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Effects of PRF on Regeneration of Muscle, Bone, and Intervertebral Disc

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Abstract

Platelet concentrates including platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) are becoming innovative tools of regenerative medicine. Autologous PRP is derived from an individual's whole blood then centrifuged to remove red blood cells and leucocytes. The remaining plasma has a 5- to 10-fold higher concentration of growth factors than whole blood. These growth factors have been found to promote natural healing responses, and hence PRP is widely used in orthopedic and sports medicine to relieve pain in musculoskeletal injuries. Platelet-rich fibrin (PRF) is a newer generation of platelet concentrates and has being used as an adjunctive autologous biomaterial to promote bone and soft tissue healing and regeneration. This thesis included an in vitro study and 3 clinical trials, and the objectives were to investigate the roles of PRF on regeneration of muscle, tendon, bone, and intervertebral disc (IVD). In vitro study, we investigated the biologic effects of pig PRF on the proliferation of different cells, including rat L8 myoblasts, mouse RAW 264.7 monocytic cells which are precursors of osteoclasts, mouse L929 fibroblasts, and porcine endothelial cells (PEC). L8 cells and L929 cells cultured with PRF showed a decrease in cell number when compared to the control group in which horse serum was used as a control. RAW 264.7 cells and PEC cultured with PRF, on the other hand, increased cell number. These results suggested that PRF had negative effect on the proliferation of L8 and L929 cells, however, expressed positive regulation on RAW 264.7 cells and PEC. In clinical trials, we investigated the clinical effects of PRF on bone fusion in spinal fusion surgery and IVD regeneration after endoscopic discectomy. Posterior lumbar decompression and

transforaminal interbody fusion (TLIF) is a safe and effective surgery for degenerative spinal diseases. Several options of bone graft are available but autologous iliac crest bone grafting (ICBG) is still the gold standard. However, some morbidity is associated with ICBG harvesting such as infection, fracture, or donor site pain. To avoid donor site morbidity, various allograft materials have been used as a substitute but are still questionable as to the effectiveness and safety. In our study, allograft combined with autogenous bone marrow and with/without PRF was used to compare with autologous ICBG in TLIF surgery. After at least 12 months follow-up, the fusion rate in single level TLIF was 100% in both autograft group and allograft with PRF group, while the fusion rate of allograft without PRF was 77.8%. The results demonstrated positive effect of PRF on bone fusion in TLIF. Discogenic low back pain (DLBP) is associated with degeneration of IVD and usually is a complex problem regrading to diagnosis and treatment. It is suspected that the pain source of DLBP comes from the torn lesion of annulus fibrosus (AF) which is located on the outer surface of IVD. We applied percutaneous endoscope to treat torn AF in patients with DLBP and the satisfactory outcomes were more than 85% in selected patients at two years follow-up. The procedure is effective and minimally invasive because only inflammatory tissues on AF and loose nucleus debris are removed under clear endoscopic vision. To investigate the effect of PRF on regeneration of IVD, we inserted PRF into IVD in 6 patients who were diagnosed with DLBP and had undergone endoscopic treatment. Autogenous PRF was inserted into nucleus at the end of endoscopic procedure. At 12 months follow-up, 5 of 6 patients showed excellent results and MRI T2-weighted signal of involved IVD did not show further degenerative change. This adjunctive biomaterial expressed beneficial effects on clinical outcomes and radiologic results.

Key Word: Platelet-rich fibrin (PRF), Myoblast, Fibroblast, Osteoclast, Endothelial cells, Spinal fusion surgery, Disc, Endoscopic spine surgery, Discogenic back pain

富含血小板纖維蛋白對肌肉、骨骼及椎間盤再生之作用

摘要

血小板內含多種活性生長因子，釋放後可以促進血管增生及組織的再生與修復。一般組織中，血小板的濃度不夠高，而且混雜其間的紅血球及白血球可能對組織的修復產生負面的影響。血小板濃縮物，包括富含血小板血漿（platelet-rich plasma; PRP）和富含血小板纖維蛋白（platelet-rich fibrin; PRF），是藉由特定的處理程序取到純化且高濃度的血小板製品，內含更豐富的生長因子，因此理論上更能夠促進組織修復及生長。PRP 是抽取自體的血液，經儀器離心處理後，取中間層富含血小板的血漿，再重新注射回體內。PRP 在臨床的使用範圍相當廣泛，在骨科的領域裡最常用於局部肌腱、韌帶或關節的治療。PRF 是第二代的血小板濃縮技術，與 PRP 比較，PRF 製備時無需添加任何生物製劑即可獲得富含血小板及生長因子的纖維蛋白，臨床上可當再生膜使用，而有助於組織缺損的修復。然而，有關 PRF 對於肌肉、骨骼及椎間盤生物性作用的資料有限。本研究包含三大主題，第一主題是探討 PRF 對於四種不同細胞的增殖作用，包括大鼠肌母細胞 (L8)、噬骨細胞的前驅 RAW 264.7 單核細胞、L929 纖維母細胞、和 PEC 內皮細胞。我們分別將這四種細胞加入 PRF 共同培養，來模擬組織中的細胞生長實驗，並以馬的血清當對照組。結果顯示 PRF 對 L8 肌母細胞和 L929 纖維母細胞有負面的調節作用，而且細胞週期的調控應在第零間期 (G0 phase)。相反的，PRF 對 RAW 264.7 單核細胞和 PEC 內皮細胞卻有正面的調節作用。我們再探討不同濃度釋放液對 L8 細胞增殖的影響，結果顯示含有高濃度釋放液組別的肌母細胞數目反而較低濃度釋放液的組別為少。我們推測 PRF 的作用可能有細胞種類和濃度的差別。第二主題是探討 PRF 在臨床脊椎融合手術中，對脊椎融合的效益。脊椎後側椎間融合術常被運用在治療脊椎部分的疾病，例如滑脫、畸形、腫瘤等，而骨融合率決定了手術的成功率。目前移植骨骼的金標準是自體骨骼，因為有最好的骨融合率。我們將取自骨銀行的異體骨混合自體 PRF 和自體骨髓置入準備好的椎間盤間隙中，一年的骨融合率大於 90%，比使用異體骨骼為好，與完全使用自體骨骼相比，融合率則無顯著差異。這顯示 PRF 可促進脊椎融合手術中使用異體骨的融合率，但手術時間縮短，出血量較少，滿意度較高，因為節省了採自體骨的時間和避免取骨所衍生的後遺症。第三主題是探討 PRF 對椎間盤修復的效果。在脊椎的病變中，沒有神經症狀的嚴重背痛一直是很棘手的議題，不僅痛源難以診斷，治療的方式也頗有爭議。盤源性背痛，是指因椎間盤退化或發炎所導致的背痛，佔了其中六到八成，我們首先利用核磁共振和激發式椎間盤照影確定盤源性背痛的診斷，並選擇適當的病人，然後採用微創經皮脊椎內視鏡手術處理這些患者椎間盤的病變處。結果證實有很高的成功率和滿意度，其中成功的關鍵在於徹底移除椎間盤中

發炎的纖維環和退化的髓核。在之後更進一步的臨床試驗中，我們再將自體 PRF 置入六位患者因盤源性背痛接受經皮脊椎內視鏡減壓和清創手術的椎間盤內，觀察臨床結果，並利用影像學觀察椎間盤一年的變化。結果發現六位中的五位有最高的滿意度，並且置入 PRF 的椎間盤，核磁共振影像訊號未再顯示更進一步的退化，由此推斷 PRF 有修復椎間盤的效果。

關鍵字:富含血小板纖維蛋白，肌母細胞，纖維母細胞，噬骨細胞，內皮細胞，脊椎融合，椎間盤，盤源性背痛，脊椎內視鏡

Introduction

The use of the autologous products is a rapidly growing field of orthopedics focusing on manipulating growth factors and secretory proteins to improve the healing of bone and soft tissues. Research on the biology of bone, ligament, and tendon healing has led to the development of a variety of products designed to increase biologic factors and promote healing.

Platelets isolated from peripheral blood are an autologous source of growth factors. When platelets in a concentrated form are added to graft materials, a more predictable outcome is obtained. Platelet-rich plasma (PRP) is an easily accessible source of growth factors to enhance the healing of bone and soft tissue healing. PRP is derived by methods that concentrate autologous platelets and is added to surgical wounds or grafts and to other injuries in need of supported or accelerated healing. Blood clot is the center focus of initiating any soft-tissue healing and bone regeneration [1]. In all natural wounds, a blood clot forms and initiates the healing process. PRP is a simple strategy to concentrate platelets or enrich growth factors to natural blood clot, which forms in normal surgical wounds, to lead to a more rapid and complete healing process.

Platelet-rich fibrin (PRF) is a new generation of platelet agglutination, which was first developed in France by Choukroun et al. in 2001 as an autologous biomaterial [2]. PRF contains a fibrin matrix polymer, blood aggregates, leucocytes, cytokines and circulating stem cells. PRF is obtained from autologous peripheral blood by centrifugation, without adding any biological agents. Compared with PRP, PRF is produced with a simpler method; it is lower in cost and more easily available. PRF has been applied in many different clinical fields, particularly oral and maxillofacial surgery, plastic surgery, and orthopedics.

Both PRP and PRF contain autologous concentrated platelets and the use of platelet concentrates has several advantages. The properties of platelet concentrates are attributed to the production and release of multiple growth and differentiation factors upon platelet activation.

These factors are critical in the regulation and stimulation of wound healing process, and play important roles in regulating cellular processes such as mitogenesis, chemotaxis, differentiation, and metabolism [3]. Clinical studies for evaluating the effects of the injection of PRP into degenerated intervertebral discs for patients with discogenic low back pain have been reviewed, and most studies reported that PRP was safe and effective in reducing back pain [4]. In spinal fusion surgery, PRF seemed to accelerate bony deposition, promote tissue healing, and increase bone density [43].

The popularity of PRF is increasing due to its many advantages. The findings of Wiltfang et al. from a series of clinical trials are encouraging, in which they showed improvement of PRF on osteoblast proliferation and differentiation as compared with PRP [2]. Although the clinical evidence of tissue repair of intervertebral disc (IVD) by PRF treatment is currently lacking, there is a great possibility that the application of PRF has the potential to lead to a feasible intradiscal therapy for the treatment of degenerative disc diseases. Further large-scale studies may be required to confirm the clinical evidence of PRF in promotion of bone healing and intervertebral disc regeneration. In future, more histologic and clinical evaluations are required to understand the benefits of this second-generation platelet concentrate.

The aims of the present studies are to elucidate whether PRF can influence (1) the proliferation of different cells including rat L8 myoblasts, mouse RAW 264.7 monocytic cells which are precursors of osteoclasts, mouse L929 fibroblasts and porcine endothelial cells (PEC) in vitro, (2) the clinical outcomes and fusion rate in patients who underwent transforaminal interbody fusion and (3) the safety and initial efficacy of intradiscal application of autologous PRF in patients who underwent full endoscopic treatment for discogenic low back pain.

Chapter 1 Literature Review

1 Bioactive Factors in platelets

PRP or PRF potentially enhances healing by the delivery of various growth factors and cytokines from the α-granules of platelets. The basic cytokines identified in platelets include transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I, IGF-II), fibroblast growth factor (FGF), epidermal growth factor, vascular endothelial growth factor (VEGF), and endothelial cell growth factor [1]. These cytokines play important roles in cell proliferation, chemotaxis, cell differentiation, or angiogenesis (Table 1). A particular value of PRP is that these native cytokines are all present in “normal” biologic ratios. In contrast, exogenous cytokines produced by recombinant technology such as bone morphogenic protein (BMP), are delivered in high dose using a carrier vehicle and have extraordinary levels in the target tissues.

Because healing is a highly delicate and complex process, there are distinct limitations to the ability of single-factor therapy to improve tissue healing. The dense granules also contain bioactive factors including serotonin, histamine, dopamine, calcium, and adenosine (Table 2). These non–growth factors also have fundamental effects on the biologic aspects of wound healing. The 3 stages in healing process are inflammation, proliferation, and remodeling. The inflammatory stage begins with tissue injury; consequently, platelets are stimulated to aggregate and secrete growth factors, cytokines, and hemostatic factors critical in the early stages of the intrinsic and extrinsic pathways of the clotting cascade. Histamine and serotonin are released by activated platelets, and both increase capillary permeability, which allows inflammatory cells to access the wound site more easily and activates macrophages [4]. Polymorphonuclear leukocytes migrate toward the area of inflammation and, soon thereafter, begin to proliferate, whereas fibroblasts synthesize and secrete ground substance. Adenosine receptor activation by adenosine derived from platelets modulates the

inflammatory process during wound healing.

The platelets in PRP or PRF are kept in a clot, which contains several cell adhesion molecules including fibronectin, fibrin, and vitronectin. These cell adhesion molecules play roles in cell migration, and thus also add to the potential biologic activity of PRP or PRF. The clot itself can also play a role in tissue healing by acting as a conductive matrix or “scaffold” upon which cells can adhere and initiate the tissue healing process.

2. Platelet Concentrates

In general, platelet concentrates are blood-derived products used for the prevention and treatment of hemorrhages for serious thrombopenia. The development of platelet concentrates as bioactive surgical additives started since 1990. Platelet concentrates are applied locally to promote wound healing stems from the use of fibrin adhesives. Blood contains several components which are involved in the natural healing process and have the potential to accelerate healing when added to wounded tissues or surgical sites. Fibrin glue was originally described in 1970 and is formed by polymerizing fibrinogen with thrombin and calcium. It was originally prepared using donor plasma; however, the stability and quality of fibrin glue were low because of the low concentration of fibrinogen in plasma.

These adhesives can be obtained autologously from the patient or can be obtained commercially (Tisseel, Baxter Healthcare). These products are heat-treated, thus immensely reducing, but not entirely eliminating, the risk of disease transmission. Therefore, the commercially available adhesives constitute an infinitely small risk of disease transmission. PRP is an autologous modification of fibrin glue, which has been described and used in various applications with apparent clinical success. PRF is available as a fibrin clot without biochemical modification. PRP or PRF obtained from autologous blood is used to deliver growth factors in high concentrations to the site of bone defect or a region requiring augmentation [5].

3. Platelet-Rich Plasma (PRP)

Platelets are small, irregularly shaped anuclear cells, 2-4 μm in diameter, which are derived from fragmentation of precursor megakaryocytes. The life span of a platelet ranges between 8 and 12 days. Platelets play a fundamental role in hemostasis and are a natural source of growth factors. Growth factors stored in the α-granules of platelets include platelet-derived growth factor, insulin-like growth factor, vascular endothelial growth factor, and transforming growth factor-β [Table 1]. The release of growth factors is triggered by the activation of platelets, which may be initiated by a variety of substances or stimuli, such as thrombin, calcium chloride, collagen or adenosine 5-diphosphate [6]. In addition to these growth factors, PRP contains fibrinogen and a number of adhesive glycoproteins that enhance cell migration.

PRP can be prepared by two techniques. The techniques differ in their technical aspects and are divided into general-purpose cell separators and platelet-concentrating cell separators. General-purpose cell separators require large quantities of blood (450 ml) and generally require to be operated in a hospital setting. Blood is drawn into a collection bag containing citrate-phosphate-dextrose anticoagulant. It is first centrifuged at 5,600 rpm to separate RBCs from platelet-poor plasma (PPP) and PRP. The centrifugation speed is then reduced to 2,400 rpm to get a final separation of about 30 ml of PRP from the RBCs. With this technique, the remaining PPP and RBCs can be returned to the patient's circulation or discarded. Platelet-concentrating cell separators are more widely used than general-purpose cell separator because this equipment can be accommodated in a clinic or operation room setup. These technologies permit the procurement of PRP using smaller quantities of blood. Several studies have compared the efficacy of these systems [7, 8]. Although traditionally a double-spin technique has been used, a single spin technique has been proposed. The preparation and processing of PRP is quite similar in most of the platelet-concentrating systems though the anticoagulant used and the speed and duration of centrifugation may differ among different systems.

In the PRP preparation, venous blood is drawn into a tube containing

anticoagulant to avoid platelet activation and degranulation. The first centrifugation is called "soft spin", which allows blood separation into three layers, namely bottom-most RBC layer (55% of total volume), topmost acellular plasma layer called PPP (40% of total volume), and an intermediate PRP layer (5% of total volume) called the "buffy coat". Thereafter, using a sterile syringe, the operator transfers PPP, PRP and small amount of RBCs are transferred into another tube without an anticoagulant. This tube will undergo a second centrifugation, which is longer and faster than the first, called "hard spin". This allows the platelets to settle at the bottom of the tube with a very few RBCs, which explains the red tinge of the final PRP preparation. The acellular plasma, PPP (80% of the volume), is found at the top. Most of the PPP is removed with a syringe and discarded, and the remaining PRP is shaken to mix well. This PRP is then mixed with bovine thrombin and calcium chloride at the time of application. This results in gelation of the platelet concentrate. Calcium chloride nullifies the effect of the citrate anticoagulant, and thrombin helps in activating the fibrinogen, which is converted to fibrin and cross-linked [9, 10].

4. Potential Risks of Using PRP

Sanchez et al. have elaborated on the potential risks associated with the use of PRP [11]. The preparation of PRP involves the isolation of PRP after which gel formation is accelerated by using calcium chloride and bovine thrombin. It has been discovered that the use of bovine thrombin may be associated with the development of antibodies to the factors V, XI and thrombin, resulting in the risk of life-threatening coagulopathies. Bovine thrombin preparations have been shown to contain factor V, which could result in the stimulation of the immune system when challenged with a foreign protein. Other methods for safer preparation of PRP include the utilization of recombinant human thrombin, autologous thrombin or perhaps extra-purified thrombin. Landesberg et al. have suggested that alternative methods of activating PRP need to be studied and made available to the dental community [12].

5. Platelet-Rich Fibrin (PRF):

Preparation and Properties

Overcoming the restrictions in the French law related to the reimplantation of blood-derived products, PRF was first developed in France by Choukroun et al. This second-generation platelet concentrate eliminated the risks associated with the use of bovine thrombin. A report of clinical trials comparing the growth factor content of PRF and PRP indicated that PRF and PRP application led to similar level of growth factors [3].

Preparation

A major advantage of PRF is the simple preparation protocol. Blood is drawn from the patient using a sterile 10 ml vacutainer (2-12 tubes) just before or during surgery (Fig. 1-1 A). The tubes with collected blood samples are immediately (within 2 min after blood collection) centrifuged. The clinical success of the PRF protocol is dependent on the quick blood collection of blood and its transfer to the centrifuge because blood will spontaneously start to coagulate after 1-2 min and make it difficult to obtain the required clot quality [4]. Failure to accomplish the quick preparation of PRF could result in a diffuse polymerization of fibrin, which is not ideal for tissue healing.

Centrifugation

The centrifuge tubes containing blood should always be balanced by opposing two tubes to equilibrate the centrifugation forces and to prevent vibrations during the centrifugation process (Fig. 1-1 B). After the centrifugation, the caps are removed and the tubes are placed in a sterile tube holder (Fig. 1-1 C). The blood sample with clot is allowed to

rest/mature for approximately 4-8 min before extracting the clot from the tube (Fig. 1-1 D). The centrifugation process activates the coagulation process and separates the blood sample into three different layers: an acellular plasma at the top of the tube, a strongly polymerized fibrin clot in the middle, and blood cells (red corpuscle base) at the bottom of the tube [4, 5].

Centrifugation protocols currently used

There are various centrifugation processing-protocols that are currently being used [6].

1. Original Choukroun's PRF protocol (standard protocol): 3000 rpm / 10 min
2. Dohan Ehrenfest's Group - Leukocyte- and Platelet-Rich Fibrin (L-PRF): Speed 2700 rpm / 12 min)
3. Choukroun's advanced PRF (A-PRF), enriched with leukocytes: 1300 rpm / 8 min
4. Choukroun's i-PRF (solution/gel): 700rpm/3 min

Effect of centrifugation protocols on the optimum fibrin clot/cell ratio

Current data have shown that there is a differential distribution of red blood depending on the centrifugal force used. The clinical efficacy of different centrifugation protocols, however, still need to be independently validated with controlled clinical trials. In vitro studies showed that the longer centrifugation protocol (2,700 rpm) produces a denser (stronger) fibrin clot with less inter-fibrous space containing less cells compared to the shorter centrifugation protocol of A-PRF (1300 rpm) that produced a less dense fibrin clot with a looser inter-fibrous structure containing more cells. Dohan Ehrenfest and coworkers found in their in vitro studies that the original L-PRF protocol produces larger clots and membranes, and a more intense release of growth factors than the modified A-PRF protocol. Based on the findings of their study they suggested that centrifuge characteristics and protocols may have a very significant impact on the cell, growth factors, and fibrin architecture of a PRF clot and membrane [6]. In contrast, another recent in-vivo study

showed that Choukroun's new formulation of PRF (A-PRF) obtained a more gradual release of growth factors, up to a 10-day period, and the PRF stimulated significantly higher growth factor release over time when compared to Choukroun's standard PRF. Weibrich concluded that A-PRF may prove clinically beneficial for future regenerative procedures. The clinical effectiveness and implications of above mentioned findings still have to be validated through robust randomized controlled clinical trials [7].

Effect of the type of test tube and compression on the clot quality

Research data suggested that the type of vacutube used (i.e. dry glass or glass-coated plastic tubes) and the compression process of the clot (forcible or soft) do not seem to influence the architecture of this autologous biomaterial. However, both parameters could influence the growth-factor content and the matrix properties of the product [8]. The influence of these preparation factors requires further analysis and the effect on the clinical efficacy needs to be validated with good quality clinical trials.

Preparation of PRF membranes

Each fibrin clot concentrates most platelets (97%) and more than half of the leukocytes from a 9-ml blood harvest. The PRF clot is removed from the tube with a sterile tweezer. The fibrin clot is separated from the red blood cell fragment, approximately 2 mm below the interface, using a scissor (Fig. 1-1 D). The section of the blood clot attached to the fibrin clot contains stem cells. The PRF clots are placed in the BoX grid (Process, France) or Xpression Kit (Intra-Lock, Boca- Raton, FL, USA) and covered with the lid (Fig. 1-1 E). The PRF membranes are ready for use after 2 min. It provides a 3-dimensional matrix or scaffold that contains high concentrations of platelets, leukocytes, and growth factors (Fig. 1-1 F). A PRF membrane remains usable many hours after preparation, as long as the PRF is prepared correctly and conserved in physiologic conditions. Moreover, the use of the PRF Box, is a user-friendly and inexpensive tool, allows standardized preparation of homogeneous PRF membranes with higher growth factor content, to avoid the dehydration or death of the leukocytes living in the PRF clot, and also to prevent the shrinkage of the

fibrin matrix architecture. Because of the absence of anticoagulant, blood begins to coagulate as soon as it comes in contact with the glass surface. Therefore, speedy blood collection and immediate centrifugation before the clotting cascade is initiated is absolutely essential for successful preparation of PRF [9].

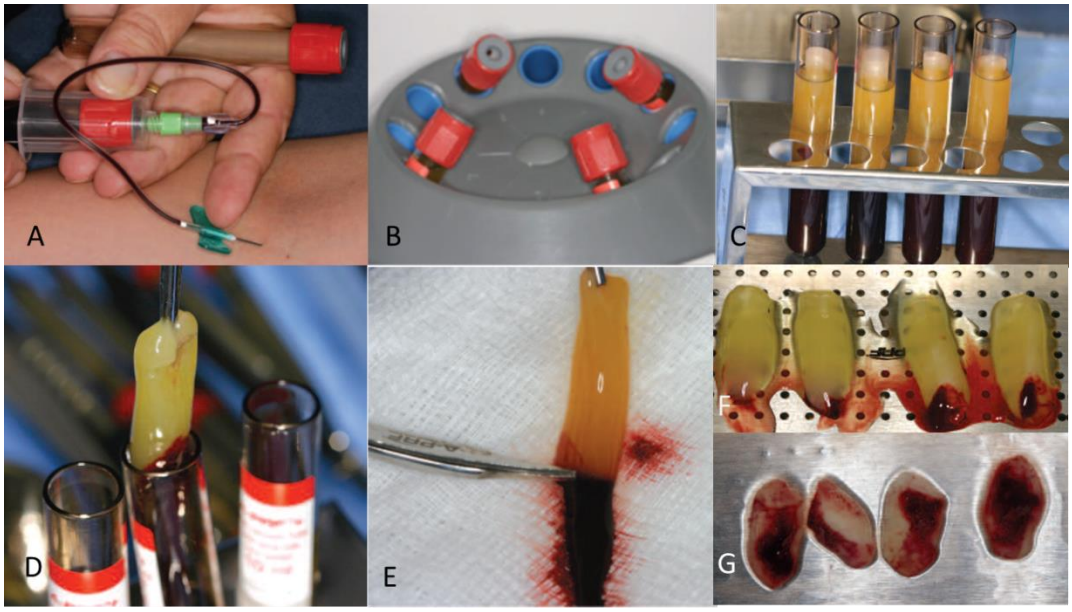

Figure 1-1 PRF preparation.
(A) Blood is drawn from a patient using a sterile 10 ml vacutainer just before or during surgery. (B)Tubes are balanced to equilibrate the centrifugation forces and to prevent vibrations during the centrifugation process. (C) After centrifugation, the caps are removed and the tubes placed in a sterile tube holder. (D) Extracting the PRF clot from the tube with a sterile tweezer. (E) The fibrin clot is separated from the red blood cell fragment using a scissor. (F) The PRF clots are placed in the BoX grid or Xpression Kit and covered with the lid. (G)The PRF membranes are ready for use after 2 min.

Advantages of PRF

PRF is a form of platelet gel and can be used in conjunction with bone grafts, which offers several advantages including promoting wound healing, bone growth and maturation, graft stabilization, wound healing and hemostasis, and improving the handling properties of graft materials. PRF can also be used as a membrane. Clinical trials suggested that the combination of bone grafts and growth factors contained in PRP or PRF may be suitable to increase bone density [9]. In an experimental trial, the growth factor content in PRP or PRF aliquots was measured using Elisa kits. The results suggested that the growth factor content (PDGF and TGF-β) was comparable in both [10]. Another experimental study used osteoblast cell cultures to investigate the influence of PRP and PRF on proliferation and differentiation of osteoblasts. The affinity of osteoblasts to the PRF membrane appeared to be superior [11]. A study was designed to evaluate the effect of biologic characteristics of platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) on proliferation and differentiation of rat osteoblasts. The exudates of PRP and PRF were collected at the time points of 1, 7, 14, 21, and 28 days. The levels of platelet-derived growth factor AB (PDGF-AB) and transforming growth factor beta1 (TGF-beta1) were quantified in PRP and PRF. When the exudates of PRP and PRF were supplemented to culture of rat calvarias osteoblasts, it was showed that PRP released the highest amounts of TGF-beta1 and PDGF-AB on the first day, followed by significantly decrease at later time points [12]. PRF released the highest amount of TGF-beta1 on day 14 and the highest amount of PDGF-AB on day 7. The exudates of the PRP supplement on day 1 and the PRF supplement on day 14 expressed maximal alkaline phosphatase (ALP) activities. The cells treated with exudates of PRF collected on day 14 reached peak mineralization significantly more than those of both negative control and positive control groups. The study also showed that PRF was superior to PRP, in terms of expression of ALP and induction of mineralization [13].

In clinical applications, PRF also has many advantages superior to PRP. The usage of PRF eliminates the redundant process of adding anticoagulant and the need to neutralize it. The addition of bovine-derived thrombin to promote conversion of fibrinogen to fibrin in

PRP is also eliminated. The elimination of these steps considerably reduces the time required for the biochemical handling of blood and the risks associated with the use of bovine-derived thrombin. The slow conversion of fibrinogen into fibrin due to small quantities of physiologically available thrombin present in the blood sample may result in a physiologic architecture favorable to the healing process.

Table 1-1 Growth Factors Identified Within Platelets and Their Physiologic Effect

Factor	Target cell/Tissue	Function
PD-EGF	Blood vessel cells, Fibroblasts, and many other cell types	Cell growth, recruitment Differentiation, skin closure Cytokine secretion
PDGF A + B	Fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, mesenchymal stem cells	Potent cell growth, recruitment Blood vessel growth, granulation Growth factor secretion; matrix formation with BMPs (collagen and bone)
TGF-β1	Blood vessel tissue, outer skin cells Fibroblasts, monocytes TGF gene family includes the BMPs Osteoblasts	Blood vessel (±), collagen synthesis Growth inhibition, apoptosis (cell death) Differentiation, activation
IGF-I, II	Bone, blood vessel, skin, other tissues Fibroblasts	Cell growth, differentiation, recruitment Collagen synthesis with PDGF
VEGF, ECGF	Blood vessel cells	Cell growth, migration, new blood vessel growth Anti-apoptosis (anti–cell death)
bFGF	Blood vessels, smooth muscle, skin Fibroblasts, other cell types	Cell growth Cell migration, blood vessel growth

PD-EGF, platelet-derived epidermal growth factor; PDGF, platelet-derived growth factor; BMP, bone morphogenetic protein; TGF, transforming growth factor; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; ECGF, endothelial cell growth factor; bFGF, basic fibroblast growth factor.

Reference: Kellie K. Middleton et al., Iowa Orthop J. 2012; 32: 150–163

Table 1-2 Bioactive Molecules Found in the Dense Granules of Platelets

Specific Molecules	Biologic Activities
Serotonin	Vasoconstriction, increased capillary permeability, macrophage attraction
Histamine	Increased capillary permeability, attract and activation in macrophages
Dopamine	Regulation of heart rate and blood pressure, neurotransmitter
ADP	Promotion of platelet aggregation
ATP	Participates in platelet response to collagen
Ca^{++}	Cofactor for platelet aggregation and fibrin formation
Epinephrine/ Norepinephrine	Sympathomimetic hormones released by the adrenal glands in response to stress

ADP, adenosine diphosphate; ATP, adenosine triphosphate

Reference: Voja Pavlovic et al., Open Med 2016; 11(1): 242–247.

7. Effects of platelet concentrate on soft tissues

The healing of connective tissues, such as tendon, ligament, and muscle, experiences 3 phases: inflammation, proliferation, and remodeling. Various cytokines act during each of these phases of wound and bone healing. Cytokines play fundamental roles in wound healing by binding to transmembranous receptors on the local and circulating cells, initiating the intracellular signaling that ultimately affects nuclear gene expression. The result is the expression of proteins regulating cell proliferation, cell chemotaxis, angiogenesis, cellular differentiation, and extracellular matrix production. The cytokines and other bioactive factors released from PRP are known to affect these fundamental metabolic processes in soft tissues of the musculoskeletal system including tendon, ligament, and muscle [14].

Tendon

Several recent studies have clearly shown that platelet concentrate positively affects gene expression and matrix synthesis in tendon and tendon cells. PRP increases the cell proliferation and total collagen production in human tenocytes cultured with slight increase in expression of matrix degradation enzymes, matrix metalloproteinase (MMP)-1 and MMP-3 [14,15]. Equine flexor digitorum superficialis tendon explants cultured with PRP showed increased gene expression of type I collagen, type III collagen, and cartilage oligomeric matrix protein with no concomitant increase in the catabolic molecules MMP-3 and MMP-13. In vivo study revealed that the platelet concentrate injected percutaneously into the hematoma 6 hours after creation of defect in a rat Achilles tendon increased tendon callus strength and stiffness [14]. Another study showed that the PRP injected into a patellar tendon injury site increased recruitment of circulation derived cells to the injury site, with a concomitant increased collagen production [15].

Muscle

Several cytokines contained in PRP have a positive effect on muscle healing. For example, basic FGF (bFGF) and IGF-I improved muscle healing in a gastrocnemius muscle laceration model in mice. Muscles treated with IGF-I and bFGF showed improved healing and significantly increased fast-twitch and tetanus strength. [16] In a mouse gastrocnemius contusion injury model, autologous conditioned serum injection at 2, 24, or 48 hours after injury resulted in accelerated satellite cell activation and increased diameter of regenerating myofibers [17].

8. Effects of platelet concentrate on bone healing

A number of studies have focused on how platelet concentrate affects osteoblasts, osteoclasts, and mesenchymal osteoprogenitor stem cells. It is hypothesized that platelets can act as an exogogenous source of growth factors that potentially stimulate bone formation [18]. Gruber et al. suggested that platelets can stimulate the formation of osteoclast-like cells, and hence promote bone growth and remodeling [18]. When osteoblasts were cultured with PRF membrane, cell proliferation and expression of alkine phosphatase (ALP), collagen type I and osteoprotegerin (OPG) were significantly promoted [19]. Another study revealed that PRF significantly stimulated mesenchymal stem cell (MSC) proliferation and osteogenesis [19]. Platelet concentrate has been shown to be osteopromotive, rather than osteoinductive [20]. Growing evidence has suggested that PRP has a limited or even negative efficacy in certain delivery vehicles [20]. PDGF was shown to inhibit intramuscular osteoinduction and chondrogenesis by demineralizing bone matrix in immunocompromised mice [20]. PRP also reduced the osteoinductivity of active demineralized bone matrix [21]. However, more data have shown the improved efficacy of PRP and bone graft materials on human bone marrow stromal cell activity, with bone formation being significantly modified by adding the agents in combination.

The bone regeneration initiates with the release of PDGF and TGF-β from the degranulation of platelets in the PRC. PDGF stimulates mitogenesis of marrow cells and endosteal osteoblasts. PDGF also initiates angiogenesis by inducing capillary budding into the surgical site through endothelial cell mitosis. TGF-β activates fibroblasts, induces pre-osteoblasts to proliferate, and triggers differentiation of pre-osteoblasts to mature osteoblasts [21]. TGF-β also induces osteoblasts to lay down bone matrix [22]. Fibroblasts deposit a collagen matrix to support capillary ingrowth, which are visualized by day 3. By day 14, capillaries permeate the regenerative bone site. When this cellular activity is going on, growth factors are relied on to rapidly increase the numbers of these cells and promote their activity during the time of injury or surgery. As the platelets come to the end of their life cycle, the growth factors have already activated chemotaxis, and macrophages have been triggered to replace the platelets as the primary source of factors. Macrophage-derived growth and angiogenic factors become the primary cellular drivers of bone healing. Marrow stem cells secrete TGF-β to self-stimulate bone formation. Once the site is revascularized (after about 4 weeks), it is self-sustainable. Maturation of the bone now is attributed to the morphogenic protein (BMP) produced by the bone matrix. When the matrix is formed and mineralized, BMP is laid down within the matrix. BMP is released by osteoclast during resorption of normal bone remodeling, and enhances stem cells to increase and differentiate into osteoblasts. This process of bone healing can be potentially accelerated by triggering the cascade of events early in the healing with the use of PRP [21, 22].

9. Effects of platelet concentrate on regeneration of intervertebral disc (IVD)

Studies showed that PRP has great potential to stimulate cell proliferation and metabolic activity of IVD cells in vitro [23]. Several animal studies revealed that the injection of PRP into degenerated IVDs is effective in restoring structural changes (IVD height) and improving the matrix integrity of degenerated IVDs as evaluated by magnetic resonance

imaging (MRI) and histology. The results of this basic research have shown the great possibility that PRP or PRF has significant biological effects for tissue repair to counteract IVD degeneration [35].

10. Clinical Applications of PRF

Wound healing

In clinic, a lot of literatures reported the effect of PRF in wound healing. After the discovery on characteristics of PRF, Lundquist et al. suggested that the PRF could be beneficial for the healing of recalcitrant wounds [42]. Jorgensen et al. also indicated the consistent results [22]. A pilot trial on 15 patients, with 16 lower extremity chronic wounds of varying etiologies, was performed with a positive outcome [22]. In a study on the efficacy of PRF in a randomized controlled clinical trial (RCT) of wound healing, Chignon-Sicard demonstrated that a single PRF application on fresh postoperative hand wounds showed an improvement on the 5th day compared with the standard treatment [23].

Facial plastic surgery

The hemostatic, fibrogenic, and angiogenic properties of PRF have been used in some procedures such as rhytidectomy, rhinoplasty and facial implants, in which rapid healing, minimal edema and reduction of ecchymosis are desired. Sclafani et al. applied the PRF in facial plastic surgery, including periorbital treatments (tear troughs, suborbital hollows, and glabellar furrows), midface and lower face treatments (malar augmentation, zygomatic arch enhancement, nasolabial folds, acne scars, and boxcar acne scars) and adjuvant use of facial plastic surgery (facelift, rhinoplasty, and facial implants) and found that only a small percentage (10% or less) of patients do not generate a tissue response sufficient to be clinically acceptable [24]. Because of the angiogenic ability, PRF was co-injected during autologous fat transfer to

enhance the viability and survival of the fat [25]. Evidence from the work of Sclafani and McCormick suggests that PRF can also induce an anabolic state in mature adipose tissue and potentially promoting more rapid vascularization of the transferred adipose tissue [26].

Orthopedic Applications

Platelet concentrate has a significant potential in the treatment of pathologic conditions of cartilage, tendon, ligament, and bone. A number of orthopedic studies have been performed or are currently underway using platelet-rich concentrate.

(1) Tendinitis

In the first in vivo study for human autologous PRP as a treatment for chronic severe elbow tendinosis in patients who had failed non-operative treatment, the data suggested that buffered PRP can be considered as a potential alternative to surgery in patients with this disorder. In Mishra's series of studies [27], all fifteen PRP-treated patients with recalcitrant lateral epicondylitis displayed significant improvement with a single injection with no reported complications. Six-month data in a prospective study showed that PRP injection with the autologous blood product compared with cortisone injection obtained significant improvement in chronic tennis elbow, consisting with previously findings by Mishra's [27]. A study was undertaken to determine the effectiveness of PRP compared with corticosteroid injections in patients with chronic lateral epicondylitis with a 2-year follow-up. The result showed that PRP treatment reduces pain and increases function significantly, and is superior to the effect of corticosteroid injection even after a follow-up of 2 years [28].

(2) Acute Ligament Injuries

Recently, the use of PRP in the treatment of acute ligament injuries in athletes has gained in popularity among sports medicine specialists. After a ligament injury, the medical care of an elite athlete is focused on the safe and expeditious return of the athlete to competition. Medial collateral ligament injuries of the knee are a common problem in sports.

Two of the authors evaluated a group of professional soccer players experienced grade II acute medial collateral ligament injuries who were treated with a single PRP injection within 72 hours after injury treated with standard rest and rehabilitation. The results showed that PRP shortened the return-to-play time by 27% compared with the control group [29].

(3) Acute Muscle Injuries

An injury to muscle tissue can be attributed to a direct blow, or from tearing of the muscle fibers because of eccentric load when the muscle is contracting. These mechanisms can cause a spectrum of injury ranging from contusion to significant muscle tear (strain). The most commonly injured muscle groups in the athlete include the hamstrings, gastrocnemius, and quadriceps. Muscle healing experiences the stages of inflammation, proliferation, and remodeling, which are coordinated by cellular interactions. As in other parts of the body, healing is dependent upon the vascularity of the injured tissue. The speed of recovery depends on the severity of the injury, the post-injury treatment, and the patient's inherent ability to heal soft tissue injuries. The usual recommendation for a muscular injury is rest, ice, compressive dressings, and elevation of the affected extremity. Several techniques have been employed in an effort to shorten the return to play intervals. PRP has been suggested as a potential intervention for athletes with acute muscular injuries. An in vitro study suggests that growth factors may influence muscle regeneration after injury. PRP treatment after an acute muscular injury may benefit the athlete by decreasing the duration to recovery; however, there are no randomized controlled human studies regarding the use of PRP for muscle injuries [29]. Despite the reported success in expediting return to play after muscular injury in the athlete, other researchers found that PRP derivatives could induce a fibrotic healing response in muscle tissues [30]. This theoretical deleterious side effect of PRP is based on the elevation of TGF-β levels after its injection into muscle. Basic studies have demonstrated that platelet granules can release TGF-β when stimulated. TGF-β has been shown to stimulate fibrosis in muscle tissue in vitro. It is hypothesized that fibrotic healing following muscular injury may lead to an increased incidence of reinjury. Therefore, researchers have advocated caution when considering PRP injections for athletes with muscular injury. The same group of

researchers who raised the concerns regarding PRP argues that the introduction of anti-TGF-β agents such as suramin, decorin, γ-interferon, and relaxin may be helpful in reducing fibrosis and thus expediting muscle healing [31].

(4) Bone Defects

The use of platelets concentrate to help restore bone defects in different surgical settings, including orthopedic surgery, maxillofacial surgery, and plastic surgery, has been studied. The use of isolated cells with a biocompatible matrix in combination with PRP maximizes the effects of growth factors on these cells. Yamada demonstrated in a canine model that the combination of mesenchymal stem cells with PRP resulted in neovascularization and a higher maturation of bone when compared to the control subjects receiving stem cells only [32]. Similarly, Kitoh reported on a case series with distraction osteogenesis in which 17 patients were treated with PRP while 29 patients did not receive PRP treatment [32]. The authors concluded that the PRP group did not delay in consolidation and has fewer complications. Currently, it is common to combine the platelet-rich material with autograft, allograft, demineralized bone matrix, or other graft material. When PRP was applied in conjunction with autogenous bone graft, the rate of bone formation doubled and bone density increased by 25% compared with controls [33].

(5) Osteoarthritis

Platelet-rich product has been used as a gel either to fill cartilage defects or to slow the progression of arthritis in animal models. Findings from current clinical trials suggest that PRP may have the potential to fill cartilage defects to enhance cartilage repair, attenuate symptoms of osteoarthritis and improve joint function, with an acceptable safety profile. The effects of PRP injection on the recovery of osteoarthritis of the knee were determined in several clinical studies. In a study in which 40 patients with osteoarthritis of the knee were treated with intra-articular PRP injection, the clinical outcome measures revealed significant improvements in visual analog pain scale, International Knee Documentation Committee scores and subjective evaluations at 6-month follow-up [34]. Although current evidence appears to favor PRP over hyaluronic acid for the treatment of osteoarthritis, the efficacy of PRP

therapy remains unpredictable owing to the highly heterogeneous outcome of reported studies and the variation in composition of the PRP preparations. Future studies are critical to elucidate the functional activity of individual PRP components in modulating specific pathogenic mechanisms.

(6) Discogenic Low Back Pain (DLBP)

Previous clinical reports suggested that an intradiscal injection of various preparations of PRP into degenerated discs of patients with LBP provided positive effects on pain relief [35]. Although there was only one double-blind randomized controlled trial, most studies reported that PRP was safe and effective in reducing back pain [35]. Although the clinical evidence of tissue repair of IVDs by PRP treatment is currently lacking, there is a great possibility that the application of PRP or PRF has the potential to lead to a feasible intradiscal therapy for the treatment of degenerative disc diseases. Further large-scale studies may be required to confirm the clinical evidence of PRP or PRF for the treatment of DLBP.

11. Problems of PRF

The question of the leucocyte content within platelet concentrates for surgical use is an old debate. However, there is no actual proof that the leucocytes within these surgical preparations might cause undesirable side effects. On the contrary, several studies showed that L-PRPs have antimicrobial effects [36], and no undesirable inflammatory reactions have been observed with PRPs up to date, even in immune-sensitive applications [37,38]. The influence of leucocytes injected with surgical platelet concentrates is actually a relevant way of research, and no study claims that their influence is negative [39]. All statements on this matter should be carefully and scientifically discussed and proven [40]. The influence of the leucocytes on the biology of each product and its potential benefits should be carefully analyzed because it could explain many controversial data from the literatures.

12. Conclusion

The therapeutic use of PRF for accelerating tissue healing and regeneration has increasingly grabbed the attention because this biomaterial is of natural origin, readily available, easy to prepare and use, and widely applicable, whilst being financially realistic for the patient and the clinician with virtually no risk of a rejection reaction. The 3D architecture of fibrin provides the PRF membrane with great density, elasticity, flexibility, and strength, which are excellently suitable for handling, manipulation and suturing. Optimal PRF membrane quality and treatment success is dependent on: quick collection of blood and transfer to the centrifuge; use of proper centrifugation protocol; maturation of the clot for 5 min before use; preparation of the membrane using a standardized preparation technique; and appropriate conservation of the membrane before use. The PRF can be used as a membrane, gel, plug or fragments, and applied either in stand-alone therapies, additive therapies (i.e. added or mixed to bone substitutes), or used in combination therapies with other biomaterials (i.e. protective barrier). More importantly, the use of PRF enables local delivery of a fibrin matrix, cells, growth factors and proteins that provide unique biological properties and cues for promoting new blood vessel formation, and potentially accelerating wound healing and tissue regeneration, whilst at the same time reducing adverse events. Consequently, the benefits of PRF in wound and bone healing, the antibacterial and anti-hemorrhagic effects, the low risks with its use, and the availability of easy and low cost preparation methods should encourage more clinicians to adopt this technology in the practice for the benefit patients. One of the clinical limitations to deal with is the heterogeneity in the quality and quantity of platelets and blood components due to use of different PRF preparation protocols. There is not a single randomized controlled clinical trial to compare the effectiveness of any of the PRF protocols. Furthermore, in vitro studies that claim superiority or inferiority of a specific PRF preparation have not been validated by independent clinical trials. PRF preparation protocols and the effectiveness in bone or soft tissues thereof still have to be validated.

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Chapter 2 Effects of PRF on Musculoskeletal Cell Proliferation and Migration

Abstract

Platelet-rich plasma (PRF) is a new generation of platelet concentrates and possesses a lot of growth factors and cytokines, which have the potential to have differentiative or proliferative influence on different cells tested. This study was conducted to investigate the role of PRF in the process of the proliferation of myoblast, osteoclast, fibroblast and endothelial cells. Porcine blood without anticoagulant was centrifuged immediately after collection to produce the PRF. The PRF was added to the medium for the culture of rat L8 myoblasts, mouse RAW 264.7 monocytic cells, mouse L929 fibroblasts, and porcine endothelial cells (PEC). The cell proliferation and migration were evaluated. The L8 cells and L929 cells cultured with PRF showed a decrease in cell number after 24-hour-culture compared to the control group, however, RAW 264.7 cells and PEC showed an increase in cell number. Flow cytometric analysis showed that PRF induced the apoptosis in L8 and L929 cells. After scratching on the cell layer, the PEC cells treated with PRF migrated faster than the control cells. It is concluded that the proliferation of RAW and PEC cells is promoted by PRF, but the proliferation of L8 and L929 cells is inhibited by PRF due to the induction of apoptosis. The enhancement of PEC cell migration implies PRF promotes angiogenesis.

Introduction

Most musculoskeletal tissues have the ability to heal after injury, but the process may take between weeks to months to heal [1]. For some tissues such as muscle or ligament, the repaired tissue is functionally inferior to normal tissue and is accompanied by poor performance after healing and increased risk of further injury [2]. The impact of musculoskeletal pathologies is profound but the treatment of musculoskeletal lesions is often hindered by limited options of successful and evidence-based treatments [3, 4]. New treatment strategies, such as the use of autologous growth factors, might improve healing. Platelets contain different growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, insulin-like growth factor (IGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF), which are released from their α-granules upon platelet activation and delivered to the injured site to facilitate healing [5,6]. Autologous platelet concentrates including platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) have been developed, and the actions of platelet-derived factors imply that PRP or PRF may have significant potential in the treatment of pathological conditions of muscle, tendon, bone, and cartilage. However, recent clinical studies reported on a higher level of evidence failed to show consistent positive results for the use of PRP or PRF [7]. The aim of this study was to examine the effect of pig PRF on rat L8 myoblasts, mouse RAW 264.7 monocytic cells which were the precursor of osteoblasts, mouse L929 fibroblasts, and porcine endothelial cells (PEC) in vitro.

Materials and methods

Pig PRF preparation

Peripheral blood was collected from healthy pigs. To prepare the PRF, the blood samples were immediately (within approximately 2 min after

blood collection) centrifuged by a centrifugation system at 2400 rpm (400 x g) for 12 min. The resulting PRF preparations were picked up with forceps, and the red thrombus (the fraction of red blood cells) was eliminated with scissors along the interface. The PRF was cut into 2 parts along midline with scissors. The upper part contained little amount of platelets because most of platelets were concentrated at the lower part after centrifugation (Fig. 2-1).

Cell culture

L8 rat myoblast, L929 fibroblasts and RAW 264.7 osteoblasts were obtained from American Type Culture Collection (Rockville, MD, USA). Porcine endothelial cells (PEC) was isolated from porcine artery and maintained in our laboratory [9]. Cells were grown in DMEM with 10% FBS, 100 units of penicillin/ml, 100 units of streptomycin/ml, 44 mM sodium bicarbonate in humidified atmosphere of 5% CO_2 and 95% air at 37◦C. The medium was changed every 2 days. After cells reached 80% confluent, the medium was replaced with mitogen-depleted medium (DMEM + 2% HS) to induce differentiation. PRF was embedded in medium to determine the effect on proliferation. In the control group, cells were incubated in DMEM supplemented with horse serum. The difference between the half upper part and half lower part of fibrin in response was also determined. At critical time intervals, i.e. at the undifferentiated stage and at 2 days of differentiation, the cells were observed with a reverted microscope (RM) and photographed with a digital DN system.

Giemsa staining

Giemsa staining and microscopic examination was used to monitor cell differentiation. For Giemsa staining, medium was removed and the plates were washed three times with phosphate buffered saline (PBS). After adding methanol, the plates were incubated for 20 min to fix the cells. The cells were stained with Giemsa stain solution for 20 min and

destained by rinsing with distilled water.

Trypan Blue Exclusion Assay

Cells were harvested and resuspended in 2 ml of ice-cold PBS. An equal volume of 0.4% trypan blue stain solution was added to the cell suspension and the numbers of dead and live cells were counted by microscopic examination. Cell viability was expressed as the percentage of live cells.

Flow Cytometric Analysis

To examine the effect of PRF on cell cycle phase distribution, both detached and adherent cells were harvested, washed with ice-cold PBS, and resuspended in 8 ml of ice-cold 70% ethanol to fix overnight at −20◦C. Centrifugation was performed at 300 × *g* for 5 min at 4◦C the next day and 0.5 ml of 0.5% Triton X-100 and 2 μl of RNase A were added to resuspend the cell pellets. The mixture was incubated in a water bath at 37◦C for 30 min, and the supernatant was discarded following centrifugation at 300 × *g* for 5 min at 4◦C. Then 0.5 ml of propidium iodide (PI) stock solution (20 μg/ml in PBS) was added to the pellet and this mixture was incubated for 10 min on ice. PI+ cells represent secondary necrosis or late apoptosis.

The wound assay

To examine the effect of PRF on the cell migration, the wound assay was performed [42]. PEC were cultured in DMEM with 10% FBS in 6 mm petri dish to create a confluent monolayer. Yellow tip (200 ul) was used to scrape the cell monolayer in a straight line. The cell debris was removed by wash 2X with growth medium. The medium was replaced by fresh DMEM with 2% FBS and then placed the dish in a tissue culture incubator at 37 1C for 8–18 h. After the incubation, the dish was

placed under a phase-contrast microscope, matched the reference point, and aligned the photographed region.

Statistical analysis

Data collected was analyzed using the Statistical Package for Social Sciences (SPSS) version 16. All of the results from this study are expressed as the mean +/- S.D. The differences between means were considered statistically significant if $p < 0.05$. Comparison between groups was made by a Student's t test.

Results

Pig PRF preparation

The PRF clot was produced in glass-coated plastic tubes. The clot was collected and changed into membranes.

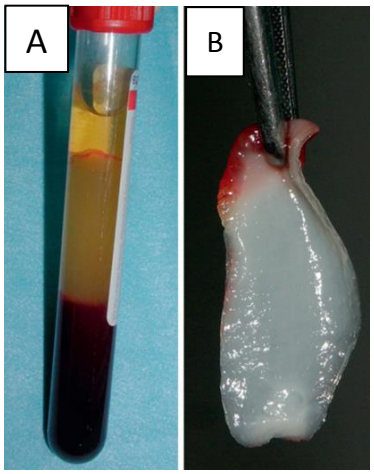


Figure 2-1 (A) The PRF clot was produced in glass-coated plastic tubes. (B) The clots were changed into membranes.

This biomaterial was built with fibrin, platelets, and leukocytes .The micrographs showed the differences of platelet numbers between upper part and lower part of PRF. Fig. 2-2(A) showed the fibrin network with little amount of platelets in upper part of PRF, and Fig. 2-2(B) showed the fibrin network with leukocytes and a great amount of platelets in lower part of PRF.

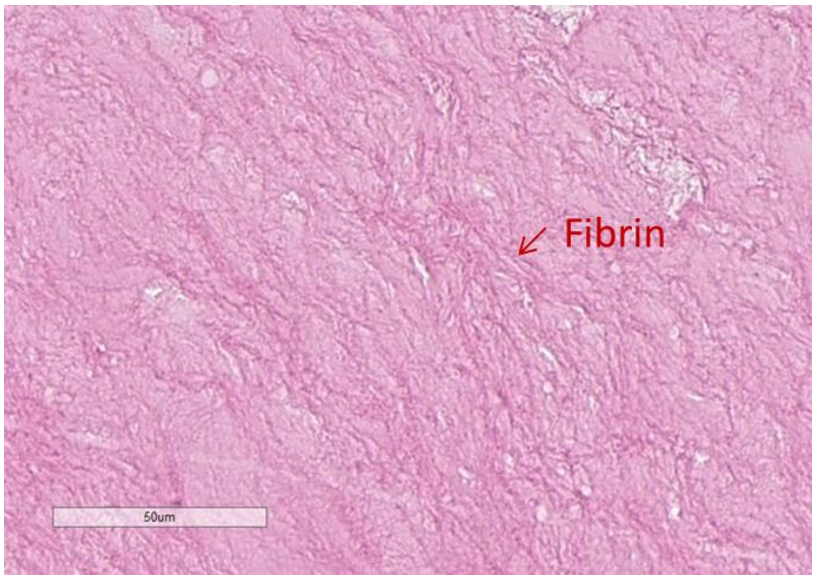
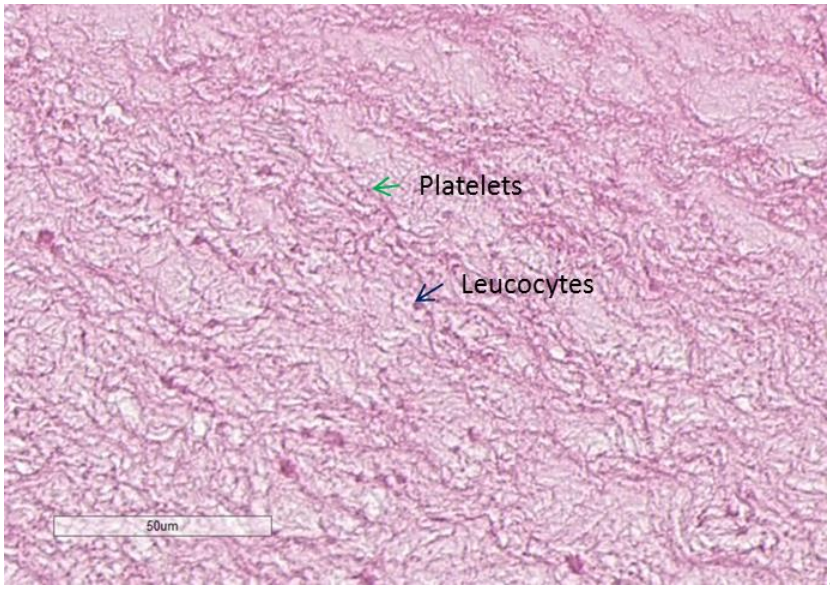


Figure 2-2 Micrograph of upper part of PRF showed the network structure of fibrin with little amount of platelets.



200X

Figure 2-3 Micrograph of lower part of PRF showed the fibrin network with leukocytes and a great amount of platelets.

Effect of PRF on cell proliferation and cell cycle

Rat L8 myoblast cells, L929 fibroblasts, RAW 264.7 osteoblast cells and porcine endothelium cells PEC were used to investigate the effect of PRF on the cell proliferation. The result showed PRF inhibited the proliferation of L8 and L929 cells. However, PRF promoted the proliferation of RAW and PEC cells (figure 2-4). To examine the inhibition effect of PRF on the cell proliferation, L8 and L929 cells were collected and then assay by Coulter Epics XL-MCL Flow cytometer, the result

showed PRF induced the apoptosis in L8 and L929 cells (Figure 2-5).

Effect of PRF on cell migration

PEC is porcine endothelial cells and involved in the angiogenesis. In cell migration assay, PEC cells treated with PRF migrated faster than the control cells (Figure 2-6). PRF treated cells migrated to cover the scratch space within 12 h, while the control cells needed 24 h to cover the scratch space (Figure 2-7).

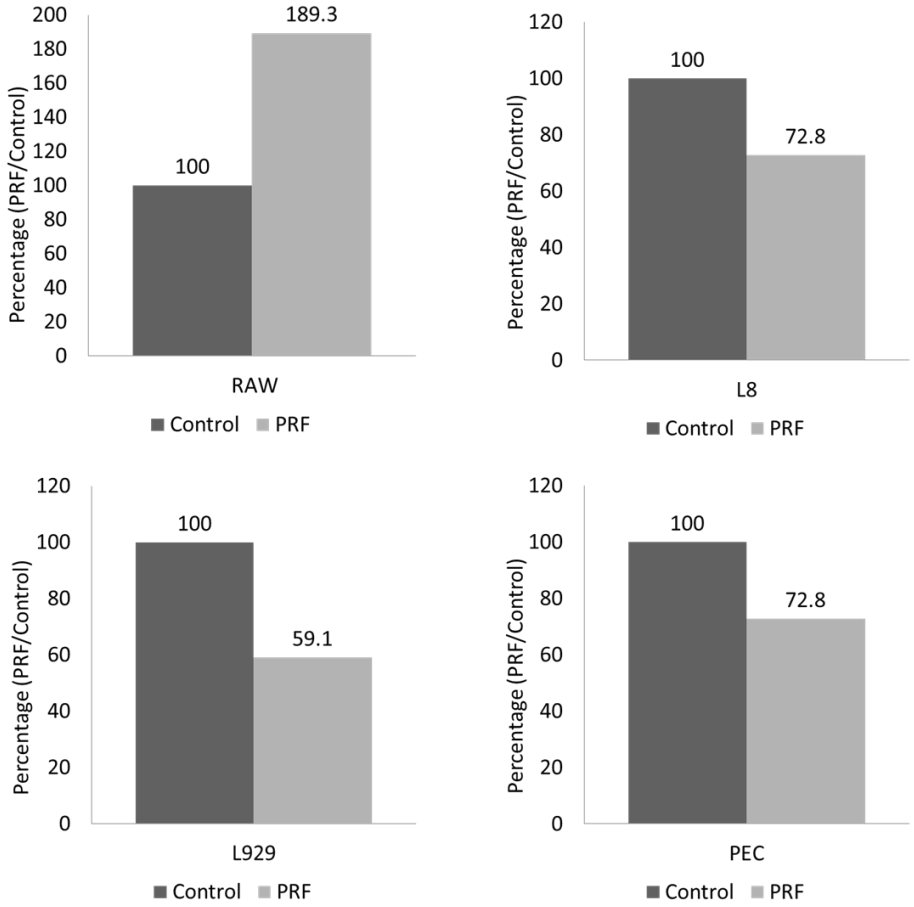


Figure 2-4 Effect of PRF on cell proliferation. Four cell lines were treat with PFR for 24 h and the cell number were counted by hemocytometer.

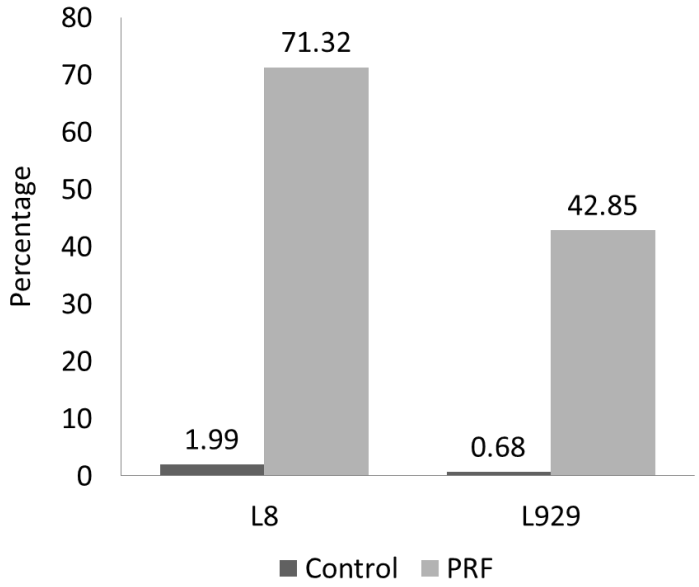
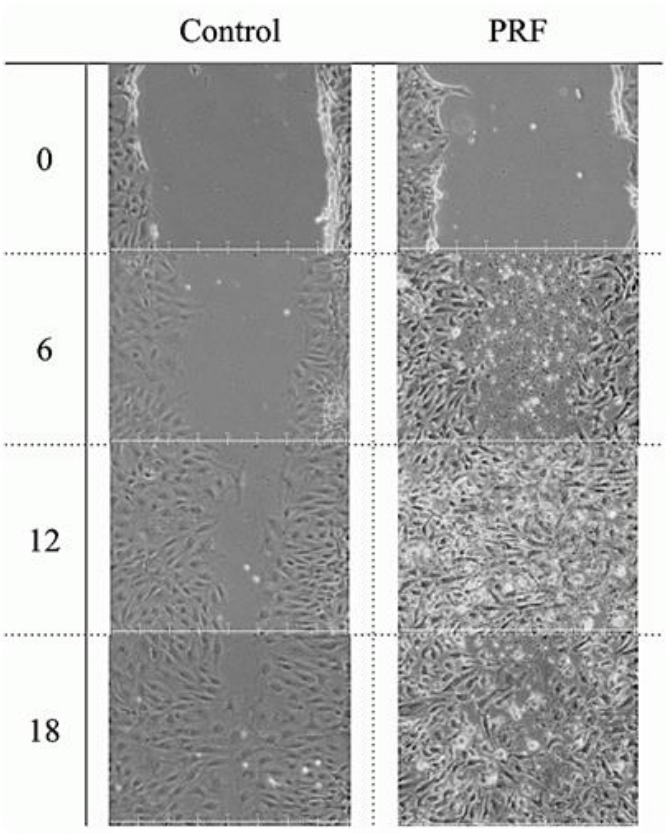


Figure 2-5 Effect of PRF on apoptosis in L8 and L929 cells.

A



B

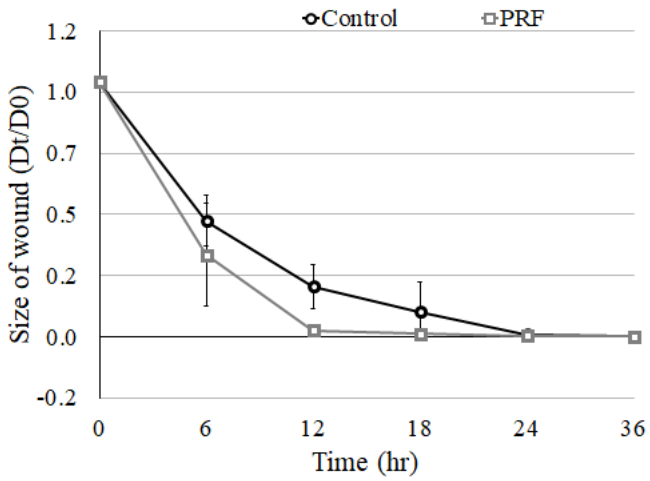


Figure 2-6 Effect of PRF on the PEC cell migration. A: photo of cell migration in different period; B: size of wound (Distance at time 0/Distance at measure time)

Discussion

The use of PRF to augment the healing of musculoskeletal injuries is only beneficial if the contents have advantageous effects on the tissue-specific cells of the injured site. In this study, we aimed to investigate the role of PRF in the process of the proliferation and migration of cells. The results showed that the PRF had negative effect on L8 and L929 cell proliferation. It is meaningful to speculate the specific effects of the various contents of PRF on these target cells. PRF contains high concentrations of growth factors that provide the potential to modulate the healing of bone, muscle, and tendon through interactions with specific cells in these respective structures [8].

The process of bone regeneration involves a series of events dependent on resident bone cells and the extracellular environment that includes mesenchymal stromal cells (MSCs), growth factors, and vascular structures [9, 10]. PRF contains pro-osteogenic factors including PDGF, TGF-β, EGF, VEGF, and bFGF that may play a critical role in the process of bone regeneration [11]. These factors make PRP a potential therapeutic agent to be used either alone or in combination with MSCs to promote the regeneration of bone [12, 13].

For muscular injuries, specific growth factors involved in repair are not completely understood at the present time; however the effects of growth factors present in PRF are believed to have the potential to improve healing and clinical outcomes [14]. The underlying basis of this proposed benefit is that a muscle contusion, strain, or laceration undergoes a repair process that includes three overlapping phases: inflammation, regeneration, and repair, followed by fibrosis and remodeling [15, 16]. The transition between phases of this repair process is controlled by PDGF, bFGF, TGF-β, and HGF [14, 17, 18]. Based on the presence of these growth factors and the key roles they play in the muscular repair process, the therapeutic goal of PRP or PRF for muscular injury is to shorten the sequence of the healing cascade [14]. Similar to its effects on muscle healing, the intrinsic properties of PRF also regulate the phases of tendon healing. The effects that growth factors have on tendon healing are still under investigation; however, it is possible that the intrinsic characteristics of PRF may augment the healing process [13, 14].

A study reported on the potentially beneficial effects of PRF on target cells (osteoblasts, myoblasts, fibroblast), it remains unclear as to which platelet concentration or PRF preparation is considered the optimal treatment of various cell types, and that a “more is better” theory for the use of higher platelet concentrations could not be supported [19]. Anitua et al. evaluated the effects of scaffolds prepared from preparations rich in growth factors (PRGF) with increasing amounts of platelets on fibroblast cell cultures harvested from three different anatomical sites [20]. They observed an increase in cell proliferation for all PRGF types and preparations compared to the controls. However, adding scaffolds containing a platelet concentration of 2-4 times above baseline resulted in the highest proliferation rate [20]. In addition, fibroblasts harvested from different origins demonstrated variable angiogenic responses with regards to their anatomical origin.
Proliferation of tenocyte-like cells was affected positively by the addition of PRP products in a number of in vitro studies to evaluate tendon-like cells [21, 22]. However the in vitro models fail to conclusively show if this increased proliferation has positive or negative effects.
Recently, increasing tendencies to use PRP to improve skeletal muscle regeneration after injury raise concerns especially because of one PRPs specific growth factor TGF-β, which is known to impair the process of muscle regeneration [23]. In this study, we aimed to investigate the role of PRF in the process of the proliferation L8 cells. The results showed that the PRF has negative effect on L8 cells proliferation. The leucocyte content or growth factor TGF-β within PRF may be the problem in this cell culture study. These findings correlate well with a few previous publications about the controversial effects of PRP and various individual growth factors on skeletal muscle regeneration [24, 25]. The inhibitory effects on TGF-β caused by PRP were also significantly higher when compared to decorin, which was identified as a powerful regulatory agent of muscle regeneration [26, 27]. Our findings suggest that preparations of autologous growth factors to act as a relevant therapeutic option for skeletal muscle injuries should be cautious, because of the additional source of TGF-β.
The effects of platelet concentrates on bone healing also are controversial [28]. Graziani et al. investigated the biological rationale of PRP by evaluating its effect at different concentrations on fibroblasts and osteoblasts activity in vitro. It was found that PRP preparations exerted a

dose-specific effect on oral fibroblasts and osteoblasts. Increased concentrations resulted in a reduction in proliferation and a suboptimal effect on osteoblast function primarily [29]. Griffin et al. showed in a systematic review that although early clinical results suggest the use of PRP is safe and feasible, however presents with no clinical benefit in either acute or delayed fracture healing was observed and therefore its use in bone regeneration was not determined [30]. While other models have also shown favorable results on new bone formation with platelet concentrates [31, 32], the results from our study showed that PRF had positive influence on osteoclast. PRF has important effects not only on osteoblastic differentiation but also on osteoclast differentiation during bone formation. However, the present study did not examine the interaction between osteoblasts and osteoclasts in bone formation. Further study is therefore required to identify additional details regarding this potential association.

Results obtained in this study confirm that plasma rich in growth factors technology stimulates the processes related to tissue regeneration in fibroblasts. The addition of PRF to a conventional DMEM culture medium specifically allowed for a favorable modulation of the fibroblasts proliferation. These results are consistent with findings reported by a number of other studies [33, 34]. Soft tissue breakdown is thought to be one of the main reasons associated with infection and implant loosening in joint replacement and the ability for PRF to improve collagen synthesis during the regenerative phase would theoretically improve the ability for host tissues to resist incoming bacterial pathogens [35]. Furthermore, since PRF contains leukocytes, its use for the treatment of peri-implant disease is hypothesized to be of value since leukocytes are thought to actively resist pathogens found in peri-implants [35]. Nevertheless, future clinical research is certainly necessary to validate these hypotheses as the resolution of peri-implant disease remains one of the most challenges.

The ability to regenerate lost soft or hard tissue by tissue engineering technology has been speculated. Blood supply is very important for the survival and differentiation of stem cells in tissue regeneration [36]. PECs are vascular endothelial cells that participate in vascular repair under ischemic or apoptotic stimuli [37]. Their role in promoting angiogenesis has been verified [38]. In one study, PRP not only maintained the growth and proliferation of cells but also promoted vasculogenesis [39]. Tube

formation assay confirmed that PRP promoted endothelial cells to form vessel-like structures [39]. In another study demonstrated the potential role of activated platelets in homing of human endothelial progenitor cells to subendothelial matrix [40]. In this study, PECs cultured with PRF showed an increased in cell number and induced a 2-4 fold significant increase in cell migration at 12 hr. According to our results, PRF should be helpful to promote angiogensis in the early pro-inflammatory phase during soft tissues or fracture healing.

Conclusion

We showed that PRF exerted various effects on 4 different cell types. PRF had negative regulation on L8 and L929 cells, however, expressed positive regulation on RAW 264.7 cells and EPCs. The influence of leucocytes embedded in PRF and related key growth factors were relevant points for research. Further research including standardized and efficient preparation of PRF is needs to illustrate the mechanism of growth factors involved in the regulation of tissue regeneration.

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Chapter 3 Allograft Mixed with Autologous Bone Marrow or Platelet-Rich-Fibrin (PRF) Versus Autograft in Transforaminal Lumbar Interbody Fusion

Abstract

Study Design
Retrospective analysis of prospectively collected data.

Objectives
To compare clinical and radiological outcomes of transforaminal lumbar interbody fusion (TLIF) using allograft with autologous bone marrow aspirate (BMA) and platelet-rich-fibrin (PRF) versus autograft.

Background
TLIF is a proven technique to achieve fusion in symptomatic spinal deformities and instabilities. Allografts as bone substitutes for TLIF have been studied with varying results. Stem cells contained in bone marrow and growth factors in the PRF are favorable for bone growth. Adjunction of bone marrow aspirate (BMA) and PRF to allograft may create better condition and may increase bone growth.

Methods
From Oct 2012 to April 2014, 107 patients (135 levels) who underwent single-level or 2-level TLIF with a minimum 1-year follow-up were included. Group 1 contained 38 patients (24 single- [group 1A] and 14 two-level [group 1B]) who underwent TLIF using PRF and BMA on allograft. Group 2 included 12 patients (7 single- [group 2A] and 5 two-level [group 2B]) with procedures using BMA on allograft. Group 3 consisted of 48 patients (30 single- [group 3A] and 18 two-level [group

3B]) with procedures using autograft. Demographic, surgical, and clinical data were collected from medical records. Time to solid fusion mass formation, fusion rate, complications, and clinical outcomes were evaluated. Oswestry Disability Index, neurogenic symptom score, the 36-Item Short Form Health Survey, and visual analogue scale scores for back and leg pain were obtained before surgery, and 6 months and 1 year after surgery. Fusion rates were assessed by Hackenberg classification.

Results

All single level fusion in Group 1A and Group 3A achieved 100% fusion; whereas Group 2A had a fusion rate of 77.8%. In single level fusion, Group 3A developed a solid fusion mass earlier than Groups 1A and 2A. In 2-level fusion, Group 1B and Group 3B also achieved high fusion rates. Group 3B developed solid fusion earlier than Group 1B and Group 2B. Groups 1 and 3 had a similar overall fusion rate.

Conclusion

TLIF using allograft combined with MBA and PRF is comparable with TLIF using autograft in terms of midterm clinical outcomes and fusion rates with the additional benefits of less initial postoperative pain, less blood loss, earlier rehabilitation, and shorter hospitalization.

Introduction

Autologous iliac bone graft (ICBG) is considered a “gold standard” for fusion in lumbar vertebral surgery. ICBG autograft contains 3 vital components for a successful fusion: an osteoconductive scaffold, osteogenic factors, and cellular elements. The reasons for its diminished use include morbidity of donor site, chronic pain at harvest site, and limited tissue supply. It is also associated with an increase in operative time, blood loss, risk of infection, cosmetic deformity, and arterial and nerve injury [1-3].

These limitations have fueled research in the exploration of bone graft substitutes and extenders, such as growth factors and cell-based therapies to enhance bone formation and improve fusion rates [4]. Bone

marrow contains hematopoietic components and osteogenic precursor cells [3]. Mesenchymal stem cells (MSCs) are present in bone marrow and are known to differentiate into a variety of tissue types, including bone. MSCs also secrete numerous autocrine and paracrine factors that may have trophic effects on surrounding tissues [4]. Bone marrow aspirates used in conjunction with allograft (BMAA) as a substitute to ICBG have also achieved satisfactory results in multiple orthopedic applications. By combining the osteogenic factors in bone marrow to the osteoconductive factors in allograft, BMAA may have the potential to be an ICBG alternative in spine fusion [5-6].

A number of growth factors, namely, transforming growth factor β, basic fibroblast growth factor, insulin-like growth factor I, vascular endothelial growth factor, and platelet derived growth factor, have a positive influence in bone repair. However there are limitations with the use of these products mainly due to high costs, short preservation period, and limited clinical availability. The aforementioned growth factors are released from the alpha granules of activated platelets following injury and the process of inflammation and tissue repair is initiated [7].

Various forms of platelet concentrates can be considered as a rich source of autologous growth factors and could be used as an alternative of commercially available products [8-9]. Platelet rich fibrin (PRF) is a second generation platelet concentrate which has many advantages over the first generation platelet rich plasma (PRP). PRF is produced by collection of autologous blood after immediate centrifugation in glass tubes without any anticoagulant. The resultant product is a clot containing fibrin, platelets, and leukocytes with a high concentration of growth factors. PRF does not require activation prior to use and the growth factors are released slowly over a sustained period [10].

The aim of this study was to compare the effectiveness of allograft plus bone marrow aspirate (BMA) and PRF with autologous ICBG in TLIF.

Materials and methods

Patients with degenerative spondylolisthesis with neuronal compression between L3 and S1 were included. Patients were included only if symptoms persisted for more than 6 months despite conservative

treatment. Inclusion criteria were patient age between 40 and 80 years, objective radiographic significant evidence of significant disc degeneration and neuronal compression at lumbar level involving L3/L4, L4/L5 and L5/S1 as confirmed by plain films and magnetic resonance imaging (MRI). Exclusion criteria were the presence of infection of spine, tumor, or metabolic bone disease in the lumbar spine.

From Oct 2012 to April 2014, 107 consecutive patients (135 levels) who underwent single-level or 2-level TLIF with a minimum 1-year follow-up were included in this study. Patients were divided into 3 groups according to the kind of bone grafts used in the procedure. In Group 1, 38 patients (24 single- [group 1A] and 14 two-level [group 1B]) underwent TLIF using PRF and BMA on allograft. In Group 2, 12 patients (7 single- [group 2A] and 5 two-level [group 2B]) used BMA on allograft. In Group 3, 48 patients (30 single- [group 3A] and 18 two-level [group 3B]) used autograft. All cases employed transpedicular instrumentation. Demographic, surgical, and clinical data were collected from medical records. Time to solid fusion mass formation, fusion rate, complications, and clinical outcomes were evaluated. Oswestry Disability Index (ODI), neurogenic symptom score, the 36-Item Short Form Health Survey, and visual analogue scale scores for back and leg pain were obtained before surgery, and 6 months and 1 year after surgery.

TLIF-cage

The TLIF-cage used in this study was the Novel® SD cage (Alphatec Spine, CA, USA) which is a Polyether ether ketone (PEEK) cage (Fig. 3-1). Its surfaces are designed to match vertebral anatomy and restore lordosis to reliably normal spinal alignment, stability and provide optimal conditions for fusion. Primary stability is achieved by sharp teeth on the superior and inferior surfaces of the cage. The Novel SD cage was implanted in all patients. This cage was prefilled with allograft which was vacuum-impregnated with bone marrow aspirate gained from the iliac crest before implantation.

Figure 3-1 Novel® SD PEEK cage.

Bone marrow aspirate

A trocar was intruded into the posterior iliac crest after skin incision, and 20 ml of bone marrow were aspirated with a Jamshidi needle and applied into the vacuum perfusion device (Fig. 3-2).

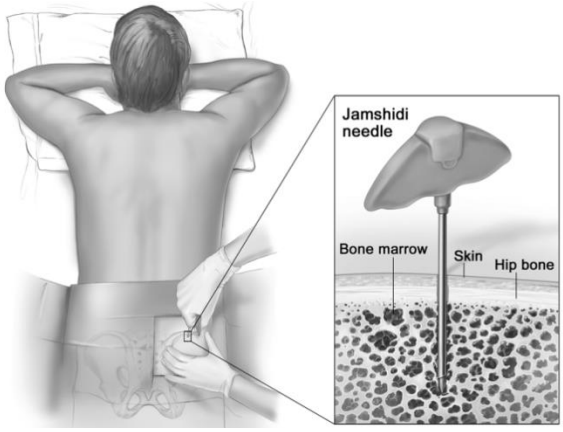

Figure 3-2 Bone marrows were aspirated from posterior iliac crest with a Jamshidi needle.

PRF preparation

Peripheral blood 10 ml was collected by a butterfly needle. To prepare the PRF, the blood samples were immediately centrifuged to 2,400 rpm (400 x g) for 12 min. The resulting PRF preparations were picked up with forceps, and the red thrombus (the fraction of red blood cells) was eliminated with scissors along the interface. The PRF was placed into intervertebral disc space anteriorly before the insertion of cage in Group 2 patients (Fig.3-3).

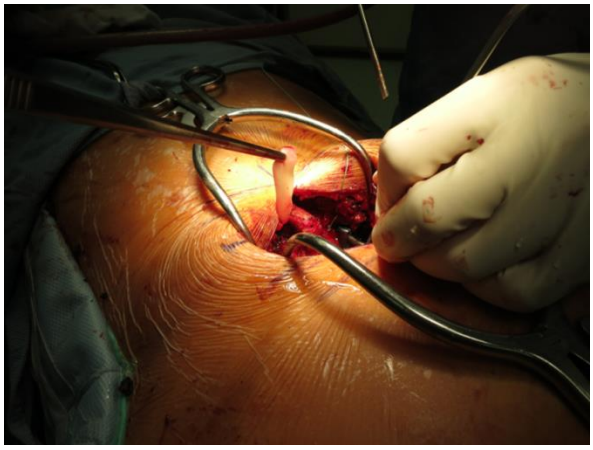

Figure 3-3 PRF is placed into intervertebral disc space.

Surgical approach

All TLIF procedures were performed by the same surgeon. The TLIF technique was performed in a standardized technique. First, the pedicle screws (SmartLoc spinal fixation system, Taiwan) are placed under fluoroscopic control. The extent of dorsal decompression was adapted to the clinical and anatomic necessities based on preoperative imaging and intraoperative judgement. A medial facetectomy was performed and the nerve root was moved medially to get access to the disc space. A window was created into the disc, which was large enough to place the appropriate cage avoiding excessive traction on the root. After removal of the disc, the endplates of the vertebrae were denuded, without weakening the subchondral bone plates to avoid implant subsidence. Cage implantation was carried out in all cases according to a standardized operative procedure. The cages are prefilled with allograft in group 1 and group 2 patients, and autograft in group 3 patients. The small quantity of local bone was incorporated in all patients. All allograft was perfused with bone marrow aspirate from the iliac crest. In group 1 and group 2, additional allograft was applied to fill the disc space medial and anterior to the cage (Fig.3-4).

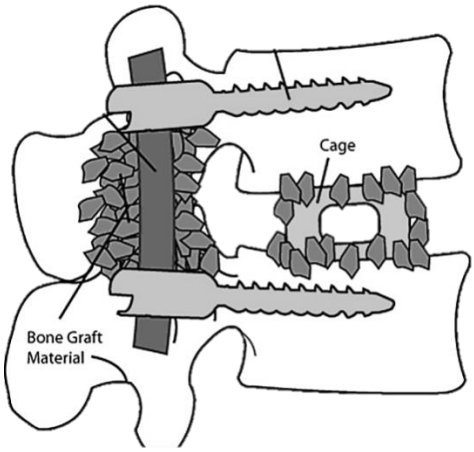

Figure 3-4 Transforaminal lumbar interbody fusion (TLIF). Disc is removed and replaced with a Novel SD cage pre-filled with bone graft.

Clinical and radiological assessments

The Oswestry Disability Index (ODI) and the visual analogue scale (VAS) were used to assess patients preoperatively at discharge and 3, 6, and 12 months follow-up. Postoperative radiological evaluations were carried out at discharge, and 3, 6 and 12 months after surgery. Clinical and radiological evaluations were performed by an independent observer. All subsequent surgeries in the lumbar spine of any type were documented. All intraoperative and postoperative complications such as infection, vascular injury, dura leakage, hematoma and adjacent disc disease were documented. Intraoperative data, such as blood loss, duration of hospital stay and cut-suture time were recorded.

The assessment of fusion and non-union was only performed for the intervertebral area and not for the interspinous region. CT scans were obtained 12 months after surgery. Bony fusion in the plane X-rays was assessed according to an established method described by Hackenberg [11]. Criteria for fusion were bony bridging, bony continuity between endplates, trabecular structure in anterior bone bridge, and lack of radiolucent lines around implants. Fusion rate was classified as ‘‘fused 3’’ (3 criteria positive), ‘‘probably fused 2’’ (2 criteria positive), ‘‘probably not fused 1’’ (1 criterion positive) and “pseudoarthrosis’’ (0 criteria positive and evidence of radiolucent lines) (Fig. 3-5).

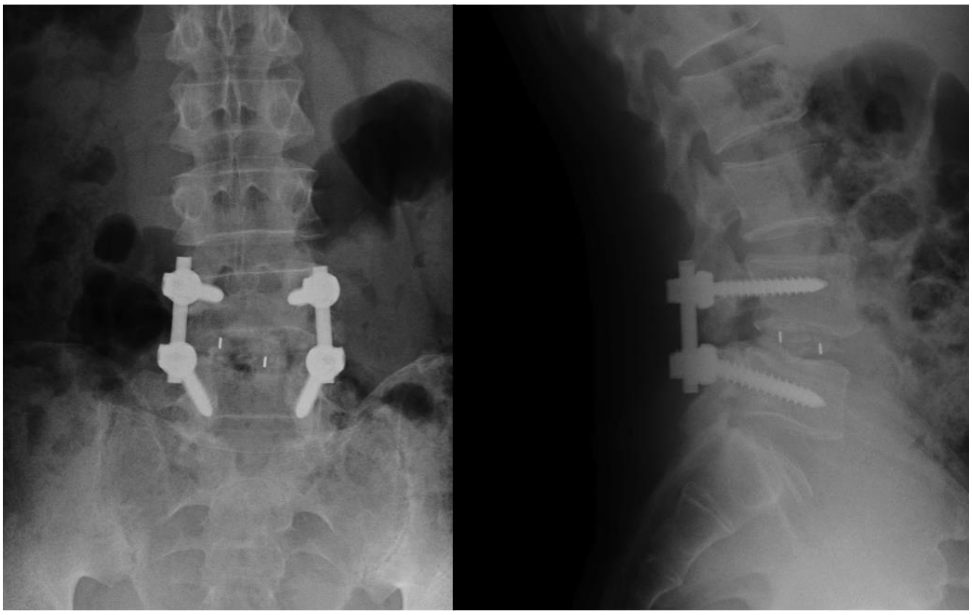

Figure 3-5 X-radiographs show the fusion meet 3 criteria: bony continuity between endplates, trabecular structure in anterior bone bridge and lack of radiolucent lines around implants.

Qualitative criteria were used for assessment of fusion in the CT scan. The classification described by Faundez et al. was used to assess anterior fusion in CT scan [9]. Approximately, 30 % of endplate to endplate bridging bone surface was required to consider the interbody fusion to be radiologically fused (A-1). A level was probably fused when bony bridging of less than 30 % was seen (A-2). The interbody fusion was indeterminate when no apparent bony bridges could be detected (A-3). Either no bony bridges between the two endplates or marginal lucency revealed in the CT scan was classified as probably not fused (A-4). If the CT scan showed cystic lucency, graft fragmentation and marginal lucency on screws the level was classified as pseudoarthrosis (A-5).

Fusion was also compared between patients with single level TLIF and patients with multilevel TLIF. Additionally, we investigated whether any of the patient characteristics available (age, gender, occupational status, smoker) had any impact on the outcome. As outcome we defined fusion A-3 or better evaluated by anterior CT scans at 12 months follow-up.

Results

Clinical evaluation

There were no significant intraoperative or postoperative complications or adverse effects due to the bone marrow aspiration. There were no infections or neurological deficits in any patient and no returns to the operating room within the first year. There was a significant decrease between preoperative and 1-year postoperative VAS scores in all groups. Group 3 had the longest operative time because the additional procedure was required to harvest the autograft from posterior iliac crest. Group 1 and Group 2 started to ambulate earlier than Group 3. Group 1 and Group 3 also showed a shorter hospital stay. The donor site morbidity in Group 1 might delay patients for ambulation and discharge when compared with patients in Group 1 and Group 2 (Table 3-1).

Radiologic evaluation

All single level fusion in Group 1A and Group 3A achieved 100% fusion, (24/24) and (32/32), respectively; whereas Group 2A had a fusion rate of 77.8% (7/9). In single level fusion, Group 3A developed a solid fusion mass earlier than Groups 1A and 2A, with a mean of 265.3 days in Group 3A versus 280.5 days in Group 1A, versus 295.7 days in Group 2A. In 2-level fusion, Group 1B and Group 3B also achieved high fusion rates (87.5%, and 90.0% respectively); whereas Group 2B showed a fusion rate of 62.5%. Group 3B developed solid fusion earlier than Group 1B and Group 2B. Groups 1 and 3 had a similar overall fusion rate (Fig. 3-6).

Outcome assessment and statistical analysis

All of the results from this study are expressed as the mean +/- S.D. One-

or two-way analysis of variance (ANOVA) was used to analyze results for the fusion rate, time to fusion, Op time, blood loss, ambulation day and hospitalization day. The differences between means were considered statistically significant if $p < 0.05$. Outcome was evaluated by visual analogue scale (from 0 to 10) scores for back pain and Oswestry Disability Index (from 0 to 100) scores for functional disability. The modified MacNab criteria were used for clinical global outcome assessment. Patients were asked to complete these questionnaires at pre-operation, and 2 weeks, 1 month, 3 months, 6 months, and 12 months post-operatively. Pre-op and post-op scores in VAS, ODI and Modified MacNab criteria were compared by Wicoxon Signed-rank test. Results were considered to be statistically significant if the P value was less than 0.05. Statistical analysis was performed using the SPSS 13.0 software (SPSS Inc, Chicago, IL).

Table 3-1 Demographic Data

	Group 1 (BMA + PRF + Allograft)	**Group 2** (BMA + Allograft)	**Group 3** (Autograft)	P-value
Fusion Rate				
Single-level (A)	100% (24/24)[a]	77.8% (7/9)[b]	100% (30/30)[a]	<.05
2- level (B)	87.5% (14/16)[a]	62.5% (5/8)[b]	90.0% (18/20)[a]	<.05
Time to Fusion (day)				
Single-Level (A)	280.5±65.3[a]	295.7±68.2[b]	265.3±60.3[a]	<.05
2-Level (B)	295.5±75.4[a]	315.8±78.5[b]	280.3±79.5[a]	<.05
Op Time (min)				
Single-Level (A)	95.5±10.2[a]	92.6±8.7[a]	112.5±15.5[b]	<.05
2-Level (B)	120.6±18.5[a]	115.6±17.6[a]	145.3±20.4[b]	<.05
Blood Loss (ml)				
Single-Level (A)	120.6±45.6[a]	130.4±55.6[a]	164.5±62.4[b]	<.05
2-Level (B)	180.5±68.5[a]	178.6±80.5[a]	220.4±95.6[b]	<.05
Ambulation (day)				
Single-Level (A)	1.9±0.2	1.8±0.3	2.4±0.8	0.315
2-Level (B)	2.5±1.2	2.7±1.6	3.2±2.1	0.225
Hospitalization (day)				
Single-Level (A)	5.2±2.4	5.4±3.2	6.3±2.1	0.106
2-Level (B)	6.3±2.7	6.8±3.5	7.2±3.4	0.361

ANOVA was used to analyze results for the fusion rate, time to fusion, Op time, blood loss, ambulation day, and hospitalization day. [a, b] means with different superscript differ significantly ($p<0.05$).

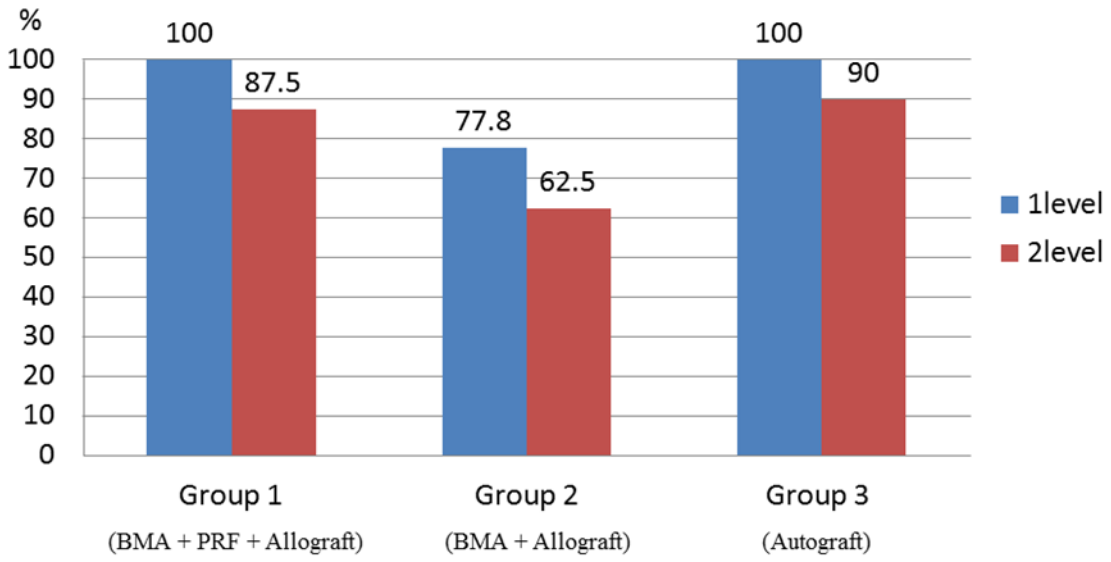

Figure 3-6 The one year fusion rate in 3 groups.

Discussion

Bone graft substitutes have caught the interest of spine surgeons over the last few years with the introduction of numerous potential alternatives. The significant potential limitations associated with the use of autogenous bone graft include significant donor site morbidity, longer rehabilitation times, and increase in operative time and blood loss. Preservation of posterior elements in TLIF procedure poses the additional challenges of a limited available quantity of autogenous bone grafts. The ability of red marrow to form bone in rabbits was first observed by Goujon in 1869[12]. In 1965, Urist observed that it was bone marrow that gave osteogenic properties to iliac crest bone [13]. Connolly et al, in 1989, showed that bone marrow osteogenesis correlated to the cell concentration [14]. This observation corroborated by Hernigou et al in the treatment of tibial nonunions [15]. Curlyo et al, in a rabbit model found that fusion rate of iliac crest bone mixed with peripheral blood was lower than that of iliac crest mixed with BMA; the rates were 25% and 61%, respectively [16].

In this study, we compared autologous iliac crest bone with a progenitor cell preparation in a human model. Results showed that allograft

combined with autologous BMA achieved good fusion rates in single and 2-level TLIF when compared with previous studies using allograft alone. Application of PRF further improved the results. The fusions rates of allografts with BMA and PRF were nearly equivalent to that of autologous iliac crest bone at 1 year follow-up.

Conclusion

TLIF using allograft combined with MBA and PRF is comparable with TLIF using autograft in terms of 1-year clinical outcomes and fusion rates with the additional benefits of less initial postoperative pain, less blood loss, earlier rehabilitation, and shorter hospitalization.

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Chapter 4 Using Percutaneous Endoscopic Outside-In Technique to Treat the Selected Patients with Refractory Discogenic Low Back Pain

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Abstract

Study Design
A prospective study and retrospective observation was performed on 24 consecutive patients with a minimum two year follow-up.

Objective
The purpose of this study is to evaluate the clinical results of percutaneous endoscopic treatment for annular tear in the selected patients with discogenic low back pain (DLBP) by using outside-in technique.

Background
Controversy usually remains to make the diagnosis of DLBP and locate the pain source of symptomatic disc in patients with DLBP. Various techniques, from minimally invasive procedure to fusion surgery, are used to treat chronic DLBP, but the clinical outcomes are variable. Percutaneous endoscopic discectomy by transforaminal or interlaminar approach is considered to be an effective method to treat DLBP, but the evidence is limited and that might be associated with patient selection and surgical technique.

Methods
Twenty-four consecutive patients with a single-level DLBP diagnosed by positive high intensity zone (HIZ) on MRI, positive provocative discography and block test underwent percutaneous endoscopic procedure from 2014 January to 2015 December. Transforaminal approach or interlaminar approach was selected according to the location of annular tear. The torn lesions were visualized directly and

treated by puncture and debridement of the inflammatory tissues from outer annulus fibrosus to inner nucleus using outside-in technique. The visual analogue scale score and Oswestry Disability Index score were evaluated before and after surgery. The clinical global outcomes were assessed on the basis of modified MacNab criteria.

Results
These patients included 13 males and 11 females with a mean age of 43.8 years (ranged between 32 and 55 years). There were 15 lesion levels at L4/L5 and 9 lesion levels at L5/S1. Among them, 15 levels were accessed by transforaminal approach and 9 levels by interlaminar approach. No serious complications were observed during the follow-up periods. All except 2 patients experienced significant symptomatic and functional improvements at 2 year follow-up with the success rate of 91.7%.

Conclusion
Percutaneous endoscopic procedure provides a safe and effective treatment for the selected patients with DLBP. Outside-in technique allows the surgeons to visualize and treat the torn or inflammatory lesions directly, and the success rate was high at 2 year follow-up.

Key words: transforaminal, interlaminar, outside-in technique, endoscopic discectomy, discogenic low back pain

Introduction

Discogenic low back pain (DLBP) is a complex medical problem and accounts for 26%-42% of the patients with chronic low back pain attributed to the annular tear of intervertebral disc [1]. The pathologic examination of discs from the patients with DLBP showed the formation of vascularized granulation tissue on the outer part of annulus fibrosus with extensive innervation in fissures extending into the nucleus pulposus. Magnetic resonance imaging (MRI) may identify a degenerative disc and an annular tear, but it cannot clearly differentiate between a pathologically painful disc and a physiologically aging disc.

High-intensity zone (HIZ) on the T2-weighted MRI is identified in the posterior annulus fibrosus [2]. Aprill and Bogduk reported high sensitivity (71%) and specificity (89%) rates for the correlation between HIZs and concordant pain during discography [3]. However, there was a controversy regarding the diagnostic value of HIZ because high prevalence (25%) of HIZ lesions occurred in asymptomatic individuals [4]. Signal intensity may account for such variabilities. The true HIZ defined as at least 50% bright as adjacent cerebrospinal fluid was considered to be a reliable marker for DLBP, suggesting high intensity signal rather than any white spot noted on MRI to represent annular tear [5].

Provocative discography, despite the invasiveness and high false positive rates (33-35%), still is the gold standard for diagnosis of DLBP [6]. The test is positive when presents contrast leakage and reproduces patients' concordant pain during the procedure. A study showed that positive provocative discography screening significantly improved surgical outcomes [7]. Combined MRI and discography may provide more information in patient selection for surgery. The evidence against the stand-alone use of discography was reported in selecting patients for fusion due to poor results [8]. A higher surgical success rate (75%) was noted in symptomatic patients with positive MRI and positive discography versus the lower rate (50%) in symptomatic patients with negative MRI and negative discography [9].
Fluoroscopically directed epidural injections with or without steroids under local anesthetics provide the short-term improvements in back and lower extremity pain for patients with DLBP [10]. A review showed that transforaminal epidural injection with steroid for DLBP results in 68% pain relief at 2 months, 56% at 6 months, and 59% at 12 months [11]. Therefore, epidural injection has been referred as a block test for diagnosis and predictive test for surgical prognosis of DLBP [12].

Percutaneous endoscopic lumbar discectomy (PELD) through foramen and thermal annuloplasty has been used to treat DLBP but the satisfaction varied widely among different studies attributed to both patient selection and surgical technique [13-15]. With the improvements of endoscopic instrument and technique, bony procedure such as foraminoplasty is performed to enlarge working space and the disk can be examined thoroughly. Percutaneous endoscopic interlaminar

approach provides another access to the L5-S1 disc, especially in patients with high iliac crest [16]. Based on the levels of disc and location of lesion, transforaminal or interlaminar approach is selected to visualize and manage the lesion comprehensively.

Patients and methods

The hypothesis of this study is that the pain sources of DLBP are the fissures or vascularized granulation tissues located on the annulus fibrosus and can be relieved by complete excision or debridement of those lesions. Concerning about the ‘outside-in’ technique, the endoscope does not enter into the disc initially after foramenoplasty, but stays on the surface of annulus to find out pathological area. This method manages the torn lesion precisely without disturbing the remaining healthy parts. On the contrary, the 'inside-out’ technique creates a cavity in the disc for viewing and manipulating endoscopic tools intradiscally for debridement [17]. This study was approved by the Research Ethics Committee of Dalin Tzu Chi General Hospital, Taiwan (IRB ID number: 10504004). All of the medical records were anonymous, and no patient information was extracted except for research purposes.

Patient selection

From 2014 January to 2015 December, there were 52 patients received MRI due to severe back pain without neurologic symptoms. True HIZs were detected in 42 patients. Among them, 36 patients underwent discography in which contrast medium leakage was detected in 32 patients and concordant pain was induced in 34 patients. Most patients (35 of 36) who received discography experienced some degree of back pain relief after epidural injection of local anesthesia but the period of relief varied from 3 days to 12 months. Among them, 24 patients including 11 women and 13 men met the criteria and underwent percutaneous endoscopic lumbar disc surgery with L4/5 being involved in 15 cases and L5/S1 in 9 cases (Table 1). Their age ranged from 32 to 55

years (mean 43.8years). The inclusion criteria involved: (1) chronic low back pain that failed at least 6 months of conservative treatment (including medication, exercise, and physical therapy), (2) HIZ on a single-level disc, (3) positive discography of contrast medium leakage and concordant pain, (4) positive transforaminal epidural injection test (at least short-term palliation of back pain after local epidural infiltration of anesthesia and steroid). The exclusion criteria were: (1) clinical manifestation of neurological deficits, (2) more than one level of involvement, (3) significant disk herniation, segmental instability, or spinal stenosis that resulted in lower extremity pain greater than low back pain, (4) spinal infection, tumor or fracture, (5) history of lumbar spinal surgery, and (6) coexisting psychological diseases.

Procedure and surgical technique

A 2-staged protocol was arranged for those patients with HIZ lesions.

1. Provocative discography and transforaminal epidural injection test

The purpose of the first stage is to make an accurate diagnosis. The procedure involves provocative discography and block test by transforaminal epidural injection. Discography was performed on the level with HIZ. Under fluoroscopy, a 22G needle was inserted and directed into the central nucleus of each intervertebral disc. One to 3 mL iohexol (Omnipaque; GE Healthcare, Piscataway, NJ) was injected slowly into the nucleus. Concordant pain was defined as provoked low-back pain of similar character, location, and intensity. Annular tear was proved when extravasation of radio-opaque contrast from disc was noted in fluoroscopy. The provocative discography was positive when both concordant pain and extravasation of contrast presented. Patients with positive discography were considered as real DLBP patients. After discography, the needle was withdrawn a short distance and its tip was placed just outside the annulus fibrosus. A 4 mL of mixture with 2 mL xylocaine (0.25%) and 80 mg triamcinolone acetonide was injected. The block test was positive when palliation of back pain occurred.
The second-staged surgery of outside-in technique was performed for

the patients who still suffered from back pain after a previous study showed positive findings on provocative discogram and block test. This procedure involved 2 steps: (1) percutaneous endoscopic discectomy by either transforaminal or interlaminar approach, depending on the level of disc and location of HIZ, and (2) endoscopic thermal annuloplasty with bipolar coagulator.

2. Percutaneous endoscopic discectomy and annuloplasty in the outside-in technique

Transforaminal approach

The surgery is performed under local anesthesia in the prone position. The skin entry point and the angle of trajectory were decided by pre-operative planning according to the location of HIZ. After local anesthesia, an 18-gauge spinal trocar punctured the disc and 2 mL methylene blue was injected to dye the nucleus pulposus and the displaced fragment blue. A guide wire was inserted through the puncture needle. A dilator was then inserted and docked on the facet joint. Next, an 8-mm working channel was inserted and stayed outside the disc. An endoscope (SPINEDOS GmbH, Germany) was then inserted. Foraminoplasty was applied in some cases to enlarge the working space by removal of some bony tissue using reamer, Kerrison punch or high-speed burr. The entire annulus was examined and the lesion of annular tear could be detected after thorough examination or palpation with a probe. The lesion was debrided with punch. Some debris and embedded disc material were removed with disc grasp through the hole of tear. We tried to make the torn lesion as small as possible for prevention of possible subsequent herniation. After intradiscal debridement and decompression, a thermal annuloplasty around the lesions was performed to make the size of tear smaller. Patients are observed for 6 to 12 hours postoperatively before discharge.

Interlaminar approach

The surgical procedure is performed under general anesthesia. The entry point was targeted on the superolateral corner of interlaminar window. After a small skin and fascia incision, a dilator was introduced and docked to the lateral edge of the interlaminar window. A working sheath was introduced through the dilator and the final position was checked by

the fluoroscope. The surgery was performed after introducing an endoscope (SPINEDOS GmbH, Germany) to incise ligamentum flavum 3-5 mm, and went to the epidural space. A little bony structure including lamina or facet joints was resected to create an enough working space. With nerve hook to probe the nerve root shoulder, the neural structures were then retracted medially and protected by rotating the beveled opening inwards to expose the disc clearly [16]. The inflammation tissues were debrided, the embedded disc fragments were removed, and thermal annuloplasty was performed. Wound closure was performed after endoscope removal.

Outcome assessment and statistical analysis

Outcome was evaluated by visual analogue scale (0-10) scores for back pain and Oswestry Disability Index (0-100) scores for functional disability. The modified MacNab criteria were used for clinical global outcome assessment. Patients were asked to complete these questionnaires at pre-operation, and 2 weeks, 1 month, 3 months, 6 months, 12 months and 24 months post-operatively. Pre-op and post-op scores in VAS, ODI and Modified MacNab criteria were compared by Wicoxon Signed-rank test. Results were considered to be statistically significant if the P value was less than 0.05. Statistical analysis was performed using the SPSS 13.0 software (SPSS Inc, Chicago, IL).

Results

General information

Twenty-four patients with 11 (45.8 %) female and 13 (54.2%) male were enrolled in the present study to manage 15 lesion levels (62.5 %) at L4/L5 and 9 lesion levels (37.5 %) at L5/S1. Patient age averaged 43.8 years (range, 32-55) at surgery. The mean duration of pain was 3.2 years (range, 1.5–20 years). Comorbidity included 4 (16.7 %) smoker and 3 (12.5%)

diabetes. The averaged skin-to-skin duration was 65.2 minutes (range, 55-80 minutes), and blood loss was minimal. On average, the hospital stay was 1.2 days. No patients encountered major complications such as wound infection or nerve root injury (Table 4-1).

Patient follow-up

Of the total 24 patients undergoing percutaneous endoscopic surgery, 2 patients still suffered from back pain within 1 year after operation. One patient underwent fusion surgery under the impression of segmental instability, and the other patient underwent repeated percutaneous endoscopic surgery on the same level. Both of them improved back pain after additional surgery. VAS scores, ODI scores, and Modified MacNab Criteria were available for all patients preoperatively and at least 24 months follow-up. VAS and ODI scores significantly improved from preoperative baseline to the times at 2 weeks, 1 month, 3 months, 6months, 12 months and ≥24 months post-surgery ($p<0.05$)(Fig. 4-1).

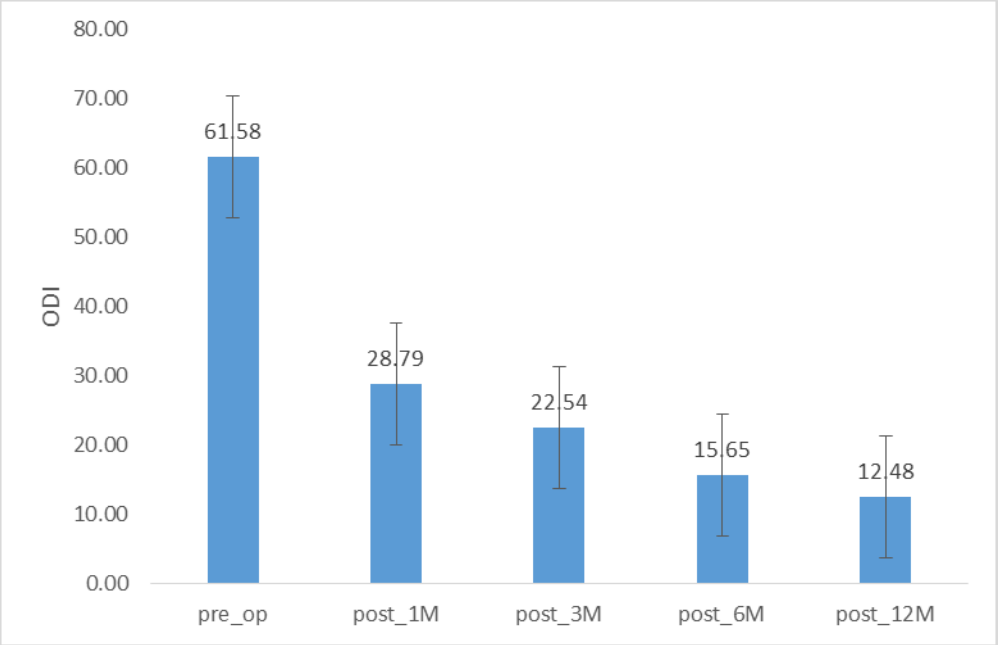


Figure 4-1 Pre-operative and postoperative ODI. *P<0.001, compared by Wicoxon Signed-rank test.

Patient demographics and outcome results between male and female group are summarized in Table 4-2. Modified MacNab criteria were used to assess the overall outcomes and the success rate (excellent and good)

was 87.5% (21/24) at one-month and 91.7% (22/24) at 3-month, 6-month and more than one-year follow-up. The 2 patients who underwent additional operations were rated poor (Table 4-3).

Table 4-1 ***Demographic data of 24 patients with HIZ lesion***

	Number	Median	Range
Gender			
Male	13 (54.2%)		
Level			
L4/5	15 (62.5%)		
L5/S1	9 (37.5%)		
Age (year)	24	43.8 ±7.58	32-60
VAS			
Pre-op	24	6.83 ±0.86	5-8
Post_2week	24	3.42 ±.081	2-5
Post_1month	24	3.17 ±0.70	2-5
Post_3month	23	2.88 ±0.79	1-4
Post_6month	23	1.96 ±0.70	1-3
Post_12month	22	1.50 ±0.59	0-1
ODI			
Pre-op	24	61.58 ±5.37	51-72
Post_1month	24	28.79 ±5.54	22-50
Post_3month	23	22.54 ±7.33	13-45
Post_6month	23	15.65 ±4.89	11-35
Post_12month	22	12.48 ±2.33	10-20

VAS: visual analogue score, ODI: Oswestry disability index

Table 4-2 ***Comparison between groups***

	Female (n=11)			Male (n=13)			
	Median	SD	95%CI	Median	SD	95%CI	P-value
Age	42.00	6.10	40.4-48.6	40.00	8.84	37.8-48.5	0.494
VAS							
Pre_op	7.09	0.83	6.53-7.65	6.62	0.75	6.09-7.14	0.228
Post_2week	4.00	1.07	2.85-4.24	3.00	0.75	2.85-3.76	0.569
Post_1month	3.00	0.90	2.67-3.88	3.00	0.49	2.78-3.38	0.649
Post_3month	3.00	1.00	2.33-3.67	3.00	0.59	2.41-3.13	0.733
Post_6month	2.20	0.78	1.64-2.76	1.77	0.59	1.41-2.13	0.257
Post_12month	2.00	0.56	1.49-2.31	1.00	0.38	0.92-1.41	0.009 *
ODI							
Pre_op	62.00	6.78	57.26-66.37	60.00	4.11	58.90-63.87	0.531
Post_1month	30.00	7.13	26.12-35.70	27.00	3.00	25.19-28.81	0.106
Post_3month	26.00	9.23	18.53-30.93	21.00	4.88	17.74-23.65	0.361
Post_6month	14.50	3.28	12.75-17.45	14.00	5.95	12.48-19.67	0.832
Post_12month	12.00	1.77	11.13-13.67	12.00	2.75	10.87-14.20	0.832

*P<0.05, compared by Mann-Whitney U test.

Table 4-3 Surgical outcome assessment using Modified MacNab Criteria

Grade	2 Weeks	1 Month	3 Month	6 Month	≥ 12 Months
	Pt (%)	Pt (%)	Pt (%)	Pt (%)	Pt (%)
Excellent	4 (16.7)	6 (25.0)	15 (62.5)	17 (70.8)	18 (75.0)
Good	17 (70.8)	15 (62.5)	7 (29.2)	5 (20.8)	4 (16.7)
Fair	3 (12.5)	3 (12.5)	1 (4.2)	1 (4.2)	0 (0)
Poor	0 (0)	0(0)	1 (4.2)	1 (4.2)	2 (8.3)

Case report 1

A 51-year-old male labor suffered from low back pain for more than 5 years. T2-weighted MRI showed degeneration of L4/5 disc and HIZ in sagittal and axial images (Fig 4-2-1). Provocative discography showed contrast leakage (Fig. 4-2-2) and concordant low back pain, which could be relieved by L4/5 transforaminal epidural steroid injection. He experienced another episode of severe low back pain 3 months later, and underwent percutaneous endoscopic surgery on the basis of outside-in technique. The transforaminal approach was selected and an entry point was made about 12 cm from the midline. The endoscopic views were shown in fig. 3, and histology disclosed the penetration of vessels to the inner annulus and nucleus in fig. 4. The DLBP resolved within 1 month of surgery. He returned to work 3 months after surgery without recurrence at 24 months follow-up.

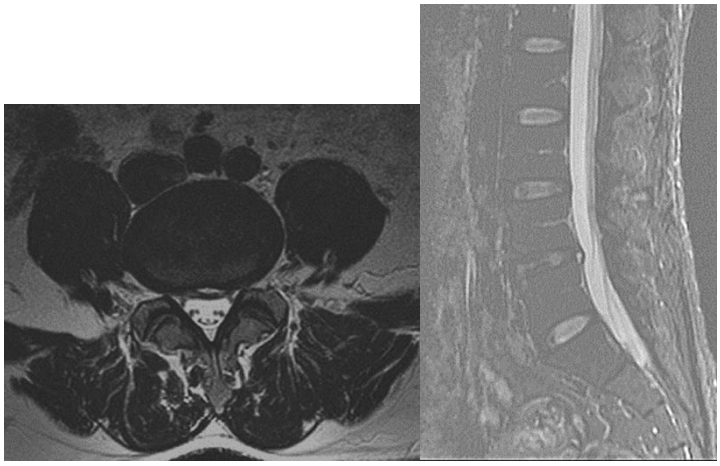


Figure 4-2-1 Sagittal and axial images on T2-weighted MRI showed HIZ in L4/5 disc.

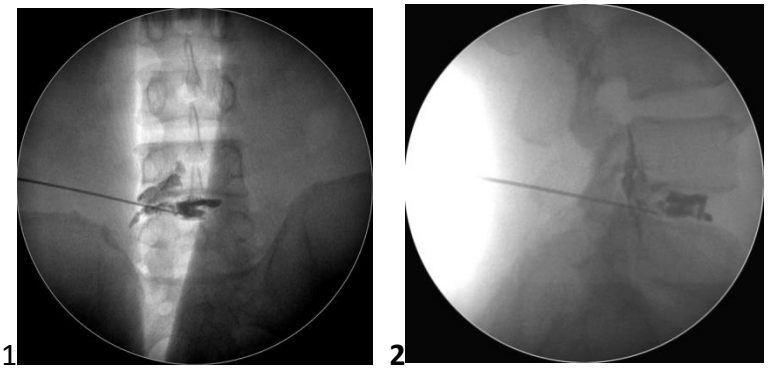


Figure 4-2-2 Discography showed leakage of contrast media. The patient reported concordant back pain, which was completely relieved by transforaminal epidural injection.

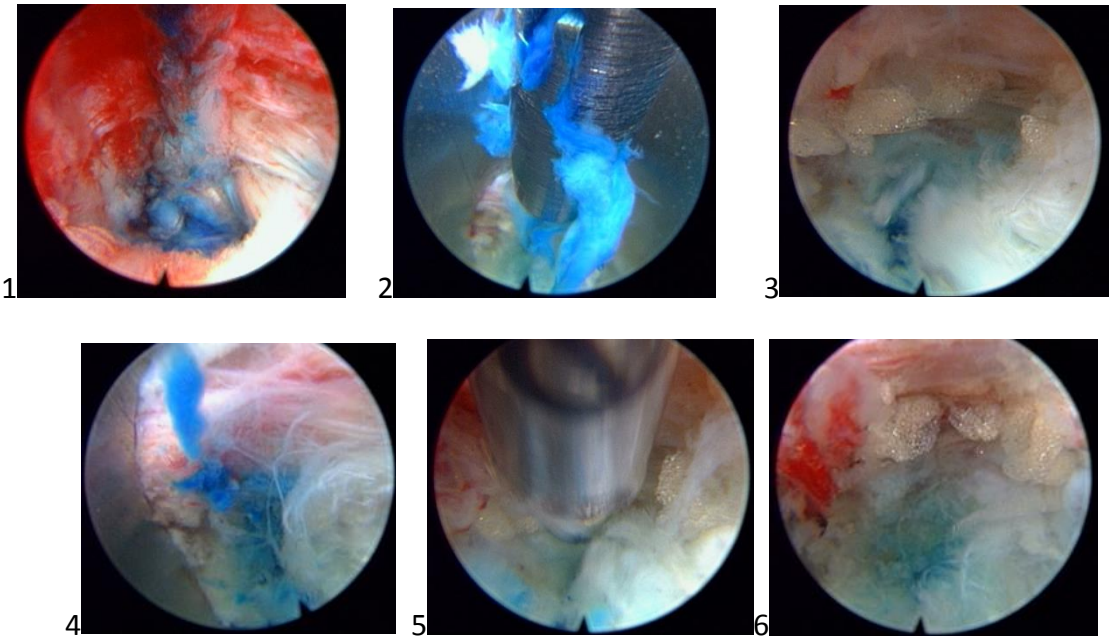


Figure 4-3 Endoscopic views during surgery.

Fig. 4-3-1: The endoscope was placed on the outer surface of annulus to visualize the lesion of annular tear and the migrated NP dyed blue.

Fig. 4-3-2: The granulation tissues, loose fragments and displaced NP were removed with a punch.

Fig. 4-3-3: The hole of annular tear was not enlarged during procedure. Loose fragments of disk material were removed through the original tear lesion.

Fig. 4-3-4: The annular tear became evident after debridement.

Fig. 4-3-5: The tear site was ablated with a radiofrequency coagulator.

Fig. 4-3-6: The size of annular tear became smaller after thermal abrasion.

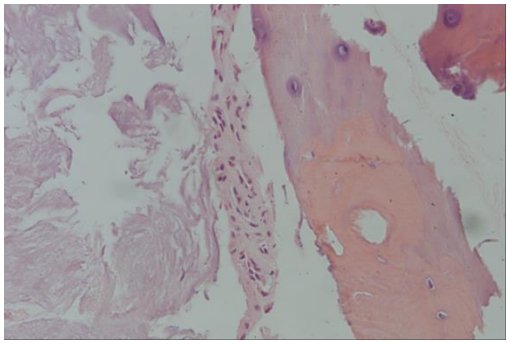


Figure 4-4 Histological findings: Hematoxylin and eosin staining showed penetrating vessels between AF and NP. (200 x)

Case report 2

A 31-year-old male truck driver suffered from chronic low back pain for more than 10 years. T2-weighted MRI showed HIZ in L5/S1 disc (Fig. 4-5). Provocative discography and block test were positive. The interlaminar approach was selected because of the high iliac crest and transforaminal approach might not reach the lesion in L5/S1 (Fig. 4-6). Endoscopic views were illustrated in Fig.4- 7, and histology disclosed the infiltration of vessels in annulus fibrosus (Fig. 4-8). His back pain subsided within one month after surgery. He started to drive a truck 2 month later, and did well at 18 months follow-up.

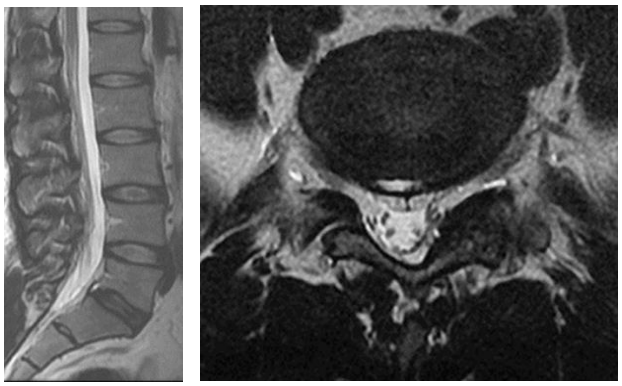


Figure 4-5: HIZ was obvious on posterior area of L5/S1 disc.

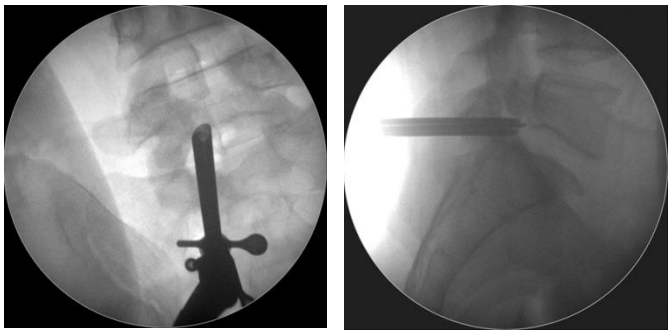
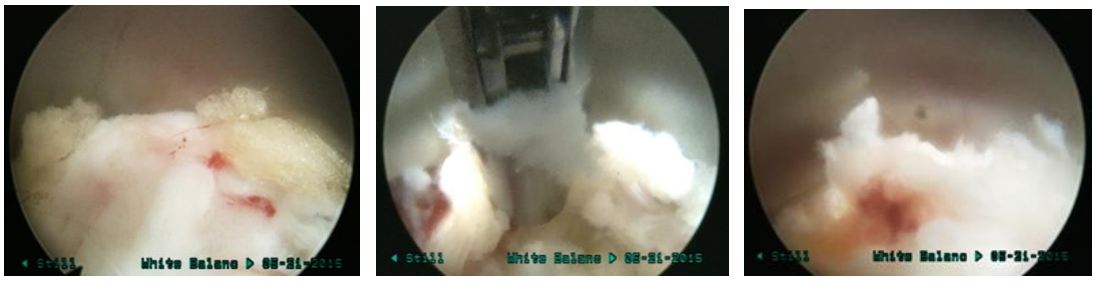
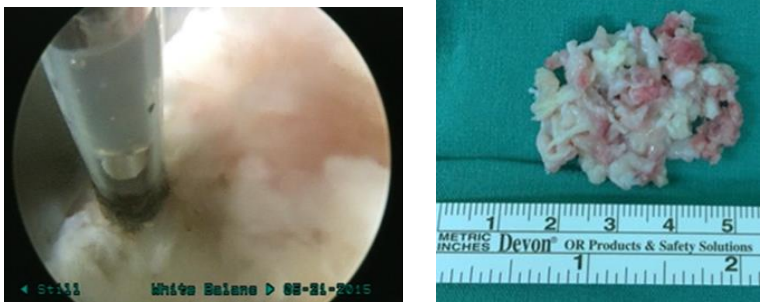


Figure 4-6: Interlaminar approach was selected for the L5/S1 lesion.



7-1 7-2 7-3



7-4 7-5

Figure 4-7: **Endoscopic views and the removed disc material during surgery.**

Fig. 4-7-1: The lesion was covered with vessels and red granulation tissues.

Fig. 4-7-2: Those inflammatory tissues were removed with a punch.

Fig. 4-7-3: The vessels and red granulation tissues also could be found on the inner layer of annulus and in NP suggesting penetration of new vessels into the annulus and NP.

Fig. 4-7-4: The lesion was ablated with a radiofrequency coagulator.

Fig. 4-7-5: The removed disc material appeared slightly red in color.

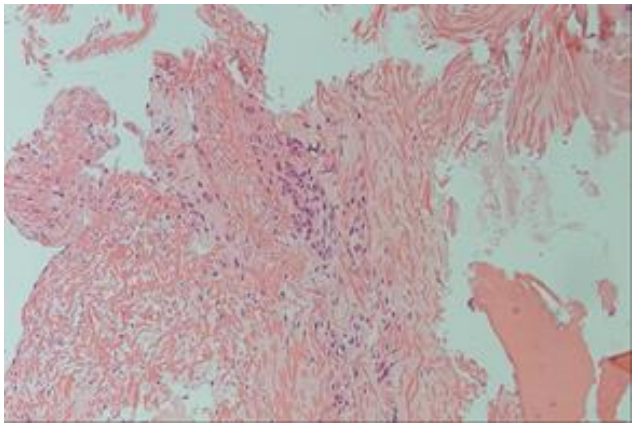


Figure 4-8 Hematoxylin and eosin staining show infiltration of vessels in outer and inner layer of annulus fibrosus (100X).

Discussion

DLBP is a very challenging condition to treat because the diagnosis is really controversial to begin with. Diagnostic protocol should be helpful to make an accurate level. High intensity signal rather than low or medium intensity signal is the reliable marker in MRI for diagnosis of DLBP. The true HIZs should be recognized as at least 50% bright as CSF. Provocative discography still is the gold standard to make the diagnosis, but the technique is concerned due to the invasive nature and associated complications such as discitis or disc degeneration [18]. In this study, after the screening of MRI, discography was performed on the target level only and the procedure-related complications should be declined. Concordant pain induced by provocative discography, and relived by injection of local analgesic and steroid provided a clear clue to localize the pain source and predicted the prognosis of treatment. Therefore, a combination of positive MRI findings, positive discography and block test may increase the accuracy of diagnosis for DLBP.

The treatment for DLBP traditionally has been limited to either conservative management or lumbar fusion surgery. Lumbar fusion is the surgical procedure of choice in patients who failed aggressive nonsurgical program but the results varied considerably in different studies [19-21]. The surgical invasiveness and complications after fusion should be considered. Persistent symptoms may be associated with patient selection and approach-related muscle denervation, disruption of the facet joints or pseudoarthrosis. Over the past decade, many percutaneous techniques such as intradiscal electrothermal therapy, annuloplasty, nucleoplasty and percutaneous disc decompression have been advocated to treat DLBP [22, 23]. These procedures aimed at altering the internal mechanics or innervation of the disc, but limited or poor evidence existed for their benefits. Randomized trials did not support the effectiveness of percutaneous intradiscal thermal procedure for the treatment of DLBP [24].

Percutaneous endoscopic treatment for chronic DLBP has been reported since 1991 but the success rates ranged from 41.6% to 91% in different studies [13, 14, 25]. Patient selection and surgical technique should be

the reasons. Transforaminal inside-out technique was widely used in previous studies. In the inside-out technique, a needle was inserted to the disc directly and a cavity in the disc must be created for viewing and manipulating the endoscopic tools intradiscally. The annular tear will be visualized after removing disc material inside the disc followed by cutting the annulus and changing trajectory. The healthy part of annulus and nucleus may be damaged. Besides, the lesion when located on central part is not easy to deal with by inside-out technique. On contrary, an outside-in technique can be accessed directly in a shallow trajectory and targeted below the facet with the landing of instruments being located outside the annulus. For visualizing the entire annulus, access can be dilated by foraminoplasty. Percutaneous interlaminar approach provides another access for L5-S1 disc level especially in patients with high iliac crest. The outside-in technique used in either transforaminal or interlaminar approach allows the surgeons to visualize the annulus thoroughly, ablate aberrant nerve endings, and remove inflammatory tissues clearly.

In this study, we detected the inflammatory granulation tissues or annular defect showing the true HIZ on MRI in every patient. After adequate debridement of inflammatory tissues, all 24 patients experienced relief in back pain to some degree. Only 2 patients received additional surgery within one year; of whom one underwent fusion surgery under the impression of segment instability, and the other one repeated endoscopic surgery due to recurrence of DLBP. According to the pre-op and post-op scores in VAS, ODI and Modified MacNab outcome assessment for back pain and functional activity, 22 in 24 patients achieved significant improvements at 24 months follow-up.

Conclusion

Combination of clinical symptoms, true HIZ, positive discography and block test provided strict criteria to make the accurate diagnosis of DLBP. Patients who met the above selection criteria received percutaneous endoscopic management could experience symptomatic and functional improvements at short term and two-year follow-up. The outside-in technique allows the surgeon to visualize and treat the lesion directly.

The significant limitation of this study is its nonrandom format and small sample size. Future research may focus on the controlled prospective studies with larger sample sizes and long term follow-up to examine the validity of this protocol.

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Chapter 5 Intradiscal Application of Autologous Platelet-Rich Fibrin (PRF) Following Endoscopic Discectomy to Treat Discogenic Low Back Pain

Abstract

Study Design: Prospective clinical trial.

Back Ground: Controversy exists in the treatment of discogenic low back pain (DLBP). Various techniques, from minimally invasive procedures to fusion surgery, are used to treat chronic DLBP, but the clinical outcomes are variable. Percutaneous endoscopic discectomy by transforaminal or interlaminar approach is considered to be an effective method to treat DLBP. PRF, which is composed of autologous growth factors and cytokines, has been widely used in the clinical setting for tissue regeneration and repair.

Objectives: To determine the safety and initial efficacy of intradiscal application of autologous platelet-rich fibrin (PRF) following full endoscopic discectomy in patients who were diagnosed with discogenic low back pain.

Methods: Inclusion criteria for this study included patients who underwent full endoscopic discectomy for discogenic low back pain. PRF was inserted into the nucleus pulposus following endoscopic procedure. Outcome measures included the use of a visual analog scale (VAS) and Oswestry disability index (ODI), as well as X-ray and MRI (T2-quantification).

Results: Data were analyzed from 6 patients (3 man and 3 women; mean age, 40.2 years). The average follow-up period was 12 months. Following treatment, no patient experienced adverse events or significant narrowing of disc height. The mean pain scores before treatment (VAS, 6.8±1.1; ODI, 60.0±10.1) were significantly decreased at one month, and

this was generally sustained throughout the observation period (6 months after treatment: VAS, 2.1±1.4, ODI; 13.7±4.5 and 12 months: VAS, 1.8±2.8; ODI, 11.5±3.8; $p < 0.01$, respectively). The mean T2 values did not significantly change after treatment.

Conclusions: Intradiscal insertion of autologous PRF following endoscopic procedure in patients who were diagnosed with discogenic low back pain is safe, with no adverse events observed during follow-up. The endoscopic procedure is to debride and remove inflammatory tissues of intervertebral discs (IVDs). Application of PRF after endoscopic discectomy might provide additional effects to promote regeneration of IVDs. Future randomized controlled clinical studies should be performed to systematically evaluate the effects of this therapy.

Introduction

Low back pain is a complex medical problem and significantly associated with lumbar disc degeneration [1]. Intervertebral disc degeneration is clinically characterized by decreased signal intensity on T2-weighted magnetic resonance imaging (MRI). Biochemical characteristics of degenerated IVDs include degradation of the extracellular matrix, with loss of proteoglycan and water content in the nucleus pulposus (NP) and collagen degeneration in the annulus fibrosus (AF). These degenerative changes may lead to internal disc disruptions, including radial and circumferential tears, cracking, and fissuring, all of which are associated with pain sources [2]. The degenerated IVD has limited potential for self-repair because of poor blood flow and lack of IVD cells [3]. Abnormal tissue remodeling processes identified with disc disruptions, including the ingrowth of vascularized granulation tissue and nociceptive nerve fibers are implicated in causing pain [4]. The pathomechanism of discogenic low back pain is generally attributed to IVD degeneration and tear of AF, which involves the enhanced expression of proinflammatory cytokines, neurotrophins, and the formation of internal disc disruptions [5]. In response to cellular activation, platelets can release the contents of intracellular granules, which include growth factors, coagulation proteins, adhesion molecules, cytokines, and inflammatory molecules,

which are known to promote wound healing [6]. Platelet-rich plasma (PRP), which contains autologous growth factors and cytokines, has been widely used clinically for tissue regeneration and repair [6-8]. PRP has also recently been experimentally applied to degenerated IVDs to enhance self-repair [9]. However, intradiscal injection of PRP and its effect on disc regeneration has been met with controversy [10-12] because of the possibility that the injected PRP might release growth factors or cytokines in an unstable manner and at different rates [9]. In vitro and in vivo studies has shown that the soluble releasate isolated from PRP (PRP releasate) stimulated matrix metabolism of porcine IVD cells [13]. Platelet-rich fibrin (PRF) is a new generation of platelet concentrate, which was first developed in France by Choukroun et al. in 2001 as an autologous biomaterial [14]. PRF contains a fibrin matrix polymer, blood aggregates, leucocytes and cytokines as well as the involvement of circulating stem cells. PRF is obtained from autologous peripheral blood by centrifugation, without adding any biological agents. Compared with PRP, PRF is produced with a simpler method, lower in cost and more easily available. PRF has been applied in many various clinical fields, particularly oral and maxillofacial surgery, plastic surgery, as well as orthopedics. Therefore, we hypothesized that intradiscal application of PRF exerts a direct stimulatory effect on the repair of degenerated discs. The aim of our study was to evaluate the safety and initial efficacy of intradiscal application of autologous PRF following endoscopic procedure for treat discogenic low back pain.

Materials and Methods

1. Study design

This study was a prospective clinical feasibility study, primarily a safety assessment, conducted from January 2016 to June 2016.

2. Patients

Patients who underwent full endoscopic lumbar discectomy for discogenic low back pain from January 2016 to June 2016 were recruited. Among 10 patients who were diagnosed with DLBP and underwent endoscopic procedure, 6 were included in this study. Inclusion criteria for this study were age between 20 and 55 years and having (1) chronic low back pain without leg pain for more than 3 months, (2) single lumbar disc (L3/L4 to L5/S1) with evidence of degenerative changes or tear of AF noted in MRI (disc degeneration was defined as more than grade III via the Pfirrmann disc degeneration grade/classification system [15], and (3) only one symptomatic disc confirmed using standardized provocative discography and disc block. Exclusion criteria included neurological symptoms resulted from lumbar spinal stenosis or spondylolisthesis and inflammatory arthritis.

3. Diagnosis of discogenic low back pain

Provocative discography and subsequent disc block were performed to diagnose discogenic low back pain as previously reported [16]. Discography was performed on the level with the high intensity zone (HIZ). Under fluoroscopy, a 22-gauge needle was inserted and directed into the central nucleus of each intervertebral disc. One to 3 mL iohexol (Omnipaque; GE Healthcare, Piscataway, NJ) was injected slowly into the NP. Concordant pain was defined as provoked low back pain of similar character, location, and intensity. Annular tear was proved when extravasation of radiopaque contrast from the disc was noted during fluoroscopy. The positive provocative discography was recognized as a real DLBP when both concordant pain and contrast extravasation presented. After discography, the needle was withdrawn a short distance and its tip was placed just outside the annulus fibrosus. A 4-ml mixture of 2 mL xylocaine (0.25%) and 80 mg triamcinolone acetonide was injected. The block test was positive when palliation of back pain occurred.

4. Full endoscopic discectomy and annuloplasty

The full endoscopic surgery using the outside-in technique was performed for the patients who still suffered from back pain after a previous study showed positive findings on the provocative discogram and block test. This procedure involved 2 steps: (1) full endoscopic discectomy by either transforaminal or interlaminar approach, depending on the level of disc and location of the HIZ; and (2) endoscopic thermal annuloplasty with bipolar coagulator.

Transforaminal approach

The surgery was performed under local anesthesia in the prone position. The skin entry point and the angle of trajectory were decided by preoperative planning according to the location of the HIZ. After local anesthesia, an 18-gauge spinal trocar was punctured into the disc and 2 ml of methylene blue was injected to dye the nucleus pulposus and the displaced fragment blue. A guide wire was inserted through the puncture needle. A dilator was then inserted and docked on the facet joint. Next, an 8-mm working channel was inserted and stayed outside the disc. An endoscope (SPINENDOS GmbH, Germany) was then inserted. Foraminoplasty was applied in some cases to enlarge the working space by removal of some bony tissue using reamer, Kerrison punch, or high-speed burr. The torn annulus could be found by visualization and palpation; it was usually bulging and surrounded by reddish inflammatory tissues or vessels. The lesion surface was soft, thin, and loose, and could be distinguished from an intact annulus by palpation with a probe. The stained blue material representing NP debris was visible around the torn annulus when the tear was complete or when the outer surface became thinned. When the tear of the annulus was not complete, the stained blue NP was clearly visualized after debridement and removal of the torn annulus. Some debris and embedded disc material were removed with a disc grasp through the hole of the tear. We tried to make the torn lesion as small as possible for prevention of possible subsequent herniation. The thermal annuloplasty was performed after the torn annulus was debrided and the granulation tissues and displaced NP were removed. A bipolar probe was applied to the outer surface of the annulus adjacent to the torn lesion for

hemostasis and ablation of inflammatory soft tissues. The annular defect could be altered physically and became smaller in size by thermal effect.

Interlaminar approach

The surgical procedure was performed under general anesthesia. The entry point was targeted on the superolateral corner of the interlaminar window. After a small skin and fascia incision, a dilator was introduced and docked to the lateral edge of the interlaminar window. A working sheath was introduced through the dilator and the final position was checked by the fluoroscope. The surgery was performed after introducing an endoscope (SPINENDOS GmbH, Germany) to incise the ligamentum flavum 3-5 mm and enter the epidural space. A little bony structure, including lamina or facet joints, was resected to create enough working space. With a nerve hook to probe the nerve root shoulder, the neural structures were then retracted medially and protected by rotating the beveled opening inwards to expose the disc clearly. The inflammation tissues were debrided, the embedded disc fragments were removed, and thermal annuloplasty was performed. Wound closure was performed after endoscope removal.

5. PRF preparation

Peripheral blood 10 ml was collected by use of butterfly needles. To prepare the PRF, the blood samples were immediately centrifuged at 2400 rpm (400 x g) for 12 min. The resulting PRF preparation was picked up with forceps, and the red thrombus (the fraction of red blood cells) was eliminated with scissors along the interface. The PRF was transferred into intervertebral disc space anteriorly after full endoscopic discectomy procedure.

6. Procedure for application of PRF

PRF was applied just after procedure of full endoscopic discectomy and decompression was complete. The PRF was inserted to NP with grasp through the torn lesion of AF under endoscope. The insert position of PRF was near the center of NP to prevent it outer migration from the

torn lesion and might lead to compression on neural structures.

7. Efficacy assessment

The efficacy of this treatment was assessed by a visual analog scale (VAS) [17] for back pain and the Oswestry Disability Index (ODI) [18] for back pain-related disability at baseline and at 1, 3, 6 and 12 months after the treatment. A neurological assessment, including motor strength, sensory function, and reflexes, was also performed at the same time points.

8. Radiographic evaluation of the lumbar spine

Lateral lumbar spine radiographs of each patient were taken centered on the L3 vertebrae in the standing position before and after treatment as well as every second month until the end of the study. The anterior and posterior heights and the depths of the intervertebral discs were measured, and disc height index (DHI) was calculated as previously reported [19]. The % DHI was calculated as the rate of change in DHI compared to the baseline [(DHI at follow-up–DHI at baseline)/DHI at baseline] ×100%. The lumbar lordosis angle (the angle between the planes of the superior end plate of the first lumbar vertebra and the superior line of the sacrum) was also determined.

9. MRI analysis

MRI analysis was performed before treatment and at 12 months after treatment. MRI was performed using a 3.0-Tesla scanner with a SENSE-Spine coil. Sagittal T2-weighted (time to repeat, or TR, of 2,500 milliseconds; time to echo, or TE, of 90 milliseconds) fast spin-echo images were used to classify lumbar discs into five grades of degeneration using the Pfirrmann disc degeneration grading scheme [15]. Quantitative T2 mapping was performed using a multiecho spin-echo

sequence in the sagittal plane. Scanning parameters were TR=2,500 milliseconds; TE=15 to 300 milliseconds (15 TEs); field of view=280 mm; slice thickness= 4 mm; image matrix=512×512; number of excitations= 1; and total scanning time=7 minutes and 20 seconds. To automatically segment and analyze regions of the NP usually unapparent in degenerate discs, a template based method [20] was used. Briefly, MRI data from cadaveric human spines containing grade 1 discs (n=11) with visibly distinct NP regions were analyzed using Otsu's method [21] to threshold and segment NPs. Individual masks of the NP and whole disc were registered (rigid body with scaling) and averaged to create a single template. The template was applied to all discs by affine registration of the template to match the mask of the target discs. The registered template was applied to T2 maps to determine average T2 values of the NP and AF from each disc. To reduce variability between patients, all T2 values were normalized to the values at L3/4, which were usually much less degenerate compared to the injected levels. Among the 6 patients, quantitative T2-mapping analysis was performed in 4 patients who provided consent.

10. Safety assessments

The safety of this treatment was evaluated in terms of neurological changes; radiological examination, including changes in disc height; the lumbar lordosis angle; and the MRI T2 value. The presence or absence of adverse events associated with this treatment was also evaluated throughout the follow-up period.

11. Outcome assessment and statistical analysis

Outcomes were evaluated using the VAS (0-10) scores for back pain and ODI (0-100) scores for functional disability. The modified MacNab criteria were used for clinical global outcome assessment. Patients were asked to complete these questionnaires at pre-operation, and 2 weeks, 1 month, 3 months, 6 months, and 12 months postoperation. Preoperative and postoperative scores on the VAS, ODI, and modified MacNab criteria

were compared using the Wilcoxon signed-rank test. Results were considered to be statistically significant if the P value was less than .05. Statistical analysis was performed using SPSS Version 13.0 (SPSS Inc., Chicago, IL).

Results

1. Patient population

Data were analyzed from 6 patients (3 men and 3 women; mean age, 40.2 years; age range, 35-47 years). Six discs were studied in this study; patient characteristics are summarized in Table 1. All 6 patients were available for the follow-up examination at 1, 3, and 6 months, and for the final examination at 12 months. The targeted discs were L4/L5 (in 4 cases) and L5/S1 (in 2 cases). The Pfirrmann disc degeneration grade was 3 in 4 patients and grade 4 in 2 patients. All 6 patients underwent single procedure of full endoscopic lumbar discectomy. During the follow-up period, 1 patients temporarily used NSAIDs (16.7%).

2. Measures of efficacy (VAS and ODI scores)

More than 50% reduction of LBP, as evaluated by VAS scores, was observed in 83% (5/6) of patients within four weeks after procedure; this was generally maintained throughout the observation period. The mean pain score (VAS) significantly decreased after procedure (baseline, 6.8; 1 month, 3.3; 3 months, 2.8; 6 months, 2.2; 12 months, 1.8; all $p<0.01$ vs. baseline) (Fig. 5-1). Improvement in physical disability scores (ODI scores) was relatively better than that in VAS scores. Eighty-three percent of patients (5/6) showed a significant reduction (more than 50%) in ODI scores 3 months after procedure. This was maintained for 12 months after treatment. The mean ODI score significantly decreased after injection (baseline,60.0; 3 months, 17.2; 6 months, 13.7; 12 months, 11.5; all $p<0.01$ vs. baseline) (Fig. 5-2).

3. Radiographic assessment

For all patients, their lumbar lateral radiographs showed no significant progression of disc height narrowing and ossification of the targeted disc following the procedure (Fig. 5-3). The % DHI of targeted discs and the control (L3/L4) discs had similar changes during the follow-up period. Analysis of variance revealed no significant interaction between disc treatment and time point. In addition, the angle of lumbar lordosis (L1–S angle) did not change significantly during the evaluation period (Fig. 5-4).

4. Quantitative MRI assessment

Sagittal T2 maps suggested no significant changes in the normalized T2 value of the NPs between pre-operation and the follow-ups in all six patients (Fig. 5-5).

5. Adverse events

None of the patients exhibited neurological deterioration or symptoms of discitis. No other adverse events associated with treatment were observed.

Discussion

In this study, we evaluated the safety and initial efficacy of intradiscal insertion of PRF for patients with discogenic low back pain after full endoscopic discectomy. No apparent adverse effects were identified during the follow-up period. Treatment with endoscopic procedure significantly improved low back pain as evaluated via VAS and ODI scores. Insertion of PRF further improved the results. Radiographic analyses, including lumbar radiography or MRI, showed no significant changes after PRF insertion compared to baseline. PRP has been used to treat

musculoskeletal pathologies [6], and particularly, there have been recent applications in orthopedic surgery, especially regarding sports-related injuries [7, 8]. Although a number of basic research studies have shown the stimulatory effect of PRP on tissue repair, there has been a wide range of outcomes among clinical studies. To note, the variability in PRP preparation is considered a primary, potential cause of the differential clinical effects of PRP. Classically, for the preparation of platelet concentrates in transfusion, the two major methods for isolating platelets from whole blood are the platelet sedimentation (PS) and (buffy-coat) BC methods [23]. Principally, both methods consist of a two-stage centrifugation; however, the order of soft and hard spins is different between the two methods. Platelet concentrates isolated from the BC method (hard to soft spins) are less activated during the preparation process [24] and contain fewer leucocytes [25], and thus, lower concentrations of proinflammatory cytokines compared to those found using the PS method [23]. The PRP preparation process by BC method is classified as pure PRP" (P-PRP), which contains fewer leucocytes, and is usually utilized for isolating autologous PRP for intradiscal injection therapy [8, 26]. The reparative effect of PRP itself with gelatin hydrogel has been demonstrated in pre-clinical animal studies [11, 12]. A prospective, randomized, and controlled clinical study to evaluate the effectiveness of intradiscal injection of autologous PRP has recently been reported [27]. Chronic low back pain patients who received intradiscal PRP showed significant improvement in pain and function scores. Activated platelets are known to release the stored intercellular mediators and cytokines from three types of granules: alpha granules, dense granules, and lysosomes [28]. Platelet secretion is dependent on the intensity of activation stimuli (energy-dependent) because lysosomal secretion needs the greatest stimulation, while alpha and dense granules require less stimulation [28]. On the other hand, in this study, PRF was inserted into the targeted degenerated discs. Compared to PRP, PRF eliminates the redundant process of adding anticoagulant as well as the need to activate it. For PRF, current data show that there is a differential distribution of red blood depending on the centrifugal force used. The clinical efficacy of different centrifugation protocols however still need to be independently validated with controlled clinical trials. In vitro studies showed that a longer centrifugation protocol (2,700 rpm) produces a denser (stronger) fibrin

clot with less inter-fibrous space containing less cells compared to the shorter centrifugation protocol of A-PRF (1300rpm) that produced a less dense fibrin clot with a looser inter-fibrous structure containing more cells. Dohan Ehrenfest and coworkers found in their in vitro studies that the original L-PRF protocol produces larger clots and membranes, and a more intense release of growth factors than the modified A-PRF protocol. Based on the findings of their study they suggested that centrifuge characteristics and protocols may have a very significant impact on the cell, growth factors and fibrin architecture of a PRF clot and membrane [29]. In contrast, another recent in-vivo study showed that Choukroun's new formulation of advanced PRF (A-PRF) had a more gradual release of growth factors, up to a 10-day period, and stimulated significantly higher growth factor release over time when compared to Choukroun's standard PRF. The consideration of A-PRF is that the contained enriched leukocyte and the influence of leucocytes injected with surgical platelet concentrates is actually a relevant way of research, and no study can claim that their influence is negative. Therefore, in this treatment, L-PRF was utilized and applied to degenerated discs after full endoscopic procedure.

The primary purpose of our study was to assess the safety of PRF; therefore, we aimed to objectively evaluate changes in disc degeneration following PRF insertion by radiographic image analyses. Radiographically, no remarkable progression of disc height narrowing was observed after intradiscal injection of PRP releasate. Although a significant reparative effect on disc height was not observed in this study, as we previously reported [14], our findings suggested that PRF did not have negative impact on disc height. The MRI T2-mapping technique may quantitatively evaluate changes in the molecular composition and structural organization of the intervertebral disc [30]. In the present study, we examined whether PRF insertion affects the T2 value of both AF and NP tissues separately using a template-based segmentation method [20]. This method has been developed to segment the AF and NP tissues of a degenerated IVD based on the template of a normal cadaveric lumbar spine. PRF had no effect on T2 values of both AF and NP tissues; these results were similar to those of previous study in animals [14]. Thus, intradiscal injection of PRP releasate did not negatively affect the matrix of degenerated IVDs.

Conclusions

We demonstrated that intradiscal insertion of PRF to treat patients with low back pain and degenerated IVDs is safe and feasible. This treatment would be especially suitable for young adults or pre-middle age patients who generally are not recommended for spinal fusion surgery. Surgical l treatment options in orthopedic field are expanding and new techniques are constantly being developed. Present treatment modalities can assist with the stimulation of tissue formation after surgical procedures, leading to various results. Disc regeneration is a complex process, and the placement of PRF may enhance the healing of nucleus following discectomy procedure. PRF is an advantageous technique that provides optimal results when used in conjunction with many orthopedic procedures. Further researches are needed to validate these treatment strategies in evidence-based clinical practice.

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Table 5-1 *Demographic data of 6 patients with discogenic back pain*

Patient	Sex	Age	Level
#1	F	39	L5/S1
#2	F	38	L4/5
#3	M	35	L4/5
#4	M	47	L4/5
#5	F	42	L5/1
#6	M	40	L4/5

Table 5-*2* *Surgical outcomes assessment using modified MacNab criteria*

Grade	Post Op	Post 1 M	Post 3 M	Post 6 M	Post 12 M
	n (%)	n (%)	n(%)	n(%)	n(%)
Excellent	2 (33%)	4 (67%)	4 (67%)	4 (67%)	5 (83%)
Good	4 (67%)	2 (33%)	2 (33%)	2 (33%)	1 (17%)
Fair	0	0	0	0	0
Poor	0	0	0	0	0

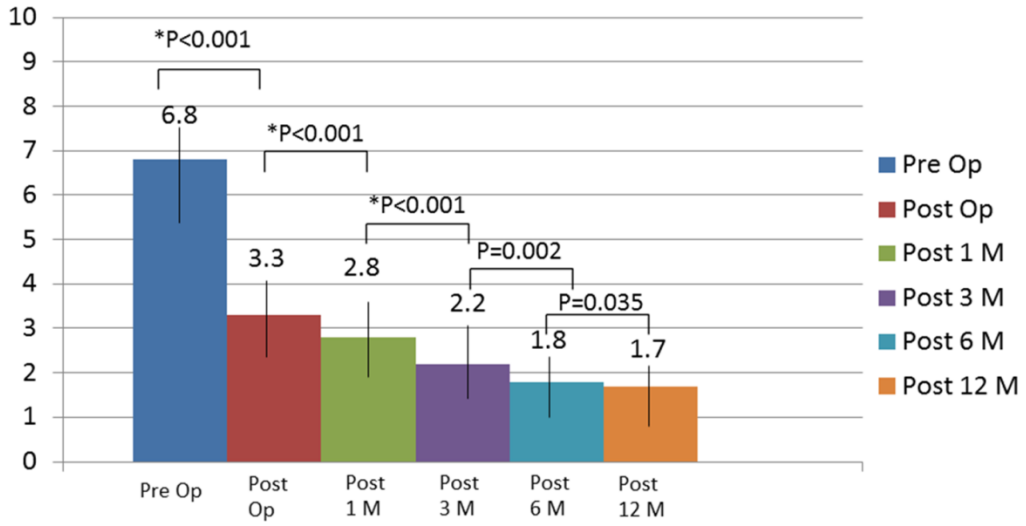

Figure 5-1 Preoperative and postoperative Visual Analog Scale (VAS) scores. *P < 0.001, compared by Wilcoxon signed-rank test

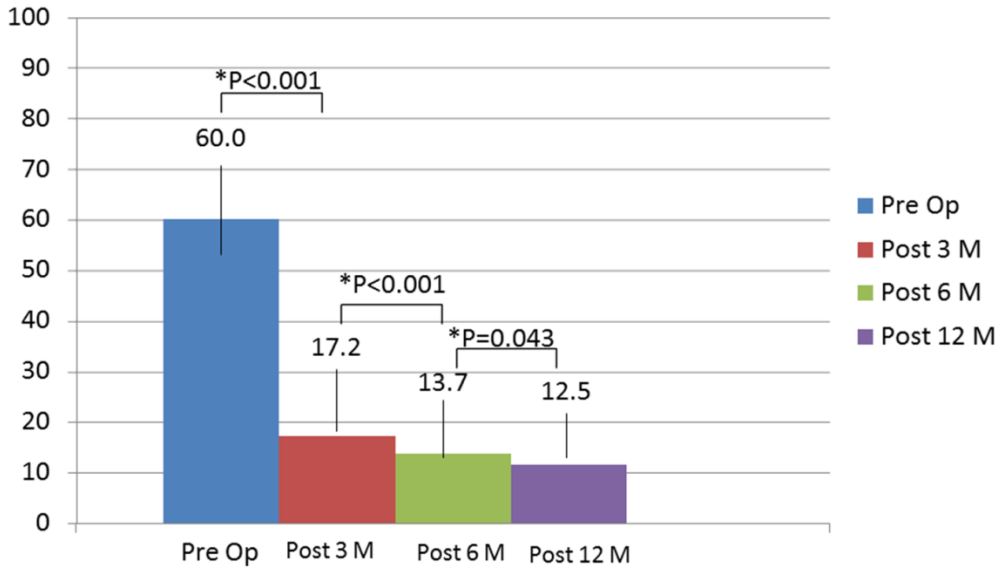

Figure 5-2 Preoperative and postoperative Oswestry Disability Index (ODI). *P < 0.001, compared by Wilcoxon signed-rank test.

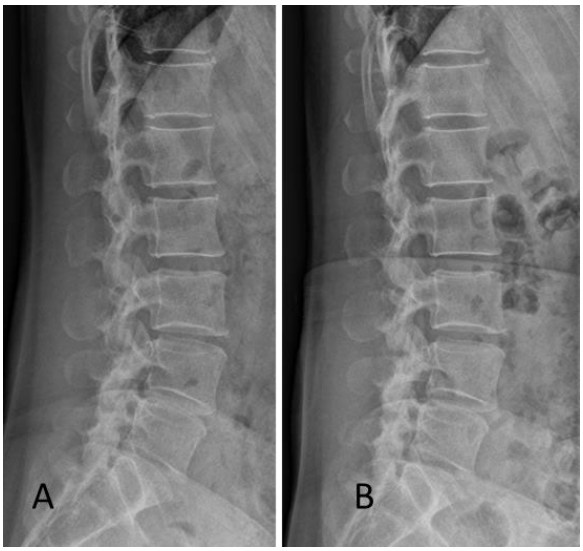

Figure 5-3 Representative lumbar radiographs before and after full endoscopic discectomy followed by intradiscal insertion of PRF. Lumbar lateral radiographs of patient #2. Pre-treatment (A), and 12 months (B) after procedure.

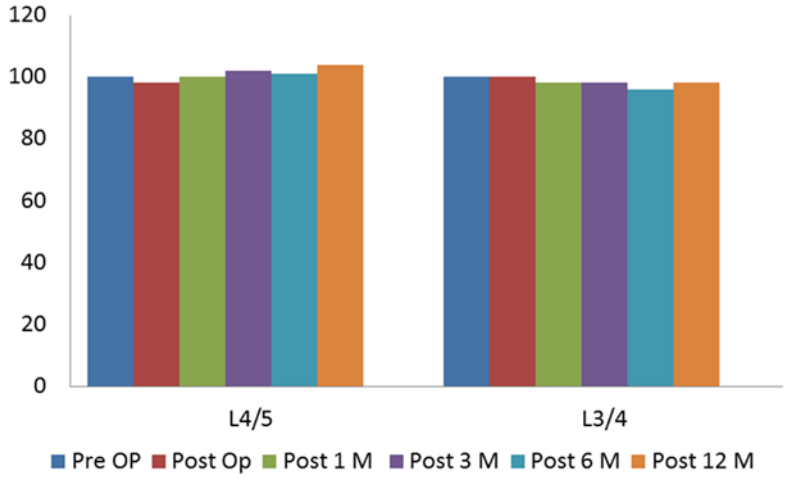

Figure 5-4 Radiographic assessments of change in disc height. The % DHI of PRF inserted discs and the control (L3/L4) discs had similar changes during the follow-up period.

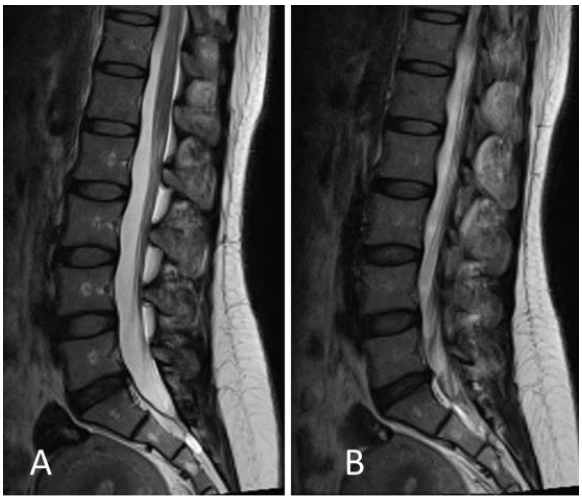

Figure 5-5 Magnetic resonance imaging of patient 1. Representative sagittal T2 weighted signal of L5/S1 IVD before operation (A) and 12 months after operation (B). There were no significant changes in normalized T2 values of both nucleus pulposus and annulus fibrosus tissues.