

Volker Gassling
 Timothy Douglas
 Patrick H. Warnke
 Yahya Açil
 Jörg Wiltfang
 Stephan T. Becker

Platelet-rich fibrin membranes as scaffolds for periosteal tissue engineering

Authors' affiliations:

Volker Gassling, Timothy Douglas, Patrick H. Warnke, Yahya Açil, Jörg Wiltfang, Stephan T. Becker, Department of Oral and Maxillofacial Surgery, University of Schleswig-Holstein, Kiel, Germany

Patrick H. Warnke, Faculty of Health Sciences and Medicine, Bond University, Gold Coast, Qld, Australia

Corresponding author:

Volker Gassling
 Department of Oral and Maxillofacial Surgery
 University of Schleswig-Holstein
 Campus Kiel, Arnold-Heller-Straße 3
 Haus 26, 24105 Kiel, Germany
 Tel.: + 49 431 597 2791
 Fax: + 49 431 597 4084
 e-mail: gassling@mkg.uni-kiel.de

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Abstract

Objectives: Platelet-rich fibrin (PRF)-based membranes have been used for covering alveolar ridge augmentation side in several *in vivo* studies. Few *in vitro* studies on PRF and no studies using human periosteal cells for tissue engineering have been published. The aim is a comparison of PRF with the commonly used collagen membrane Bio-Gide® as scaffolds for periosteal tissue engineering.

Material and methods: Human periosteal cells were seeded on membrane pieces (collagen [Bio-Gide®] and PRF) at a density of 10⁴ cells/well. Cell vitality was assessed by fluorescein diacetate (FDA) and propidium iodide (PI) staining, biocompatibility with the lactate dehydrogenase (LDH) test and proliferation level with the MTT, WST and BrdU tests and scanning electron microscopy (SEM).

Results: PRF membranes showed slightly inferior biocompatibility, as shown by the LDH test. The metabolic activity measured by the MTT and WST tests was higher for PRF than for collagen (BioGide®). The proliferation level as measured by the BrdU test (quantitative) and SEM examinations (qualitative) revealed higher values for PRF.

Conclusion: PRF appears to be superior to collagen (Bio-Gide®) as a scaffold for human periosteal cell proliferation. PRF membranes are suitable for *in vitro* cultivation of periosteal cells for bone tissue engineering.

After tooth loss, the reconstruction of alveolar ridge defects is still a challenge. The aim is the cultivation of bone grafts to reconstruct the jaw bone so that dental implants can be placed. Thus far, vital autograft has been the gold standard for repair of large bone defects, because of its ability to stimulate new bone formation by recruitment of mesenchymal stem cells (MSC). Kostopoulos & Karring (1995) and other research groups (Stevenson 1999) showed that the periosteum and especially the MSC contained in the cambium layer play a crucial role in bone autograft healing by differentiation into bone cells. Based on

these findings, periosteal cells are currently a prime focus in bone tissue engineering. A main problem in bone transplantation research is the application of these cells *in vivo*.

One of the pioneers of tissue regeneration was Buser et al. (1993), who introduced the term 'guided bone regeneration (GBR)' to describe the support of bone augmentation by a barrier membrane. Many membrane systems, both non-resorbable and resorbable, have been investigated in the past few years. Until now, bioresorbable membranes have been preferred because they permit a single-stage

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procedure without removal and **less bacterial colonization** (Rothamel et al. 2005). Hence, a main goal in tissue engineering is **to create a resorbable membrane system to grow periosteal cells for bone tissue engineering**. In general, these membranes could be of **synthetic origin** or **derived from natural materials like collagen** (BioGide®), a membrane composed of **porcine type I and type III collagen fibers**. Arnold et al. (2002) revealed that a **synthetic resorbable composite of PGLA polymer (poly[glycolic acid-co-lactic acid]), β-TCP and fibrin led to enhanced proliferation and osteogenic differentiation of periosteal cells *in vitro***. It could be shown that **periosteal cell/matrix composites can be spatially transferred with fibrin glue from culture dishes to recipient sites in animal models without loss of viability of cultured tissue** (Isogai et al. 2000).

Thus, the use of **vital fibrin as an autologous scaffold for periosteal cell or stem cell transplantation and consequently for bone tissue engineering** is an obvious option. It is **biocompatible, bioresorbable** and plays an essential role in wound repair, not only for hemostasis but also **provides a matrix for migration of tissue-forming cells like fibroblasts and endothelial cells, which are involved in angiogenesis and that are responsible for remodeling of the new tissue**. In the normal wound-healing process, **platelets** are trapped within the fibrin matrix and are **subsequently activated** so that growth factors like **platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and insulin-like growth factor I (IGF-I)** are set free (Schliephake 2002), which could stimulate the mitogenic response of the periosteum during bone repair (Gruber et al. 2003).

The **essence of platelet-rich fibrin (PRF) modified by Choukroun and colleagues (Dohan et al. 2006a) is a fibrin matrix in which the platelet cytokines and cells are trapped and may be delivered after a certain time** (Mosesson 2005). Although there are several studies on the use of Choukroun's PRF *in vivo* (Choukroun et al. 2006a, 2006b), there are relatively few studies concerning cell culture on PRF *in vitro* (Choukroun et al. 2007; Dohan et al. 2007; Gassling et al. 2009). It could be shown **that PRF can serve as a resorbable membrane that can be used in preprosthetic surgery as well as in implantology**

to cover bone augmentation sites (Choukroun et al. 2006b). These, and other studies involving other PRF preparations (Lundquist et al. 2008), have concentrated on the effect of PRF added to two-dimensional cell cultures on cell culture polystyrene and not on direct culture of cells on PRF. To the authors' best knowledge, there are no *in vitro* studies comparing PRF with other membranes (e.g., the commonly used collagen membrane, BioGide®) or involving the use of periosteal cells that are commonly used in bone tissue engineering applications.

With a view to developing new scaffold materials, in this study, **biocompatibility and ability to support and promote the proliferation of human periosteal cell proliferation** was measured for the first time on PRF membranes produced using the method of Choukroun and colleagues (Dohan et al. 2006a) and compared with the conventional collagen membrane BioGide® *in vitro*.

Material and methods

Isolation and cultivation of cells from the periosteum

Human periosteum biopsy was harvested from one patient (male, 24 years old) during the course of oral surgery (wisdom tooth removal). The study (AZ 417/07) was approved by the Ethics Commission of the Christian-Albrechts-University of Kiel, Germany. The cells were cultivated using an osteogenic medium consisting of **Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IE penicillin/ml, 100 µg streptomycin/ml and 1 mmol/l ascorbic acid at 37°C with 5% CO₂**. Cell seeding was performed after the second passage. During passaging, **cells were detached from 75 cm² cell culture flasks using 5 ml of a 0.05% trypsin/0.02% EDTA solution in phosphate-buffered saline (PBS)**. After a 1 : 1 dilution of the cell suspension with DMEM containing 10% FCS and centrifugation at 3200 g for 3 min, **cells were resuspended in DMEM containing 10% FCS**, counted and reseeded at a density of 10⁵ cells/75 cm² cell culture flask. Cells were cultured in the same medium used for cell seeding in a humidified atmosphere with 5% CO₂ at 37°C. **Medium change took place every 3 days.**

Membranes

BioGide® (Geistlich AG, Baden-Baden, Germany) (**porcine collagen types I and III, non-crosslinked**) and **PRF** produced using the method of Choukroun and colleagues (Dohan et al. 2006a) served as scaffolds for the cultivation of human periosteum cells.

PRF membranes were produced in the following way:

Briefly, one healthy study participant (**male, 35 years**) donated **40 ml** of his whole blood in 10 ml tubes without an anticoagulant (Vacuette 455092, Greiner Bio-One, Frickenhausen, Germany). Ethical approval (AZ 118/07) was granted by the Ethics Commission, Christian-Albrechts-University of Kiel, Germany. **Blood samples were immediately centrifuged for 12 min at 2700 r.p.m. (approximately 400 g)**. After activation of the coagulation cascade by contact of blood platelets with the tube walls, a fibrin clot was obtained in the middle between the plasma at the top and red blood cells at the bottom of the receptacle. PRF clot was separated from the red blood cells using a sterile syringe and scissors and then **transferred onto a sterile compress**. A stable fibrin membrane was obtained by squeezing serum out of the PRF clot (Fig. 1a–d).

Both membranes, BioGide® and PRF, were cut into quadratic pieces of side length 7 mm and placed in 24-well cell culture plates (Nunc, GmbH, Langenselbold, Germany). **Cells were seeded on membrane pieces at a density of 10⁴ cells/well**. Cells were cultured on membrane pieces in 2000 µl of the same medium used for cell seeding in a humidified atmosphere with **5% CO₂ at 37°C**. Medium change took place every 3 days. At these points, cultures were checked microscopically.

Assessment of cell vitality

Cell vitality was assessed by **fluorescein diacetate (FDA) and propidium iodide (PI) staining**. Staining was performed on cells cultured in an eluate from membranes after incubation for 10 min, 1 or 24 h in a serum-free cell culture medium. 5 × 10³ cells in cell culture medium with 10% FCS were seeded on eight-well objectives. After 1 day of culture, 200 µl eluate from membranes immersed in a serum-free cell culture medium for 10 min, 1 or 24 h was added to cells. After a 24 h incubation at 37°C and

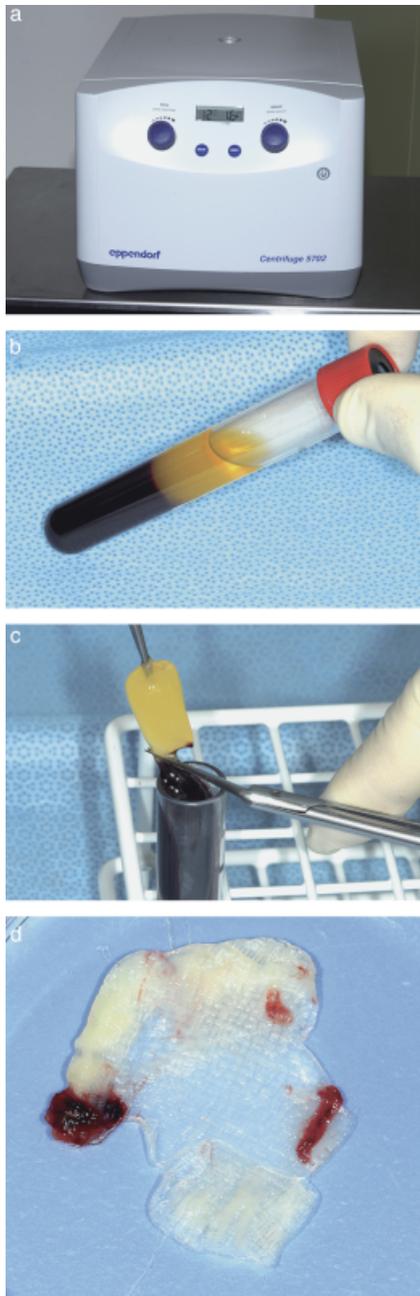


Fig. 1. (a) Centrifugation of whole blood (12 min at 2700 r.p.m. [approximately 400 g]) in an Eppendorf Centrifuge 5702 (Germany). (b) Fibrin clot in the middle of the tube, between the red corpuscles at the bottom and acellular plasma at the top. (c) Separation of platelet-rich fibrin (PRF) clot from the red blood cells using a sterile syringe and scissors. (d) Resistant autologous fibrin membrane.

5% CO₂, cells were rinsed with PBS and immersed in an FDA solution prepared by diluting 30 µl × 1 mg FDA/ml acetone in 10 ml PBS. After incubation for 15 min at 37°C in the dark, the FDA solution was removed by suction and replaced with a PI solution prepared by diluting 500 µl × 1 mg/ml PI in 10 ml PBS. After

incubation for 2 min at room temperature in the dark, scaffolds were rinsed twice in PBS. While still immersed in PBS, scaffolds were then subjected to fluorescence microscopy with excitation at 488 nm and detection at 530 nm (FDA, green) and 620 nm (PI, red).

Biocompatibility and proliferation tests

Lactate dehydrogenase (LDH) and 5-bromo-2-deoxyuridine (BrdU), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) and WST tests

The LDH test can show cell death and lysis. Cells were seeded in 96-well cell culture plates (Nunc) in 100 µl DMEM at a concentration of 5×10^3 cells/well. After a 24 h culture in a humidified atmosphere with 5% CO₂ at 37°C, medium was removed and replaced with 150 µl eluate from the membranes. Cells cultured in 2% Triton-X 100 in serum-free DMEM served as high controls. Cells cultured in serum-free DMEM served as low controls. After a 24 h incubation, 100 µl eluate was transferred to another 96-well cell culture plate. Extracellular LDH activity was measured with the help of an LDH detection kit (Roche Diagnostics, Mannheim, Germany, Catalogue No. 11644793001). Absorbance was measured at 490 nm. The remaining 850 µl eluate per well remaining in the cell culture plate was removed and replaced with 100 µl DMEM containing 10% FCS, 100 IE penicillin/ml, 100 µg streptomycin/ml and 1 mmol/l ascorbic acid. After 5 days of incubation, proliferation was measured with the help of a BrdU Cell Proliferation ELISA kit (Roche Diagnostics, Cat. No. 11647229001). This method is based on the incorporation of BrdU instead of thymidine into newly synthesized DNA of proliferating cells. Absorbance was measured at 450 nm.

After a 24 h incubation with an eluate from the membranes, proliferation was assessed with the aid of an MTT Cell Proliferation Kit (Roche Diagnostics, Catalogue No. 11465007001). The detection of cell vitality is based on the reduction of a yellow-colored dye MTT to blue-violet Formazan. Calibration curves of 0.16–10 × 10³ cells/well served as standards. Absorbance was measured at 450 nm.

As mentioned previously, membrane pieces were seeded at a density of

10⁴ cells/piece. After 7 days of culture in 2000 µl cell culture medium, proliferation was assessed with the aid of a Cell Proliferation Reagent WST-1 (Roche Diagnostics, Cat. No. 116446807001). The evaluation of cell proliferation is based on the cleavage of tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Briefly, 200 µl WST-1 reagent was added to each well at a 1:10 ratio to cell culture medium. After a 4 h incubation in a humidified atmosphere with 5% CO₂ at 37°C, medium was transferred to 96-well plates and absorbance was measured at 450 nm. Cells cultured in wells without membrane pieces at a density of 10⁴ cells/well served as controls.

Scanning electron microscopy (SEM) examinations

SEM investigations were carried out 1 week after cell seeding using an XL30CP device (Phillips Electron Optics GmbH, Kassel, Germany) operating at 10–25 kV, as used by Yang et al. (2006). As preparation for the SEM investigation, cell-seeded membranes and membranes without cells as control were first rinsed using PBS to remove cell culture medium. Cells were then fixed using 3% glutaraldehyde in PBS at pH 7.4 for 24 h. After removal of the glutaraldehyde solution, cells were dehydrated by incubating scaffolds in a series of ethanol solutions of increasing concentration. Scaffolds were immersed for 5 min in each of the following ethanol solutions: 50%, 60%, 70%, 80%, 90% and 100%. Subsequently, critical point drying was performed using a K850 Critical Point Dryer (Emitech, EM Technologies Ltd, Ashford, UK), followed by gold sputtering with an SCD 500 device (CAL-Tec, Ashford, UK).

Statistical evaluation

Each sample (value) represents one well. Absorbance values of the tests were related to the mean results obtained without membrane materials. All values of the LDH test were divided by 0.07 (mean of low control), the values of the MTT test by 0.26, the WST results by 1.22 and the BrdU values by 1.52. These results were analysed by ANOVAS for each test with the factors material (PRF, BioGide®) and time (10 min, 1 and 24 h). Least squared means and 95% confidence intervals are presented in the text and figures.

Results

Assessment of cell vitality on membranes

After 10 min, 1 and 24 h, all samples showed viable periosteal cells. The pronounced green color of the cells due to FDA staining demonstrated their vitality on both membranes, whereas the absence of a red color despite PI staining indicated that no cells died as a result of eluate from membranes. These results are illustrated in Figs 2 and 3.

Biocompatibility tests

In the case of the LDH test, cytotoxicity after treatment with an eluate from the collagen membrane after incubation in a serum-free cell culture medium was similar to the low control (zero cytotoxicity). In the case of PRF, the LDH test results were around five times as high as the low control ($P < 0.0001$; see Fig. 4 and Table 1a). The values were especially high after 1 h (5.06 compared with 1.47 after 10 min and 2.76 after 24 h; $P < 0.0001$). The MTT test gives a measure of cellular metabolic activity dependent on living cells, proliferation, viability and cytotoxicity. At a later point than MTT, the WST test indicates the metabolic activity of cells. In the case of the MTT test, metabolic activity on both membranes was slightly higher than the control (> 1) and the materials showed different results ($P = 0.0007$; see Fig. 5 and Table 1b). No differences were seen for the time effect ($P = 0.95$) (results not shown). The WST test revealed values of around 0.35 for collagen and 2.21 for PRF membranes ($P < 0.0001$; see Fig. 6 and Table 1c). The high values of the PRF group indicate a high metabolic activity. BrdU tests show cell proliferation by incorporation of BrdU during DNA synthesis. The results were between 0.89 (collagen) and 1 (PRF), ($P < 0.0001$; see Fig. 7 and Table 1d).

SEM investigations of cell morphology on membranes

Periosteal cells formed layers covering the surfaces of both membranes 7 days after seeding. The close-up images in Fig. 8 show that both membranes were almost completely covered by cells, which had an elongated morphology with numerous cell pseudopodia, suggesting good biocompatibility.

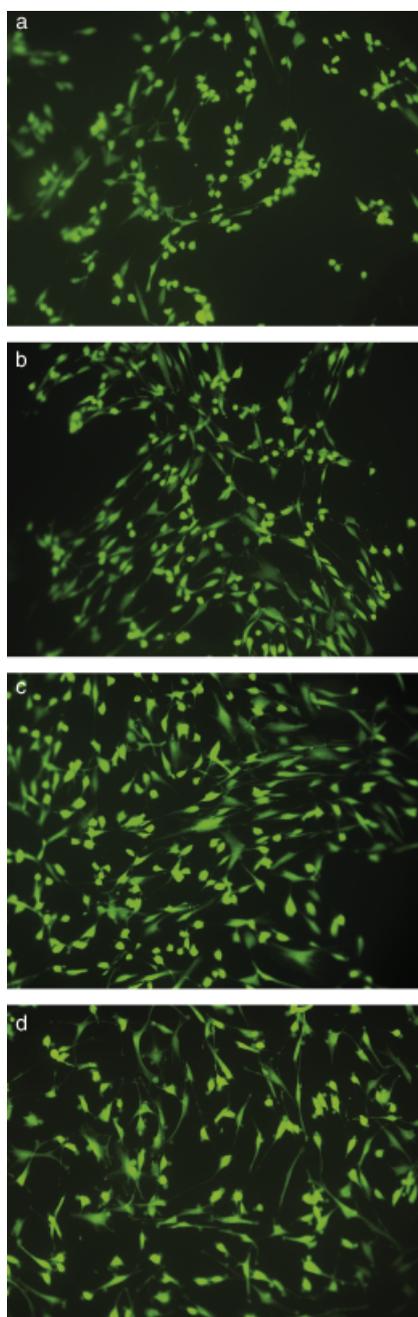


Fig. 2. Fluorescence microscopy images of human periosteal cells on collagen (BioGide®) after staining with fluorescein diacetate (FDA) and propidium iodide (PI): note the green color due to staining with FDA, indicating living cells. The lack of a red color despite staining with PI indicates the absence of dead cells. (a) 10 min, (b) 1 h, (c) 24 h; and (d) control.

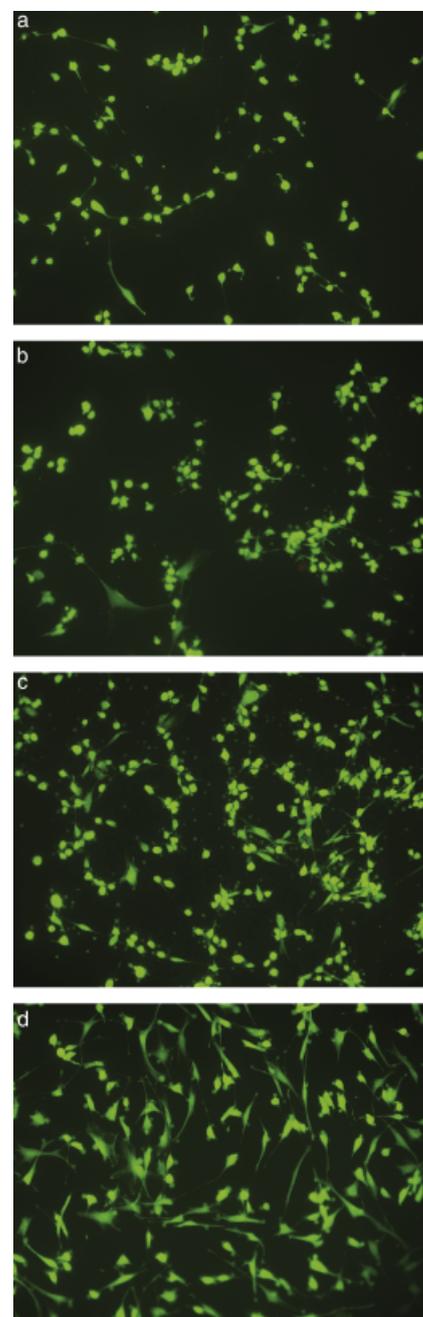


Fig. 3. Fluorescence microscopy images of human periosteal cells on platelet-rich fibrin (PRF) after staining with fluorescein diacetate (FDA) and propidium iodide (PI): note the green color due to staining with FDA, indicating living cells. The lack of a red color despite staining with PI indicates the absence of dead cells. (a) 10 min, (b) 1 h, (c) 24 h and (d) control.

Discussion

Tooth loss often leads to alveolar ridge atrophy. For stable insertion of dental implants, adequate jaw bone is an essential precondition. Thus, the primary aim in implantology is the regeneration of lost

bone tissue e.g., through scaffold–cell constructs grown outside the body (Vacanti & Vacanti 2000). The perfect scaffold material for cultivation of periosteal cells or stem cells has not yet been found.

Membranes have been used for many years in guided tissue regeneration (GTR)

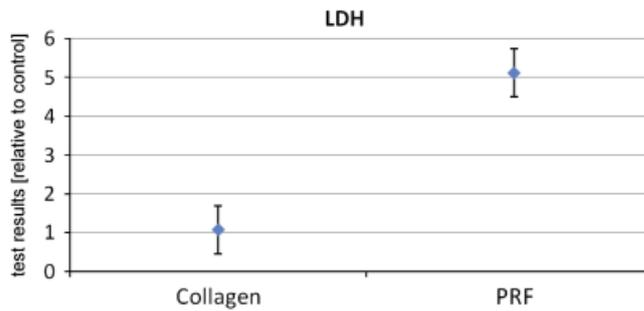


Fig. 4. Lactate dehydrogenase (LDH) test results (LSM \pm 95% CIs): note that the values of platelet-rich fibrin (PRF) were five times as high as the low controls, whereas collagen (BioGide[®]) values were at the same level as the controls. LSM, least squared mean, CI, confidence interval.

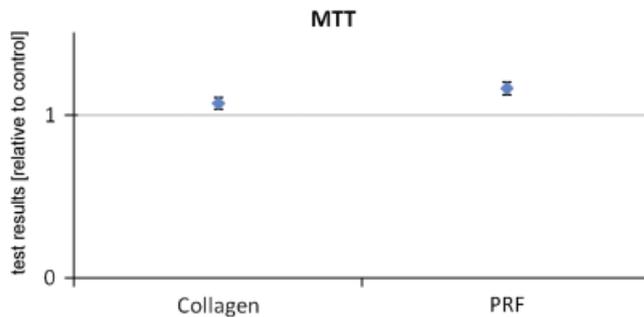


Fig. 5. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) test results (LSM \pm 95% CIs): metabolic activity on both membranes was slightly higher than the control. LSM, least squared mean, CI, confidence interval.

Table 1. Mean, SDs and number of samples for the two treatment groups for the three time points

Membrane	Time	N	Mean	SD
(a) LDH test				
Collagen	1	8	1.25	0.37
Collagen	10	8	0.97	0.21
Collagen	24	8	0.99	0.33
PRF	1	8	8.87	0.73
PRF	10	8	1.96	0.55
PRF	24	8	4.53	0.5
(b) MTT test				
Collagen	1	8	1.11	0.09
Collagen	10	8	1.12	0.09
Collagen	24	8	0.99	0.07
PRF	1	8	1.14	0.03
PRF	10	8	1.11	0.03
PRF	24	8	1.24	0.05
(c) WST test				
Collagen	1	8	0.49	0.04
Collagen	10	8	0.35	0.07
Collagen	24	8	0.21	0.03
PRF	1	8	2.2	0.18
PRF	10	8	2.48	0.79
PRF	24	8	1.96	0.12
(d) BrdU test				
Collagen	1	8	0.93	0.07
Collagen	10	8	0.78	0.08
Collagen	24	8	0.96	0.04
PRF	1	8	0.93	0.06
PRF	10	8	0.91	0.04
PRF	24	8	1.16	0.09

LDH, lactate dehydrogenase; BrdU, 5-bromo-2-deoxyuridine.

to heal periodontal defects and to regenerate peri-implant defects in GBR (Fritz 1996). Collagen membranes composed of bovine or porcine collagen type I and III are commonly used (Bunyaratavej & Wang 2001). The use of collagen as a scaffold material in tissue engineering is justified by several characteristics like tissue integration, dimensional stability and biocompatibility. Warnke et al. (2009) investigated the biocompatibility of different collagen membranes on periosteal cells. It could be shown that these cells were able to proliferate and formed layers covering the surface of different collagen membranes. The influence of collagen on osteogenesis was demonstrated by Taguchi et al. (2005). They revealed that collagen fibers of BioGide[®] appeared to have an osteoinductive effect, whereby the underlying mechanism is still unclear. It is believed that the collagen fibers function as a 'trap' for osteoinductive factors such as bone morphogenetic protein (BMP), TGF- β , IGF-I and other cytokines and thus mediate osteogenic differentiation. The investigation of the biological effects of different GBR materials on osteoblastic cell migration

showed that for mouse osteoprogenitor cells, collagen is a favorable scaffold for osteoblastic cell proliferation (Takata et al. 2001).

The rationale for a cell culturing system based on fibrinogen is due to its ability to enhance cell attachment (Gorodetsky et al. 1998), proliferation (Sporn et al. 1995) and differentiation (Huang et al. 2002). Several publications have judged fibrin to be a suitable scaffold material for colonization of human MSC (HMSC) (Catelas et al. 2006; Trombi et al. 2008) and HMSC are able to adhere, spread and proliferate, depending on different fibrinogen concentrations (Bensaid et al. 2003). Perka et al. (2001) developed a bioresorbable alginate-fibrin vehicle that ensured an initial cell proliferation and differentiation to establish a stable matrix structure for transplantation of different cell types like periosteal cell and chondrocytes.

There may be varying abilities of fibrin- and collagen-based substrates to support proliferation and topographical differences. Unfortunately, there are only a few studies in the literature that deal with this context. The equation of fibroblast and keratinocyte proliferation in collagen and fibrin gels revealed no differences after 2 days of cultivation (Hojo et al. 2003). Another publication revealed superior growth of placental trophoblasts on collagen I-coated cell culture plastic compared with fibrin coatings after 24 and 72 h (Farmer & Nelson 1992). Both these studies may suggest that the superior proliferation on PRF is not due to fibrin. However, it is known that smooth surfaces promote proliferation better than rough surfaces. The surface of PRF membranes is considerably smoother than that of the bone-friendly side of collagen (BioGide[®]) (Fig. 8), which may partly explain the above-mentioned higher proliferation on PRF.

Another possible explanation for higher cell proliferation on PRF membranes compared with collagen (BioGide[®]), as shown by the results of the BrdU test (Fig. 7) and SEM investigation (Fig. 8), is the above-mentioned release of growth factors from platelets in PRF, which influences proliferation. The relationships between platelet-released supernatants and mitogenic activity of periosteal cells were shown by Gruber et al. (2003). They could show that factors set free from the activated platelets

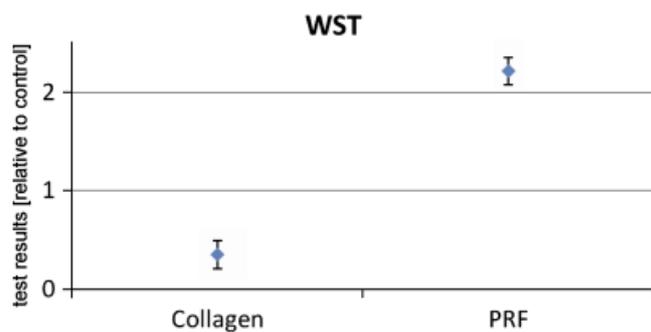


Fig. 6. The WST test (LSM \pm 95% CIs) showed values of 0.35 for collagen (BioGide[®]) and 2.21 for platelet-rich fibrin (PRF) membranes (compared with controls). The high values of the PRF group indicate a high metabolic activity. LSM, least squared mean, CI, confidence interval.

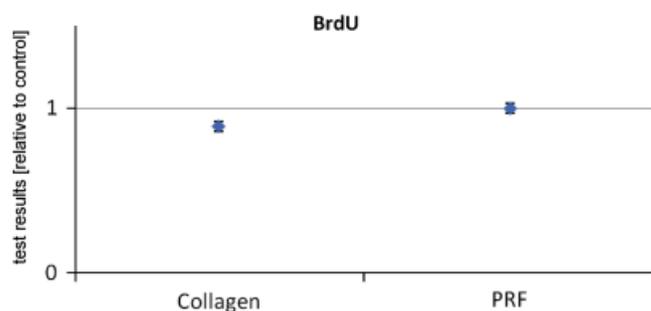


Fig. 7. The BrdU test (LSM \pm 95% CIs) revealed cell proliferation rates of 0.89 for collagen (BioGide[®]) and 1 (same as controls) for platelet-rich fibrin (PRF). LSM, least squared mean, CI, confidence interval.

like PDGF and basic fibroblast growth factor (bFGF) increased the number of proliferating periosteum cells. Both Choukroun's PRF (Dohan et al. 2006c; Gassling et al. 2009) and other PRF preparations (Lundquist et al. 2008) are known to contain platelets and thus growth factors such as PDGF, TGF- β and IGF-I, which can promote cell proliferation.

Stem cells reside in a unique microenvironment composed of extracellular matrix and resident cells, also known as a *niche* (Dawson et al. 2008). Such vital microenvironments composed of extracellular matrix and entrapped platelets containing cytokines were found in PRF, which was first described by Choukroun and colleagues (Dohan et al. 2006a). The good results for PRF suggest that one possible explanation is the more physiological natural and progressive polymerization during centrifugation of PRF like nullifies by Choukroun and colleagues (Dohan et al. 2006b). The fibrin network thus formed presents a particularly homogeneous three-dimensional organization, even more highly coherent than natural fibrin clots. Moreover, a progressive polymerization mode signifies increased incorporation of the circulating

cytokines into the fibrin meshes (intrinsic cytokines). In contrast, in collagen membranes there is no vital microenvironment. BioGide[®] is composed of porcine collagen fibers without growth factors. Despite the fact that collagen fibers are the major components in extracellular bone matrix that mediate cell-matrix attachment of osteogenic cells, the lack of bone-specific proteins leads to a purely physical scaffold nature of BioGide[®]. Until now, there is only a slight assumption that collagen fibers may trap osteoinductive factors from the bone (Koda & Bernfield 1984).

Conclusion

Here, for the first time, we could show that PRF is a suitable scaffold for breeding human periosteal cells *in vitro*, which may be suitable for bone tissue engineering applications. Nevertheless, both systems, PRF and collagen (BioGide[®]), seem to be suitable as scaffold materials for the generation of periosteum layers. However, it should be borne in mind that the vital property of PRF is a benefit not only because of

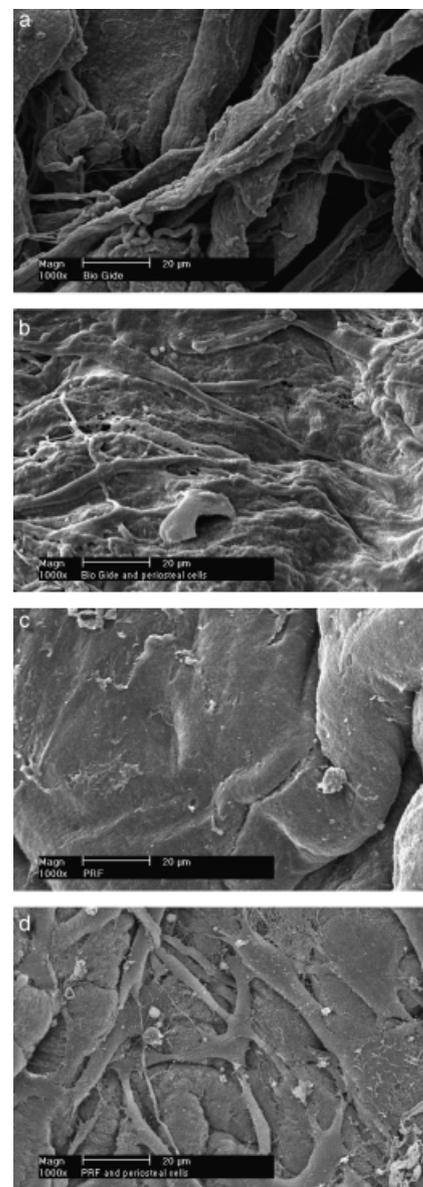


Fig. 8. Close-up scanning electron microscopy images of membranes seeded with human periosteal cells and controls 7 days after seeding. The membranes were almost completely covered by cells, with an elongated morphology and numerous cell pseudopodia, suggesting good biocompatibility (a) collagen (BioGide[®]) without cells, (b) collagen (BioGide[®]) control with cells, (c) platelet-rich fibrin (PRF) without cells, and (d) PRF control with cells (scale bar 20 μ m).

biocompatibility but also because its use results in lower costs.

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