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Benefit of Percutaneous Injection of Autologous Platelet-Leukocyte-Rich Gel in Patients with Delayed Union and Nonunion

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Key Words
Platelet-rich plasma · Platelet-leukocyte-rich plasma · Bone-healing disturbances · Growth factors · Gel · Nonunion · Delayed union infection

Abstract
This article reports the efficacy of percutaneous autologous platelet-leukocyte-rich gel (PLRG) injection as a minimally invasive method alternative to open grafting techniques. Each of 32 participants was followed on a regular basis with clinical examinations, roentgenograms, dual-energy X-ray absorptiometry examinations. In the delayed union group, the average time to union was 9.3 weeks after PLRG injection and the union was achieved in all cases. In the nonunion group, the union was observed in 13 of 20 cases and the average time to union was 10.3 weeks after PLRG injection. Interestingly, in patients in whom union was not achieved, the average time from the fracture and/or from the last operation was >11 months. This is our initial experience with the use of PLRG as biologic treatment for delayed union or nonunion. Our investigation showed that percutaneous PLRG injection in delayed union is a sufficient method to obtain union, which is less invasive procedure than bone marrow injection. Percutaneous PLRG grafting can be also an effective method for the treatment of selected cases of nonunion. The essential factor is the average time from the initial surgery to PLRG injection for nonunion; <11 months seems to be critical for good outcomes.

Introduction

Despite continuous advances in the treatment of long bone fractures, disturbances of healing processes remain a difficult challenge. Approximately 10% of treated fractures will require further surgical procedures because of impaired healing [1, 2]. The preferred management of delayed union and nonunion is to provide the essential elements for bone formation. Because autologous cancellous bone and bone marrow grafting has become the standard treatment, alternative treatments must be not only equally successful in achieving union but also should provide some additional benefit to justify their use [1].

Recent advances in cellular and molecular biology have led to the identification of specific cytokines that mediate cellular activities [2]. The ability to control cellular activities becomes a powerful tool in management of orthopedic disorders. Among others, platelet-leukocyte-rich gel (PLRG) enriched with growth factors may enhance bone formation in vitro [3, 4] and in vivo [5–7].

Percutaneous administration of substances with osteoinductive and osteogenic properties offers the advantage of decreased morbidity associated with the classic open grafting techniques [8]. Additional advantages are decreased costs and hospitalization time [1, 8, 9]. Previous clinical studies have shown that local application of bone marrow and bone morphogenetic protein-7 is capable of increasing bone defect healing [10–12]. Platelet

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Table 1. Clinical characteristics of the delayed union group

No.	Sex	Age	Type of bone-healing disturbances	Delayed union site	Time from fracture to PLRG injection months	Fracture site operations	Time from the last fracture site operation to PLRG injection months	Previous infections	Present infection	Healing	Time to union weeks
1	M	39	D	Tibia	6	0	n/c	N	N	Y	8
2	M	30	D	Tibia	6	0	n/c	N	N	Y	12
3	F	42	D	Tibia	6	0	n/c	N	N	Y	8
4	M	52	D	Tibia	6	0	n/c	N	N	Y	8
5	M	37	D	Tibia	6	0	n/c	N	N	Y	8
6	M	41	D	Tibia	9	0	n/c	N	N	Y	12
7	M	55	D	Fibula	9	0	n/c	N	N	Y	8
8	F	49	D	Fibula	7	0	n/c	N	N	Y	12
9	M	51	D	Fibula	8	0	n/c	N	N	Y	8
10	M	38	D	Tibia	8	0	n/c	N	N	Y	12
11	M	56	D	Tibia	9	0	n/c	N	N	Y	12
12	F	45	D	Tibia	8	0	n/c	N	N	Y	8

D = Delayed union; n/c = not concerned.

concentrates rich in growth factors represent a novel osteoinductive therapy that could be valuable as adjunct of bone healing [13, 14]. Platelets retain numerous growth factors such as platelet-derived growth factor, transforming growth factor-β, insulin-like growth factors I and II, and epidermal growth factor [15–17]. By concentrating platelets, higher levels of growth factors can be reached which might stimulate the prematurely terminated bone-healing processes [5, 6]. Platelets, once activated in the presence of thrombin, release growth factors and begin to form gelatinous scaffold for the developing fibrin mass – so-called PLRG [18].

This article reports on the efficacy of percutaneous autologous PLRG injection as a minimally invasive method for the treatment of delayed unions and nonunions, preventing open grafting techniques.

Material and Methods

Between October 2003 and February 2006, 15 patients diagnosed with delayed union and 22 with nonunion participated in this study at the Department and Clinic of Orthopedics in Sosnowiec of the Medical University of Silesia, Poland. The following inclusion criteria were applied for this study: (1) established delayed union >6 months after injury which has not progressed to full bony union (delayed union group) [19]; (2) established nonunion >6 months after injury or time from last fracture site operation, which has not shown progressive evidence of healing process throughout the past 4 months (nonunion group) [8, 19]; (3) no surgical treatment after fracture in the delayed union group; (4) a good general health status, and (5) regular visits in the outpatient clinic.

Patients with open fractures, diabetes, platelet count $<130 \times 10^9$/l, age >60 years or taking medicines known to influence platelet function were excluded from the study.

Three patients from the delayed union group and two from the nonunion group did not attend more than 1 from 7 routine outpatient visits and were excluded from statistical analyses (tables 1, 2).

Following the outpatient procedure, each participant was followed on a regular basis with clinical examinations, roentgenograms, dual-energy X-ray absorptiometry (DEXA) examinations and functional evaluations.

The University Ethics Committee approval for performing investigations was obtained (opinion No. NN-013-196/I/03 from July 2, 2003).

All examinations were performed at day 3 as well as 3, 5, 8, 12, 18 and 24 weeks after percutaneous PLRG injection. X-ray films were taken in two views: anteroposterior and lateral. DEXA examination was carried out with a Lunar DPX scanner once per visit. Scan regions were located at the fracture site. The standard regions of interest were 4.8 mm long and covered the whole width of the bone (fig. 1). Areas containing soft tissue or metal were excluded (Cuttermole's method [20]). The rectangle was located in the fracture site with minimal bone mineral density. The University Ethics Committee did not allow for more than one scan per visit. In 1 patient with the nonunion of the clavicle (case 17) DEXA examination was not performed.

Union was determined on strict roentgenographic criteria. A patient was considered healed when 75% of the circumference of the bone at the defect site was resolved [8]. In cases where we did not observe completion of the bone-healing process, the observation period was prolonged up to 10 months (42 weeks).

Table 2. Clinical characteristics of the nonunion group

No.	Sex	Age	Type of bone-healing disturbances	Nonunion site	Time from fracture to PLRG injection months	Fracture site operations and type of operation	Time from the last fracture site operation to PLRG injection months	Previous infections	Present infection	Healing	Time to union weeks
1	F	47	NU-O	Humerus	24	2 – RN, AL	11	N	N	Y	8
2	M	50	NU-A	Femur	12	0 – n/c	n/c	N	N	N	n/c (42)
3	M	38	NU-A	Tibia	17	1 – BM	11	N	N	Y	12
4	M	54	NU-A	Humerus	28	1 – AU+AL	13	N	N	N	n/c (42)
5	M	54	NU-A	Tibia	10	0 – n/c	n/c	Y	Y	Y	12
6	M	39	NU-H	Tibia	6	0 – n/c	n/c	N	N	Y	12
7	M	28	NU-H	Tibia	8	0 – n/c	n/c	N	N	Y	12
8	M	49	NU-A	Femur	33	2 – ND, AU	12	Y	Y	N	n/c (42)
9	M	46	NU-O	Radius	12	0 – n/c	n/c	N	N	N	n/c (42)
10	F	30	NU-O	Radius	7	0 – n/c	n/c	N	N	Y	12
11	M	31	NU-H	Tibia	13	1 – BM	7	N	N	Y	12
12	M	46	NU-H	Tibia	23	2 – ND, AU	8	Y	N	Y	18
13	F	22	NU-O	Tibia	15	0 – n/c	n/c	N	N	N	n/c (42)
14	M	43	NU-O	Fibula	9	0 – n/c	n/c	N	N	Y	12
15	M	60	NU-O	Humerus	8	0 – n/c	n/c	N	N	Y	12
16	M	19	NU-A	Tibia	29	2 – BM, AL	7	N	N	Y	12
17	M	21	NU-O	Clavicle	8	0 – n/c	n/c	N	N	Y	12
18	M	36	NU-O	Tibia	35	3 – ND, BM, AU	14	N	N	N	n/c (42)
19	M	41	NU-H	Tibia	26	2 – BM, AU	10	N	N	N	n/c (42)
20	F	36	NU-O	Tibia	10	0 – n/c	n/c	Y	Y	Y	18

NU-O = Oligotrophic nonunion; NU-A = atrophic nonunion; NU-H = hypertrophic nonunion; n/c = not concerned; RN = reamed with interlocking nailing; AL = operation in open technique with using allograft; AU = operation in open technique with using autograft; BM = bone marrow injection; ND = nail dynamization.

PLRG Preparation Procedure

Platelet-leukocyte-rich plasma (PLRP) was prepared by extracting blood from the patient's basilic vein by using 19-gauge needle and two 60-ml syringes. 108 ml of whole blood with 12 ml of anticoagulant (sodium citrate) was drawn into two sterile tubes and centrifuged for 12 min at 3,200 rpm (GPS I Platelet Concentration System; Biomet, Inc., Warsaw, Ind., USA). Following centrifugation, the blood was separated into three basic components: red blood cells, PLRP, and platelet-leukocyte-poor plasma. 12 ml of PLRP was obtained and mixed with 3 ml of 1,600 U/ml bovine thrombin (Biomed, Lublin, Poland) in a 10% calcium chloride solution (Polfa, Lódz, Poland) at room temperature to form PLRG.

The platelet and leukocyte counts in peripheral blood and PLRP were measured in hematology analyzer (Advia 120, Bayer, Germany).

Surgical Procedure

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 delayed union or nonunion under fluoroscopic guidance (fig. 2). In all cases, PLRP and thrombin solution (a total of 15 ml) was injected by dual syringe applicator system (Biomet Inc.) into the disturbed bone-healing area forming a gelatinous mass (fig. 3). A second injection into the gap or operation with bone grafts was not performed.

Statistical Analysis

Statistical analysis was performed using Statistica for Windows 6.1 Version (Statsoft). Statistical differences were evaluated using Mann-Whitney's U-test and χ^2 test with Yate's correction. The Spearman ratio (r) was used to estimate the correlation between parameters. A simulation program for estimating the statistical power of Cox's proportional hazards model assuming no specific distribution for the successful treatment was used. Probability values $p \leq 0.05$ were considered significant. To the statistical analysis in cases from the nonunion group where union was not achieved in 'time to union', number 42 was written (table 2).

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 (fig. 3). A few patients had moderate discomfort at their donor vein site, which generally resolved within a few hours.

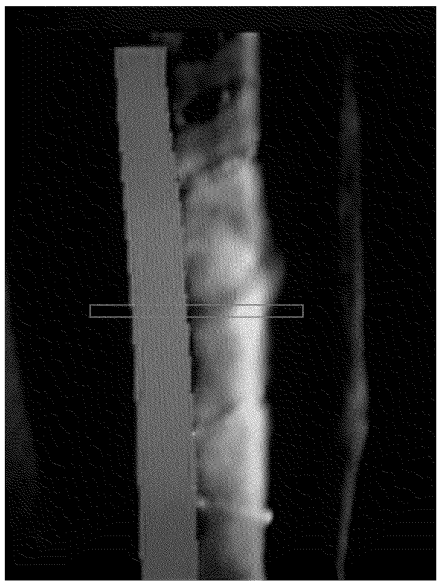

Fig. 1. DEXA examination with rectangle located in the fracture site.

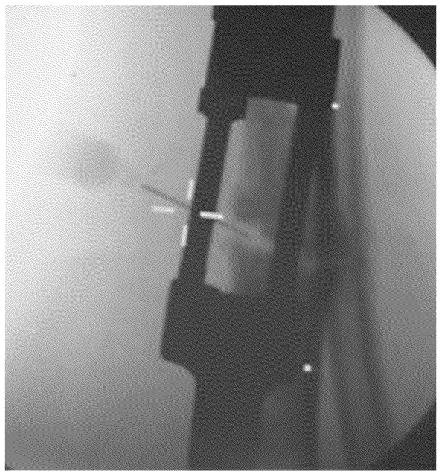

Fig. 2. Percutaneous PLRG injection into the nonunion gap of the right crus under fluoroscopic guidance.

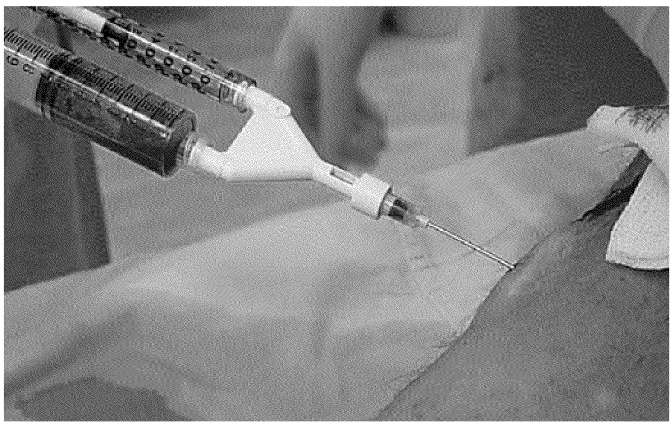

Fig. 3. Subcutaneous swelling at the injection site.

In the delayed union group, the average hospital stay per patient was 1.9 days. Union was observed in all cases. The average time to union was 9.3 weeks (range 5–12 weeks) after PLRG injection (table 1).

In the nonunion group, the average hospital stay was 1.8 days per patient. Union was observed in 13 of 20 cases. The average time to union was 10.3 weeks (range 8–18 weeks) after PLRG injection (table 2). In 2 patients (cases 2 and 8) destabilization of the fracture occurred after 5 months and 5 weeks, respectively. In 4 cases, in which we did not observe bone-healing processes, the observation period was extended to 10 months and during visits only X-ray scans were performed. Despite the prolonged observation time, union was not achieved. Notably, in 1 patient (case 4) in whom union did not occur, there was a mass of new callus in the humerus. Nevertheless, the outcome was classified as a failure, because of the strict endpoint of this study.

The mean platelet count was $241 \pm 64 \times 10^9$/l and mean leukocyte count was $7.6 \pm 2.57 \times 10^9$/l in blood. Platelet counts were increased by 720% and leukocyte counts were increased by 760% on average. No correlation was observed between treatment results and concentrations of platelets and leukocytes in blood and PLRP.

Most patients exhibited an increase in bone mineral density in DEXA examinations (tables 3, 4). A maximum value was reached at the last check-up point (24th week) in all cases with delayed union. In the nonunion group, 1 patient (case 1) with humeral nonunion reached a maximum value in the 18th week while the remaining cases at the last check-up point. In 7 cases where bone-healing processes were not observed, the last scans showed minimal increase or even decrease of bone density in comparison with the first examination (third day after operation).

No correlation was observed either between the age of patients and increase in bone mineral density, or between body mass index and density of the bone. In the nonunion group, the number of fracture site operations, type of nonunion and fracture localization did not significantly influence treatment results. Significant correla-

tion was noted between successful bone healing and the time from the last operation ($p \leq 0.001$) and the time from injury ($p \leq 0.05$) (table 5). Notably, the union did not occur in all cases where the time from last operation or time from injury in patients who had no operation of fracture site was >11 months.

To examine the influence of particular parameter (age, type of bone-healing disturbances, nonunion site, number of the fracture site operations, time from the last fracture site operation, time from fracture, previous infections, and present infection) on treatment outcome, the Cox's statistic was performed. It demonstrated no significant correlations between parameters.

Union was achieved in 2 out of 3 patients with active methicillin-sensitive *Staphylococcus aureus* infection. In a single patient with methicillin-resistant *S. aureus* infection (case 8), fracture destabilization occurred during the 5th week of follow-up. Nevertheless, we did not find a significant correlation between active infection and success of the treatment.

Table 3. BMD increase in the delayed union group

BMD increase	Values, %	Mean ± SD	Median
3rd week	–1 to 4	1.8 ± 1.5	1.5
5th week	–1 to 9	3.7 ± 3.3	4.5
8th week	–3 to 13	7.5 ± 5.1	8.0
12th week	2 to 24	12.3 ± 6.6	14.0
18th week	–1 to 31	18.6 ± 11.4	22.5
24th week	3 to 41	25.6 ± 13.4	30.5

Table 4. BMD changes in the nonunion group

BMD increase	Values, %	Mean ± SD	Median
3rd week	–1 to 14	3.5 ± 4.2	2.0
5th week	–2 to 28	7.1 ± 8.1	4.0
8th week	–2 to 50	12.9 ± 15.3	7.0
12th week	–2 to 83	21.3 ± 24.6	13.0
18th week	–12 to 112	26.9 ± 32.6	19.0
24th week	–7 to 98	33.7 ± 34.1	26.0

Table 5. Relation between successful treatment and particular features in the nonunion group

Features	p value
Time from fracture	0.023227
Fracture site operations	0.53107
Type of nonunion	0.57727
Nonunion site	0.38252
Time from the last fracture site operation[1]	0.000037

[1] Time from injury in patients who had no operation of fracture site.

Discussion

The use of growth factors in combination with tissue engineering seems to be the most promising future treatment method of bone and cartilage defect [2]. Traditionally applied autologous bone grafting in principle provides three vital local components – osteoconductive matrix, osteoinductive growth factors, and osteogenic cells [8, 9]. According to some authors, an alternative technique must also provide these three components for successful treatment of disturbed bone-healing processes [1, 11]. In recent decades, application of percutaneous bone marrow therapy has been in common use, which has mainly osteoinductive and osteogenic properties. Connolly [21] and Healey et al. [22] have demonstrated that percutaneous injections of autologous bone marrow can successfully treat between 78 and 95% of nonunions. However, the level of the osteoprogenitor cells in aspirated bone marrow is highly variable per patient. Hernigou et al. [23] injected bone marrow concentrates containing between 60 and 6,120 progenitors/cm^3 into a gap of noninfectious atrophic nonunion. They achieved union in all cases where obtained concentrates had concentrations of osteoprogenitor cells of >1,500/cm^3. They reported that the fibrous tissue interposed between the bone ends ossified after the injection of bone marrow. However, they could not explain the exact mechanism that allows the transformation of fibrous tissue into callus [23]. Autologous platelets as a source of healing factors have been shown to promote tissue repair in many other clinical situations in orthopedic surgery [13, 24–26].

Most authors report the usage of the platelet-rich plasma (PRP) in their investigations [4, 6]. However, what is injected into a fracture site is usually a combination of PRP and thrombin. After activation of clotting system by thrombin, a series of proteolytic reactions is initiated in PRP that ultimately results in platelet degranulation and the conversion of soluble fibrinogen into insoluble fibrin [27]. Moreover, thrombin itself stimulates fibroblast proliferation, synthesis of type IV collagen and active mediators such as NF-κB, etc. [27]. This is why we have em-

phasized using the name, which contains both active substances, PRP and thrombin, i.e. platelet-rich gel (PRG) [27]. Some authors prefer to use the term platelet-rich fibrin interchangeably with PRG [28]. Rarely, PRP could be administered without thrombin, e.g. in chronic severe elbow tendinosis [26].

Several reports have indicated that PRP contains a substantial amount of leukocytes. Neutrophils and lymphocytes are important elements of the immune system and they play an important role in healing processes [27]. They also synthesize substances which earlier were viewed as megakaryocyte- and platelet-specific, e.g. platelet basic protein and platelet factor 4 [29]. Therefore, it might be more appropriate to apply the terms PLRP and PLRG for this osteoinductive biomaterial rich in platelets, leukocytes and related active substances.

The usage of PLRG to enhance bone regeneration and soft tissue maturation has also increased in the field of maxillofacial surgery over the last decade [30]. Lowery et al. [25] used PLRG with allogeneic 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 [13] showed a case report of PLRG treatment in a patient who suffered from disturbances of bone-healing processes. Kitoh et al. [24] 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 [24].

Some authors have reported that PLRG alone cannot produce the desired stimulatory response because a substantial amount of vital bone cells is needed [31]. However, in the delayed union site, the regeneration process is significantly retarded, so the application of the critical growth factors can still stimulate the local osteogenic cells. Our investigation showed that percutaneous PLRG injection is a sufficient method to treat delayed union and is less invasive procedure than bone marrow injection. In contrast, union was never achieved in patients with nonunion, if they were treated with PLRG >11 months after last operation. Probably the fibrous tissue in the gap interposing the bone ends becomes more ossified with time and the vascularization diminished, so the PLRG cannot induce the bone-healing processes. Therefore, a time span <11 months after initial surgery seems to be critical to achieve a good outcome with percutaneous PLRG injections for nonunion. Interestingly, in all cases of femoral nonunion, the time from the last operation was at least 12 months and the union was never reached. In tibial nonunion cases the time from the last operation was <11 months and the treatment was successful. Nevertheless, the association between fracture localization and the outcome did not reach statistical significance. In contrast, we found a significant inverse correlation between the time from the fracture and successful outcome.

The autologous percutaneous bone marrow injection offers the advantage of decreased morbidity associated with the classic open grafting techniques [1, 23]. 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 [1, 22, 23]. The time of operation including bone marrow aspiration and application is too long to use short-term intravenous general anesthesia. The application of PLRG under fluoroscopic guidance lasts about 1–2 min and can be performed under short-term intravenous general anesthesia. PLRG injection into the healing disturbances site is a minimal invasive surgical procedure and can be easily done in the outpatient clinic.

In contrast to previously published studies [32], we applied bovine thrombin at significantly higher concentrations, which resulted in immediate formation of the gelatinous mass. The applied volume of 12 ml PLRP with 3 ml thrombin solution was quite high as compared to the size of a gap. Several patients, particularly those with fibular and tibial healing disturbances, developed subcutaneous swelling of several centimeters in diameter at the injection site, which resolved over several hours. Therefore, it is very difficult to define a minimal, therapeutic volume.

Some authors reported that PLRG might not produce the desired stimulatory response when autologous bone is not present in the graft or when the defect is of a large volume, because vital bone cells are needed for this stimulation to occur [31]. Marx et al. [30] reported that 5 ml of PLRP with 1 million platelets/μl is required to induce bone- and soft tissue-healing processes. Since we applied PLRG without bone marrow grafts, we decided to increase PLRG volume to 15 ml. Similarly, in the study of Cieslik-Bielecka et al. [33], the odontogenic cysts were

filled up with PLRG without autologous bone or bone marrow grafts. Nevertheless, bleeding occurring during cyst removal and the wall curettage could induce additional release of growth factors stimulating progenitor cells localized in bone matrix. PLRG application was sufficient to increase bone-healing processes in smaller and bigger cysts despite the lack of osteogenic cells from harvested bone grafts [33]. In the present study, bone ends in a gap were not subjected to a curettage procedure. Since formation of the fibrous tissue between bone ends occurs, breaking down of this barrier is essential to achieve the union. In nonunions treated after >11 months from the initial surgery, even the large PLRG volume of 15 ml was too low to reach union. Most probably for such cases simultaneous application of PLRG and autologous bone grafts should be required.

This is our early experience with the use of PLRG as biologic treatment for delayed union or nonunion. To our knowledge, this is the first study looking at the effect of percutaneous PLRG delivery to the fracture site in delayed union and nonunion. We believe that percutaneous autologous PRG grafting can be an effective and safe method for the treatment of delayed union and for selected cases with nonunion.

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