Antibacterial effect of autologous platelet gel enriched with growth factors and other active substances

AN IN VITRO STUDY

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Platelet-rich plasma is a new inductive therapy which is being increasingly used for the treatment of the complications of bone healing, such as infection and nonunion. The activator for platelet-rich plasma is a mixture of thrombin and calcium chloride which produces a platelet-rich gel.

We analysed the antibacterial effect of platelet-rich gel in vitro by using the platelet-rich plasma samples of 20 volunteers. In vitro laboratory susceptibility to platelet-rich gel was determined by the Kirby-Bauer disc-diffusion method. Baseline antimicrobial activity was assessed by measuring the zones of inhibition on agar plates coated with selected bacterial strains.

Zones of inhibition produced by platelet-rich gel ranged between 6 mm and 24 mm (mean 9.83 mm) in diameter. Platelet-rich gel inhibited the growth of Staphylococcus aureus and was also active against Escherichia coli. There was no activity against Klebsiella pneumoniae, Enterococcus faecalis, and Pseudomonas aeruginosa. Moreover, platelet-rich gel seemed to induce the in vitro growth of Ps. aeruginosa, suggesting that it may cause an exacerbation of infections with this organism. We believe that a combination of the inductive and antimicrobial properties of platelet-rich gel can improve the treatment of infected delayed healing and nonunion.

Despite advances in surgical techniques, the treatment of open fractures continues to be associated with a high rate of delayed union and nonunion. When the healing of a fracture is delayed, a secondary intervention may be needed which may itself be associated with increased morbidity and a reduced quality of life. Consequently, the goal of the initial treatment should be to increase the likelihood of union.1,2

Several factors influence the development of nonunion. These include the location and type of fracture, the skill of the surgeon, and complicating infection. The success of secondary treatment depends upon various factors, including the type of nonunion, the application of osteoinductive and osteoconductive biomaterials, the method of stabilisation used, any concomitant infection and the general health of the patient. Deep infection may not respond to surgical management and infection precludes the use of biomaterials. The conventional methods for the treatment of nonunion require considerable improvement.1

In recent years the application of platelet-rich plasma to enhance bone regeneration and soft-tissue maturation has increased in maxillofacial and orthopaedic surgery.3,7 Platelet α-granules contain over 30 growth factors. The most important are platelet-derived growth factor, transforming growth factor-β, vascular endothelial growth factor, insulin-like growth factor and epidermal growth factor.8-10 By concentrating platelets, higher levels of growth factors may be reached, which then stimulate the healing processes. The activator for platelet-rich plasma is a mixture of thrombin and calcium chloride which produces platelet-rich gel. Apart from converting fibrinogen into fibrin, thrombin also directly stimulates cells, for example, by inducing cell proliferation.6,11

The linking of osteoinductive and antimicrobial activity may be critical to the treatment of infected nonunion. For this reason we have investigated the in vitro antimicrobial activity of platelet-rich gel.

Patients and Methods

Our study group comprised 20 healthy volunteers examined between February and November 2005. All were free from infection and none had taken any medication during the previous two months. From each we collected
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54 ml of whole blood into a syringe containing 6 ml of citrate solution. The whole blood was drawn into a sterile tube and centrifuged for 12 minutes at 3200 rpm (GPS I Platelet Concentration System; Biomet, Warsaw, Indiana). This caused separation of the blood into its three basic components: red blood cells, platelet-rich plasma (sometimes referred to as ‘buffy coat’), and platelet-poor plasma. Because of differing densities, the layer of red blood cells was formed at the lowest level, platelet-rich plasma comprised the middle layer and platelet-poor plasma the upper layer. The centrifuge separated each layer from the least dense to the most dense with platelet-poor plasma first and platelet-rich plasma second, leaving the residual red blood cells. Subsequently, the platelet-poor plasma component was removed into a 30 ml syringe. The now platelet-poor plasma-free tube was shaken vigorously by hand for 30 seconds in order to suspend the platelets. We then obtained 6 ml of platelet-rich plasma from the tube, and stored it with 1.5 ml of 1600 U/ml bovine thrombin in a 10% calcium chloride solution (Trombina 400; Biomed, Lublin, Poland) at room temperature.

The platelet and leucocyte counts in the peripheral blood, and in the platelet-rich plasma were measured in a haematology analyser (Advia 120, Bayer, Leverkusen Germany).

In vitro laboratory susceptibility to platelet-rich gel and thrombin was determined by the Kirby-Bauer disc-diffusion method12 on Mueller-Hinton agar (Becton Dickinson Cockeysville, Maryland). Agar plates were coated with one of the following bacterial strains: methicillin-resistant Staphylococcus aureus (MRSA), methicillin-sensitive Staph. aureus (MSSA), Escherichia coli (Extended Spectrum Beta Lactamase, ESBL), E. coli, Klebsiella pneumoniae (ESBL), Enterococcus faecalis and Pseudomonas aeruginosa. We created two groups. In the first, standard 6 mm discs12 were coated with 10 µl of platelet-rich plasma and 2 µl of thrombin in calcium chloride by using separate micropipettes, thereby forming platelet-rich gel. In the second, 12 µl of thrombin were used. The agar plates were then kept in an incubator at 35˚C. Baseline antimicrobial activity was assessed after 16 to 18 hours by measuring the zones of inhibition, which were recorded as the diameter in millimetres across the centre of the embedded discs.

Statistical analysis. Statistical analysis was performed using Statistica for Windows software, version 6.1 (Statsoft, Kraków, Poland). Statistical differences were evaluated using the Mann-Whitney U test with a p-value ≤ 0.05 considered to be significant.

Results
Table I shows the results of the baseline tests for the platelet-rich gel and thrombin-coated discs against various bacteria. Zones of inhibition produced by platelet-rich gel ranged between 6 mm and 24 mm (mean 9.83) in diameter. Platelet-rich gel dramatically inhibited the growth of MSSA and 2 µl of thrombin in calcium chloride by using separate micropipettes, thereby forming platelet-rich gel. In the second, 12 µl of thrombin were used. The agar plates were then kept in an incubator at 35˚C. Baseline antimicrobial activity was assessed after 16 to 18 hours by measuring the zones of inhibition, which were recorded as the diameter in millimetres across the centre of the embedded discs.

Table I. Zones of inhibition for the platelet-rich gel and thrombin groups in mm

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<th>Sample</th>
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* MRSA, methicillin-resistant Staphylococcus aureus; MSSA, methicillin-sensitive Staphylococcus aureus; ESBL, extended spectrum beta lactamase.
plate coated with *Ps. aeruginosa* we observed the growth of bacteria under the disc containing platelet-rich gel and for better visualisation in the next 19 cases, platelet-rich plasma with thrombin was applied directly on the agar plate with *Ps. aeruginosa*. The growth of this strain on platelet-rich gel was observed (Fig. 3). We did not see any antibacterial effects induced by thrombin itself. The differences in the antimicrobial activity between platelet-rich gel and thrombin against *Staph. aureus* and *E. coli* were highly significant (p < 0.001).

The mean platelet number was 228 ± 59 SD × 10⁹/l and the mean leucocyte number 6.679 ± 2.201 SD × 10⁹/l. Platelet counts were increased by a mean of 760% (710% to 780%) and leucocyte counts by 790% (720% to 830%). We found no correlation between antimicrobial activity and the concentration of platelets and leucocytes in the blood and the platelet-rich plasma.

**Discussion**

Clinical studies have shown that application of platelet-rich gel during surgery enhances soft-tissue healing.¹³,¹⁴ Autologous platelets have been shown to promote tissue repair in several clinical situations in orthopaedic surgery, including lumbar spinal fusion, nonunion and tennis elbow.⁵,⁶,¹⁵ Recently, publications have demonstrated an improved outcome during cardiac surgery when platelet-rich gel was applied during closure of the sternal wounds.¹⁶,¹⁷ Despite a wide spectrum of available diagnostic techniques and potent antimicrobials, osteomyelitis associated with disorders of bone healing remains a challenging problem. *Staph. aureus* is a major cause of hospital-acquired infection of both surgical wounds and infections associated with indwelling medical devices.¹⁸ In our study, we have found a strong effect of platelet-rich gel on MSSA. This activity is comparable with that of gentamicin and oxacillin.¹²

*E. coli* is also a common cause of wound and bone infections.¹⁸ Our study has shown that platelet-rich gel is active against this organism. However, the differences in antimicrobial activity between platelet-rich gel and thrombin were significant for the two strains of *Staph. aureus* (MRSA and MSSA), and the two strains of *E. coli* which we studied. We observed this antimicrobial activity in 18 of 20 subjects although in two, antimicrobial activity was not detected. In contrast, we found no activity of platelet-rich gel against *K. pneumoniae*, *E. faecalis*, or *Ps. aeruginosa*. Indeed, in all cases we observed growth of bacteria under the disc containing platelet-rich gel in an agar plate coated with *Ps. aeruginosa*. Additionally, in 19 cases, platelet-rich gel was applied directly onto the agar plate with *Ps. aeruginosa* (Fig. 3). We observed that platelet-rich gel induced growth of this bacterial strain, suggesting that it may induce a flare-up of infection from *Ps. aeruginosa*.

*Staph. aureus* and *Ps. aeruginosa* are the most common bacterial species resident in chronic ulcers.¹⁹ In particular, ulcers infected with *Ps. aeruginosa* are reported to be significantly larger than those not infected with this strain.¹⁰ Gjodsbol et al.²⁰ suggested that the presence of *Ps. aeruginosa* in venous leg ulcers can induce enlargement of the ulcer and delayed healing. Our *in vitro* tests suggest that the value of treating chronic ulcers with platelet-rich gel in the presence of a co-existing infection with *Ps. aeruginosa* is uncertain and, in our view, such treatment should be contraindicated.

We could not find any reports on the antibacterial properties of platelet-rich gel in the available literature, suggesting that its antibacterial mechanism is not yet fully understood. Existing evidence suggests that platelets have many functions in the antimicrobial host defence systems.²¹-²³ These include navigation toward the inflammatory chemoattractant N-MetLeuPhe, expression of immunoglobulin-G Fc receptors and for C3/C5 complement fragments, and the capacity to generate antimicrobial oxygen metabolites including superoxide, hydrogen peroxide, and hydroxyl free radicals.²²,²⁴ Moreover, platelets interact directly with micro-organisms, contribute to the
clearance of pathogens from the bloodstream, and actively participate in antibody-dependent cell cytotoxicity against microbial pathogens. Numerous investigators have sought to isolate platelet-specific antimicrobial molecules from animal and human platelets. Yeaman et al characterized antimicrobial polypeptides from thrombin-induced released materials and acid extracts of rabbit platelets. Tang et al reported the isolation and tentative identification of seven antimicrobial peptides from human platelets after stimulation by thrombin as follows: fibrinopeptide A, fibrinopeptide B, thymosin β-4, platelet basic protein, connective-tissue-activating peptide 3, RANTES and platelet factor 4. They tested in vitro antimicrobial activities of these peptides against E. coli, Staph. aureus, Candida albicans and Cryptococcus neoformans. They were more potent against bacteria than fungi, and antimicrobial activities were dose-dependent. These findings suggested a direct relationship between the platelet concentration and the antimicrobial effect.

Platelet α-granules contain not only growth factors and antimicrobial peptides, but also catecholamines, serotonin, osteonectin, von Willebrand factor, proacce, and other substances. These are released in high concentrations after platelet aggregation and may also have antibacterial effects. Platelet-rich plasma is also a concentration of leukocytes. We, and others, have found markedly increased leukocyte counts (more than sevenfold) compared with baseline levels. This applied to both neutrophils, which are involved in direct bacterial killing, and lymphocytes which are responsible for the antigen-specific immune response.

In the treatment of an infected nonunion, the goal is to eradicate the infection and to obtain both union, and a functional limb. Most authors consider that radical debridement and stable external fixation are necessary for good results. However, resistant infections may not respond to surgical management. Furthermore, operations which use biomaterials may induce a flare-up of a previous infection. Because of the lack of improvement in the conventional methods of treatment we suggest that a new alternative should be found, such as the linking of the osteoinductive properties and antimicrobial activity of platelet-rich gel. This may be of substantial value in the treatment of infection-related delayed healing and nonunion. Platelet-rich gel can also be used prophylactically to cover wounds and bone defects in open fractures since its gelatinous mass forms a barrier against microbes. Its value in infection with Ps. aeruginosa may be uncertain, since our in vitro data suggest that this may exacerbate the inflammatory process. Further studies are necessary in order to clarify fully the antimicrobial effects of platelet-rich gel.

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References