The prophylactic effects of platelet-leucocyte gel in osteomyelitis
AN EXPERIMENTAL STUDY IN A RABBIT MODEL

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Platelet-leucocyte gel (PLG), a new biotechnological blood product, has hitherto been used primarily to treat chronic ulcers and to promote soft-tissue and bone regeneration in a wide range of medical fields. In this study, the antimicrobial efficacy of PLG against Staphylococcus aureus (ATCC 25923) was investigated in a rabbit model of osteomyelitis. Autologous PLG was injected into the tibial canal after inoculation with Staph. aureus. The prophylactic efficacy of PLG was evaluated by microbiological, radiological and histological examination. Animal groups included a treatment group that received systemic cefazolin and a control group that received no treatment.

Treatment with PLG or cefazolin significantly reduced radiological and histological severity scores compared to the control group. This result was confirmed by a significant reduction in the infection rate and the number of viable bacteria. Although not comparable to cefazolin, PLG exhibited antimicrobial efficacy in vivo and therefore represents a novel strategy to prevent bone infection in humans.

Bone and joint infection remains a challenging problem in orthopaedic surgery. Various prophylactic measures have been used to reduce the incidence of osteomyelitis, including choice of operation, aseptic and minimally invasive techniques, and peri-operative antibiotics. However, prolonged and widespread use of antibiotics has created adverse reactions, such as Clostridium difficile-associated diarrhoea and antibiotic-resistant bacterial strains, which present new challenges. Several antimicrobial drug delivery systems, including cement and biodegradable organic materials, have been developed in order to generate higher local concentration of antibiotics and lower systemic toxicity. However, the additional operations to remove the cement and the uncertainty and high cost-benefit ratio associated with organic materials are obstacles to their clinical use.

Platelet-rich plasma (PRP), a new biotechnological blood product, has been used mainly to promote soft tissue and bone regeneration. Platelet-leucocyte gel (PLG), prepared by thrombin stimulation of PRP, contains high concentrations of platelets and leucocytes, which are active in opsonophagocytosis, chemotaxis and oxidative microbicide. Furthermore, microbicidal peptides, released from platelets and neutrophils through contact with pathogens or stimulation by thrombin, possess dual antimicrobial and chemotactic properties which attract immune cells. Therefore, we thought that PLG might exhibit antimicrobial and growth-stimulating properties in vivo and present a viable option for the management of infection. Also, the ease of preparation of non-immunogenical high-quality PLG and its low cost make it an ideal ‘biological antibacterial’.

The purpose of this study was to investigate the antimicrobial activity of PLG against Staph. aureus in vivo.

Materials and Methods
Following the guidelines of the Local Animal Welfare Committee, 36 healthy, pathogen-free adult rabbits (weighing 2.85 kg (2.75 to 3.03) SD 0.11) were randomly assigned to four groups: PLG (n = 10), cefazolin (10), untreated controls (10) and sham-operated, using phosphate buffer saline, group (6).

Methicillin-sensitive Staph. aureus (ATCC25923) was used to induce infection in the rabbit tibiae. The bacteria were streaked and incubated on blood agar plates (Oxoid Ltd, Basingstoke, United Kingdom), and five colonies were transferred into brain/heart infusion broth (Oxoid Ltd) and incubated at 37°C for 24 hours. The bacteria were washed twice and aliquots of the organisms were frozen at -80°C. Pre-operatively, a frozen aliquot was thawed and a bacterial suspension containing 1 × 10^7
colony-forming units (CFU) per ml in PBS prepared. A volume of 0.1 ml (1 × 10⁶ CFU) was injected into the medullary cavity to induce osteomyelitis. Post-operatively, the same inoculum was serially diluted, plated on blood agar, and after incubation at 37°C for 24 hours, the number of bacteria was confirmed by colony counts.

In order to obtain PLG, PRP was prepared under aseptic conditions immediately prior to the test. Autologous whole blood (10 ml) was collected by puncture of the auricular vein and added to a 15 ml centrifuge tube (Corning Incorporated, New York). To prevent clotting, 1.5 ml of anticoagulant citrate dextrose-A solution (ACD-A) was added and the tubes were gently agitated to ensure mixing.

The blood underwent two-step centrifugation in an automated table-top centrifuge (Ankel TDL-5-A, Shanghai Anting Scientific Instrument Factory, Shanghai, China) at 250 g for ten minutes to separate the blood into three components based on density. Red blood cells formed at the lower level, platelets and leucocytes (the "buffer coat") in the middle and plasma at the top. The top two layers were transferred into an identical centrifuge tube and centrifuged at 1000 g for ten minutes to separate the platelet-poor plasma (PPP) in the upper layer from the PRP at the bottom. Approximately three-quarters of the PPP layer was extracted and the remaining PRP shaken vigorously by hand for 30 seconds. Shortly before administration, the PRP was activated by bovine thrombin (1000 IU/mL 10% calcium chloride solution) in a 10:1 ratio to produce PLG.

Subsequently, platelet and leucocyte counts in samples of whole blood, PRP and PPP were measured with a fully automated haematology analyser (Sysmex, XS-800i, Kobe, Japan).

The operations were performed under strict aseptic conditions. Rabbit weights were recorded and PRP was prepared according to the method described above. Ketamine (35 mg/kg) and xylazine (5 mg/kg) were injected subcutaneously. The proximal area of the right tibia was shaved, disinfected with povidone-iodine and covered by sterile drapes. Lidocaine (5 mg/kg) was injected into the operation area for local anaesthesia. The following operations were based on a previous model described by Nijhof et al. In summary, a 2.0 cm skin incision was made on the anterolateral surface of the right proximal tibia and the cortex of the metaphysis was partially exposed. After drilling a hole in the cortex using a 2.0 mm Kirschner wire, an 18-gauge needle was inserted into the medullary canal, which was subsequently irrigated with saline after bone marrow extraction. Then, 0.1 ml of 5% sodium morrhuate, 0.1 ml of bacterial suspension (1 × 10⁶ CFU/ml) and 0.1 ml of saline (0.9%) were injected sequentially into the medullary cavity. The PLG group continued to be injected with 1.0 ml of PLG. The cefazolin group received a 72-hour regimen of cefazolin (30 mg/kg every eight hours) injected through the auricular vein beginning one hour pre-operatively. The control group received no further injection. The PBS group was injected with equal volumes of 5% sodium morrhuate and PBS instead of bacterial suspension. Finally, the holes were sealed with sterile bone wax to prevent leakage of the injection. The fascia, subcutaneous layer and skin were irrigated and closed with 4/0 Vicryl.

The post-operative follow-up period was 28 days, during which the animals were housed in individual cages and fed a normal diet. All were monitored daily for general condition, including activity, body temperature, weight and wound appearance. Also, blood samples were taken pre-operatively and on days 3, 7, 14, 21, 28 post-operatively for measurement of the leucocyte count and ESR.

On day 28, blood samples were extracted and radiographs taken. All animals were killed by an intravenous injection of pentobarbital sodium. The operation site was prepared in the same way as at the first operation. The tibiae were excised, freed from soft tissues and, using a swing saw under continuous normal saline irrigation, they were cut into three parts, the proximal metaphysis (section A, 1.0 cm long), diaphysis (section B) and distal metaphysis (section C, 1.5 cm long). A 1.0 cm part of the proximal section B (section B1) was sawn off for microbiological analysis and all remaining parts of the tibiae were used for histological examination (Fig. 1). All these procedures were performed under aseptic conditions.

For all animals, standard radiographs, including anteroposterior and lateral views, were taken on days 1, 7, 21 and 28 post-operatively. All radiographs were randomised and scored by an orthopaedic surgeon (Z-PX) in a blinded fashion, according to the severity score system for osteomyelitis described by Norden, Myerowitz and Keleti. Total tibial length was taken into account and a composite score obtained. Animals with scores ≥ 3 were diagnosed with osteomyelitis. Similarly, conventional pathological sections stained with haematoxylin and eosin were prepared for
bone samples and, in a blinded fashion, were assessed by a
pathologist (JZ) according to the scoring system of Smeltzer et al.\textsuperscript{16} The animals with four points or more were diag-
nosed with osteomyelitis.

A portion of bone (0.86 g (SD 0.08)) was cut from section
B1 and homogenised in 30 ml sterile PBS at 10 000 rpm for
five minutes using a tissue homogeniser. A series of tenfold
dilutions were prepared in saline and 10 $\mu$l of each dilution
was plated on a blood agar plate and incubated at 37°C for
48 hours. Finally, the colonies were counted and the
amount of CFU/g of bone was obtained. For statistical
analysis, the negative cultures were calculated conserva-
tively as $2 \times 10^3$ CFU/g, which is the detection limit value.
All tests were carried out in triplicate under aseptic condi-
tions. Also, in order to identify \textit{Staph. aureus} based on
16S rDNA genotyping of bacteria, we used polymerase
chain reaction (PCR) and pulsed-field gel electrophoresis
(PFGE) as described by Schabereiter-Gurtner et al.\textsuperscript{17}

\textbf{Statistical analysis.} The Statistical Package for the Social
Sciences (SPSS) (SPSS Inc., Chicago, Illinois) version 11.0
for Windows was used to analyse the results. Non-
parametric tests for independent samples (the Mann-
Whitney tests) were performed to compare radiological and
histological scores between groups at 28 days, as well as
the CFU count in the culture of bone samples among
groups. The paired Student’s \textit{t}-test was used to compare
body temperature, weight, leucocyte count and ESR at dif-
ferent times within a group. The one-way ANOVA (least
significance difference \textit{post hoc} test) was used for compar-
ison among groups at autopsy. Chi-squared tests were
used to evaluate infection rates among groups. The results
were reported as mean SD, and p-values of 0.05 or less were
considered to be significant.

\textbf{Results}

For the determination of PRP approximately 1.0 ml of PRP
was obtained from each rabbit. The platelets and leucocyte
counts of the whole blood were 381(SD 25) $(10^6$/ml) and
8.2 (SD 1.1) $(10^6$/ml), respectively. After PRP preparation
these numbers increased to 2636 (SD 356) $(10^6$/ml) and
48.3 (SD 4.3) $(10^6$/ml), representing an average 6.9-fold and
5.9-fold increase in platelets and leucocytes. There were
few platelets or leucocytes in the PPP. One week post-
operatively, one rabbit in the PLG group and one in the
control group died from severe infection. The remaining
animals recovered with no obvious signs of systemic com-
lications in the 28-day follow-up. Therefore, a total of
34 rabbits were included in the statistical analysis.

Although there were fluctuations in the follow-up period,
there were no significant differences in the measurement of
body temperature, weight, leucocyte count and ESR at
autopsy compared to the pre-operative levels (Figs 2 and 3).

Radiographs showed more severe osteomyelitis in the
control group, with eight of nine animals having a score of
three or above. Four of nine and two of ten animals in the PLG and cefazolin groups, respectively, had a score of three or above at autopsy and no infection was observed in the PBS group (Fig. 4). The total scores of the control group were significantly higher than in the PLG group \( (p = 0.036) \) and the cefazolin group \( (p = 0.002) \). There was no statistically significant difference between the PLG and cefazolin groups \( (p = 0.058, \text{Table III}) \).

Histological results further confirmed the varying severity of osteomyelitis among the groups (Fig. 5). Typical signs of osteomyelitis, including intraosseous acute inflammation, intraosseous chronic inflammation, periosteal inflammation and bone necrosis, could be seen in some animals, and partial signs were noted in others. A severity score of four or more was found in five of nine, three of ten, and eight of nine animals in the PLG, cefazolin and control groups, respectively. The control group showed a significantly higher score than the PLG \( (p = 0.044) \) and cefazolin groups \( (p = 0.001) \). A statistically significant difference was also found between the PLG and cefazolin groups \( (p = \)
Table III. Results of radiological, histological scoring and culture of tibial specimens at autopsy

<table>
<thead>
<tr>
<th>Group</th>
<th>Radiological scores (SD)</th>
<th>Histological scores (SD)</th>
<th>Outcome of bacterial culture</th>
<th>Bacterial quantity (log CFU/g) (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLG</td>
<td>2.78 (1.39)†</td>
<td>5.89 (3.92)†</td>
<td>4/9†</td>
<td>4.44 (1.36)†</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1.55 (1.48)†</td>
<td>3.00 (2.31)†</td>
<td>2/10†</td>
<td>3.39 (0.23)†</td>
</tr>
<tr>
<td>Control</td>
<td>4.17 (1.25)‡</td>
<td>10.33 (4.03)‡</td>
<td>8/9</td>
<td>5.83 (1.50)‡</td>
</tr>
</tbody>
</table>

CFU/g, colony-forming units per gram of bone
* p < 0.05 compared to control group
† p = 0.059 compared to cefazolin group
‡ p = 0.042 compared to cefazolin group
§ p = 0.252 compared to cefazolin group
¶ p = 0.138 compared to cefazolin group

Photomicrographs of longitudinal sections of the tibiae in (a) the control group, showing destruction of cortical bone, sequestrum formation (white arrow), remodelled bone (black arrow), necrotic adipocytes (white arrowhead), fibrosis (black arrowhead) and periosteal new bone formation (black block arrow) (haematoxylin and eosin × 20), (b) sequestrum surrounded by the granulocytes and proliferated foamy histocytes (black block arrow) and severe inflammation with intramedullary abscess (white block arrow) were also observed in the control group (haematoxylin and eosin × 40) (c) the platelet-leucocyte gel-treated group (left) showing mild destruction and calcification of cortical bone and intramedullary new bone formation without signs of severe inflammation (haematoxylin and eosin × 40) and the cefazolin-treated and PBS groups (right) showing no histological signs of bone infection (haematoxylin and eosin × 40). Sequestrum surrounded by the granulocytes and proliferated foamy histocytes (black block arrow) and severe inflammation with intramedullary abscess (white block arrow) were also observed in the control group (haematoxylin and eosin × 40).

0.042) (Table III). No pathological changes were observed in the PBS group.

On microbiological examination MSSA was recovered from four of nine, two of ten and eight of nine bone specimen from the PLG, cefazolin and control groups, respectively. No bacterium was found in the sham-operated group. Further quantitative data on bacterial culture showed significantly fewer viable bacteria in the PLG (p =
0.026) and cefazolin (p = 0.004) groups than in the controls. No significant difference was found between the PLG and cefazolin groups (p = 0.138, Table III). The bacterial strains cultured from samples with positive culture results at autopsy were confirmed to be identical to those used for induction of osteomyelitis. Therefore, no contamination by other bacterial strains during the operations was observed.

Discussion

Various prophylactic measures have been used with varying success to reduce the incidence of bone infection, and perioperative antibiotic prophylaxis has become the standard treatment in orthopaedic surgery. Although antibiotics are effective in preventing bone infection,18 their long-term use often causes adverse systemic reactions. Also, the emergence of many resistant strains makes antibiotic selection difficult.

Platelet-rich plasma has been used to promote soft- and bone-tissue repair owing to the substantial amount of growth factors released from platelets. However, evidence showing the antimicrobial activity of PLG is very limited. Recently, Bielacki et al19 found that PLG could inhibit the growth of methicillin-sensitive and methicillin-resistant Staph. aureus and Escherichia coli in vitro. A similar antimicrobial property of PLG against Staph. aureus in vitro was reported by Moojen et al.20 Two clinical studies have also demonstrated the bactericidal potential of PLG in arthroplasty and cardiac surgery.21,22 To our knowledge this study is the first to investigate the antimicrobial capability of PLG in a rabbit model of osteomyelitis.

In our preparation of PLG the two-step centrifugation of whole blood allowed for the enrichment of leucocytes and platelets owing to their similar sedimentation coefficients. Our results showed that an average increase of 6.9-fold of platelets and 5.9-fold of leucocytes were concentrated from whole blood of rabbits. Similarly, in a study by Everts et al,23 PRP was reported to consist not only of platelets but also of concentrated leucocytes, including neutrophils, monocytes and lymphocytes. The multiple abilities of platelets and leucocytes in host defence include antimicrobial properties. Neutrophils and monocytes are responsible for the phagocytosis and elimination of foreign pathogens by releasing antimicrobial enzymes, as well as by generating reactive oxygen derivatives.9

Platelets represent a large component in PLG and are indispensable in antimicrobial host defence. Yeaman et al24 found that platelets can adhere directly to and inhibit the growth of bacteria and fungi in vitro, and can also enhance clearance of organisms from the bloodstream. Kriegsveld et al25 also concluded that platelets can eliminate pathogens from the bloodstream by actively participating in antibody-dependent cell cytotoxicity reactions. Platelets can exert chemotactic responses to neutrophils and monocytes by expressing selectins, immunoglobulin G (IgG) Fc receptors and C3a/C5a complement fragments.11 Meanwhile, many growth factors contained within the α granules of platelets, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and insulin-like growth factor (IGF) can be released when PRP is activated by thrombin.7 In addition to promoting tissue regeneration, these active proteins can also act as chemokines to attract neutrophils and monocytes to the site of infection.26 The chemotactic property of platelets to inflammatory cells contributes to the production of oxygen metabolites and eventually the elimination of the pathogen.

Neutrophils and platelets can also release a variety of small cationic peptides, which play a significant role in antimicrobial host defence via non-oxidative mechanisms.10-12 Yeaman et al27 and Tang, Yeaman and Selsted28 isolated and identified several types of microbial peptide from rabbit and human platelets. Four main defensins (HNP-1-4) have also been found in humana neutrophils.10 Further studies indicate that these peptides share similar features, such as voltage-dependent membrane permeabilisation and functional membrane perturbation, whereby they become microbicidal by disrupting the cytoplasmic membrane and inhibiting macromolecular synthesis of target organisms.12 Many in vitro and in vivo data show that these peptides exert potent microbicidal activities against pathogens, including Staph. aureus, E. coli, Streptococcus viridans, Candida albicans and Cryptococcus neoformans.29-31 Also, these peptides can act in synergy with conventional antibiotics and are less prone to inducing bacterial resistance, owing to the difficulty of changing the bacterial membrane structure.32,33 Therefore, when using PLG, the dose of antibiotic can be considerably reduced.

Recent studies have shown that some antimicrobial peptides from human platelets and neutrophils also possess chemotactic properties for immune cells such as neutrophils, monocytes, T cells and mast cells.34 This discovery indicates that there are overlapping functions between inflammatory chemotactic factors and antimicrobial peptides, thereby establishing a synergistic net between innate immune mechanisms and antimicrobial peptides.35

Another property, fibrinogen in PRP, is converted to a network of fibrin gels by the addition of thrombin in 10% calcium chloride solution. The antimicrobial components and growth factors released from platelets and leucocytes are trapped within the fibrin network and not liberated immediately.36 Because of this barrier, PLG not only protects normal host tissue from direct invasion by pathogens, but also contributes to persistent antimicrobial activity as a slow-release system.

The 89% infection rate (eight of nine rabbits) in the control group compared to the PBS group, with no significant difference of body temperature, weight, leucocyte count and ESR among the different groups on day 28, indicates that the infection was localised in the tibia and confirmed the validity of this animal model.

We found a significant difference between PLG and cefazolin in histological scores (p = 0.042), and an almost significant difference in radiological scores (p = 0.059), which indicated that the efficacy of PLG for infection prophylaxis in vivo was not comparable to that of cefazolin. The lack of
We wish to thank J. Zhou and W. Tan for their help in processing bone samples, which are necessary to confirm the clinical value of PLG.

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References