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BIOFILM FORMATION BY STAPHYLOCOCCI ON FRESH, FRESH-FROZEN AND PROCESSED HUMAN AND BOVINE BONE GRAFTS

Martin Clauss[1, 2,*], Ulrika Furustrand Tafin[2], Alain Bizzini[2], Andrej Trampuz[2] and Thomas Ilchmann[1]

[1]Clinic for Orthopaedic and Trauma Surgery, Kantonsspital Baselland Liestal, Liestal, Switzerland
[2]Infectious Diseases Service, Department of Medicine, University Hospital Lausanne (CHUV), Lausanne, Switzerland

Abstract

Biofilm formation is a multi-step process influenced by surface properties. We investigated early and mature biofilm of *Staphylococcus aureus* on 4 different biological calcium phosphate (CaP) bone grafts used for filling bone defects. We investigated standardised cylinders of fresh and fresh-frozen human bone grafts were harvested from femoral heads; processed human and bovine bone grafts were obtained preformed. Biofilm formation was done in tryptic soy broth (TSB) using *S. aureus* (ATCC 29213) with static conditions. Biofilm density after 3 h (early biofilm) and 24 h (mature biofilm) was investigated by sonication and microcalorimetry. After 3 h, bacterial density was highest on fresh-frozen and fresh bone grafts. After 24 h, biofilm density was lowest on fresh bone grafts ($p < 0.001$) compared to the other 3 materials, which did not differ quantitatively ($p > 0.05$). The lowest increase in bacterial density was detected on fresh bone grafts ($p < 0.001$). Despite normal shaped colonies, we found additional small colonies on the surface of the fresh and fresh-frozen samples by sonication. This was also apparent in microcalorimetric heat-flow curves. The four investigated CaP bone grafts showed minor structural differences in architecture but marked differences concerning serum coverage and the content of bone marrow, fibrous tissue and bone cells. These variations resulted in a decreased biofilm density on fresh and fresh-frozen bone grafts after 24 h, despite an increased early biofilm formation and might also be responsible for the variations in colony morphology (small colonies). Detection of small colony variants by microcalorimetry might be a new approach to improve the understanding of biofilm formation.

Keywords: Biofilms; *Staphylococcus aureus*; bone grafts; bacterial colonies.

*Address for correspondence:
Martin Clauss M.D.
Clinic for Orthopaedic and Trauma Surgery
Kantonsspital Baselland Liestal
Rheinstrasse 26
CH-4410 Liestal, Switzerland
Telephone Number: 41 61 925 2525
FAX Number : +41 61 925 2808
Email: martin.clauss@ksli.ch

Introduction

Bone defects are a frequent problem in every day clinical work (Tadic and Epple, 2004). Bone grafts are increasingly used to fill bone defects in orthopaedic and trauma surgery (Ketonis *et al.*, 2010). Autologous cancellous bone grafts are typically harvested from the iliac crest or the distal radius and transplanted as *fresh* bone grafts during the same operation (De Long *et al.*, 2007). Alternatively, allogeneic cancellous bone grafts can be harvested from femoral heads during primary hip replacement surgery and stored as fresh-frozen bone grafts in a bone bank (Van de Pol *et al.*, 2007; Kappe *et al.*, 2009). Another strategy to fill bone defects is the use of processed cancellous bone grafts *(*human or bovine*)* or synthetic/artificial bone grafts such as β-tricalcium phosphate (β-TCP) or hydroxyapatite (HA) (Bohner, 2010).

For structured allogenic bone grafts, mainly used in reconstructive procedures after removal of a bone tumour, bone graft infection is a frequent complication with reported infection rates up to 12 % (Lord *et al.*, 1988; Aro and Aho, 1993). The infection rates of unstructured bone grafts to our knowledge are unknown.

Bone grafts represent a temporary foreign body lacking vascularisation and are therefore of increased susceptibility to infection, which may be introduced either intraoperatively or after surgery by contiguous route. Infections may occur despite proper surgical site preparation and systemic perioperative antimicrobial prophylaxis (Trampuz and Zimmerli, 2006a). In elective orthopaedic surgery, infection rates are reported between 0.7-4.2 % (Crockarell *et al.*, 1998), whereas they may increase up to 30 % after open fractures (Ostermann *et al.*, 1994). Bone grafts with a low propensity for biofilm formation should therefore lead to superior clinical results especially in cases where infection rates are high. The majority of infections associated with orthopaedic foreign bodies are caused by staphylococci (70-90 %) (Gristina, 1987; Trampuz and Zimmerli, 2006b; Trampuz and Zimmerli, 2008).

Bone graft-associated infections are due to biofilm formation on the surface of the bone graft (Costerton *et al.*, 2005; Trampuz and Zimmerli, 2006b) and often require removal of the infected bone graft with substantial patient morbidity (Zimmerli *et al.*, 2004; Ketonis *et al*., 2010).

Biofilms are currently one of the main topics in biomaterial and orthopaedic infection research. With regard to pathogenesis, biofilm development is a multi-step process including (i) early biofilm formation within 3 h and (ii) the development of a mature biofilm after 24 h containing of an additional extracellular matrix (slime

envelope) with nutrition channels and quorum sensing communication (An and Friedman, 1998; MacKintosh *et al.*, 2006; Patel *et al.*, 2007). Infections associated with biofilms on foreign material are more difficult to treat than the ones caused by bacteria growing in free-living (planktonic) form and eradication is often only possible by removal of the foreign body and long-term antimicrobial treatment (Ehrlich *et al.*, 2005; Ketonis *et al*., 2010).

Whereas infection of bone grafts is a well-recognised complication, the particular risks and infection dynamics associated with either fresh, fresh-frozen or processed human or bovine cancellous bone grafts are unknown. Aim of this study was a quantitative and qualitative analysis of the formation of (i) early biofilm after 3 h of incubation and (ii) mature biofilm after 24 h using a standard laboratory strain of *Staphylococcus aureus* (ATCC 29213) on the surface of (i) fresh and (ii) fresh-frozen human bone grafts out of femoral heads and commercially available cancellous bone grafts of processed (iii) human or (iv) bovine bone.

Materials and Methods

The study protocol regarding sampling of human cancellous bone grafts was approved by the institutional review board (EKBB 79/09). Donors of femoral heads gave informed consent prior to surgery.

As donors, we included two otherwise healthy male patients (77 and 73 years of age) who were scheduled for total hip replacement (THR) due to primary coxarthritis without any lifetime history of bacterial coxitis and a one year history without bacterial infection due to *S. aureus*. Antibiotic prophylaxis was postponed until harvesting of the femoral heads, in order to avoid a carryover of antibiotics that might have interfered with biofilm formation. Afterwards, the patients received a single shot of Cefuroxime 1.5 g (Sandoz, Basel, Switzerland) and the implantation of the THR was continued without any further modifications of the routine setting. Both patients had an uneventful wound healing and postoperative rehabilitation.

Bone graft samples

Fresh (fr) human cancellous bone grafts were harvested as cylinders (6.5 x 10 mm) out of femoral heads during total hip replacement in the operating room using a hollow drill system (SDS, Zimmer, Winterthur, Switzerland). Perioperative antibiotic prophylaxis was postponed until the femoral head was harvested. Afterwards, processed samples were transferred to 10 mL Falcon tubes pre-filled with 1 % phosphate buffered saline (PBS). Samples were transferred to the laboratory and experiments started within 2 h.

Fresh-frozen (ff) human cancellous bone grafts were harvested out of the same femoral head in the same way as fr cylinders, but samples were transferred to sterile Eppendorf tubes and immediately deep-frozen at -80 °C in the in-house bone bank. After a storage period of 10 days, samples were transferred deep-frozen to the laboratory, thawed within the Eppendorf tubes filled with 2 mL of 1 % PBS at room temperature and processed within 2 hours.

Processed human (ph) cancellous bone grafts (Tutoplast™, Novomedics, Zürich, Switzerland) were obtained as ready-to-use cylinders (6.5 x 10 mm) from the manufacturer. Tutoplast™ is a commercially available human bone graft material processed through a multi-step chemical treatment (osmolysis, NaOH, H_2O_2, and acetone) to obtain a sterile product (Tadic and Epple, 2004). After processing samples consist of almost 45 wt% of carbonated HA, 9 wt% H_2O and 34 wt% of soft tissue and organic bone matrix (BM) (Tadic and Epple, 2004).

Processed bovine (pb*)* cancellous bone grafts (Tutobone™, Novomedics, Zürich, Switzerland) were obtained in the same shape as ph and are produced in the same way as mentioned above but out of bovine bone. Final samples contain a higher percentage of HA (57 wt%) and less soft tissue and organic BM (26 wt%) as compared to ph blocks (Tadic and Epple, 2004).

Bacterial strains

S. aureus ATCC 29213 (a methicillin-susceptible strain) was used in all experiments. This strain represents a commonly used laboratory reference strain for investigation of biofilms (Ceri *et al.*, 1999). The strain was stored at -70 °C by using a cryovial bead preservation system (Microbank, Por.Lab Diagnostics, Richmond Hill, Ontario, Canada). For preparation of the inoculum, a single bead was grown on sheep blood agar overnight. Bacterial stock solution was prepared from individual colonies which were resuspended in 1 % PBS to a McFarland turbidity of 0.5 representing a bacterial concentration of about $1x10^8$ colony-forming units (cfu)/mL, which was diluted 1:100 for further experiments.

Biofilm formation

50 mL Falcon tubes were pre-filled with 2700 µL tryptic soy broth (TSB, Becton Dickinson, Basel, Switzerland). Five replicates of each test material were placed separately onto the surface of the broth with a sterile forceps to allow a homogeneous soaking of the porous blocks over 30 min (Stahli *et al.*, 2010). At the end of the period, samples were completely submerged due to the additional water content in the pores. Then 300 µL of bacterial stock solution were added to the media. One set of 5 samples was incubated at 37 °C in ambient air under static conditions for 3 h, and another set of 5 samples for 24 h.

Biofilm analysis

Sonication

After either 3 h (early biofilm) or 24 h (mature biofilm) incubation, samples were transferred with a sterile forceps to a new 50 mL Falcon tube and washed 3 times with 5 mL of 1 % PBS. Afterwards samples were vortexed (Vortex Genie 2, Scientific Industries, Bohemia, NY, USA) for 10 s at maximum power, sonicated in a bath tub type sonicator (BactoSonic, Bandelin, Berlin, Germany) at 40 kHz and acoustic power of 0.2 W/cm^2 for 5 min and vortexed again for 10 s (Trampuz *et al.*, 2007a) with the samples completely immersed. Sonication fluid was transferred to a 10 mL Falcon tube and the samples harvested for further investigation.

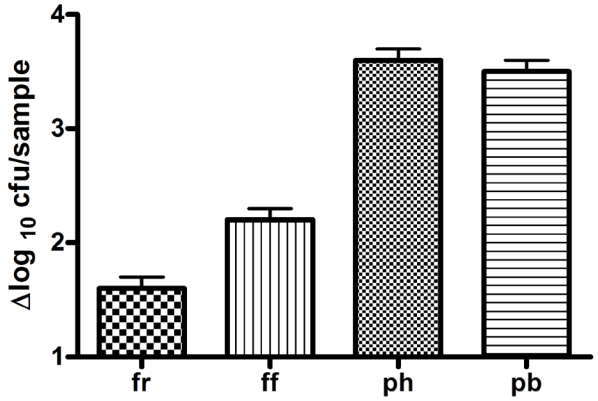

Fig. 1. Evaluation of bacterial density in sonication fluid. Change in bacterial density (Δlog_{10} cfu/sample ± SD) on the surface of fr, ff, ph and pb bone grafts. Differences between all groups are significant ($p < 0.001$) except between ph and pb ($p > 0.05$)

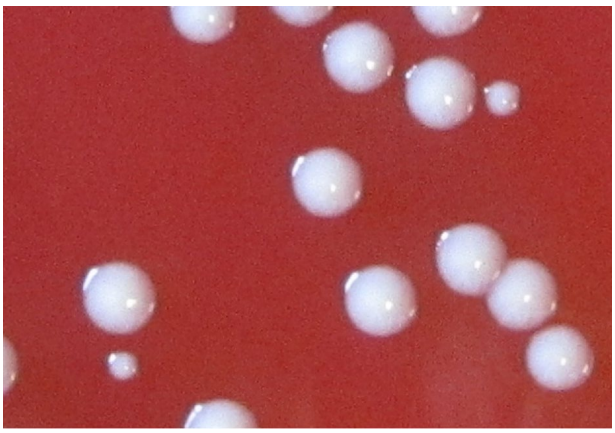

Fig. 2. Differences in colony morphology growing from sonication fluid (representative area).

The dislodged biofilm (sonication fluid) was serially diluted in Eppendorf tubes and aliquots of 100 µL were plated on sheep blood agar and incubated at 37 °C aerobically for 24 h. Bacterial counts were enumerated and expressed as cfu/sample. Differences in colony counts between the 3 h and 24 h incubations were calculated.

Plates were examined for variations in colony morphology (colour, size). Colonies of different sizes were Gram stained to assess the presence of Gram-positive cocci and re-plated on blood agar (incubated at 37 °C with ambient air for 24 h) for further analysis. Colonies with different size were further analysed by microcalorimetry (see below).

Microcalorimetry

After sonication, test samples were transferred with a sterile forceps into microcalorimeter glass ampoules pre-filled with 1 mL TSB. Ampoules were sealed with rubber cups and transferred into the microcalorimeter to analyse the remaining biofilm (Clauss *et al.*, 2010). Heat was measured in a 48-channel batch microcalorimeter (thermal activity monitor, model 3102 TAM III; TA Instruments, New Castle, DE, USA) at 37 ± 0.0001 °C with a sensitivity of 200 nW. Heat was measured every 10 s and expressed as heat-flow over time (in microwatts [µW]). Calorimetric time to detection (TTD) was defined as the time from insertion of the ampoule into the calorimeter to an exponentially rising heat-flow signal exceeding 100 µW. Choosing the detection limit at 100 µW ensures that only replicating bacteria are detected. TTD indirectly quantifies the amount of bacteria with a shorter TTD representing a higher amount of bacteria. Quantitative differences in bacterial load (ΔTTD) between the mature and early biofilm were calculated.

The theoretical detection limit of the microcalorimetric assay is one viable cfu/mL (Trampuz *et al*., 2007b). The peak heat-flow (PHF) was defined as the maximum heat-flow after an exponential rise of the signal and the time to reach the peak heat-flow (ttPHF) was recorded. Whenever multiple peaks were recorded during the experiment they were enumerated sequentially (PHF1, PHF2) and the different time intervals to reach peak heat-flow (ttPHF) recorded. The total produced heat (TPH, in joules [J]) was determined by integration of the area below the heat-flow-time curve from time 0 until 24 h (Trampuz *et al*., 2007c).

Colonies with different morphology assessed after plating of the dislodged biofilm (sonication fluid) were resuspended in 1 % PBS to a McFarland turbidity of 0.5 and 50 µL transferred in microcalorimetry ampoules containing 2 mL fresh TSB to investigate potential differences in bacterial metabolism.

Statistical analysis

All experiments were performed in sets of five to calculate the mean ± standard deviation. Statistical analysis was done by Kruskal-Wallis test with Dunn´s multiple comparison tests using Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The significance level was set at $\alpha = 0.05$ and a p-value of < 0.05 was considered significant.

Results

Sonication (detecting dislodged biofilm)

After 3 h incubation (early biofilm), bacterial counts in the sonication fluid (log_{10} cfu/sample) were highest on the ff bone grafts (5.5 ± 0.3) compared to fr (5.1 ± 0.3, $p > 0.05$), ph (4.0 ± 0.3, $p < 0.01$) and pb (4.5 ± 0.2, $p < 0.01$) bone grafts. Bacterial counts (log_{10} cfu/sample) after 24 h (mature biofilm) were lowest on the fr bone grafts (6.7 ± 0.2, $p < 0.01$) but no difference between the 3 other groups was observed (ff 7.8 ± 0.2, ph 7.6 ± 0.2, pb 8.0 ± 0.4; $p > 0.05$). The lowest increase in bacterial density (Δlog_{10} cfu/sample) between early and mature biofilm was detected on fr bone grafts (1.6 ± 0.1, $p < 0.001$, Fig. 1). The plating of the ph and pb bone grafts on agar plates showed identical *S. aureus* colony morphologies. In contrast, colonies with 2 different diameters (small and large) were found on the plates of the fr (early biofilm

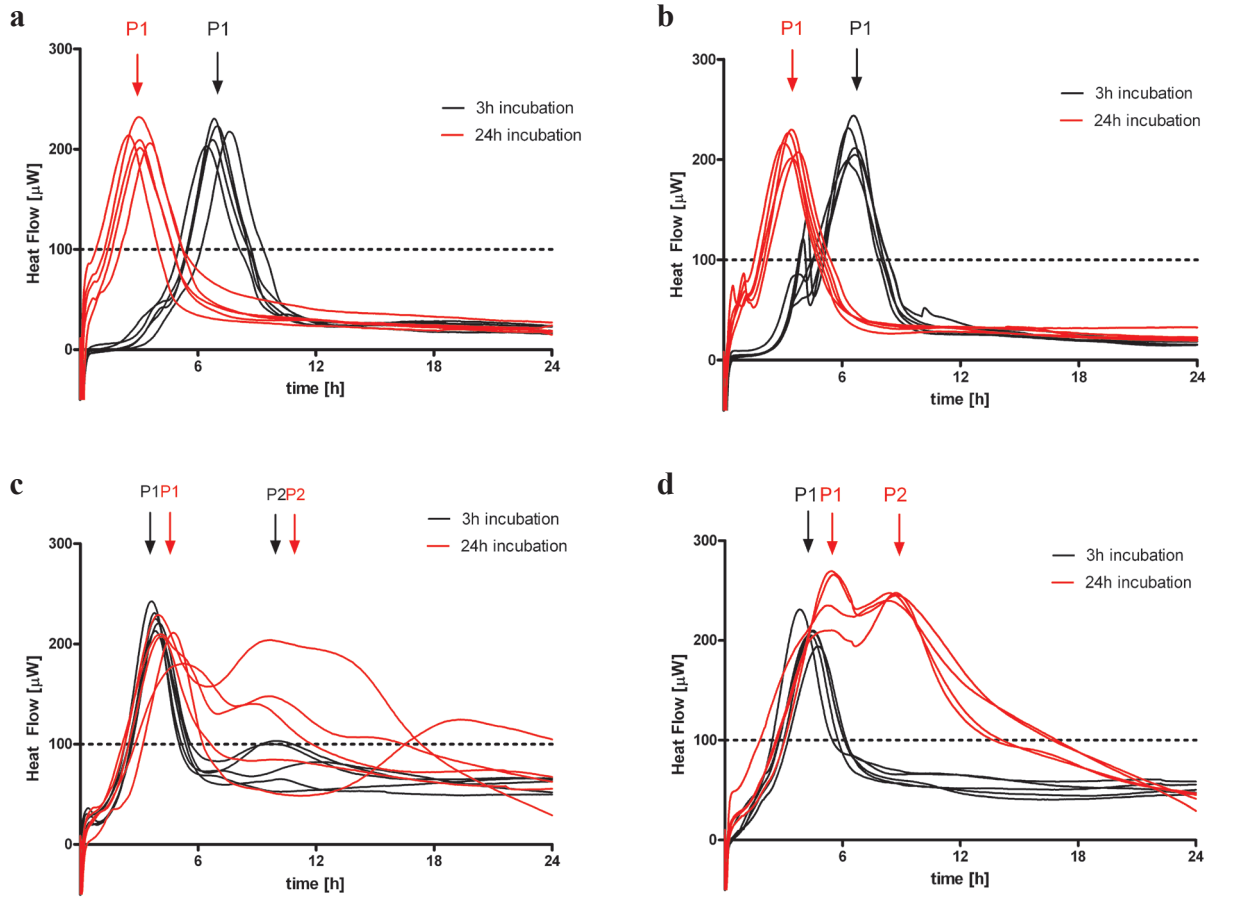

Fig. 3. Heat-flow curves for: (**a**) processed human (ph), (**b**) processed bovine (pb), (**c**) fresh (fr), (**d**) fresh-frozen (ff) bone grafts. P1 and P2 represent calculated peak number 1 and 2 out of all curves (Table 1). Heat-flow curves show a batch signal of heat produced in the ampoules. The initial thermal equilibration of the ampoules (negative values within the first 15 min) is followed by an exponentially rising signal in the ampoules, reflecting exponential growth of the bacteria. After depletion of nutrient inside the ampoules (PHF) the signal turns and shows a negative slope.

and mature biofilm) and ff bone grafts (mature biofilm, Fig. 2). Gram staining showed a homogeneous pattern of Gram-positive cocci (data not shown). After re-plating, all colonies reverted to large colonies of *S. aureus* only.

Microcalorimetry (remaining biofilm)

The microcalorimetric analysis of the remaining biofilm after sonication showed two different shapes of heat-flow curves. The ph (Fig. 3a) and pb (Fig. 3b) bone grafts had one single peak exceeding 100 µW for early biofilm as well as for the mature biofilm. The fr bone grafts (Fig. 3c) had a double peak for each of the two investigated time points. One peak was found for the ff bone grafts after 3 h incubation (early biofilm), while two peaks were observed analysing the remaining mature biofilm after 24 h incubation (Fig. 3d). The highest number of bacteria (shortest TTD) in the remaining mature biofilm (24 h incubation) was found on the surface of the ph and pb bone grafts. A lower number of bacteria (i.e., longer TTD) was found on the surface of fr and ff bone grafts incubated for 3 h ($p < 0.05$) and 24 h ($p < 0.05$) with no differences in bacterial density between fr and ff bone grafts ($p > 0.05$) independent of whether incubated for 3 h or 24 h ($p > 0.05$). The lowest number of bacteria (longest TTD, $p < 0.01$) was found on the ph and pb bone grafts incubated for 3 h. Microcalorimetric results (remaining biofilm) corresponded well with the quantitative cultures of the removed biofilm after sonication and plating (Fig. 1). The quantitative increase in bacterial density (ΔTTD) on the surface of the samples was 3.9 ± 0.2 h ($p < 0.001$) for ph and 2.9 ± 0.1 h ($p < 0.001$) for pb, respectively. fr (0.1 ± 0.2 h, $p > 0.05$) and ff (0.1 ± 0.4 h, $p > 0.05$) showed no ΔTTD, but an additional second peak reflecting an additional number of bacteria (Table 1, Fig. 3c,d). These findings corresponded well with the increase in bacterial counts from the removed biofilm in sonication fluid (Fig. 1).

PHF of the first peak was independent of the length of incubation and the test material and reached an average of 219 ± 20 J ($p > 0.05$). PHF is directly related to the detected germ, thus potential contaminations in the ampoules can be excluded. A second peak was only seen in ff and fr, it was higher on ff than on fr bone grafts ($p < 0.01$, Table 1). After 3 h incubation there were no differences ($p > 0.05$) in the total produced heat (TPH) comparing fr and ff bone grafts but between fr and ph ($p < 0.05$) and between fr and

pb ($p < 0.05$). TPH was highest on ff bone grafts incubated for 24 h (Table 1) and TPH was higher compared to fr ($p > 0.05$), pb ($p < 0.05$), and pb ($p < 0.01$) bone grafts.

Microcalorimetric analysis of resuspended (planktonic) bacteria of different colony morphology showed a delayed heat-flow curve with a decreased peak heat-flow for the small colonies compared to the heat-flow curves of normal sized colonies (Fig. 4). The time points for the ttPHF in Fig. 4 are matching with the ttPHF in Fig. 3 c and d for both the small and large cfu. The increased height of the curves in Fig. 4 is due to the fact that the planktonic (resuspended) bacteria are metabolically more active than their biofilm counterparts. In summary, we found the largest increase in biofilm density from 3 h incubation to 24 h incubation was observed on the surface of ph and pb bone grafts both by sonication and microcalorimetry. After 24 h incubation the absolute amount of biofilm was also highest on ph and pb grafts. Small colonies found in the sonication fluid of fr and ff grafts were also detected by microcalorimetry (expressed by the double peak of the heat-flow curve).

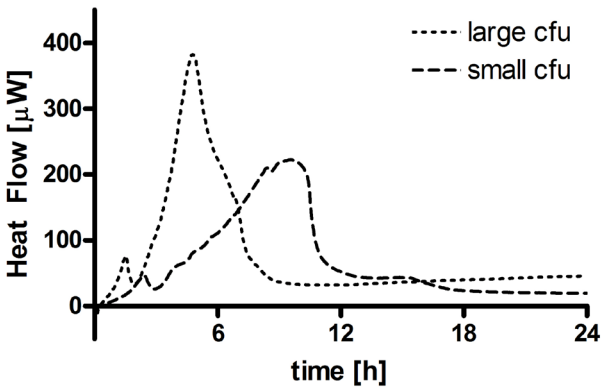

Fig. 4. Microcalorimetric analysis of resuspended (planktonic) bacterial colonies with two different morphologies.

Discussion

Biofilm formation is a multi-step process (Gristina, 1987; An and Friedman, 1998) and extensive research on medical devices and their propensity for biofilm formation by staphylococci has been performed during the last years (Harris and Richards, 2006). Several factors, such as surface hydrophobicity (Jakubowski *et al.*, 2008), surface charge (Oga *et al.*, 1988), surface roughness (Harris *et al.*, 2007) and surface chemistry (Abraham and Jefferson, 2010; MacKintosh *et al*., 2006; Patel *et al.*, 2003; Patel *et al*., 2007) have been shown to influence biofilm formation. But the effect of these findings on bone grafts in clinical practice is unclear and the use of *fresh* autologous bone grafts is still regarded the standard procedure to fill bone defects (Delloye *et al.*, 2007; De Long *et al*., 2007; Ketonis *et al*., 2010).

To our knowledge, there are no data comparing the formation of early and mature biofilm on the surface of fresh (fr), fresh-frozen (ff) or processed human (ph) and bovine (pb) cancellous bone grafts. The aim of our study was to quantitatively and qualitatively evaluate (i) the early biofilm within 3 h and (ii) the mature biofilm within 24 h of *S. aureus* (ATCC 29213) on fr, ff, ph and pb bone grafts under standardised *in vitro* conditions.

The fr, ff and ph bone grafts are harvested out of human femoral heads and thus have the same architecture of the CaP skeleton. The pb bone grafts differ by means of the CaP content which is slightly higher in the bovine samples (Tadic and Epple, 2004). Despite these minor structural differences, the four investigated materials harbour marked differences. The surfaces of fr and ff bone grafts are covered by serum proteins, whereas proteins are completely removed during processing in ph and pb bone grafts (information from manufacturer). There are only minor differences in the composition of serum proteins but a reduced content of water in the ff bone grafts compared to the fr bone grafts, due to freezing and storage at -80 °C (Nade, 2002). Additionally, fr and ff bone grafts are partly filled with bone marrow containing granulocytes, fibroblasts and connective fibrous tissue, which is not present in the ph and pb bone grafts. It is

Table 1: Microcalorimetric evaluation of biofilm formation on the different bone grafts. Data represent sets of five samples and are presented as mean ± SD. Time to detection (TTD) was measured at 100 µW. Peak heat-flow (PHF) reflects the peaks of the heat-flow curve after an exponential rise of the signal and time-to-PHF (ttPHF) the time period until PHF was reached. Peaks are enumerated sequentially in case of multiple peaks (Fig 3a-d). Total produced heat (TPH) represents the total amount of energy produced during the 24 h interval and is calculated by integrating the area under the heat-flow curve.

incubation time	Sample	TTD [h]	PHF 1 [µW]	ttPHF 1 [h]	PHF 2 [µW]	ttPHF 2 [h]	TPH [J]
3 hours	*fr*	2.5 ± 0.2	226.3 ± 10.0	3.8 ± 0.1	83.2 ± 16.1	13.3 ± 5.5	6.6 ± 0.5
	ff	2.8 ±0.3	209.8 ± 12.0	4.4 ± 0.3	-	-	5.8 ± 0.5
	ph	5.5 ± 0.4	216.5 ± 9.7	6.9 ± 0.4	-	-	3.8 ± 0.2
	pb	4.8 ± 0.2	218.2 ± 16.8	6.5 ± 0.2	-	-	4.1 ± 0.1
24 hours	*fr*	2.6 ± 0.4	207.4 ± 15.6	4.4 ± 0.5	140.2 ± 38.6	11.4 ± 4.0	8.8 ± 1.7
	ff	3.0 ± 0.9	249.5 ± 23.5	5.7 ± 0.5	245.0 ± 3.2	8.5 ± 0.2	10.6 ± 1.1
	ph	1.6 ± 0.3	207.2 ± 14.3	3.1 ± 0.2	-	-	4.3 ± 0.8
	pb	1.9 ± 0.2	216.2 ± 11.0	3.4 ± 0.2	-	-	4.3 ± 0.2

questionable whether the cells are still viable at the end of the 3 h incubation in the fr bone grafts setting, but these cells should die during 24 h incubation (fr bone grafts) or freezing and thawing (ff bone grafts). Bone cells, that are initially present in the fr and ff bone grafts too, die once living bone is separated from its blood supply (Nade, 2002), thus living bone cells are not present in any of the investigated samples.

The use of ultrasound is a standard procedure to dislodge biofilms from devices (Ceri *et al.*, 1999; Monsen *et al.*, 2009) and was adapted to the sample sizes (Clauss *et al*., 2010) from our recently published protocol (Trampuz *et al*., 2007a). Our results demonstrate an increased early biofilm formation at 3 h as shown both by direct CFU counts as well as microcalorimetry experiments on the serum covered surface of fr and ff bone grafts but a decrease after 24 h. Abraham and Jefferson (Abraham and Jefferson, 2010) have recently shown that a low molecular weight component of serum inhibits the formation of *S. aureus* biofilms. In contrast, MacKintosh *et al*. (MacKintosh *et al*., 2006) and Patel *et al.* (Patel *et al*., 2007) found a significant reduction of the early biofilm (2-12 h) but an increased *S. epidermidis* biofilm formation within 24 h on the surface of polymers. Both studies (MacKintosh *et al*., 2006; Patel *et al*., 2007) investigated the effect of various incubation media (human serum, TSB, PBS) on biofilm formation on different surfaces. We only used TSB as growth media to provide the same substrates to the bacteria (An and Friedman, 1998) to focus on the influence of the surface modifications and proteins on biofilm formation.

Analysing colony morphology, we found small colonies of *S. aureus* on the plates of fr (3 h and 24 h incubation) and ff (24 h incubation) bone grafts but not on the plates of the ph and pb bone grafts. Small colony variants (SCV) represent a subpopulation of naturally occurring, slow growing phenotypes with a distinct pathogenic trait and a colony size of one-tenth of "normal" *S. aureus* (Von Eiff, 2008; Sendi and Proctor, 2009). A persistence of SCV of *S. aureus* has been documented in lysosomes of living cells showing a great resistance of this life-form in tissue culture models (Schroder *et al*., 2006) and explaining the difficulties eradicating SCV infections *in vivo*. Anyhow, an intracellular growth as a potential explanation for the occurrence of SCV in our experimental setting can be excluded because no viable eukaryotic cells are present even on the surface of the fr bone grafts incubated for 3 h (Nade, 2002). The observed reversion of SCV to a "normal" morphology size is well described (Sendi and Proctor, 2009). Normally, SCV become visible on growth media at least 72 h after normal-sized colonies (Sendi and Proctor, 2009), unexpectedly we found small colonies within 24 h after plating.

Microcalorimetry has been shown to be an ideal tool to evaluate biofilm formation (Lerchner *et al.*, 2008; Buchholz *et al.*, 2010; Clauss *et al*., 2010) and we had two interesting findings: Firstly, we found an expected increase in bacterial density (shorter TTD) in the remaining biofilm with longer incubation for both processed materials (ph and pb), but there was no change in the TTD for fr and ff bone grafts comparing 3 h and 24 h incubation. TTD correlated well with the cfu counts after sonication, thus these results are not a consequence of different amounts of bacteria being dislodged during the sonication procedure; Secondly, heat-flow curves with two peaks were only seen with fresh (3 h and 24 h incubation) and fresh-frozen (24 h incubation) samples and small colonies were only found for these samples, too. Heat flow curves represent an unspecific sum signal of exothermal and endothermal reactions within the calorimetry ampoules and might be affected by oxygen depletion and accumulation of metabolic waste (Braissant *et al.*, 2010). Our experimental setting provided the same amount of TSB and oxygen to all samples, thus variations in the heat-flow curves should not be related to varying conditions in the ampoules. The microcalorimetric analysis of the two different colony types showed a slower/reduced metabolism (delayed TTD) for the small colonies. The second peak in the heat-flow curves of biofilm analysis seems to be related to the presence of small colonies on these samples. The small colonies might have grown because of the presence of human serum proteins, cells and fibrous tissue on these two non-cleaned and non-processed samples.

We found significant differences for TPH between samples containing small colonies and those which do not. Microcalorimetry gives a sum signal of all endothermal and exothermal reactions in the ampoules. It is well established that SCV are related to a reduced and modified nutrition (Von Eiff, 2008; Sendi and Proctor, 2009), which is described for bacteria growing in biofilm too (Costerton *et al.*, 1999). Therefore, differences between TPH must be attributed to the ability of the bacteria growing in small colonies to use different/additional sources of energy in the TSB. The presence of small colonies and biofilm formation might be interconnected and research in that field might provide further understanding in foreign body infection.

The limitations of this study should be appreciated:

1. Variations in the bone architecture of the samples might have influenced the results to a certain extent. To normalise these variations an important number of samples from different individuals would be necessary. As the perioperative antibiotic prophylaxis prior to harvesting of the femoral heads during total hip replacement was postponed for this study, patients were exposed to an increased risk of perioperative infection (Trampuz and Zimmerli, 2006a), and the number of examined femoral heads had to be kept small. Therefore, all fr and ff samples were taken from the same femoral head limiting the possible variation in architecture. In contrast ph and pb samples were manufactured out of various donors. As the range in the results was small we believe that our results were not affected by these variations in architecture.
2. We only investigated one bacterial strain. *S. aureus* ATCC 29213 is a reference strain for *in vitro* biofilm research especially when incubated in TSB for 24 h (Ceri *et al*., 1999) but results might differ with other strains.

Clinical implication and outlook

More than one million patients per year need a bone grafting procedure to repair a bone defect resulting from

a trauma or a bone disease (Bohner, 2010). Infection rates for elective orthopaedic surgery are up to almost 5 % (Crockarell *et al*., 1998) and even higher in case of severe trauma, where bone loss also is more common (Ostermann *et al*., 1994). Thus, more than 50,000 infected bone grafts/ year must be expected. Treatment of infected bone grafts is a time consuming, costly endeavour, and frequently leads to extraction of the graft material (Geurts *et al.*, 2011). During recent years synthetic bone grafts have appeared for specific use in infection treatment (Geurts *et al*., 2011). Despite extensive research in the field, none of the synthetic bone grafts matches the unique architecture and composition of bone (Delloye *et al*., 2007).

All 4 investigated bone grafts have almost the same CaP architecture and results can be compared against fr bone grafts, which still is regarded as the gold standard for bone grafting procedures (Delloye *et al*., 2007; Bohner, 2010; Ketonis *et al*., 2010). Even taking into account the limitations of the *in vitro* setting fr bone grafts seem to represent the gold standard concerning resistance against staphylococcal biofilm formation. But the SCV found on the fr and ff grafts remain a concern.

Further studies are needed confirming our findings *in vivo* before advocating the use of one of the 4 tested material in clinical cases were the risk of infection is high.

Conclusion

We found an increased initial bacterial adhesion of *S. aureus* ATCC 29213 on the surface of serum covered fr and ff bone grafts as compared to ph and pb bone grafts. While there was only a slight increase of bacterial density over time on the surface of fr and ff bone grafts we found a tremendous increase on both the ph and pb bone grafts.

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The authors report no competing interests.

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Discussion with Reviewers

Reviewer I: What are the clinical implications of the study regarding selection of bone graft in reconstructive surgery?
Authors: From the laboratory setting, it is difficult to draw clinical conclusions as biofilm formation was done in TSB. Anyhow, we believe that in cases with a high risk of infection, autologous human bone still represents the gold standard.

Reviewer II: According to the protocol as it is written, it seems unlikely that a 50 mL tube with 2.7 mL of TSB would be enough to submerge the bone graft sample. Were the bone grafts submerged in TSB, or were they floating "on the surface of the broth"?
Authors: The experimental setting was optimised and results presented in our previous study (Clauss *et al*., 2010, text reference). The 50 mL tubes give enough volume to (i) submerge the samples (see Fig. D1.), (ii) manipulate them with a forceps in the tube without contamination and (iii) to get an ideal position of the tube in the sonicator to obtain an equal amount of energy on all samples. We agree, however, that the setting in a first glance seems somewhat unlikely.

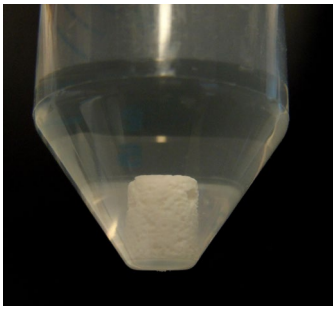

Fig. D1. Example of submerged CaP sample illustrating the experimental setting. *N.B.* the sample shown has the same dimensions, but is another CaP block and the image was not part of this study.