Platelet rich plasma and fresh frozen bone allograft as enhancement of implant fixation
An experimental study in dogs

T.B. Jensen [a,*], O Rahbek [a], S. Overgaard [a,b], K. Søballe [a,b]

[a] Orthopaedic Research Group, Institute of Experimental Clinical Research, Aarhus University Hospital,
Aarhus Kommunehospital, 8000 Aarhus C, Denmark
[b] Department of Orthopaedics, Aarhus University Hospital, Denmark

In memory of Dr. Lai-Martine Schiøtz (1972–2000)

Abstract

Platelet rich plasma (PRP) is an autologous source of growth factors. By application of PRP around cementless implants alone or in combination with bone allograft chips, early implant fixation and gap healing could be improved.

We inserted two porous HA coated titanium implants extraarticularly in each proximal humerus of eight dogs. Each implant was surrounded by a 2.5 mm gap. Four treatments were block randomized to the four gaps in each dog: Treatment 1: empty gap, treatment 2: PRP, treatment 3: fresh frozen bone allograft, treatment 4: fresh frozen bone allograft + PRP.

PRP was prepared from each dog prior to operation by isolating the buffycoat from centrifuged blood samples. Platelet count in PRP was increased 670% compared to baseline level. Calcium/thrombin was added to degranulate platelets and form a gel.

Three weeks after surgery, push-out test and histomorphometri was performed.

After three weeks, the non-allografted implants had poor mechanical properties. Bone grafting significantly increased implant fixation, bone formation in the gap and bone growth on the implant surface. We found no significant effect of PRP alone or mixed with bone allograft on implant fixation or bone formation.

In conclusion, we showed the importance of bone allografting on early implant fixation and bone incorporation but we found no effect of PRP. More studies are needed to investigate the effect and possible clinical applications of platelet concentrates which are now being commercialised.
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Introduction

Since early subsidence of femoral components is an important predictor for later loosening [5], primary stability followed by bone incorporation of non-cemented implants is of great interest. HA coating is one approach to increase bone growth on bone grafted and non-grafted implants which is now well established in clinical use. Another approach is local delivery of growth factors. Previous studies show, that local application of growth factors such as OP-1 and TGF-β alone or mixed with bone allograft is capable of increasing gap healing [2,4,9–11,18]. Platelet α-granules contain growth factors such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), insulin-like growth factor (IGF) and epidermal growth factor (EGF) [8,17]. Those factors are important in initiating fracture healing. By concentrating platelets and delivering them locally, a higher level of growth factors might be reached which could stimulate the healing process in bone in a more physiological way than delivery of a single growth factor [6,15]. In a recent clinical study, PRP was capable of increasing bone regeneration in mandibular defects in combination with autograft [15]. In rabbits PRP increased bone formation in combination bovine cancellous bone in calvarial defects [6].

In the present study, we investigated the influence of PRP and bone grafting on implant fixation and bone formation.

* Corresponding author. Tel.: +45-8949-4134; fax: +45-8949-4150.
E-mail address: tbj@webspeed.dk (T.B. Jensen).

Materials and methods

Study design

8 skeletally mature Labrador dogs with an average weight of 27.5 kg [5,7–27] were used for implantation. Two additional dogs served as bone graft donors. Animal handling was performed according to Danish laws. Observation time was three weeks. A gap of 2.5 mm (0.66 cc) surrounded each implant as previously described [4,22]. Each gap was randomly allocated to the following treatments: Treatment 1: empty gap, treatment 2: PRP, treatment 3: fresh frozen bone allograft, treatment 4: fresh frozen bone allograft + PRP.

Implants

Titanium alloy (Ti 6Al 4V) implants cylindrical in shape with a plasma sprayed Ti porous coating were manufactured by Biomet® Inc. (Warsaw, IN, USA) (Fig. 1). Mean pore diameter was 480 μm and porosity 44%. A 50 μm hydroxyapatite (Ca/P ratio 1.67) coating was plasma sprayed on the porous Ti-alloy coating by BioInterfaces Inc. (San Diego, CA, USA). Final dimension of implants was a length of 10 mm and a diameter of 6 mm. The surface roughness (Ra) of HA coated implants was 41 μm. Implants were sterilized by 35 kGy Co-60 gamma irradiation. A standardised 2.5 mm gap was obtained by a footplate and a washer (Figs. 1 and 2).

Surgery

Surgery was done under halothane anaesthesia. Proximal humerus was exposed through a lateral extraarticular approach under sterile conditions. A 1.8 mm guide wire was inserted and an 11 mm hole was drilled using a cannulated drill. The implants of 6 mm in diameter were inserted in the overdrilled holes (Fig. 2). The most proximal implant was inserted immediately distal of the greater tubercle. The most distal implant was inserted 9 mm distal to the proximal drill hole. Before and after each operation, 1 g Ampicillin (Anhypen; Gist-Brocades, Delft, Holland) was administered. The dogs were killed by Methohexital (Brietal; Lilly, Denmark) sedation and an overdose of pentobarbital.

Bone allograft

Proximal humerus, proximal femur and proximal tibia were harvested from two dogs not included in the study. Bone graft was put into double plastic containers used for bone banking and stored at −80 °C.

After three weeks the bone graft was thawed and soft tissue and cartilage was removed. Using the finest grater in a standard bone mill, the graft was milled to chips applicable in a 2.5 mm gap. Most cortical bone was assorted during the milling process. During surgery, the graft was used with or without PRP according to the study design. It was
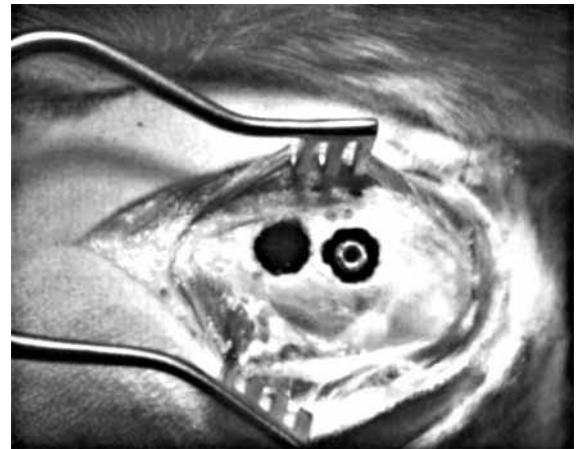
Fig. 2. Two holes 11 mm in diameter were drilled extraarticularly in each proximal humerus. An implant with a footplate was inserted in the hole (right hole). The 2.5 mm gap surrounding the implant was treated according to the treatment groups. After treatment of the gap, a washer was put on the top of the implant to seal the gap from the surrounding tissue (Fig. 1).

stamped into the gap to make it as tight as possible. Bacterial cultures were taken and the bone chips were stored at −80 °C in portions of 0.66 cc.

Platelet rich plasma

Before each operation peripheral venous blood was drawn into Vacutainer® EDTA (ethylene-diamine-tetra-acetic acid) coated test tubs. One 3 ml test tube and twelve 9 ml test tubes were filled. The twelve 9 ml test tubes were centrifuged (Hettich Universal 30 RF) at 4000 RPM for 20 min. Centrifugation divided the blood into its three components: Erythrocytes, buffycoat and platelet poor plasma (PPP). PPP was collected by a pipette and disposed. PRP was prepared by isolating the buffycoat and the adjacent 1 mm of PPP and erythrocytes. Approximately 0.6 ml of PRP was collected from each 9 ml test tube. PRP from all 12 samples was pooled in a 9 ml test tube. Three ml pooled PRP and 3 ml venous blood was analysed in a Coulter counter to determine concentrations of platelets, erythrocytes and leucocytes (Fig. 4).

In the operation room, 0.5 ml of PRP was mixed with 0.125 ml bovine thrombin/calcium chloride (2000 units of thrombine (Biofac, Kastrup Denmark) per ml) solution to allow formation of a gel. The PRP-gel was either used alone or mixed with bone allograft before application in the gap around the implants.

Preparation of tissue samples

Two implant/bone sections were cut of each implant perpendicular to the long axis of the implant using a water cooled band saw (Exact Apparatebau, Nordenstedt, Germany). One section with a thickness of 3.5 mm was frozen at −20 °C and later used for mechanical testing. Another section with a thickness of 5.0 mm was put into 70% alcohol and prepared for histomorphometry (Fig. 3). All specimens were blinded during preparation and evaluation.

Histomorphometry

After dehydrating the specimens in 70–100% ethanol containing 0.4% basic fucsin, each specimen was embedded in methylmethacrylate (Technovit 7200 VLC, Exakt, Germany). Four sections of 25 μm thickness were cut on a microtome (Leiden, Holland) and stained with 2% light green [3]. By this staining method, mineralized tissue is stained green other tissue is red (Figs. 2 and 3).

The vertical section method [16] was used: Each implant was randomly rotated around a vertical axis of the implant prior to sectioning
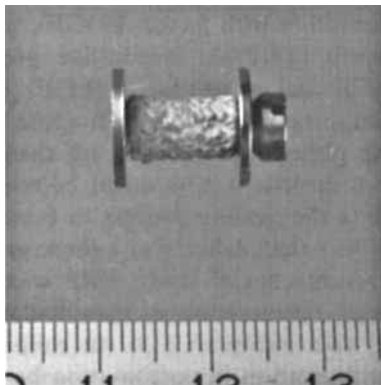

Fig. 1. Porous coated titanium alloy implant coated with hydroxyapatite (HA). The length of the cylindrical implant was 10 mm and the diameter was 6 mm. A footplate and a washer with a diameter of 11 mm secured a 2.5 mm gap.
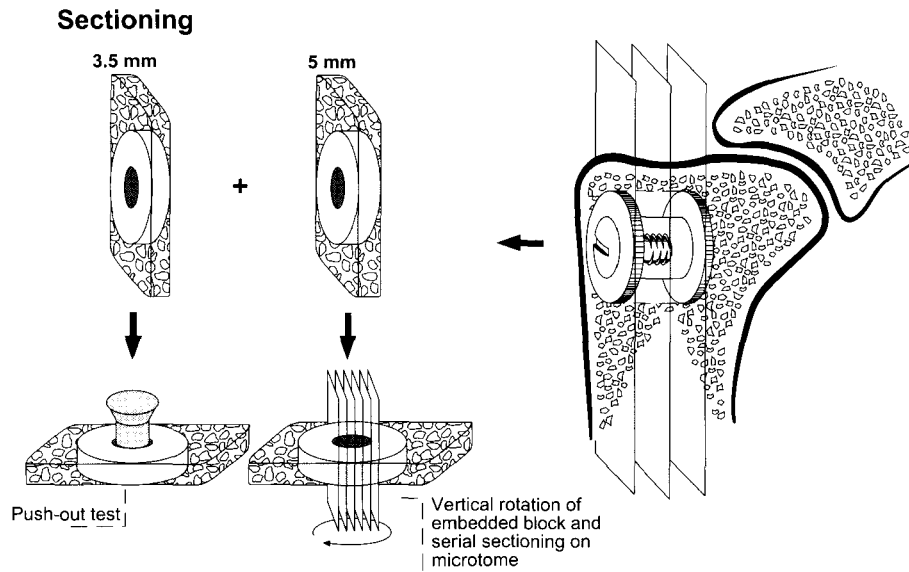
Sectioning

Fig. 3. Preparation of bone implant specimens. A water cooled band saw was used to make two implant/bone specimen. The first specimen was used for push-out testing. The second specimen was prepared for histomorphometry.

and serial sections were made parallel to that axis. Quantification was performed using an image-analysis system (Grid, Olympus, Denmark). The microscope fields were transmitted to a monitor and user-specified grids were superimposed randomly according to the vertical section method for unbiased estimates [16].

Volume fractions of woven bone, grafting material and other tissue in the gap were determined in two well-defined zones: Respectively 0–1 and 1.5–2.5 mm from the implant at 100× magnification. 250 points were counted in each of the two zones bone on every section. 250 intersections between a line grid and the surface of each section were used to estimate bone ongrowth.

Mechanical testing

Push-out test was performed using an Instron Universal test machine (Instron Ltd., High Wycombe, UK). The specimen was placed on a metal support jig and the implant, with a diameter of 6 mm in diameter, was centralised over a circular opening with a diameter of 7 mm. A preload of 2 N defined the contact position for the start of the test. The implant was displaced at a velocity of 5 mm/min. Data was transferred to a spread sheet (Excel, Microsoft, US) and load–deformation curves were obtained. Ultimate shear strength (σ_u) was determined from the maximal force (F) and was calculated as $\sigma_u = F/\pi DL$ where D is diameter and L the length of the implant. Apparent shear stiffness was obtained from the slope of the straight-line part of the load–displacement curve and calculated as $E = (\delta F/\pi DL)/\delta L$. Energy absorption was calculated from the area beneath the curve until failure [22].

Statistical analysis

Data on PRP are presented as mean and standard error of mean in brackets (SEM) (Table 1) all other data are presented as median (interquartile ranges). Non-parametric tests were used to compare gap treatments, since not all data was normal distributed. After applying Kruskal–Wallis one way analysis of variance (ANOVA) on ranks, gap treatments were all pairwise compared using Student–Newman–Keuls test. Volume fractions of graft were compared using a Wilcoxon signed rank test. P-values less than 0.05 (two-tailed) were considered significant.

Results

Surgery

All dogs were mobilised after two days. There were no signs of infections around any of the implants.

Four dogs had subcutaneous oedema aspirated from the deltoid region. The fluid was clear without signs of infections. All bacterial cultures from the bone graft were negative.

Analyses of PRP

Platelet count was increased by in average 670% and leukocyte count was increased by 720% compared to base line level (Table 1).

Table 1
Analyses of PRP and whole blood (mean (SEM), $n = 8$)

Group	Baseline count	PRP	PRP/whole blood
Platelets ($\times 10^9$/l)	246 (20)	1884 (178)	7.7 (0.4)
Leukocytes ($\times 10^9$/l)	8.1 (0.9)	64.2 (10.1)	8.2 (1.1)
Erythrocytes ($\times 10^{12}$/l)	5.9 (0.3)	9.0 (0.4)	1.6 (0.1)

PRP/whole blood was calculated for every single dog.

Table 2
Push-out data (median values (interquartile ranges))

Group	Ultimate shear strength (MPa)	Energy abs. (J/m²)	Apparent stiffness (MPa/mm)
Empty gap	0.03 (0.00–0.04)	6 (0–14)	0.05 (0.00–0.10)
PRP	0.07 (0.03–0.13)	14 (7–38)	0.15 (0.10–0.70)
Allograft	1.59 (1.38–1.76)*	331 (209–512)*	7.50 (4.55–9.65)*
Allograft + PRP	1.56 (0.75–2.31)*	275 (88–397)*	6.95 (4.90–10.95)*

*P < 0.05 compared to no empty gap or PRP treated gaps.

Mechanical tests

Four out of sixteen non-bone grafted implants could not achieve a preload of 2 N. Of those four implants, three was non-PRP treated.

Adding PRP to an empty gap markedly increased all mechanical parameters however none of those differences were statistical significant (Table 2).

Bone allografting significantly increased implant fixation. Adding PRP to FFBA did not change mechanical parameters significantly (Table 2).

Histomorphometry

Qualitatively analysis: In the non-bone grafted gaps, new bone was seen in both zones. The degree of bone growth on the implant surface varied a lot from implant to implant, but was not associated with PRP treatment. All bone in the gap was woven. Non-mineralized tissue was mainly cell rich and bone marrow was seen. A membrane with fibres parallel to the implant surface was found in contact with implant surface in some sections (Fig. 4).

In the bone grafted gaps, a large number of bone chips was found in both zones. They could be distin-guished from newly formed bone in the lamellar appearance and also, the green colour was lighter than that of newly formed bone (Fig. 5). Newly formed bone was found in both zones and bone growth into the HA coating was common.

Quantitative analysis: PRP had no influence on bone formation in the grafted or non-grafted group (Table 3). Bone allografting significantly increased the growth of bone into the HA coating and also bone formation in the gap (Table 3).

Discussion

In the present study, we found strong effect of bone grafting of HA coated implants after three weeks. Previously, bone grafting has shown increase in fixation of non-HA coated but not HA coated implants in a similar model after six weeks [22]. The present study emphasizes the importance of grafting on the very early implant fixation even when using HA coated implants.

We found no effect of PRP treatment on bone grafted and non-grafted implants. Application of a platelet concentrate at the site of a fracture or a gap surrounding an implant could increase the local concentration of growth factors and thereby stimulated bone incorporation of
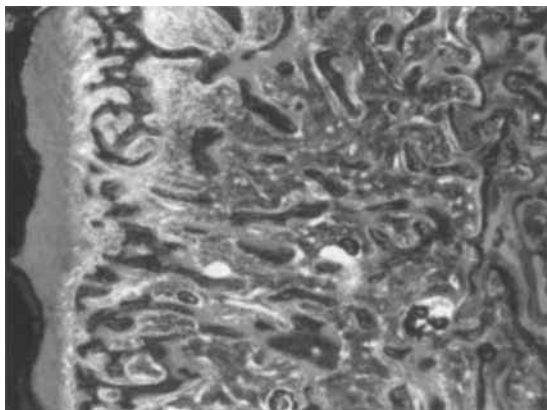

Fig. 4. Section from group with no PRP or bone graft (empty gap) (50× magnification, light green and basic fucsin). Implant with HA coating is seen to the left, bone surrounding the gap is seen to the right. Bone in the gap is stained green. A fibrous membrane surrounds the implant surface, no bone is in contact with the implant. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)
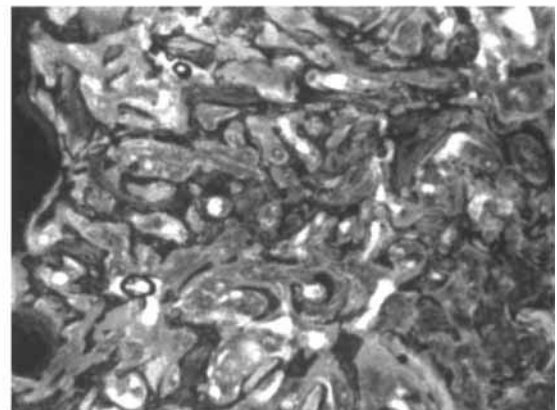

Fig. 5. Section from bone allografted group without PRP (50× magnification, light green and basic fucsin). Bone chips and newly formed bone is seen in the gap. Newly formed bone is in contact with the implant surface and no fibrous membrane is seen. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Bone ongrowth and volume fractions of woven bone, bone graft and non-mineralized tissue in percentages of total area (median values (interquartile ranges))

	Bone ongrowth	Woven bone		Graft		Non-mineralized tissue	
		Zone 1	Zone 2	Zone 1	Zone 2	Zone 1	Zone 2
Empty	0 (0–8)	13 (7–15)	15 (13–18)	–	–	87 (85–93)	86 (82–87)
PRP	0 (0–3)	11 (6–15)	14 (11–18)	–	–	89 (85–95)	87 (82–89)
Allograft	38 (33–45)*	18 (14–19)*	19 (15–20)*	30 (29–33)	27 (23–30)	53 (49–55)*	55 (50–63)*
Allograft + PRP	26 (15–51)*	16 (11–20)*	21 (19–25)*	31 (26–34)	22 (19–25)	54 (50–59)*	54 (52–61)*

* P < 0.05 compared to non-allografted groups.

the implant. Platelet concentrate stimulates osteoblast proliferation in vitro [17]. A number of articles describes the use of platelet rich plasma to enhance bone regeneration and healing of soft tissue in vivo [1,7,23,25,27] however only one clinical trial using PRP to enhance bone healing has been published by Marx et al. [15]. In a prospective randomized clinical study, 88 patients with mandibular defects were randomly treated with morselized autograft alone or autograft + PRP. PRP was increased by 238% compared to base line. After six months more bone was found in defects treated with PRP and radiographic examination showed enhanced maturity and bone consolidation of the PRP treated graft. Furthermore, PRP increased in combination with bovine cancellous bone allograft increased bone formation in calvarial defects in rabbit [6].

Several factors might explain why we found no effect of PRP. The way we isolated PRP differed from Marx. He drew 400–450 ml of whole blood from each patient and used a cell separator to divide the blood into its three basic components. The cell separator added citrate phosphate dextrose (CPD) to achieve anticoagulation. By isolating the buffycoat and the upper layer of the red blood cells Marx produced PRP containing 785×10^9 platelets/ml. In our setting we used EDTA as an anticoagulant, which compared to citrate phosphate dextrose gives a higher concentration of platelets [8]. EDTA might fragment platelets which according to Marx could decrease effectiveness of the growth factors [8]. PRP prepared with the use of EDTA was effective in another study [6].

In our study we reached an average platelet count of 1884×10^9 platelets/ml. This is higher than previously reported and was 770% of the baseline level. However, since 0.6 ml PRP was isolated from each test tube containing 9 ml blood, this increase could have been even higher since very few platelets are found in the plasma fraction [1,8]. By isolating the PRP from 12 test tubes from each dog, we managed to get a small standard deviation on the PRP/whole blood ratio (Fig. 4). This method could be used in clinic, if only limited volumes of PRP are needed as previously described [1].

PRP consist of concentrated platelets in a fibrin gel [1,15,27]. Theoretically, the fibrin network could keep the platelets on location and serve as an osteoconductor

[24]. Fibrin sealant makes bone chips easier to handle [15,24], however the influence on bone healing is controversial [13].

We determined the concentration of platelets in PRP prior to implantation but since bleeding from the gap might dilute the PRP we can not predict the final concentration of platelets in the gap. Also, as the bleeding from the bone supplied the graft with platelets when it was packed in the gap, the bone allograft used without PRP will be mixed with some platelets and leukocytes. Yet, the number would be expected to be less than if the graft was mixed with PRP prior to implantation.

A combination with bone allograft is clinical relevant since grafting of the bone bed is often necessary in the revision of THA. It has been suggested, that bone graft chips would serve as a carrier of the platelets and keep the growth factors on location [26].

We used an implant gap model with good healing capacity. HA coating alone is a strong stimulator of gap healing [19–22]. In such model, it might be difficult to further improve gap healing by addition of PRP especially when the gap is grafted. In a previous study, Soballe et al. found no effect of OP-1 in a loaded primary implant model with a bone grafted gap. In contrast, OP-1 increased fixation when the implants where inserted in a revision model with impaired bone healing capacity [18].

The concept of an autologous platelet concentrate in a fibrin gel has now been commercialised. Products such as AGF (Interpore, Irvine, CA, US) and SmartPReP (Harvest Autologous Hemobiologics, Norwell, Mass) are now marketed and used clinically [12,14]. However indications, minimum level of platelets and the benefit of using commercialised products have not yet been well elucidated.

Acknowledgements

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