PRF Applications in Endodontics

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I dedicate this book to my wife, Parvin, and my children, Sara and Ali, with love.

–MS

I would like to thank my mentors, Dr Ronald J. Nicholson and Dr Chutima Mangkornkarn for inspiring me to become a better clinician and person, and to Dr Calvin Tae Nam for sharing his knowledge about PRF.

–ESL

To the soul of my dear father whom we lost to cancer early in his and our lives.

–MT

Contents

Foreword

The use of platelet-rich fibrin (PRF) has seen a rapid increase over the past decade, owing to its ability to rapidly release autologous growth factors harvested quite easily from peripheral blood. While original case studies dating back nearly two decades focused primarily on its use in medicine for the treatment of hard-to-heal wound ulcers, it is now well known that its inclusion of high concentrations of platelets and leukocytes has served several benefits in dentistry. Specifically, platelets are largely responsible for the release of various regenerative growth factors favoring wound healing, whereas leukocytes (white blood cells) participate in host defense against incoming pathogens. The ability to concentrate both cell types found within PRF has shown pronounced benefits in the oral cavity, an area particularly concentrated with various oral bacteria.

Over the years, several research articles focused on the use of PRF for multiple applications in regenerative dentistry; more recently, publications have begun to emerge dealing specifically with its use in endodontics. I have the great pleasure to announce the launch of this new book, *PRF Applications in Endodontics*, which addresses this topic in extensive detail.

The book begins by providing background knowledge on various cell types found in regenerative medicine with particular focus on stem cells. Thereafter, the book rapidly enters into a variety of chapters dedicated to PRF with a brief history regarding its scientific background, including growth factors, armamentarium, and protocols utilized to fabricate PRF. The discussion then transitions to nonsurgical applications in regenerative endodontics, as well as its use in dentistry, particularly for the formation of a bone grafting material complex including bone grafting particles and autologous PRF (aka "sticky bone"). Its use as an alternative to bone grafts and other biomaterials is further discussed in later chapters dealing with endodontic surgery. These include various endodontic procedures indicated following common human "accidents" (accidental tooth loss and replantation, for instance), for improvements in furcation-involved teeth as a result of iatrogenic procedures, for root-end resection procedures, and for the management of surgical cysts.

This textbook is for both the beginner as well as the advanced endodontist and practicing dentist working in the field of endodontics wishing to further improve their practice by adopting some of the latest regenerative protocols. It is certainly a first of its kind and a must-read in the field of endodontics, highlighting the benefits of autologous blood concentrates specifically dedicated to endodontic procedures.

Colleagues will certainly enjoy this read, and it will undoubtedly open many avenues of future research on the topic!

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Preface

As endodontists and periodontists, we are all familiar with the potential applications of platelet-rich plasma (PRP) in medicine and dentistry. But in the past few years, platelet-rich fibrin (PRF) has emerged as an alternative material in its own right. One of our first opportunities to observe the effects of PRF was in discussion with colleagues using it in oral surgery procedures. Their patients experienced remarkable hard and soft tissue healing with minimal postoperative discomfort. Intrigued, we dug further and discovered the widespread applications of PRF in dentistry and medicine.

The appeal of PRF stems from the fact that it is made from a patient's own blood. It is easy to prepare and can be used for many kinds of procedures, making it cost-effective. PRF has many potential applications in endodontics. It can be used in surgical endodontics and adjunctive surgical procedures such as root amputation and hemisection. In addition, it can be used for root perforation repair, vital pulp therapy, and regenerative endodontics. Furthermore, it can be used as a bone graft binder during socket preservation to create "sticky bone" for the closure of surgical sites.

When the three of us first met, the idea of sharing these various applications of PRF was an immediate common ground. We were working with residents at the time and knew how much they could benefit from learning about PRF. After using PRF and observing successful outcomes in several cases, we decided to take things to the next level. We brought together some of the most forward-thinking endodontists, periodontists, oral surgeons, and general practitioners to share our thoughts regarding potential use of this material in endodontics and other fields of dentistry.

This book, representing a collaboration of like-minded clinicians, is the first to introduce the idea of PRF and cord blood stem cells in endodontics. It contains an overview of PRF itself with up-to-date information on tissue regeneration, as well as step-by-step instructions on how to use PRF in a variety of endodontic and oral surgery procedures. We have been using this knowledge for years to improve tissue healing for our patients, and we hope this book will help you on your quest to improve healing for your patients.

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Introduction

Recent studies using novel biomaterial scaffolds that contain host endogenous growth factors represent a departure from traditional clinical approaches and may result in better and more predictable regenerative solutions in medicine and dentistry. As early as 1966, Rule and Winter published a case report regarding continued root formation and apical closure in an immature human premolar tooth using pulp bleeding as a scaffold. Nygaard-Ostby et al, Nevins et al, Iwaya et al, Banchs and Trope, as well as others reported pulp revascularization in teeth with necrotic pulps and immature apices that showed continuous root maturation, dentinal wall thickening and, in some cases, a positive response to vitality tests. In 2011, we reported a case of pulp revascularization using platelet-rich plasma (PRP) in a second maxillary premolar with immature root that had been accidently extracted and then replanted. After removing the necrotic pulp, irrigating it with 5.25% sodium hypochlorite, and medicating it with a triple antibiotic paste for 3 weeks, we prepared PRP from the patient's blood and injected it into the canal space. Mineral trioxide aggregate (MTA) was placed over the clotted PRP and double-sealed with Cavit (3M) and amalgam. Radiographic examination of this tooth 5.5 months later showed resolution of the periapical lesion, further root development, and continued apical closure. Vitality tests elicited positive responses like those found in the first premolar tooth. The shortcomings of PRP include the need to draw blood from the patient and the complexity of centrifugtion and purification in a clinical setting.

Platelet-rich fibrin (PRF) is an autologous product that contains high concentrations of nonactivated, functional intact platelets within a fibrin matrix that release a relatively constant concentration of growth factors/cytokines over a few days. It is easier to produce but it has to be used immediately after blood drawing and centrifugation. PRF is a potential substitute for PRP in regenerative endodontics and other regenerative procedures involving reconstruction of hard tissues, such as surgical endodontics and adjunctive surgical procedures like root amputation, hemisection, and repair of root perforations.

The main purpose of *PRF Applications in Endodontics* is to stimulate research in regenerative procedures in endodontics and encourage clinicians to use PRF to improve healing of their patients and save natural dentition. The book has seven chapters and starts with the history of stem cells in regenerative medicine and its possible applications in endodontics, followed by PRF armamentarium and description of how to make PRF, use of PRF in nonsurgical endodontic procedures, its soft tissue applications, hard tissue applications, surgical endodontics, and finally socket preservation. It is assembled by well-known scientists and clinicians who are experts in their fields and interested in the use of innovative materials and techniques to improve human lives.

Mahmoud Torabinejad, DMD, MSD, PhD

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LEARNING OBJECTIVES

- Gain a better understanding of stem cell biology and how it relates to regenerative medicine, specifically dentistry
- Compare different sources of stem cells and the relative strengths and weaknesses associated with each source
- Gain a better understanding of a mesenchymal stem cell secretome and why it is important therapeutically

Stem Cells in Regenerative Medicine

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Regenerative medicine, also commonly known as *tissue engineering*, is a discipline of medicine that is focused on restoring native tissue structure and functionality to an afflicted tissue. Dentistry has traditionally been at the forefront of regenerative medicine, commonly employing novel bioactive materials to stimulate bone growth and regeneration. Recently, stem cells and other cell-based therapies have attracted significant attention in this space due to their ability to not only treat patients' symptoms but to improve physiologic activity and restore native tissue structure.

Stem cells are characterized by a capacity for self-renewal while maintaining an undifferentiated state and, given the proper stimulus, the ability to differentiate into various types of specialized somatic cells. Stem cells are further classified by their relative differentiation potential. Stem cells that can differentiate into any cell type in the body are termed *totipotent* and have the widest differentiation potential. *Mesenchymal stem cells* (MSCs) are multipotent stem cells that are most closely associated with the mesodermal lineage and are known to differentiate into chondrogenic, osteogenic, myogenic, and adipogenic cell types.[1]

The discovery of stem cells and their multipotent potential has encouraged the development of the whole field of research, projected to have reached $170 billion by 2020. In particular, the multipotent MSCs, with their stem-like quality to differentiate into mesodermal cell types, have been a focus. Indeed, overall revenue for MSC products was projected to be $10.9 billion from 2010 to 2020. Alongside the possibilities of therapeutic successes (ranging from treating graft-versus-host disease, Crohn disease, spinal cord injury, and use in support of hematopoietic stem cell treatments) comes the inherent ethical and logistic dilemmas behind obtaining stem cells. This chapter focuses on MSCs due to their popularity for regenerative applications.

Mesenchymal Stem Cells

Stem cell sources

First isolated in bone marrow, bone marrow–derived MSCs were found to be precursors to multiple cell types and could be viably cultured while retaining their capacity for multilineage differentiation. Obtained from an invasive bone marrow harvesting procedure, bone marrow–derived MSCs avoid the ethical concerns as well as tumorigenicity of embryonic stem cells and have subsequently been used in a nearly exponential increase in research studies and trials.[2] Unfortunately, bone marrow–derived MSCs are relatively low yield and limited to autologous use, requiring in vitro expansion that increases the risk of contamination. Additionally, harvesting the cells requires a surgical procedure with associated donor morbidity and risk, and the potency (ie, "stemness") has been questioned when compared with more recently discovered sources of MSCs.[3]

One of these sources is umbilical cord blood (also known as *cord blood*), collected via venipuncture of the typically discarded umbilical cord. Painless and without morbidity, cord blood is considered superior to human bone marrow stem cells in its harvesting and yield. Cord blood is cryopreserved in two main methods using dimethyl sulfoxide (DMSO): (*1*) red cell reduction, which is less expensive to store and easier to defrost; and (*2*) plasma depletion, which is more economical to process. Public cord blood banks cost about $1,500 to $2,500 per unit stored, while private banks typically charge an initial processing fee of $1,400 to $2,300 plus annual storage costs of $115 to $150. However, MSCs only represent a small proportion—1,000 to 5,000 MSCs in one 100-mL unit of cord blood—of the cell types within cord blood, which includes hematopoietic cell types, endothelial and progenitor cells, as well as MSCs.[4]

There has also been recent attention toward Wharton's jelly (WJ) within umbilical cords. WJ was found at the turn of the century to contain a multipotent, fibroblast-like MSC population with greater multipotent potency, faster proliferation, and longer life spans than adult bone marrow–derived MSCs.[3] This is a result of reduced telomere length. Telomeres shorten with age, eventually resulting in cellular senescence. MSCs isolated from cord blood are much younger than adult MSCs and possess significantly longer telomeres.[5]

MSCs in WJ are an entity apart from cord blood MSCs and endothelial cells from the umbilical vein. The plentiful presence of MSCs in WJ is theorized to either be due to the trapping and retaining of fetal MSCs during the two waves of migration of fetal MSCs in early development or to the fact that the cells in WJ are actually primitive MSCs that originate from mesenchyme already present in the umbilical cord matrix. More research has been focused on not only the characterization and usage of these WJ-derived MSCs, but also on discrete differences of the stem cell populations depending on the anatomical region of the WJ.[6]

The most recent development is that what was once thought to be a single mass providing uniform MSCs is actually more anatomically distinct. There are six different zones of the cord with cells in various stages of differentiation: (*1*) the surface (amniotic) epithelium, (*2*) subamniotic stroma, (*3*) clefts, (*4*) intervascular stroma, (*5*) perivascular stroma, and (*6*) vessels. However, the descriptors separating these zones are not clear. It is thought that WJ is composed mainly of perivascular progenitors but may possibly include nonperivascular progenitors as they move away from the vasculature.[6] In addition to the anatomical differences, there is concern that the MSCs may differ lengthwise and that the mother end of the umbilical cord may have different mesenchymal features than the fetus end of the umbilical cord.[7]

In addition to harvesting and potency advantages, cord blood and WJ-derived MSCs are also not limited to autologous use. Due to their excellent immunomodulatory properties and universally tolerated surface marker profiles, MSCs isolated from cord blood and WJ can be made available to patients as allografts.[8,9] Using cells isolated from birth tissue as "off-the-shelf" allografts greatly simplifies the manufacturing process of MSCs for therapeutic use, providing a standardized, scalable method of producing cells that does not need to be personalized for each patient.

Importance of the secretome

The therapeutic effectiveness of MSCs is well documented, especially as it pertains to wound healing. However, the mechanisms of action are not well understood. *Stem cells* are partially defined as cells that are capable of differentiation into a

variety of specialized somatic cells, and this knowledge has fueled speculation that cell differentiation upon engraftment is responsible for the observed therapeutic effects. On further investigation, it would appear that this is not the case.

Recent research has shown that MSCs introduced therapeutically primarily function through trophic and immunomodulatory signaling pathways, and the stem and progenitor cells of the host actually do most of the work.[10] This is why the *secretome*, or the collection of bioactive molecules secreted from the cells, has been receiving more attention from researchers. Rich in growth factors and cytokines that are associated with modulating inflammation and promoting angiogenesis, the MSC secretome seems perfectly suited to enhance wound healing. This is evidenced in human physiology by the ability of MSCs to zero in on areas of inflammation and injury and secrete bioactive factors.[11]

The regenerative effects of growth factors and cytokines have been well documented in dentistry. Peptides in the transforming growth factor β (TGF-β), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and interleukin (IL) families are crucial components driving regeneration, especially as it relates to bone growth.[12] These factors stimulate host cells in regenerative pathways, but it can be challenging to maintain dosing and ensure efficient cell uptake of these factors therapeutically. The MSC secretome is rich in many of these peptides, suggesting that the secretome could be responsible for some of the observed therapeutic effects.[13]

Stem Cells in Regenerative Endodontics

The potential utility of cord blood MSCs has not yet been fully realized in the relatively new field of regenerative endodontics. Since its development in 2004 by Banchs and Trope, regenerative endodontics has been employed as a root-preserving alternative to a root canal, utilized to eradicate pulp infections in immature permanent teeth, thus permitting further root development and preservation of teeth in patients who are still growing.[14] Liao et al[15] revealed the presence of osteogenic MSCs in both inflamed pulp tissue and inflamed periapical tissues, and further investigation by Chrepa et al[16] revealed that the evoked bleeding step demonstrated an increase in local accumulation of undifferentiated MSCs even in a mature tooth. Because of these revelations, the recommended American Association of Endodontists protocol was revised to use irrigants that are less toxic to stem cells and propose the use of platelet-rich plasma, platelet-rich fibrin, and autologous fibrin matrix in place of the simple blood clot.

However, there is no evidence in the literature of regeneration of the dentin pulp complex. The effect of noninflamed apical residual tissue in regeneration

of pulp has been investigated recently in an animal model. Torabinejad et al[17] conducted a study providing evidence that noninflamed apical residual pulp has the capability to regenerate the normal pulp. Given the discovery of the presence of stem cells involved in the restoration of the root, studies are now underway to assess if implantation of stem cells may in fact accelerate pulp tissue regeneration and healing and perhaps shorten the wait time. Tissue engineering permitted two strategies: (*1*) the direct implantation of freshly isolated stem cells with or without biodegradable scaffolds, and (*2*) implantation of preassembled tissue constructs containing in vitro cultured cells in the scaffold.[18] Studies showed that the implantation of stem/progenitor cells isolated from a human root in a mouse model resulted in formation of a pulp-like tissue with a layer of dentin-like tissue along the canal wall.[19]

Unfortunately, retrieval of autologous dental stem cells is difficult, especially in the common case where all other teeth are healthy. It is even more difficult to obtain one of the five subtypes of dental stem cells: (*1*) dental pulp stem cells (DPSCs), (*2*) stem cells from human exfoliated deciduous teeth (SHEDs), (*3*) stem cells from apical papilla (SCAPs), (*4*) periodontal ligament stem cells (PDLSCs), and (*5*) tooth germ progenitor cells (TGPCs).[20]

As such, recent efforts have investigated the use of MSCs derived from other origins, such as human bone marrow–derived MSCs, which have potential for osteogenesis as a more multipotent stem cell than the differentiated dental stem cells. However, bone marrow–derived MSCs are associated with risks, mortality, and high cost of bone marrow harvesting and processing. Induced pluripotent stem cells have also been investigated,[21,22] which studies show contribute to mesenchymal progenitors to create early cells in the osteogenic lineage. Unfortunately, the study by Sueyama et al[23] using a rat model found that implanting MSCs alone showed incomplete dentin bridges, while coimplantation of MSCs with endothelial cells resulted in pulp healing with complete dentin bridge formation.

As such, a viable strategy to allow osteogenic regeneration involves the use of cord blood MSCs. This avoids the ethical concerns of embryonic stem cells and the morbidity of bone marrow acquisition while retaining the multipotency required for the regeneration of complex endodontic tissue. In 2018, Chen et al[24] showed that MSCs derived from cord blood were able to achieve successful osteogenic and angiogenic properties, in addition to density, when cocultured with human umbilical vein endothelial cells. These cord blood MSCs had similar capabilities and performance as human bone marrow–derived MSCs and human embryonic stem cells.[24]

Currently, the literature does not indicate any effect of cord blood stem cells used in combination with residual dental pulp tissue on the ability of a tooth to regenerate pulpal tissues. The authors propose that cord blood stem cells will enhance the ability of residual pulp tissue to regenerate.

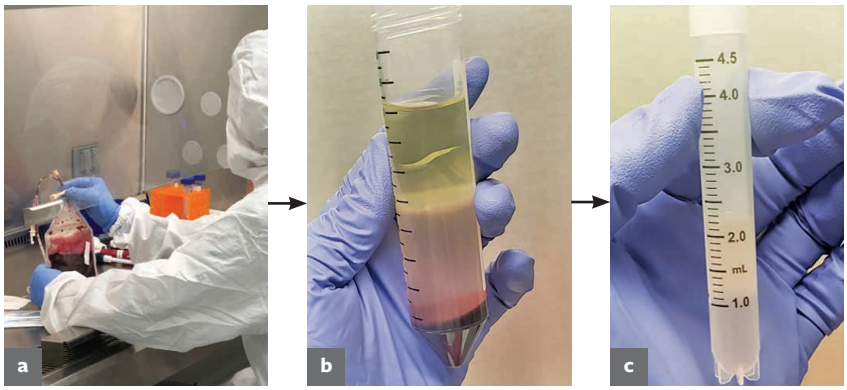

FIG 1-1 The different stages of cord blood processing. (*a*) Blood bag as it was received being processed under aseptic conditions. (*b*) Conical tube after centrifugation. Note plasma layer (*top*), buffy coat layer (*middle*), and red blood cells (*bottom*). (*c*) PBMCs suspended in Stem-Cellbanker ready for storage in a cryovial.

MSC Isolation Methods

Cord blood–derived and WJ-derived MSCs are an excellent option for therapeutic use because they are easily collected, readily available, and highly proliferative. They can also be used as allografts. Procedures used to isolate MSCs from these sources are described in the following sections.

Cord blood

Cord blood is collected from eligible donors at the time of delivery and transported to the processing facility on ice (2°C to 8°C) in a blood bag. Upon arrival, the blood is processed immediately under aseptic conditions (Fig 1-1a).

The blood bag is first drained into conical tubes and centrifuged at 1500 × g to separate components. This results in distinct layers in the conical tube, with plasma rising to the top and red blood cells forming a pellet at the bottom of the tube. There is also a distinct buffy coat layer below the plasma that contains the cells of interest. The buffy coat layer is then collected and diluted with a phosphate-buffered saline (PBS) solution before undergoing density gradient centrifugation. The buffy coat and PBS mixture is added to Ficoll-Paque (GE Healthcare) density gradient media and then centrifuged at 409g. This results in an isolation of peripheral blood mononucleated cells (PBMCs) in the resulting buffy coat (Fig 1-1b).

The PBMCs are then collected and diluted once again with PBS before being centrifuged at 409 × g. This wash step is repeated until the resulting supernatant is no longer cloudy or hazy, and the resulting pellet is then resuspended in Stem-Cellbanker (Amsbio). Cell count, viability, and surface marker profile are then tested using flow cytometry. This information is used to aliquot the appropriate cell number into cryovials, and the cells are immediately stored at –80°C (Fig 1-1c).

Wharton's jelly

Umbilical cords are collected from eligible donors at the time of delivery and transported to the processing facility on ice (2°C to 8°C) in Dulbecco's Modified Eagle Media (DMEM). Cords are processed immediately under aseptic conditions, and MSCs are collected for culture according to the procedure described below.

To start a primary explant culture, a roughly 1 × 1–cm segment of cord is obtained using sterile forceps and scalpel. This segment is dissected to remove blood vessels and isolate the WJ. The resulting segments of WJ are then added to 10 mL of a collagenase-DMEM solution and incubated at 37°C for 4 hours. The collagenase-DMEM solution is prepared to a strength of 300 collagenase degrading units (CDU) per mL. Digested pieces of tissue are then collected using sterile forceps and transferred to a T-25 flask containing 10 mL of MSC-Brew Xeno-Free Media (Miltenyi Biotec). Cultures are then incubated for 72 hours at 37°C, at which point the media is replaced and the pieces of tissue are removed from the flask.

Once the primary culture has been established, regular media changes occur every 2 to 3 days, and cells are allowed to grow to 80% to 90% confluency. At this point, cells are passaged using trypsin-EDTA solution (0.25%) and reseeded into a T-75 flask. The culture is maintained this way until the target number of cells has been reached, at which point passaged cells are suspended in Stem-Cellbanker and frozen at –80°C.

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