

# The Influence of Leukocyte and Platelet Concentrate Enrich in Stem Cells on Bone Regeneration Processes: A Clinical and Flow Cytometry Study

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## Abstract

This article reports the efficacy of percutaneous autologous leukocyte- and platelet-rich plasma (L-PRP) injection into delayed union site as a minimally invasive method alternative to bone marrow aspirate and open grafting techniques. Each of 15 participants was followed on a regular basis with clinical examinations, roentgenograms. The average time to union was 8.4 weeks after L-PRP injection and the union was achieved in all cases. CD34+/45+ cells counts were increased by 410% and CD34+/45- cells counts were increased by 488% on average in L-PRP. Our investigation showed that L-PRP enrich in stem cells can produce the desired stimulatory response despite a substantial amount of vital bone cells from mesenchymal line. We believe that the using of L-PRP enriched in stem cells, growth factors and antimicrobial proteins might be a promising treatment method in regenerative medicine.

## Keywords

Healing, Growth Factors, Induction, Stimulation, Osteogenesis

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## 1. Introduction

Due to their numerous key properties, platelets started to be used in the contemporary medicine in the strategies of stimulation of tissue healing, as an autologous cell and plasma fraction extracted from the peripheral blood [1]. The autologous whole blood undergoes centrifugation in order to obtain a concentrate of platelets—called platelet-rich plasma (PRP) like in transfusion medicine—which could be transformed into a fibrin gel after activation by the addition of thrombin and calcium ions (or equivalent technologies). Most authors believe that the gel activation and the release of the growth factors lead to the acceleration of the wound healing process [2]. The impact of platelet growth factors was particularly intensively investigated and debated in trauma and maxillofacial applications [3]-[13], and PRP/PRF technology for regenerative medicine treatments is one of the most active fields of research in the dental/maxillofacial and orthopedic literature, with implant surfaces/designs and bone bioengineering [14]-[20].

Since the early development of these technologies, researchers were mostly focused on the platelet count in the concentrates. However, PRPs can contain a significant number of leukocytes and other unknown cells. In the present study, we report the influence of L-PRP on regeneration of tibial fractures. To our knowledge, this is the first report of concentrated stem cells in L-PRP harvested from blood.

## 2. Material and Methods

The study group consisted of 15 patients treated at our clinic with delayed union after tibial open fracture (II stage in Gustilo-Anderson scale) and blocked intramedullary nail fixation. Basic characteristics of the study population are summarised in **Table 1**.

For the study, 108 ml of whole blood was collected from each subject into a syringe containing 12 ml of citrate solution. Whole blood was drawn into two sterile tubes and centrifuged for 15 minutes at 3200 RPM (GPS II Platelet Concentration System, Biomet, Warsaw, USA). This resulted in blood separation into its three basic components: red blood cells, L-PRP sometimes referred to as “buffy coat”, and acellular plasma (ACP). Subsequently, ACP component was removed into 60 ml syringe. Next the tube was shaken vigorously for 30 seconds to suspend platelets and 12 ml of L-PRP was obtained.

### 2.1. Surgical Procedure

Before investigation informed consent was obtained from the patients. The surgical procedure was performed in the operating room under general anesthesia. An 18-gauge or biopsy needle was introduced immediately into the gap of nonunion under fluoroscopic guidance. In all cases, L-PRP and 3 ml of autologous thrombin solution (TPD, Biomet, Warsaw, USA), a total of 15 ml, was injected by dual syringe applicator system (Biomet Inc.) into the disturbed bone-healing area forming a gelatinous mass. A second injection into the gap or operation with bone grafts was not performed.

### 2.2. Cell Preparation, Flow-Cytometric Analysis, and Cell Sorting

Blood and L-PRP samples were processed under standardized and optimized conditions within less than 5 hours after collection. For each sample two 8-fold staining with fluorochrome-conjugated mouse anti-human monoclonal antibodies were performed.

The antibody set was designed to allow leukocyte analysis, so the antibodies against lineage specific progenitor stem cells surface markers were used: hematopoietic line (CD34+/CD45+) and peripheral blood-derived endothelial progenitor cells (CD34+/CD45-).

After staining step, erythrocyte lysis and fixation was performed with FACSlyse Solution (Becton Dickinson). Subsequently the sample was washed with Cell Wash solution (Becton Dickinson) and finally resuspended in FACSFlow solution (Becton Dickinson).

**Table 1.** Characteristics of the study population.

Variable	Mean + SD or n (%)
Age (years)	36 ± 7
Female/male	8 (53%)/7 (47%)

Acquisition of data was performed in 8-color 3-laser flow cytometer FACSCanto II (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The data were acquired and analyzed with Diva software (Becton Dickinson).

The Silesian Medical University Ethics Committee approval for performing investigation was obtained.

### 3. Results

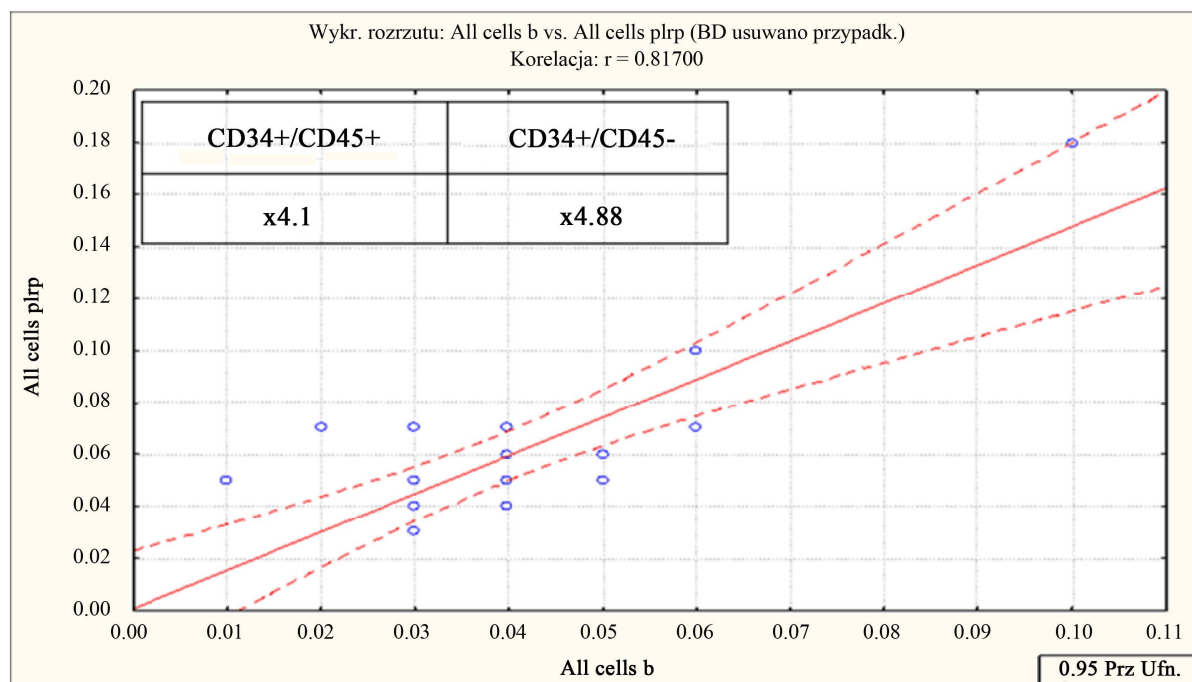
No complications related to surgical technique were observed. Several patients, particularly with fibular and tibial healing disturbances, developed subcutaneous swelling of few centimeters in diameter at the injection site. These areas were first evident during injection and resolved within several hours. A few patients had moderate discomfort at their donor vein site, which generally resolved within a few hours. The average hospital stay per patient was 1.9 days. No infection complicating bone healing was observed. Union was observed in all cases. The average time to union was 8.4 weeks (range 5 - 12 weeks) after L-PRP injection.

We found markedly increased platelet and leukocyte counts (more than sevenfold) compared with baseline levels. Adult stem cell counts were also increased. **Figure 1** shows the results of the adult stem cell from hematopoietic line measurements for the blood and L-PRP. Peripheral blood-derived endothelial progenitor cells were also detected in blood and L-PRP samples.

### 4. Discussion

Most authors use platelet separation systems, which allow to collect leukocytes [21]-[24]. However, some authors exclude leukocytes from L-PRP, so they obtain pure PRP [14] [25]. In Aspenberg and Virchenko studies L-PRP was irradiated at 25 Gy to inactivate the WBCs, and truly platelet concentrate was reached [25]. For non-advanced reader the distinction between two similar, but not the same biomaterials could be unfulfillable. The truth is that many authors do not mention about leukocytes concentration even though they use standard separator system and focus only at platelets and growth factors [26]. That is why Everts *et al.* and Bielecki *et al.* proposed term “platelet-leukocyte-rich plasma or gel (PLRP/PLRG)”, independently [23] [27], and in 2012 Dohan Ehrenfest study team consisting from blood concentrate specialists reached consensus in terminology and leukocyte- and platelet-rich plasma/fibrin “L-PRP/L-PRF” term is finally used. [1].

The usage of L-PRP to enhance bone regeneration and soft tissue maturation has also increased in the field of



**Figure 1.** CD34+/45+ cells counts were increased by 410% and CD34+/45- cells counts were increased by 488% on average.

orthopaedic and trauma surgery over the last decade [27]. Lowery *et al.* [28] used PLRG with allogenic grafts in lumbar spinal fusion with good results. They did not observe any radiological or clinical evidence of pseudoarthrosis in all patients. Bielecki and Gazdzik [10] showed a case report of L-PRP treatment in a patient who suffered from disturbances of bone-healing processes. Kitoh *et al.* [29] reviewed clinical results of distraction osteogenesis with transplantation of marrow-derived mesenchymal stem cells and PLRG in 3 patients. A mixture of PLRG and osteoblast-like cells were injected into the callus. In 1 case they had to increase the distraction to 1.5 mm/day between days 34 and 47, because callus formation was likely to consolidate prematurely. Enhanced callus formation was observed radiographically after first transplantation of marrow-derived mesenchymal stem cells and PLRG in all 3 cases. However, they applied a combination of two osteoinductive biomaterials and we do not know to what extent PLRG influenced bone formation in these cases [29].

Our investigation showed that percutaneous L-PRP injection is a sufficient method to treat delayed union and is less invasive procedure than bone marrow injection. Additional advantages include decreased hospitalization costs and duration. Although autologous bone marrow collection is thought to be a relatively simple procedure, it can be associated with numerous complications such as biopsy site bleeding, hematoma and/or infection [7] [27]. The application of L-PRP under fluoroscopic guidance lasts about 1 - 2 min and can be performed under short-term intravenous general anesthesia. L-PRP injection into the healing disturbances site is a minimal invasive surgical procedure and can be easily done in the outpatient clinic.

Until now, no one has published the adult stem cells in L-PRP in patients with bone healing disturbances. We know that leukocytes and their progenitors are cells of the immune system defending the body against both infectious disease and foreign materials. Particularly T-lymphocytes, might mediate rejection and destruction of allogenic grafts, which are widely used in orthopaedic surgery [30]. Taking into account significant numbers of progenitors in L-PRP, immunogenic allograft might retard healing processes or even induce local immune response causing bone destruction [30].

Most progenitors (adult stem cells) are described as multipotent [31]. They are in the “center” between stem cells and fully differentiated cell. Like stem cells, mostly, they are formed and transported in a colony, with the right conditions for them to grow and differentiate into their target tissues [31] [32]. Our study showed that after blood separation process the higher level of CD34+ cells is reached. It opens a new direction of investigations. It is possible that platelet-derived growth factors can induced progenitor cells from L-PRP in first stage to excrete numerous growth factors, which can stimulate bone healing by autocrine, paracrine and juxtacrine interactions. In consequence, osteogenic cells from bone matrix are activated and regeneration processes can occur. However, further studies are necessary to fully elucidate the role of stem cells from L-PRP.

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