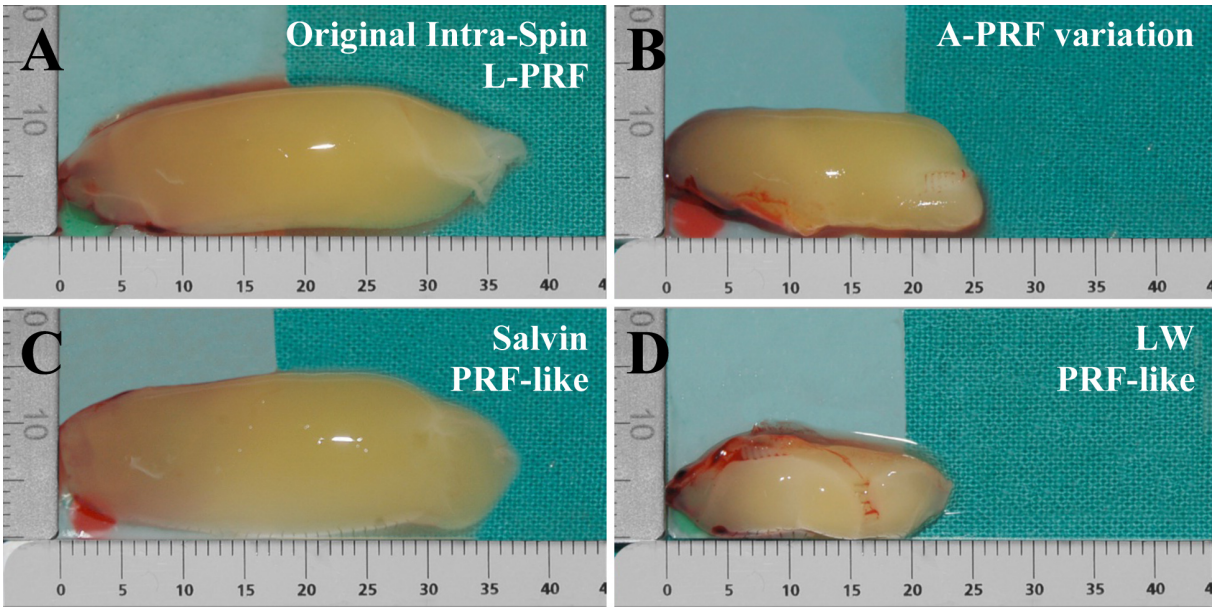

Figure 2. Macroscopic evaluation of the PRF clots produced with the 4 different centrifuges: original Intra-Spin L-PRF system **(A)**, A-PRF system **(B)**, Salvin centrifuge **(C)** and LW centrifuge **(D)**. Obvious differences can be observed in terms of size and aspect, the original L-PRF **(A)** being always denser and heavier (and in most cases larger) than the others.

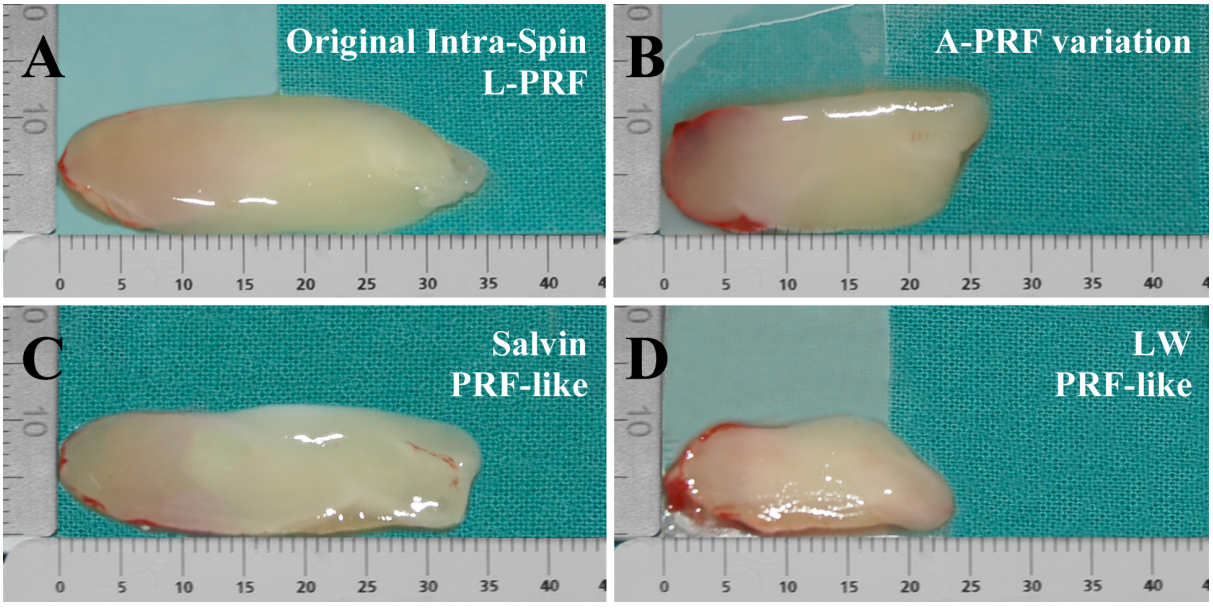

Figure 3. Macroscopic evaluation of the PRF membranes produced with the 4 different centrifuges: original Intra-Spin L-PRF system **(A)**, A-PRF system **(B)**, Salvin centrifuge **(C)** and LW centrifuge **(D)**. Obvious differences can be observed in terms of size and aspect, the original L-PRF **(A)** being always denser and in most cases larger than the others.

2.5. Scanning Electron Microscopy (SEM) Procedure

A morphologic evaluation of the L-PRF membranes was done with a scanning electron microscope. The membranes were fixed in 2.5% glutaraldehyde for 24 hours at 4°C and treated for gradual desiccation. The specimens were sputter-coated with 20nm gold

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(Edwards S-150, Crawley, UK) and examined in a scanning electron microscope (JEOL JSM-6380LV, JEOL Ltd, Tokyo, Japan). Photographs were taken with 15 to 20kV using 2,000 to 5,000X magnifications. This study was mainly descriptive.

3. Results

3.1. Macroscopic analysis

All the macroscopic results are presented in the **Table**. The numeric values are clearly illustrated by the observation of the clots and membranes in the **Figures 2 and 3**.

For the temperature of the tubes, Intra-Spin allowed to keep the lowest temperature among the 4 tested machines. A-PRF and Salvin were both associated with a significant increase of temperature in the tube.

For the clot and exudate weights, Intra-Spin produced by far the heaviest clot and quantity of exudate among the 4 techniques. Salvin remains high but far behind. Finally A-PRF and LW produced very light and small clots. For the membranes weights, Intra-Spin and Salvin presented similar weight. The A-PRF and LW membranes were significantly lighter.

In terms of clot and membrane length and width, the clots and membranes from Intra-Spin and Salvin presented similar sizes. The A-PRF and LW clots and membranes were significantly shorter and more narrow.

Finally, the Intra-Spin L-PRF clot was the heaviest clot to be produced with an initial blood harvest of 9ml.

	IntraSpin	A-PRF	Salvin	LW
Variable	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Final T° of Tube (°C)	27.5 (0.66)	28.83 (0.67)	28.8 (0.66)	27.88 (0.57)
Clot Weight (g)	2.09 (0.19)	1.38 (0.24)	1.73 (0.27)	0.74 (0.15)
Membrane Weight (g)	0.62 (0.15)	0.48 (0.17)	0.6 (0.19)	0.3 (0.25)
Exudate Weight (g)	1.47 (0.13)	0.9 (0.21)	1.12 (0.27)	0.44 (0.26)
Clot Length (mm)	35.69 (3.43)	26.56 (4.25)	35.25 (4.1)	20.12 (4.29)
Clot Width (mm)	12.81 (0.75)	10.93 (1.08)	13.06 (0.94)	9.12 (1.13)
Membrane Length (mm)	34.81 (2.95)	26.81 (3.38)	34.43 (2.87)	21.5 (2.39)
Membrane Width (mm)	12.25 (0.71)	10.37 (0.92)	11.93 (0.78)	9.12 (0.64)
Weight ratio(%) Clot/Blood sample 10ml	20.94 (2.4)	13.98 (2.6)	17.42 (2.63)	7.41 (1.45)

Table. Results of the macroscopic analysis of the clots and membranes produced with the 4 tested centrifuges. Values expressed in Mean and Standard Deviation (SD).

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3.2. Light microscopy analysis

In light microscopy (**Figure 4**), most cell bodies (stained in dark purple for the nuclei) were concentrated in the proximal (head-face) area of each membrane: with Intra-Spin, A-PRF and Salvin, the 3/4 of the cell bodies were observed in the proximal area, the last 1/4 was observed in the center; the distal part had only residual traces of cell bodies. With LW, the cell bodies appeared more spread all over the membrane (40% proximal, 48% center and 12% distal), as the clot and membranes were particularly small and shrunk. Light microscopy did not allow to observe in more details the exact state of these cell bodies.

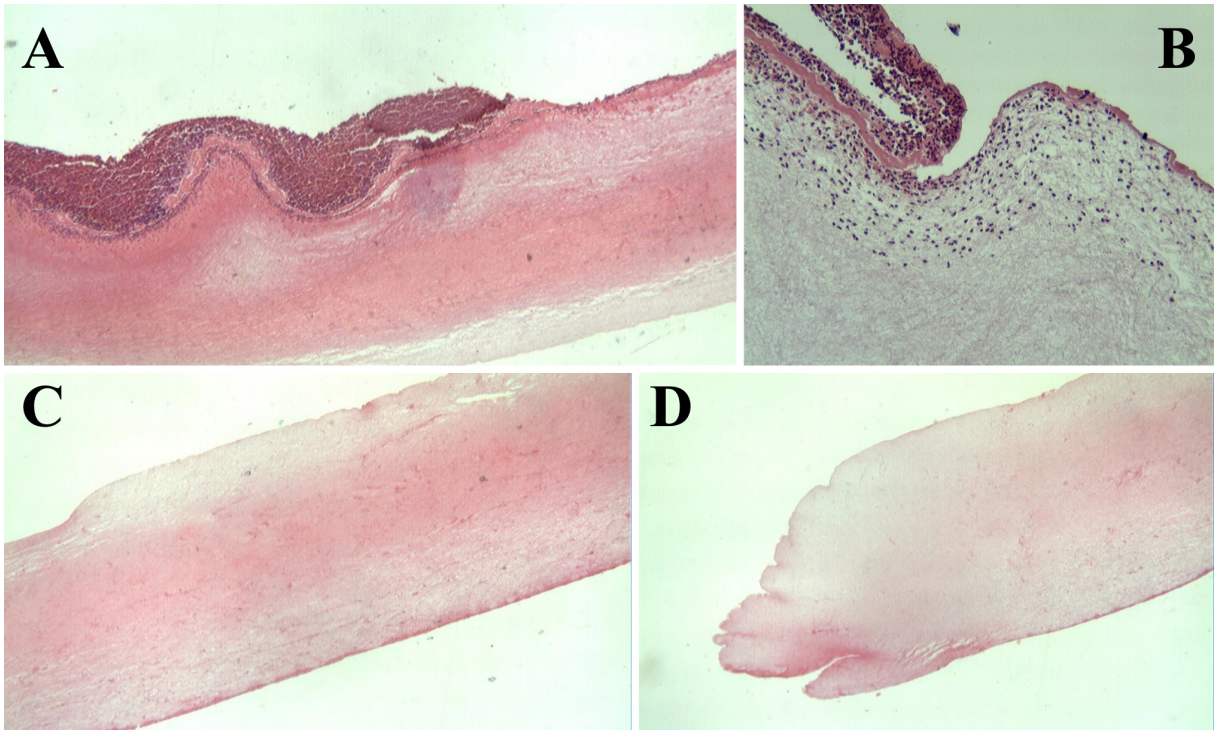

Figure 4. Microscopic evaluation of the PRF membranes produced with the 4 different centrifuges in light microscopy (hematoxylin eosin). The different membranes showed similar organization in light microscopy, with a concentration of most visible cell bodies (75%) in the first 1/3 proximal part of the membrane (**A**, x2; **B**, x80), the remaining in the central 1/3 part (**C**, x2) and only residual bodies in the last 1/3 distal part (**D**, x2). Illustration obtained here from an original L-PRF membrane (Intra-Spin). The LW PRF-like membrane was the only one with a different distribution, mostly due to the strong shrinking of the membrane.

3.3. SEM analysis

The SEM analysis allowed to evaluate in details the aspect of the fibrin network and of the cell content of each membrane (**Figures 5 and 6**).

The original L-PRF produced through Intra-Spin presented a strongly polymerized fibrin matrix with thick fibrin fibers. Moreover, all observed cells appeared alive with a normal shape. Lymphocytes presented typical textured surface aspect observed in activated lymphocytes. This observation corresponds to the exact characterization of an original L-PRF clot done in previous works, and can serve as a standard to evaluate the 3 other types of L-PRF produced in this study

The A-PRF, Salvin and LW PRF-like membranes presented a lightly polymerized fibrin gel with slim fibrin fibers, clearly very different from the original L-PRF. Moreover, all the visible cell bodies appeared squashed or shrunk. No cell body with a normal cell shape or even an activated cell shape could be detected. It was considered that the whole cell population was completely damaged and almost destroyed.

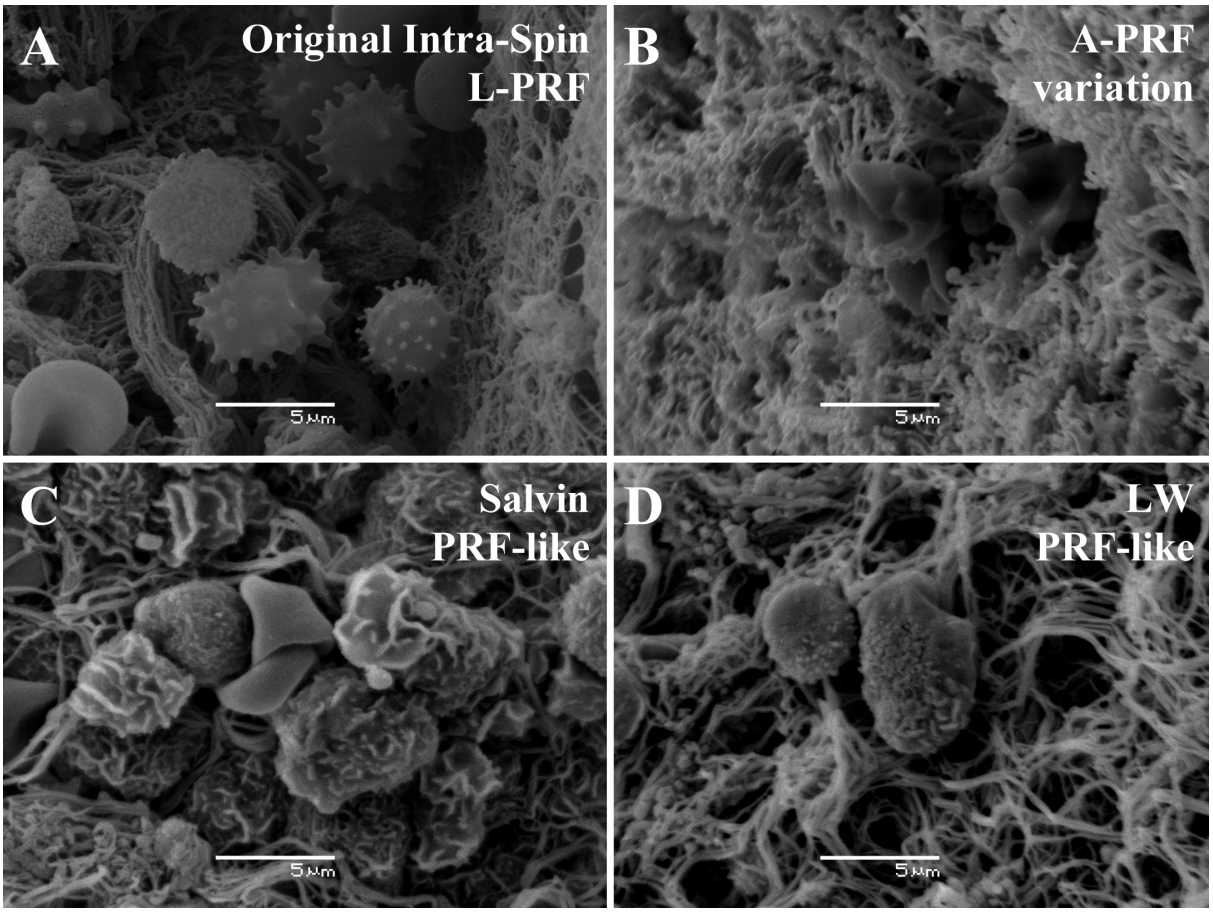

Figure 5. SEM Microscopic evaluation of the PRF membranes produced with the 4 different centrifuges. The different membranes showed very different aspects during SEM analysis. The original L-PRF membrane (Intra-Spin, **A**) presented a strongly polymerized fibrin network and the presence of a large living cell population appearing in good shape. The PRF-like membranes produced with the A-PRF **(B)**, Salvin **(C)** and LW **(D)**, all presented a slimmer and more disorganized fibrin network, and all cells appearing severely damaged, shrunk or squashed.

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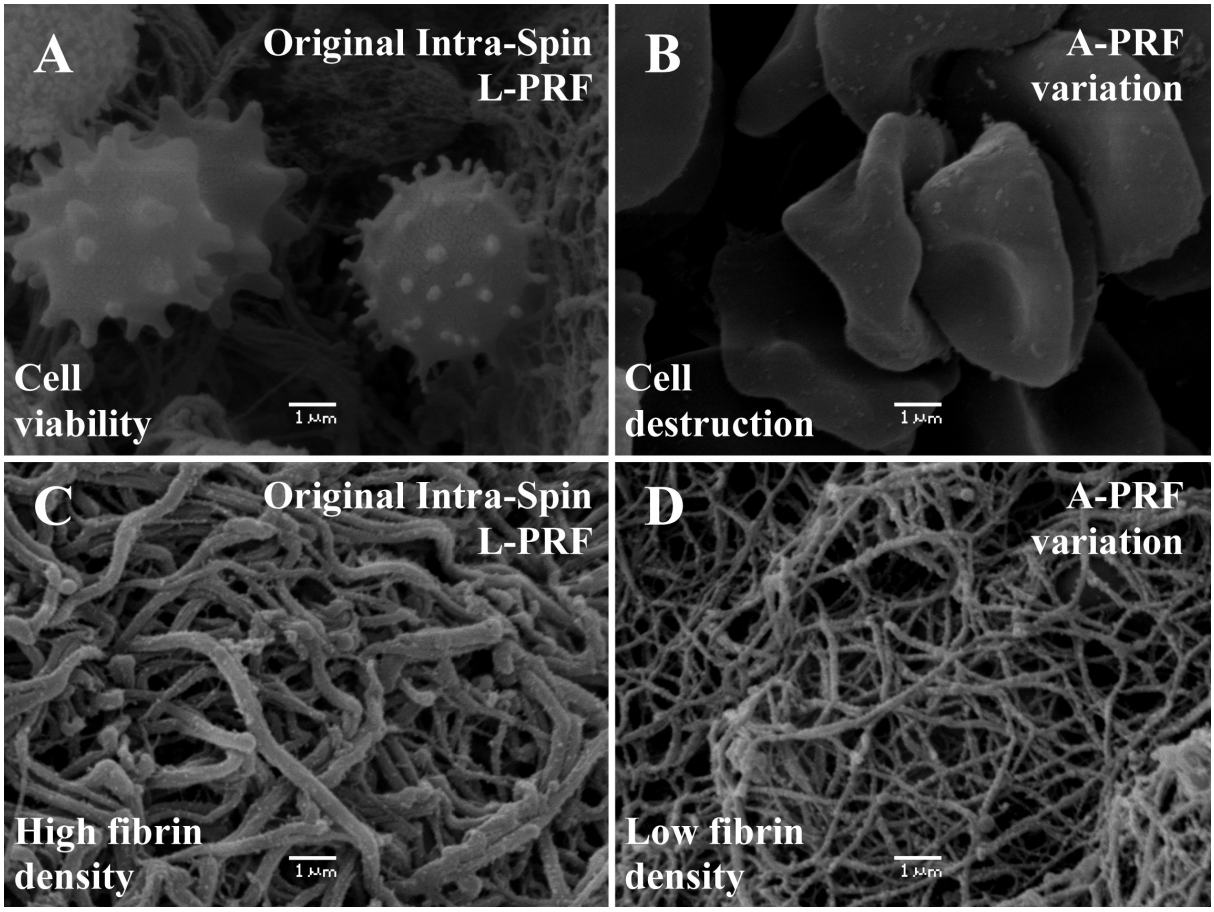

Figure 6. SEM Microscopic evaluation and comparison of the PRF membranes produced with 2 different centrifuges. The original Intra-Spin L-PRF membranes **(A, C)** presented a large cell population **(A)**, and all observed cells appeared alive with a normal shape. Lymphocytes presented typical textured surface aspect observed in activated lymphocytes. Moreover, the fibrin matrix appeared strongly polymerized with thick fibrin fibers **(C).** On the contrary, in the A-PRF membranes **(B, D),** all the visible cell bodies appeared squashed or shrunk **(B)**, and the fibrin gel presented a lightly polymerized fibrin matrix with slim fibrin fibers **(D)**.

4. Discussion

4.1. Impact of the vibrations on the fibrin polymerization and cell content

The original L-PRF materials and protocols were carefully selected in order to reach the best possible result. The development was not empirical, but based on a significant feedback of observation and experience. In the literature, much confusion started to appear **[18,28-30]**, as many studies did not use the same hardware (centrifuge and tubes) and did not get the same product, even if the protocols appeared identical (same g force and centrifugation time).

In this study, we tried to highlight how the centrifuge characteristics may impact the L-PRF architecture and composition. Blood collection materials, tubes and protocol were strictly identical. The centrifugation parameters were also standardized (same g force than the original L-PRF protocol, calculated to fit each machine, and same centrifugation time). Therefore the only difference between the 4 products was the hardware (the centrifuge). After

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blood centrifugation in the 4 different centrifuges was completed, L-PRF clots were observed to be not identical in terms of weight, volume, fibrin architecture and cell content.

Having verified that the g forces were almost identical in the 4 centrifuges, the hypothesis was that mechanical vibrations may be responsible of differences between the final products. This vibration variable appeared as the main (and most logical) parameter to evaluate. It was proven in the first part of this series of articles that the vibration levels (both radial and vertical) were very different between the commercially available centrifuges used for L-PRF. As vibrations level was the only variable between the 4 products, it is therefore possible to associate the differences of L-PRF weight, volume, fibrin architecture and cell content between the 4 systems to this level of vibrations, even if other parameters may be considered in the future.

In this study, all membranes were produced using a 400g centrifugation force. This corresponds to a 2700 rpm with the original L-PRF centrifuge (Intra-Spin), resulting in a parasite acceleration (vibration) level of 0.75 $m.s^{-2}$ (see article 1), so far under the threshold of 1. For the 3 other centrifuges, the rpm speed used to stay in the 400g centrifugation forces were all associated with a vibration level much higher than 1: 2.2 $m.s^{-2}$ (LW), 3 $m.s^{-2}$ (A-PRF) and 4.5 $m.s^{-2}$ (Salvin). It is interesting to point out that the PRF-like products created with these 3 machines had all in common the damage or destruction of the cell content. This observation reinforces logically the theory that there is an integrated mathematical threshold for resonance located around 1 $m.s^{-2}$ in parasite acceleration, and that this limit should be avoided as much as possible to avoid the destruction of the cell content within the tube. The triggering moment for a resonance phenomenon within the tubes, that could damage the cell content and damage the fibrin organization, is anyway clearly located in this range of vibrations.

4.2. Without cells, A-PRF, Salvin and LW are in fact not L-PRF

The wide and diverse cell content living within the strong fibrin matrix is one of the most important characteristics of a L-PRF clot **[25,27]**. It was clearly pointed out in vitro through various cell studies where the significant tissue engineering results obtained with L-PRF were clearly connected to the slow release of growth factors **[21]**, direct contact induction of the fibrin and the interactions of cells in coculture with leukocytes **[20,24]**. The presence of activated cells is also what make the L-PRF to be considered as a real tissue, that can be used in tissue engineering approach (what was termed leukocyte-driven tissue engineering)**[19]**. Moreover, the biological signature of the L-PRF presented a strong slow release of growth factors **[22]**, and it was shown that this release was probably even increased by a mediator production from leukocytes **[21]**. Therefore, the damage or destruction of all cells within a L-PRF clot raises very significant concerns about its biological and clinical potential **[25]**.

Finally, in case all cells are not destroyed but only damaged, it raises even deeper concerns as damaged cells are releasing per definition many pro-inflammatory mediators. While L-PRF activated and preserved cell content was considered clinically to regulate the inflammatory process, it is impossible to know the effects of a damaged cell population, and it is anyway difficult to claim a necrotic cell population as a positive characteristic.

These observations of the cell content in fact allow to claim that the PRF-like products obtained with the A-PRF, LW and Salvin machines can not be classified in the L-PRF family **[1]**. Without preserved cell content, they are more likely to be classified as a kind of Pure

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Platelet-Rich Fibrin (P-PRF), therefore from the same family than the Fibrinet PRF matrix for example **[2]**. In all cases, the literature about L-PRF cannot be applied to the products created with the A-PRF, LW and Salvin devices, and this should be clear for all readers to avoid more confusion in the scientific literature. A-PRF, LW and Salvin centrifuges are not suitable for the production of original L-PRF clots and membranes.

Finally this result opens a considerable debate about the way PRPs and PRFs have been produced and tested since years, as it is the first time that it is proven that the quality of the hardware is directly impacting the architecture and composition of the platelet concentrates, and therefore also their own definition, type, biological and clinical characteristics. This observation may point out a major flaw in a large quantity of the publications in this field **[32,33]**.

5. Conclusion

This study definitively illustrated and clarified what is exactly a L-PRF clot or membrane, and that the centrifuge characteristics (particularly the vibrations) are directly impacting the architecture and cell content of a L-PRF clot. The original L-PRF clot (Intra-Spin) used and validated since years presented very specific characteristics, which appeared completely distorted when using centrifuges with a higher vibration level. A-PRF, LW and Salvin centrifuges produced PRF-like materials with a damaged and almost destroyed cell population through the standard 400g protocol developed initially for the L-PRF, and it is therefore impossible to classify these products in the L-PRF family. A-PRF, LW and Salvin centrifuges are not suitable for the production of original L-PRF clots and membranes at 400g. To conclude this series of studies, it would be interesting to evaluate how the changes of the protocol (for example reduction of the g forces) alone may influence the biological signature of the L-PRF clots and membranes, independently from the characteristics of the centrifuge.

Disclosure of interests

The authors have no conflict of interest to report.

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Author Contributions

All authors participated to the technical design and organization of the study, the treatment of data and to the elaboration of the manuscript. NP, AP, PJ and MQ were in charge of the collection of samples and raw data.

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Research article

The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors and fibrin architecture of a Leukocyte- and Platelet-Rich Fibrin (L-PRF) clot and membrane. Part 3: comparison of the growth factors content and slow release between the original L-PRF and the modified A-PRF (Advanced Platelet-Rich Fibrin) membranes

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Abstract

Background and Objectives. L-PRF (Leukocyte- and Platelet-Rich Fibrin) is one of the 4 families of platelet concentrates for surgical use and is widely used in oral and maxillofacial regenerative therapies. The objective of this third article was to evaluate how the changes of the L-PRF protocol may influence its biological signature, independently from the characteristics of the centrifuge.

Materials and Methods. In each volunteer donor, veinous blood was taken in 2 groups, respectively Intra-Spin 9ml glass-coated plastic tubes (Intra-Lock, Boca-Raton, FL, USA) and A-PRF 10ml glass tubes (Process, Nice, France). Tubes were immediately centrifuged at 2700 rpm (around 400g) during 12 minutes to produce L-PRF clots, or at 1500 rpm during 14 minutes to produce A-PRF clots. All centrifugations were done using the original L-PRF centrifuge (Intra-Spin system, Intra-Lock), as recommended by the 2 manufacturers. All clots were collected into a sterile surgical box (Xpression kit) and compressed into membranes. Half of the membranes were placed individually in culture media and transferred in a new tube at 7 experimental times: 20 minutes, 1 hour, 4h, 24h, 72h, 120h and 168h. The releases of Transforming Growth Factor β-1 (TGFβ-1), Platelet Derived Growth Factor AB (PDGF-AB), Vascular Endothelial Growth Factor (VEGF) and Bone Morphogenetic Protein 2 (BMP-2) were quantified using ELISA kits at these 7 experimental times. The remaining membranes were used to evaluate the initial quantity of growth factors of the L-PRF and A-PRF membranes, through forcible extraction.

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Results. The slow release of the 3 tested growth factors (TGFβ-1, PDGF-AB and VEGF) from original L-PRF membranes was significantly much stronger (more than twice stronger, $p<0.001$) at all experimental times than the release from A-PRF membranes. No trace of BMP2 could be detected in the A-PRF membrane. A slow release of BMP2 was detected during at least 7 days in the original L-PRF. Moreover, the original L-PRF clots and membranes (produced with 9mL blood) were always significantly larger than the A-PRF clots and membranes (produced with 10mL blood). The A-PRF membranes dissolved in vitro after less than 3 days, while the L-PRF membrane remained in good shape during at least 7 days.

Discussion and Conclusion. The cumulative curves are defining the biological signatures of the tested product. The original L-PRF signature is always more than twice stronger than the A-PRF signature. The same centrifuge was used for both products in this study; only the protocol (particularly the centrifugation forces) was different. The original L-PRF protocol allowed producing larger clots and membranes and a more intense release of growth factors than the modified A-PRF protocol. The exact impact of the tubes should also be investigated in the future. Both protocols are therefore very significantly different, and the clinical and experimental results from the original L-PRF shall not be extrapolated to the A-PRF. Finally, the comparison between the total released amounts and the initial content of the membrane (after forcible extraction) highlighted that the leukocytes living in the fibrin matrix are involved in the production of significant amounts of growth factors.

Keywords. Blood platelets, growth factors, leukocytes, platelet-rich plasma, regenerative medicine, wound healing.

1. Introduction

Leukocyte- and Platelet-Rich Fibrin (L-PRF) is one of the 4 main families of platelet concentrates for surgical use **[1-4]**, and it is frequently used in oral and maxillofacial surgery as a surgical adjuvant to improve healing and promote tissue regeneration**[5-12]**. The original L-PRF protocol was open-access, very simple and inexpensive **[1]**: blood is taken in 9ml tubes without anticoagulant and immediately centrifuged at 2700 rpm during 12 minutes. The blood coagulation during centrifugation allows the formation of the L-PRF clot in the middle of each tube **[13]**. The L-PRF clot can be then used directly **[14,15]** or compressed into a membrane **[16,17]** or a cylinder plug **[18,19]**, depending on the expected application. The original system is nowadays marketed in only one form as a CE and FDA cleared inexpensive material under the name Intra-Spin L-PRF (Intra-Lock, Boca-Raton, FL, USA). In the first description of the architecture and properties of this regenerative material, it was shown that the L-PRF clot or membrane contains most platelets and half of the leukocytes from the initial blood harvest, with an increased ratio of lymphocytes **[13]**. Because of this specific cell composition within a strong fibrin network, the L-PRF acronym was justified and L-PRF started to be described as a real autologous tissue and an optimized blood clot **[5]**.

The release of growth factors is one of the main objectives justifying the use of platelet concentrates in regenerative medicine **[20-22]**, even if other parameters shall not be neglected (matrix and cell content)**[23-25]**. In previous works, it was shown that the L-PRF membrane presents in vitro during at least 7 days a very intense slow release of many growth factors (particularly the growth factors released by the platelets) and related molecules **[18,26,27]**, all involved in the acceleration and control of healing and tissue regeneration: Transforming Growth Factor β1 (TGFβ1), Platelet-Derived Growth Factor AB (PDGF-AB), Vascular Endothelial Growth Factor (VEGF), Thrombospondin-1 (TSP-1), Fibronectin and

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Vitronectin. This intensity and pattern of release were compared with other forms of platelet concentrates (particularly with some Platelet-Rich Plasma gel)**[27]**, and it was claimed that this growth factors slow release profile can be considered as a biological signature of each platelet concentrate gel as a regenerative healing biomaterial **[27,28]**. From these studies, it was shown than the natural combination of growth factors, platelets and adequate fibrin network was the key to obtain a strongly sustained release of these factors. It was also advocated that the presence of cells in L-PRF was participating to some supplementary growth factors production **[26]**.

With the development of this method worldwide, some authors tried to prepare L-PRF using different centrifuges or even protocols. The protocols, even when they may appear often identical (same g force and time) did not use the same hardware (centrifuge and tubes). Using different centrifuges and kits, L-PRF clots were not identical in terms of weight and volume. The main reason of these changes of hardware was economic, the main idea behind was to use a cheaper centrifuge and to try to get an acceptable result with it. Unfortunately, these changes of materials and/or methods are now a source of considerable confusion and bias in the literature **[29-31]**, as these changes are affecting considerably the architecture and cell content of the L-PRF material **[18]**.

The original L-PRF was developed as an open-access protocol, but the material was initially selected carefully, using some high-quality very stable centrifuge in order to reach the best possible clot and result. In the first 2 parts of this series of articles, we demonstrated that 3 low-cost laboratory centrifuges (A-PRF, Salvin, LW) frequently encountered for the production of L-PRF (even if not CE/FDA cleared for this application) presented in fact a much higher level of vibrations than the original L-PRF system (Intra-Spin). These vibrations were directly responsible of the perturbation of the fibrin architecture and the critical damage of the cell content of the L-PRF clots and membranes. Moreover, size and weights of the clots and membranes were very different with these centrifuges in comparison to the original L-PRF. It was concluded that A-PRF, LW and Salvin centrifuges were not suitable for the production of original L-PRF clots and membranes through the standard 400g protocol developed initially for the L-PRF.

To conclude logically this series of studies, the objective of this third article was to evaluate how the changes of the protocol alone (for example reduction of the g forces) may influence the biological signature of the L-PRF clots and membranes, independently from the characteristics of the centrifuge. To reach this objective, the slow release of some growth factors from an original L-PRF membrane was compared with the slow release of an A-PRF (Advanced Platelet-Rich Fibrin) membrane, as both products can be prepared using the original L-PRF machine.

2. Materials and methods

2.1. Preparation of L-PRF and A-PRF

For the production of L-PRF and A-PRF clots and membranes, blood collection was carried out on six volunteer donors, 3 males and 3 females, non-smokers, aged between 30 and 40 years old, with no history of recent aspirin intake or any medication neither disease correlated with the coagulation process. For each volunteer, eight tubes of blood were obtained without anticoagulant from the antecubital vein, respectively 4 Intra-Spin 9ml glass-coated plastic tubes (Intra-Lock International Inc., Boca-Raton, FL, USA) and 4 A-PRF 10ml glass tubes (Process, Nice, France).

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The blood was collected quickly (17 seconds mean value, less than 20 seconds per tube) and immediately (before 1 minute) centrifuged at room temperature at 2700 rpm (around 400g) during 12 minutes to produce L-PRF clots, or at 1500 rpm during 14 minutes to produce A-PRF clots. All centrifugations were done using the original L-PRF centrifuge (Intra-Spin system, Intra-Lock, Boca Raton, FL, USA), as recommended by the manufacturers of both A-PRF (Process) and Intra-Spin L-PRF (Intra-Lock). The A-PRF was initially developed on the original centrifuge (Intra-Spin), before to become an independent technique with its own centrifuge. The use of the same centrifuge allowed to neutralize the parameter related to the quality of the centrifuge, as it was shown in the first part of this research that the Intra-Spin centrifuge was by far the most stable machine and the only one without significant vibrations.

Four Intra-Spin L-PRF clots were produced for each donor: 2 were used to quantify the release of molecules during the experiment, and 2 were used for immediate extraction by force and quantification. Four Process A-PRF clots were produced for each donor: 2 were used to quantify the release of molecules during the experiment, and 2 were used for immediate extraction by force and quantification. The clots were finally collected carefully into a sterile adapted surgical box (Xpression kit, Intra-Lock, Boca-Raton, FL, USA) and compressed into membranes for the next step of the study.

2.2. Sample preparation

In the release quantification group, each L-PRF or A-PRF clot was gently pressed into a membrane, and placed in a 10 mL tube with 4 mL of sterile DMEM (Dulbecco's Modified Eagle's Medium). Then, at each experimental time, the membrane was transferred in a new tube of 4 mL sterile DMEM, and the previous 4 mL were stored at -80°C before ELISA quantification. The membrane transfer was done at 7 experimental times: 20 minutes, 1 hour, 4 hours, 24 hours (day 1), 72 hours (day 3), 120 hours (day 5) and 168 hours (day 7). This procedure was done separately for the 4 membranes (2 L-PRF, 2 A-PRF) of each donor, thus 24 membranes were separately treated, in order to calculate means and standard deviations.

In the group for immediate extraction by force, each L-PRF or A-PRF membrane was cut in small pieces and homogenized in 1 mL sterile DMEM using a Polytron extraction-dispersing machine (Polytron, Kinematica AG, Lucerne, Switzerland). Then, a final centrifugation (15000rpm during 10 minutes) was performed in order to remove residual particulates. About 1 mL of solution was then collected and stored at -80°C before ELISA quantification. This procedure was done separately for the 4 membranes (2 L-PRF, 2 A-PRF) of each donor, thus 24 membranes were separately treated, in order to calculate means and standard deviations.

2.3. ELISA quantification and data collection

When all the samples were collected, quantifications of 4 molecules were performed by using classically available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA): Transforming Growth Factor β-1 (TGFβ-1), Platelet Derived Growth Factor AB (PDGF-AB), Vascular Endothelial Growth Factor (VEGF) and Bone Morphogenetic Protein 2 (BMP-2). Absorbances were read using a microplate reader ELISA X500, then concentrations were calculated. For intra- and inter-study comparisons, all the results were finally referred to a 1 mL volume, and then expressed as total weight of molecules (nanograms for TGFβ-1 and

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PDGF-AB, picograms for VEGF, picograms for BMP2). For each molecule and each experimental period, means and standard deviations were calculated. Differences at each time between L-PRF and A-PRF data were assessed using a paired t-test ($p<0.01$).

Finally, for each tested molecule, the total released amounts were calculated and these results were then compared to the initial amount forcibly extracted from the membrane soon after L-PRF and A-PRF preparation. The ratio between the total released quantity and the initial extracted quantity was calculated.

3. Results

As a first macroscopic observation, the original L-PRF clots and membranes produced with 9ml blood were always much larger than the A-PRF clots and membranes produced with 10ml blood (**Figure 1**). The difference was not calculated, but it appeared systematically that the L-PRF were at least 30% bigger than the A-PRF clots and membranes.

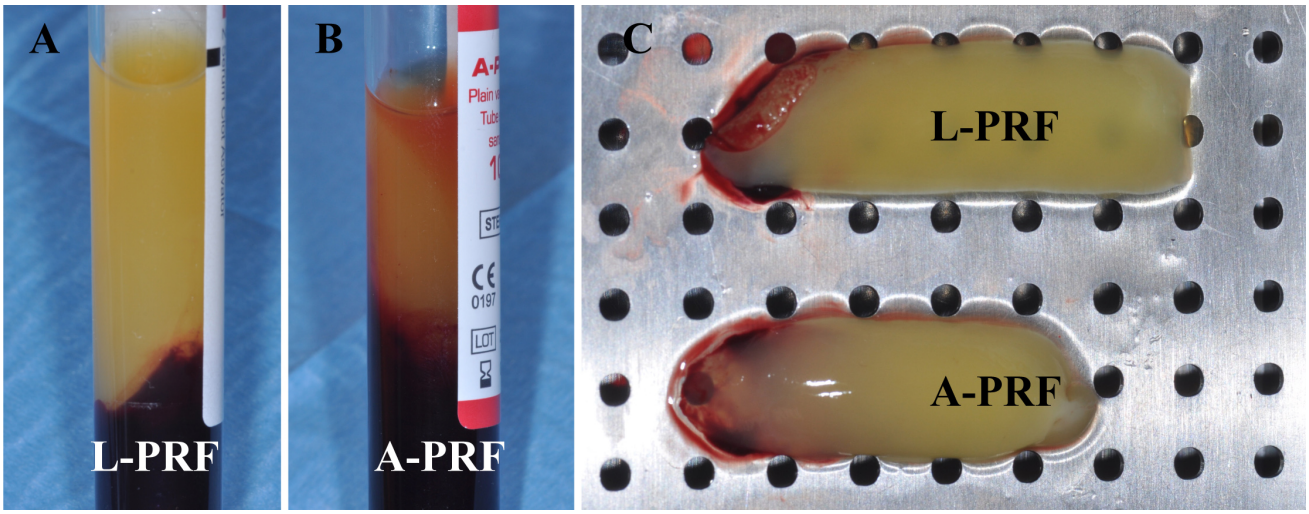

Figure 1. Original Intra-Spin L-PRF **(A)** and A-PRF **(B)** clots in their respective official tubes just after centrifugation. The aspect of the 2 kinds of clots in the tube was since the beginning very different. Clots were then collected and placed in the PRF surgical box (Xpression, Intra-Lock) for compression into membranes **(C)** to be used for the slow release test. The original Intra-Spin L-PRF membrane was produced with the original L-PRF protocol (2700rpm, 12 minutes) and 9mL blood. The A-PRF membrane was produced with the specific A-PRF protocol (1500rpm, 14 minutes) and 10mL blood. However, in comparison to the original L-PRF membrane, the A-PRF clots and membranes appeared obviously much smaller, more fragile and not so clearly separated from the red blood cell part.

During the test, the original L-PRF membrane remained in good shape up to the last experimental time (7 days), while the A-PRF membrane completely dissolved in the medium between the first and the third day. For this reason, the last A-PRF value was measured at the day 3 experimental time. Significant amounts of TGFβ-1, PDGF-AB and VEGF were found at each experimental times, even 7 days after production with the original L-PRF membrane and up to 3 days with the A-PRF membrane (**Figure 2**). These amounts of molecules presented a specific slow release kinetic. TGFβ-1, PDGF-AB and VEGF releases showed similar general profiles, characterized by a quick increase of the release during the first 24

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hours. For original L-PRF, a significant but slower release until day 5 (120 h) was observed; during the last 2 days of the experiment, membranes continued to release significant amounts of these molecules, but very slowly. For the A-PRF, the release also slowed down after the first day, but the release stopped quickly thereafter with the complete dissolution of the A-PRF membrane.

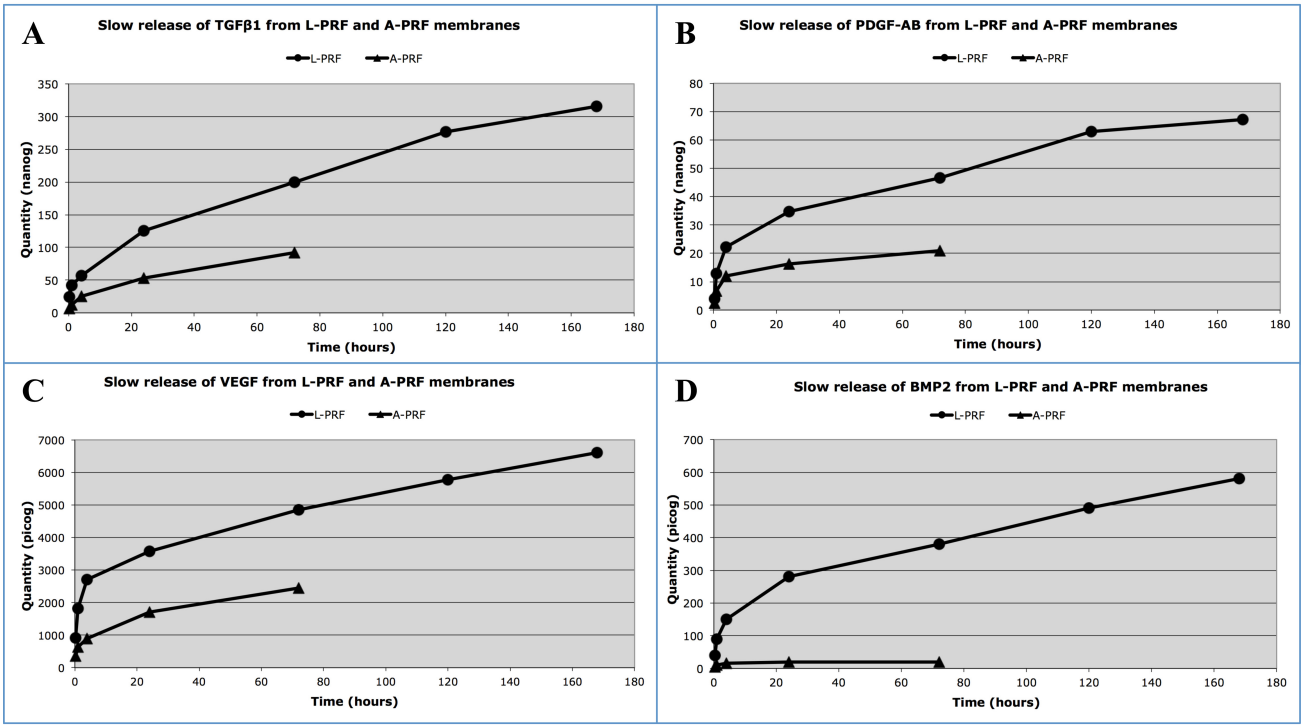

Figure 2. Slow release of TGFβ1 **(A)**, PDGF-AB **(B)**, VEGF **(C)** and BMP2 **(D)** from an original L-PRF membrane and from an A-PRF membrane during 7 days in vitro. Values are expressed as the cumulative mean quantity of molecules at 20 minutes, 1 hours, 4 hours, 24 hours, 72 hours (3 days), 120 hours (5 days) and 168 hours (7 days).

The slow release of TGFβ-1, PDGF-AB and VEGF from an original L-PRF membrane (Intra-Spin) was always significantly much stronger ($p<0.001$) at all experimental times than the release from an A-PRF membrane. All results were presented as graphs (**Figure 2**) to follow the cumulative released mean amounts of each molecule during the first 168 hours after L-PRF and A-PRF membrane preparation respectively. The gradient of the curves revealed the force of the slow release during the experimental periods. These curves were defining the biological signatures of the original L-PRF membrane produced with Intra-Spin system and of the A-PRF membrane. The original L-PRF signature was always more than twice stronger than the A-PRF signature.

For the evaluation of BMP2, no traces of BMP2 could be detected in the A-PRF membrane, and the values represented in the **Figure 2** are considered as the unavoidable experimental background noise. On the contrary, a slow release of BMP2 was clearly detected during at least 7 days in the original L-PRF, even if the quantities remained quite small.

For the original L-PRF, the total quantity of released factors was in all cases significantly higher than the total amounts extracted just after membrane preparation (**Table**). However the ratios between these values (total slow release/initial quantity) were

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very different according to the molecule: TGFβ-1, VEGF and BMP2 following the same high ratio (around 7), while PDGF-AB ratio was much closer to 1.

For the A-PRF, the total quantity of released factors and the total amounts extracted just after membrane preparation were significantly smaller than for the L-PRF membrane (**Table**). In A-PRF, the ratios between these values (total slow release/initial quantity) were also very different according to the molecule: TGFβ-1 and VEGF following the same high ratio (around 4.5 or 5, lower than for L-PRF), while PDGF-AB ratio was much closer to 1 (similar to L-PRF).

Tested molecule		**TGFβ-1 (nanog)**	**VEGF (picog)**	**PDGF-AB (nanog)**	**BMP-2 (picog)**
Total released after 168h (sum of the amounts measured at each experimental time)	L-PRF	315.5 (±21.1)	6602 (±704)	67.1 (±9.8)	580 (±73)
	A-PRF	92.1 (±25.4)	2445 (±782)	20.9 (±8.1)	NA
Total extracted at To from the membrane	L-PRF	44.4 (±3.7)	994 (±159)	44.4 (±4.2)	79 (±10)
	A-PRF	18.6 (±4.8)	514 (±188)	15 (±5.8)	NA
Ratio between slow released and extracted molecules	L-PRF	7.1	6.64	1.51	7.34
	A-PRF	4.95	4.76	1.39	NA

Table. Comparison between the total released quantity at the end of the experimental time (after 168 hours) and the initial extracted quantity of each tested molecule in an original L-PRF membrane and in an A-PRF membrane. Results are expressed as means and standard deviations.

4. Discussion

This study compared accurately the biological signatures of 2 kinds of L-PRF materials, the original L-PRF (Intra-Spin) and the modified protocol A-PRF. A-PRF is in fact a variation of the original L-PRF using a much lower centrifugation speed, a slightly longer centrifugation time, and glass tubes **[32]**. This technique was initially proposed on the original L-PRF centrifuge (using 1500 rpm) before it was definitively associated with the specific A-PRF centrifuge (using 1300 rpm) tested in the 2 previous parts of this series of articles. It was therefore a perfect model to compare the impact of the change of protocol alone on the biological signature of a PRF membrane, as both original L-PRF and A-PRF can be produced using the exact same centrifuge.

The main observation of this experiment was that A-PRF clots showed a much lower release of growth factors and a weaker biological signature than the original L-PRF. Moreover, the A-PRF clots dissolved quickly in the tubes, while the original L-PRF remained in good shape even after 7 days in vitro. The second observation was that all A-PRF clots and membranes (produced with 10ml blood) were at least 30% smaller than the original L-PRF clots and membranes (produced with 9ml blood). It was previously proven in this series of articles that the vibrations of the A-PRF centrifuge were leading to the formation of an even much smaller clot and membrane. In this study, the same stable original centrifuge (Intra-

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Spin) was used to produce both L-PRF and A-PRF clots to neutralize the centrifuge vibrations variable, and the main difference that could explain these differences of size of the clots and of biological signature of membranes was the change of the protocol, mostly the forces of centrifugation, but also the proprietary type of tubes and the time of centrifugation.

In a previous work, it was shown that the production of L-PRF clots did not seem to be affected by the use of glass tubes or glass coated plastic tubes **[13]**, therefore the differences of tubes between A-PRF and L-PRF may not explain the observed differences. However, this shall be confirmed in future research, as there are many kinds of glass tubes and glass coated plastic tubes. The Intra-Spin tubes were selected very specifically following the long L-PRF experience to fulfill CE and FDA clearance, while nothing is known about the source of the A-PRF tubes (except they are for in vitro diagnostic and made in China). This difference may have an impact in the results and should be investigated. Moreover, it was shown that there is very little impact in using longer centrifugation time with an original L-PRF, as it is common to centrifuge during 18 minutes when patients are under anticoagulant treatment **[19]**. The increase of centrifugation time mostly gives a bit more time for a fibrin clot to polymerize. It was not needed to last longer than 12 minutes for the original L-PRF in most cases, but A-PRF seems to need this supplementary time to finish its gel polymerization (14 minutes in total).

As a conclusion, these differences of size, aspect and biological signature of the clots and membranes between the original L-PRF and A-PRF can be probably associated with the change in the centrifugation forces. It confirms the need for using forces around 400g (2700 rpm in the original centrifuge), in order to do a proper separation of the blood constituents with an adequate gradient of centrifugation and collect a large and proper L-PRF clot. The use of a lower g force and speed (1500 rpm for A-PRF) did not seem enough for a proper separation of the blood constituents and lead to the preparation of a clot (A-PRF) of much smaller size, weaker biological signature and lower fibrin polymerization, even when the tubes were larger (10ml) and if an adequate stable centrifuge was used.

In previous publications, it was advocated that the cell population of a L-PRF membrane was responsible of the production of new growth factors **[26]**. Indeed the total release quantities after 7 days of many growth factors were always much higher than the total quantities detected after forcible extraction from the whole membrane just after preparation **[26]**. In this new study, the same observation can be done for L-PRF, and the result obtained with A-PRF somehow confirmed it. The comparison of the results between L-PRF and A-PRF also highlighted that cells must be placed in a specific environment to massively produce more molecules. TGFβ-1, VEGF and BMP2 presented the same high ratio (around 7 for L-PRF) between total slow release / initial quantity, revealing somehow the activity of production of these molecules by the cells within the clots. The lower ratios of A-PRF revealed also a lower production activity. On the contrary, PDGF-AB ratio was quite stable around 1 for both products, as this molecule is mostly contained and released by platelets initially collected in the sample. Therefore this study also confirmed the need to protect the viability of the cells and even pointed out the need for an activation of the cells by the centrifugation process. This notion of activation is the most logical explanation for the very strong differences of biological signatures between original L-PRF and A-PRF, particularly the interesting result with BMP2.

BMP-2 is an important osteoinductive molecule belonging to the TGF-β superfamily of proteins and playing particularly an important function in bone development. For this reason, recombinant forms of this molecule were marketed in a few countries for the

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treatment of bony defects in orthopaedic and maxillofacial surgery, with mixed or controversial results **[33]** related to the difficult control of the effects of this molecule in a direct therapeutical approach. The release of small quantities of BMP2 from the original L-PRF probably contributed in some way to the stimulation of bone cell proliferation and differentiation observed in vitro by the L-PRF **[34,35]** and to the positive clinical effects of L-PRF during bone regeneration **[14]**. However, its importance in the global equation of the L-PRF (combining, many cells, many growth factors into a specific fibrin matrix) is impossible to point out at this time **[23,27,34]**.

It is interesting to notice that BMP2 was not detected with A-PRF (detected as a noise, probably under the detection threshold of 29 pg/mL), while the company marketing it used the release of BMP2 as a commercial argument for the A-PRF protocol. The exact origin of the BMP2 detected in the L-PRF is difficult to point out, as BMP2 is a molecule specific to bone cells, the ELISA kit itself was designed mostly for bone tissue extracts and bone cell culture supernatants. Small quantities of BMP2 can be detected in the blood in some conditions **[36]**, but its overexpression blood is often associated with various pathologies **[37,38]**. As BMP2 is not supposed to be released by platelets, consequently the different cell populations (mostly leukocytes) living in the L-PRF clot released BMP2. As BMP2 levels were quite low after forcible extraction from the initial L-PRF clot, consequently the L-PRF cells released and produced this molecule step by step during the experiment. The combination of these observations in both L-PRF and A-PRF supported the conclusion that the quantity and state of the cell population within the L-PRF clot defines a large part of its biological signature.

Finally, the evaluation of the slow release of key growth factors from a PRP gel or a L-PRF membrane appeared again as a quite simple method of characterization of the biological signature of an activated platelet concentrate gel. It was already used in several publications **[27,28]**, and it illustrated quite well the differences of growth factor content, cell content and fibrin architecture of different products. The slow release pattern is so characteristic from a product, that it should be evaluated systematically in all kinds of products to define their exact biological signature prior to compare them or analyze their clinical effects.

5. Conclusion

The slow release of the 4 tested growth factors from original L-PRF membranes was much stronger than the release from A-PRF membranes. Moreover, the original L-PRF clots and membranes (produced with 9mL blood) were always significantly larger than the A-PRF clots and membranes (produced with 10mL blood). The A-PRF membranes dissolved in vitro after less than 3 days, while the L-PRF membrane remained in good shape during at least 7 days. The same centrifuge was used for both products in this study; only the protocol (particularly the centrifugation forces) was different between the original L-PRF and the A-PRF. Consequently, it can be concluded that the original L-PRF protocol allows to produce larger clots and membranes and a more intense release of growth factors than the modified A-PRF protocol. The exact impact of the tubes should also be investigated in the future. As a general conclusion for this series of articles, it was clearly proven that the centrifuge characteristics and centrifugation protocols have a very significant impact on the cell, growth factors and fibrin architecture of a L-PRF clot and membrane, and that any modification of the original L-PRF material and method shall be clearly investigated and identified separately from the original methods, in order to avoid to create confusion and inaccurate results in the literature.

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Disclosure of interests

The authors have no conflict of interest to report.

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Author Contributions

All authors participated to the technical design and organization of the study, the treatment of data and to the elaboration of the manuscript. DDE, NP, MDC and BSK were in charge of the collection of the materials, samples and raw data.

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