Heating or freezing bone

Effects on angiogenesis induction and growth potential in mice

Michael Leunig, Fan Yuan, David A Berk, Leo E Gerweck and Rakesh K Jain

We have characterized the effect of bone graft treatment by heating or freezing (with or without dimethyl sulfoxide (DMSO)). Tissue culture and dorsal skinfold chambers in mice were used as sites to quantify the effect on angiogenesis, growth and calcification of neonatal femora. Fresh femora increased in both length and cartilage diameter (calcification in vivo only), but cryopreservation or heating abolished the increase in femoral dimensions. In vivo, femora of all experimental groups elicited an angiogenic response from the host tissue, which was most pronounced for fresh femora, weaker for DMSO-preserved frozen bone and poor for unprotected frozen bone and boiled femora. Freezing in the presence of a cryopreservative (DMSO) was found to preserve the angiogenic potential of frozen bone, whereas unprotected heating or freezing significantly impaired angiogenesis induction and growth potential.

Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA. Correspondence: Dr. Michael Leunig, Department of Orthopedic Surgery, University of Berne, Inselspital, CH-3010 Berne, Switzerland. Tel +41 31-632 2222. Fax -382 3561
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Transplanted bank bone is often associated with complications such as local inflammation, nonunion or fatigue fracture (Horowitz and Friedlaender 1991, Mankin et al. 1992). The exact mechanism(s) underlying these clinical failures are not known. To identify factors possibly involved, we have experimentally evaluated the effect of tissue pretreatment (freezing or heating) on the biological potential of bone by means of a recently developed tissue culture and animal model.

Animals and methods

Femora

52 femora were obtained from 35 neonatal NCr/Sed (nu/nu) nude mice (1.6 g b.w.) bred and maintained in a specific pathogen germ-free colony at the Massachusetts General Hospital (Boston, MA). After killing by decapitation, mice were maintained for 5 min in 70% ethanol to reduce possible skin contamination by pathogens. Both femora were blunt-dissected in 10 mL Hanks' balanced salt solution (HBSS, Gibco Laboratories, Grand Island, NY) at room temperature (22 °C) and cleansed of soft tissues. Thereafter, femora were transferred to a second dish containing fresh HBSS at 22 °C room temperature and subjected to either the in vitro (n 24) or the in vivo (n 28) part of this study. Only femora lacking signs of tissue damage (cartilage damage, fracture), as verified by a stereomicroscope, were used for further analysis (52 of 70).

Tissue culture

For the in vitro studies, one femur each was cultured in 6-well plates (Multiwell, Falcon 3046; Becton Dickinson & Company, Lincoln Park, NJ) containing 3 mL of serum-free Dulbecco's modified Eagles medium (DMEM, Cellgro; Meditech, Herndon, VA) and 10% fetal calf serum. The pH was adjusted to 7.4 in an atmosphere of 95% humidified air and 5% CO_2 at 37 °C; the medium was supplemented with a combination of antibiotics (50,000 U/mL penicillin G, 50 µg/mL streptomycin and 100 µg/mL neomycin, Sigma Chemical Company, St. Louis, MO).

Dorsal skinfold chambers

For the in vivo studies, one femur (isograft) was transplanted into each microcirculatory preparation implanted into the dorsal skin of 28 NCr/Sed (nu/nu) nude mice (male, 3–4 months, 26–32 g) (Figure 1). In brief, nude mice were anesthetized (ketamine hydrochloride 7.5 mg and xylazine 2.5 mg per 100 g b.w.) and two symmetrical titanium frames, which are mirror-images of each other were implanted so as to sandwich the extended double layer of the skin (Leunig et al. 1994). One of the two skin layers was removed in a circular area of approximately 15 mm in diameter and the remaining layer, consisting of epidermis, subcutaneous tissue and striated muscle, was covered with a removable coverslip incorporated into one of the frames. After a recovery period of 24–48 h from surgery, femora were implanted into chambers

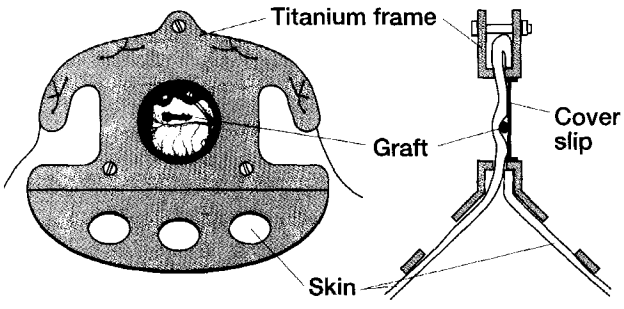

Figure 1. Dorsal skinfold chamber preparations in mice served as the host tissue for the visualization of angiogenesis and growth of grafted bone. (Lateral view and cross-section through the center of a chamber.)

Figure 2. Experimental protocol

- **Harvesting**
  - Decapitation
    - 5 min 70% ethanol
  - Dissection
    - 30–55 min HBSS
- **Pretreatment**
  - Untreated
  - Boiled
    - 5 min at 100 °C in 0.9% NaCl
    - 25 min in HBSS
  - Frozen, unprotected
    - 30 min at 4 °C, no preservation
    - 7 days at –60 °C
    - 1 h at 22 °C in HBSS
  - Frozen, protected (DMSO)
    - 30 min at 4 °C in 10% DMSO
    - 7 days at –60 °C
    - 1 h at 22 °C in HBSS
- **Experiments**
  - *In vitro*
  - *In vivo*

fulfilling the criteria of intact microcirculation (Sewell 1966). For implantation of femora, the coverslip of the chamber was removed and femora were implanted onto the upper tissue layer of the chamber preparation (striated skin muscle). Thereafter, the access chamber was sealed again by the coverslip. This preparation allows visualization of growth, calcification and angiogenesis of transplanted bone for more than 2 weeks.

Experimental groups

All femora of the in vitro and in vivo parts of this study were pretreated (Figure 2). 6 femora were assigned to each in vitro group, 7 to each in vivo group. Fresh femora were used 60 min after procurement, without any further treatment. In the boiling group, 30 min after decapitation of the mice, explanted femora were heated (100 °C) for 5 min in 0.9% NaCl and implanted after keeping the bone for a further 25 min in HBSS. The cryopreservation protocol was designed as follows. After 30 min in HBSS, femora were maintained for 30 min at 4 °C, then frozen to –60 °C and stored at that temperature for 7 days in sterile microcentrifuge tubes. To the second freezing group, DMEM containing 10% dimethyl sulfoxide (DMSO) (No. D-5879, Sigma Chemical Company, St. Louis, MO) was added for 40 min at 4 °C prior to freezing. After 7 days at –60 °C, femora of both groups were rapidly thawed to room temperature (22 °C), transferred in HBSS for 1 h and subsequently transplanted.

Experimental procedure

In vitro. Observations on changes in femoral dimensions were performed under a microscope with a 1.25× objective (0.035, Plan Neofluar, Zeiss), using brightfield transillumination with a resulting 36-fold magnification on a TV screen (PVM-13420, Trinitron, Sony, Tokyo, Japan). On days 0, 8 and 16, the medium was changed and supplemented by 100 µg/mL oxytetracycline (OTC; Liquamycin LA-200, Pfizer, New York, NY). 30 min later, the culture medium was replaced by a fresh one and OTC-fluorescence persisting in the calcified femur fraction was excited by epiillumination, using an excitation filter (356-418 nm) and a dichroic mirror (450 nm). The fluorescence after a barrier filter (495 nm) was detected by an intensified CCD camera (C2400-88, Hamamatsu Photonics K.K., Hamamatsu, Japan) to quantify femoral calcification.

In vivo. Unanesthetized mice were positioned in a polycarbonate tube of approximately the same diameter (25 mm) as the mice in their crouched position. The chamber preparation containing the transplanted bone was mounted on the stage of a microscope (Axioplan, Zeiss, Oberkochen, Germany). For intravital microscopy, transillumination technique (12 V, 100 W Halogen Lamp, Zeiss), employing a green filter to enhance black/white photomicrography and a conversion filter for converting artificial light of 3200 K into daylight of 5500 K was used. On days 0, 8 and 16, 100 mg/kg b.w. oxytetracycline was injected i.p. and 6 hours later, when OTC was readily cleared from non-calcified tissues, OTC-fluorescence was excited by epiillumination. This dose of OTC given on days 0, 8 and 16 did not inhibit angiogenesis induced by femora (Leunig et al. 1995). All observations were recorded on tapes, using a video casette recorder (AG-6500, Panasonic, Secaucus, NJ) at the rate of 30 frames per sec and analyzed off-line by means of an image analysis system (Sun Workstation, 3/260, Sun Microsystem; MVP-VME, Video Digitizer, Matrox International Corp.).

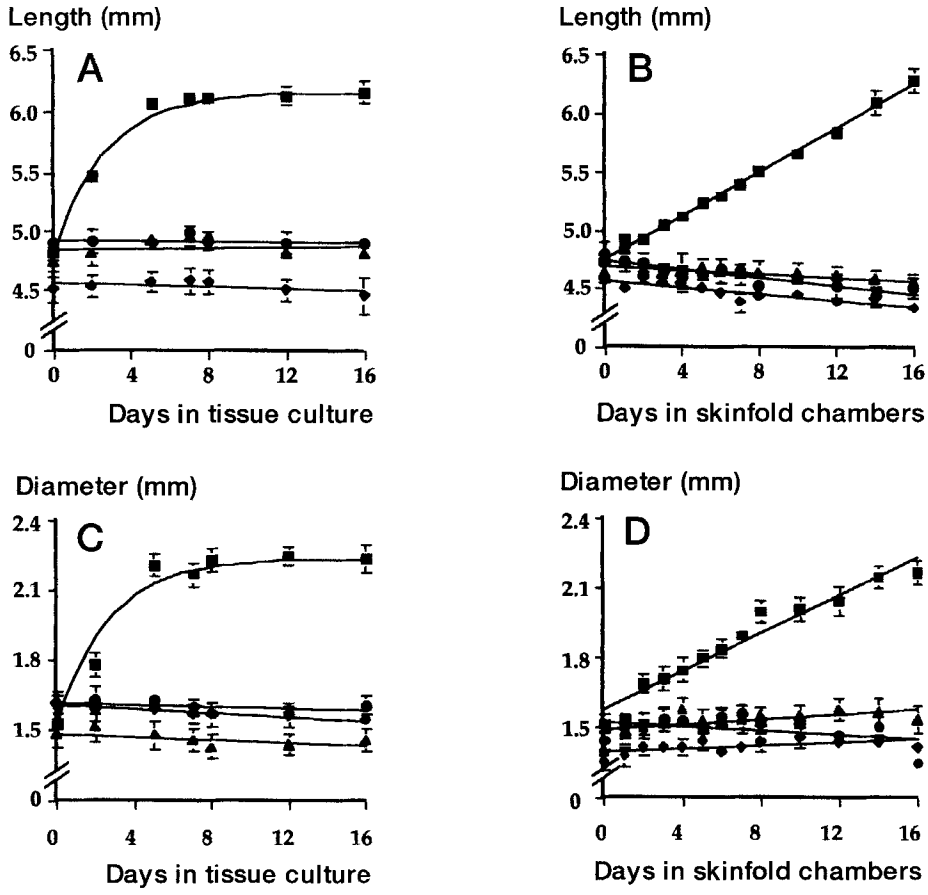

Figure 3. Measurements of overall femur length in vitro (A) and in vivo (B) demonstrated striking differences in the growth rates and sizes reached for fresh isografts and the three pretreated groups ($p < 0.001$). Only fresh femora (■) were capable of significant elongation. Freezing in the presence (◆) or absence of DMSO (●) similarly to boiling of femora (▲) abolished growth. Measurements of the transverse diameter at the proximal and distal femur ends in vitro (C) and in vivo (D) revealed that only fresh transplanted tissue was capable of cartilage growth.

Data analysis

Femoral dimensions are given in mm and are defined as follows. Length is the distance of the femur in the longitudinal axis from the proximal to the distal end; diameter is the distance of the cartilage in the transverse axis of the femur at the proximal and distal ends; and length of the calcified fraction is reflected by the oxytetracycline-labeled femur fraction. The error in these measurements was less than 2.5%.

Angiogenesis was quantified by monitoring the time of initial appearance of newly formed blood vessels on the surface of the transplanted femora and the time when first flow observed in these vessels.

All in vitro and in vivo measurements were performed at the same time on each day throughout the experimental period.

Statistics

Data are presented as mean and standard error of the mean (SEM). Data were tested for significant differences between the groups, using the Kruskal-Wallis-test and U-test for independent samples or Friedman-test and Wilcoxon-test for related samples. Differences were considered as significant at $p < 0.05$.

Results

In organ culture (in vitro), untreated (fresh) femora increased significantly in length and cartilage diameter, which was characterized by a saturable growth kinetic (Figure 3). At day 5 in tissue culture, untreated femora attained the plateau phase of longitudinal and transverse cartilage growth. Cryopreservation with or without DMSO or boiling completely abolished fem-

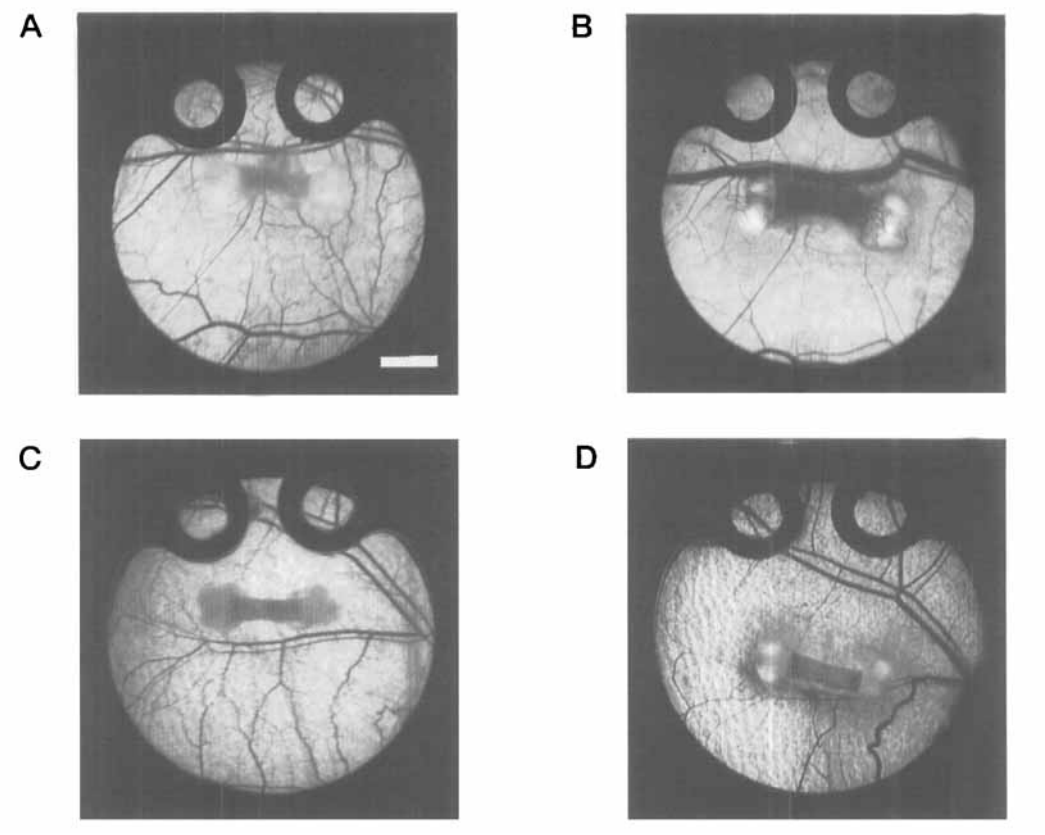

Figure 4. Photographs taken at day 0 (A and C) and day 8 (B and D) after implantation of neonatal femora in dorsal skinfold chambers. Femora significantly revascularized and grew when transplanted fresh (A and B), whereas only a minor angiogenic reaction occurred after freezing femora prior to implantation (C and D); growth was completely abolished after unprotected freezing. (The bar represents 2 mm).

Table 1. ***In vitro:*** **Length of OTC-stained calcified femoral fraction on days 0, 8 and 16. Mean** ***SEM***

	Length in mm (n 6)		
Pretreatment	Day 0	Day 8	Day 16
Fresh	2.47 *0.05* [a]	2.40 *0.04* [a]	2.24 *0.04*
DMSO	2.48 *0.05* [b]	2.46 *0.07*	2.23 *0.07*
Frozen	2.40 *0.05* [c]	2.44 *0.04* [c]	2.22 *0.05*
Boiled	2.42 *0.04* [d]	2.35 *0.07* [d]	2.10 *0.07*

At days 0, 8 and 16, no significant differences were detected between the pretreatment groups, whereas the calcified femoral fraction had decreased in all groups by day 16.
[a] $p < 0.05$ vs fresh (day 16)
[b] $p < 0.05$ vs DMSO (day 16)
[c] $p < 0.05$ vs frozen (day 16)
[d] $p < 0.05$ vs boiled (day 16)

Table 2. ***In vivo:*** **Length of OTC-stained calcified femoral fraction on days 0, 8 and 16. Mean** ***SEM***

	Length in mm (n 7)		
Pretreatment	Day 0	Day 8	Day 16
Fresh	2.47 *0.06*	2.93 *0.05* [e]	4.24 *0.07* [e]
DMSO	2.42 *0.06*	2.44 *0.05* [f]	2.43 *0.06* [g]
Frozen	2.41 *0.05*	2.48 *0.05* [f]	2.44 *0.05* [g]
Boiled	2.41 *0.05*	2.36 *0.04* [f]	2.38 *0.05* [g]

Only in fresh femora did the calcified femoral fraction significantly increase in length.
[e] $p < 0.05$ vs fresh (day 16). At days 8 and 16, the calcified femoral fraction was significantly increased in fresh femora, compared to pretreated femora.
[f] $p < 0.05$ vs fresh (day 8)
[g] $p < 0.05$ vs fresh (day 16)

oral elongation. Independent of pretreatment, femoral calcification quantified by measuring the OTC-stained femur fraction did not enlarge, but rather decreased slightly between days 8 and 16 by approximately 10% ($p < 0.05$) (Table 1). In the dorsal skinfold chamber (in vivo), the growth rate of fresh femora was linear throughout the observation period of 16 days (Figures 3 and 4) and the femora were significantly calcified ($p < 0.05$) (Table 2), whereas physical pretreatment abolished the increase in length and cal-

Table 3. Statistics for Figure 5. Effect of physical pretreatment on initial appearance of newly formed blood vessels and first flow in these vessels. Figures are p-values

	Initial vessel			
First flow	Fresh	DMSO	Frozen	Boiled
Fresh	–	0.6	0.004	0.003
DMSO	0.05	–	0.004	0.003
Frozen	0.002	0.06	–	0.02
Boiled	0.003	0.003	0.03	–

Non-parametric statistics (Kruskal-Wallis-test and U-test)

cification in vivo as observed in vitro.

Angiogenesis was observed in the vicinity of all transplanted femora, however, in varying degrees (Figure 5). Newly formed blood vessels appeared nearly simultaneously in fresh or protected frozen (+ DMSO) femora, whereas unprotected freezing or boiling significantly delayed neovascularization (Table 3). The following rank order was observed: Fresh > DMSO > Frozen > Boiled. First blood flow in these vessels was established 2 or more days later, revealing the same sequence as seen for neovascularization: Fresh > DMSO > Frozen > Boiled (Figure 5). The least angiogenic response was observed after boiling of femora, with delayed vessel appearance and flow, as compared to unprotected frozen femora (Table 3).

Discussion

Unprotected freezing, currently the most widely used method for long-term storage of bone, was demonstrated to impair neovascularization of grafted bone, as does heat pretreatment. In the presence of DMSO, which is clinically used for freezing of osteochondral allografts to enhance tissue viability (Tomford et al. 1987), angiogenesis was partially restored, but the growth potential could not be preserved. What causes the varying degrees of biological impairment subsequent to bone pretreatment by heating and freezing? Untreated (fresh) femora suffered little, if any, tissue or cellular damage from transplantation. Thus, both angiogenesis and growth were maintained. In femora frozen in the presence of DMSO, superficial structures may have survived freezing and thawing, however, "organ integrity" was impaired. The lost growth capability indicates severe damage to the function of the growth plate. However, some cells, most probably at the femoral surface, resist cell damage and sustain its capability of expressing mitogenic signals, which explains the significantly enhanced angiogenic response, compared to the unprotected frozen bone. Diffusion limitations of DMSO (Maroudas 1976) and heat transfer restrictions are most probably responsible for the forfeited organ function. In unprotected frozen as well as boiled femora, most viable organ structures are fatally damaged. Only preexisting factors stored in the mineralized matrix of femora hypothetically remain for eliciting angiogenesis or growth (Hauschka et al. 1986). The insignificant angiogenic response induced by boiled femora may be caused by

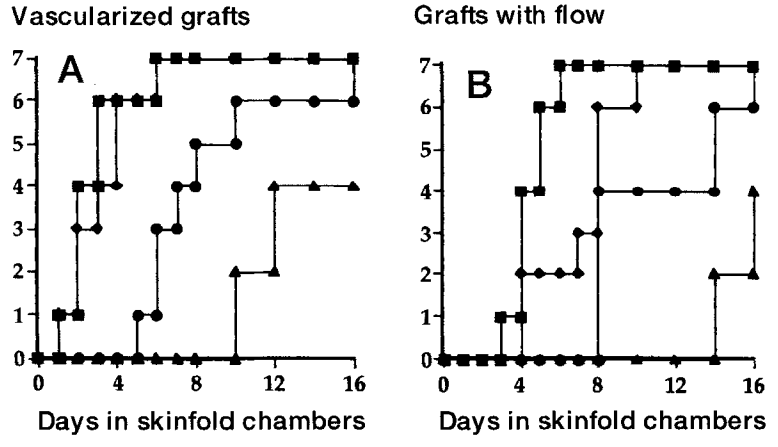

Figure 5. Cumulative frequency of initial appearance of newly formed blood vessel (A) and first observed flow in these vessels (B) at the surface of transplanted femora. Within 6 days, all transplanted fresh (■) and DMSO-preserved femora (◆) were reached by newly formed blood vessels, whereas this process was significantly delayed up to 16 days for the frozen femora (●) and boiled femora (▲). For statistical comparisions see, Table 3.

inactivation of growth factors trapped in the matrix of bone (Hauschka et al. 1986) and similar to that seen for nonbiologic tissue, such as synthetic vascular grafts (Menger et al. 1990).

Comparable to other tissues, bone allografts can evoke a significant immune reaction by the host (Horowitz and Friedlaender 1991). Graft processing has been instituted to reduce antigenicity rather than manipulate the immune system of the host. For more than 30 years deep-freezing (freeze-drying) has been used in most bone banks (Tomford et al. 1987, Kakaiya and Jackson 1990, deBy, 1991) to attenuate the immune response towards bone allografts (Mankin et al. 1992). The rate of clinical failures after bone allografting ranges between 20% and 50% (Horowitz and Friedlaender 1991, Mankin et al. 1992). Apart from immunological phenomena, this failure rate may be due to the biological impairment by current methods of bone banking. One possible way to reduce tissue antigenicity and simultaneously increase tissue viability is the selective reduction of certain cell populations ("passenger leukocytes") (Lafferty et al. 1983), which appears to play an important role in the immunomodulation by cryopreservation (Faustman et al. 1981). Rapid freezing of tissue (> 50 °C per min) causes destruction and immunological inactivation of leukocytes transplanted with the graft, whereas tissue viability remains intact (Taylor and Bank 1988, Taylor et al. 1990). Particularly in elderly patients possessing reduced bone repair capacity, a contribution of the grafted tissue to the osseous integration and subsequent consolidation might improve the treatment outcome.

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