Optimal handling of fresh cancellous bone graft

Different peroperative storing techniques evaluated by in vitro osteoblast-like cell metabolism

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ABSTRACT We investigated the influence of three peroperative handling techniques on the quality of autogenous bone graft by means of osteoblast-like cell metabolism in vitro.

Cancellous bone was harvested from the iliac crest of 12 4-month-old female pigs. Osteoblast-like cell cultures were established, using the tissue-explant method: (1) immediately after harvest of bone, (2) after storage of bone in saline at room temperature for 2 hours and (3) after "dry" storage of bone at room temperature for 2 hours. Proliferation was assessed by ^{3}H-thymidine incorporation. Differentiation was assessed by alkaline phosphatase activity and procollagen I production (PICP).

We found that osteoblast-like-cell proliferation was higher, when cultures were started shortly after harvesting of bone, or else stored in saline for 2 hours, as compared to bone left "to dry" for 2 hours. Basal alkaline phosphatase and PICP production did not differ in the three groups.

These in vitro results suggest the superiority of harvest of autogenous bone graft shortly before the grafting procedure, or else temporary storage of the graft in saline for up to 2 hours.

The efficacy of bone grafting procedures has been investigated in several clinical and experimental studies, but theories differ regarding the fate of the bone graft components and the origin of the newly formed bone induced by the grafting procedure (Nade 1970, Burchardt 1987, Steiner and Ramp 1988, Lang et al. 1989, Gross et al. 1993, Stevenson 1999, Gould et al. 2000).

The interacting factors believed to affect bone grafting include the type, quality, quantity, fixation and preparation of the bone graft material, as well as the blood supply and site and states of the host bed (Nade 1970, Albrektsson 1980, Gray and Elves 1981, Gross et al. 1993, An et al. 1995, Toribatake et al. 1998, Davy 1999, Kim et al. 1999, Perry 1999, Sandhu and Grewal 1999, Gould et al. 2000, Laursen 2001). Albrektsson et al. (1980) emphasized that minimizing trauma to a fresh bone graft enhanced the survival of the cells in the graft and resulted in faster revascularization and remodeling of the graft at the host site. Moreover, experimental evidence suggests that, under optimal conditions—namely, proper techniques of bone storage and efficient and rapid transplantation to a vascular bed—fresh autocancellous bone graft components actively contribute to the synthesis of new bone (Gray and Elves 1981, 1982, Stevenson 1999, Gould et al. 2000). These graft components are osteoblasts, osteoprognitor stem cells, osteoconductive hydroxyapatite collagen matrix and a series of osteoinductive growth factors, of which the BMPs are the most prominent.

It seems feasible to hypothesize that varyious peroperative handling and storage techniques might affect the osteogenic potential and the cells of graft origin. In relation to one-stage spinal fusion procedures, fresh cancellous bone graft is handled

mainly by 3 methods: 1) harvest of the graft in close relation to the implantation, 2) harvest followed by storage of the graft at room temperature, or alternatively, 3) in saline for a few or more hours preceding implantation in the fusion bed.

We investigated how these three procedures affected the baseline metabolism of osteoblast-like cells derived from cancellous bone in vitro.

Animals and methods

Trabecular bone was obtained from the iliac crests of 12 anesthetized 4-month-old female Danish Landrace pigs, with a mean weight of 44 (39–51) kg.

Surgical technique

Cancellous bone was harvested via a posterior-lateral approach, from both of the iliac crests to ensure enough bone for the tissue-explant experiments. Contamination by the growth plates, periosteum and cortical bone in the bone specimen was avoided by excising the upper 2 cm of the rim and by harvesting the cancellous bone only within the limits of cortical bone.

Experimental groups

The bone specimen from the 2 iliac crests of each pig was mixed, divided into 3 equal portions and handled according to 3 protocols:

1) Preparation of the bone sample for explant cultures was started within 45 minutes of harvesting—"0 h" (reference time: time zero);
2) The bone was stored in saline at room temperature for 2 hours before starting to prepare the culture—"NaCl 2 h"; and
3) The bone was left "dry" at room temperature for 2 hours before starting to prepare the culture—"Dry 2 h".

We recorded the time when the preparation of "0 h"-cultures was started; this was taken as the reference time for the experiments.

Bone for grafting and cultures was harvested by means of chisel and curettes, thereby ensuring optimal conditions for cell viability. High-speed tools, which increase the surgical trauma and the local temperature above body temperature, affect the survival of graft bone cells (Albrektsson 1980).

Cell culture

Osteoblast-like cell cultures were established by the explant technique, as described elsewhere (Beresford et al. 1984, Robey and Termine 1985, Kassem 1993). Briefly, cancellous bone fragments were rinsed in phosphate-buffered saline (PBS), cut into 1–2 mm pieces and washed in PBS × 3. The initial washing procedure was followed by collagenase type IV (Sigma, USA) treatment for 2 hours at 37 °C. The collagenase-treated bone pieces were washed in PBS × 1, medium × 1 and then, in medium supplemented with 10% fetal calf serum (FCS) × 1. The extensive washing procedures before and after the collagenase treatment were done to avoid fat and blood cell contamination of the explant cultures. The washed and collagenase-treated bone specimen was weighed, and exactly 1.0 g of bone pieces was cultured in 80 cm^2 culture flasks, in Earle's MEM supplemented with 10% FCS, 50 μg/mL streptomycin and 100 μg/mL penicillin G. The medium was changed after 24 hours and thereafter twice weekly. Cells were grown for 3 weeks and released from culture by 120 sec. trypsinization. Proliferation and differentiation were assayed in terms of ^{3}H-thymidine incorporation, procollagen C-terminal propeptide (PICP (^{125}I)) and alkaline phosphatase (AP) activity. The cells used for experiments were from the first passage. Assays of AP activity and ^{3}H-thymidine incorporation were done in triplicate × 2. The PICP assay was performed by assessing the pooled conditioned medium from triplicate wells twice. One change in serum was used for all experiments.

In vitro assays

Cell count. The concentration of cells in all cell suspensions after culture was determined by using a hemocytometer.

DNA synthesis assay. Osteoblast-like cell proliferation was evaluated in terms of the incorporation of ^{3}H-thymidine into DNA. Cells were plated at a density of 1×10^4 in 96 well plates in 200 μL Earle's MEM containing 10% FCS and allowed to adhere for 24 hours. After 24 hours of adherence, the medium was changed to Earle's MEM with 1% and 10% FCS for an additional 48 hours. ^{3}H-thymidine (46 Ci/mmol) was added for the last 16 hours of the incubation time (0.15 μCi/well). The

Summary of findings. Median (range)

	^{3}H-thymidine, cpm × 10^3		Mitogenic response	AP (nmol/10^4 cells/min)		PICP (μg/L)
	1%	10%		1%	10%	10%
0 h	2.10 (4.74)	7.25 (8.15)	3.5	1.2 (4.5)	2.5 (7.90)	217 (315)
NaCl 2 h	1.51 (4.74)	3.97 (8.45)	2.6	1.2 (4.5)	3.2 (10.3)	180 (348)
Dry 2 h	1.51 (6.58)	3.75 (7.99)	2.5	1.9 (7.0)	3.4 (20.5)	241 (308)

Mitogenic response: (cpm (FCS 10%)/cpm (FCS 1 %)).

incorporation of ^{3}H-thymidine into trichloroacetic acid-precipitable DNA was measured by liquid scintilography (Beta-counter, Wallac, Finland). The mitogenic response was estimated by the proliferation ratio in wells supplemented with 10% FCS and 1% FCS (cpm (10%)/cpm (1%)).

Alkaline phosphatase activity. To assay baseline alkaline phosphatase activity, osteoblast-like cells were plated in 96 well plates with 4 x 10^4 cells/well in a 200 μL medium containing 10% FCS and allowed to adhere for 24 hours. The culture medium was then changed to Earle's MEM with 1% (control) and 10% FCS and maintained for another 48 hours. AP activity was measured in the cell layer after 30 minutes of incubation with p-nitrophenol phosphate as a substrate at 37 °C (Sigma Diagnostics). Absorbance of p-nitrophenol was determined with a microspectrophotometer at 405 nm. Phenotype expression was estimated by the ratio of AP in wells supplemented with 10% FCS and 1% FCS.

In addition, histochemical staining for AP was done randomly in other cultures. The cells were fixed and incubated with substrate containing varium blue salts and sodium naphthyl-phosphate at 4 °C for 5 min. They were then counterstained with Mayer's hematoxylin for 30 sec.

Procollagen type I synthesis. Basal procollagen type 1 C-terminal (PICP) propeptide was measured in the conditioned medium from 96 wells (1 × 10^4 cells/well, 200 μL/well) after incubation for 48 hours in Earle's MEM containing 10% FCS. PICP was quantitated in conditioned medium by means of a commercial radioimmunoassay, using an antibody that recognizes procollagen C-terminal propeptide (PICP (^{125}I) (Orion Diagnostica, Finland).

Statistics

Nonparametric statistics was used, as the material did not comply with the assumptions for normally-distributed data. Friedman's rank test for comparing k-related groups, considering a two-way analysis of variance of the ranks, was used to compare the three pre-culture bone storage groups. If significance was reached with Friedman's test, additional analysis within dual groups was done by means of the Wilcoxon signed ranks test. The Wilcoxon test was also used to compare osteoblast metabolism in wells supplemented with 10% and 1% FCS. The intra-assay coefficient of variation (CV) was calculated with the formula: SD/mean. Values entered into the statistical tests were the mean of triplicate wells (AP and ^{3}H-thymidine) or duplicates (PICP) for each individual (n = 12) in all conditions. A result of $p < 0.05$ (two-tailed) was considered significant.

Ethical considerations

The experiments complied with the Danish Law on Animal Experiments and were approved by the Danish Ministry of Justice, J.no.1998-561-67.

Results

All cultures from 1 bone donor were excluded because of bacterial contamination; thus, the results are based on 11 bone donors.

Proliferation (Table, Figure 1). Cultures from the 11 bone donors all showed a positive mitogenic response. The proliferation rate (10% FCS) differed ($p = 0.04$) among cultures, which were started using the three different preculture handling techniques. Osteoblast-like cell proliferation was lower in cultures in which bone was initially

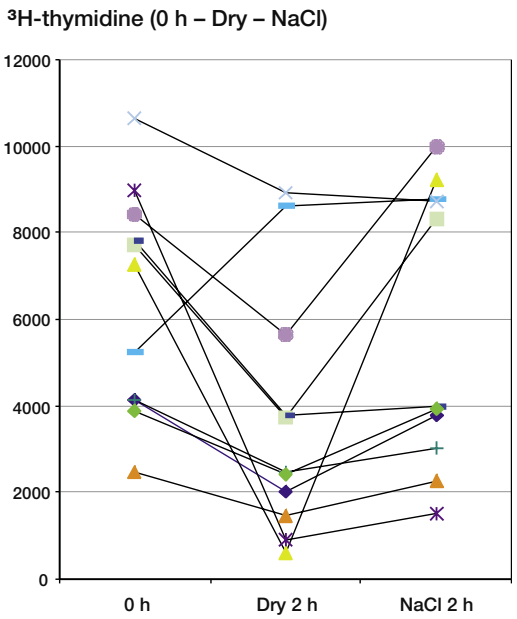

Figure 1. Proliferation. ^{3}H-thymidine incorporation (cpm) according to preculture handling of the bone explant. (Cultures supplemented with 10% FCS). n = 11, mean of triplicate wells.

stored "dry" for 2 hours than in those in which trabecular bone was prepared for culture immediately after harvest ($p = 0.03$), or stored in saline for 2 hours before starting preparation of the culture ($p = 0.008$). In cultures from 1 bone donor, the proliferation assays showed the opposite tendency in the two pre-culture conditions "0 h" and "Dry 2 h", as compared to all the other cultures. The intra-assay CV was, on average, 13.1% (0 h), 11.8% (NaCl 2 h) and 16.8% (Dry 2 h).

Basal AP activity and PICP production (Table). In cultures supplemented with 10% FCS, a significant increase in basal AP activity was seen, as compared to cultures supplemented with 1% FCS. Histochemical staining for alkaline phosphatase was done randomly in donor cultures; in these cultures, staining was positive, but not uniform (Figure 2). No significant differences were found in alkaline phosphatase activity and PICP production (10% FCS) in the three culture groups ($p < 0.32$ and $p < 0.61$, respectively). The intra-assay CVs (AP) were, on average, 11.5 % (0 h), 9.2%(Dry 2 h), and 10.4% (NaCl 2 h) and the intra-assay CVs (PICP) were, on average, 11.7% (0 h), 15.4% (Dry 2 h) and 15.6% (NaCl 2 h).

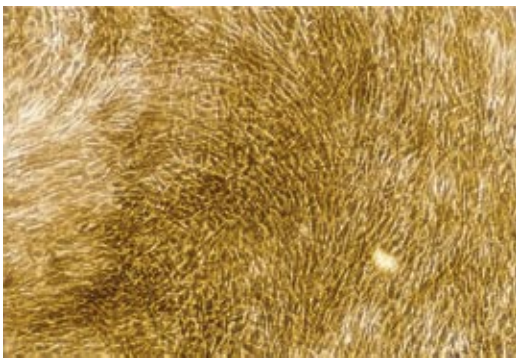

Figure 2. Histochemical staining for alkaline phosphatase. (×10).

Despite the fact that the donors were of the same age and gender, the inter-individual variations were grater in cultures from different donors (Table).

Discussion

In the incorporation of bone autografts, an important, but less often studied matter concerns the effect on osteoblast viability of temporary peroperative methods of storing fresh bone-autografts.

In our in vitro study, the proliferation of osteoblast-like cells was affected by various preculture handling techniques, resembling different peroperative handling methods of cancellous bone for grafting purposes. It could be theorized that bone explant-derived osteoblast-like cells preserve a higher proliferative capacity and a lower rate of necrosis when the bone is prepared immediately for culture or placed in saline for up to 2 hours. Unlike the proliferation potential, we found no statistically significant differences in the basal AP activity among the three groups. In the current study, cultures were maintained for 3 weeks; at this time, they were actively proliferating, which may have affected the expression of differentiation markers in the assay. In most cell systems, active cell proliferation affects the potential for a highly differentiated expression of phenotype characteristics. The baseline AP production was relatively low due to the high rate of cellular proliferation and therefore, small changes in production were not detectable.

We found no statistically significant differences in the basal type 1 collagen production and AP

activity was not significant in the three groups. The PICP release may reflect the tendency in the proliferation assays, as found by Eklou-Kalonji et al. (1998). They divided the in vitro development of porcine osteoblast cells into two periods: 1) active proliferation—i.e., an increase in thymidine incorporation during which PICP release had increased and this was followed by 2) a period of cell differentiation during which thymidine incorporation and the release of PICP declined and AP activity gradually increased.

The intra-assay variation seen in our study may have been caused by a difference in cellular activity between wells, and the manual steps when preparing assays.

The bone explant method for obtaining homogeneous osteoblast-like cell cultures, synthesizing type 1 collagen and with phenotype characteristics of osteoblasts—i.e., osteocalcin production and alkaline phosphatase activity—is a well-described and documented method for use in humans and various species including the porcine (Beresford et al. 1984, Auf'mkolk et al. 1985, Robey and Termine 1985, Marie et al. 1989, Kassem 1993, Denis et al. 1994, Jonsson et al. 1999). The porcine is thought to be a good physiological model for studies of bone metabolism and assessment of the effects of osteotropic agents on bone remodeling (De Vernejoul et al. 1987, Denis et al. 1994). As regards the evaluation of the in vitro effects of various handling techniques of trabecular bone in human bone graft surgery, it would have been better to avoid species-induced differences. However, taking into account the amount of bone, needed to do this study, harvesting trabecular iliac crest bone in patients undergoing grafting procedures would not be ethically acceptable. Despite the use of an experimental animal model in which confounding factors related to living conditions, age and gender were reduced, the results of this study showed a high inter-individual variation in the cultures. This variation can hardly have been caused by differences in the basic experimental set-up, which was stringently controlled. However, the variation may have been partly due to a variation in the percentage of osteoblastic cells derived from each bone donor, since contamination with cells of marrow origin can not be excluded. Moreover, the bone donors in this study were young animals with a high growth potential. This could contribute to the large inter-individual variation, as reflected by the variations in the animals' weights.

It has been generally accepted that the transplanted cells associated with a fresh bone graft were already, or soon became, necrotic and unable to proliferate (Nade 1970). However, it is disputed (Albrektsson 1980, Gray and Elves 1981, Burchardt 1987, Lang et al. 1989, Gross et al. 1993, Gould et al. 2000) whether the presence of viable osteogenic cells and a healthy blood supply, although not indispensable for success, play a role in the induction of osteogenesis (Albrektsson 1980, Gray and Elves 1982, Toribatake et al. 1998, Stevenson 1999, Gould et al. 2000). Moreover, osteogenic elements may survive in fresh autografts to the extent that the bone transplant may be considered active in its own rearrangement (Burchardt 1987). Few previous studies have evaluated the effects of temporary peroperative storage of fresh cancellous bone grafts in vivo and in vitro (Gray and Elves 1981, Steiner and Ramp 1988). Steiner and Ramp (1988) investigated the in vitro effects on glucose metabolism and collagen synthesis by means of short-term storage of bone obtained from embryonic chick tibiae. Their findings suggested that, unlike the storage of bone in distilled water, storage in saline or Ringer's solution for up to 5 hours did not significantly affect glucose metabolism or collagen synthesis. Gray and Elves (1982) also investigated the effect of various temporary preimplantation storage methods on strontium gamma activity in experimental isografts implanted for 12 days in male rats. They found that storage for 6, 12 or 24 hours in air (dry) or in saline before implantation of these isografts significantly reduced the strontium uptake after 12 days of implantation, as compared to fresh implanted isografts. Even storage in saline for 3 hours produced a smaller, but significant, decline in strontium uptake. Unlike the findings in the current study, dry storage of the graft for 3 hours before implantation had no significant effect on the osteogenetic potential.

To mimic the clinical situation, bone samples were stored at room temperature in the current study. It seems likely that cell viability would be affected by differences in storage-temperatures. However, Gray and Elves (1982) reported no dif-

ference in cell viability after storage for 4 hours at 4, 25 or 37 °C. Despite obvious differences in study design and outcome parameters, all these authors suggest that temporary peroperative methods of storage, influence graft cells in the experiments. Harvest of autogenous bone in close relation to the grafting procedure may also be advantageous.

In addition, Albrektsson (1980) emphasized that by reducing the trauma to the bone graft, the survival of the graft could be extended, which would result in more rapid revascularization and bone remodeling. He suggested a causal relationship between graft incorporation and the presence of viable cells, as shown by histochemical tests. Recently, Gould et al. (2000) found that abundant graft-derived cells contributed to all states of the fusion mass, from the immediate postoperative state to the final remodeling one. They developed two chimeric mouse isograft models, which involved placing male-derived bone grafts into syngeneic female host fusion beds. By in situ hybridization for Y-chromosome sequences present only in the graft (male) cells, they could track the graft-cell contribution in early and late states of graft incorporation. Lang et al. (1989) studied the contribution of living cells to the improvement in bone regeneration by using the explant technique to establish osteoblast-like cell cultures after reimplantation. Histological examination of the reimplantation showed regeneration activity, since new bone formation was found in the center of the defect and at the defect margins. The growth pattern differed from that of the control groups (no implantation therapy), as the controls mainly showed peripheral growth activity. They also found that the implanted osteoblast-like cells survived, because the cells implanted were explanted again after 2 days and could be recultivated in vitro. The above experimental studies, in which Gould et al. (2000) provided substantial evidence, suggest that viable graft cells play a role in graft incorporation. An extrapolation of these findings concerns the effect of peroperative storage/handling on the viability of graft cells.

In summary, our in vitro findings in porcine osteoblast-like cells suggest that it is advantageous to harvest autogenous bone grafts shortly after the grafting procedure, or temporarily store them in saline for up to 2 hours.

No competing interests declared.

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